EFFECT OF THE CELL POLARIZATION ON ENDOCYTIC PROPERTIES OF THE
HUMAN BRAIN ENDOTHELIAL CELLS

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Tiivistelmä/Referat – Abstract

Blood-brain barrier (BBB) is a physical barrier between the blood and the brain. BBB restricts drugs transport from blood stream to the brain, which sets challenges in drug delivery to the brain. Nanoparticles can be utilized in drug delivery to the central nervous system (CNS). Nanoparticles are internalized via endocytosis. However it remains unknown which endocytic pathways are active in brain endothelial cells. The characterization of BBB cells would help light on the exact mechanism of nanoparticle delivery into the brain, which would enable the design of targeted nanoparticles to deliver drugs to the CNS. In present study we characterized human brain endothelial cells, hCMEC/D3, which are widely utilized as BBB in vitro model. As brain endothelial cells are polarized *in vivo,* the aim of the study was to demonstrate the cell polarization of hCMEC/D3 cells and to study the activity and functionality of different endocytic pathways as a function of cell polarization.

The level of cell polarization in cells grown on transwell permeable supports was characterized at multiple timepoints with four different methods: transepithelial electrical resistance (TEER) measurement, lucifer yellow permeability assay, alkaline phosphatase expression and ZO-1 expression. To characterize hCMEC/D3 cells for the presence of specific endocytic pathways, proteins involved into each pathway were selected. Expression of these proteins at mRNA level was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). For clathrin-mediated endocytosis, mRNA level of CHC protein was further correlated with the protein level of this protein, and the activity of clathrin-mediated endocytosis was analyzed by fluorescence activated cell sorting (FACS).

Our results showed that hCMEC/D3 cells are best polarized after growing on transwell permeable support for 7 days. At the later timepoints, the cell polarization started to decrease, probably due to multilayer formation. We concluded that measuring TEER alone is not a reliable method to determine polarization status of the cells. mRNA levels of endocytosis-related proteins did not change remarkably as a function of cell polarization. In case of clathrin-mediated endocytosis, there was lack of correlation between CHC mRNA and protein level, but good correlation between mRNA level and activity of the pathway.

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Tiivistelmä/Referat – Abstract

Veriaivoeste on fyysinen este veren ja aivojen välillä. Veriaivoeste estää joidenkin lääkkeiden kulkeutumisen verenkierrosta aivoihin, mikä asettaa haasteita annosteltaessa lääkkeitä aivoihin. Nanopartikkeleita voidaan käyttää hyödyksi lääkkeiden kuljettamiseksi keskushermostoon. Nanopartikkelit kulkeutuvat soluihin endosytoosin avulla. Tarkkaan ei tiedetä, mitkä aivojen endoteelisolujen endosytoosireitit ovat aktiivisia. Veriaivoestesolujen karakterisointi voi auttaa löytämään oikean endosytoosimekanismin, johon nanopartikkeli voitaisiin kohdentaa ja näin saada lääkeaine kuljetettua keskushermostoon kohdennettujen nanopartikkelien avulla. Tässä tutkimuksessa karakterisoimme ihmisen aivojen endoteelisoluja, hCMEC/D3:a, joita on laajalti käytetty veriaivoesteen *in vitro* mallina. Koska aivojen endoteelisolut ovat polarisoituneet *in vivo*, tutkimuksen tarkoituksena oli osoittaa hCMEC/D3 solujen polarisoituminen sekä tutkia eri endosytoosireittien aktiivisuus ja toiminnallisuus polarisaation eri tasoilla.

Puoliläpäisevällä kalvolla kasvatettujen solujen polarisaatioaste karakterisoitiin useassa aikapisteessä, neljää eri metodia käyttäen: transepiteelisen elektronisen resistanssin (TEER) mittaus, lucifer yellow permeaatio, alkaalisen fosfataasin ilmentyminen ja ZO-1:n todentaminen. hCMEC/D3 soluille tunnusomaiset endosytoosireitit määritettiin reitille ominaisien proteiinien avulla. Näiden proteiinien ilmentyminen tutkittiin mRNA-tasolla kvantitatiivisen reaaliaikaisen polymeraasiketjureaktion (qRT-PCR:n) avulla. Klatriiniriippuvaiselle endosytoosimekanismille ominaisen proteiinin, CHC:n, mRNA tuotantoa verrattiin proteiinituotantoon ja tämän endosytoosimekanismin aktiivisuus analysoitiin fluoresenssiaktivoidun solulajittelun (FACS) avulla.

Tulostemme perusteella hCMEC/D3 solut ovat parhaiten polarisoituneita, kun ne ovat kasvaneet 7 päivän ajan puoliläpäisevällä kasvatusalustalla. Tämän jälkeen polarisaatio heikkeni todennäköisesti siksi, että solut alkoivat kasvaa päällekkäin. Tulosten perusteella päättelimme, että TEER yksinään ei ole riittävä metodi määrittämään solujen polarisaatiota. Endosytoosiriippuvaisten proteiinien mRNA tasot eivät muuttuneet merkittävästi polarisaation muuttuessa. Klatriiniriippuvaisen endosytoosin kohdalla havaitsimme huonon korrelaation CHC:n mRNA ja proteiinituotannon välillä, mutta hyvän korrelaation proteiinin mRNA tuotannon ja endosytoosireitin aktiivisuuden välillä.

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veriaivoeste, solujen polarisoituminen, endosytoosi, nanopartikkeli

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1 INTRODUCTION

Blood-brain barrier (BBB) is a physical barrier between the blood and the brain, which function is to maintain brain homeostasis by transporting vital nutrients to the brain but at the same time protecting from harmful compounds (de Lange, 2010). The main component of the BBB is a monolayer of polarized endothelial cells sealed with tight junctions. Many potential drugs for the treatment of diseases of the central nervous system (CNS) are unable to reach the brain in sufficient amounts because of this barrier. Number of new drug candidates for the treatment of CNS diseases has grown, but only few of them enter the market (Pardridge, 2002).

Drugs that are intended to act in the CNS can be administered systemically if they are able to pass the BBB or otherwise they need to be introduced directly in the CNS by invasive methods. Polymeric, biodegradable nanoparticles can provide one possibility to deliver drugs to the CNS by noninvasive way (Wolfart et al, 2012). Nanoparticles are submicron sized particles that can be associated with a drug without any modification of the drug molecule. Many studies have shown that nanoparticles are able to overcome the BBB as well as to produce pharmacodynamic effect on the CNS.

Because of the relatively large size, nanoparticles cannot cross the cell membrane and therefore are internalized via endocytosis (Hillaireau and Couvreur, 2009). Endocytosis in the brain endothelial cells is not fully understood. It remains unclear, which endocytic mechanisms are present in brain endothelial cells, which are their functions and how they are regulated (Smith and Gumbleton, 2006 ; Sandvik et al, 2011). Brain endothelial cells are polarized *in vivo*. It is known that cell polarization regulates endocytic processes and endocytic activity in the brain endothelial cells is rather low. In this study, we wanted to characterize the effect of cell polarization on endocytic pathways in human blood brain barrier *in vitro* model. The characterization of BBB cell model for endocytosis aids to employ nanoparticles for targeted brain delivery of molecules across the BBB.

2 ENDOCYTOSIS AND ITS ROLE IN DRUG DELIVERY

2.1 Types of endocytosis

Endocytosis is an energy-dependent process of cellular ingestion in which the plasma membrane folds inward to bring extracellular molecules into the cell (Doherty and McMahon, 2009). The main function of the endocytosis is uptake of nutrients, but it also controls the composition of the plasma membrane and how the cells interact with and respond to their environments. Endocytosis is typically classified into phagocytosis, restricted to specialized mammalian cells, and pinocytosis, occurring in all cell types. The phagocytosis is uptake of solid particles with diameter of few micrometers and pinocytosis is uptake of fluids containing particles in the nanosize range.

The most modern classification of endocytosis in based on lipid rafts (El-Sayed and Harashima, 2013). Clathrin-mediated endocytosis (CME) was first described endocytic mechanism and it was long thought to be the only pathway (Sandvik et al, 2011). CME is also the best studied endocytic mechanism, which takes place in non-lipid raft regions of the cell membrane. Lately more pathways have been discovered, such as lipid raft dependent endocytosis, which includes caveolae-mediated endocytosis, flotillin-dependent endocytosis, CLIC/GEEC endocytosis, Arf6-dependent endocytosis and RhoA-dependent endocytosis. Phagocytosis and macropinocytosis are endocytic mechanisms that transport large particles and are expected to contain both lipid raft and non-raft regions of the membrane.
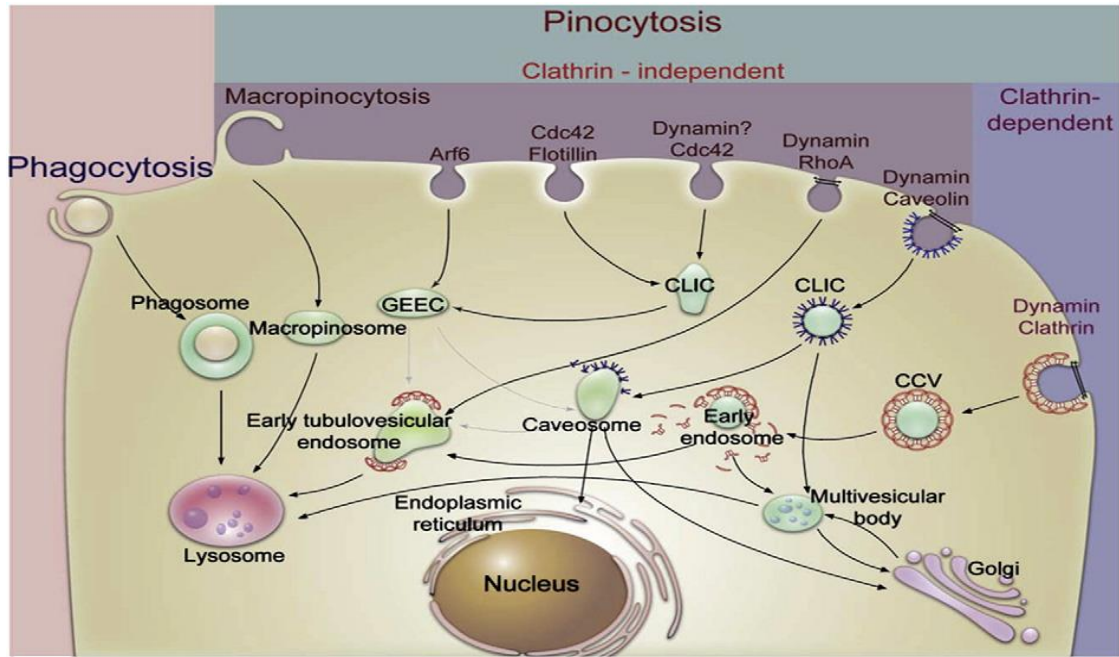
Figure 1, Model of different endocytotic mechanisms and intracellular transport in the cell. Cargo taken up by endocytosis are enclosed in the vesicles, such as clathrin coated vesicles (CCVs), GPI-anchored protein-enriched compartment (GEEC), clathrin-independent carriers (CLIC), micropinosomes, early endosomes or phagosomes. These vesicles with cargo can be matured down the degradative pathway and become multivesicular bodies (MVB) which fuse with lysosomes or transported back to its final destination. Phagocytosis is restricted to specialized mammalian cell and pinocytosis occurs in all cell types (adapted Sahay et al, 2010).

2.1.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the most studied and the best known endocytic mechanism and it is common in most mammalian cell types (Doherty and McMahon, 2009). CME is vesicular in morphology and it takes place in non-lipid raft regions of the cell membrane (Figure 1). Clathrin is a coat protein that surrounds the cargo in endocytotic process and forms coated pits which can be up to 200 nm in size (McMahon and Boucrot, 2011). The main scaffold components of the clathrin coat are the proteins called clathrin heavy chain (CHC) and clathrin light chain (CLC) (Ungewickell, 1983).

In CME, cargo binds to the transmembrane receptor and is then packaged with the use of adaptor and accessory proteins into clathrin-coated vesicles (CCVs), which is then internalized (Schmid et al, 2006). Most important adaptor protein is AP2, which links

cargo to nucleating clathrin. Accessory proteins participate in CME through helping membrane deformation in the membrane bud by recruiting other participating proteins, or by performing scaffolding or coordination functions within the endocytic process (Doherty and McMahon, 2009). For instance, N-BAR and BAR domain-containing proteins, such as amphiphysin, can generate membrane curvature, bind clathrin and AP2, and recruit dynamin to the budding vesicle (David et al, 1996). However, different receptors can use alternative adaptor and accessory proteins for the clustering and stimulation of the endocytosis (Doherty and McMahon, 2009). Adaptor and accessory proteins can also control the internalization of different cargoes.

Dynamin, the membrane scission protein, is large GTPase, which forms a polymer around the vesicle neck, upon hydrolysis (Praefcke and McMahon, 2004). Dynamin mediates the fission of the vesicle from the plasma membrane as well as helps releasing the clathrin-coated vesicle (CCV) into the interior of the cell. The clathrin basket is released from the vesicle by auxilin and hsc70. Vesicle drifts within the cell and delivers its cargo to intracellular compartment.

In most cases cargo taken up by CME ends up in lysosomes but it can also have a role in transcytosis (Tuma and Hubbard, 2003). In the cytosol, CCVs begins to lose its clathrin coat following formation of an early endosome (El-Sayed and Harashima, 2013). Cargos inside the endosome can be recycled back to the cell membrane or early endosome can be matured into late endosome. Then fusion between late endosome and lysosomes occurs to form endolysosome, where degradation of the cargo takes place.

CME takes part of internalization of wide variety of cargoes. Most of the endogenous proteins are internalized by CME (Conner and Schmid, 2003). For instance many nutrients, such as cholesterol low density lipoprotein (LDL), are taken up into the cell by CME (Schmid, 1997). Transferrin is also an example of a cargo that is transported through the cell membrane by CME. Transferrin binds to the transferrin receptor and is then internalized. Transferrin receptor and transferrin itself are then transported back to the surface of the cell membrane for reuse (Harding et al, 1983).

2.1.2 Caveolae-dependent endocytosis

Caveolae-dependent endocytosis is common in various cell types, such as fibroblast and endothelial cells (Couet et al, 2001). Caveolae-mediated endocytosis is vesicular or tubuvesicular in morphology (Figure 1). Caveolae are flask-shape, remarkably stable plasma membrane invagitations in size of 50-100 μm. These invagitations are associated with lipid rafts. Caveolins, integral membrane proteins, constitute the major protein component of caveolae membrane and have a structural role in the formation of caveolae. Caveolins also interact with a number of signaling molecules. Caveolin 1 is enriched in caveolae and it binds to cholesterol in lipid raft. Caveolin 1 together with caveolin 3 is reguired in caveolae biogenesis (Lipardi et al, 1998). Caveolin 2 is not necessary in caveolae formation but it can co-localize with caveolin 1 to form a hetero-oligomeric complexes (Mora et al, 1999). Cavin, another protein that is included in caveolae endocytic machinery, stabilizes caveolae and induces membrane curvature, dynamin, which enables vesicle fragment (Hansen et al, 2009; Nabi, 2009).

At the cell surface, caveolae forms a stable functional unit that is generated by oligomerized caveolin and associated proteins and lipids (Couet et al, 2001). Also during endocytosis caveolar unit is maintained stable (Pelkmans et al, 2004). In caveolae-dependent endocytosis, caveolae is first stimulated, for instance by SV 40 virus or sterols (Sharma et al, 2004). Process is regulated by dynamin, protein kinase C and tyrosine kinases (Parton and Simons, 2007). Caveolae form caveolar carriers that fuse with the early endosome, or can fuse back to the plasma membrane without the involvement of an endosomal intermediate, therefore it may have a role in transcytosis (Tuma and Hubbard, 2003).

Folic acid use caveolae-dependent endocytosis to be internalized (Chang et al, 1992). Also glycosylphosphatidylinositol (GPI)-enchored proteins, CTB and Shinga toxin are mainly internalized through caveolae-dependent endocytosis (Kirkham et al, 2005). Even though caveolae is taking a part in endocytosis, it has many other functions, such as calcium signaling and other signal transduction events. Some studies suggest that under normal, non-stimulated conditions, caveolae are not involved in endocytosis to

any significant degree (Thomsen et al, 2002; van Deurs et al, 2003). However, more studies are required to understand the whole mechanism and function of caveolae-dependent endocytosis (Parton and Howes, 2010).

2.1.3 Macropinocytosis

Macropinocytosis is a specific, larger scale pinocytosis that occurs in all cell types with only a few exceptions, such as macrophages and brain microvessel endothelial cells (Doherty and McMahon, 2012). Macropinocytosis is highly ruffled in morphology and GTPase, rac, and actin dependent (Figure 1). In macropinocytosis the invagitation of the cell membrane forms a pocket, macropinosome, which pinches off into the cell to form a vesicle up to 10 µm in size. Pocket, sized 0.5 - 10 µm, is filled with a large volume of extracellular fluid and molecules within it. Macropinosome membrane is formed both of lipid raft and non-lipid raft membrane domains (Manes S et al, 1999; Grimmer et al, 2002).

In macropinocytosis GTP-bound rac stimulates accumulation of actin filaments at the plasma membrane and that is involved in plasma membrane ruffling induced by growth factors (Ridley et al, 1992). Cholesterol is required for the recruitment of activated rac to these sites (Grimmer et al, 2002). Rac activates the Arp2/3 protein complex and WAVE, which is a process involved in ruffling (Gao et al, 2007). Many proteins, such as ras and Src, kinase PAK1 and arf6, one of the small GTPases, are known to be involved in macropinocytosis (Dharmawardhane et al, 2000; Donaldson, 2003; Yarar et al, 2007). Solute macromolecules and nutrients are known to be internalized via this pathway.

In the cytosol internalized macropinosome loses its actin filaments on its surface. In the majority of the cells, such as macrophages and brain microvascular endothelial cells, macropinosomes mature in early endosome, then late endosome or degrade with lysosomes (Racoosin and Swanson, 1993; Liu et al, 2002; Mercer and Helenius, 2012). In A531 human carcinoma cells, cargo is recycled back to the cell exterior through fusion with the cell plasma membrane (Hewlett et al, 1994). Macropinocytosis can also

be followed by transcytosis, for instance Shinga toxin through intestinal epithelial cells (Lukyanenko et al, 2011).

