TRANSMIGRATION OF BREAST CANCER AND MELANOMA CELLS THROUGH THE BLOOD-BRAIN BARRIER: SIMILARITIES AND DIFFERENCES

Ph.D. Thesis

Judit Molnár

Supervisors: István Krizbai M.D., Ph.D., D.Sc.
Imola Wilhelm M.D., Ph.D

Doctoral School of Theoretical Medicine

Institute of Biophysics, Biological Research Centre
Hungarian Academy of Sciences

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List of Publications

Full papers directly related to the subject of the thesis

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3. Mallareddy, J. R., Toth, G., Fazakas, C., **Molnar, J.**, Nagyoszi, P., Lipkowski, A. W., Krizbai, I. A., Wilhelm, I. (2012). Transport characteristics of endomorphin-2 analogues in brain capillary endothelial cells. Chemical Biology and Drug Design 79 (4):507-13. (IF2012: 2.469)

4. Vegh, A. G., Fazakas, C., Nagy, K., Wilhelm, I., **Molnar, J.**, Krizbai, I. A., Szegletes, Z., Varo, G. (2012). Adhesion and stress relaxation forces between melanoma and cerebral endothelial cells. European Biophysics Journal with Biophysics Letters 41 (2):139-45. (IF2012: 2.274)

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Abbreviations

ABC	- ATP Binding Cassette
ARHGAP22	- Rho GTPase activating protein 22
BBB	- Blood-Brain Barrier
bFGF	- basic Fibroblast Growth Factor
BCL-2	- B-Cell Lymphoma 2
BSA	- Bovine Serum Albumin
CAR	- Coxsackie and Adenovirus Receptor
CDKN2A	- Cyclin-Dependent Kinase Inhibitor 2A
CEC	- Cerebral Endothelial Cell
CK5/6	- Cytokeratin 5/6
CNS	- Central Nervous System
CRB3	- Crumbs Homolog 3
D3 (hCMEC/D3)	- human Cerebral Microvascular Endothelial Cells, clone D3
DOCK3	- Dedicator Of Cytokinesis 3
EC	- Endothelial Cell
ECM	- Extracellular Matrix
EDTA	- Ethylenediaminetetraacetic Acid
EGFR	- Epidermal Growth Factor Receptor
ER	- Estrogen Receptor
ESAM	- Endothelial cell-Selective Adhesion Molecule
FBS	- Fetal Bovine Serum
GAP	- GTPase Accelerating Protein
GEF	- Guanine Nucleotide Exchange Factor
GUK	- Guanylate Kinase
HER2	- Epidermal Growth Factor Receptor 2
HUVECs	- Human Umbilical Vein Endothelial Cells
JAM	- Junctional Adhesion Molecule
MAPK	- Mitogen Activated Protein Kinase
MC1R	- Melanocortin 1 Receptor
MMP	- Matrix Metalloproteinase
NEDD9	- Neural precursor cell Expressed Developmentally Down-regulated protein 9
PBS	- Phosphate-Buffered Saline
PDMS	- Poly-Dimethylsiloxane
PDZ	- Psd95/Discs large 1/Zonula Occludens
PI3K	- Phosphoinositide 3-Kinase
PIP2	- Phosphatidylinositol (4,5)-bisphosphate
PIP3	- Phosphatidylinositol (3,4,5)-trisphosphate
PR	- Progesterone Receptor

PTEN	- Phosphatase and Tensin homologue
P-gp	- P-glycoprotein
P-Rex1	- PIP3-dependent Rac Exchange factor 1
RBEC	- Rat Brain Endothelial Cell
RIPA	- Radioimmunoprecipitation Assay
ROCK	- Rho-associated protein Kinase
SLC transporters	- Solute Like Carrier transporters
SH3	- Src Homology 3
TBS-T	- Tris-Buffered Saline and Tween-20
TEER	- Transendothelial Electrical Resistance
TGF-β2	- Transforming Growth Factor-beta 2
TJ	- Tight Junction
VEGF	- Vascular Endothelial Growth Factor
WAVEs	- WASP-family verprolin-homologous proteins
ZO	- Zonula Occludens

