



MASTERARBEIT

Titel der Masterarbeit

„Differential proteome analysis of blood-brain barrier (BBB)
endothelial *in vitro* models“

1 von 1

Verfasserin ODER Verfasser

DI (FH) Christiane Gebhard, Bakk.tech

angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2011 05.09

Studienkennzahl lt. Studienblatt: A >066 834<

Studienrichtung lt. Studienblatt: Master Molekulare Biologie

Betreuerin / Betreuer: Univ. Prof. Mag. Dipl. Ing. Dr. Christian R. Noe

Acknowledgments

I would like to give thank to O.Univ.-Prof. Dipl.-Ing. Mag. Dr Christian R. Noe, for giving me the interesting topic in proteome research and offering the possibility to join the BBB research group of Pharmaceutical University Vienna, Department of Medicinal and Pharmaceutical Chemistry.

Thanks a lot to Professor Fricker for the possibility to work in his research team at the Department for Pharmaceutical Technology and Biotechnology, Karl Ruprechts University, Heidelberg

Thanks a lot to Dr. Johannes Winkler, for your support and your patient way how to explain experimental questions.

I would like to thank Dr. Martina Stessl for reading my thesis and giving me valuable comments.

Thanks to Dr. Winfried Neuhaus and DI (FH) Elisabeth Strizsik to made contact for cooperation with Prof. Gert Fricker from University Heidelberg and Dr. Martina Marchetti-Deschmann from Vienna University of Technology.

Special thanks to my family and my friends for being in my life, giving me great support and to cheer me up in difficult times.

Index

1	INTRODUCTION.....	8
1.1	WHAT IS THE BLOOD- BRAIN BARRIER?	9
1.1.1	<i>Historical approach.....</i>	9
1.2	MORPHOLOGY AND PHYSIOLOGY OF THE BBB	10
1.2.1	<i>The components of the BBB</i>	11
1.2.2	<i>The tight junction complex.....</i>	13
1.2.3	<i>Tight junction molecules.....</i>	14
1.3	TRANSPORT SYSTEMS ACROSS THE BBB	17
1.4	PATHOLOGY IN BBB.....	19
1.5	CELL CULTURE MODELS FOR <i>IN VITRO</i> BBB EXPERIMENTS	20
1.6	PROTEOMIC APPROACH	20
1.6.1	<i>What is the application of proteomics?.....</i>	21
2	AIM OF THE WORK.....	22
3	MATERIALS AND METHODS	24
	MATERIALS.....	24
3.1	CHEMICALS	24
3.2	CELL CULTURE MATERIALS.....	26
3.3	PROTEOME MATERIALS.....	27
	METHODS	28
3.4	CELL CULTURE.....	28
3.5	EXPERIMENTAL CELL LINES	28
3.5.1	<i>Media and solutions</i>	31
3.5.2	<i>Cell freezing</i>	35
3.5.3	<i>Cell thawing.....</i>	35
3.6	TRANSWELL STUDIES.....	36
3.6.1	<i>Coating of the Transwell insert.....</i>	37
3.6.2	<i>Cell seeding</i>	37
3.6.3	<i>Media exchange on Transwell model.....</i>	38
3.6.4	<i>Transendothelial Electrical Resistance (TEER) measurements</i>	38
3.7	CELL LYSIS	39
3.8	BRADFORD ASSAY	40

3.9	DIGE 2D-ELECTROPHORESIS.....	40
3.10	2-D ELECTROPHORESIS	42
3.11	TRYPTICAL <i>IN-GEL</i> DIGESTION.....	45
3.12	MALDI TOF MS	46
3.13	ISOLATION OF PRIMARY PBCECs.....	47
4	RESULTS	48
4.1	TEER MEASUREMENTS	48
4.2	PROTEOMICS.....	55
4.2.1	<i>Comparison of PBMEC/C1-2 and HUVEC.....</i>	<i>55</i>
	Uninduced cultivation models	55
	Induced cultivation models.....	56
	Co-culture models.....	57
	Protein list of significantly changed proteins	58
	MALDI TOF identification.....	65
	PMF spectrum and Mascot Search Results.....	68
5	DISCUSSION.....	72
6	ZUSAMMENFASSUNG	78
7	SUMMARY	80
	REFERENCES	82
	Appendices	87
A	CV /Lebenslauf.....	88
B	PMF analysis data.....	91

In memory to my childhood friend, Bernard Flemmich

List of Abbreviations

2-D	Two dimension
2-DE	Two dimensional gel electrophoresis
2-DE	Two dimensional gel electrophoresis
ACM	Astroglial/Astrocyte conditioned medium
AD	Alzheimer disease
AJs	Adherence junctions
AMT	Adsorptive-mediated transcytosis
A β	β -amyloid
BBB	Blood – brain barrier
C6	Rat glioma cells
CNS	Central nervous system
CSF	Cerebrospinal fluid
DIGE	Difference gel electrophoresis
EC	Endothelial cells
et al.	<i>et alii</i>
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	hour
H ₂ O ₂	hydrogen peroxide
Hsp	Heat shock protein
HUVEC	Human umbilical vein endothelial cells
IEF	Isoelectric focussing
ISF	Interstitial fluid

JAMs	Junctional adhesion molecules
MALDI	Matrix assisted laser desorption ionization
min	minutes
MS	Mass spectrometry
PBCECs	Porcine brain capillary endothelial cells
PBMEC	Porcine brain microvessel endothelial cells
pI	Isoelectric point
PMF	Peptide mass fingerprint
RMT	Receptor-mediated transcytosis
RT	room temperature
SDS	Sodium dodecyl sulfate polyacrylamide gel
sec	seconds
SLC	Solute carriers
TEER	Transendothelial electrical resistance
TJs	Tight junctions
TOF	Time of flight
ZO	Zonula Occludens Protein

1 Introduction

The investigation of this **pilot study** based on the modern system pharmacological research field about dysfunction analysis of the blood-brain barrier (BBB). The main topics of the BBB research group at the Department of Medicinal/Pharmaceutical Chemistry from University Vienna, head Univ. Prof. Dr. Mag. DI Christian R. Noe are the pharmaceutical research on the biological system BBB. Three major topics are involved in the research of drug discovery on BBB models:

- Transport studies
- Drug impact studies
- BBB as drug target

This work is part of the BBB drug target research based on modern **system pharmacology** approaches Using human primary cells (HUVEC) and immortalized porcine brain cells (PBMEC/C1-2) for different BBB models to identify via mass spectrometric methods **molecules of interests**. Those molecules should be starting points for further pilot studies in **BBB dysfunction research**.

The BBB is a system for it selves with a variety of important function as:

- regulatory element
- gate keeper to the central nervous system (CNS)
- transport barrier for nutrition

Many dysfunctions especially due to aging processes lead to neurodegeneration and pathological circumstances in the brain. In this study the overall purpose is to understand further mechanism of the BBB as pharmacological treatment element. The next parts show an overview of the physiology and morphology of the BBB, their transport pathways and the pathology.

1.1 What is the Blood- Brain Barrier?

1.1.1 Historical approach

The existence of the blood-brain barrier has been recognized for more than 100 years and was first experimentally demonstrated by the German scientist Paul Ehrlich. He conducted experiments of dye injections administered into the circulation system. Ehrlich observed that certain dyes were rapidly taken up into the body with the exception of the spinal cord and brain (Ehrlich 1904). First he thought that these differences could be interpreted as a lack of affinity for these dyes and the cerebral vascular endothelium within the nervous system. Edwin E. Goldman, an assistant of Ehrlich, demonstrated that the same dyes injected into the cerebrospinal fluid readily, stained solely nervous tissue (Engelhardt and Sorkin 2009). With these findings the understanding of a barrier inside the brain began (Bradbury 1979) and was firstly named “blood-brain barrier” by Lewandowsky in the year 1890.

The importance of the blood-brain barrier was shown in many further experiments revealing the signaling functions between glia cells and endothelium responsible for the regulation capacity of the BBB. Ultrastructure studies pointed out that BBB endothelial cells (ECs) differ fundamentally from most peripheral tissues. They have very few endocytotic vesicles which cause a limited amount of transcellular flux (Rubin and Staddon 1999). Specialized features and properties of the BBB are induced by cell-cell interactions. The BBB is definitely a dynamic system with complex pathways of modulation and transport alterable due to outer circumstances.

The blood-brain barrier (BBB) is part of the brain and has an important protective function for the central nervous system (CNS). The BBB acts as physical and metabolic barrier and is formed by endothelial cells (lining cerebral microvessels). It separates the systemic blood circulation from the CNS. The CNS is a highly sensitive system in the body and has to be protected from toxic and pathogenic agents found in the blood, which are capable of disturbing neuronal functions. Overall, there are three barriers described inside the brain with the main function of regulating the molecular exchange at the interfaces between blood and neural tissue (Abbott 2006). The BBB is formed by the cerebrovascular endothelial cells, the choroid plexus epithelium between blood and ventricular CSF and the arachnoid epithelium between

blood and subarachnoid CSF. Another important function of the brain barriers is the aspect that they are involved in the control mechanisms of cerebral homeostasis. This kind of regulation of the brain microenvironment is necessary for healthy functions of the CNS. The position of the three brain barriers in the CNS are shown in figure 1.

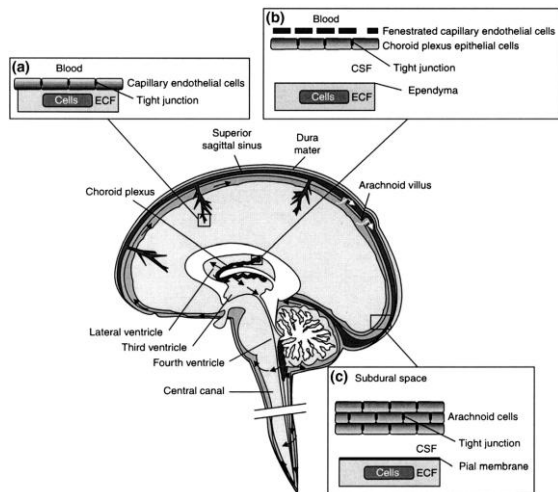


Figure 1: Scheme of the barriers in the brain (Picture is adapted from Abbott et al 2010)

The localizations of the three brain barriers are represented. a) The BBB, the largest surface area for exchange with a size of 12 to 18 m² in adult humans, b) the blood-CSF barrier at the choroid plexus in the lateral third and fourth ventricles of the brain and c) the arachnoid barrier, which lies under the dura, enveloped by the arachnoid membrane (Abbott et al. 2010)

1.2 Morphology and Physiology of the BBB

The blood-brain barrier acts as a physical barrier with tight junctions between endothelial cells to enable molecular transport. Small gaseous molecules as well as small lipophilic agents can diffuse freely through the lipid membrane (Abbott et al. 2006). In the region of the luminal and abluminal membranes of the BBB there are specific transport systems that regulate ionic traffic permitting entry of required nutrients or efflux of potential harmful substances. Tight junctions limit the crossover of large molecules and hydrophilic drugs into the brain and protect them from an influx of toxic compositions. Basically the BBB is located in all brain regions. Exceptions in this context are the circumventricular organs including area postrema, median eminence, neurohypophysis, pineal gland, subfornical organ and lamina terminalis. The blood vessels of these brain areas are supplied with blood-borne

molecules which pass the vessel wall through diffusion. These unprotected fields of the brain are separately regulated by the autonomic nervous system and endocrine glands (Ballabh et al. 2003).

Hence, the BBB provides the brain with essential nutrients and mediates efflux of many waste products. It restricts ionic and fluid movements between the blood and the brain by ion transport systems and contributes to the production of brain interstitial fluid (ISF) which guarantees neuronal function in an optimal medium.

Specific characteristics of the BBB endothelium, such as tight junctions, astrocytes and neurons, enable the correct function of CNS processes. The BBB is formed by capillary endothelial cells, enveloped by basal lamina and astrocytic perivascular endfeet. The connection between basal lamina and neurons is provided by astrocytes. Pericytes are distributed inside the basal lamina and microglia are part of the surroundings of the astrocytic endfeet. All these components [Figure 2] form the “neurovascular unit” that has an enormous influence on health and proper function of the CNS (Engelhardt and Sorokin 2009).

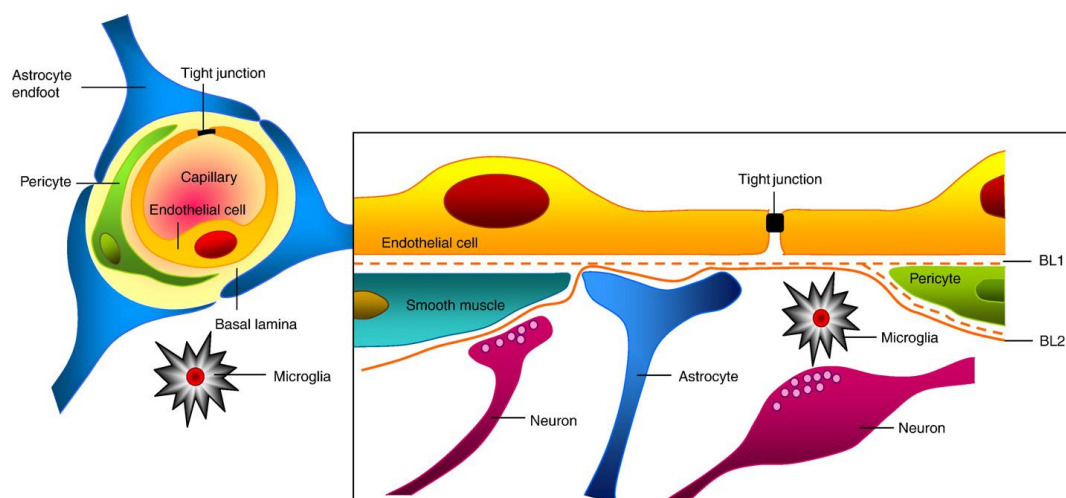


Figure 2: Scheme of the “neurovascular unit” (Picture is adapted from Abbott et al. 2010)

The main components and the structure of the blood brain barrier are shown. Adjacent capillary endothelial cells form tight junctions, surrounded by basal lamina, pericytes and astrocytic endfeet. Astrocytes make the link to the neurons. (Abbott et al. 2009).

1.2.1 The components of the BBB

Main task of the BBB is the maintenance of the homeostasis in the central nervous system (CNS). The main components of the BBB are the endothelial cells which line the lumen of the blood capillaries in the brain. The properties of the endothelial cells

of the BBB are determined by their environment. Signaling from neighbored pericytes, astrocytes, neurons and probably shear stress influence the functionality of the BBB.

Endothelial cells

BBB endothelial cells harbor tight junctions within their cell-cell contacts. Tight junctions are typical hallmarks of the BBB. The junction complex consists of adherens junctions (AJs) and tight junctions (TJs). They regulate the paracellular flux pathway and effectively block penetrations of macromolecules and other harmful substances. In contrast, small molecules like O₂ and CO₂ can enter through diffusion across the membrane along their concentration gradient. Lots of junction molecules are involved in the regulation of the permeability process in endothelial cells [see also chapter 1.2.2 The tight junction complex].

The electrical resistance of measured transendothelial cells was about 1500-2000 Ωcm^2 . That seems rather high compared to other endothelial cells as human placenta cells with the resistance of 22-52 Ωcm^2 (Huber et al 2001, Abbott et al. 2003). The effect of that strong resistance is the impediment of ionic movement, the protection of the brain and the maintenance of homeostasis.

Basal lamina

The basal lamina forms a layer around cerebral endothelial cells. Inside the basal lamina 1 [Figure 2] pericytes and smooth muscle cells are enclosed. The basal lamina 2 forms an extracellular matrix providing contact among cerebral endothelial and surrounding cells.

Astrocytes

Astrocytes supply stimulating factors to the basal surface of brain capillary ECs. These factors are important for complete BBB formation, so astrocytes might induce ECs to adopt a brain phenotype (Rubin and Staddon 1999, Grant et al 1998). Many experiments showed that endfeet of astrocytes are close to the capillary endothelium releasing chemical signals for induction and maintenance of the BBB.

Neurons and Microglia

Axonal projections from neurons onto arteriolar smooth muscles contain vasoactive neurotransmitters and peptides which regulate local cerebral blood (Abbott et al. 2010). Microglia are resident macrophages of the brain and spinal cord and act in the active immune system as key cellular mediators in inflammatory processes.

1.2.2 The tight junction complex

Endothelial cells are joined by the junctional complex area which is a substantial junctional cleft. The junctional complex is built up by tight junctions (TJs) and adherens junctions (AJs). Both form a continuous network of parallel, interconnected, intramembrane strands of proteins representing a range of multiple barriers (Huber et al. 2001). The graphics below [Figure 3] demonstrates the structure of the tight junction complex.

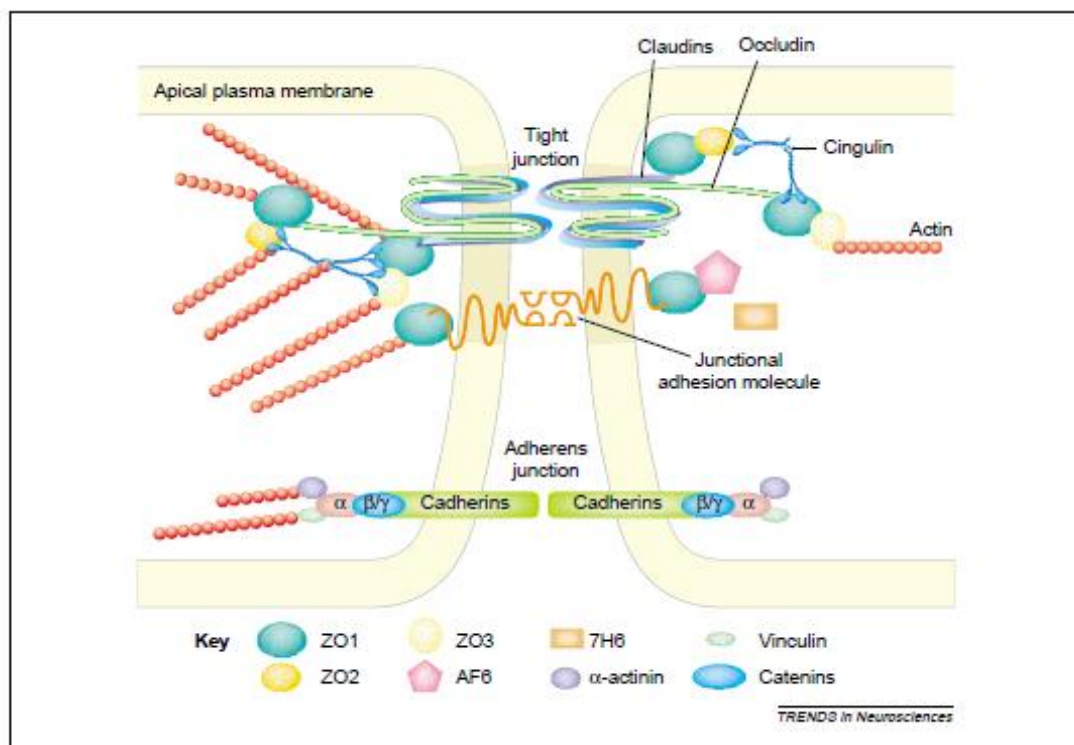


Figure 3: The tight junction complex with its components is shown in this graphic. (Picture is adapted from Huber et al. 2001) The two main structures are the adherens junctions (AJs) and the tight junctions (TJs). Other important regulation elements are junctional proteins with their multiple functions of development and maintenance of the BBB.

Adherens junctions (AJs) play an important role in development and formation of tight junctions. The contact between adjacent cells is dependent on membrane proteins named **cadherins**. Cadherins are single-pass transmembrane glycoproteins

that interact homotypically in the presence of Ca^{2+} (Rubin and Staddon 1999). Cadherins associate with other proteins termed **catenins**, which consist of three different types (α , β and γ Catenin). Catenin β and γ are combined and connected with α catenin that links them to the actin-based cytoskeleton. Via this pathway signaling components may be activated to allow communication between adherens and tight junctions. Within the basolateral membrane endothelial cells are joined by tight junction proteins, namely claudins and junctional adhesion molecules. These proteins form the backbone of TJs (Huber et al. 2001). They are linked to an actin-based cytoskeleton allowing TJs the capacity of rapid modulation and regulation.

1.2.3 Tight junction molecules

Occludin

Occludin was the first tight junction transmembrane protein which was discovered in 1993. It is a 65 kDa integral membrane phosphoprotein with four transmembrane domains that exist exclusively in tight junction strands. The real function of occludin is more complex than previously expected. However, some experiments in occludin-deficient mice showed that tight junctions were morphologically not affected and transepithelial resistance in small and large intestine epithelial cells did not differ from measurements in wild type mice. Abnormalities, such as chronic inflammation and hyperplasia of the gastric epithelium, calcification in the brain, testicular atrophy, loss of cytoplasmic granules in striated duct cells of the salivary gland, and thinning of the compact bone, were found in several tissues (Saitou et al. 2000). These results are indices for the complex function of tight junctions (TJs). Phosphorylated occludin proved to have only low influence on TJ regulation (Wolburg and Lippoldt 2002), nevertheless extensive phosphorylation of occludin occurs when the cytoplasmic domain is localized within the tight junction. In MDCK cells occludin exists in many phosphorylation states. It was shown that especially serin phosphorylation is responsible for the stabilization of membrane associated occludin (Kniesel and Wolburg 1998). It was observed that occludin is not required for the formation of tight junction strands but that it is responsible for the sealing of tight junctions (Sakakibara et al. 1997; Lacaz-Vieira et al. 1999). Furthermore, occludin was shown to be necessary for mature cells to gain the right properties for barrier function. Additionally, the presence of the regulatory protein in the BBB is correlated with

increased electrical resistance across the barrier and decreased paracellular permeability (Huber et al. 2001).