2.1.4 CLIC/GEEC-type endocytosis

Clathrin-independent carrier (CLIC) / GPI-AP-enriched early endosomal compartment (GEEC) endocytosis is dynamin-independent uptake mechanism which takes place in lipid rafts of the cell membrane (Lundmark et al, 2008). Mechanism is mediated by primary carriers, CLICs, which fuse to form tubular early endocytic compartments, GEECs. The small GTPases, Cdc42 and GRAF1, and ADP-ribosylation factors, arf1 and arf6, induce CLIC/GEEC-type endocytosis (Sabharanjak et al, 2002; Kumari and Mayor, 2008; Lundmark et al, 2008). After formation of CLICs, they become acidified and acquire Rab5 and EEA1 before fusing to other GEECs or early endosome (Kalia et al, 2006). GPI-anchored proteins (GPI-APs) are internalized via this CLIC/GEEC uptake mechanism.

2.1.5 Flotillin-dependent endocytosis

Flotillin-dependent endocytosis is common in mammalian cells (Glebov et al, 2006). Flotillin-dependent endocytosis is vesicular in morphology and it takes place in raft membrane domains (Figure 1). Lipid raft proteins, flotillin 1 and flotillin 2, are oligomerized in distinct membrane microdomains, but are not enriched in caveolae. Flotillin-dependent endocytosis appears to be both dynamin dependent and independent (Glebov et al, 2006; Frick et al, 2007). Cargo is colocalized with flotillin vesicles and after internalization it is delivered into late endosome and lysosomes (Payne et al, 2007; Riento et al, 2009). Proteoglycan-binding ligands, PEI, LF and PA, enter the cells by flotillin-dependent endocytosis.

2.1.6 Phagocytosis

Phagocytosis is an endocytic mechanism that is common in specialized mammalian cells, such as macrophages, neutrophils and dendritic cells (Underhill and Goodridge,

2012). It transports large solid particles (> 5 μm) into coated vesicles, phagosomes, through mechanism that requires actin polymerization. Phagosomes are formed of lipid rafts, which are enriched by sphingomyelin and ceratine (Magenau et al, 2011). Phagosomes, are shaped according to the shape of particle they engulf. Cargoes can be recognized by the "eat me" signals or through opsonisation of the cargo. In cytosol, phagosomes fuse to early endosomes and late endosomes, and then fuse to the lysosome to form a phagolysosome, where the digestive processing of the cargo takes place (Flannagan et al, 2012).

2.2 The role of endocytic pathway in drug delivery

Endocytosis occurs in all cell types in the body and is used to internalize vital molecules as well as therapeutic molecules (Doherty and McMahon, 2009). Small and lipophilic therapeutic molecules can permeate across the cell membrane by passive diffusion or by carrier-mediated diffusion, therefore they do not need endocytosis to be internalized. Macromolecular and hydrophilic drugs, such as proteins and peptides, are too large to enter the cell by diffusion, therefore they cannot readily cross the cell membrane but are internalized via endocytosis. Macromolecular and poorly soluble drugs can also be loaded into nanoparticles to be protected or to be intended for targeted drug delivery (Hillaireau and Couvreur, 2009).

Certain cell types, such as blood-brain barrier endothelial cells and intestinal endothelial cells, carry certain receptors on their surface, which provides the possibility for drug targeting (Hillaireau and Couvreur, 2009). Therapeutic molecule or nanoparticle can be associated with specific binding moiety to be targeted to the specific receptor and internalized via receptor-mediated endocytosis (Wohlfart et al, 2012). For instance folic acid and transferrin has been widely used as targeting ligands for nanoparticles (Qian et al, 2002; Hilgenbrink and Low, 2005). Different endocytic pathways may lead to different endocytic routing and intracellular destiny of the cargo, therefore designing nanoparticles specifically targeted to a certain pathway may result in the more specific delivery of the drug to certain intracellular compartments. Most of the endocytic pathways lead to the delivery of the cargo to endolysosomal compartment (Doherty and

McMahon, 2009). Therefore, for cytoplasmic delivery of the drug, it has to be able to be released from the endosomes.

In drug delivery, it is important to know whether the drug is endocytosed or further transcytosed. For example, for brain drug delivery of intravenously injected drug, it must be transcytosed through the brain endothelial cells in order to reach the brain (Gabathuler, 2010). Several pathways including clathrin- and caveolae-mediated endocytosis were shown to lead to transcytosis of the drug (Tuma and Hubbard, 2003). However, the regulation of transcytosis is poorly understood. For instance in clathrin-mediated endocytosis, cargo can be degraded in lysosomes or it can be further transcytosed through the cell.

2.3 Asymmetry of endocytic processes in polarized cells

Cell polarity is an asymmetrical organization of cellular components and functions and it develops through the localization of specific proteins to specific areas of the cell membrane (Mellman and Nelson, 2008) (Figure 2). It is implicated in the differentiation, proliferation and morphogenesis of cellular organisms and it enables cells to perform specialized functions. One example of a polarized cell type is endothelial cells, which demonstrates apical - basolateral polarity providing adsorptive and protective function in tissue. The establishment and maintenance of the cell polarity involves many processes including signaling cascades, membrane trafficking events and cytoskeletal dynamics. All these processes need to be well coordinated.

Cell polarity requires polarity regulators, proteins that show roles in polarization (Shivas et al, 2010). These proteins form three different complexes at the plasma membrane, PAR, Crumbs and Scribble complexes (Mellman and Nelson, 2008). Each of these complexes are localizes to a distinct sub-domain of polarized cell. Also endocytosis is regulated by different proteins, as described previously. These two systems, intracellular trafficking and polarity control, seems to work together by crosstalk between polarity proteins and endocytic regulators (Shivas et al, 2010). For instance, the PAR complex may promote dynamin-dependent endocytosis, while

dynamin-dependent endocytosis may be required to maintain correct localization of the PAR complex.
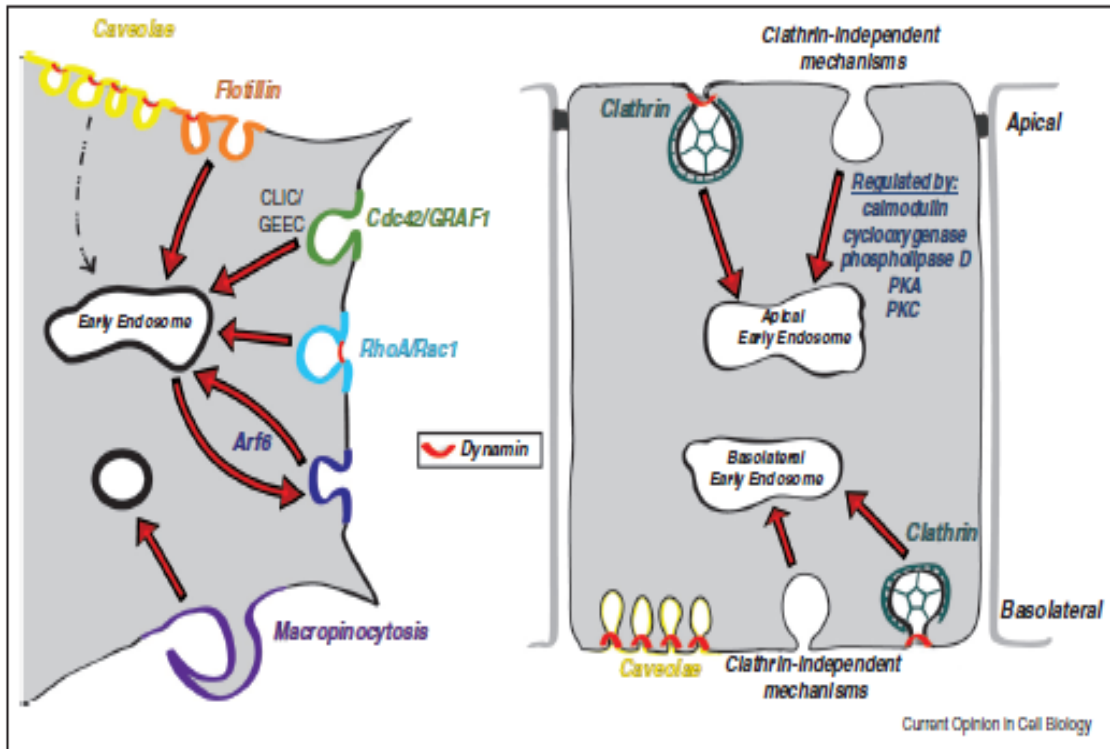

Figure 2, Endocytosis in polarized cell (right) is more complex than in non-polarized cell (left). In non-polarized cell, different endocytic mechanisms are not regulated as much as in polariced cell. As we can see from the picture: caveolae-mediated endocytosis is dependent of caveolae, flotillin-dependent endocytosis reguires flotillin, clathrin-independent carrier (CLIC) / GPI-AP-enriched early endosomal compartment (GEEC) endocytosis (GLIC/GEEC-endocytosis) is dependent of Cdc42 and GRAF1 proteins, RhoA-dependent endocytosis reguires RhoA and rac1 proteins, Arf6-dependent endocytosis reguires arf6 and macropinocytosis occurs by invagination of the cell membrane. Dynamin is GTPase, which is related to several of these endocytic pathways. In polarized cell, endocytosis is regulated different manner on apical and basolateral membrane of the cell. On apical membrane, clathrin-independent endocytosis is known to be regulated by number of factors, such as calmodulin, cyclooxygenase, phospholipase D, PKA and PKC, which do not effect on endocytosis in basolateral pole. Also caveolae is found only at the basolateral side of the polarized cell (adapted Sandvik et al, 2011).

In polarized Madin Darby canine kidney (MDCK) cells, clathrin-independent endocytosis have been proven to go through differential regulation on the apical and

basolateral poles (Eker et al, 1994) (Figure 2). Apical clathrin- independent endocytosis is under complex regulation, and can be regulated independently of uptake at the basolateral side. Apical clathrin-independent endocytosis can be regulated by a number of factors, for example protein kinase A, protein kinase C, phospholipase D, cyclooxygenase and calmodulin, that do not affect basolateral uptake.

Most of the knowledge about endocytic mechanisms derives from cells in culture. Studies of endocytic mechanisms in cell lines do not directly reflect uptake mechanisms *in vivo* (Sandvik et al, 2008). Cells cultured in on plastic support do not go through polarization but on filters polarization occurs. To which extent different endocytic mechanisms vary depending on cell polarization is to a large extent not known.

3 BLOOD-BRAIN BARRIER AND ITS ROLE IN DRUG DELIVERY

3.1 Structure and function of Blood-Brain Barrier

Blood-Brain Barrier (BBB) consists of specialized capillary endothelial cells surrounded by astroglial endfeet and pericytes (de Lange, 2012) (Figure 3). Specialized capillary endothelial cells (BBB) form barrier function by tight junctions between the cells. BBB intercepts the blood and the brain protecting the brain from harmful compounds but allowing vital nutrients to pass. It therefore has a role to maintain the neuroparenchymal microenviroment.
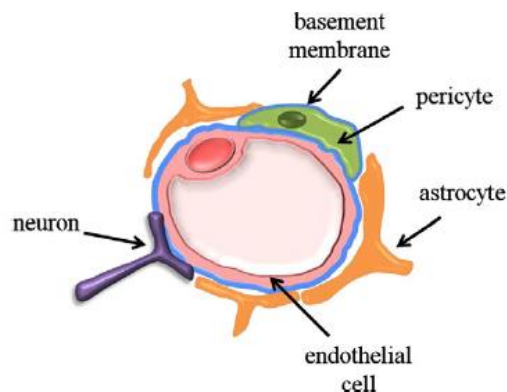

Figure 3. The blood-brain barrier (adapted Cordoso et al, 2010).

Capillaries in the brain are around 50-100 times tighter than in the periphery due the tight junctions tightly sealing polarized endothelial cells (Abbott et al, 2008). Tight junctions are composed of transmembrane proteins and peripherally tight junction associated proteins, such as zonula occludens proteins (ZO-1, -2 and -3), occludins, claudins and adhesion molecules (Wolburg and Lippoldt, 2002). ZO-proteins are submembranous tight junction-associated proteins that take a part in signal transduction and in anchoring the transmembrane tight junction proteins to the cytoskeleton. Occludins are not required for the formation of tight junctions, but rather needed for regulating the barrier properties by sealing the tight junctions (Lacaz-Vieira et al, 1999). Claudins are important in formation of barrier properties (Tsukita and Furuse, 1999; Tsukita and Furuse, 2000). Adhesion molecules, such as the junctional adhesion molecules (JAM) are involved in organizing the tight junctional structure (Martin-Padura et al, 1998; Dejana et al, 2000)

Many signalling pathways have been shown to be involved in tight junction regulation (Wolfburg and Lippoldt, 2002). All these proteins together with capillary endothelial cells form a tight barrier with transendothelial resistance (TEER) over 1000 $\Omega \cdot cm^2$ (Butt et al, 1990). TEER of other capillaries is much less. For instance human umbilical endothelial cells (HUVEC) reach TEER of 25 $\Omega \cdot cm^2$ (Ali et al, 1999). Tight junctions regulate paracellular transport as well as also help on maintenance of cell polarity in BBB.

Cerebral endothelial cytoplasm has many enzymes, which have important role in blood-brain barrier as a metabolic barrier (de Lounge, 2012). Cytochrome P450 enzymes (CYPs), CYP1B1 and CYP2U1, are the most expressed enzymes in brain microvessels (Dauchy et al, 2008). The role of these enzymes is not yet fully understood, but they take a part in drug metabolism. Differences in the amount of various enzymes at both sides of the cell membrane seem to contribute to the polarity of endothelial function in the control of the BBB (de Lounge, 2012). Plasma membrane of brain endothelial cells exhibits many transporters, such as P-glycoprotein (P-gp), multidrug resistance

associated protein (MRP) 1 and 4, and breast cancer resistance protein (BCRP), to maintain the homeostasis of the brain.

Basement membrane (also called basal lamina) is a thin layer of a specialized extracellular matrix that form a supporting structure surrounding the brain capillary endothelial cells which consist of number of collagens, laminin, fibronectin, antactin, thrombostin, heparin sulfate and chondroitin sulfate proteoglycans (Vorbrodt et al, 1988; Paulsson, 1992; Erickson and Couchman 2000). It provides mechanical support for cell attachment and it can act as a barrier to the passage of macromolecules. It also provides base for the cell migration and separates adjacent tissue

Astrocytes are glial cells that provide cellular link to the neurons (Abbott et al, 2006). Astrocytes participate on induction of blood-brain barrier properties of endothelial cells and they also have a role in synaptic activity, cerebral blood flow and metabolism. Pericytes are contractile cells surrounding endothelial cells of capillaries. Pericytes help to sustain blood-brain barrier, but also maintain homeostatic and hemostatic functions (Krueger and Bechmann, 2010). They play a role in blood-brain barrier integrity, angiogenesis, neovascularization and regulation of cerebral blood flow.

3.2 Significance of BBB in drug delivery

The number new drugs developed for treatment of CNS diseases has grown, but only few are on the sale (Pardridge, 2002). This is due the permeation limiting BBB, which restricts transport of many potential drugs to the brain in sufficient concentrations. The main reason for the limited transfer of drugs to the brain is tight junctions. Also enzymatic barrier and efflux transporters expressed in the cerebral endothelium restrict drugs transport to the brain.

Potential drugs can permeate across the brain endothelium by several routes (Abbott et al, 2008). Nonpolar, small lipophilic drugs and gaseous molecules can diffuse passively through the BBB (Abbott et al, 2004). Drugs that cannot be diffused, can be transported to the brain via carrier-mediated transporters or via endocytosis (Pardridge 2007;

Dobson and Kell, 2008). For instance, glucose and small peptides can be transported to the brain via carrier-mediated influx transporters and macromolecules via transcytosis (Abbott et al, 2008). Some diseases, such as brain tumors and multiple sclerosis (MS), can affect barrier properties of the BBB. Disease state can modulate the tight junctions and open the paracellular pathway and therefore increase the transport of drugs to the brain.

Therapeutic molecules, which are bigger than 500 Daltons and hydrophilic, can be delivered to the brain by effective drug delivery strategies. Invasive drug delivery methods include temporary increase of the blood-brain barrier permeability, for instance by the osmotic disruption, and intra - cerebroventricular (ICV) and intracerebral implants (Neuwelt et al, 1979; Chauhan, 2002; Westphal et al, 2006). These strategies are rather expensive and they are associated with high risk of complications. Also most of the invasive strategies rely on diffusion, which is poor mode of drug delivery to the brain (Pardridge, 1997).

Another strategy to overcome the BBB is the lipidization of the drug molecule. Hydrophilic drug can be conjugated to lipid carriers (Prokai –Tatrai et al, 1996). Lipidization increases the uptake of the drug in the brain, but also to other tissues in the periphery, which is a disadvantage of this strategy. Small hydrophilic drugs can be transported to the brain via carrier-mediated transport within the brain capillary endothelial plasma membrane, if the drug can be structurally altered to mimic an endogenous nutrient (Pardridge and Oldendorf, 1977). Large molecules, such as insulin, can be transported through the BBB by receptor-mediated transport. Drug molecule can be bound to endogenous ligands, but many times these ligands have serious side effects. As described further, good strategy to take advantage of endogenous transport systems, is the use of nanoparticles, which are internalized via endocytosis.

3.3 In vitro models of blood-brain barrier

As discovery and development of CNS drugs are extensively growing, the development of comparatively simple, stable and reproducible *in vitro* BBB permeability model is an

increasing need (Alavijeh et al, 2005). *In vivo* preparations maintain the brain cellular and fluid compartments, as well as the cerebral blood flow. However, *in vivo* preparations involve surgical skill and are relatively low throughput. Animal models are also expensive and they are involved in many ethical issues. In vitro BBB cell models provide high throughput and can be used in many kind of drug testing, for instance in drug distribution and drug permeability studies. Blood-brain barrier *in vitro* models are also important in structural studies of BBB. Evaluating the ability of the drug to penetrate via BBB is important not only for the targeting the drug to CNS, but also for other drugs due to toxicity issues.

Good and well predictive in vitro BBB cell model to be exploited for the transendothelial BBB permeability screening of potential CNS drugs must exhibit many requirements. BBB cell model must display reproducible solute permeability and restrictive paracellular barrier properties (Gumbleton and Audus, 2001; Abbott et al, 2008). It allows ease of culture meeting the time and technical requirements, such as grow time, good viability and show little change in phenotype following passage. Good *in vitro* BBB cell model also has realistic cell morphology, high cell purity and functional expression of most important transporters.

3.3.1 Primary/low passage cultures

Primary or low passage cultures of brain capillary endothelium are close to the *in vivo* cell phenotype and can provide remarkable amount of isolated brain capillary endothelial cells (Gumbleton and Audus, 2001). Mostly used species are bovine and porcine. These cultures generate a restrictive paracellular barrier properties as TEER is approximately over 500 $\Omega \cdot cm^2$. However, time and technical resource required for isolation as well as reproducibility in phenotypic properties makes the primary/low passage cultures challenging. Also non-human origin makes molecular characterization difficult.