1 Introduction

1.1 Formation of brain metastases

Brain metastases of malignant tumors have a very poor prognosis and are frequent complications for cancer patients. Only in the United States, about 170,000 metastatic brain tumors are diagnosed annually (Platta et al, 2010), whereas primary brain tumors represent 17,000 new cases/year. During metastasis formation, tumor cells successfully infiltrating the brain parenchyma overcome several obstacles, including survival in the circulation (Lorger & Felding-Habermann, 2010), extravasation through brain capillaries and resisting deleterious signals of the reactive brain stroma (Valiente et al, 2014). However, cancer cells able to migrate into and to survive in the brain will benefit of a supportive and protective microenvironment, including the dense vasculature with the opportunity of vessel co-option (Bugyik et al, 2011) and chemoprotection mediated by astrocytes and endothelial cells (Kim et al, 2014). As a consequence, brain metastases have a poor prognosis. The survival of patients with central nervous system (CNS) metastases is low: the median survival time is 4-6 months, and only 20-40% of patients are alive at 1 year after the diagnosis (Pestalozzi, 2009).

The most common cancers that spread to the brain are lung cancer (40-50% of brain metastases originate from lung carcinomas), breast cancer (15-25%) and melanoma (5-20%) (Wen et al, 2011).

Breast cancer is the second most common type of cancer, the most frequent in women and worldwide causes more than half a million deaths annually. Usually breast cancer either begins in the cells of the lobules or in the ducts, these cancer types are carcinomas. Less commonly, breast cancer can begin in the stromal tissues, these cancer types are sarcomas. Within carcinomas, there are many different types of breast cancer. In situ carcinoma is "pre-invasive" carcinoma that has not yet invaded the breast tissue. Invasive carcinomas have the potential to spread to other sites of the body, including the brain.

Breast cancer metastases to the CNS include the clinically distinct situations of multiple brain metastases (78%), solitary brain metastases (14%) and leptomeningeal metastases (8%) (Pestalozzi, 2009). CNS metastases occur in 10-16% of stage IV patients, while they are found in 30% of patients in autopsy series (Pestalozzi, 2009). In most cases, brain metastases represent a late relapse in breast cancer patients who already have liver, lung

or bone involvement. Previous studies have identified several risk factors of brain metastases. Especially, estrogen receptor (ER) negative status is associated with high incidence of brain metastases. Overexpression of epidermal growth factor receptor 2 (EGFR2, also known as HER2) is reported as another risk factor. Some other factors were also reported to associate with brain metastases of breast cancer: p53 positivity, high EGFR expression, lower BCL-2 expression and expression of basal CK5/6 (cytokeratin 5/6) (Chang et al, 2003; Hicks et al, 2006; Stemmler et al, 2006). Triple-negative breast cancers, which are very aggressive tumors, are characterized by lack of ER, of progesterone receptor (PR), and of HER2 overexpression. They are typically associated with poor prognosis and give brain metastases with high frequency. This type of breast cancer has only partial response to chemotherapy and to targeted therapies (Cetin & Topcul, 2014).

Melanoma is a malignant tumor which develops from melanocytes. This is one of the most aggressive types of skin cancer. Melanomas are usually caused by DNA damage resulting from exposure to ultraviolet (UV) light from the sun, but genetics also plays an important role. A number of rare mutations, which often run in families, greatly increase melanoma susceptibility. One class of mutations affects the gene CDKN2A (cyclin-dependent kinase inhibitor 2A), which acts as tumor suppressor. MC1R (melanocortin 1 receptor) gene mutation also increases the risk. Proteins involved in the MAPK (mitogen activated protein kinase) pathway that regulate transcription of genes involved in cell proliferation and survival also take part in melanoma development. Mutations in NRAS and BRAF, two proteins in the MAPK pathway, are found in 20% and 60% of melanomas, respectively. The most commonly seen BRAF mutation is a substitution of valine with glutamate (V600E). It has also been demostrated that upregulation of the PI3K (phosphoinositide 3-kinase) pathway (PTEN/phosphatase and tensin homolog deletion or Akt activation) and subsequent inhibition of apoptosis also increases the risk of melanoma development (Lee et al, 2014).

Based on the depth of the primary tumor and on the distance the cancer has spread to, melanoma is divided into four stages. Breslow thickness is the depth of the tumor mass calculated perpendicularly from the skin surface (in mm) (http://www.americanskin.org/resource/melanoma.php). In situ melanomas have a better prognosis after surgical excision with a sufficient surgical margin. Invasive melanomas are far more serious. Melanoma can spread to the liver, bones, abdomen or distant lymph nodes, and these tumor cells have an unexpectedly high affinity to the brain. This is partly due to the fact that the neural environment plays a key role in the protection and growth of melanoma cells,

which are of ectodermal origin, similar to the CNS.