Claudin

Claudins are 22 kDa phosphoproteins which form dimers that bind homo directly to claudine of adjacent endothelial cells. Claudins belong to the multigene PMP22/EMP/MP20 claudin family of proteins that comprise many isoforms, of which at least 24 have been identified in mammals (Huber et al. 2001). Claudin-1 and Claudin-2 are integral components of the tight junction strands. They are one of the major elements in the TJ complex and build up a primary seal of tight junction proteins (Furuse et al. 1999). The carboxy terminal of claudins binds to other proteins inside the cytoplasm like cytoplasmic accessory proteins (ZO1, ZO2). Claudin-1 and Claudin-5, together with Occludin, have an important influence in the establishment of barrier properties. Another well known member inside the claudin family is the oligodendrocyte protein (OSP), also called Claudin-11. It is a major part of the CNS myelin sheat and of Sertoli cells of the blood-testis barrier (Ballabh P et al. 2003). Functional investigations showed that claudins directly determine barrier functions. Tight junction-negative L-fibroblasts were transfected with claudins and formed tight junctions associated with P-face and E-face. They form very long and branched strands. In contrast, occludin induces short strands and was found to be localized at both fracture faces (Hirase et al. 1997; Furuse et al. 2001).

Junctional adhesion molecules (JAMs)

In general junctional adhesion molecules (JAMs) are compounds of the tight junction complex that may regulate the transendothelial migration of leukocytes, and are involved in the maintenance of the TJ, but their whole function in the BBB is still unclear (Abbott et al. 2010). The **JAM** family consists of three different types of molecules, called JAM-1, JAM-2 and JAM-3 all of them belonging to the immunoglobulin superfamiliy. Mediations of these molecules are performed by hemophilic and also heterophilic interactions. JAM-1 is a 40 kDa transmembrane protein with a large extracellular domain. This member of the IgG superfamiliy mediates the early attachment of adjacent endothelial cells through hemophilic interaction. Via a PDZ binding motif at the carboxy terminus JAM-1 binds to ZO-1, AF-6/afadin, cingulin and Cas kinase/lin-2 (Martinez-Estrada et al. 2001).

Experiments showed that JAM-1 increases the cellular resistance in cells which normally does not form tight junctions. Another effect is the reduction of paracellular permeability. Therefore JAM-1 also has an impact on the localization of occludin. It seems that the molecule has an organizing function to build up the right junctional structure (Wolburg and Lippoldt 2002). JAM-2 and JAM-3 are present in endothelial tissue and lymphatic cells with the exception of epithelial cells (Hawkins and Davis 2005).

Membrane-Associated Guanylate Kinase-Like Proteins (MAGUK)

Membrane-Associated Guanylate Kinase-Like Proteins (MAGUK) are accessory proteins in the cytoplasm of BBB endothelial cells. Three different types of MAGUK proteins have been identified in the tight junction complex, named zonula occludens (ZO-1, ZO-2 and ZO-3). Zonula occludens (**ZO**) are adapter proteins that bind to intramembrane proteins. ZO-1 is a 220 kDa phosphoprotein expressed not only in endothelial cells but also in cells without TJs. The main action of ZO-1 is the linkage of transmembrane proteins at TJs to the actin based cytoskeleton via ZO-2 or ZO-3. This interaction is important for proper structure conditions within the cytoplasm and for an efficient function of TJs. Absence of ZO-1 at the junctional complex has increased permeability effects on the BBB. This accessory protein acts as response molecule in intracellular signal transduction (Hawkins and Davis 2005). ZO-2 is a 160 kDa phosphoprotein that shows a high sequence homology to ZO-1. Normally ZO-2 is located along the membrane. However, during stress and proliferation the adaptor protein is turned into the nucleus. Interestingly, ZO-2 has also been found in cells without tight junctions. The third adaptor protein, ZO-3, has a molecular weight of 130 kDa and is solely found in endothelial tight junctions, where it regulates the interaction of cytoplasm components with the actin-based cytoskeleton.

Other Accessory Proteins

Other accessory proteins are elements of the endothelial cytoplasm within the tight junctional complex. Among these proteins there are **AF6** and **7H6** which both interact with junctional adhesion molecules. **Cingulin** is connected with ZOs, JAMS and myosin for passing on the signals from TJs to the cytoskeleton and vice versa. Inside the adherens junctions **α -actinin** and **vinculin** interact directly with the actin-based cytoskeleton and transfer the information from the adherens junction by Catenins.

1.3 Transport systems across the BBB

For the maintenance of homeostasis in the CNS, the blood-brain barrier selectively transports nutrients into the brain via multiple transport mechanisms (Huber et al 2001). Several pathways across the blood-brain barrier are shown below in figure 4.

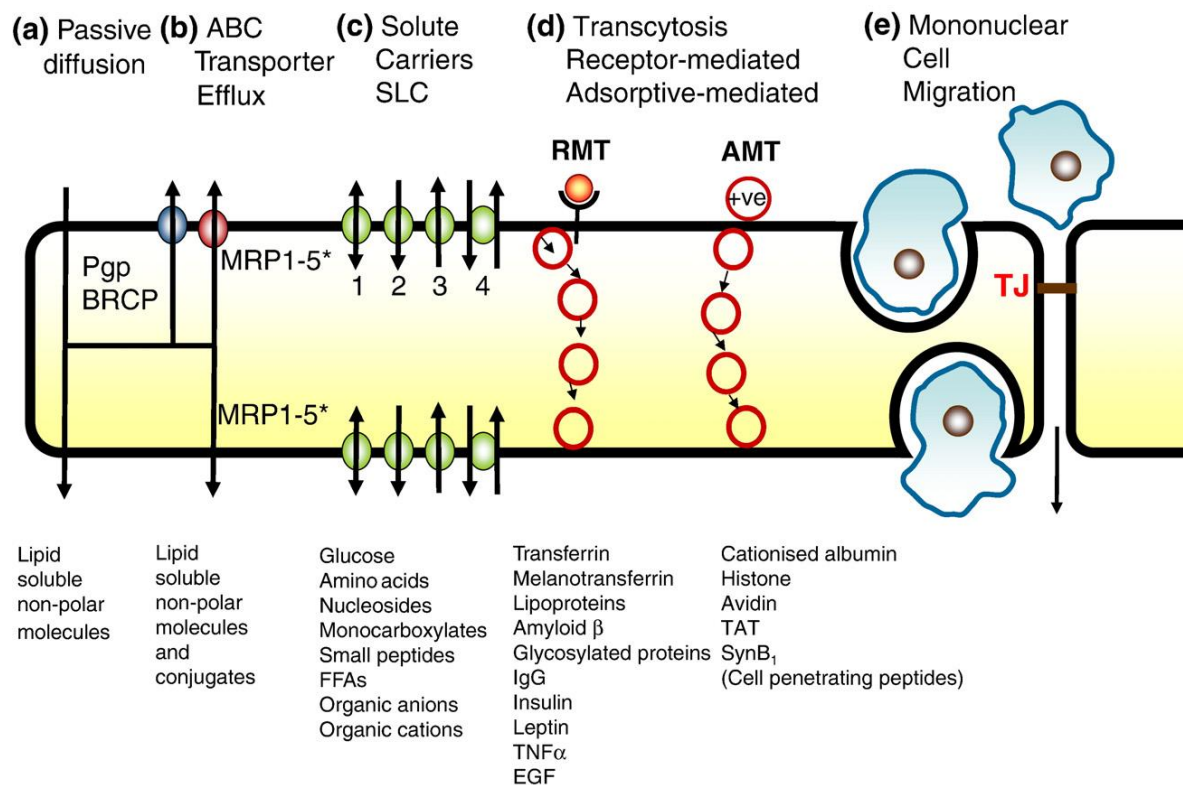


Figure 4: Scheme of the transport pathways through the BBB (Picture is adapted from Abbott et al. 2010). **a)** Passive diffusion: Solutes pass the membrane by passively diffusing into the cell. **b)** ABC Transporter Efflux: The ATP-binding cassette transporters (ABC transporters) in the BBB actively pump neurotoxic substances out of the brain against a concentration gradient. For this process ATP is required. **c)** Solute Carriers (SLC): The SLC transport substances act on a bi-directional way in or out of the brain. The transport direction depends on the substrate concentration gradient. **d)** RMT and AMT: Receptor-mediated transcytosis (RMT) needs a receptor on the surface of the cell that recognizes the ligand and subsequently transports the molecule inside the vessel through the membrane. (Adsorptive-mediated transcytosis) AMT: Instead of the receptor the positively charged macromolecule triggers the start of endocytotic mechanisms. **e)** Immune respondent cells are able to penetrate the membrane under pathological or pharmacologically induced conditions.

The transport pathways through endothelial tight junctions have been divided in five subtypes. (a) **Passive diffusion** directly through the membrane (Fig. 1.3a) represents the transport of soluble, lipid and non-polar molecules, such as water, oxygen and carbon dioxide. The endothelium contains several proteins with carrier functions for the transport of nutrients such as glucose, amino acids and other

substances into the brain and specific efflux carriers which eliminate toxic substances from the brain.

(b) **ABC transporters** are active efflux carriers that pump molecules either directly out of the cell or on their way to cross the membrane. Members of the ABC transporter system in BBB are the *Multidrug Resistance Proteins* (MDRs), the *Multidrug Resistance-related Proteins* (MRPs) and *Breast Cancer Resistance Proteins* (BRCP). The first identified MDR protein was **P-glycoprotein** (p-gp, MDR1) an ATP dependent efflux carrier that prevents the traverse of xenobiotics and drugs (e.g. lipid glucocorticoid, cholesterol, peptides and drugs) into the brain. The major role of ABC transporters is the active efflux of various lipid-soluble compounds out of the brain. In this process the compounds are pumped out of the brain against a concentration gradient, for which the required energy is delivered by ATP (Carvey et al. 2009, Abbott et al. 2010).

There are specific requirements for a substance that determine the way of transport through the endothelial membrane such as structural features, lipid solubility and electrical charge. Efflux systems play an important role in establishing and maintaining the homeostatic level of the CNS. An effective efflux can inhibit a drug to become potentially neurotoxic and/or to develop a therapeutic effect inside the CNS (Banks 1999).

(c) **SLCs** (solute carriers) enable the entrance of polar nutrients that are important for the CNS metabolism. These proteins are located at the apical and basal membrane side. SLC proteins transport substances on a bi-directional way in or out of the brain. The transport direction depends on the substrate concentration gradient. There are four different SLC pathways: (1) unidirectional, meaning either into or out of the cell, (2/3) substrate exchange, one substrate gets in the brain and another is pumped out in a parallel manner, and (4) reversible transport which depends on an electrochemical gradient (Abbott. et al 2010).

(d) **RMT** and **AMT** (Transcytosis Receptor-Mediated and Adsorptive-Mediated Transport). RMT is an important way how large molecules can be moved into the cell. The molecule binds to a receptor at the luminal side of the endothelium initiating the process of transcytosis which means that the ligand is transported across the membrane inside the vesicle. Examples of these receptors are the transferrin

receptor, the insulin receptor or the LDL receptor (Carvey et al. 2009). **AMT** is not receptor-mediated and specific for positively charged macromolecules, which can be transported across the membrane through endocytotic mechanisms.

(e) **Mononuclear Cell Migration** is a method where immune cells are able to pass the membrane under pathological or pharmacologically induced conditions. Leukocytes, monocytes and macrophages are attached to the area of inflammation. They penetrate the blood-brain barrier near the tight junction region and form cuffs to get inside the perivascular space (Davoust et al. 2008, Abbott et al. 2010).

1.4 Pathology in BBB

Under healthy conditions the blood-brain barrier is highly permeable and acts as defense wall for toxic substances. Unfortunately, the permeability changes extremely under pathological conditions. Some chemicals mediate an increase of permeability that leads to an entrance of unwanted molecules into the brain. The wide range of barrier dysfunctions can range from mild or transient tight junction openings to chronic barrier breakdown which induce abnormalities in the molecular transport system cross the membrane (Abbott et al. 2010). Under hypertension, inflammatory mediated exposure of cytokines or leukocytes and ischemia conditions the BBB is obviously damaged in its structure (Moody 2006). The CNS is normally protected by their surrounding cells namely astrocytes, pericytes and microglia. Interestingly, under pathological circumstances these cells are able to enhance inflammation and trigger cellular damage. This process is mediated by modified regulation of neurotoxins and neurotrophins (Ballabh et al 2003). In cases of a brain tumor the complete blood-brain function is disturbed. The down regulation of claudin-1 leads to the hypothesis that the molecule acts as an early marker in barrier dysfunctions (Liebner et al 2000). Also diseases such as multiple sclerosis (MS) or stroke are related to barrier break down and down regulation of claudin-1 and claudin-3. Shear stress has an influence on the expression of junctional compounds in endothelial cells especially in hypertension induced stroke (Wolburg and Lippoldt 2002). Changes in alternated nutrition transport through the membrane are caused by disruption of the blood-brain barrier. Often these abnormalities occur by changes in the metabolic system of the CNS (Banks 1999). In many times increased BBB permeability is an indication of pathology for example brain injury or stroke (Hawkins and Davis 2005). Neuro pathological studies showed that blood-brain barrier

endothelial cells are protected from hypoxic stress through their surrounding cells like astrocytes and pericytes (Fischer et al 2002, Hayashi et al 2004). During drug abuse symptoms of dementia are amplified. Also the consumption of nicotine modifies the transport of ions which leads to an increase of the BBB permeability (Hawkins and Davis 2005). These events underline the great importance of studies with the aim to get more information about the connection between tight junction pattern and signaling processes in healthy versus disease circumstances (Wolburg and Lippoldt 2002)

1.5 Cell culture models for *in vitro* BBB experiments

Lots of different cultivation models are presently available. The most common is the Transwell system in which mono- or co-culture *in vitro* experiments can be realized. In the early 90th Jochen Neuhaus and colleagues found out that astrocytes are mediators for the induction of BBB characteristics in brain endothelial cells. These observations could be shown by *in vitro* test models where bovine brain endothelial cells were co-cultured with astrocytes from rats. The cells were seeded in transfilter culture systems on both sides of the membrane but without direct contact between astroglial cells and bovine cells. In presence of astrocytes the permeability change of tight junctions were shown by a reduced sucrose penetration (Neuhaus et al. 1991). Another publication of Fred Arthur in the year 1987 exemplifies the effect of astrocyte conditioned media (ACM) on rat brain cells. The conclusion of this study was that ACM also induce tight junction formation in brain endothelial cells (Arthur et al. 1987). Astrocytes induce factors which influence the building up of tight junctions and their permeability. Both methods (ACM and co-culture) show efficient results to induce TJ formation in brain endothelial cells. Astrocytes are able to induce tight junction properties in endothelial cells, also if they are not of nervous system origin. Additionally, it is suggested that astroglial factors have to be highly conserved because their mechanism of action is not based on the same species from where the astrocytes were taken from (Janzer and Raff 1987).

1.6 Proteomic approach

Proteomics describes techniques and analyses of the whole proteome, which includes the entirety of proteins of one organism. There are several methods for proteome analyses. The most common fields for proteome research are the analysis of protein expression pattern, protein sequencing, protein identification and protein

function assays. In this study the influence of different culture conditions on blood-brain barrier cells was investigated using a classical proteome approach, including 2-DE, tryptic *in-gel* digestion and subsequent MALDI-TOF MS. Further details about the used techniques are described in chapter [Materials and Methods].

1.6.1 What is the application of proteomics?

The genetic code of an organism stays stable during its whole lifetime. Not all of the information present in the cellular genome will be expressed on the protein level. On the other hand the proteome pattern gives a more detailed insight in intracellular functions and mechanisms since the proteome is dynamic and underlies lots of structural changes. One gene can be transcribed into many proteins. Proteins exist in many forms and their functions are characterized by their modifications, which often define localization, structure and turnover number of proteins. By adapting the proteomic pattern an organism tries to cope with changing environmental conditions, which can be effects of biodiversity. Using mass spectrometry (MS) biomolecules, especially proteins and peptides can be detected. MS provides mass measurements of proteins up to 100 kDa and more. Furthermore, it is capable of identifying peptide masses after proteolytic digestion with high sensitivity. The sequence analyses of peptides, especially of species with less MS data availability, are of great importance. The results from mass measurements can be directly compared to sequence databases by the use of search engines to identify and characterize the proteins of interest. (Liebler 2002)

2 Aim of the work

The **dysfunction of the BBB** has a major role in many diseases which is a hallmark for medical treatment. The properties of brain endothelial cells are strongly dependent on their surrounding cells which induce multiple factors, such as growth factors. These molecules have influence in the proper building of tight junctions which in turn are potential regulators of the brain permeability. Many BBB *in vitro* models exist and are more or less suitable to answer specific questions regarding brain pathology. Porcine brain endothelial cells (PBEC) are experimentally well established as *in vitro* models in human drug discovery research and are routinely used in clinical research. Nevertheless limited sequence data are available in protein databases about porcine endothelial cells.

Main points of this **pilot study** are:

- The cultivation of **human primary cells** (HUVEC) and **immortalize porcine cells** (PBMEC/C1-2) on Transwell filter systems
- The identification of statistical results of changes in the proteome pattern by different cultivation conditions
- The analysis of **molecules of interests** via 2D-DIGE
- The detection of the significantly changed proteins via MALDI TOF MS
- The indication of the biological function of the identified proteins

The overall purpose of this project is the investigation of three different cell models (PBEC, HUVEC) in terms of how they are differently influenced by cultivation conditions using a proteomic approach. The main intension of this thesis is the **observation of changes in the proteome pattern due to the following cultivation conditions (a) “uninduced system” (cultivation in respective growth media corresponding to the control), (b) “co-culture” (using a special experimental setup) and (c) “induced system” (culture in respective media including astroglial factors)**. Another aspect is the comparison of two different endothelial cell lines either of human or of porcine origin in terms of their suitability for proteomic investigations. In other words:

- Will it be possible to identify changes in the proteome pattern due to different cultivation conditions?
- Are porcine BBB endothelial cells (PBEC) comparable with human endothelial cells (HUVEC) for the investigation of specific brain issues using proteome analysis?

Obtained experimental results will be starting points for further detailed analyses to identify specific BBB target proteins.