Bovine brain microvessel endothelial cells (BBMEC) are commonly used as a primary or low passage culture in BBB *in vitro* model (Abbott et al, 2008). Bovine brains yield

around 100 viable cells and monolayer of BMEC maintains many morphological and biochemical properties as the BBB *in vivo* (Audus and Borchardt, 1986; Audus and Borchardt, 1987; Pal et al, 2007). BMEC are functionally polarized as bovine brain microvessel endothelial cells *in vivo* and it can develop restrictive paracellular barrier. These features are found to decline in culture and following passage (Abbott et al, 2008). Co-culture BMEC with astrocytes or exposure to astrocyte-conditioned medium, can induce and maintain the tightness of the monolayer and this type of model has been the most successful. TEER of the BMEC has reported to be approximately 180 $\Omega \cdot cm^2$ but when co-cultured with astrocytes it was 10 times higher (Wolburg et al, 1994).

Porcine brain microvessel endothelial cells (PBMEC) are one of the most used primary/low passage cultures of BBB *in vitro* model (Abbott et al, 2008). PBMEC can develop tight cell monolayer when serum is removed and replaced with hydrocortisone (Franke et al, 1999). Hydrocortisone seems to increase the tight-junctional barrier. TEER of the PBMEC have been approximately 600-800 $\Omega \cdot cm^2$. As the other primary and low passage cultures, PBMEC exhibits many characteristics similar to the BBB *in vivo*.

Murine brain microvessel endothelial cells (MBMEC) co-cultured with rat astrocytes provide *in vitro* BBB cell model with transendothelial resistance of 200 $\Omega \cdot cm^2$ (Shayan et al, 2011). MBMEC also show high barrier properties in sodium fluorescein permeability test and in expression of specific tight junction proteins, such as occludin and claudin. Astrocytes seem to be responsible of these barrier properties in this model. MBMEC provide relevance to *in vivo* models in drug industry, but the TEER is many times lower than in other primary/low passage cultures.

3.3.2 Immortalized cell lines

A number of immortalized brain capillary endothelial cell lines have been generated (Gumbleton and Audus, 2001). Immortalization allows for ease of culture. So far, immortalized cell lines are rather leaky due the absence of the necessary restrictive paracellular barrier properties which does not allow their effective use in

transendothelial permeability screening. However, these cell lines can be good for drug uptake and efflux studies, because they express the typical BBB transporters. For instance, immortalized cell lines can be used in nanoparticle uptake studies as nanoparticles are too big to penetrate in between the cells even though the tight junctions are not so tight (Hillaireau and Couvreur, 2009).

bEND3 and bEND5 cells, derived from mice, are commercially available and can be used in transport studies (Gumbleton and Audus, 2009). Well characterized, rat derived RBE4 cell line expresses the typical endothelial markers and shows differentiation of a brain endothelial phenotype in the presense of astroglial factors (Gumbleton and Audus, 2001). Human based SV-HCEC cell line expresses many properties of primary/low passage brain capillary cells, but does not develop a sufficient paracellular barrier (Muruganandam et al, 1997; Kannan et al, 2000). However, all these immortalized cell lines have failed to develop the necessary paracellular barrier that would allow its use as a good *in vitro* BBB transendothelial permeability tool.

So far, immortalized human brain endothelial cell line hCMEC/D3 is the best characterized human BBB cell line (Poller at al., 2008). hCMEC/D3 cells has been developed by immortalization of primary human brain capillary endothelial cells (BCEC) through coexpression of h-TERT antibody and SV40 large T antigen via a lentiviral vector system (Weksler, 2005). Human brain tissue has been obtained from the temporal lobe of an adult female.

Weksler and collegues (2005) perfomed the detailed characterization of hCMEC/D3 cell line. Morfology of the hCMEC/D3 cell line appeared to be similar to primary cultures of brain endothelial cells. Cell line showed no senescence or dedifferentiation during over 100 population doublings. Maintenance of nontransformed phenotype is significant characteristic of this cell line. No other human derived cell line has showed to maintain nontransformed phenotype during doublings (Gumbleton and Audus, 2001). Expression of many endothelial markers, such as cellular junction associated proteins PECAM-1 and VE-cadherin and cytoplasmic granules of von Willebrand factor, has been showed (Weksler et al, 2005). Also β- and γ-catenins and F-actin were expressed.

No change was detected in endothelial markers when passaging the cells. Based on these results, hCMEC/D3 cell line displays close phenotype to normal primary endothelial cells.

Even though hCMEC/D3 has a leakier phenotype than the *in vivo* BBB, hCMEC/D3 cell line has a good permability correlation (R=0.938) between *in vitro* and *in vivo* data (Weksler et al, 2005). Correlation has been evaluated by *in vitro/in vivo* comparisons for some markers of low and high passice permeability. Permeability range in the cell line have been roughly $0.5 - 5.5 \cdot 10^{-3}$ cm/s, which is far from the *in vivo* situations (Poller et al, 2008). However, it has been noticed that human serum (HS) increases the tightness of the monolayer, when changing the fetal calf serum (FCS) to HS (Poller et al, 2008).

hCMEC/D3 expresses many of the most important transporters similar to that in isolated brain microvessels (Weksler et al, 2005; Poller et al, 2008; Dauchy et al, 2009). ATP-binding cassette (ABC) efflux transporters, which includes P-glycoprotein (P-gp, also called ABCB1 and MDR1), breast cancer resistant protein (BCRP, also called ABCG2) and multi resistant proteins (MRPs), such as MRP1, MRP3, MRP4 and MRP5, are expressed in hCMEC/3 cells (Dauchy et al, 2009). At least P-gp, BCRP and MRP1 have been proved to be functional (Dauchy et al, 2005; Weksler et al, 2005).

Some phase 1 cytochrome P450 (CYP) enzymes has been detected from hCMEC/D3 cells (Dauchy et al, 2009). CYP2U1 is main enzyme expressed in the cells. Also CYP2S1 and CYP2R1 are expressed but lesser extent. CYP2U1 is involved in arachidonic acid and other long chain fatty acid metabolism while CYP2S1 is metabolizing naphthalene and CYP2R1 is taking a part in vitamin D metabolism. The role of these enzymes is not fully understood. As hCMEC/D3 cells exhibits many of the characteristics that are essential for good and predictive BBB in vitro model, it can be one of the most promising tool for BBB studies.

In addition, also surrogate, immortalized cell lines have been used *in vitro* studies of active transport (Summerfield et al, 2007; Hellinger et al, 2012). Continuous cell lines

of non-cerebral origin are commercially available and they exhibit at least some of the criteria required for an *in vitro* BBB permeability model. Canin renal epithelial cell line, MDCK, transfected with the human P-glycoprotein (Pgp) gene, MDR1, has been used as a surrogate BBB model (Summerfield et al, 2007; Hellinger et al, 2012). Human colon carcinoma derived Caco-2 cell naturally express Pgp and it has been used in permeability and efflux studies (Hellinger et al, 2012). Both cell lines have better TEER values than the cells obtained from the brain tissue and they have shown good integrity, but unnatural transporter expression and epithelial lineage makes it questionable if these cell lines gives reliable data.

3.3.3 Dynamic three-dimensional BBB model

Fluid flow through the endothelial cells *in vivo* exerts shear stress across the apical surface and induces factors from astrocytes, which have influence on functional differentiation of brain endothelial cells (Rizzo et al, 1998; Santaguida et al, 2006). Dynamic three-dimensional *in vitro* BBB model mimics functionally and anatomically the microvasculature and enables the co-culture of brain endothelial cells and astrocytes inside porous fiber tubes or scaffolds, and incorporation of fluid flow pathway via the lumen. This model generates TEER of roughly 2000 $\Omega \cdot cm^2$. The model appears also to display polarized functional expression of transporters and receptors characteristic in the BBB.

Bovine aortic endothelial cells co-cultured with glial cells were first used in this kind of dynamic BBB cell model (Stanness et al, 1997; Cucullo et al, 2002). Good BBB characteristics has been demonstrated also with mouse bEnd3 cells with and without astrocytes (Booth and Kim, 2012; Prabhakarpandian et al, 2013). Also the hCMEC/D3 cells have been shown to perform well in this system (Cucullo et al, 2008; Griep et al, 2013). Three-dimensional BBB model would have great benefit in cell studies, but due the technical demands, it may not be the best model for a high throughput permeability screening.

3.3.4 Human endothelial cells derived from human pluripotent stem cells

Recently, endothelial cells derived from pluriporent stem cells (hPSCs) have been demostrated as an *in vitro* BBB cell model (Lippmann et al, 2012). hPSC cells have been co-differentiated with neural cells and followed by purification of the BBB-like endothelial population on selective matrix. These endothelial cells have many BBB properties similar to those of brain microvessel endothelial cells (BMECs). They develop tight-junctions and express the most important transporters as well as polarized efflux transporters. Most importantly, these endothelial cells could be capable on transport studies, due the barrier and transport properties. BBB endothelial cells differentiated from hPSCs have transendothelial electrical resistance 1450 (±140) $\Omega \cdot cm^2$ and they are relatively easy to culture, which makes this type of models potentially promising.

3.3.5 Human umbical vein endothelial cells

Langford and colleagues (2005) have demonstrated a human based blood-brain barrier *in vitro* cell model using human umbical vein endothelial cells (HUVEC). These cells are derived from the endothelium of veins from the umbilical cord. HUVEC were cultured with fibroblast growth factor 2 (FGF2) which is produced by astrocytes. FGF2 promotes endothelial cell fitness, angiogenesis and maintains the integrity of the BBB. It seems that FGF2 is needed to produce tight barrier properties in HUVEC. There is lack of information about this cell line, but the advantage of this cell line is the human origin.

Overall, development of a well predictive BBB cell model has proven hard. Primary or low passage cultures exhibits many of the characteristics of the BBB *in vivo,* but they have inter-batch variability in expression of tight junctions, transporters, enzymes and receptors (Abbott, 2004; Abbott, 2007). Immortalized cell lines provide ease of culture but fail to generate tight monolayer (Gumbleton and Audus, 2001). Tridimensional models develop tight monolayer, express the most important transporters and enzymes, but are not suitable for high throughput screening.

3.4 Nanoparticles as tools for drug delivery through BBB

Nanoparticles are submicron sized particles, colloidal carriers, which can be associated with a drug molecule (Faraji and Wipf, 2009). Variety of materials, including polymers, lipids and inorganic compounds, can be used in formation of nanoparticles (Figure 4). Nanoparticles can improve the pharmacokinetic properties of the drug due the longer self-lives, higher stability and possibility to better control of drug release, which leads to better efficacy and reduction in side effects (Kreuter et al, 1995). Nanoparticles, which are intended for parenteral use, have to be biodegradable and nontoxic.
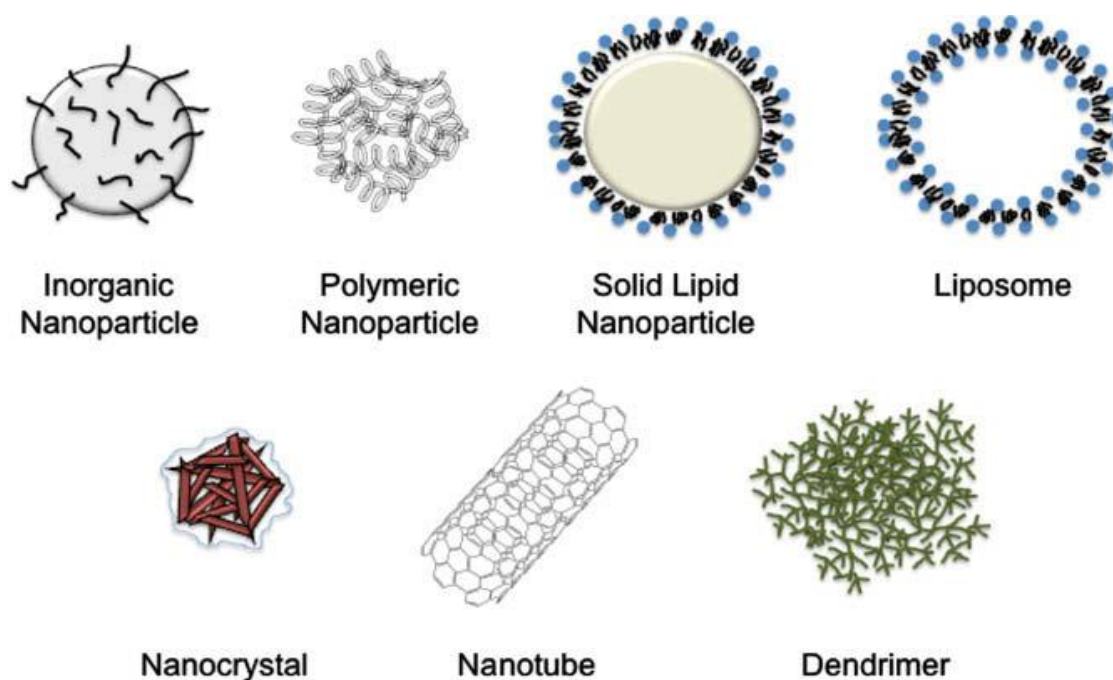

Figure 4, Different kind of nanoparticles used in drug delivery. (adapted Faraji and Wipf, 2009).

Due to relatively large size and hydrophilic nature, nanoparticles cannot cross the cell membrane and they are mainly internalized via endocytosis. Successful size seems to be 150-300 nm and this size is mainly internalized by clathrin-mediated endocytosis (Hillareu and Couvreur, 2009). Nanoparticles over 500 nm are using caveolae-mediated endocytosis. Internalization mechanism depends on particle size, surface charge and hydrophobicity of the particle (Wohlfart et al, 2012). Positively charged nanoparticles

are internalized faster because of the negatively charged cell membrane. Endocytosis of the nanoparticle seems to be also cell type dependent (Sahay et al, 2010). Surface modification of the nanoparticle is important, because it have an effect to the solubility, stability and a targeting of the nanoparticle. Nanoparticles (NPs) can serve a promising, non-invasive tool to deliver drugs through the BBB. Many studies have proven that different kinds of surface modified nanoparticles are able to entry into the brain after intravenous injection via endocytosis (Wohlfart et al, 2012). This strategy does not involve any modification of the drug molecule, which is also important advantage.

3.4.1 Polymeric nanoparticles

Polymeric nanoparticles are biodegradable and biocompatible (Faraji and Wipf, 2009) (Figure 4). Polymeric nanoparticles can be engineered various ways and usually they are surface modified for instance with gelatin, chitosan and polyethylene glycol (PEG). Surface modification improves the pharmacokinetic control, stability and the efficacy of the nanoparticle. PEG reduces immunogenicity and phagocytosis of the macrophages, which increases the blood level of drug in organs, such as in the brain (Alyautdin et al, 1997; Alyautdin et al, 2001; Kreuger et al, 2003). Polymer matrix prevents drug degradation and can provide controlled drug release.

Poly(butyl cyanoacrylate) (PBCA) is the most studied nanoparticle to be used drug transport to the brain (Wilson, 2009). PBCA is low molecular weight polymer with relatively low toxicity and very rapid biodegradability. PBCA with polysorbate 80 (PS 80) has been shown to pass the BBB both *in vitro* and *in vivo* and it has been used to deliver wide variety of drugs to the brain (Kreuter et al, 1995; Kreuter et al, 2002, Wilson, 2009). Uptake mechanism is not fully understood, but it has been proposed to happen via receptor-mediated endocytosis (Kreuter et al, 2005). Absorption of apolipoprotein associated PS 80 coated PBCA has been also demonstrated *in vivo* (Kreuter et al, 2002). Effect of PBCA seems to be dependent of the surface modification as PBCA without PS 80 does not have any effect on internalization of drugs to the brain.

A few studies have been performed using poly(lactide-co-glycolide) (PLGA) as a nanocarrier in brain delivery of drugs (Wohlfart et al, 2012). PLGA is a synthetic material which internalization seems to be cell type and surface charge dependent (Vasir and Labhasetwar, 2008). Surfactant coated PLGA-PEG-PLGA (PEP) nanoparticles showed improved transport of loperamide through the BBB *in vitro* and *in vivo* (Cheng et al, 2013). Internalization occurred by receptor mediated endocytosis. Cheng et al also demonstrated that PEP without surfactant coating did not improve the internalization of loperamide. Also poly(methoxypolyethyleneglycol cyanoacrylate-co-hexadecylcyanoacrylate (PEG-PHDCA) have been investigated to be transported to the brain *in vitro* and shown to be internalized via clathrin-mediated endocytosis (Kim et al, 2007).

3.4.2 Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are lipid-based submicron sized colloidal carriers that consist of lipid core surrounded by monolayer of phospholipids and further stabilized by surfactants (Faraji and Wipf, 2009) (Figure 4). Due to ease of biodegradation, solid lipid nanoparticles are less toxic than polymeric nanoparticles and their pharmacokinetic profile can be easily engineered with core design. Surfactant amount affects to the release profile of the drug payload.

Many studies have been made to demostrate that solid lipid nanoparticles are able to overcome the BBB. For instance, risperidone-loaded solid lipid nanoparticles have been investigated to be transported to the brain by intranasal route *in vivo* (Patel et al, 2011). Venishetty and collegues (2013) demonstrated the increased transport of betreliesoxybutyric acid (HBA) grafted docetaxel loaded solid lipid nanoparticles (HD-SLNs) to the brain via monocarboxylic acid transporter (MCT1) *in vitro* and *in vivo*. Also Martin et al (2012) demonstrated the uptake of antitumor medicine, camptothecin, loaded SLNs to the brain both *in vitro* and *in vivo*.

3.4.3 Liposomes

Liposomes are bilayered vesicles surrounded by a phospholipid membrane (Faraji and Wipf, 2009) (Figure 4). Liposomes are amphiphilic in nature, easy to surface modifiable and biocompatible. They are also able to deliver large amounts of drug to the tissue. Disadvantage of liposomes is the limited biological stability, which can be though improved by PEGylation, which allows longer residence time and better control of drug delivery. Wide variations of drugs have delivered to the brain by liposomes. Targeted liposomes have been investigated to deliver drugs to the brain (Schnyder and Huwyler, 2005). Boado and Pardridge (2011) have also demonstrated efficient gene transfer to the brain by Trojan horse liposomes (THL), which are pegylated liposomes containing plasmid DNA in the interior of the liposome. THLs were specifically targeted to the receptors on the BBB by peptidomimetic monoclonal antibody and were transported through the BBB via receptor-mediated transcytosis as well as endocytosed into the cell and the brain cell nuclear compartment.