Melanoma brain metastases are diagnosed in 40-50% of the patients, which, after autopsy, increases with an additional 30-40% (Fidler et al, 1999). Melanoma causes 4% of all skin cancers but is responsible for 74% of skin cancer deaths, and this is mainly due to the high incidence of brain metastases. Of melanoma brain metastases 49% are intraparenchymal, 22% are leptomeningeal and 32% are dural (Fidler et al, 1999). The number of diagnosed melanoma cases is constantly increasing (Douglas & Margolin, 2002). Mean survival from diagnosis of melanoma brain metastasis varies from 2 to 16 months, while the mean 1-year survival is estimated to be only about 20% (Fidler et al, 1999).

Approximately 60% of patients with brain metastases have subacute symptoms. Symptoms are usually related to the location of the tumor and may include the following: headache, seizure, nausea, vomiting, nuchal rigidity, photophobia, cognitive dysfunction and motor dysfunction (Saha et al, 2013). Longer survival, improved quality of life and stabilization of neurocognitive function for patients with brain metastasis is the goal of treatments. Unfortunately not even these goals are completely met by current therapies, which include surgery, whole-brain radiation therapy, chemotherapy, stereotactic radiosurgery and targeted therapies (Leone & Leone, 2015). In most tumor types chemotherapy shows limited or no activity in brain metastases, because many systemically used chemotherapeutic agents do not cross the blood-brain barrier (BBB), and therefore do not reach a therapeutically relevant concentration in the brain metastatic lesion. Others may transiently weaken the BBB (Blecharz et al, 2015). Trastuzumab – a monoclonal antibody against HER2, approved for the first line treatment of HER2 positive breast cancers – is not able to cross the BBB, thus it is inefficient in inhibiting the growth of brain metastatic lesions of breast cancer. Some new chemotherpeutic agents have been developed which can pass the BBB and target tumor cells, e.g. temozolomide, an alkylating agent, which is a basic compound in the therapy of brain metastases. According to clinical studies, the combination of temozolomide with radiotheraphy or with other cytotoxic agents shows a few months prolonged median survival (Owonikoko et al, 2014). However, these results are far from the final goal of total remission; therefore, it is necessary to further optimize current methods. In the treatment of melanoma one of the most important targeted therapeutic agents is vemurafenib, which is an inhibitor of V600E BRAF mutation in metastatic melanoma, and is often used in combination with radio- and immunotherapy (Owonikoko et al, 2014). It has been recently shown that vemurafenib can be safely and effectively used in patients with brain metastatic melanoma (Dummer et al,

2014). However, the responses are frequently unsatisfactory, rangig from substantial side-effects and toxicity to no impact of the treatment on the metastatic lesion. Therefore, prevention of the formation of brain lesions would be of great clinical benefit.

Since the CNS parenchyma lacks a lymphatic circulation, the only possibility for cancer cells to reach the brain is via the blood stream. Brain metastases can be formed both in the parenchyma and the meninges. Leptomeningeal metastases resulting from solid tumors occur late and usually coexist with CNS parenchymal disease. Metastatic cells invading the CNS parenchyma, however, have to pass the BBB. During transmigration through the BBB, arrest of tumor cells was found to take place at the level of capillaries and postcapillary venules, where the diameter of the vessels is comparable to those of the metastatic cells, predominantly at vessel branches (Kienast et al, 2010).

1.2 The blood-brain barrier

1.2.1 Cellular structure of the blood-brain barrier

The BBB is located at the level of cerebral capillaries in the forefront of the defense line of the CNS and restricts the free movement of solutes and cellular elements between the systemic circulation and neuronal tissue. The most important cellular elements of the BBB are endothelial cells, astrocytes and pericytes, which together with the extracellular matrix and neurons form the neurovascular unit (Figure 1A).

Endothelial cells lining brain capillaries are thin, flat cells interconnected by tight junctions (TJs) (Brightman & Reese, 1969) and characterized by a high number of mitochondria (Oldendorf et al, 1977) and low number of caveolae (Nag, 2003). The contact region of brain endothelial cells is usually overlapping, and the border between the apical and basolateral cell membranes is interconnected by a continuous line of tight junctions. This limits the free transport of different solutes and cellular elements between adjacent cells. Cerebral endothelial cells (CECs) share common features with other endothelia (presence of factor VIII, high alkaline phosphatase and γ-glutamyl transpeptidase activity, uptake of acetylated-low density lipoprotein) and epithelia as well (high transendothelial electrical resistance (TEER), continuous line of TJs, low level of pinocytosis), these latter being indispensable for the barrier function.