3 Materials and Methods

Materials

3.1 Chemicals

Amphotericin B, A2942 Sigma-Aldrich, Inc. (St. Louis, MO, USA)

Bicarbonate, sterile, 7%, 25080, Invitrogen Life technologies (Gibco TM, Carlsbad, CA, USA)

Bromophenol blue, Na salt, 15375, Serva Electrophoresis GmbH (Heidelberg, Germany)

Bovine serum albumine BSA, A-8806, Sigma- Aldrich, Inc. (St. Louis, MO, USA)

Calcium chloride solution, 1 M 21114, Sigma-Aldrich, Inc (St. Louis, MO, USA)

Collagen bovine, 150703, MP Biomedicals (Irvine, CA, USA)

Coomassie Brilliant Blue G250 pure Serva Electrophoresis GmbH (Heidelberg, Germany)

Dextran av. mol wt. 15.000-30.000, D 4626, Sigma-Aldrich, Inc. (St. Louis, MO, USA)

DMEM (41966, plus 4.5 g/L glucose, L-glutamine and pyruvate; Gibco TM, Life Technologies, Carlsbad, CA, USA)

DMSO, 1.02952.1000, MerckKGaA (Darmstadt, Germany)

DTT research grade, 20710, Serva Electrophoresis GmbH (Heidelberg, Germany)

Earle´s salt solution L1915 Biochrom (Berlin, Germany)

EBM, CC-3156, Lonza Walkersville, Inc. (Walkersville, MD, USA)

EDTA disodium, 11280, Serva Electrophoresis GmbH (Heidelberg, Germany)

Ethanol 70%

FCS, sterile, 10108-157, Invitrogen Life technologies (Gibco TM, Carlsbad, CA, USA)

Fibronectin human, 356008, BD Biosciences (Bedford, MA, USA)

Gelatine from bovine skin, Type B, G9391, Sigma-Aldrich, Inc. (St.Louis, MO, USA)

Glycerol, 23176, Serva Electrophoresis GmbH (Heidelberg, Germany)

Glycine analytical grade 1kg, 23390, Serva Electrophoresis GmbH (Heidelberg, Germany)

Ham's F12, sterile, 21765-029, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

HBSS, 10x, sterile filtered, H4641, Sigma-Aldrich, Inc. (St. Louis, MO, USA)

Heparin sodium salt from porcine intestinal mucosa, 51551, Fluka (Buchs, Switzerland)

Hepes, 1 M, sterile, 15630-049, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

IMDM, sterile, 21980-032, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

Kanamycin (10 mg/ml) 15160-047, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

L-Glutamine, 200 mM, sterile, 10x, 25030-024, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

M199, sterile, 31153-026, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

M199 sterile F0615 Biochrom (Berlin, Germany)

Magnesium chloride solution, 1M, M1028 Sigma-Aldrich, Inc. (St. Louis, MO, USA)

NCS, sterile, 26010-74, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

PBS, sterile, 14190-094, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

Penicillin/Streptomycin, 100x, sterile, 15140-122, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

Protease inhibitor cocktail, P8340, Sigma-Aldrich, Inc. (St. Louis, MO, USA)

Puromycin A11138-03, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

TEMED research grade, 35925, Serva Electrophoresis GmbH (Heidelberg, Germany)

Transferrin holo, for cell culture, T-0665, Sigma-Aldrich, Inc. (St. Louis, MO, USA)

TRIS research grade, 37190, Serva Electrophoresis GmbH (Heidelberg, Germany)

Triton X- 100 pure, 37240, Serva Electrophoresis GmbH (Heidelberg, Germany)

Trypsin/EDTA solution, 10x, sterile, 15400-054, Invitrogen Life technologies (Gibco™, Carlsbad, CA, USA)

Tween 20, 37470, Serva Electrophoresis GmbH (Heidelberg, Germany)

3.2 Cell culture materials

6- well Tissue Culture Plates, 35046, BD Falcon (Franklin Lakes, NJ, USA)

Cell Cultureware

Product Number: 353046

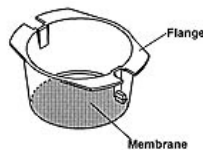


353046 - BD Falcon™ 6-well Multiwell Plate. Tissue-culture treated polystyrene, flat bottom, with low-evaporation lid. (50/ca)

Cell culture inserts for 6-well plates, 353102, BD Falcon (Franklin Lakes, NJ, USA)

Cell Cultureware

Product Number: 353102



353102 - BD Falcon™ Cell culture inserts for 6-well plates. 1.0 µm pores, Transparent PET Membrane. (6/sp, 48/ca)

Autoclave bags, 75x15 and 21x42, Schinko-Neuroth (Wolfsberg, Austria)

Autoclave band, 84-200-048-CC, steam A, Schinko-Neuroth (Wolfsberg, Austria)

Biofine tubing, ID 5mm, wall thickness 1 mm, Fresenius Medical Care (St. Wendel, Germany)

Biofuge tubes, 15 ml, Cellstar 188271, Greiner Bio-One GmbH (Frickenhausen, Germany)

Biofuge tubes, 50 ml, Cellstar 227261, Greiner Bio-One GmbH (Frickenhausen, Germany)

Cell scraper, sterile, 541080, Greiner Bio-One GmbH (Frickenhausen, Germany)

Eppendorf vials, Eppendorf AG (Hamburg, Germany)

Eppendorf Research pipettes, Eppendorf AG (Hamburg, Germany)

Pipetman Pipette, 100 µl-1000 µl, 20 µl-200 µl, 1 µl-10 µl, 2 µl-20 µl, 0,2 µl-2 µl, Gilson (Middleton, USA)

Sterile filter flasks – Filtration System/Vacuum Driven GP Millipore Express (Billerica, MA, USA)

Sterile pipette, Volume, 25 ml, 10 ml, 5 ml, 2 ml, Sarstedt Serological Pipette

Tissue Culture Fasks, surface area 25 cm², 75 cm², 175 cm², 690160, 658170, 660160, Greiner Bio-One GmbH (Frickenhausen, Germany)

Thoma, counting chamber, Superior Marienfeld (Lauda-Königshofen, Germany)

3.3 Proteome materials

2-D Difference Gel Electrophoresis (2-D DIGE)

DIGE Scanner

Scanner Ettan DiGe Imager, GE Healthcare (Amersham, UK)

Electrophoresis

Ettan IPGphor 3, GE Healthcare (Amersham, UK)

Ettan Dalt six, GE Healthcare (Amersham, UK)

IPG strips, pl 3-11 non linear, GE Healthcare (Amersham,UK)

IpGphor cup loading strip holders

Labelling Kit, CyDye Dige, 25-8010-65, GE Healthcare (Amersham,UK)

Software

DeCycler 2D 7.0

Two Dimensional Gel Electrophoresis (2-DE)

BioRad - Protean II si Cell (Hercules, CA, USA)

Ettan IPGphor 3, GE Healthcare (Amersham, UK)

IPG strips, pl 3-11 non linear, GE Healthcare (Amersham,UK)

MALDI TOF MS

MALDI TOF/RTOF (AXIMA ToF²TM and CFRTM) (Shimadzu Biotech Kratos Analytical, Manchester, UK)

Methods

3.4 Cell culture

3.5 Experimental cell lines

Human umbilical vein cells (HUVEC)

HUVEC are primary cells isolated from umbilical cords of newborns. The morphology of these cells is shown in figure 5. These cells were a kind gift from Dr. Renate Hofer-Warbinek, Medical University Vienna. During the studies the cells were obtained directly after isolation. To enable the same conditions for the whole test, two cell doublings were conducted until the cells seeded on Transwell culture system with passage number 3.

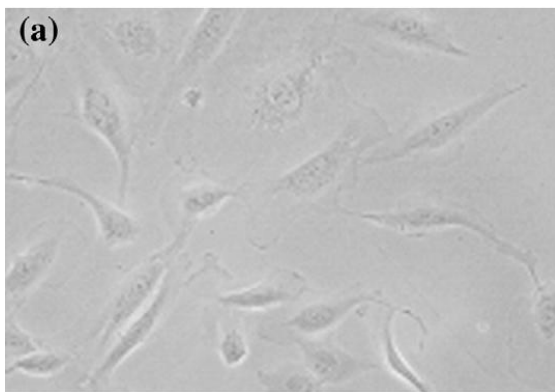


Figure 5: Characteristic cell structure of (untreated) human umbilical vein cells with spread cell branches. The cell body is clearly visible. The picture is adapted from Yang Jin Park et al. 2008. (In this graph (a) shows the control, untreated HUVEC).

Porcine brain microvascular endothelial cells (PBMEC/C1-2)

PBMEC/C1-2 are immortalized brain endothelial cells. Teifel and Friedel (1996) established and characterized these cells. *The experimentally used PBMEC/C1-2 cells to conduct this study were a kind gift of them.* Many specific endothelial cell markers have been described, such as van Willebrand factor (vWF), lectin binding receptors for UEA-1, acetylated LDL as well as typical BBB properties. The picture [figure 6] shows the morphology of porcine brain endothelial cells with their stretched branches. Passage numbers up to more than 190 population doublings can be used without losing significant characteristics.. Immortalized PBMEC cells are well

appropriate for many cell doublings without losing specific characteristic properties up to passage 198 (Suda et al 2001). The highest passage number used in these studies was 79 including 35 cell doublings.

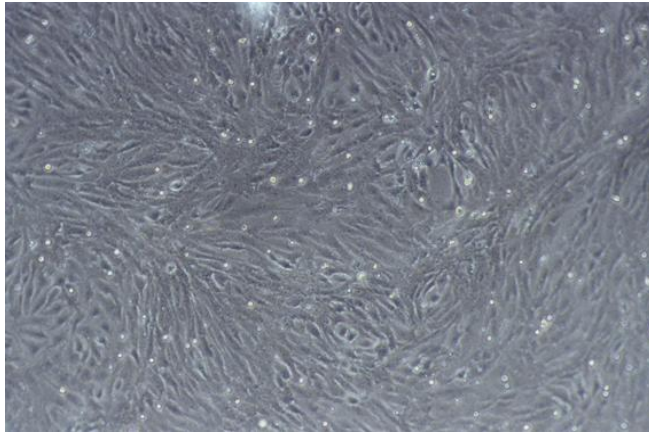


Figure 6: Picture of a confluent cell layer with PBMEC/C1-2 cells. (Adapted from doctoral thesis, Winfried Neuhaus 2007)

Primary porcine brain capillary endothelial cells (PBCECs)

PBCECs are primary endothelial cells directly isolated from porcine brains. The isolation of capillary endothelial cells was done during a research stay at the University of Heidelberg.

The morphology of primary porcine brain endothelial cells is comparable to that of PBMEC/C1-2. Under confluent conditions the cells have long stretched cell extensions without branches. The cell body is microscopically well observable.

Rat glioma cells (C6)

C6 cells are brain tumor cells from abnormal proliferating brain cells. They are isolated directly out of the rat brain. It is described that in most cases C6 cells are originally cancer stem cells¹. Glioma cells were cultured to obtain astrocyte conditioned media (ACM) also named glioma conditioned media (GCM). Conditioned media was necessary for the cultivation experiments under induced conditions, where astrocytic factors inside the media have influence on the experimental cells to produce barrier properties. C6 glioma cells were received from Cancer Research

¹ <http://cancerres.aacrjournals.org/content/67/8/3691.abstract> (Accessed 16.03.2011)

Center Heidelberg. The used passage numbers of C6 rat glioma cells in these studies ranged from 21-33 cell doublings.

Astroglial cells

Astrocytes are glia cells located in the brain. They release factors which cause changes in the structure of endothelial cells. These properties are important for blood-brain barrier functions. In these test series astrocytic cells (which were a kind gift from Dr. Michael Berger, from Brain Research Center Vienna) were used for co-cultivation tests. For experiments the astrocytes were taken from rat brain. The passage number normally ranges up to approximately ten cell doublings. During the study it could be observed that the passage number goes up to eight doublings with a loss of cells. The optimal density is reached between passage 0 and 10. After passage numbers ten or higher astrocytes needed more time to get confluent in comparison to cells with lower passage number. Therefore, in this study experiments were conducted with maximal five cell doublings. Working with primary cells requires experience in cell cultivation.

The cell culture studies were conducted under aseptic conditions in the lamina air flow working bench. The following cell lines were used for cell culture experiments, except primary porcine capillary endothelial cells (PBCECs) which were gained by isolation studies during my research stay at University Heidelberg. Detail information about the main characteristics of those cells is explained in chapter 1. Introduction [1.6 Experimental cell lines].

1. *Human umbilical vein cells (HUVEC)*
2. *Porcine brain microvascular endothelial cells (PBMEC/C1-2)*
3. *Rat glioma cells (C6)*
4. *Astroglial cells*

Human umbilical cells and porcine brain microvascular cells were used for Transwell studies under every of the three different cultivation condition whereas rat glioma cells were taken to produce astroglial conditioned media (ACM). Astroglial cells were directly seeded at the bottom of each Transwell to conduct co-culture studies.

Sterile working conditions were ensured by using the laminar air flow working bench. All materials (sterile pipettes, tubes, media and solutions) were cleaned with Ethanol 70% (v/v) before use. After finishing the daily tests the bench was cleaned with Ethanol 70% (v/v) and sterilized over night with ultraviolet radiation.

Cell culture flasks had to be coated with sterile gelatine solution 1% (v/v). The volume of the coating solution is dependent on the flask size. In general for standardized tissue culture flasks T25 - 2 ml, for T 75 - 6 ml and for T175 - 10 ml of gelatine solution were taken to cover the surface area. After 25 min of incubation time in the incubator (37 °C, 5 % CO₂ and 96% humidity) the gelatine solution was removed and the experimental cells could be seeded for growth into the culture flasks.

3.5.1 Media and solutions

For cell culture the following **media** and **solutions** were taken for the experiments. All media were sterile filtered inside the lamina air flow before usage.

Trypsin/EDTA solution

(For 200ml)

20 ml	10x Trypsin/EDTA solution
20 ml	10x HBSS
154 ml	sterile PBS
2 ml	1 M HEPES
2 ml	7.5 % (v/v) bicarbonate
2 ml	10.000 Units/ml penicillin; 10 mg/ml streptomycin

Cryomedium

DMSO was added to sterile filtered NCS to reach a final concentration of 7 % (v/v).

C6 medium

C6 medium was used for cultivation of C6 cells and PBMEC/C1-2. The medium consists of a growth media mixture (ratio 1:1 of IMDM and Ham's F12) and the following substances: 7.5 % (v/v) NCS, 7 mM L-glutamine, 5 µg/ml transferrin, 0.5 U/ml heparin, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B.

(For 1000 ml)

439 ml	Ham's F12
439 ml	IMDM
35 ml	200 mM L-glutamine solution
500 µl	10 mg/ml humane holo-transferrin
500 µl	1000 U/ml heparin
75 ml	NCS
10 ml	10.000 Units/ml penicillin; 10 mg/ml streptomycin
1 ml	250 µg/ml amphotericin B

Astrocyte conditioned medium (ACM)

Astrocyte conditioned medium was gained from C6 glioma cells. The supernatant of growing cells was collected in sterile bottles (25 ml per day).

PBMEC/C1-2 cell culture media

At the beginning all PBMEC/C1-2 were cultured in **C6** medium (see above). Those cells which were used for the cultivation condition (a) "not induced system" were further cultivated in C6 medium. Condition (b) "co-culture" was conducted in C6 medium either. For experiments under condition (c) "induced system" cells were transferred to **minus FIB medium (see below)** which includes astroglial factors (ACM). In this thesis the terms minus FIB medium is used in the sense of astrocyte conditioned medium (ACM) without fibronectin.

minus FIB medium (with astrocytic factors)

Minus FIB medium consists of about 50 % C6 media and 50 % astrocyte conditioned media (ACM). Additionally, L-glutamine, holo-transferrin, heparin, penicillin, streptomycin and amphotericin B were added.

(For 500ml)

104 ml	Ham's F12
104 ml	IMDM
250 ml	ACM (astrocyte conditioned media)
17.5 ml	200 mM L-glutamine solution
250 µl	10 mg/ml humane holo-transerine
250 µl	1000 U/ml heparin
19 ml	NCS
5 ml	10.000 Units/ml penicillin; 10 mg/ml streptomycin
500 µl	250 µg/ml amphotericin B

Cell shift from standard growth media to ACM for “induced culture test systems”:

1st. day: 80 % standard growth media plus 20 % ACM

2nd.day: 60 % standard growth media plus 40 % ACM

3rd. day: 40 % standard growth media plus 60 % ACM

4th. day: 100 % ACM

HUVEC cell culture media

The following media were used for cell culture of human umbilical vein cells: Cells tested under conditions (a) “uninduced system” and (b) co-culture” have been cultured in basal endothelial medium without astrocytic factors, named **EGM**, whereas for condition (c) the “induced system” the conditioned medium **minus EGM** was used.

EGM (Endothelia growth medium)

The medium consists of a growth media mixture (ratio 1:1 of IMDM and EBM) and the following substances: 7.5 % (v/v) NCS, 7mM L-glutamine, 5 µg/ml transferrin, 0.5 U/ml heparin, 100 U/ml penicillin, 100 µg/ml streptomycin and 0,25 µg/ml amphotericin B were added.

(For 1000 ml)

439 ml	EBM
439 ml	IMDM
35 ml	200 mM L-glutamine solution
500 µl	10 mg/ml humane holo-transferrin
500 µl	1000 U/ml heparin
75 ml	NCS
10 ml	10.000 Units/ml penicillin; 10 mg/ml streptomycin
1 ml	250 µg/ml amphotericin B

Minus EGM (with astrocytic factors)

Minus FIB medium consists of about 50 % C6 media and 50 % astrocyte conditioned medium (ACM). Additionally, L-glutamine, holo-transferrin, heparin, penicillin, streptomycin and amphotericin B were added.

(For 500ml)

104 ml	EBM
104 ml	IMDM
250 ml	ACM (astrocyte conditioned media)
17, 5 ml	200 mM L-glutamine solution
250 µl	10mg/ml humane holo-transferrin
250 µl	1000 U/ml heparin
19 ml	NCS
5 ml	10.000 Units/ml penicillin; 10 mg/ml streptomycin
500 µl	250 µg/ml amphotericin B

Media were changed every second day. Before changing the media the solutions were prewarmed at 37 °C for 30 min to avoid a temperature shock of cells. After removal of consumed media, fresh media were added. The volumes of added media depend on the size of tissue culture flask. Generally following volumes for media exchange have been taken:

<i>Size of tissue culture flask</i>	<i>Exchange volume</i>
T 25	5 ml
T 75	10 ml
T 175	25 ml

Cell passaging

Labeled flasks (name, date, passage number, cell line) were placed into an incubator (37°C, 5% CO₂ and 96% humidity). Cells were checked in the microscope for confluence and adherence and passaged if necessary, around every 5 days. Old media were removed and the cells were washed twice with sterile PBS. Afterwards trypsin/EDTA solution (2 ml) was added. After morphological control under the microscope the trypsin/EDTA solution was removed and cells were detached from the surface. Cells were resuspended in fresh media and the required volume of the cell suspension depending on the splitting factor was transferred into a new cell culture flask, which had been coated with gelatine solution before. Usually the ratio of 1: 10 (cell suspension to growth medium) was used. At the end the flask was filled up with 4.5 ml growth media and stored in the incubator.

3.5.2 Cell freezing

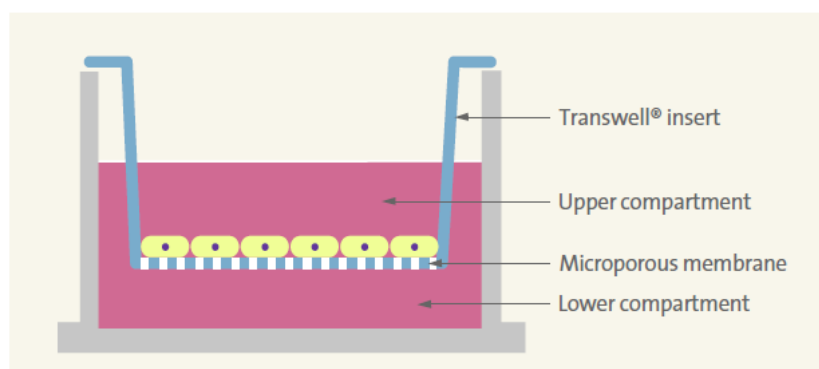
All cells were stored at -80°C prior to culture tests in order to expose them to similar conditions. After trypsinisation, 2 ml of cryomedium were added to one T 25 flask and the cell suspension was transferred into a cryotube. After 15 min incubation at +4°C the cells were stored in the freezer (- 80°C).

3.5.3 Cell thawing

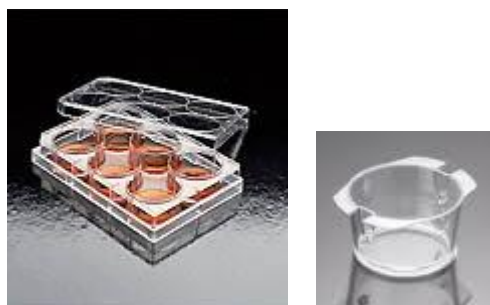
Frozen cells in cryotubes were put into the water bath (37 °C) for a few seconds. When the melting process has started the whole solution is quickly put into 5 ml of prewarmed medium. The final cell solution was pipetted into a gelatin-coated T 25 flask and stored in the incubator. On the following day medium was changed.

3.6 Transwell studies

Transwell filter systems are common *in vitro* test methods to study the physiology of the blood-brain barrier. Today three different types of *in vitro* models are used in research: *in silico* models, the Transwell model and the dynamic *in vitro* model of the blood-brain barrier. The advantages of the Transwell model are easy handling, the possibility to conduct multiple tests at the same time, low acquisition costs, and the possibility to perform transport experiments (Cucullo et al. 2005). These transport studies are conducted on Transwell filter systems [figure 9 and figure 10].



² **Figure 9:** The picture shows the mechanism of inserts inside the Transwell plate. The blue framed line shows the insert, which is submerged in medium. On the bottom of the insert is a porous membrane where cells are grown.



³ **Figure 10:** The whole 6-well Tissue Culture model is demonstrated on the left side. On the right side a single insert is shown. The insert will be put inside the well of the plate as described in figure 9.

The Transwell model consists of the 6-well tissue culture plate and appropriate inserts. The insert is placed into the well which is filled up with growth medium. The

² http://catalog2.corning.com/Lifesciences/media/pdf/transwell_guide.pdf (Accessed 16.03.2011)

³ <http://catalog.bd.com/bdCat/viewProduct.do?customer=353046> (Accessed 18.03.2011) <http://www.ultident.com/scientific/categories.php?category=Cell-and-Tissue-Culture/Cell-Culture-Inserts-and-Companion-Plates> (Accessed 18.03.2011)

model is divided in two separated compartments. The inserts represent the donor chamber (upper compartment) whereas the well acts as acceptor chamber (lower compartment). The upper area inside the insert represents the lumen of brain microvessels and the surrounding as lower compartment represents the brain. The main characteristics of that model are the side by side diffusion effect through the porous semi-permeable insert membrane. The growing area at the bottom of the insert is 4.2 cm^2 and the membrane pore size is $1 \text{ }\mu\text{m}$ which is close enough to prevent cell migration.

3.6.1 Coating of the Transwell insert

The coating procedure was done under the laminar air flow cabinet. The inserts were put in the 6-well plate with sterilized forceps. Each insert was filled with $150 \text{ }\mu\text{l}$ of sterile collagen solution at the concentration of 0.14 mg/ml . After one hour storage in the incubator the collagen supernatant was removed and the plate was dried for 15 min before $150 \text{ }\mu\text{l}$ fibronectin solution were added into the insert. Again a one hour incubation step followed and subsequently the supernatant was removed from each insert.