3.4.4 Inorganic nanoparticles

Inorganic nanoparticles are typically composed of silica and alumina, but also metals, metal oxides and metal sulfides are used (Faraji and Wipf, 2009) (Figure 4). Particle size, shape and porosity can be modified when producing inorganic nanoparticles of these materials. Relatively easy surface modification of these nanoparticles and stability in broad range of temperature and pH, are the major advantages of inorganic nanoparticles. For instance, Yim and coworkers (2012) have demonstrated the penetration of serum albumin surface coated magnetic metal ferrite nanoparticles through the BBB, both *in vitro* and *in vivo*. Also gold nanoparticles have been demonstrated to pass the blood-brain barrier *in vivo* (Sonavane et al, 2008). Based on results, penetration of gold nanoparticles to the brain was particle size-dependent. Only smaller nanoparticles in size of 15 and 50 nm were able to penetrate through the BBB, while bigger nanoparticles in size of 100 and 200 nm were not able to pass the BBB. However, the non-biodegradability and slow dissolution limits the use of these nanoparticles as a drug delivery tool to CNS.

3.4.5 Other nanoparticles

Nanotubes are sheets of atoms arranged in tubes and usually they are made of soluble fullerene derivatives (Faraji and Wipf, 2009) (Figure 4). Nanotubes serve large internal volumes and the external surface can be easily functionalized. However, due to the potential toxicity, they are not useful in nanoparticulate brain delivery. Dendrimers are macromolecules formed from monomeric or oligomeric units of polymers. They provide many advantages, such as ease of modification, size control and the potential to create an isolated active site core area. Dendrimers have been proven to across the BBB *in vitro* but they need to be improved in biocompatibility and cytotoxicity profiles (Dhanikulaet al, 2008). Nanocrystals are molecule aggregates that can be combined into a crystalline form of the drug surrounded by a coating of surfactant (Faraji and Wipf, 2009). Also nanocrystals are potentially toxic, but it can be reduced by surfactant coating. Use of nanocrystals as nanomedicines in drug delivery is limited due the limited stability and the requirement of crystallization of the therapeutic compound.

4 AIM OF THE STUDY

The aim of this work was to characterize the effect of cell polarization on endocytic pathways in human blood brain barrier *in vitro* model. The more specific aims were (1) to demonstrate the cell polarization and (2) to characterize the expression, localization and functionality of endocytic pathways in polarizing human brain endothelial cells. Immortalized, human hCMEC/D3 brain endothelial cells were chosen for this study as this cell line displays well the *in vivo* characteristics of BBB and is suitable for nanoparticle uptake studies (Gumbleton and Audus, 2001).

Four different approaches were utilized to characterize the level of cell polarization at multiple time points. First, tightness of the endothelial layer was evaluated by lucifer yellow permeability assay and by measurement of transendothelial electrical resistance (TEER). Second, expression of alkaline phosphatase, the marker enzyme of

differentiated BBB phenotype, was demonstrated. Third, expression of specific tight junction protein, ZO-1, was observed by confocal microscopy after immunostaining.

To characterize hCMEC/D3 cells for the presence of specific endocytic pathways, proteins involved into each pathway were selected. Expression of these proteins at mRNA level was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). For clathrin-mediated endocytosis, mRNA level of clathrin heavy chain (CHC) was further correlated with the protein level, and the activity of clathrin-mediated endocytosis was analyzed by fluorescence activated cell sorting (FACS).

Present study yields valuable information about the endocytic pathways in the blood-brain barrier. Better understanding of the endocytosis, their activity and functionality, in BBB aids developing efficient drug delivery systems to the brain. Knowledge of the active endocytic pathways present in BBB, enables development of nanoparticles that are targeted to use these active endocytic pathways. Targeted nanoparticles can serve a considerable tool to overcome the blood-brain barrier.

5 MATERIALS AND METHODS

5.1 Cell culture

Cell culturing was performed in aseptic conditions. Laboratory ware and all the reagents were sterile. Cells were incubated in HeraCell 150, Thermo electron incubator at 37 ºC in 5 % CO_2.

5.2 Cell maintenance

Cells were cultured on 75 cm² flasks at a density of 25 000 cells per cm², in a growth medium containing 5 % fetal bovine serum (FBS), 1 % penisillin-streptomycin, 0.05 % hydrocortisone, 0.5 % acid ascorbic, 1% chemically defined lipid concentrate, 1 % HEPES and 0.5 % human basic fibroblastic growth factor (bFGF) in endothelial basal

medium (EBM-2)). Flasks were 1 hour beforehand coated with the 150 µg/ml rat collagen I (3443-100-01, Cultrex). Growth medium was changed every second or third day. After 3-4 days cells reached confluence and they were either passed or used for experiments (until passage 35). Confluent cells were trypsinized with 0.25 % Trypsin/EDTA (25200, Gibco). Cell counter (Cedex XS, Innovatis) was used to determine the number of cells after trypsinization.

5.3 Cell polarization studies

For differentiation, cells were seeded on collagen coated 6 well PET cell culture inserts (3450, Costar) at 50 000 cells per cm². Medium was changed every second day.

5.3.1 Lucifer yellow permeability

To evaluate the tightness of the endothelial layer, the transmonolayer permeability of lucifer yellow (LY) was determined. In the transwell inserts, culture medium was replaced by 2.5 µg/ml Lucifer yellow solution (0259, Sigma) in the transport buffer (1 % of HEPES 1M (15630-080, Gibco), 1 % of sodium pyruvate 100 mM (11360, Gibco) in 1XHBSS (14025, Gibco)). The inserts were transferred at 10, 25 and 45 minutes to a new well containing transport buffer. Lucifer yellow fluorescence in sample corresponding to each timepoint was determined with a plate reader (Varioskan Flash 2.4.3, Thermo Scientific; excitation wavelength 425 nm, emission wavelength 535 nm). Calibration curve was made and concentration of the marker molecules was calculated.

5.3.2 Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was measured to evaluate the tightness of the cell monolayer. TEER of cells grown for 1, 3, 7 and 10 days on transwell permeable support was measured in Endohm cup (ENDOHM-24SNAP, World precision instruments) with epithelial voltohmmeter (EVOM, World precision instruments).

5.3.3 Immunocytochemistry

Formation of tight junctions, cell proliferation and localization of CHC protein was evaluated using immunocytochemistry (ICC). hCMEC/D3 cells were cultured on transwells for 1, 3, 7 and 10 days as described in section 5.3. Cells were washed with 1X phosphate buffered saline (PBS) two times, fixed with 1 ml of 4 % paraformaldehyde (from Biocenter) per well for 10 minutes and washed three times with 1XPBS for 1 minute. Membranes were cut from transwells and stored in 1XPBS prior to further analysis. To permeabilize the cells, 1 ml of 0.1 % Triton-X100 (T8787, Sigma) in 1XPBS was added on the membrane and incubated for 3 minutes. Cells were washed three times with 1XPBS for 1 minute. To reduce unspecific binding cells were incubated with 1 ml of blocking solution containing 2 % goat serum (S26, Sigma) and 1 % bovine serum albumin (A2153, Sigma) in 1XPBS for 1 hour. Primary antibodies, ZO-1 (1:100, 10 µg/ml, 40-2200, Invitrogen), CHC (1:400, 2.5 µg/ml, Ab21679, Abcam), Ki67 (1:200, 5 µg/ml, Ab15580, Abcam) and negative control IgG (1:200, 5 µg/ml, sc-2027, Santa Cruz biotechnology) were diluted in blocking solution and incubated with the cells for two hours in moist chamber at room temperature (200 µl/membrane). Cells were washed three times with 1XPBS for 5 minutes. Then cells were incubated with secondary antibody (1:200, 5 µg/ml, Alexa Fluor 594, A11012, Invitrogen), for 1 hour in moist chamber at room temperature protected from light. Cells were washed three times with 1XPBS for 5 minutes, briefly rinsed in water and incubated with 200 µl of DAPI solution (1:400, 2.5µg/ml, D3417, Sigma) to stain the nucleus. Membranes were briefly rinsed in water, transferred to microscopic slides and mounted in VECTASHIELD hard set mounting medium (H-1400, Vector laboratories). Slides were stored at 4 ℃. Imaging was performed with Leica TCS SP5II HCS A confocal microscope with air objectives (HC PL APO 10x/0.4 and HC PL APO 20x/0.7 CS) and immersion objective (HCX PL APO 20x/0.7 Imm Corr).

5.3.4 Alkaline phosphatase expression

The expression of alkaline phosphatase, the marker enzyme for a differentiated blood brain-barrier phenotype, was determined to evaluate the polarization level. hCMEC/D3

cells were cultured on transwells 1, 3, 7 and 10 days as described in Section 5.3. Sample collection was performed by lysing the cells with 1XRLB buffer (E397A, Promega). 5 µl of sample of each day were transferred in duplicate to 96 well plate and 95 µ of dilution buffer (Great EscAPe SEAP Chemiluminescence Kit 2.0, 631736-8) were added to each sample well. Samples were incubated with 100 µl of SEAP substrate solution (Great EscAPe SEAP Chemiluminescence Kit 2.0, 631736-8) for 30 minutes in dark. Luminescence was measured with a plate reader (Varioskan Flash, 2.4.3, Thermo Scientific).

5.4 RNA isolation

hCMEC/D3 cells were cultured for 1, 3, 7 and 10 days of differentiation on transwells as described in Section 5.3 and then disrupted with 350 µl of RLT buffer (1015750, Qiagen), homogenized and kept at -20 C prior to further analysis. The total RNA was extracted by using Qiagen RNAeasy mini kit (74104) according to manufacturer's instructions. Briefly 350 µl of ethanol was added to the lysate. The sample was applied to the RNeasy mini spin in which total RNA binds to the membrane. Contaminants are efficiently washed away with RW1 buffer (1014567, Qiagen) and RPE buffer (1018013,Qiagen). High-quality RNA was eluted in 30 µl of RNase-free water (1017979, Qiagen). Bind, wash and elution steps were performed by centrifugation in a microcentrifuge. The concentration of RNA was determined by Nanodrop (Nanodrop ND-1000 spectrophotometer, Thermo scientific).

5.5 RNA convertion to cDNA

RNA samples were converted to cDNA by using the High Capacity RNA-to-cDNA Kit (4387406, Applied biosystems) according to manufacturer´s instructions. For each sample, 900 µg of RNA was converted to cDNA.

5.6 Protein extraction

Protein samples were extracted for Western blot assay. hCMEC/D3 cells were cultured on transwells for 1, 3, 7 and 10 days before extraction as described in section 5.3. Cells were washed twice with ice-cold 1XPBS. To lyse the cells, 100 µl of cold RIPA buffer (89900, Thermo Scientific) containing protease inhibitor cocktail (78410, Thermo Scientific) was added to each well for 5 minutes. Lysate was transferred to a microcentrifuge tube and centrifuged at 15 000 g in cold for 15 minutes to pellet the cell debris. Supernatant was transferred to a new tube and stored at $-$ 20 ℃ prior to further analysis.

5.7 Quantitative real-time polymerase chain reaction

Quatitative real-time polymerase chain reaction (qRT-PCR) was used to evaluate the expression of specific endocytosis related proteins on mRNA level. hCMEC/D3 cells were cultured on transwells for 1,3,7 and 10 days as described in Section 5.3. Before the experiment, RNA was isolated and converted to cDNA as described in sections 5.4 and 5.5. 18 µl of mixture (120 µl of nuclease free water (NFW, 1012888, Qiagen), 60 µl of forward primer, 60 µl of reverse primer and 300 µl of FAST SYBR green master mix (4385612, Applied Biosystems)) was added to the wells of Micro Amp® Fast optical 96 well reaction plate (Applied biosystems). Then 2 µl (10 ng) of each sample were added to the same wells with the mixture. 2 µl of NFW were added to the control wells. Plate was covered with an optical adhesive cover (4360954, Applied Biosystems) and run by qRT-PCR system (StepOne system, Applied biosystems). Primers which were used are described in Table 1. CHC, Caviolin 1 and RPLP0 were ordered from Sigma and other primers were from Oligomer. Results were normalized with RPLP0. Relative mRNA expression was calculated and the values were normalized to housekeeping gene RPLP0. Functions of each protein studied are presented in Table 2.

Table 1, Primer sequences. Abbreviations: F = forward primer, R = reverse primer

Primer	Sequence
CHC	F:TCTCTACTGATGAGCTTGTTGCTG
	R:GCTCCTCACAGCCCTCATG
Caveolin 1	F:CGACGCGCACACCAAG
	R:TCTGGTTCTGCAATCACATCTTC
Cavin 1 (Polymerase I and transcript release factor)	F:AGATCAAGAAGCTGGAGGTCAA
	R:CTCCGACTCTTTCAGCGATTT
Flotillin 1	F:CGAGGCAGAGAAGTCCCAACTA
	R:TGGCAAAGGCCTCAGCTT
Flotillin 2	F:GGTGGTGTATCTCCGACACTC
	R:GATCTTCACCTGGGCGACAC
AP2A1 (Adaptor-related protein complex 2, alpha 2 subunit)	F:TGTAAGAGCAAAGAGGCGGAAA
	R:GGCCAAGCAGGAAGATGAAAA
AP2A2 (Adaptor-related protein complex 2, alpha 2 subunit)	F:GCAGCAGGTGGTCAACATAGAG
	R:GCCCCCATACCTGAACTGAA
AP2B1 (Adaptor-related protein complex 2, beta 1 subunit)	F:TCGCCCTGAGGAACATCAA
	R:TCTGCCAGAACCTGAGCAATG
AP2M1 (Adaptor-related protein complex 2, mu 1 subunit)	F:CCTCACTGCTGGCTCAGAAGA
	R:TGCACCCCGCTTGTGTT
Cdc42 (cell division cycle 42)	F:AGTGTGTTGTTGTGGGCGAT
	R:CTCAGCGGTCGTAATCTGTCA
GRAF1	F:CAGGAGTCTCGGGTCTCTGA
	R:GAGTGGGTCCAAACACCACA
RhoA	F:TCGTTAGTCCACGGTCTGGT
	R:GTCTTTCCACAGGCTCCATCA
Arf 1 (ADP-ribosylation factor 1)	F:CCATTCCCACCATAGGCTTCA
	R:CATTGCTGTCCACCACGAAGAT
PAK 1 (P21 protein (Cdc42/Rac)-activated kinase 1	F:TGAGGGAAAACAAGAACCCAAA
	R:TTCCATAACAACCCACAGCTCAT
RPLP0	F:AATCTCCAGGGGCACCATT
	R:CGCTGGCTCCCACTTTGT

Table 2. Characteristic proteins of specific endocytosis pathways studied in this project.
CME = clathrin-mediated endocytosis; CavME = caveolin-mediated endocytosis;
FlotME = flotillin-mediated endocytosis.

Protein	Pathway	Functions
CHC	CME	Forming lattices during clathrin-mediated membrane traffic, involved in mitosis
AP2	CME	Links clathrin to plasma membrane
Caveolin 1	CavME	Role in cell polarization and migration functions, scaffolding of signaling proteins, cholesterol binding and homeostasis, regulation of endocytic trafficking
Cavin 1	CavME	Necessary for caveolae formation; regulates cell polarization and migration
PAK 1	Macropinocytosis	Involved in numerous cellular processes, several of which are through its interactions with the cell cytoskeleton modulators; involved in RhoA endocytosis
Flotillin 1	FlotME	Involved into cell-matrix adhesion, phagocytosis, exocytosis, and several signaling pathways
Flotillin 2	FlotME	The same as for flotillin 1
Cdc42	CLIC-GEEC	RhoGTPase. mediates cell polarity and filipodia, also regulates phagocytosis
Arf1	CLIC-GEEC	GTPase. A central role in vesicle formation
GRAF1	CLIC-GEEC	Not clear. Coating protein?
RhoA	RhoA-dependent E.	RhoGTPase

5.8 Western Blotting

Protein concentration of the hCMEC/D3 samples was determined by the kit (Pierce™ BCA Protein Assay Kit, 23227, Thermo Scientific). The samples were diluted two times with a sample buffer, heated for 5 min at 95 ºC for protein denaturation and frozen prior to further studies. Protein samples from hCMEC/D3 cells and molecular weight markers (high molecular weight marker (161-0309, Biorad) and low molecular weight marker (161-0305, Biorad) were separated by gel electrophoresis and then transferred to nitrocellulose membrane (162-0145, BioRad). Non-specific binding sites were blocked by 5 % milk in 1 X Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) containing 0.1 % Tween 20 (P5927, Sigma) for 1 hour at room temperature. Membrane was incubated with primary antibody, CHC (1:2000, 0.5 µg/ml, 610499, BD) in 5 % milk in 1 X Tris-buffered saline with Tween (TBST; 25 mM Tris, 150 mM NaCl, 2 mM KCl, Tween pH 7.4), for 1 hour at room temperature. Horseradish peroxidace (HRP) -conjugated Goat-anti-mouse ZyMax (1:2000, 0.5 µg/ml, 816520, Zymed) was applied as secondary antibody for 45 minutes at room temperature. Between incubations, membranes were washed with 1XTBST first 15 minutes and then 3 times 5 minutes. To reveal immunoreactive bands, the blots were incubated in ECL Plus reagent for 1 min (RPN 2106, GE Healtcare Lifesciences) and detected by camera after 1 min and 5 min expose (M35 X-OMAT Processor, Kodak).

5.9 Fluorescence-activated cell sorting

Uptake of transferrin (specific marker of clathrin-mediated endocytosis) was determined by fluorescence-activated cell sorting (FACS). hCMEC/D3 cells were seeded to transwells 10, 7 and 1 day prior to uptake experiment. Uptake experiment was made to both directions (from apical to basolateral and basolateral to apical direction). Cells were washed with warm HEPES medium (25 mM of 1M HEPES (15630-056, Invitrogen) in EBM-2 medium (190860, Lonza)). 500 µl of HEPES medium containing 5 µg/ml of transferrin (F0895, Sigma) was added to apical compartments for apical to basolateral uptake study and 2000 µl to the basolateral compartments for basolateral to apical uptake study. Opposite compartment was filled with HEPES medium. After 5

minutes incubation at 37 ºC, cells were washed two times with ice-cold HEPES buffer (1M, 15630-056, Invitrogen) to remove excess of transferrin and to stop endocytosis. To remove the surface-bound transferrin and detach the cells, 600 µl of ice-cold pronase (2 mg/ml (P5147, Sigma) in HEPES medium) was added to the wells for 1 hour. Detached cells were transferred to eppendorf tubes. Samples were centrifuged twice and pellet resuspended in ice-cold HEPES buffer. Cells were analyzed by using a FACS BD LSR II Flow Cytometer (BD, San Jose, CA, USA) operating under the DIVA software (Version 6.0). For each sample, 10 000 events were collected after gating of the living cell population by forward/side scatter, and the mean fluorescence of the living cells was determined at 488 nm.