3.6.2 Cell seeding

In this study three different types of cultivation methods were used, namely (a) the “uninduced system”, (b) the co-culture and (c) the “induced system”. They are divided in monoculture (a) and (c) and co-culture (b) seeding conditions. The “uninduced system” consisted of one experimental cell line which was seeded directly on the coated insert membrane. The “induced system” and the “uninduced system” have the same seeding conditions and are monocultures. The “co-culture system” consists of the experimental cell line at the membrane surface but in addition on the well surface a confluent layer of astrocytes is grown. The seeding density of astrocytes was chosen with $60.000 \text{ cells/cm}^2$ per well. This type of co-culture system is named non direct contact co-culture because astrocytes were not seeded on the lower insert membrane area.

Generally cells were seeded on Transwell filter inserts with a density of $80.000 \text{ cells/cm}^2$. The cell number was determined in a Thoma chamber. The chamber was filled with $12 \text{ }\mu\text{l}$ cell suspension and cells were counted under the light microscope. The average number was multiplied by 10^4 to calculate the cell number per ml. In general one well was filled up with 3 ml of preheated growth media and inside the

insert 2 ml of the prepared cell suspension was added. The plate was labeled and stored in the incubator (37 °C, 5% CO² and 96% humidity). Four wells per plate were used with test cells (*probe*) and the two remaining were used for controls (*blank*). In the following scheme [figure 11] the well classification between probe and *blank* is shown.

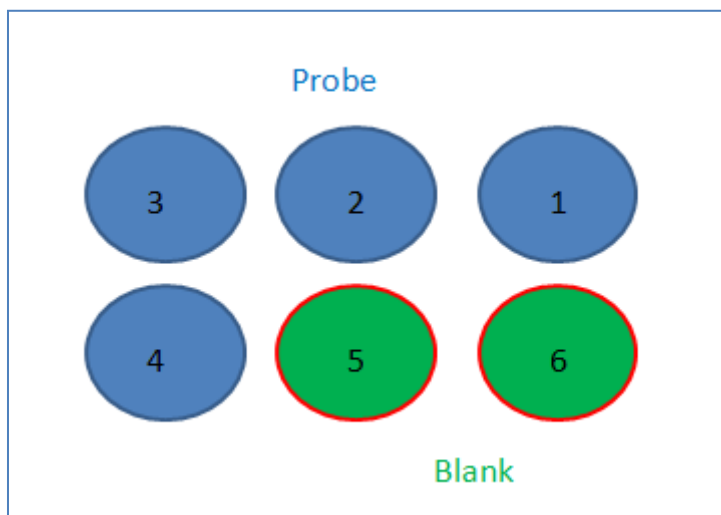


Figure 11: The graphic represents the setup of the Transwell experiments: Well number 1 to 4 contained cells whereas well 5 and 6 were only filled with media

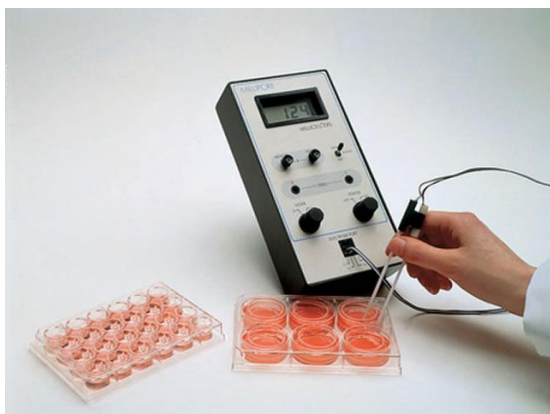
The coated Transwell inserts with number 5 and 6 were used as standards (*blank*).

3.6.3 Media exchange on Transwell model

Regular medium exchange is necessary for the cell cultivation inside the Transwell model. The culture medium was changed every day. Cell growth was controlled daily in the light microscope and in parallel TEER measurements were conducted [see 3.5.4 TEER measurements]. All plate inserts were picked with sterile forceps and the consumed medium was removed. During the medium removal from the plate, inserts were placed into an empty 6-well. Afterwards 3 ml of fresh medium were added into each plate well and 2 ml of fresh medium were placed in each insert. The labeled plate was placed back into the incubator.

3.6.4 Transendothelial Electrical Resistance (TEER) measurements

TEER was controlled by Millipore Millicell Electrical Resistance system (ERS) which is pictured in figure 12.



⁴ **Figure 12:** This pictures shows an ERS voltmeter for TEER measurement

Two electrodes are connected with the voltmeter which displays the measured resistance of the Transwell system: a shorter one for the insert and a longer one for the well. Both electrodes were equilibrated for 30 min in prewarmed media before use. Afterwards they were placed directly into the media for TEER record, whereby the steadying of the electrodes was a critical issue. In between every record the electrode was cleaned with ethanol. The resistance is inversely proportional to the cell permeability. TEER values were calculated by the formula that derives from Ohm's law.

$$\text{TEER } [\Omega \times \text{cm}^2] = (\text{TEER}_{\text{cell}} - \text{average TEER}_{\text{blank}}) \times \text{surface area } (4, 2 \text{ cm}^2)$$

Cell lysis was performed in case the measured value was about +/- 20 $\Omega \times \text{cm}^2$ or higher and furthermore if density observation by light microscope showed a highly confluent cell layer.

3.7 Cell lysis

Lysis buffer

8 M Urea
2 M Thiourea
0.5 % Triton X-100
2 % Chaps
5 mM EDTA

Before use 2 mg DTT and 4 μl protease inhibitor cocktail were added to each aliquot of 500 μl lysis buffer. The whole cell lysis was done outside the lamina flow cabinet

⁴ http://www.reiss-laborbedarf.de/uploaded/image/shop/millipore/MB330-06%5b1452-ALL%5d_big.jpg
(Accessed from 14.03.2011)

but cooled on ice. The medium was removed from the inserts and each insert was washed twice with PBS. PBS was removed carefully to prevent any dilution. The cell monolayer of one insert was incubated with 50 µl lysis buffer for 3 min on ice. For cell lysis in cell culture flasks 200 µl of lysis buffer were used. Cells were scraped and the lysate was transferred into Eppendorf tubes and centrifuged at 12.000 rpm for 10 min. The supernatants were stored at -80 °C until they were used for protein analysis.

3.8 Bradford assay

Bradford solution

(For 100 ml)

10 mg (0.01 %) Coomassie Brilliant Blue G250

5 ml (5 %) Ethanol, 96 %

10 ml (10 %) H₃PO₄ , 85 % (v/v)

85 ml H₂O bidest.

Determination of protein concentrations was performed by the Bradford assay (Bradford 1976). Protein samples and BSA standards (1 mg/ml) were mixed with Bradford solution and after five minutes incubation time at room temperature (RT) extinctions were measured at 595 nm. Protein concentrations were determined using a BSA calibration curve (0.025, 0.05 and 0.1 mg BSA/ml) which was performed for every measurement. If necessary samples were diluted with H₂O bidest. to receive a value within the linear range of BSA (0.2 to 0.7 mg/ml).

3.9 DIGE 2D-Electrophoresis

DIGE solutions

Labeling

- Standard cell wash buffer, 10 mM Tris (pH 8,0), 5 mM magnesium acetate. Stored in aliquots at -15°C to -30°C.
- Lysis buffer, 30 mM Tris base, 7M urea, 2 M thiourea, 4 % (w/v) CHAPS
- Lysine, 10 mM L-lysine
- pH indicator strips (GE Healthcare)

Sample buffer (2x)

8M Urea
130 mM DTT
4 % (w/v) CHAPS
2 % (v/v) Pharmalyte TM 3-10 NL for IEF

Rehydration buffer

8 M Urea
4 % (w/v) CHAPS
1 % (v/v) Pharmalyte TM 3-10 NL for IEF
13 mM NaOH

Sodium Hydroxide

50 mM NaOH

Gel loading buffer (2x)

120 mM tris (pH 6.8)
20 % (v/v) (87 % glycerol, 4% SDS 200 mM DTT and a few grains of bromophenol blue)

12.5 % Acrylamide gel

40 % acrylamide/bis-acrylamide
25 ml tris (1.5 M)
1 ml SDS 10 % (v/v)
1 ml ammonium persulfate 10 % (w/v)
40 ml TEMED
(make up to 100 ml with H₂O bidest.)

SDS electrophoresis running buffer (1x)

25 mM tris
192 mM glycine
0.2 % SDS

Two dimensional difference gel electrophoresis (2D-DIGE) was used for quantitative proteomics. In this method the proteins are labeled with fluorescence dye markers (CyDye DIGE Fluors). This technique makes it possible to separate up to three different protein samples on one 2-D gel. The internal standard was labeled with Cy2

(yellow) whereas protein samples were labeled with Cy3 (red) and Cy5 (blue) according to the manufacturer's instructions (Product Booklet for Ettan Dige, Ge Healthcare).

25 µg of each labeled sample (Cy3, Cy5) and 25 µg of the internal standard (Cy2) were diluted with rehydration buffer to a total volume of 350 µl. For the first dimension, *isoelectric focusing* (IEF) was performed on 18 cm, non linear IPG strips with a pI range from 3-11. The rehydration step was performed for 12 h at 20 °C and IEF was carried out to a total of 80 kVhr (Stessl et al. 2009).

The CyDye Fluors bind covalently to the epsilon amino group of lysine due to their reactive NHS ester group. Minimal labeling was used, meaning that only 1-2% of sample proteins were labeled with the respective dyes. The IPG strip was placed on a homogenous 12.5 % polyacrylamide gel. The proteins were separated at a constant voltage of 50 mA per gel in the second dimension.

DIGE Scan was performed with an Ettan Dige Imager at 100 µm resolution. The evaluation was conducted with the software program DeCyler version 7.0. Gels were grouped (not induced, induced and co-culture group) and analyses with the Batch program. Proteins of interest were defined using the following program settings: exclusion filter for differential in gel analysis (DIA) (slope: > 1.7, volume: minimum 50.000, peak height: 0 to 10.000). Subsequently gels were matched and proteins of interests were defined meeting the following statistical conditions: *average ratio* +/- 1.5, *Student's t-test* ($p < 0.05$) and presence in at least 9 of 12 spot maps.

3.10 2-D Electrophoresis

IEF

IEF was performed as described above without CyDye labelling.

SDS-PAGE

Gel preparation

Separation buffer (4x)

90.8 g tris (1.5 M)

2 g SDS (0.4 % (w/v))

Acrylamide solution (30.8 %, 37.5:1)

120 g acrylamide (30 % (w/v))

3.2 g N,N'-methylenebisacrylamide (0.8 % (w/v))

12.5 % separation gel

11.86 ml H₂O bidest

8.75 ml separation buffer (4x)

14.2 ml acrylamide solution (30.8 %)

21.88 µl TEMED

164 µl APS (10 %)

Stacking gel buffer (4x)

6.06 g tris (0,5 M)

0.4 g SDS (0.4 % (w/v))

Stacking gel

3 ml H₂O bidest

1.25 ml stacking gel buffer (4x)

0.65 ml acrylamide solution

5 µl TEMED

50 µl APS (10 %)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) is an important method for protein analysis to separate proteins within a gel matrix in an electrical field according to their molecular weight. Proteins are denaturated and saturated with negatively charged SDS in order to avoid separation by charge. Dependent on their molecular weight proteins are separated. The gel matrix in this study consists of polyacrylamide. The whole gel was divided in two parts: - the stacking gel which provides a simultaneous start of proteins, and the separation gel, where the ultimate separation takes place. The appropriate diluted protein extract was reduced by dithiothreitol (DTT) and separated on 12.5 % gel. Gels were silver stained according to the following staining protocol.

Silver staining (MS compatible)

The silver staining was processed according to Blum's staining protocol (Blum 1987). Following solutions were used to incubate the gels for different time periods. During this method ethanol 30 % (v/v) was taken for washing steps after the fixation step.

Fixing solution

30 % acetic acid

40 % methanol

Wash ethanol

30 % ethanol

Sensitizer solution

6.8 g sodium acetate

0.2 g $\text{Na}_2\text{S}_2\text{O}_3$

Silver solution

0.5 g silver nitrate

50 μl formaldehyde

Developing solution

30 g Na_2CO_3

5 mg $\text{Na}_2\text{S}_2\text{O}_3$

500 μl formaldehyd

Staining protocol

Fixing	1 hour
Washing with EtOH (30 %)	2 x each 20 min
Washing with MilliQ	20 min
Sensitizing	1 min
Washing with Aqua dest.	3 x each 1 min
Silver staining	20 min
Washing with Aqua dest.	3 x each 1 min
Developing	2 min
Washing with Aqua dest.	3 x each 1 min

Gels were kept in Aqua dest. until MS analysis

3.11 Trypsical *in-gel* digestion

Solutions

ABC buffer

100 mM NH_4HCO_3 buffer

Destaining solution

$\text{Na}_2\text{S}_2\text{O}_3$ (100 mM)

$\text{K}_3[\text{Fe}(\text{CN})_6]$ (30 mM)

Mixture 1:1

Reducing solution

10 mM DTT dissolved in 100 mM NH_4HCO_3 (ABC)

Alkylating solution

54 mM iodoacetamide dissolved in 100 mM NH_4HCO_3 (ABC)

In-gel digestion was conducted with the defined *proteins of interests* (POI). Protein spots for MS analysis were cut out from the gel by a scalpel and each piece was transferred into an Eppendorf tube which was filled up with bidest. H_2O . A matrix piece from blank gel regions acted as “control” spot to identify MS peaks deriving from the gel matrix. Gel spots were destained with destaining solution carefully

washed with MilliQ and 100 % acetonitrile (ACN) solution. Then they were reduced with 10 mM dithiothreitol in reducing solution for 40 minutes at 56 °C, afterwards washed twice with washing buffer and alkylated with 54 mM iodoacetamide in alkylation solution for 20 min at 20 °C in the dark. Two washing steps followed before gel pieces were dried in a vacuum centrifuge for 15 min. *In-gel* digestion was performed in the microwave for 10 min at 170 W with trypsin (Roche, Mannheim, Germany; 12.5 ng trypsin per μ l) . Peptides were extracted from gel pieces by three extraction steps with each 40 μ l of ABC buffer, ABC buffer/ ACN and 0.1 % trifluoroacetic acid (TFA)/ACN. The supernatants were frozen over night and dried on the next day in the vacuum centrifuge at 37 °C.

Peptides were desalted by Zip-Tip purification using Zip Tips C18 (Millipore, Bedford, MA, USA) according to the Millipore product protocol. α -Cyano-4-hydroxycinnamic acid (Sigma Aldrich) was used as MALDI matrix and samples were prepared using the dried droplet method.

3.12 MALDI TOF MS

The identification of proteins was measured in positive ion mode on a MALDI TOF/TOF (AXIMA ToF²™ and CFR™, respectively) (Shimadzu Biotech Kratos Analytical, Manchester, UK) using a CHCA (α -cyano-4-hydroxycinnamic acid) matrix. Calibration was conducted with a peptide mix which includes singly charged monoisotopic m/z values of standard peptides (Stessl et al. 2009). PMF spectra were evaluated by mMass software (Version 3.10⁵) (Strohalm 2010, Strohalm 2008). Keratin, matrix peaks or peaks caused by tryptic auto-digestion were identified separately (Mattow J 2004, Smirnov IP 2004) and excluded from spectra. Protein identification was performed via the public search engine Matrix Science-Mascot (Peptide Mass Fingerprint, Version 2.3.02). Following databases were used during data analysis (NCBI nr and SwissProt (Version 56.0) August 2010 –December 2010). Search criteria were defined as follows: fixed modifications (carbamidomethylation of cysteines), variable modifications (oxidation of methionines), taxonomy (mammalia), missed tryptic cleavages (1), peptide tolerance (+/- 0.5 –0.2 Da).

⁵ <http://www.mmass.org/> (Accessed 11.04.2011)

3.13 Isolation of primary PBCECs

The process to isolate primary porcine brain endothelial cells out of the brain was learned during a research stay at the Department for Pharmaceutical Technology and Biotechnology, University Heidelberg. Briefly, the process is divided into five steps which are shown in the overview picture (figure 14).

Step 1: Brain acquisition and Cortex preparation

Step 2: Capillary isolation

Step 3: Capillary separation

Step 4: Cell isolation

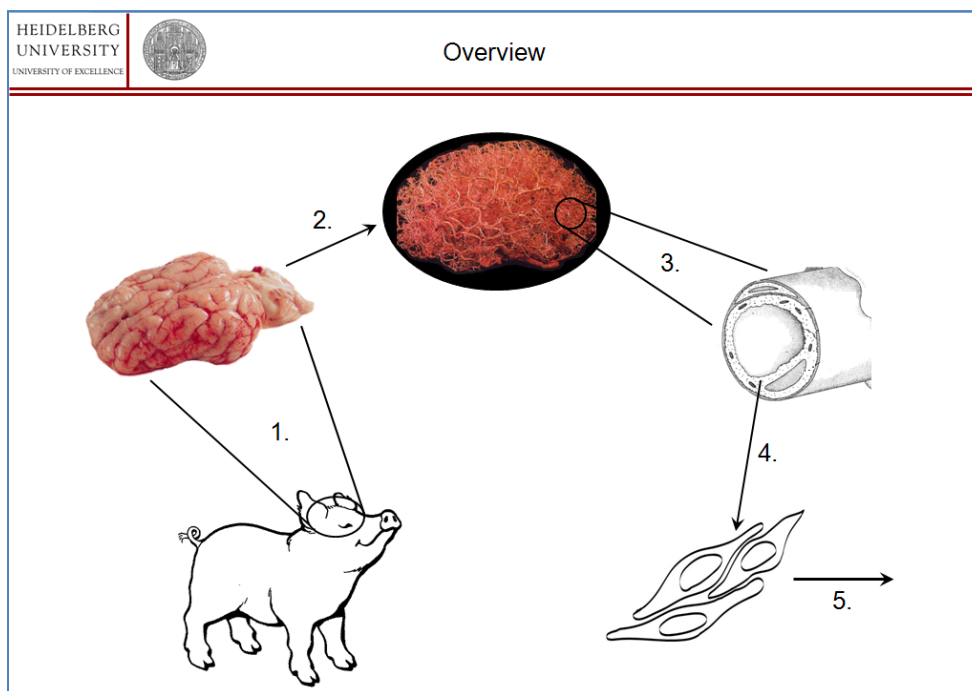


Figure 14: This picture shows an overview of the isolation procedure, separated into five steps. Step one is the brain acquisition and cortex preparation, step two the capillary isolation, step three the capillary separation and finally the cell isolation. (Helm 2010)

4 Results

Within this study the porcine BBB cell line PBEC/C1-2 (porcine brain endothelial cells) and HUVECs (primary human umbilical vein endothelial cells) were cultured onto 6 Well tissue culture plates. To determine whether soluble factors secreted by astrocytes lead to changes in barrier formation and proteome pattern, both cell lines were cultivated in standard growth medium, in medium enriched with glioma derived soluble factors, or in the presence of rat astrocytes

4.1 TEER measurements

Before cell lysis and subsequent proteomic studies, the cell density was assessed by measuring the transendothelial electrical resistance (**TEER**).

The calculation of the electrical resistance was based on the following formula:

$$\text{TEER } [\Omega\text{cm}^2] = (\text{TEER}_{\text{cell}} - \text{average TEER}_{\text{blank}}) \times \text{surface area } (4, 2 \text{ cm}^2)$$

The definition of the probe number was according to the scheme in chapter 3.5.2 Cell seeding (figure 11).

The PBMEC/C1-2 cell measurements were conducted three or four days after cell seeding (cs), until a dense cell monolayer could be observed under the microscope. The electrical resistance measurements for HUVEC were done at the following day after cell seeding and the day before cell lysis. Interestingly, the average cultivation time period from seeding to cell lysis differed between PBMEC/C-1 and HUVEC up to one or two days.

The measured TEER results are listed in the tables above:

PBMEC/C1-2 (uninduced in C6 medium) 3rd day after cell seeding (cs)			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	134	137	-12.6
3	134	137	-12.6
2	135	137	-8.4
1	138	137	4.2

PBMEC/C1-2 (cultivated in - FIB medium) 3rd day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	133	136	-12.6
3	119	136	-71.4
2	134	136	-8.4
1	136	136	0.0

PBMEC/C1-2 (co-culture in C6 medium) 4th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	620	648	-117.6
3	628	648	-84.0
2	624	648	-100.8
1	614	648	-142.8

* no significant TEER values - whiskers

PBMEC/C1-2 (co-culture in C6 medium) 4th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	411	350	256.2
3	382	350	134.4
2	352	350	8.4
1	379	350	121.8

*no significant TEER values – whiskers

HUVEC (uninduced in EGM medium) A1			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	139	143.5	-18.9
3	141	143.5	-10.5
2	141	143.5	-10.5
1	137	143.5	-27.3

*Control measurement to check the electrode function

HUVEC (uninduced in EGM medium) PL1 1 st day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	130	131	- 4.2
3	128	131	-12.6
2	133	131	8.4
1	132	131	4.2

HUVEC (uninduced in EGM medium) PL2 1 st day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	133	129	16.8
3	131	129	8.4
2	135	129	25.2
1	134	129	21.0

HUVEC (uninduced in EGM medium) PL1 3 rd day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	125	119.5	23.1
3	115	119.5	-18.9
2	124	119.5	18.9
1	123	119.5	14.7

HUVEC (uninduced in EGM medium) PL2 3 rd day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	123	120	12.6
3	118	120	-8.4
2	127	120	29.4
1	122	120	8.4

HUVEC (uninduced in EGM medium) PL1 4 th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	138	119.5	77.7
3	132	119.5	52.5
2	139	119.5	81.9
1	144	119.5	102.9

HUVEC (uninduced in EGM medium) PL2 4 th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	119	111	33.4
3	112	111	4.2
2	117	111	25.2
1	110	111	-4.2

HUVEC (co-culture in EGM medium) PL1 1 st day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	95	96	-4.2
3	96	96	0.0
2	98	96	8.4
1	110	96	58.8

HUVEC (co-culture in EGM medium) PL2 1 st day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	107	102.5	18.9
3	112	102.5	39.9
2	107	102.5	18.9
1	115	102.5	52.5

HUVEC (co-culture in EGM medium) PL1 4 th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	87	83	16.8
3	97	83	58.8
2	120	83	155.4
1	90	83	29.4

HUVEC (co-culture in EGM medium) PL2 4 th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	95	105	-42.0
3	96	105	-37.8
2	98	105	-29.4
1	110	105	58.8

HUVEC (induced in - EGM medium) PL1 5 th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	135	122	54.6
3	127	122	21.0
2	126	122	16.8
1	123	122	4.2

HUVEC (induced in - EGM medium) PL2 5 th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	136	125.5	44.1
3	140	125.5	60.9
2	139	125.5	56.7
1	135	125.5	39.9

HUVEC (induced in - EGM medium) PL3 5 th day after cs			
Probe number	Value Probe	Av. value blank	TEER [Ωcm^2]
4	139	125.5	56.7
3	139	125.5	56.7
2	134	125.5	35.7
1	139	125.5	56.7

The values of the TEER measurements show variations between the probe numbers. In parallel the tissue culture plates were checked daily after the first day from seeding the cells until lysis. The visual observation of the cell layer was an important control parameter beside the TEER measurements.

Experimentally observed time period (days) from cell seeding to cell lysis:

	PBMEC/C1-2	HUVEC
Duration in days		
Uninduced	4	5
Induced	3	6
Co-culture	4	5

Table 1: Cultivation time period of PBMEC/C1-2 and HUVECs

The results in Table 1 display differences in the period of cultivation between the two experimental cell lines (PBMEC/C1-2, HUVEC). Noticeable variation could be determined for induced system, where the cultivation days are doubled between PBMEC/C1-2 and HUVEC.

Standard variation of TEER values before cell lysis:

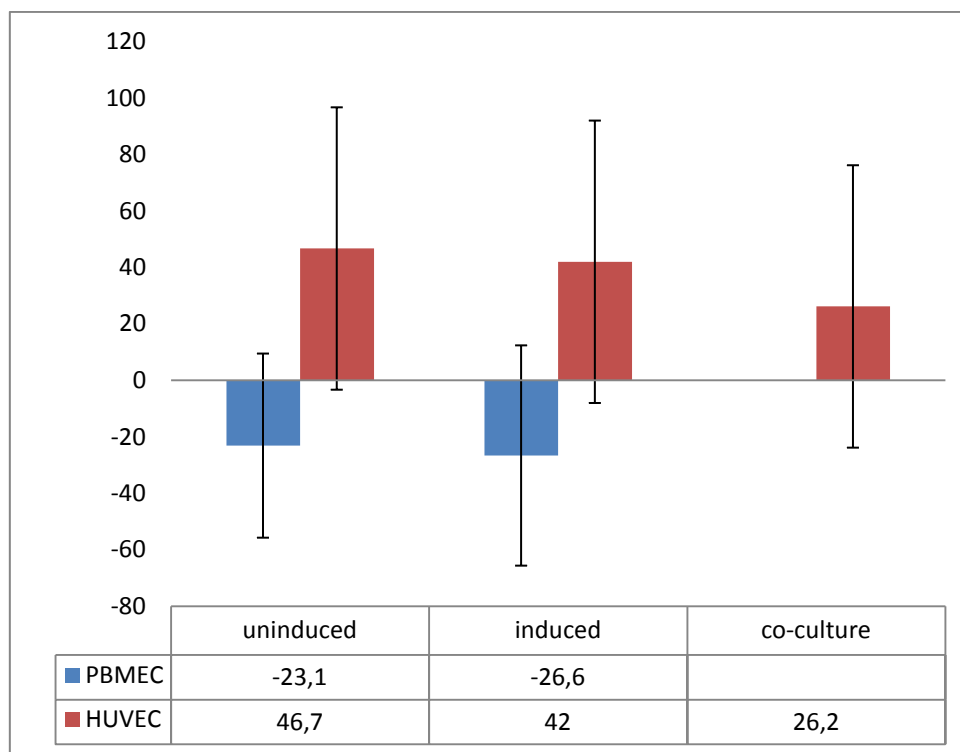


Figure 15: Diagram from standard variation of TEER values at the day before cell lysis. The co-culture values of PBMEC are not significant (whiskers) and have not been considered for the statistic calculation.

Generally, HUVECs formed a tight cell monolayer regardless of the culturing conditions, as indicated by TEER increase after four days. No significant changes between the different cultivating conditions were detected. The comparison of the time period after cell seeding (cs) and cell lyse differ from HUVEC to PBMEC/C-1. The cultivation period in tranwell filter system of PBMEC/C1-2 cells were maximum four days. PBMEC/C1-2 co-culture system and uninduced system took four days for cultivation whereas cells from the induced system were lysed after three days. Interestingly, HUVEC cells were cultivated for about five days in transwell culture plates. Both the co-culture system and the uninduced system were cultivated for five days while the induced system took for six days before the cells could be lysed. Summarizing, no significant changes inside the different cultivation conditions were determined by contrast different duration of the cultivation time between PBMEC/C1-2 and HUVEC were observed, especially for co-culture system.

4.2 Proteomics

After confirming by TEER measurements and visual inspection that a tight and stable monolayer had been formed, cells were lysed for the proteome analysis. The different culture conditions were done in triplicates (uninduced, induced and co-culture). The proteomic analyses were conducted using 2D DIGE and protein identification was performed via MALDI TOF MS. Typical gels of PBMEC/C1-2 and HUVECs are depicted in the following chapter (4.2.1 Comparison of PBMEC/C1-2 and HUVEC).

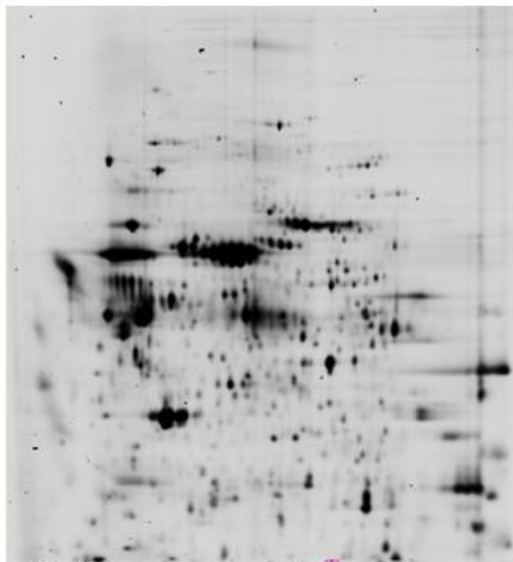
4.2.1 Comparison of PBMEC/C1-2 and HUVEC

All experiments were conducted under the same conditions inside the Transwell filter systems. The first comparison was done among uninduced models. That means the cells were cultivated in growth medium without any blood-brain barrier (BBB) inducing factors.

Uninduced cultivation models

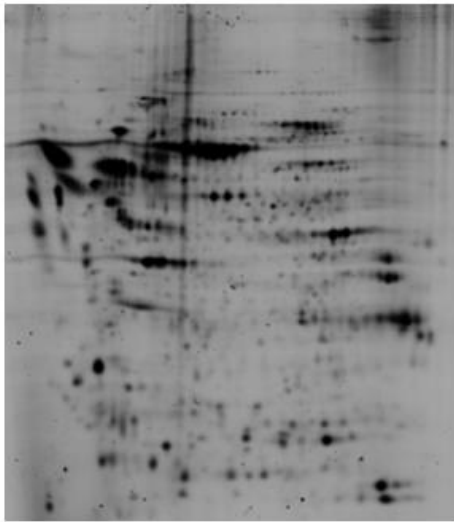
(75µg per gel, pI 3-11, 12.5% gel)

pI 3 ← → pI 11



a) Uninduced PBMEC/C1-2 Cy3

pI 3 ← → pI 11



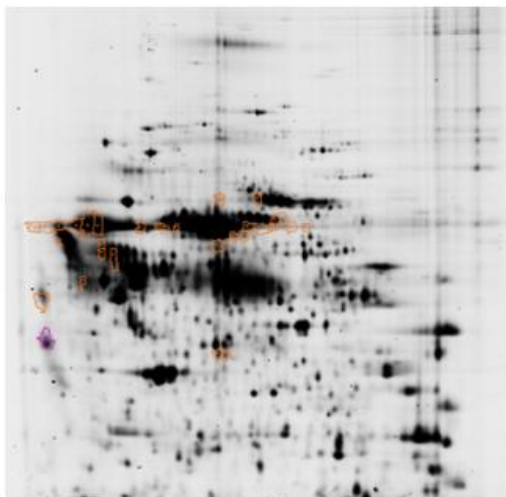
b) Uninduced HUVEC Cy3

Figure 15: Representative 2D gels of human umbilical vein cells (HUVEC) and porcine brain microvessel endothelial cells (PBMEC/C1-2) are shown in picture a) and b). The cells were grown in basal growth medium (C6 or EGM) without any blood brain barrier (BBB) properties including agents.

Induced cultivation models

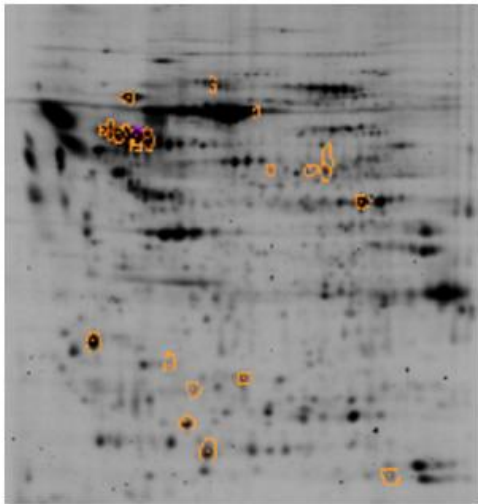
During these experiments, cells were cultivated in medium that includes astrocyte growth factors (ACM) with the aim to induce typical BBB function in endothelial cell.

pI 3 ← → pI 11



c) Induced PBMEC/C1-2 Cy5

pI 3 ← → pI 11



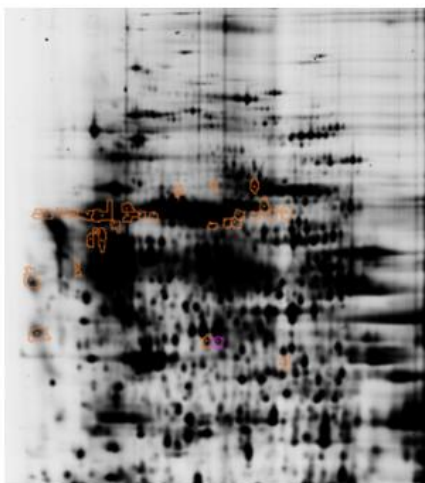
d) Induced HUVEC Cy5

Figure 16: Picture c) and d) shows the proteome pattern of induced PBMEC/C1-2 and HUVEC cells. Additionally proteins of interests are marked in orange. (75µg per gel, pI 3-11, 12. 5% gel)

Co-culture models

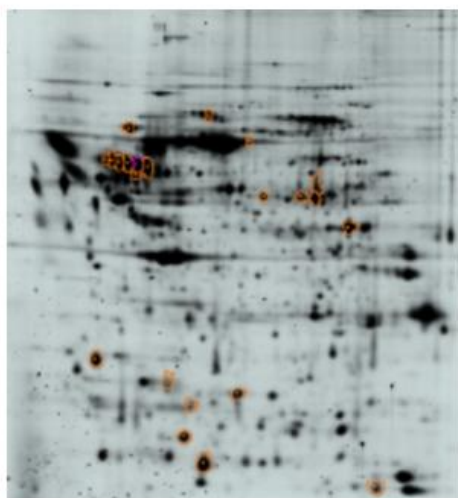
These cultivation studies included a non directly contact co-culture system with astrocyte cells from rat brains. Astrocyte conditioned medium induce BBB factors into endothelial cells leading to changes in the tight junction structure of endothelial cells.

pI 3 ← → pI 11



e) Co-culture PBMEC/C1-2 Cy5

pI 3 ← → pI 11



f) Co-culture HUVEC Cy5

Figure 17: The proteome pattern of picture e) and f) shows the separated proteins of PBMEC/C1-2 cells and HUVEC cells after cultivation in media enriched with astrocytes. (75µg per gel, pI 3-11, 12.5% gel)

Protein list of significantly changed proteins

The remarkable changed proteins (POI) were labeled and listed according to their Master ID number. In the following pictures the significantly changed proteins from HUVEC and PBMEC/C1-2 in co-culture transwell filter system are demonstrated. Decyder software version 7.0 was used to identify significantly changed protein spots with a minimum change of 1.5 fold, and statistical significance reflected by a t-test value of 0.05 or lower, all compared to the untreated control cohort. Table 1,2 shows protein spots differentially regulated in PBMEC/C1-2 cells, and table 3,4 those of HUVECs. Approximately forty spots of PBMEC/C1-2 were found to be up- or down regulated after induction with astrocyte conditioned medium and more than thirty spots after co-culturing with rat astrocytes. Fewer spots (about six after treatment with conditioned medium, more than twelve after the co-culture) were differentially regulated in HUVECs

Porcine brain microvessel endothelial cells (PBMEC/C1-2)

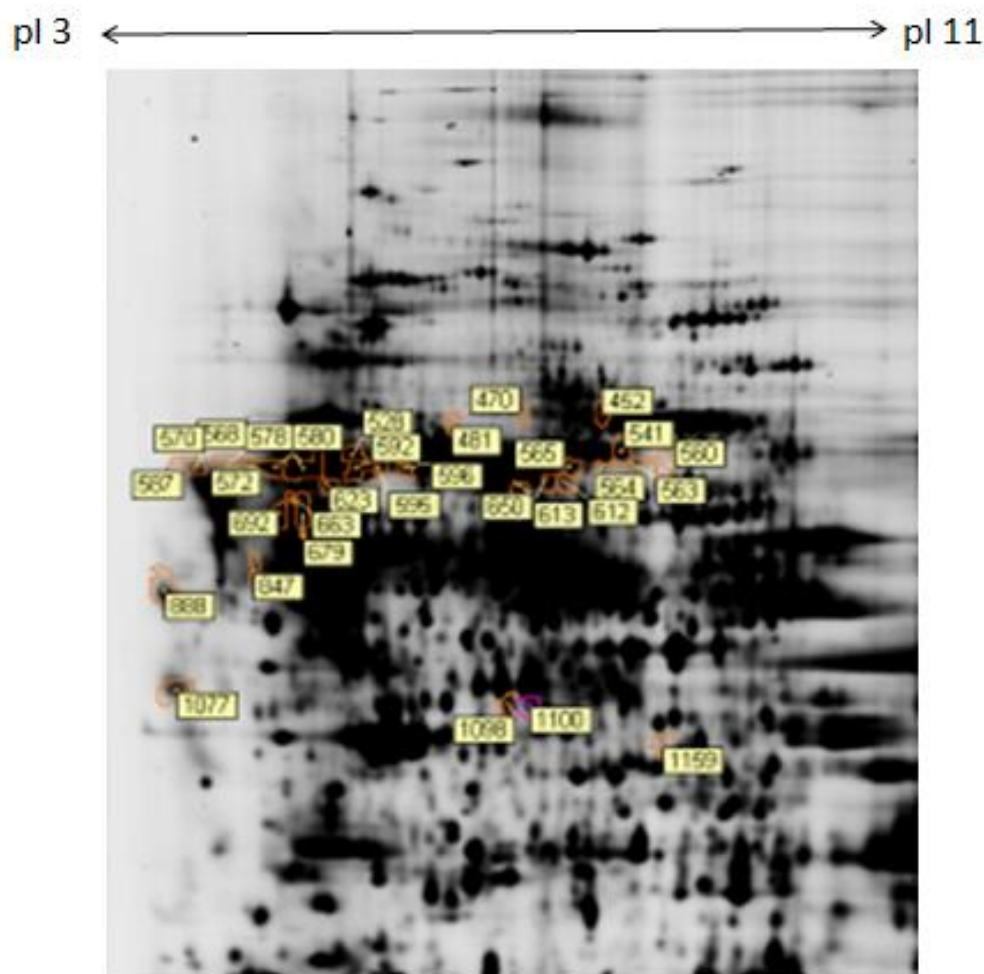


Figure 16: 2D gel image of significantly changed proteins from PBMEC/C1-2 in co-culture transwell filter system. (pI 3 -11 NL, 12.5 % SDS-Page, silver stained) The labeled proteins with their Master number are listed according to statistical parameters in the following table (Protein list: PBMEC/C1-2: Co-Culture versus ACM).

Protein list: PBMEC/C1-2: Co-Culture ACM versus uninduced system

Table of remarkable changed proteins of PBMEC/C1-2 with statistically parameters (t-value, Av. Ratio) from co-culture and induced (ACM) systems versus the uninduced system.

Co-culture			ACM	
Master No.	T-test	Av. Ratio	T-test	Av. Ratio
208	X	X	0,022	2,49
288	X	X	0,045	2,86
452	0.052	-1.80	0,0099	-2,72
525	X	X	0,0043	-3,42
532	X	X	0,046	-5,75
541	X	X	0,0055	-4,98
564	0.096	-2.25	0,028	-3,15
565	0.090	-2.15	0,018	-4,12
568	0.014	-2.03	0,0075	-3,64
570	0.029	-2.60	0,039	-2,84
575	X	X	0,0038	-3,38
578	0.20	-1.72	0,034	-4,97
580	X	X	0,033	-4,5
587	0.0091	-2.87	0,021	-2,95
592	0.20	-1.79	0,048	-4,57
594	X	X	0,018	-3,49
595	0.0033	-2.48	0,0021	-3,68
612	0.042	-2.51	0,023	-2,6
679	0.093	-1.51	0,0073	-3,71
684	0.22	-1.48	0,037	-4,15
692	0.087	-1.60	0,0086	-2,92
742	X	X	0,018	-3,47
847	0.26	-1.37	0,034	-2,34
864	X	X	0,044	-2,81
888	0.20	-1.52	0,042	-2,83
1077	0.0015	-2.22	5,50E-05	-3,03
1098	0.031	-1.84	0,0028	-2,52
1100	0.0088	-1.96	0,00028	-2,94
1202	X	X	0,038	2,14
1493	0.042	-2.27	0,0076	4,77
1508	X	X	0,02	1,71

Table 1: Values of changed protein spots of co-culture and induced (ACM) system compared to uninduced system. (X= the value does not lie inside the parameter area – no significant change detected)

Co-Culture and ACM		
Master No.	T-test	Av. Ratio
452	0,008	-2,26
470	0,034	-2,03
481	0,012	-2,3
528	0,039	-2,43
535	0,036	-3,62
541	0,03	-3,31
560	0,042	-1,54
561	0,027	-2,59
563	0,047	-2,45
564	0,011	-2,7
565	0,012	-3,14
568	0,0049	-2,83
570	0,0047	-2,72
572	0,008	-3,06
575	0,0092	-2,89
578	0,04	-3,35
580	0,033	-3,19
587	0,0015	-2,91
592	0,043	-3,18
594	0,016	-3,01
595	0,00052	-3,08
596	0,013	-2,36
612	0,0048	-2,56
613	0,036	-2,52
623	0,0084	-2,08
646	0,04	-2,46
650	0,039	-2,31
663	0,038	-2,11
679	0,031	-2,61
692	0,014	-2,26
847	0,042	-1,86
888	0,047	-2,17
1077	0,00017	-2,63
1098	0,0032	-2,18
1100	0,0013	-2,45
1159	0,05	1,54

Table 2: Table of significant changed proteins of PBMEC/C1-2,co-culture and induced system together versus uninduced system.