6 RESULTS

6.1 Lucifer yellow permeability

To evaluate the integrity of the endothelial layer, the transmonolayer permeability of lucifer yellow was determined. LY is a highly hydrophilic, low molecular weight tracer molecule that indicates paracellular transport across the tight junctions, which also indicates the polarization level of hCMEC/D3 cells. Low lucifer yellow permeability indicates tightness of the cell layer, but also high polarization. As shown in figure 5, the apparent permeability coefficient of lucifer yellow on hCMEC/D3 cells decreased from day 1 to day 7, approximately 3 fold, from 0.00194 cm/min to 0.00061 cm/min (Figure 5). On day 10, P_{app} started to increase and reached the permeability 0.00127 cm/min, which was approximately same level as on day 3.
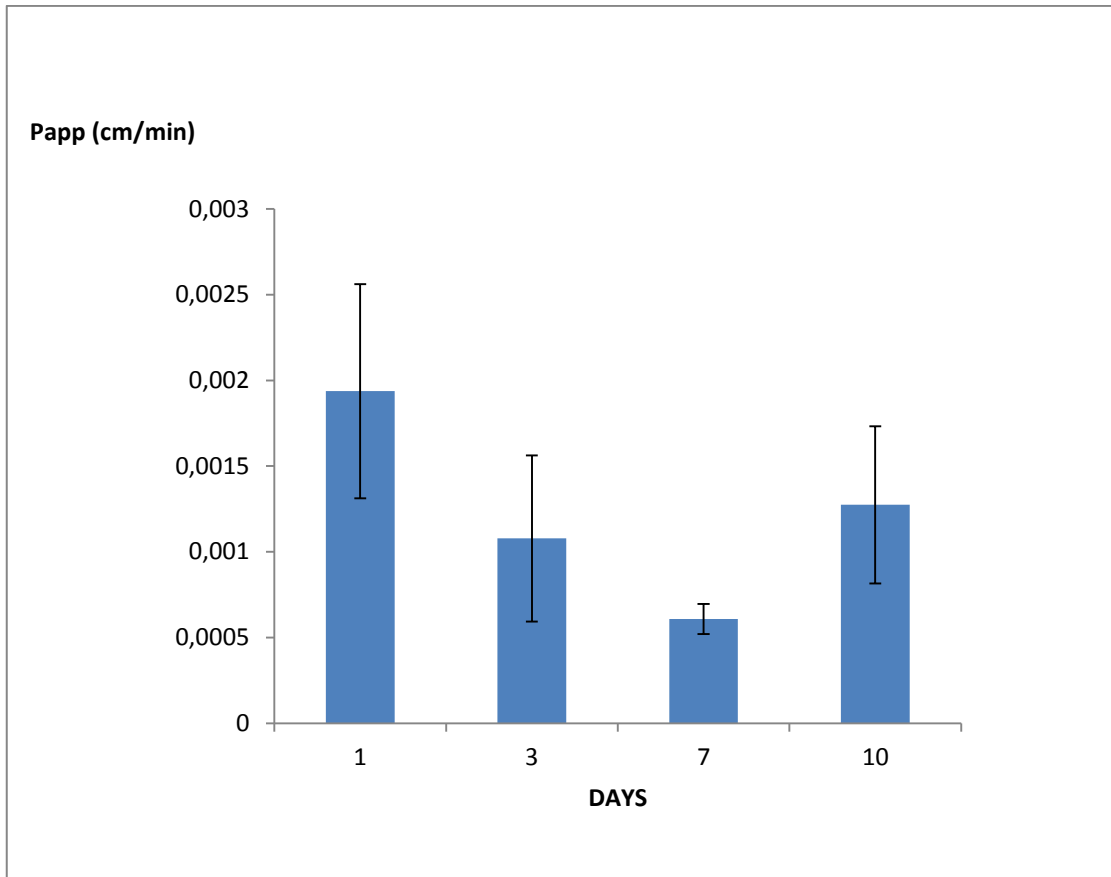
Figure 5, The apparent permeability coefficients of Lucifer Yellow on hCMEC/D3 cells after 1, 3, 7 and 10 days of differentiation on transwells (mean from 3 independent experiments ± SD).

6.2 Transendothelial electrical resistance

Transendothelial electrical resistance of hCMEC/D3 cells is indicative of the tightness of the endothelial layer, which is also related to the cell polarization level. TEER was expected to increase when cultured longer due to tight junction formation between contacting confluent cells. On day 1 the TEER value was 0 $\Omega \cdot cm^2$ as seen in the Figure 6. On day 3 TEER was 9.3 $\Omega \cdot cm^2$ and on day 7 TEER was 15.5 $\Omega \cdot cm^2$. The highest TEER was measured on day 10 and it was 35.65 $\Omega \cdot cm^2$ (Figure 6).
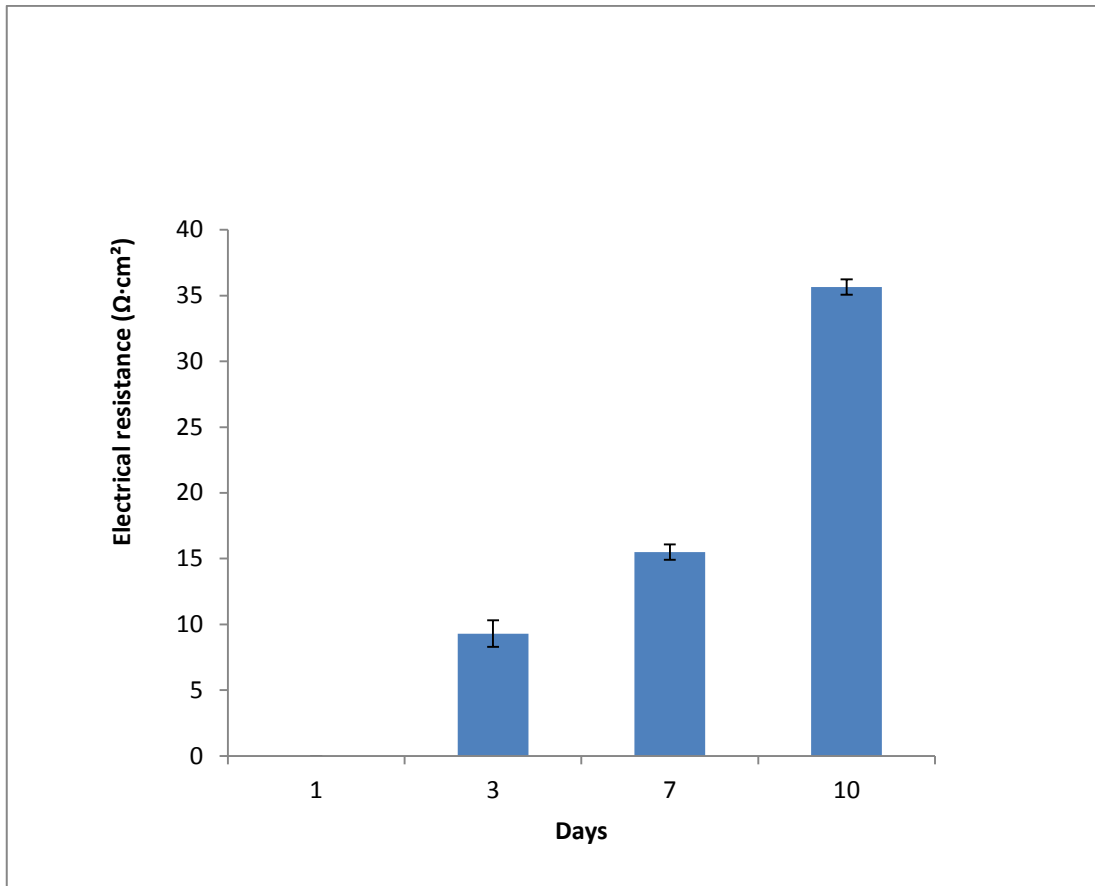
Figure 6, Transepithelial electrical resistance (TEER) of hCMEC/D3 cells after 1, 3, 7 and 10 days of differentiation on transwells (mean ± SD n = 2). TEER was measured in Ehdohm cup with epithelial voltohmmeter.

6.3 Immunofluorescence

Tight junction formation, proliferation level and clathrin-heavy chain (CHC) protein expression was characterized using immunofluorescence. Tight junction formation was analyzed by staining specific endothelial tight junction marker protein ZO-1. High expression of ZO-1 indicates higher tight junction formation as well as higher integrity of the barrier which is also indicative of higher cell polarization. As seen in Figure 7, expression of ZO-1 increased together with increased cell density from day 1 to day 10.

Proliferation level was analyzed to verify if the hCMEC/D3 cells exhibit contact inhibition, in other words, if they stop dividing when they reach confluence by staining Ki67, a nuclear protein expressed only in proliferating cells. All the hCMEC/D3 cells

were actively proliferating from day 1 to day 10 even though the cells have obviously reached the confluence already on day 7. However, on day 10 there are much less proliferating cells than on day 7 (Figure 8).

Clathrin heavy-chain (CHC) was stained to visualize the expression of this protein as a function of cell polarization. CHC is directly involved into clathrin-mediated endocytosis, therefore the expression of this protein could be indicating the presence of this pathway. As seen in Figure 9, CHC is strongly expressed in cells at all studied time points. There seem to be no remarkable differences in the level of CHC expression from day 1 until day 10.
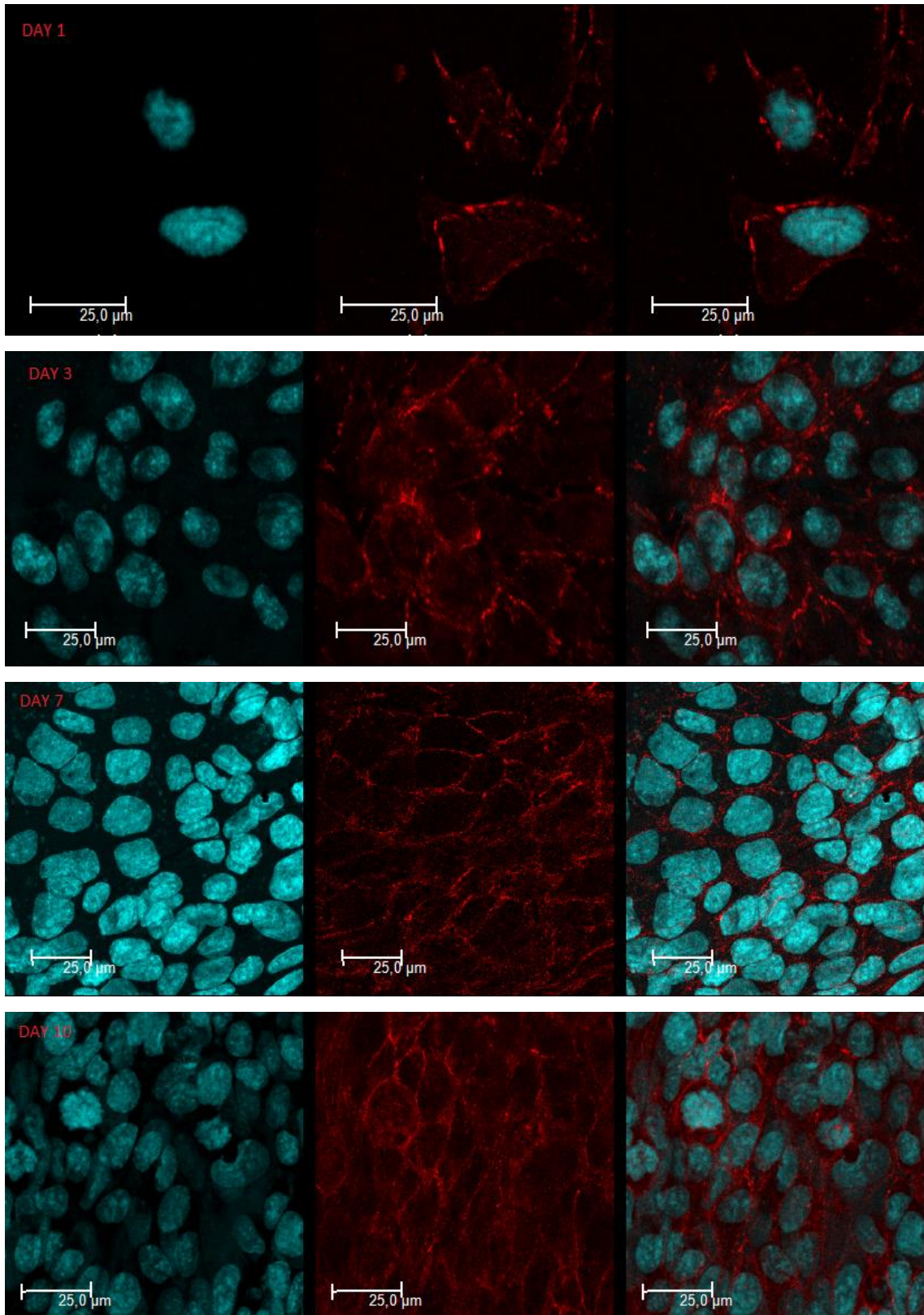
Figure 7, Confocal microscopy images of tight junctions in hCMEC/D3 cells after 1, 3, 7 and 10 days of differentiation on transwells. ZO-1 (red) was used as a marker of tight junctions. Nuclei (blue) were stained with DAPI.
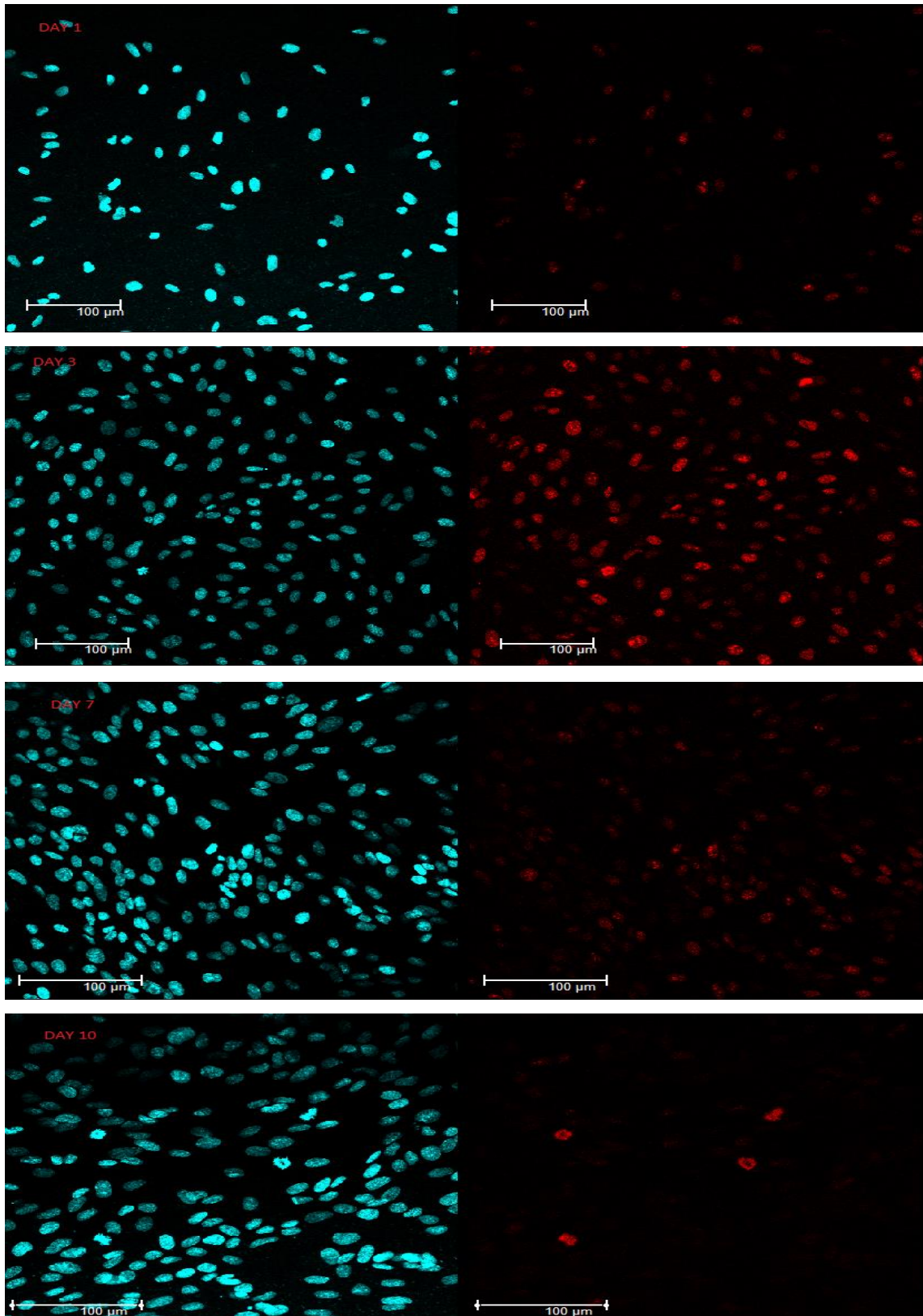
Figure 8, Confocal microscopy images of hCMEC/D3 cells after 1, 3, 7 and 10 days of differentiation on transwells. Proliferating cells (red) were stained by proliferation nuclear marker Ki67 and nuclei (blue) with DAPI.
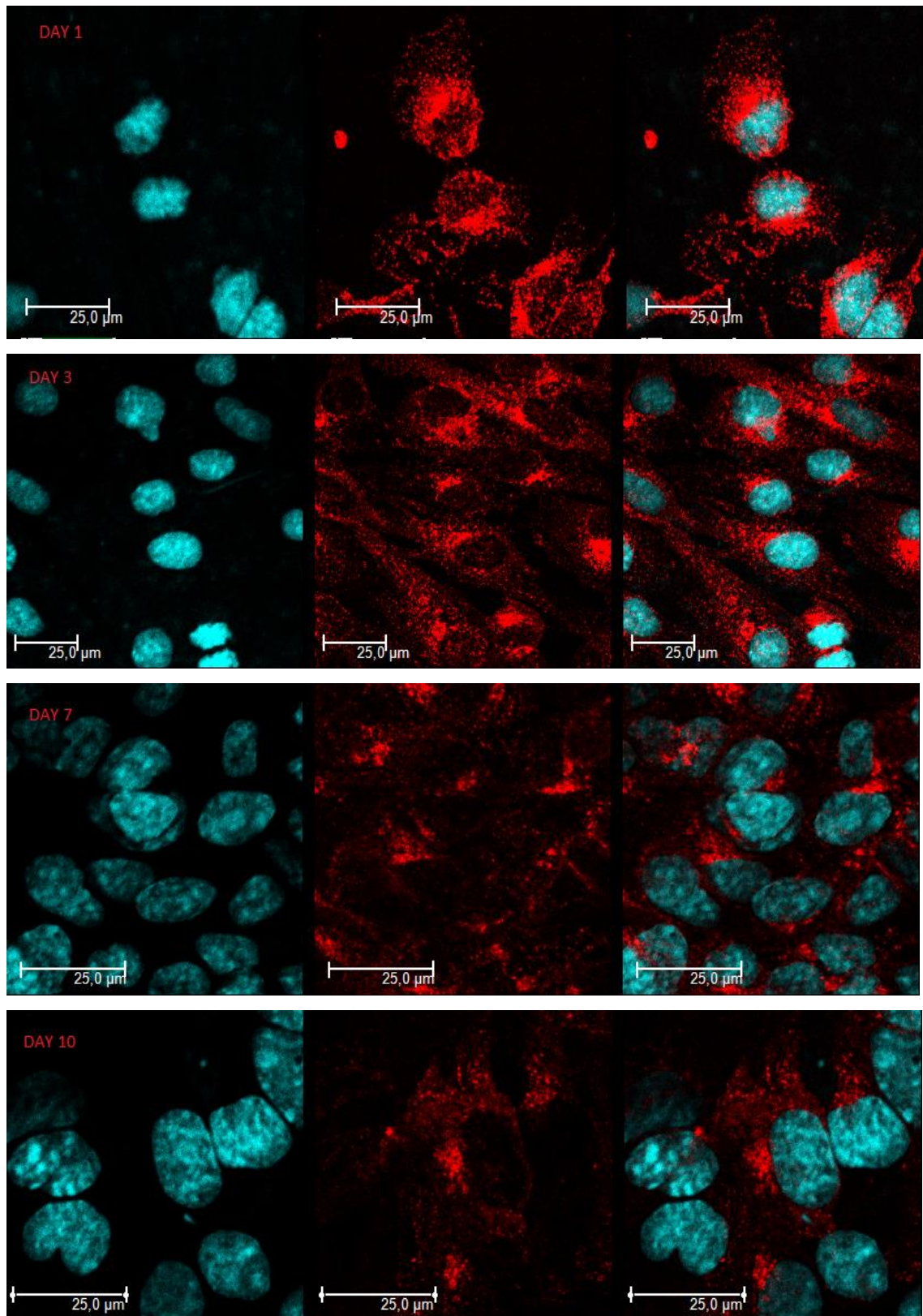
Figure 9, Confocal microscopy images of clathrin localization in hCMEC/D3 cells after 1, 3, 7 and 10 days of differentiation on transwells. Protein CHC (red) was stained with CHC specific antibody and nuclei (blue) with DAPI.