Human umbilical vein endothelial cell (HUVEC)

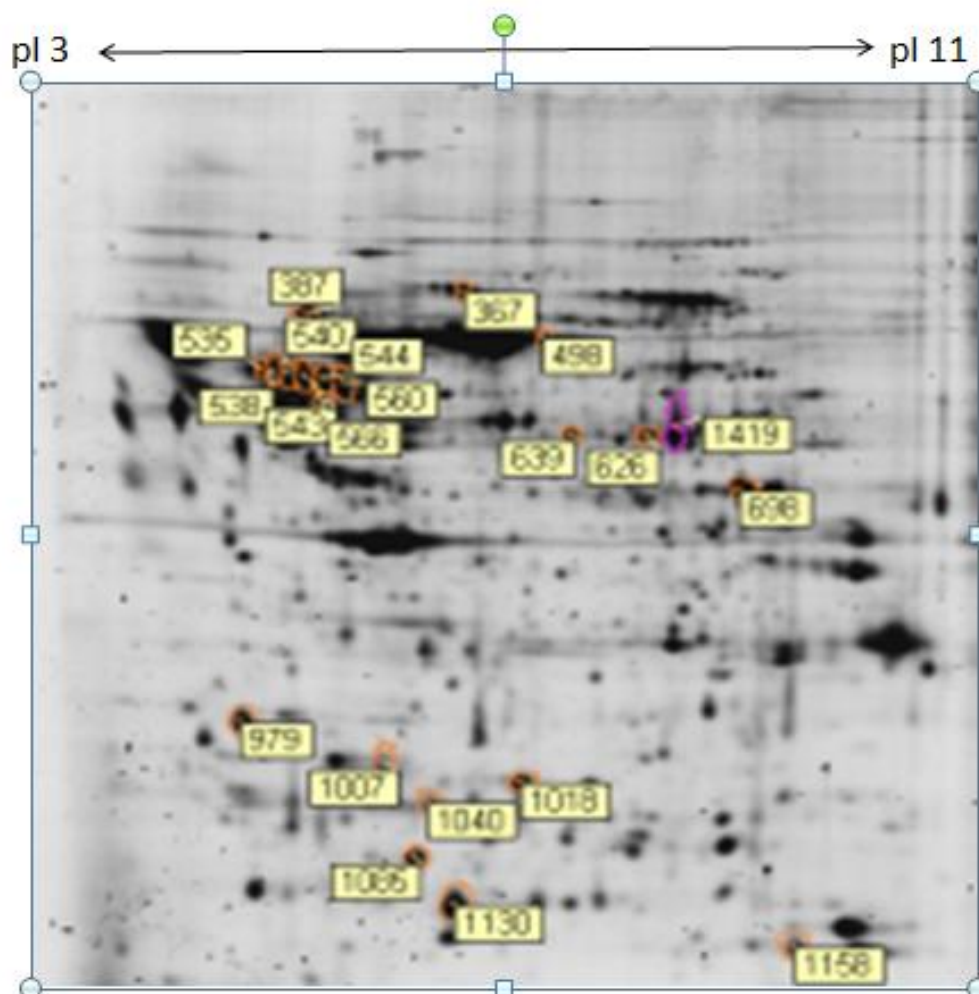


Figure 17: 2D gel image of significantly changed proteins from HUVEC in co-culture transwell filter system (pI 3 -11 NL, 12.5 % SDS-Page, silver stained).The labeled proteins with their Master number are listed according to statistical parameters in the following table (Protein list: HUVEC: Co-Culture versus ACM).

Protein list: HUVEC: Co-Culture and ACM versus uninduced system

Co-culture			ACM	
Master No.	T-test	Av. Ratio	T-test	Av. Ratio
49	0.028	-8.15	X	X
166	0.028	-6.02	X	X
176	0.044	-4.27	X	X

Master No.	T-test	Av. Ratio	T-test	Av. Ratio
209	0.027	-6.55	X	X
214	0.032	-9.47	X	X
224	0.038	-9.61	X	X
243	0.040	-12.48	X	X
367	X	X	0.018	-2.77
387	0.044	-4.25	X	X
498	0.023	-1.68	X	X
535	X	X	0.030	3.37
626	X	X	0.017	2.89
628	X	X	0.0059	-9.73
639	0.041	2.90	0.032	-1.76
669	0.028	-5.72	X	X
703	X	X	0.0062	3.41
720	0.038	-3.93	X	X
733	X	X	0.034	4.87
795	0.036	-3.31	X	X
809	0.034	-6.76	X	X
837	X	X	0.039	4.09
906	X	X	0.024	4.78
979	0.036	4.12	0.029	-1.80
1018	X	X	0.009	5.01
1419	0.025	-5.44	X	X

Table 3: Spot intensity values of changed proteins from HUVECs. Spots of co-culture and induced (ACM) system compared with uninduced system. (X= the value does not lie inside the parameter area – no significant change detected)

Co-culture and ACM		
Master No.	T-test	Av. Ratio
49	0.0096	-9.15
98	0.025	-6.43
208	0.045	-9.28
209	0.0078	-6.52
214	0.0097	-9.98
224	0.014	-11.05
243	0.013	-12.20
303	0.026	-5.87
348	0.028	-3.81
574	0.049	-1.91
617	0.031	-8.24
628	0.029	-6.01
639	0.048	-9.72
979	0.036	-8.64
1273	0.046	-12.75
1276	0.031	-13.45
1018	0.025	-5.32
1419	0.038	-3.72

Table 4: Table of significant changed proteins from HUVEC, co-culture and induced system together versus uninduced system.

MALDI TOF identification

Proteins of interest were identified by MALDI TOF analysis. Respective protein spots were cut out of silver stained gels in order to pool sufficient amount of proteins. Proteins are indicated with their Uniprot accession numbers.

Proteins of interest were searched via MASCOT (Matrix Science) in the public databases SwissProt and MSDB.

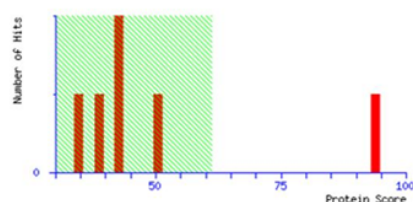
Example for the MASCOT search result of Spot no. C12 (Actin PIG), database SwissProt:

Mascot Search Results

User : CG
Email :
Search title :
Database : SwissProt 2010_12 (523151 sequences; 184678199 residues)
Taxonomy : Mammalia (mammals) (65188 sequences)
Timestamp : 29 Dec 2010 at 16:07:08 GMT
Top Score : 94 for [ACTB_SIGHI](#), Actin, cytoplasmic 1 OS=Sigmodon hispidus GN=ACTB PE=2 SV=1

Mascot Score Histogram

Protein score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event.
Protein scores greater than 61 are significant ($p < 0.05$).



Concise Protein Summary Report

Format As [Concise Protein Summary](#) [Help](#)
Significance threshold $p < 0.05$ Max. number of hits [AUTO](#)

[Re-Search All](#) [Search Unmatched](#)

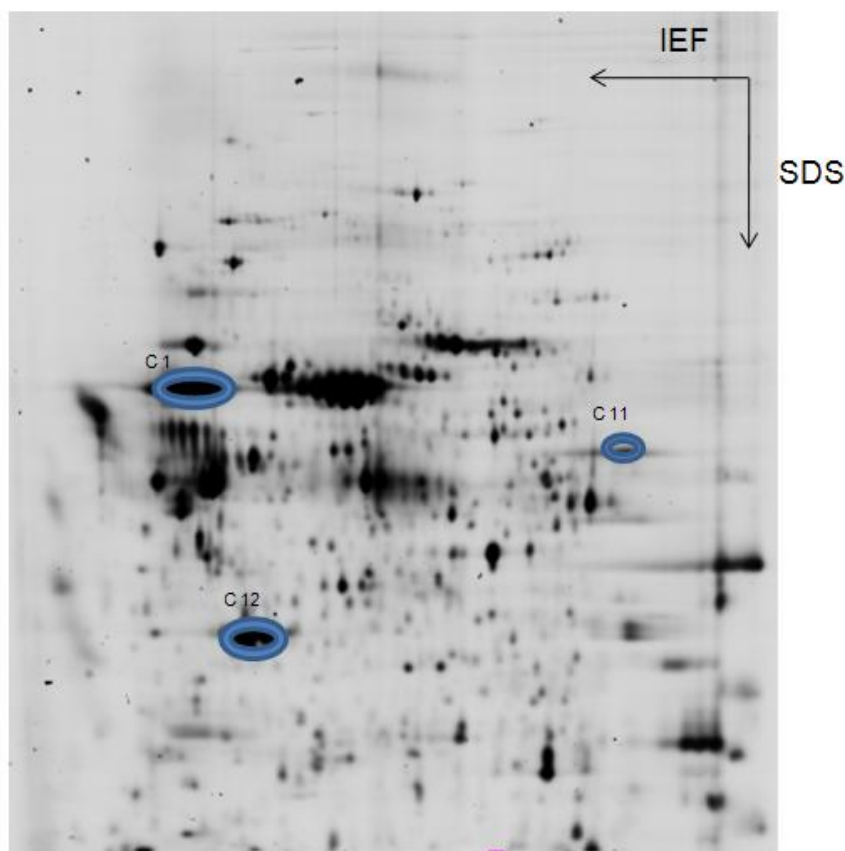
1.	ACTB_SIGHI	Mass: 42034	Score: 94	Expect: 2.7e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Sigmodon hispidus GN=ACTB PE=2 SV=1				
	ACTB_BOVIN	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Bos taurus GN=ACTB PE=1 SV=1				
	ACTB_CANFA	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Canis familiaris GN=ACTB PE=2 SV=3				
	ACTB_CAVPO	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Cavia porcellus GN=ACTB PE=2 SV=1				
	ACTB_CERAE	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Cercopithecus aethiops GN=ACTB PE=2 SV=1				
	ACTB_CRIGR	Mass: 42053	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Cricetulus griseus GN=ACTB PE=3 SV=1				
	ACTB_HORSE	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Equus caballus GN=ACTB PE=2 SV=1				
	ACTB_HUMAN	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1				
	ACTB_MACFA	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Macaca fascicularis GN=ACTB PE=2 SV=1				
	ACTB_MESAU	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Mesocricetus auratus GN=ACTB PE=1 SV=1				
	ACTB_MOUSE	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Mus musculus GN=Actb PE=1 SV=1				
	ACTB_PANTR	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Pan troglodytes GN=ACTB PE=2 SV=1				
	ACTB_PIG	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8
	Actin, cytoplasmic 1 OS=Sus scrofa GN=ACTB PE=2 SV=2				
	ACTB_PONAB	Mass: 42052	Score: 93	Expect: 3.3e-05	Matches: 8

Table 5: List of identified landmark proteins of **PBMEC/C1-2** (PMF)

Protein name	Spot ID Master no*	Acc. number	PMF Score SwissProt**	Sequ. Cov. [%]	Matching peptides	t-test value	Av. Ratio
Catalase Pig	Spot C1	O62839	64	11	5	0.075	- 0.36
Pyruvate kinase isoenzyme	Spot C11	Q9UCV6	71	8	5	0.29	1.03
Actin Pig	Spot C12	Q6QAAQ1	93	25	8	-0.82	-1.23

Master no*: defined landmark protein via Ethan Dige analyses, PMF Score**: protein score greater than 64 are significant ($p < 0.005$)

pl 3 ← → pl 11



Positions of Spot C1, C11 and C12 at the PBMEC/C1-2, 2D Dige gel uninduced –marked with blue circles (pl 3 -11 NL, 12.5 %).

Figure 18: Identified landmark proteins of PBMEC/C1-2

Table 6: List of identified proteins of **HUVEC** (PMF)

Protein name	Spot ID Ma. no	Acc. nr. MSDB	Acc. nr. Sw.Prot	PMF Score *	Sequ. Cov. [%]	Matching peptides	t-test value	Av. Ratio
Serum Albumin (bov.)	Spot C6/ 498	AAI02743	P02769	102/84	19/6	11/10	0.023	-1.68
Retinal dehydrogenase 1 (h.s)	Spot C7/ 639	DEHUE1	P00352	98/98	18/18	9/9	0.032	-1.76
α-enolase (h.s)	Spot C8/ 626	Q8WU71	P06733	200/199	32/32	15/15	0.017	2.89
Annexin A5 (h.s)	Spot C9/ 979	1HVE	P08758	221/220	44/43	16/16	0.036	4.12
Hsp 27 (h.s)	Spot C11/ 1018	HHHU27	P04792	112/112	32/32	7/7	0.009	5.01

PMF Score*: protein score greater than 64 are significant ($p < 0.005$)

Table 7: Further identified landmark proteins of **HUVEC** (PMF)

Protein name	Spot ID Master no*	Acc. number	PMF Score **	Sequ. Cov. [%]	Matching peptides	t-test value ***	Av. Ratio
GAPDH (human)	Spot C2	P04406	73	17	5	0.083	-1.36
	Spot C 3	P04406	73	14	5	0.083	-1.36
	Spot C 4	P04406	67	14	8	0.083	-1.36

Master no*: defined landmark protein via Ethan Dige analyses, PMF Score**: protein score greater than 64 are significant ($p < 0.005$) , *** t-test value: the three spots were merged to one cluster protein

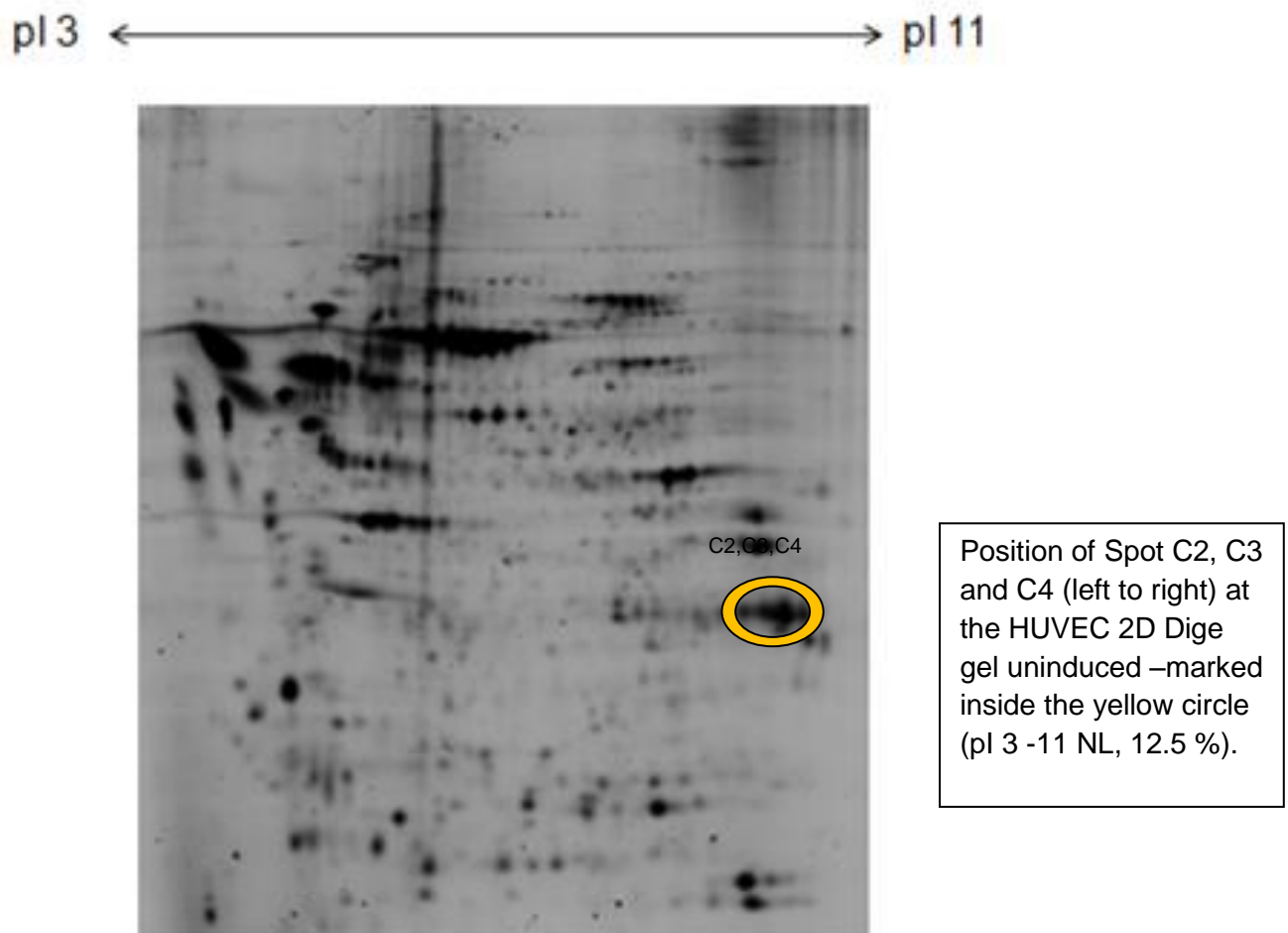


Figure 19: The proteome pattern of HUVEC cells (uninduced) shows inside the marked region (yellow circle) the spot cluster of Glyceraldehyde-3-phosphate dehydrogenase.

PMF spectrum and Mascot Search Results

Protein analysis was performed by the open source mass spectrometry tool mMass. PMF spectrometry was processed with the open source software. The identification was determined via database queries with the search engine Mascot (Matrix Science) in two different databases as Swissprot, MSDB. The protein identification was demonstrated by search results from spot C1, C12 (PBMEC/C1-2) and Spot C9 (HUVEC). The other results from the listed spots are attached in chapter Appendices (B PMF spectra and Mascot search results).

SPOT C1

Spot C1 was cut out from 12.5 % 2D gel, silver stained. The proteome pattern origin from porcine brain microvessel endothelial cells (PBMEC/C1-2).

Protein Identified:

Catalase (CATA PIG)

Search Parameters

Database:	SwissProt
Taxonomy:	Mammalia (mammals)
Enzyme:	Trypsin
Type of search:	Peptide Mass Fingerprint
Fixed modification:	Carbamidomethyl (C)
Variable modification:	Oxidation (MW), Oxidation (M)
Mass values:	Monoisotpic
Missed cleavages:	1
Mass tolerance:	+/- 200 ppm

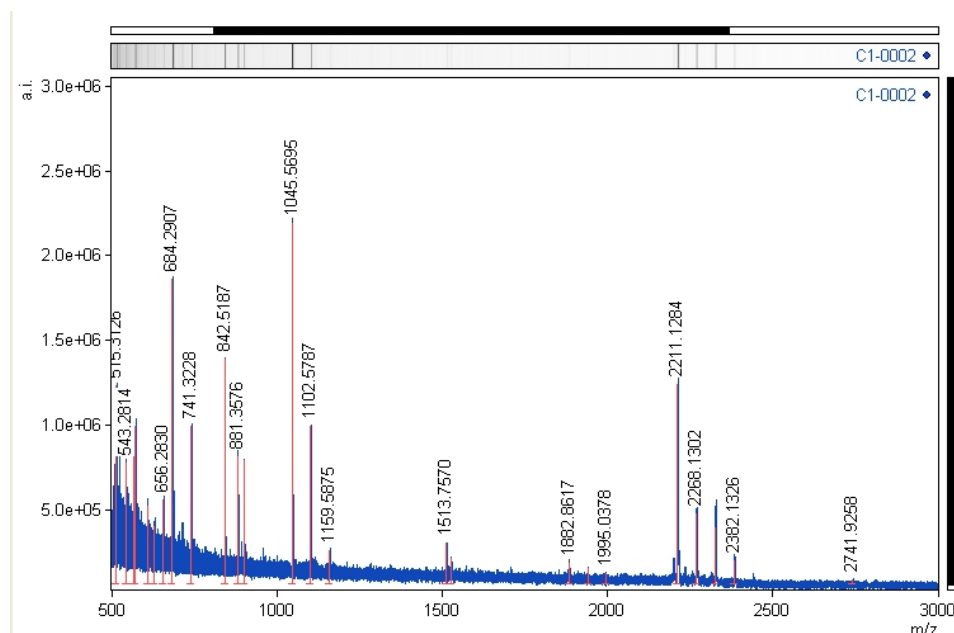


Figure 20: PMF spectrum of spot no. C1 (PBMEC/C1-2)

SPOT C12

Spot C1 was cut out from 12.5 % 2D gel, silver stained. The proteome pattern origin from porcine brain microvessel endothelial cells (PBMEC/C1-2).

Protein Identified:

Actin PIG

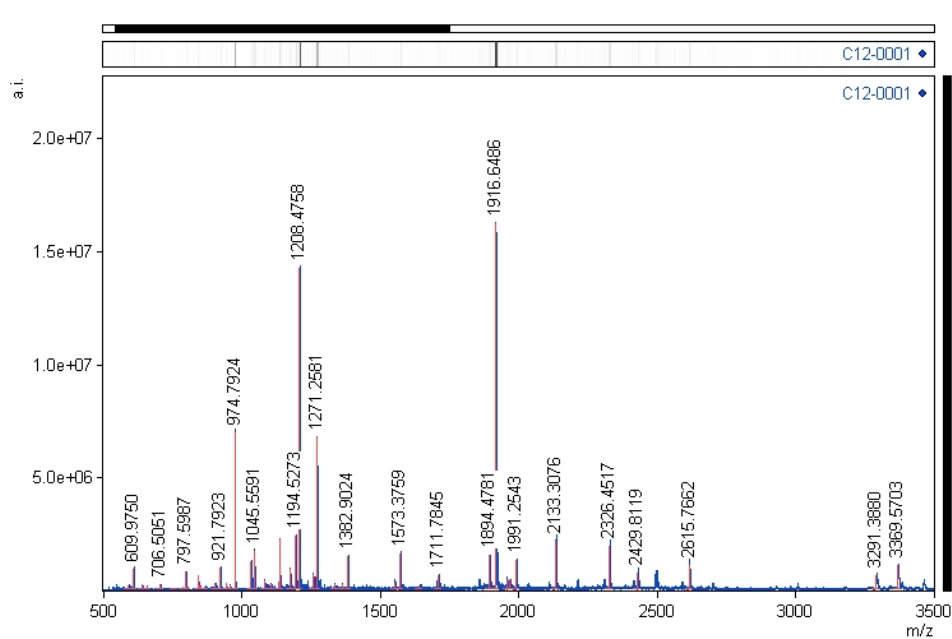


Figure 22: PMF spectrum of spot no. C12 (PBMEC/C1-2)

SPOT C9

Spot C1 was cut out from 12.5 % 2D gel, silver stained. The proteome pattern origin from human umbilical vein cells (HUVEC).