6.4 Alkaline phosphatase expression

To evaluate the differentiation of the hCMEC/D3 cells, expression of alkaline phosphatase (ALP), the marker enzyme of differentiated BBB phenotype, was determined. Cell On day 1 expression of total ALP was 6.6 µg/mg, on day 3 it was 10.6 µg/mg , on day 7 it was 10.8 µg/mg and on day it started to decrease and it was 6.7 µg/mg (Figure 10). Concentration of total protein of alkaline phosphatase was increased from day 1 to day 7 but was decreased on day 10, which is showing same kind of trend with other polarization studies, except TEER.
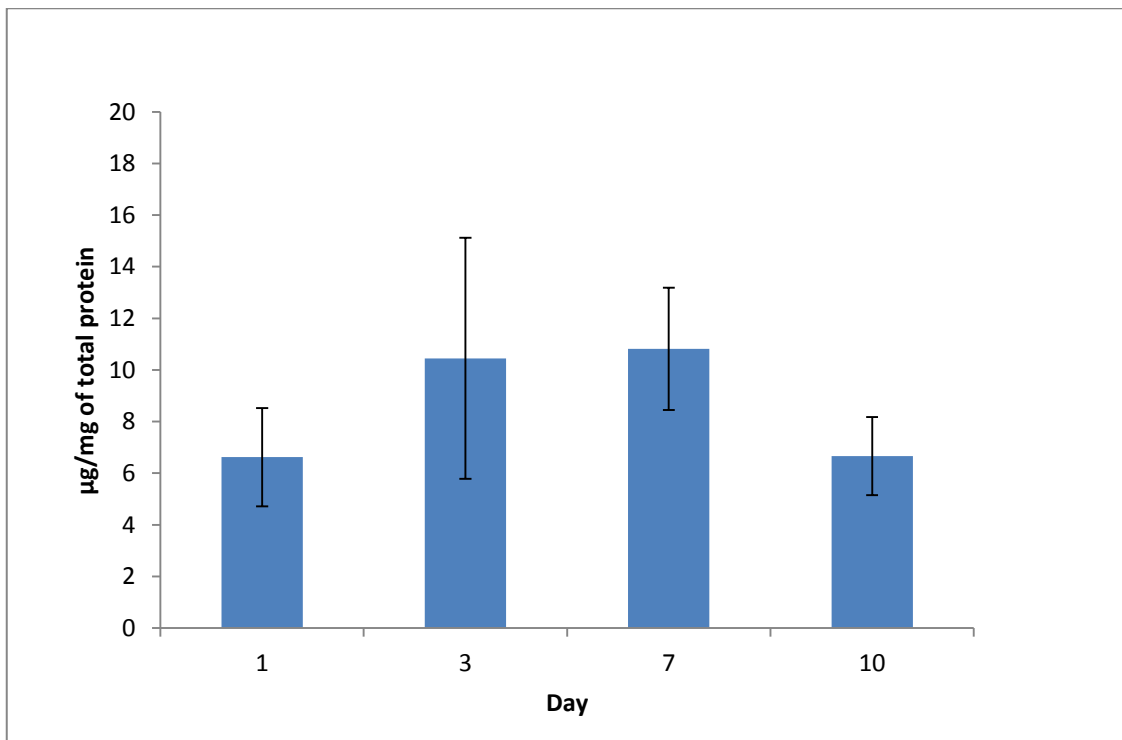

Figure 10, Expression of alkaline phosphatase in hCMEC/D3 cells after 1, 3, 7 and 10 days of differentiation on transwells (mean from 2 independent experiments ± SD).

6.5 Quantitative real-time polymerase chain reaction

Expression of proteins which are characteristic for each endocytic pathway was determined at mRNA level by quantitative real-time polymerase chain reaction (qRT-PCR). We were interested to see how polarization affects different endocytic pathways on mRNA level. To follow cell polarization in time, we were growing the cells on transwell supports for 1, 3, 7 or 10 days followed by qRT-PCR analysis..

Five proteins are related to clathrin-mediated endocytosis, clathrin heavy chain (CHC) and four adaptor protein-2 (AP2) proteins (AP2A1, AP2A2, AP2B1, AP2M1). The level of AP2 proteins remained at the same level at all tested time points. In contrast, mRNA expression of CHC increased about 30 % from day 1 to day 7 and on day 10 decreased (Figure 11). The amount of flotillin-mediated endocytosis related proteins did increase in about 50 % (flotillin 1) and 170 % (flotillin 2) on mRNA level from day 1 to day 7, but then slightly decreased on day 10. mRNA expression of macropinocytosis related protein PAK 1 increased from day 1 to day 10 about 100 % . mRNA expression of RhoA-dependent endocytosis related protein remained the same as well as clathrin-independent carrier (CLIC) / GPI-AP-enriched early endosomal compartment (GEEC) endocytosis (CLIC/GEEG) related proteins. The mRNA level of caveolin-mediated endocytosis related protein cavin 1 remained the same, whereas caveolin 1 expression decreased about 50 % between day 3 and 7. In general, mRNA expression of most of the studied proteins did not considerably change during 10 days of cell polarization on transwell permeable support.
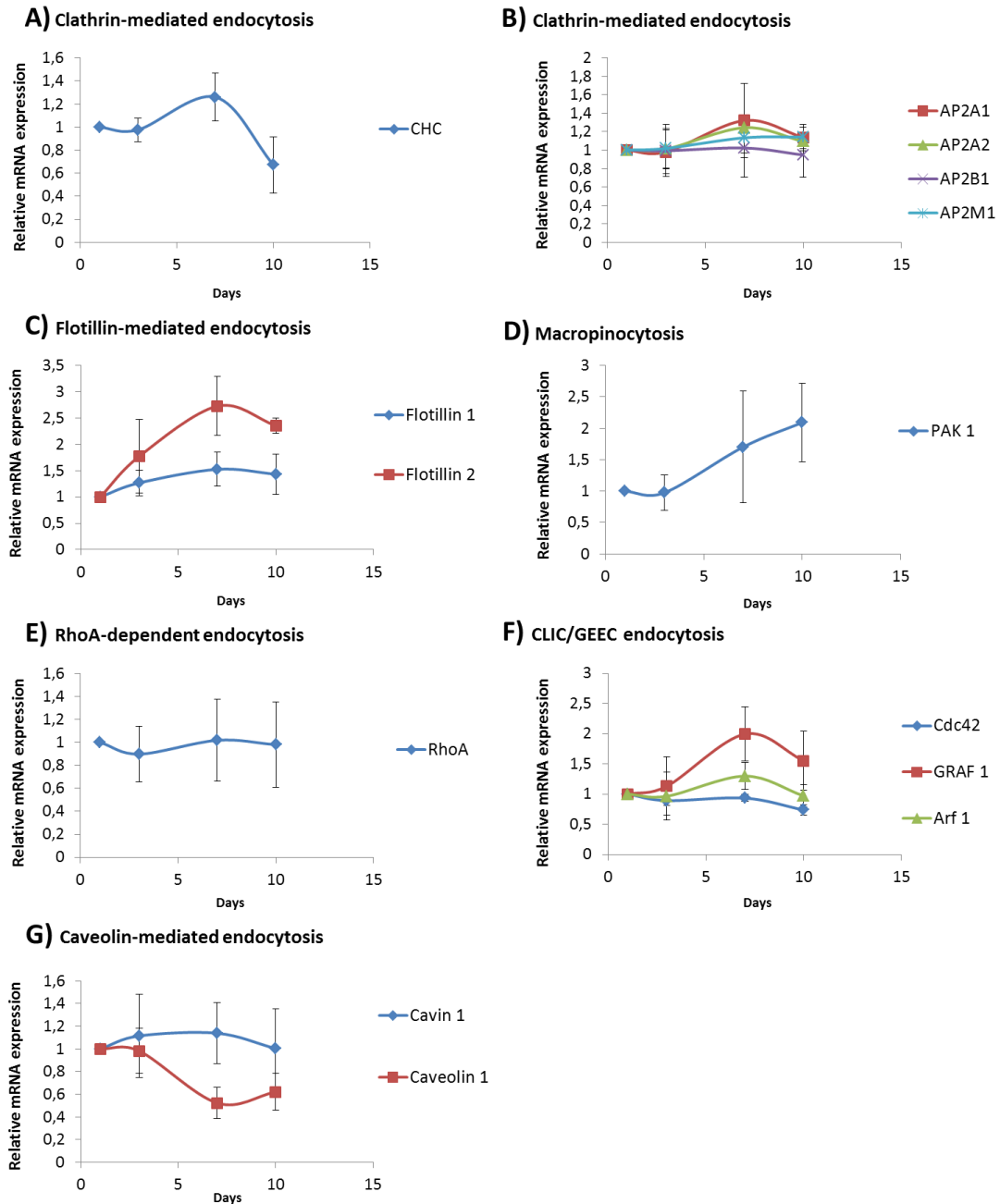
Figure 11, Relative mRNA expression of characteristic genes of endocytic pathways in hCMEC/D3 cells quantified by quantitative real-time polymerase chain reaction (qRT-PCR). A) Clathrin heavy chain (CHC) and **B)** four adaptor protein-2 (AP-2) proteins (AP2A1, AP2A2, AP2B1, AP2M1) are characteristic for clathrin-mediated endocytosis. **C)** Flotillin 1 and flotillin 2 are characteristic for flotillin-dependent endocytosis. **D)** Macropinocytosis is dependent of protein PAK 1. **E)** Protein RhoA is characteristic for RhoA-dependent endocytosis. **F)** Clathrin-independent carrier (CLIC) / GPI-AP-enriched early endosomal compartment (GEEC) endocytosis (CLIC/GEEC) is dependent of the small GTPases, Cdc42 and GRAF1, and ADP-ribosylation factor, arf1. **G)** Proteins caveolin 1 and cavin 1 are characteristic for caveolin-mediated endocytosis.

6.6 Western Blot

mRNA level of the protein does not necessarily correlate with the actual protein level. Based on the data from qRT-PCR, CHC expression decreased approximately 33 % after 10 days of differentiation in comparison to day 1. To investigate if the same trend can be seen at the protein level, we performed western blot to visualize CHC protein expression. CHC is related to clathrin-mediated endocytosis. Expression of CHC protein in hCMEC/D3 cells after 1, 7 and 10 days was not remarkably changed (Figure 12).
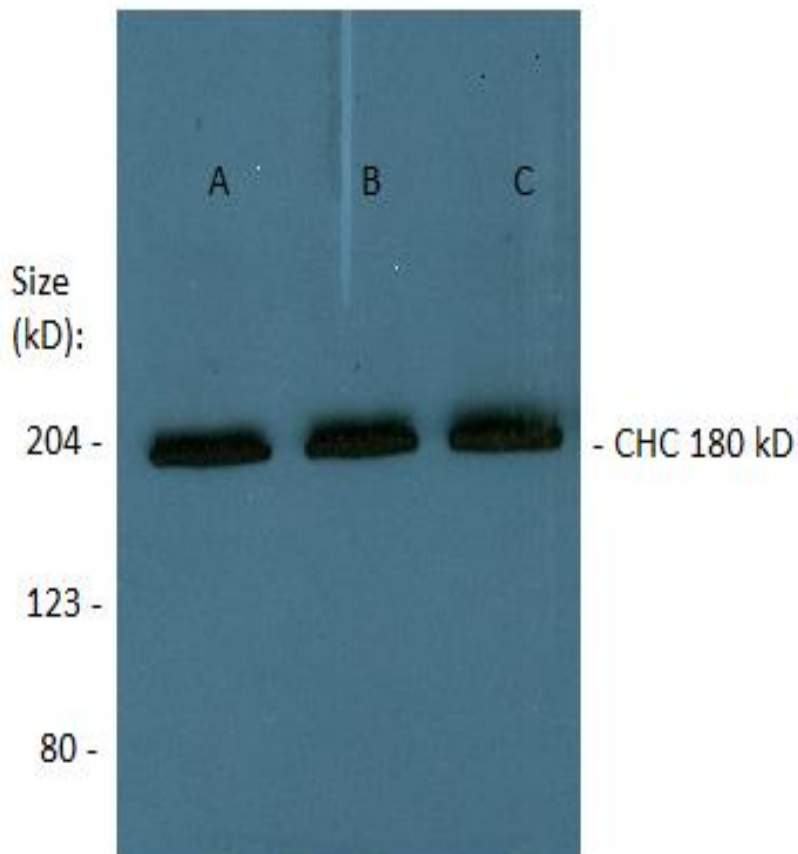

Figure 12, Expression of CHC protein in hCMEC/D3 cells visualized by western blot. Amount of the protein sample was 3,7 µg/well. Molecular weight of CHC is 180 kD. The cells were grown on transwell permeable supports for 1, 7 and 10 days in A, B, C, respectively.

6.7 Fluorescence-activated cell sorting

To study the activity of clathrin-mediated endocytosis as a function of cell polarization, uptake of fluorescently-labeled transferrin was determined using fluorescence - activated cell sorting (FACS). Transferrin is a specific marker of CME. Because the membrane supporting cells might either bind some transferrin or prevent the free access of transferrin to the basolateral side of the cells, the uptake results from one direction cannot be directly compared with the results from another direction.

According to our data, transferrin uptake in both directions was slightly (20-25 %) increased from day 1 to day 7 (Figure 13). On day 10 uptake in both apical to basolateral and basolateral to apical direction went back to the day 1 level. These results indicate light increase in the activity of clathrin - mediated endocytosis from apical to basolateral as well as from basolateral to apical direction.
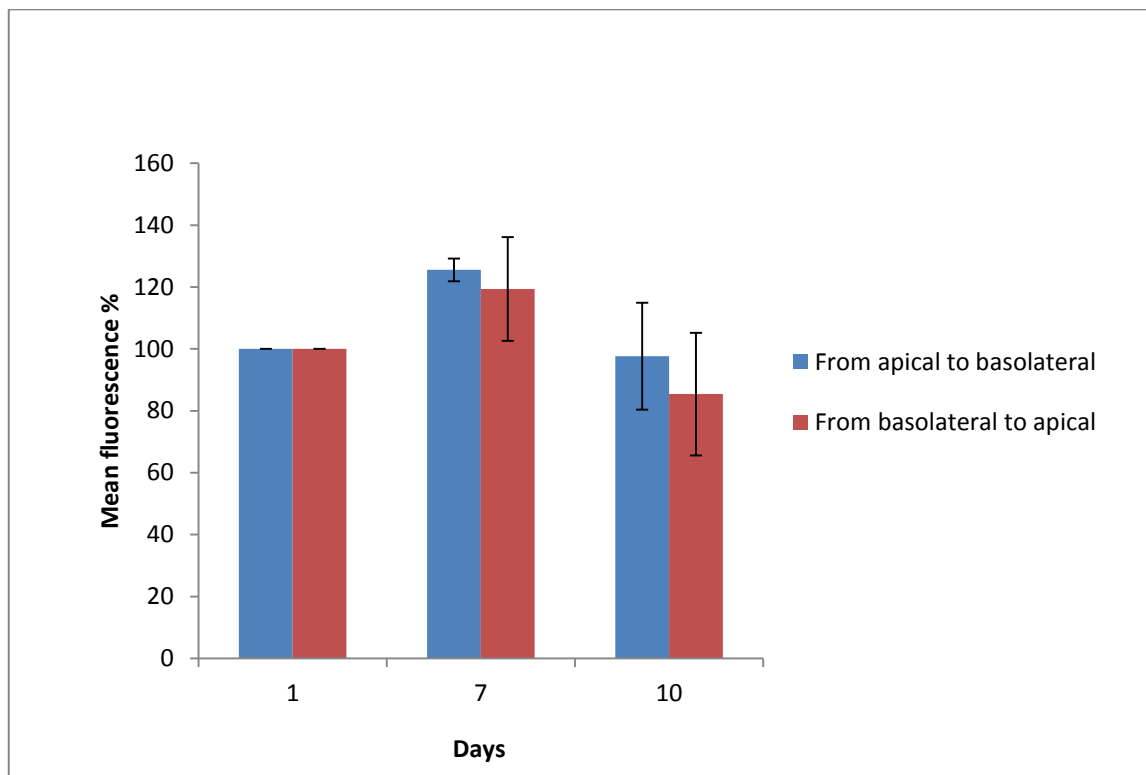

Figure 13, Transferrin uptake (specific marker of clathrin-mediated endocytosis) in hCMEC/D3 cells analysed by fluorescence-activated cell sorting after 1, 7 and 10 days of cell differentiation on transwells.

7 DISCUSSION

Nanoparticles have been shown to be promising tools to deliver drugs to the brain, but the exact endocytosis mechanism leading to efficient penetration via BBB is not fully understood. More studies are needed to set up the link between uptake mechanism and brain delivery efficiency in order to make the design of nanoparticles more effective (Smith and Gumbleton, 2006 ; Sandvik et al, 2011; Wolfart et al, 2012). In such studies careful characterization of the BBB cell model is highly important to get as reliable data as possible. Blood-brain barrier endothelial cells are known to be well polarized *in vivo* (Abbott et al, 2008). In the present study we aim to characterize polarization status of hCMEC/D3 cells, which have been widely utilized as *in vitro* BBB cell model. Furthermore, we aimed to investigate how polarization influences endocytic processes in these cells.

In our study maximum TEER observed for hCMEC/D3 cells in our study was almost 100 times lower than TEER of brain endothelial cells *in vivo*, that is approximately 1000 $\Omega \cdot cm^2$ (Butt et al, 1990). Low TEER indicates that hCMEC/D3 cells are far leakier than the brain endothelial cells *in vivo*. This leakiness is common in all immortalized cell lines due to absence of proper paracellular barrier properties (Gumbleton and Audus, 2001). Due to the leakiness of the hCMEC/D3 cells they are not an optimal model for permeability transport studies. However, nanoparticles are relatively large in size and cannot permeate through the paracellular route, therefore the leakiness is not a considerable problem in nanoparticle uptake studies (Hillaireau and Couvreur, 2009).

hCMEC/D3 cells have been widely used in nanoparticle uptake experiments (dos Santos et al, 2011, Georgieva et al, 2011; Georgieva et al, 2012). Most of the uptake experiments have been done with polarized cells but some have been made with non-polarized cells (Chattopadhyay et al, 2008; Markoutsa et al, 2011; Ragnaill et al, 2011; Pinzon-Daza et al, 2012). To polarize the hCMEC/D3 cells, they were cultured on transwells. However, polarization status has not always been confirmed, even though it

is very important, because it has been shown that nanoparticle internalization decreases when the hCMEC/D3 cells are polarized (Ragnaill et al, 2011).

In those studies where polarization level has been confirmed, it has been done by transendothelial electrical resistance measurements and sometimes also by paracellular permeability tests (Ragnaill et al, 2011: Pinzon-Daza et al, 2012). In our hands highest TEER was observed at day 10. However, the results from other polarization status studies such as lucifer yellow permeability (Figure 5), ZO-1 staining (Figure 7), alkaline phosphatase expression (Figure 10) and proliferation marker expression (Figure 8) show the same trend that polarization was increased until day 7 and then decreased on day 10. *In vivo* brain endothelial cells form a uniform well-polarized monolayer which exhibits contact inhibition (Abbott et al, 2008). Our results show that hCMEC/D3 cells continue proliferating even though they have reached the confluence (Figure 8). Therefore, the cells start growing as a multilayer, which have negative influence on their polarization. This might be the reason why lucifer yellow permeability and alkaline phosphatase activity assays indicate lower polarization of cells on day 10 in comparison to day 7, whereas TEER increases. These results indicate that TEER measurements have to be supported by other methods to demonstrate the cell polarization.

Our polarization studies are in line with the previous studies made by other laboratories. Lucifer yellow permeability was the same order of magnitude as in other studies in the literature (Poller et al, 2008). TEER in hCMEC/D3 cell line was as low in our work as in the other works (Weksler et al, 2005; Ragnaill et al, 2011). Positive staining for tight junctional protein ZO-1 has been demonstrated by the others as well as in our study (Weksler et al, 2005). The most used seeding density has been 50 000 cells per cm² (Poller et al, 2008; Ragnaill et al, 2011). Weksler and colleagues (2005) used seeding density 20 000 cells per cm². Usually hCMEC/D3 cells have been used on days 7 to10 (Ragnaill et al, 2011). In our hands hCMEC/D3 cells seeded at the density 50 000 cells per cm² are best polarized on day 7; therefore this is the optimal time point to perform studies with hCMEC/D3 cells. Overall, even though the polarization level increased to some extent, our results indicate that hCMEC/D3 cells were not well polarized due to small differences on polarization levels between early and late time points.

dos Santos et al (2011) performed the comparative study investigating the effect of particle size on the *in vitro* cell uptake in several cell lines, including hCMEC/D3 cells, HeLa cells, A549 epithelial cells, 1321N1 astrocytes and murine RAW 264.7 macrophages. hCMEC/D3 cells showed unexpectedly high uptake of nanoparticles in comparison to other cell lines. This was unexpected as brain endothelial cells *in vivo* are known to have reduced endocytic activity (Ragnaill et al, 2011). The experiments were performed in cells grown on plastic for 24 hours and incubated with nanoparticles. The use of non-polarized cells might have resulted in very different endocytic activity from highly polarized cells in BBB *in vivo*.

Nanoparticle uptake usually decreases when cells are polarized. For instance, Matsui et al (1997) demonstrated that internalization of liposome-DNA complexes in airway epithelial cells decreased when cells were differentiated. Internalization of nonviral gene delivery vectors, such as Lipofectin and polylysine, in CaCo-2 cells was decreased approximately 1000 fold in differentiated cells in comparison to non-differentiated cells (Cryan and O´Driscoll, 2003). Brain endothelial cells are known to be well polarized *in vivo* and also demonstrate very low endocytic activity (Smith and Gumbleton, 2006; Ragnaill et al, 2011). However, our data obtained in hCMEC/D3 cells demonstrated the lack of considerable changes in mRNA expression of proteins characteristic of different endocytic pathways with cell polarization in most cases. One possible explanation can be the fact that our polarization characterization studies demonstrated overall low level of polarization of hCMEC/D3 cells even after 7 days on transwell permeable support.

Furthermore, it is important to remember that mRNA levels do not necessary correlate with protein levels (Vogel and Marcotte, 2012). Therefore it is possible, that although no changes were observed at the mRNA level, the amount of corresponding protein and/or the activity of the corresponding endocytic pathways were still changed as function of cell polarization. Whereas, reduction of mRNA observed for such proteins as CHC and caveolin-1 might not lead to the decreased expression of corresponding proteins. To check this hypothesis, we chose CHC protein as an example to see the

expression of CHC on protein level and for transferrin uptake study to see the activity of the clathrin-mediated pathway.

Our results showed, that there was not remarkable difference in CHC expression on protein level on different time points, but mRNA expression of CHC was decreased approximately 30 % from day 1 to day 10. Inconsistency between CHC mRNA and protein expression might be due to gene regulation of the cell (Vogel and Marcotte, 2012). Cells are able to regulate the protein synthesis by influencing in the mRNA and probably not all mRNA is translated to protein. Gene expression is controlled many different ways, mostly by post-transcriptional, translational and protein degradation regulation. These factors explain mostly why the changes in mRNA level do not correlate with protein levels. Additionally, differences in sensitivity between methods of protein and mRNA quantification must be taken into account. Conventional Western blot is not quantitative whereas qRT-PCR is quantitative and highly sensitive method. Therefore, it is possible, that less than 2 times decrease in mRNA level between day 1 and day 10 is not big enough to be detected by western blot.

For clathrin-mediated endocytosis good correlation was observed between mRNA expression and the activity of the pathway. It would have been highly valuable to see if the same correlation can be observed for other endocytosis-related proteins. Unfortunately, there are no specific markers for most of the endocytic pathways, making it impossible to measure the activity of these pathways (Doherty and McMahon, 2009). However we can speculate that such a correlation is unlikely to be found for proteins which have multiple functions in the cell. For example Cdc42 and GRAF1 are kinases with multiple functions in regulation of cell morphology, cell cycle and migration (Hall, 1998; Erickson and Cerione, 2001; Doherty et al, 2011).

8 CONCLUSIONS

In the present study we characterized polarization status of hCMEC/D3 human brain endothelial cell line, which is widely used as BBB *in vitro* model. It was shown that

hCMEC/D3 cells are best polarized after growing on transwell permeable support for 7 days. At the later time points, the cell polarization started to decrease, probably due to multilayer formation. As the TEER of the cells growing in the multilayer is increase in comparison to monolayer cells, we conclude that measuring TEER alone is not a reliable method to determine polarization status of the cells.

RNA expression of most of endocytosis-related proteins did not change remarkably during 7 days of cell polarization on transwell permeable supports which is in a good agreement with overall poor polarization of hCMEC/D3 cell model. In case of clathrin-mediated endocytosis no correlation between CHC mRNA and protein level was observed. However, good correlation between CHC mRNA level and activity of the pathway was found. It would be interesting to study if the same trend can be observed for other endocytic pathways.

9 REFERENCES

Abbott NJ: Prediction of blood-brain barrier permeation in drug discovery *in vivo*, *in vitro*, and *in silico* models. Drug Discov Today 1: 407- 416, 2004

Abbott NJ, Rönnbäck L, Hansson E: Astrocyte-endothelial interactions at the blood-brain barrier. Neurosci 7: 41-53, 2006

Abbott NJ, Dolman DEM, Patabendige AK: Assays to predict drug permeation across the blood-brain barrier, and distribution to brain. Curr Drug Metab 9: 901-910, 2008

Alavijeh MS, Chishty M, Qaiser MZ, Palmer AM: Brug metabolism and pharmacokinetics, the blood-brain barrier and central nervous system drug discovery. NeuroRx 2: 554-571, 2005

Ali MH, Schlidt SA, Chandel NS, Hynes KL, Schumacker PT, Gewertz BL: Endothelial permeability and IL-6 production during hypoxia: role of ROS in signal transduction. Am J Physiol 277: 1057-1065, 1999

Alyautdin RN, Petrov VE, Langer K, Berthold A, Kharkevich DA, Kreuter J: Delivery of loperamide across the blood-brain barrier with polysorbate 80-coated polybutylcyanoacrylate nanoparticles. Pharm Res 14:325-328, 1997

Alyaudtin RN, Reichel A, Löbenberg R, Ramge P, Kreuter J, Begley DJ: Interaction of poly(butylcyanoacrylate) nanoparticles with the blood-brain barrier *in vivo* and *in vitro*. J Drug Targ 9: 209-221, 2001

Audus KL, Borchardt RT: Characterization of an *in vitro* blood-brain barrier model system for studying drug transport and metabolism. Pharm Res 3: 81-87, 1986

Audus KL and Borchardt RT: Bovine brain microvessel endothelial cell monolayers as a model system for the blood-brain barrier. Ann NY Acad Sci 507: 9-18, 1987

Begley DJ: The blood-brain barrier: principles for targeting peptides and drugs to the central nervous system. J Pharm Pharmacol 48: 136-146, 1996

Boado RJ, Pardridge WM: The Trojan horse liposome technology for nonviral gene transfer across the blood-brain barrier. J Drug Del 2011: 1-12, 2011

Booth R, Kim H: Characterization of a microfluidic *in vitro* model of the blood-brain barrier (μBBB). Lab Chip 12: 1725-1904, 2012

Brightman MW, Reese TS: Junctions between intimately apposed cell membranes in the vertebrate brain. J Cell Biol 40: 648-677,1969

Butt AM, Jones HC, Abbott NJ: Electrical resistance across the blood-brain barrier in anasthetized rats: a developmental study. J Physiol 429: 47-62, 1990

Cardoso FL, Brites D, Brito MA: Looking at the blood-brain barrier: Molecular anatomy and possible investigation approaches. Brain Res 64: 328-363, 2010

Chang WJ, Rothberg KG, Kamen BA, Anderson RG: Lowering the cholesterol content of MA104 cells inhibits receptor-mediated transport of folate. J Cell Biol 118: 63-69, 1992

Chattopadhyay N, Zastre J, Wong H-L, Wu XY, Bendayan R: Solid lipid nanoparticles enhance the delivery of the HIC protease inhibitor, atazanavir, by a human brain endothelial cell line. Pharm Res 25: 2262-2271, 2008

Chauhan NB: Trafficking of intracerebroventricularly injected antisense oligonucleotides in the mouse brain. Antisense Nucleic Acid Drug Dev 12: 353-357, 2002

Cheng Y-C, Hsieh W-Y, Lee W-F, Zeng D-T: Effect of surface modification of PLGA-PEG-PLGA nanoparticles on loperamide delivery efficiency across the blood-brain barrier. J Biomat Appl 27: 909-922, 2013

Conner SD, Schmid SL: Regulated portals of entry into the cell. Nature 422: 37-44, 2003

Couet J, Belanger MM, Roussel E, Drolet M-C: Cell biology of caveolae and caveolin. Adv Drug Deliv Rev 49: 223-235, 2001

Cryan SA, O´Driscoll CM: Mechanistic studies on nonviral gene delivery to the intestine using in vitro differentiated cell culture models and an in vivo rat intestinal loop. Pharm Res 20: 569-575, 2003

Cucullo L, McAllister MS, Kight K, Krizanac-Bengez L, Marroni M, Mayberg MR, Stanness KA, Janigro D: A new dynamic *in vitro* model for the multidimensional study of astrocyte-endothelial cell interactions at the blood-brain barrier. Brain Res 951: 243-254, 2002

Cucullo L, Couraud P-O, Weksler B, Romero I-A, Hossain M, Rapp E, Janigro D: Immortalized human brain endothelial cells and flow-based vascular modeling: a marriage of convenience for rational neurovascular studies. J Cereb Blood Flow Metab 28: 312-328, 2008

Dauchy S, Miller F, Couraud P-O, Weaver RJ, Weksler B, Romero I-A, Scherrmann J-M, De Waziers I, Declèves X: Expression and transcriptional regulation of ABC transporters and cytochromes P450 in hCMEC/D3 human cerebral microvascular endothelial cells. Biochem Pharm 77: 897-909, 2009

David C, McPherson PS, Mundigl O, De Camilli P: A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. Natl Acad Sci 93: 331-335, 1996

DeBault LE, Cancilla PA: Gamma-glutamyl transpeptidase in isolated brain endothelial cells: induction by glial cells *in vitro*. Science 207: 653-655, 1980

Dejana E, Lampugnani MG, Martinez-Estrada O, Bazzoni GF: The molecular organization of endothelial junctions and their functional role in vascular morphogenesis and permeability. Int J Dev Biol 44: 743-748, 2000

Dhanikula RS, Hammady T, Hildgen P: On the mechanism and dynamics of uptake and permeation of polyether-copolyester dendrimers across an *in vitro* blood-brain barrier model. J Pharm Sci 98: 3748-3760, 2008

Dharmawardhane S, Schürmann A, Sells MA, Chernoff J, Schmid SL, Bokoch GM: Regulation of macropinocytosis by p21-activated kinase-1. Mol Biol Cell 11: 3341-3352, 2000

Dobson PD, Kell DB: Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat Rev Drug Discov 7: 205-220, 2008

Doherty GJ, McMahon HT: Mechanisms of Endocytosis. Annu Rev Biochem 78: 857-902, 2009

Doherty GJ, Åhlund MK, Howes MT, Moren B, Parton RG, McMahon HT, Lundmark R: The endocytic protein GRAF1 is directed to cell-matrix adhesion sites and regulates cell spreading. Mol Biol Cell 22: 4380-4389, 2011

Donaldson JG: Multiple roles for arf6: sorting, structuring, and signaling at the plasma membrane. J Biol Chem 278: 41573-41576, 2003

dos Santos T, Varela J, Lynch I, Salvati A, Dawson KA: Quantitative assessment of the comparative nanoparticle-uptake efficiency of a range of cell lines. Small 7: 3341-3349, 2011

Eker P, Holm PK, van Deurs B, Sandvik K: Selective regulation of apical endocytosis in polarized Madin-Darby Canine Kidney by Mastoparan and cAMP. J Biol Chem 269: 18607-18615, 1994

El-Sayed A and Harashima H: Endocytosis of gene delivery vectors: From clathrin-dependent to lipid raft-mediated endocytosis. Mol Ther 21: 1118-1130, 2013

Erickson AC, Couchman JR: Still more complexity in mammalian basement membranes. J Histochem Cytochem 48: 1291-1306, 2000

Erickson JW and Richard AC: Multiple roles for Cdc42 in cell regulation. Curr Opin Cell Biol 13: 153-157, 2001

Faraji AH, Wipf P: Nanoparticles in cellular drug delivery. Bioorg Med Chem 17: 2950-2962, 2009

Flannagan RS, Jaumouille V, Grinstein S: The cell biology of phagocytosis. Annu Rev Pathol 7: 61-98, 2012

Franke H, Galla H-J, Beuckmann CT: An improved low-permeability in vitro-model of the blood-brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol. Brain Res. 818: 65-71, 1998

Franke H, Galla H-J, Beuckmann CT: An improved low-permeability *in vitro*-model of the blood-brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol. Brain Res 818: 65-71, 1999

Frick M, Bright NA, Riento K, Bray A, Merrifield C, Nichols BJ: Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. Curr Boil 17: 1151-1156, 2007

Gabathuler R: Approaches to transport therapeutic drugs across the blood-brain barrier to treat brain diseases. Neurobiol Dis 37: 48-57, 2010

Gao Y, Hubbert CC, Lu J, Lee Y-S, Lee J-Y, Yao T-P: Histone deacetylase 6 regulates growth factor-induced astin remodeling and endocytosis. Mol Cell Boil 27: 8637-8647, 2007

Georgieva JV, Kalicharan D, Couraud PO, Romero IA, Weksler B, Hoekstra D, Zuhorn IS: Surface characteristics of nanoparticles determine trein intracellular fate in and processing by human blood-brain barrier endothelial cells *in vitro*. Mol Ther 19: 318-325, 2011

Georgieva JV, Brinkhuis RP, Stojanov K, Weijers CA, Zuilhof H, Rutjes FP, Hoekstra D, van Hest JC, Zuhorn IS: Peptide-mediated blood-brain barrier transport of polymersomes. Angew Chem In Ed Engl 51: 8339-8342, 2012

Gil ES, Li J, Xiao H, Lowe TL: Quaternary ammonium beta-cyclodextrin nanoparticles for enhancing doxorubicin permeability across the in vitro blood-brain barrier. Biomacromolecules 10: 505-516, 2009

Glebow OO, Bright NA, Nichols BJ: Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Boil 8: 46-54, 2006

Griep LM, Wolbers F, de Wagenaar B, ter Braak PM, Weksler BB, Romero IA, Couraud PO, Vermes I, van der Meer AD, van den Berg A: BBB ON CHIP: microfluidic platform to mechanically and biochemically modulate blood-brain barrier function. Biomed Microdevices 15: 145-150, 2013