Protein Identified:

Annexin V (homo sapiens)

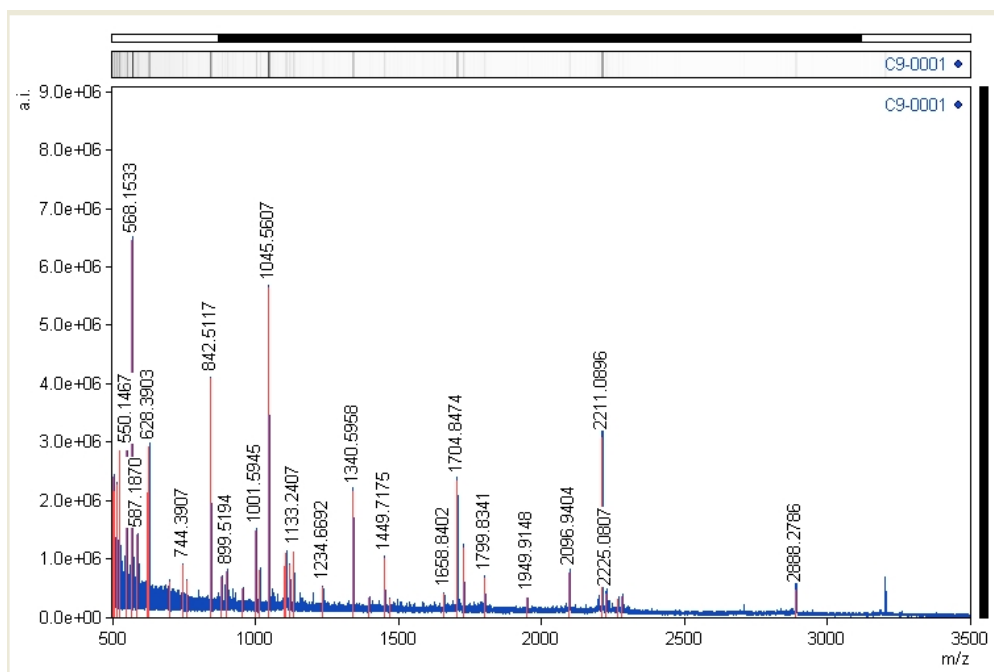


Figure 24: PMF spectrum of spot no. C9 (HUVEC)

The following PMF data were attached in chapter Appendices [B: PMF analysis data]

5 Discussion

Proteome analysis is an important method for systemic biological research. The proteome pattern and their characterization give information about a variety of proteins which carry out different functions in the organism. As example, some proteins handle structure roles, serve as essential metabolic coordinators or participate in signal transduction pathways. Additionally, a huge amount of diverse proteins exists and each has its unique structure compound that contributes to the specific function. The key issue of this work is to study the proteome pattern of proteins from two different blood-brain barrier (BBB) endothelial cells, porcine brain microvessel endothelial cells (PBMEC/C1-2) and human umbilical vein cells (HUVEC), respectively.

The permeability of the BBB has a major role in many diseases which is a hallmark for medical treatment. The properties of brain endothelial cells are strongly dependent on their surrounding cells which induce multiple factors, such as growth factors. According to literature reports (Rubin and Staddon 1999, Grant et al. 1998) it was found that astrocytes induce stimulating factors to the basal surface of brain capillary endothelial cells thereby contributing to the proper formation of the complete BBB phenotype. These chemical signals have effects on the induction and maintenance of the BBB. Additionally, those molecules influence the proper building of tight junctions which in turn are potential regulators of the brain permeability.

The overall purpose was to establish three Transwell models of PBMEC and HUVEC with different cell cultivation conditions. The main intension was the observation of changes in the proteome pattern inside the cultivation systems (uninduced, induced system and co-culture). The “uninduced system” implicates the cultivation in respective growth media and acts as control. In the “induced system” cells were cultured in astrocyte enriched media (ACM) to build up BBB specific properties. Finally the “co-culture system” has typical BBB features due to the experimental setup by co-cultivation with astroglial cells, isolated from rat brain.

Many BBB *in vitro* models exist and are more or less suitable to answer specific questions regarding brain pathology. Porcine brain endothelial cells (PBEC) are experimentally well established as *in vitro* models in human drug discovery research and are routinely used in clinical research (Neuhaus et al. 1991). General, it is

observed that astrocyte enriched media (ACM) also induce tight junction formation in BBB cells in comparison to co-cultivation with astrocytes. Both methods (ACM and co-culture) show efficient results in tight junction formation of BBB endothelial cells (Arthur et al. 1987). Moreover, astrocytes are able to induce tight junction properties in endothelial cells, also if they are not of nervous system origin (Janzer and Raff 1987). These findings are important for establishing BBB models with human endothelial cells which do not origin from brain like HUVECs.

Another aspect of this work is the comparison of the two different endothelial cell lines either of human or of porcine origin in terms of their suitability for proteomic investigations. In other words:

- 1 Will it be possible to identify changes in the proteome pattern due to different cultivation conditions?
- 2 Are porcine BBB endothelial cells (PBEC) comparable with human endothelial cells (HUVEC) for the investigation of specific brain issues using proteome analysis?

First the observation during this work with the **Transwell filter models** between the three different cultivation condition and the experimental cell lines (PBMEC/C1-2, HUVEC) will be discussed. For *in vitro* experiments cells were seeded (80.000 cells/cm²) directly on Transwell filter membranes which were embedded in suitable medium belonging to the cultivation model. The average cultivation period of PBMEC/C1-2 was about 4 days whereas HUVEC took approximately 5 days until reaching confluence, until the next step of cell lysis could follow. Interestingly, the gap between the cultivation duration lay at the induced system where the period differs up to three days. The density of the cell monolayer at Transwell filter systems was observed via visual checks under the microscope and **transendothelial electrical resistance (TEER)** measurements. Indeed, the interpretation of the TEER measurements was difficult because the values fluctuated among the probes. For co-culture system of porcine cells (PBMEC/C1-2) no valid TEER measurements could be conducted at the day before cell lysis. Probably the medium temperature between cultivation and calibration medium differed too much or another reason could be a loose connection of the meter. Independent measurements of two different operators excluded a handling error. Perhaps, a possibility to avoid fluctuation of TEER values

could be the use of an automated transepithelial/-endothelial measurement instrument such as cellZscope (nanoAnalytics, Münster, Germany). In summary no significant changes between the cultivation conditions were observed via TEER measurements. In contrast the comparison of the cell lines (PBMEC/C1-2, HUVEC) indicates changes in the cultivation period up to a maximum of six days.

Second the analysis of the **2D-DIGE** experiments from the cell line PBMEC/C1-2 and HUVEC showed a remarkable number of significantly changed proteins, within the three different cultivation systems. Further analysis of the gel series revealed a higher amount of significantly changed proteins within the proteome of the cell line PBMEC in comparison to proteome changes in HUVEC. The results showed different regulations of the proteome dependent on culture conditions and cell type indicating increased sensitivity of BBB cell line PBMEC/C1-2 to astrocytic signals compared to HUVECs. Significant changes in protein expressions of PBMEC were found for approximately 20 proteins in co-culture system versus uninduced system and about 30 proteins of induced system versus uninduced. In contrast the amount of significantly changed HUVEC proteins was smaller than of PBMEC. Almost 15 proteins could be detected for co-culture system and approximately 10 for induced system. This indicates a lower susceptibility of HUVECs for inducing factors derived from astrocytes. Some of the most remarkably changed proteins and defined landmark proteins out of Ettan DIGE software (version 7.0) were identified by MALDI-TOF MS experiments. The following table (Table 1: Biological functions of identified proteins) shows the function of the mass spectrometrically identified proteins from human and porcine cells.

Table 1: Biological function of identified proteins via MS analyses

Porcine brain microvessel endothelial cells (PBMEC/C1-2)		
Biological function	Name of protein)	Spot no.
Stress response	Catalase	Spot C1
Glucose metabolic process	Pyruvate kinase isoenzyme	Spot C11
Compound of cytoskeleton	Actin	Spot C12
Human umbilical vein endothelial cells (HUVEC)		

Biological function	Name of protein)	Spot no.
Regulation of blood pressure	Serum Albumin	Spot C6
Retinol metabolism	Retinal dehydrogenase 1	Spot C7
Glycolytic function	α -Enolase	Spot C8
Inhibition of blood coagulation	Annexin A5	Spot C9
Stress response	Hsp 27	Spot C11
Glucose metabolic process	GAPDH	Spot C2, C3, C4

Table1: List of significant changing proteins and defined landmark proteins which are detected by MALDI TOF

The listed proteins have several functions in metabolic processes or are involved in response to stress and regulation of blood homeostasis. A number of those proteins have important regulatory effects and act as biomarkers for diagnosing pathological circumstances. The following examples demonstrate the role of three identified proteins in the body under pathological disorders.

According to literature (Habib et al. 2010) **Catalase** is involved in interactions for cell protection by catalyzing hydrogen peroxide (H_2O_2). This publication indicates the correlation of accumulation from the neurotoxic β -amyloid and oxidative stress inside the brain. These effects are often described in combination with Alzheimer disease (AD). In this case the activity of catalase is blocked by the interaction with harmful $A\beta$ peptides which leads to the increase of high intracellular H_2O_2 levels. This study provides the importance of catalase for cell protection against induced oxidative stress by the interaction with $A\beta$.

Alpha-enolase belongs to the family of cytoplasmic and glycolytic enzymes. The main function of this enzyme is not clear yet but it acts in a variety of infectious and autoimmune diseases. It has a major role in glycolytic processes of eukaryotes and prokaryotes (Terrier et. al 2006).

Heat shock protein (Hsp) 27 is a stress responded protein that has repair and protective functions in the organism. Hsp27 is a common marker for tissue damage caused by oxidative stress. Corresponding to the publication (Bechtold and Brown 2003) about the induction of Hsp27 in glial cells of mammalian rat brains during

hyperthermia it is documented that hyperthermia induces a rapid influx of heat shock proteins as Hsp27. In this study it is observed that the rapid induction of Hsp 27 to glial cells promotes the repair of stress induced cellular damage in during hyperthermia.

Third the following questions which came up before starting the experimental part of this thesis, will be discussed.

1. Will it be possible to identify changes in the proteome pattern due to different cultivation conditions?

The changes of the proteome pattern inside the three different cultivation conditions could be documented via DIGE analyses. There are significant changes in the proteome pattern of PBMEC/C1-2 and HUVEC. In comparison of the two astrocytic cultivation systems (co-culture, induced) with the uninduced system remarkable changes in the protein expression structure could be demonstrated. The number of significantly changed proteins differs between co-culture system and induced system up to a maximum of ten proteins. For PBMEC/C1-2 the induced system shows a higher amount of proteins with changing expression pattern compared to the co-culture system. Interestingly, in contrast HUVEC reached the maximum of significantly changed proteins in the co-culture system. Summarizing, changes in the proteome pattern due to different cultivation conditions could be identified.

2. Are porcine BBB endothelial cells (PBEC) comparable with human endothelial cells (HUVEC) for the investigation of specific brain issues using proteome analysis?

Based on the number of changes in the proteome, human umbilical vein cells seem to be susceptible for Blood-brain barrier inducing factors, but to a lower extent than PBMEC/C1-2. During cell cultivation and mass spectrometry analyses no remarkable differences in handling the experiments could be determined. According to literature (Janzer and Raff 1987) it was documented that astrocytes are able to induce tight junction properties in endothelial cells, also if they are not of nervous system origin. This knowledge out of the publication was an important requirement in this work for choosing the proper human cell line to conduct *in vitro* BBB studies. Based on results from literature and the experiments of this study, porcine BBB endothelial cells (PBEC) are suitable to compare them with human endothelial cells (HUVEC) for the investigation of specific brain studies on the proteome.

Concluding, the results of this thesis demonstrate the a profound alteration of the proteome caused by astrocyte induction and can serve as a starting point for systemic observation of the mechanism and metabolic pathways that are involved.

6 Zusammenfassung

Zielsetzung dieser Arbeit ist die Untersuchung von **Dysfunktionsstudien** an der Blut-Hirn Schranke (BBB) um potentielle „molecules of interest“ zu identifizieren. Durch die Herangehensweise des modernen **systempharmakologischen Ansatzes** liegt der Focus auf einer systematischen Analyse der „**BBB als pharmakologisches drug target**“ für *in vitro* Modellversuche. Modellstudien dienen als Vorstufe um Krankheitsmodelle zu verstehen und diese im weiteren Sinne auf die Situation des Patienten zu rekonstruieren.

Die systempharmakologischen Studien dieser Masterarbeit liegen vor allem in der Untersuchung des Proteoms von Blut-Hirn Schranken Endothelzellen und dessen potentieller Veränderungen unter Einfluss verschiedener Kultivierungsbedingungen.

Die Experimente wurden jeweils mit humanen Nabelschnur Endothel- (HUVEC) und porcinen Hirnkapillar-Endothel- (PBMEC/C1-2) Zelllinien durchgeführt. Die getesteten Kultivierungsbedingungen wurden in folgende Ansätze unterteilt: **Co-Kultur**; **Induziert** und **Nicht induziert**. Die Systeme Co-Kultur und Induziert beinhalten Faktoren, die Blut-Hirn Schranken Eigenschaften in Endothelzellen induzieren, wodurch sich eine Veränderung der sog. „tight junctions“ innerhalb der Zellen ergibt. Bei dem System Co-Kultur wird die experimentelle Endothelzelllinie gemeinsam mit Astrozyten, welche aus Rattenhirn isoliert wurden, kultiviert. Das System Induziert wird ausschließlich mit der experimentellen Endothelzelllinie durchgeführt, jedoch in einem Astrozyten-konditionierten Kultivierungsmedium (ACM).

Mittels kontinuierlichen elektrischen Widerstandsmessungen (**TEER**) wurde die Zelldichte in den Kultivierungsmodellen bestimmt, um die Ausbildung eines dichten Monolayers zu verifizieren und anschließend die Zellen zu lysieren. Die Proteomanalyse der Zellextrakte erfolgte durch **2-D Gel Elektrophorese** und **MALDI TOF MS** Technik. Anschließend erfolgte eine gezielte Selektion der Proteine von Interesse (POI), welche signifikante Expressionsänderungen aufwiesen.

Einige dieser Proteinspots wurden massenspektrometrisch identifiziert, darunter z.B. Heat shock protein 27 (HSP27), Annexin A5, Aldehyddehydrogenase und Catalase.

Diese biologisch relevanten Proteine sind „**target Moleküle**“ bei **Neurodegeneration**, oxidativen Stress, spielen eine Rolle in Entzündungsprozessen und sind Regulationsfaktoren der endothelialen Homöostase. Die Ergebnisse dieser

Arbeit sind grundlegende Elemente einer **pilot Studie** und stellen weitere Ausgangspunkte für die fortlaufende Untersuchung von neurodegenerativen Vorgängen sowie weitere Aufklärung von Mechanismen zur Ausbildung von Blut-Hirn-Schranken-Eigenschaften in Endothelzellmodellen dar.

7 Summary

The aim of this work implicates modern **system pharmacological studies** which is the elucidation of complex physiological and pathological cellular processes and the analysis and identification of **molecules of interests**. The overall purpose of this study is to observe **dysfunction processes** of the blood-brain barrier (BBB) which observe leads to pathological circumstances caused by neurodegeneration.

This **pilot study** focuses on the **proteomic analysis** of BBB *in vitro* models, the influence of different cultivation conditions on barrier tightness and the proteomic pattern.

Experiments were conducted with human umbilical vein endothelial cells (HUVEC) and porcine brain microvascular endothelial cells (PBMEC/C1-2). The *in vitro* investigations were conducted under three different cultivation conditions to observe changes on the proteome pattern. Both cell lines were cultivated as follows (a) “**co-culture system**”, (b) “**induced system**”, (c) “**uninduced system**”. The “co-culture system” comprises of the experimental cell lines (PBMEC/C1-2 or HUVEC) and astrocytic glial cells origin from rat brain. The “induced system” consists of enriched medium containing astrocytic factors (ACM) to induce BBB characteristics by changes in tight junctions (TJ). It is known that astrocytic derived soluble factors may induce the expression of BBB properties in endothelial cells *in vitro* (Janzer and Raff 1987)

Using transendothelial electrical resistance (**TEER**) measurements to verify the tightness of the cellular monolayer in **Transwell filter systems**. Subsequently, cells were lysed for proteomic analysis with **2D DIGE** and **MALDI TOF MS**. Statistical analyses revealed differentially expressed protein spots.

The identification of “**molecules of interests**” which indicate significantly changes of the proteome pattern inside the different cultivation conditions was performed via mass spectrometry. Examples for identified proteins as heat shock protein 27 (HSP27), annexin A5, aldehyde dehydrogenase and catalase are target molecules for neurodegeneration oxidative stress, play a role in inflammatory processes and are regulation factors of endothelial homeostasis.

The results of this pilot study are profound starting points for further dysfunctional research to elucidate the biological mechanisms of the induction BBB characteristics in cell culture models.

References

- Abbott* JN, Patabendige A, Dolman D, Yusof SR, Begley DJ (2010) Structure and function of the blood-brain barrier. *Neurobiol. of Diseases* 37:13-25
- Abbott* JN, Rönnbäck L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. *Rev. Neurosci.* 7:41-53
- and angiogenesis. *Vasc. Pharm.* 49:32-36
- Arthur* FE, Shivers RR, Bowman PD (1987) Astrocyte-mediated induction of tight junctions in brain capillary endothelium: An efficient in vitro model. *Dev. Brain Res.* 36: 155-159
- Ballabh* P, Braun A, Nedergaard M (2003) The blood-brain barrier: an overview structure, regulation and clinical implications. *Neurol. Dis.* 16:1-13
- Banks* WA (1999) Physiology and pathology of the blood-brain barrier: implications for microbial pathogenesis, drug delivery and neurodegenerative disorders. *Journal NeuroVirology* 5:538-555
- Bechtold* DA and Brown IR (2003) Induction of Hsp27 and Hsp32 Stress Proteins and Vimentin in glial Cells of the Rat Hippocampus following Hyperthermia. *Neurochem. Research* 28: 1163-1174
- Blum* H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8: 93-99
- Bradbury* M (1979) The concept of a blood-brain barrier, J Wiley, Chichester
- Bradford* M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254
- Carvey* PM, Hendey B, Monahan AJ (2009) The blood-brain barrier in neurodegenerative disease: a rhetorical perspective. *Journal Neurochem.* 111:291-314

Cucullo L, Aumayr B, Rapp E, Janigro D (2005) Drug delivery and *in vitro* models of the blood-brain barrier. *Curr. Opin. Drug. Discov Devel.* 8:89-99

Deli MA, Abraham CS, Kataoka Y, Niwa M (2005) Permeability studies on *in vitro* blood-brain barrier models: physiology, pathology, and pharmacology. *Cell. Mol. Neurobiol.* 25: 59-127

Ehrlich P (1904) Über die Beziehung chemischer Konstitution, Verteilung, und pharmakologischer Wirkung. *Gesammelte Arbeiten zur Immunitätsforschung*, Berlin

Engelhardt B (2003) Development of the blood-brain barrier. *Cell tissue Res.* 314: 119-129

Engelhardt B, Sorokin L (2009) The blood–brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Semin. Immunopathol.* 31:497-511

Fischer S, Wobben M, Kleinstuck J, Renz D, Schaper W (2000) Effect of astroglial cells on hypoxia-induced permeability in PBMEC cells. *Journal Cell Physiol.* 198: 359-369

Furuse M, Furuse K, Sasaki H, Tsukita S (2001) Conversion of Zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney 1 celly. *Cell Biol.* 153: 263-272

Furuse M, Sasaki H, Tsukita S (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. *Cell Biol.* 147: 891-903

Grant GA, Abbott JN, Janigro D (1998) Understanding the Physiology of the Blood-Brain Barrier: *In Vitro* Models. *News Physiol.Sci* 13:287-293

Habib LK, Lee MTC, Yang J (2010) Inhibitors of Catalase-Amyloid Interactions Protect Cells from β -Amyloid-Induced Oxidative Stress and Toxicity *J. Biol. Chem.* 285:38933-38943

Hawkins BT and Davis TP (2005) The Blood-Brain Barrier/Neurovascular Unit in Health and Disease. *Pharmacol. Rev.* 57:173-185

Hayashi K, Nakao S, Nakaoke R, Nakagawa S, Kitagasw N, Niwa M (2004) Effects of hypoxia on endothelial/pericytic co-culture model of the blood-brain barrier. Regul. Pept. 123: 77-83