Grimmer S, van Deus B, Sandvig K: Membrane ruffling and macropinocytosis in A431 cells require cholesterol. J Cell Sci 115: 2953-2962, 2002

Gumbleton M, Audus KL: Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier. J Pharm Sci 11: 1681-1698, 2001

Hall A: Rho GTPases and actin cytoskeleton. Sci 279: 509-514, 1998

Hansen CG, Bright NA, Howard G, Nichols BJ: SDPR induces membrane curvature and functions in the formation of caveolae. Nat Cell Biol 11: 807-814, 2009

Harding C, Heuser J, Stahl P: Receptor-mediated endocytosis of transferrin and recycling of the trans ferrin receptor in rat reticulocytes. J cell biol 97:329-339, 1983
Hayashi K, Nakao S, Nakaoke R, Nakagawa S, Kitagawa N, Niwa M: Effects of hypoxia on endothelial/pericytic co-culture model of the blood-brain barrier. Regul Pept 123: 77-83, 2004

Hellinger E, Veszelka S, Toth AE, Walter F, Kittel A, Bakk ML, Tihanyi K, Hada V, Nakagawa S, Duy TDH, Niwa M, Deli MA, Vastag M: Comparison of brain capillary endothelial cell-based and epithelial (MDCK-MDR1, Caco-2 and VB-Caco-2) cell-based surrogate blood-brain barrier penetration models. Eur J Pharm Biopharm 82: 340-351, 2012

Hewlett LJ, Prescott AR, Watts C: The coated pit and macropinocytic pathways serve distinct endosome populations. J Cell Biol 124: 689-703, 1994

Hilgenbrink AR, Low PS: Folate receptor-mediated drug targeting: from therapeutics to diagnostics. J Pharm Sci 94: 2135-2146, 2005

Hillaireau H, Couvreur P: Nanocarriers´entry into the cell: relevance to drug delivery. Cell Mol Life Sci. 66: 2873-2896, 2009

Kalia M, Kumari S, Chadda R, Hill MM, Parton RG, Mayor S: Arf6-independent GPI-anchored protein-enriched early endosomal compartments fuse with sorting endosomes via a Rab5/phosphatidylinositol-3´-kinase-dependent machinery. Mol Biol Cell 17: 3689-3704, 2006

Kannan R, Chakrabarti R, Tang D, Kim KJ, Kaplowitz N: GSH transport in human cerebrovascular endothelial cells and human astrocytes: evidence for luminal localization of Na+-dependent GSH transport in HCEC. Brain Res 852: 374-382, 2000

Karlgren M, Miura S, Ingelman-Sundberg M: Novel extrahepatic cytochrome P450s. Toxicol Appl Pharmacol 207: 57-61, 2005

Kim HR, Gil S, Andrieux K, Nicolas V, Appel M, Chacun H, Desmaele D, Taran F, Georgin D, Couvreur P: Low-density lipoprotein receptor-mediated endocytosis of

PEGylated nanoparticles in rat brain endothelial cells. Cell Mol Life Sci 64: 356-364, 2007

Kirkham M, Fujita A, Chadda R, Nixon SJ, Kurzchalia TV, Sharma DK, Pagano R, Hancock JF, Mayor S, Parton RG: Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J Cell Biol 168: 465-476, 2005

Kreuter J, Alayautdin RN, Kharkevich DA, Ivanov AA: Passage of peptides through the blood-brain barrier with colloidal polymer particles (nanoparticles). Brain Res 674: 171-174, 1995

Kreuter J, Ramge P, Petrov V, Hamm S, Gelperina SE, Engelhardt B, Alyautdin R, von Briesen H, Begley DJ: Direct evidence that polysorbate-80-coated poly(butylcuanoacrylate) nanoparticles deliver drugs to the CNS via specific mechanisms requiring prior binding of drugs to the nanoparticles. Pharm Res 20: 409-416, 2003

Krueger M and Bechmann I: CNS pericytes: concepts, inconceptions and a way out. Glia 58: 1-10, 2010

Kumari S, Mayor S: ARF1 is directly involved in dynamin-independent endocytosis. Nat Cell Biol 10: 30-41, 2008

Lacaz-Vieira F, Jaeger MMM, Farshori P, Kachkar B: Small synthetic peptides homologous to segments of the first loop of occludin impair tight junction resealing. J Membr Biol 168: 289-297, 1999

Lipardi C, Mora R, Colomer V, Paladino S, Nitsch L, Rodriguez-Boulan E, Zurzolo C: Caveolin transfection results in caveolae formation but not apical sorting of glycosylphosphatidylinositol (GPI)-anchored proteins in epithelial cells. J Cell Biol 140: 617-626, 1998

Liu NQ, Lossinsky AS, Popik W, Li X, Gujuluva C, Kriederman B: Human immunodeficiency virus type 1 enters brain microvascular endothelia by macropinocytosis dependent on lipid rafts and the mitogen-activated protein kinase signaling pathway. J Virol 76: 6689-6700, 2002

Lundmark R, Doherty GJ, Howes MT, Cortese K, Vallis Y, Parton RG, McMahon HT: The GTPase-activating protein GRAF1 regulates the CLOC/GEEC endocytic pathway. Curr Biol 18: 1802-1808, 2008

de Lounge Elizabeth CM: The physiological characteristics and transcytosis mechanisms of the blood-brain barrier (BBB). Curr Pharm Biotechnol 13: 2319-2327, 2012

Lukyanenko V, Malyukova I, Hubbard A, Delannoy M, Boedeker E, Zhu C, Cebotaru L, Kovbasnjuk O: Enterohemorrhagic *Escherichia coli* infection stimulates Shiga toxin

1 macropinocytosis and transcytosis across intestinal epithelial cells. Am J Physiol Cell Physiol 301: C1140-C1149, 2011

Lundmark R, Doherty GJ, Howes MT, Cortese K, Vallis Y, Parton RG, McMahon HT: The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway. Curr Biol 18: 1802-1808, 2008

McMahon HT and Boucrot E: Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12: 517-533, 2011

Magenau A, Benzing C, Proschogo N, Don AS, Hejazi L, Karunakaran D, Jessup W, Gaus K: Phagocytosis of IgG-coated polystyrene beads by macrophages induces and requires high membrane order. Traffic 12: 1730-1743, 2011

Manes S, Mira E, Gomez-Mouton C, Lacalle RA, Keller P, Labrador JP, Martinez-A C: Membrane raft microdomains mediate front-rear polarity in migrating cells. EMBO J 18: 6211-6220, 1999

Markoutsa E, Pampalakis G, Niarakis A, Romero IA, Weksler B, Couraud P-O, Antimisiaris SG: Uptake and permeability studies of BBB-targeting immunoliposomes using the hCMEC/D3 cell line. Eur J Pharm Biopharm 77: 265-274, 2011

Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Paanzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E: Junctional adhesion molecule, a nocel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 142: 117-127, 1998

Matsui H, Johnson LG, Randell SH, Boucher RC: Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. J Biol Chem 272: 1117-1126, 1997

Mellman I, Nelson J: Coordinated protein sorting, targeting and distribution in polarized cells. Nat Rev Mol Cell Biol 9: 833-845, 2008

Mercer J, Helenius A: Gulping rather than sipping: macropinocytosis as a way of virus entry. Curr Opin Microbial 15: 490-499, 2012

Mora R, Bonilha VL, Marmorstein A, Scherer PE, Brown D, Lisanti MP, Rodriguez-Boulan E: Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae and rafts when co-expressed with caveolin-1. J Biol Chem 274: 25708-25717, 1999

Muruganandam A, Herx LM, Monette R, Durkin JP, Stanimirovic DB: Development of immortalized cerebromicrovascular endothelial cell line as an *in vitro* model of the human blood-brain barrier. FASEB J 11: 1187-1197, 1997

Neuwelt EA, Maravilla KR, Frenkel EP, Rapoport SI, Hill SA, Barnett PA: Osmotic blood-brain barrier disruption. Computerized tomographic monitoring of chemotherapeutic agent delivery. J Clin Invest 64: 684-688, 1979

Pal D, Audus KL, Siahaan TJ: Modulation of cellular adhesion in bovine microvessel endothelial cells by a decapeptide. Brain Res 747: 103-113, 1997

Pardridge WM and Oldendorf WH: Transport of metabolic substrates through the blood-brain barrier. J Neurochem 28: 5-12, 1977

Pardridge W: Drug delivery to the brain. J Cereb Blood Flow Metab 17: 713-731, 1997

Pardridge WM:CNS drug design based on principles of blood-brain barrier transport. J Neurochem 70: 1781-1792, 1998

Pardridge W: Why is the global CNS pharmaceutical market so under-penetrated. DDT 7: 5-7, 2002

Pardridge W: Drug targeting to the brain. Pharm Res 24: 1733-1744, 2007

Parton RG, Howes MT: Revisiting caveolin trafficking: the end of the caveosome. J Cell Biol 191: 439-441, 2010

Parton RG, Simons K:The multiple faces of caveolae. Mol Cell Biol 8: 185-194, 2007

Patel S, Chavhan S, Soni H, Babbar AK, Mathur R, Mishra AK, Sawant K: Brain targeting of risperidone-loaded solid lipid nanoparticles by intranasal route. J Drug Targ 19: 468-474, 2011

Paulsson M: Basement membrane proteins: structure, assembly, and cellular interactions. Cri Rev Biochem Mol Biol 27: 93-127, 1992

Payne CK, Jones SA, Chen C, Zhuang X: Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands. Traffic 8: 389-401, 2007

Pelkmans L, Bürli T, Zerial M, Helenius A: Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. Cell 118: 767-780, 2004

Pinzon-Daza ML, Garzon R, Couraud PO, Romero IA, Weksler B, Ghigo D, Bosia A, Riganti C: The association of statins plus LDL receptor-targeted liposome-encapsulated doxorubicin increases in vitro drug delivery across blood-brain barrier cells. Bri J Pharmacol 167: 1431-1447, 2012

Poller B, Gutmann H, Krähenbühl S, Weksler B, Romero I, Couraud P-O, Tuffin G, Drewe J, Huwyler J: The human brain endothelial cell line hCMEC/D3 as a human Blood-brain barrier model for drug transport studies. J Neurochem 107: 1358-1368, 2008

Praefcke GJK, McMahon HT: The dynamin superfamily: universal membrane tabulation and fission molecules? Nature 5: 133-147, 2004

Prabhakarpandian B, Shen M-C, Nichols JB, Mills IR, Sidoryk-Wegrzynowicz M, Aschner M, Pant K: SyM-BBB: a microfluidic blood-brain barrier model. Lab Chip 13: 1093-1101, 2013

Prokai-Tatrai K, Prokai L, Bodor N: Brain-targeted delivery of a leucine-enkephalin analogue by retrometabolic design. J Med Chem 39: 4775-4782, 1996

Qian ZM, Li H, Sun H, Kwokping H: Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54: 561-587, 2002

Racoosin EL, Swanson JA: Macropinosome maturation and fusion with tubular lysosomes in macrophage. J Cell Biol 121: 1011-1020, 1993

Ragnaill MN, Brown M, Ye D, Bramini M, Callanan S, Lynch I, Dawson KA: Internal benchmarking of a human blood-brain barrier cell model for screening of nanoparticle uptake and transcytosis. Eur J Pharm Biopharm 77: 360-367, 2011

Reese TS, Karnovsky MJ: Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol 34: 207-217, 1967

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A: The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70: 401-410, 1992

Riento K, Frick M, Schafer I, Nichols BJ: Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase. J Cell Sci 122: 912-918, 2009

Rizzo V, Sung A, Oh P, Schnitzer JE: Rapid mechanotransduction *in situ* at the luminal cell surface of vascular endothelium and its caveolae. J Biol Chem 273: 26323-26329, 1998

Rubin LL, Hall DE, Porter S, Barbu K, Cannon C, Horner C, Janatpour M, iaw CW, Manning K, Morales J, Tanner LI, Tomaselli KJ, Bard F: A cell culture model of the Blood-Brain Barrier. J Cell Bio 115: 1725-1735, 1991

Sabharanjak S, Sharma P, Parton RG, Mayor S: GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev Cell 2: 411-423, 2002

Sahay G, Alakhova DY, Kabanov AV: Endocytosis of nanomedicines. J Control Rel 145: 182-195, 2010

Sandvik K, Torgersen ML, Raa HA, von Deurs B: Clathrin-independent endocytosis: from nonexisting to an extreme degree of complexity. Histochem Cell Biol 129: 267-276, 2008

Sandvik K, Pust S, Skotland T, van Deurs B: Clathrin-independent endocytosis: mechanisms and function. Curr Opin Cell Biol 23: 413-420, 2011

Santaguida S, Janigro D, Hossain M, Oby E, Rapp E, Cucullo L:Side by side comparison between dynamic versus static models of blood-brain barrier *in vitro*: a permeability study. Brain Res 1109: 1-13, 2006

Schmid SL:Clathrin-coated vesicle formation and protein sorting: and integrated process. Annu Rev Biochem 66: 511-548, 1997

Schmid EM, Ford MGJ, Burtey A, Praefcke GJ, Peak-Chew S-Y, Mills IG, Benmerah A, McMahon HT: Role of the AP2 β-appendage hub in recruiting partners for clathrin-coated vesicle assembly. Plos Biol 4: 1532-1548, 2006

Sharma DK, Brown JC, Choudhury A, Peterson TE, Holicky E, Marks DL, Simari R, Parton RG, Pagano RE: Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. Mol Biol Cell 15: 3114-3122, 2004

Shayan G, Choi YS, Shusta EV, Shuler ML, Lee KH: Murine *in vitro* model of the blood-brain barrier for evaluating drug transport. Eur J Pharm Sci 42: 148-155, 2011

Shivas JM, Morrison HA, Bilder D, Skop AR: Polarity and endocytosis: reciprocal regulation. Trends Cell Biol 20: 445-452, 2010

Smith MW and Gumbleton M: Endocytosis at the blood-brain barrier: From basic understanding to drug delivery strategies. J Drug Targ 14 : 191-214, 2006

Sonavane G, Tomoda K, Makino K: Biodistribution of colloidal gold nanoparticles after intravenous administration: effect of particle size. Colloids surf B biointerfaces 66: 274-280, 2008

Stanness KA, Westrum LE, Fornaciari E, Mascagni P, Nelson JA, Stenglein SG, Myers T, Janigro D: Morphological and functional characterization of an *in vitro* blood-brain barrier model. Brain Res 771: 329-342, 1997

Summerfield SG, Read K, Begley DJ, Obradovic T, Hidalgo IJ, Coggon S, Lewis AV, Porter RA, Jeffrey P: Central nervous system drug disposition: the relationship between in situ brain permeability and brain free fraction. J Pharmacol Exp Ther 322: 205-213, 2007

Thomsen P, Roepstorff K, Stahlhut M, van Deurs B: Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. Mol Biol Cell 13: 238-250, 2002

Tsukita S, Furuse M: Occludin and claudins in tight-junction strands: leading or supporting players? Trends Cell Biol 9: 268-273, 1999

Tsukita S, Furuse M: Pores in the wall: claudins constitute tight junction strands containing aqueous pores. J Cell Biol 149: 13-16, 2000

Tuma PL, Hubbard AL: Transcytosis: Crossing cellular barriers. Physiol Rev 83: 871-932, 2003

Underhill DM, Goodridge HS: Information processing during phagocytosis. Nat Rev Immunol 12: 492-502, 2012

Ungewickell E: Biochemical and immunological studies on clathrin light chains and their binding sites on clathrin triskelions. EMBO 2: 1401-1408, 1983

van Deurs B, Roepstorff K, Hommelgaard AM, Sandvig K: Caveolae:anchored, multifunctional platforms in the lipid ocean. Cell Biol 13: 92-100, 2003

Vasir JK and Labhasetwar V: Quantification of the force of nanoparticle-cell membrane interactions and its influence on intracellular trafficking of nanoparticles. Biomat 29: 4244-4252, 2008

Venishetty VK, Samala R, Komuravelli R, Kuncha M, Sistla R, Diwan PV: β-hydroxybutyric acid grafted solid lipid nanoparticles: a novel strategy to improve drug delivery to brain. Nanomed 9: 388-397, 2013

Vogel C and Marcotte EM: Insights into the regulation of protein abudance from proteomic and transcriptomic analyses. Nat Rev Genet 13: 227-232, 2012

Vorbrodt AW, Dobrogowska DH, KimYS, Lossinsky AS, Wisniewski HM: Ultrastructural studies of glycoconjugates in brain micro-blood vessels and amyloid plaques of scrapie-infected mice. Acta Neuropathol 75: 277-287, 1988

Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO: Blood-brain barrier-specific properties of a human adult brain endothelial cell line. Faseb: 1-26, 2005

Westhal M, Ram Z, Hilt D, Bortey E: Gliadel water in initial surgery for malignant glioma: long term follow-up of a multicenter controlled trial. Acta Neurochir 148: 269-275, 2006

Wilhelm I, Fazakas C, Krizbai IA: *In vitro* models of the blood-brain barrier. Acta Neurobiol Exp 71: 113-128, 2011

Wilson B: Brain targeting PBCA nanoparticles and the blood-brain barrier. Nanomed 4: 499-502, 2009

Winkler EA, Bell RD and Zlokovic BV: Central nervous system pericytes in health and disease. Nature Neuroscience 14: 1398-1405, 2011

Wohlfart S, Gelperina S, Kreuter J: Transport of drugs across the blood-brain barrier by nanoparticles. J Control Release 161: 264-273, 2012

Wolburg H, Neuhaus J, Kniesel U, Krauss B, Schmid EM, Ocalan M, Farrell C, Risau W: Modulation of tight junction structure in blood-brain barrier endothelial cells. Effects of tissue culture, second messengers and cocultured astrocytes. J Cell Sci 107: 1347-1357, 1994

Wu D, Pardridge WM: Pharmacokinetics and blood-brain barrier transport of an anti-transferrin receptor monoclonal antibody (OX26) in rats after chronic treatment with the antibody. Drug Metab Dispos 26: 937-939, 1998

Yarar D, Waterman-Storer CM, Schmid SL: SNX9 couples actin assembly to phophoinositide signals and is required for membrane remodeling during endocytosis. Dev Cell 13: 43-56, 2007

Yim YS, Choi J-S, Kim GT, Kim CH, Shin T-H, Kim DG, Cheon J: A facile approach for the delivery of inorganic nanoparticles into the brain by passing through the blood-brain barrier (BBB). Chem Commun 48: 61-63, 2012

Zhang LW, Bäumer W, Monteiro-Riviere NA: Cellular uptake mechanisms and toxicity of quantum dots in dendritic cells. Nanomedicine 6: 777-791, 2011