Helm F (2010) Präsentation Methodenseminar, Universität Heidelberg

Hirase T, Staddon JM, Saitou M, Ando-Akatsuka Y, Itoh M, Furuse M, Fujimoto K, Tsukita S, Rubin LL (1997) Occludin as a possible determinant of tight junction permeability in ECs. Cell Sci. 110: 1603-1613

Huber JD, Richard ED, Davis TP (2001) Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. TRENDS in Neurosci. 24: 719-7125

Janzer RC and Raff MC (1987) Astrocytes induce blood-brain barrier properties in endothelial cells. Nature 325:253-256

Kniesel U, Wolburg H (1998) Tight Junctions of the Blood-Brain Barrier. Cell. and Mol. Neurobiol. 20: 57-75

Lacaz-Vieira F, Jaeger M.M.M, Farshori P, Kachar B (1999) Small synthetic peptides homologous to segments of the first loop of occluding impair tight junction resealing. Membrane Biol. 168: 289-297

Liebler DC (2002) Introduction to Proteomics – Tools for the New Biology, Humana Press

Liebner S, Fischmann A, Rascher G, Duffner F, Grote EH, Wolburg H (2000) Claudin-1 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. Acta Neuropathol. 100: 323-331

Martinez-Estrada MO, Villa A, Brevario F, Orsenigo F, Dejana E, Bazzoni G (2001) Association of junctional adhesion molecules with calcium/calmodulin-dependent serine protein kinase (CASK/LIN-2) in human epithelial Caco-2 cells. Biol. Chem. 276: 9291-9296

Mattow J, Schmidt F, Hohenwarter W, Siejak F, Schaible UE, Kaufmann SH (2004) Protein identification and tracking in two-dimensional electrophoretic gels by minimal protein identifiers. *Proteomics* 4:2927-41

Moody DM (2006) The blood-brain barrier and blood-cerebral spinal fluid barrier. *Sem. Cardiothorac. Vasc. Anesth.* 10: 28-31

Neuhaus J, Risau W, Wolburg H (1991) Induction of blood-brain barrier characteristics in bovine brain endothelial cells by rat astroglial cells in transfilter coculture. *Ann NY Acad. Sci.* 633: 578-580

Neuhaus W (2008) Doctoral thesis: Development and Validation on *In vitro* Models of the Blood-Brain Barrier

Rubin LL, Standdon JM (1999) The Cell Biology of the Blood-Brain Barrier. *Rev. Neurosci.* 22:11 – 28

Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Nodas T, Tsukita S (2000) Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands. *Mol. Biol. Cell* 11: 4131-4142

Sakakibara A, Furuse M, Saitou M, Ando-Akatsuka Y, Tsukita S (1997) Possible involvement of phosphorylation of occluding in tight junction formation. *Cell Biol.* 137: 1393-1401

Smirnov IP, Zhu X, Taylor T, Huang Y, Ross P (2004) Papayanopoulos IA, et al. Suppression of alpha-cyano-4-hydroxycinnamic acid matrix clusters and reduction of chemical noise in MALDI-TOF mass spectrometry. *Anal Chem* 76: 2958-65

Stessl M, Marchetti-Deschmann M, Winkler J, Lachmann B, Allmaier G, Noe CR (2009) A proteomic study reveals unspecific apoptosis induction and reduction of glycolytic enzymes by the phosphorothioate antisense oligonucleotide oblimersen in human melanoma cells. *Journ. Proteomic* 72: 1019-1030

Strohalm M, Hassman M, Košata B, Kodíček M: mMass data miner: an open source alternative for mass spectrometric data analysis. *Rapid Commun Mass Spec* 22 (6), 905-908 (2008).

Strohalm M, Kavan D, Novak P, Volny M, Havlicek V: mMass 3: A Cross-Platform Software Environment for Precise Analysis of Mass Spectrometric Data. *Anal Chem* 82 (11), 4648-51 (2010).

Suda K, Rothen-Rutishauser B, Günthert M, Wunderli-Allenspach H (2001) Phenotypic characterization of human umbilical vein endothelial (ECV304) and urinary carcinoma (T24) cells: endothelial versus epithelial features. *In vitro Cell. Dev. Boil.-Animal* 37, 505-514

Terrier B, Nicolas D, Philippe G, Servettaz A, Guillevin L, Mouthon L (2006) Alpha-enolase: A target of antibodies in infectious and autoimmune diseases. *Autoimmunity Reviews* 6: 176-182

Wolburg H and Lippoldth A (2002) Tight junctions of the blood-brain barrier: Development, composition and reugulation. *Vascul. Pharmacol.* 38: 323-337

Yang JP, Taeseung L, Jongwon H, In MJ, Jung KC, Sang JK (2008) Effect of nicotine on human umbilical vein endothelial cells (HUVECs) migration

[6] Anmerkung: Urheberrecht

„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“

Appendices

A CV /Lebenslauf

Lebenslauf

Persönliche Daten

Name Christiane Gebhard
Anschrift Voltagasse 43/12/1
1210 Wien

Tel: 0650/8096592
Email: gebhard_christiane@gmx.at

Geboren am 2. September 1983
Familienstand ledig

Schulbildung

1990 – 1994 Volksschule in Wien 21, Priessnitzgasse
1994 – 1998 Unterstufe am Gymnasium BRG 1 Schottenbastei
1998 – 2003 Handelsakademie des Fonds der Wr. Kaufmannschaft in
Wien 21., Franklinstrasse
Abschluss mit Matura

Studium

Universitätsstudium

2003 - 2008 Bachelorstudium Lebensmittel und Biotechnologie an der
Universität für Bodenkultur Wien

2007-2008 Mitbelegung an der Uni Wien, Absolvierung der Auflagen für
den Einstieg in das Masterstudium Molekularbiologie

2008 – 2011 Masterstudium Molekularbiologie an der Universität Wien
Spezialisierung: Neurowissenschaften am Zentrum für
Hirnforschung der Med. Uni Wien

Masterarbeit

„Differential proteome analysis of blood brain barrier endothelia
cells in vitro models“
Betreuung: Univ.Prof.DI.Dr.Christian R. Noe

Abschluss Master Science (MSc)

Fachhochschulstudium

2006- 2010 FH-Diplomstudium Bioengineering an der FH-Campus Wien
Spezialisierung: Qualitätsmanagement

Diplomarbeit

"Untersuchungen zur Bestimmung der Wiederfindung von Keimen
auf Textilien im Kontext der nosokomialen Keimübertragung"
Betreuung: Univ.Prof.DI.Dr. Rudolf F. Bliem

Abschluss

DI (FH) für technisch-wissenschaftliche Berufe

Berufliche Qualifikation

2009 Auditor für ISO 9001
Zertifizierungsstelle AuM der Technischen Universität Wien

2010 Zertifikat Quality Manager

Öffentliche Präsenz

2009 Präsentation des wissenschaftlichen Posters zum Thema der
FH- Diplomarbeit ; an der Poster Session der FH-Campus Wien;
Universität für Bodenkultur (BOKU)

2010 Komiteemitglied sowie Vortrag zu ISO 9001
„Patientensicherheit und Mitarbeiterzufriedenheit bei der
Zertifizierung öffentlicher Gesundheitseinrichtungen“;
AK Wien

2010 Präsentation des wissenschaftlichen Posters zum Thema der
Uni – Masterarbeit; bei der 4th Central and Eastern European
Proteomics Conference (CEEPC meets IMA);
Technische Universität Wien (TU)

Praktika und Berufserfahrung

2011 Forschungsaufenthalt an der Universität Heidelberg
Abteilung für Pharmazie und Molekulare Biotechnologie

2010 Universitätsassistentin (pre doc)
am Institut für Medizinisch/Pharmazeutische Chemie
Universität Wien

2010	Uni-Masterarbeit an der Pharmazeutischen Universität und Kooperation mit der Technischen Universität Institut für Medizinische/Pharmazeutische Chemie
2009 – 2010	FH- Diplomarbeit an der Universität für Bodenkultur Institut für angewandte Mikrobiologie (IAM)
2009	Trainee bei der Firma TÜV AUSTRIA CERT GmbH Bereich Lebensmittelaudit ISO 22000 und ISO 9001 Auditerfahrung im In- und Ausland (Bsp.: Kröswang, Alondo „Bio.k.“, Wozabal, „Air caterer Five Star“, Wilhelminenspital)
2008	Praktikum im Bereich der Neurowissenschaft an der Veterinärmedizinischen Universität Wien Dauer: 3 Monate
2006 - 2008	Geringfügig Beschäftigte bei der Firma Metro Langenzersdorf in der EDV-Abteilung
2004	Praktikum an der Universität für Veterinärmedizin Institut für Pharmakologie und Toxikologie Dauer: 2 Monate

Sonstige Qualifikationen

Sprachen	Deutsch, Englisch, Spanisch
EDV	Microsoft Office, SPSS,
Hobbys	Rennrad und MTB Hobbymannschaft, Klettern, Yoga, Kunst und Literatur
Soziale Interessen	Volontariat im SOS Kinderdorf Dauer: 3 Monate Betreuerin von Jugendlichen im Ferienhort am Wolfgangsee Dauer: jeweils 6 Wochen Betreuerin von verhaltensauffälligen Kindern im Jugenddorf Waldviertel Dauer: 2 Wochen

B PMF analysis data

HUVEC

Search Parameters

Database:	SwissProt /MSDB
Enzyme:	Trypsin
Type of search:	Peptide Mass Fingerprint
Fixed modification:	Carbamidomethyl (C)
Variable modification:	Oxidation (MW), Oxidation (M)
Mass values:	Monoisotpic
Missed cleavages:	1

Spot ID/ Master no.: **Spot C6 / 498**

Mass tolerance: +/- 200 ppm

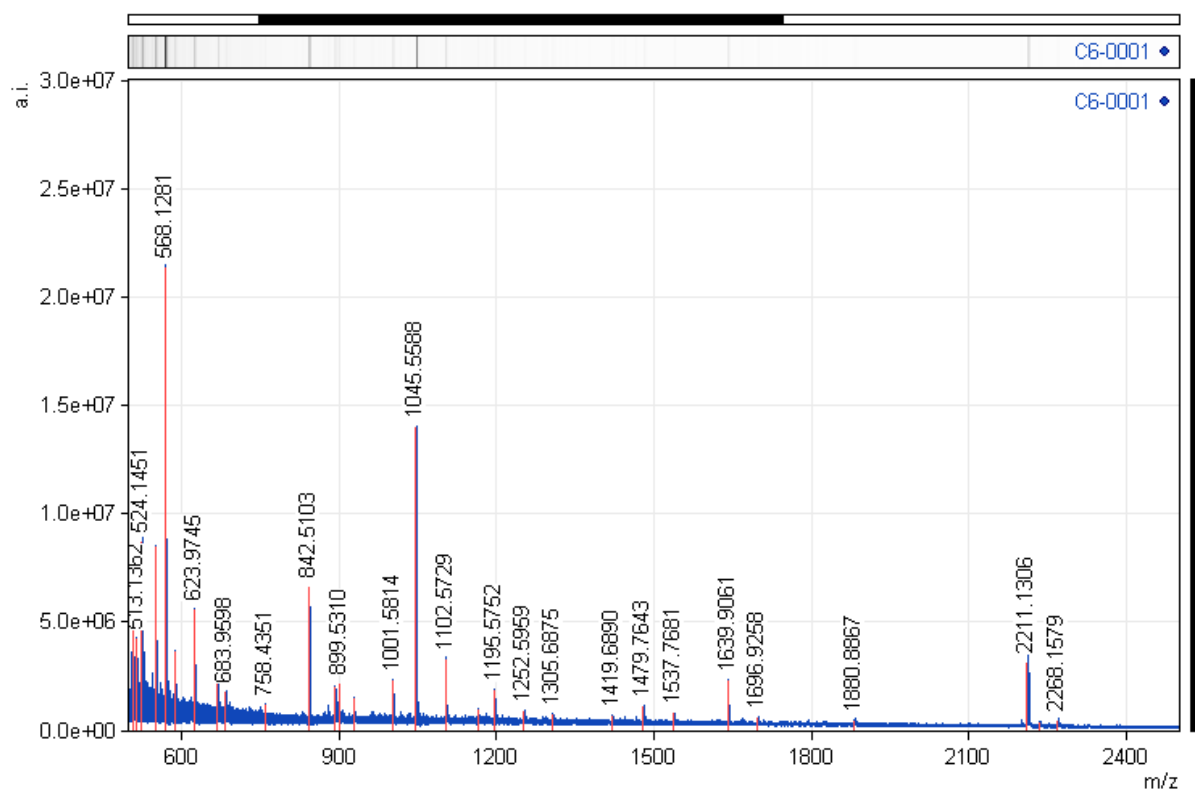


Figure: PMF spectrum of spot no. C6 (HUVEC)

Spot ID/ Master no.:

Spot C7 / 639

Mass tolerance:

+/- 100 ppm

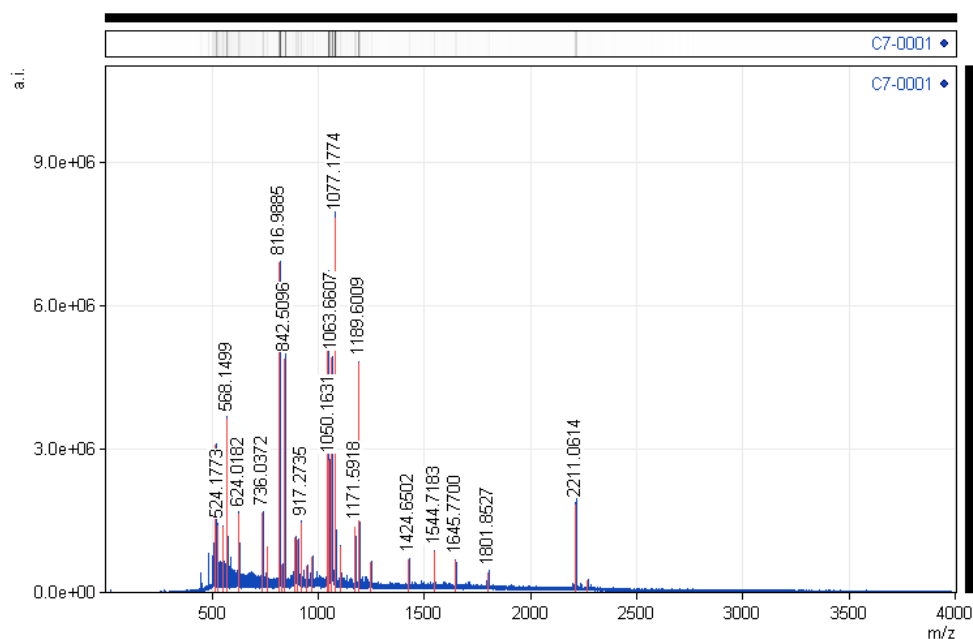


Figure: PMF spectrum of spot no. C7 (HUVEC)

Spot ID/ Master no.:

Spot C8 / 626

Mass tolerance:

+/- 200 ppm

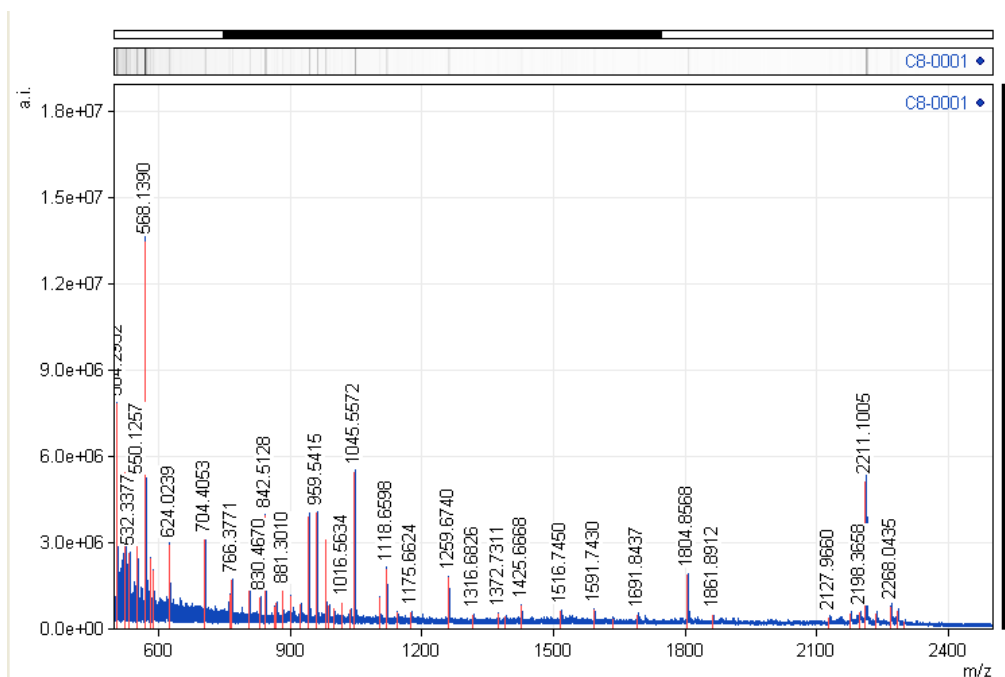


Figure: PMF spectrum of spot no. C8 (HUVEC)

Spot ID/ Master no.:

Spot C11 / 1018

Mass tolerance:

+/- 200 ppm

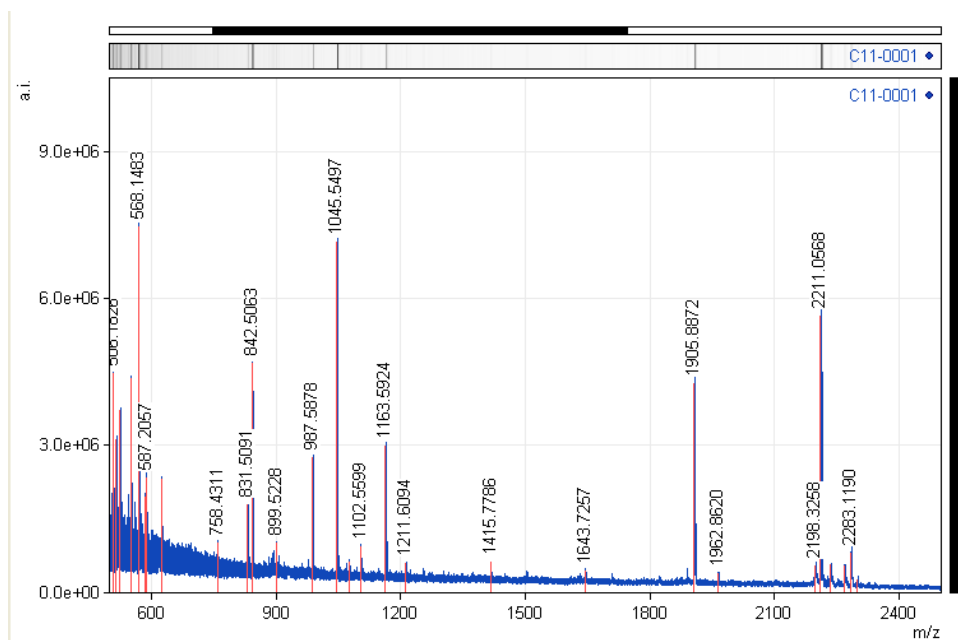


Figure: PMF spectrum of spot no. C11 (HUVEC)

PBMEC/C1-2

Spot ID/ Master no.:

Spot C11

Mass tolerance:

+/- 200 ppm

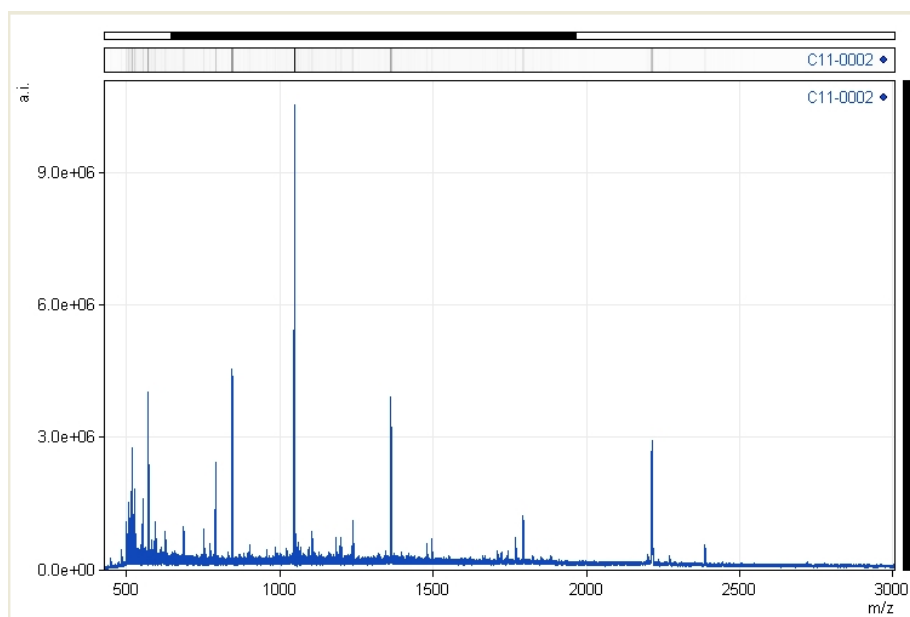


Figure: PMF spectrum of spot no. C11 (PBMEC/C1-2)