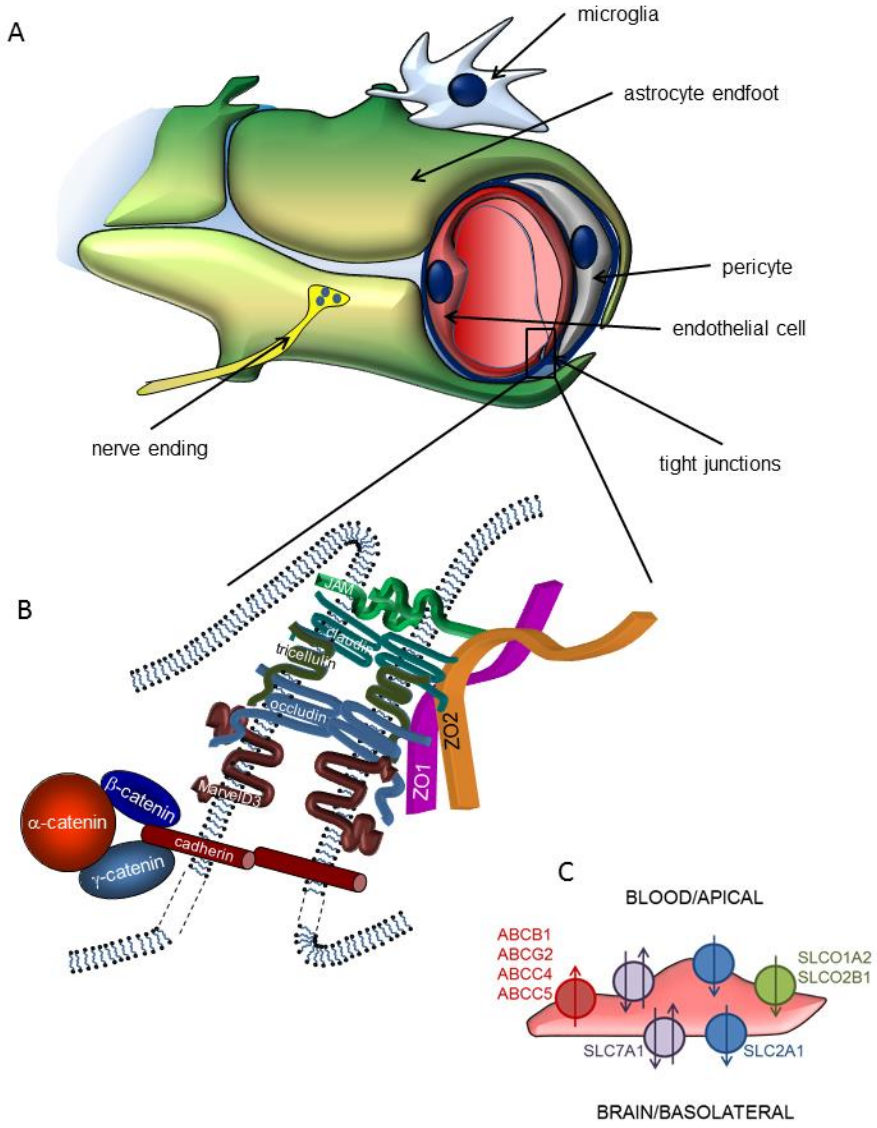


Figure 1. Schematic diagram of the blood-brain barrier. The core anatomical structure of the BBB is formed by cerebral endothelial cells, which share common basal membrane with pericytes, and are covered by astrocytic endfeet (A). Endothelial cells are interconnected by a continuous line of tight junctions. The inset illustrates the molecular structure of the junctional complex (B). ABC transporters and SLC transporters in brain endothelial cells (C).

Endothelial cells are framed by a basal membrane, which consists mainly of collagen IV, fibronectin, proteoglycans and laminin. The extracellular matrix (ECM) participates in the maintanence of endothelial barrier integrity. Disruption of the basal membrane might lead to alterations affecting the junctional proteins of the BBB (Hawkins & Davis, 2005). The integrin β1-mediated attachment of endothelial cells to the basement membrane is critical for claudin-5 stabilisation (Osada et al, 2011). Furthermore, during brain metastasis formation the basal membrane is involved in the survival of tumor cells (Carbonell et al, 2009).

Pericytes are located in the duplication of the basement membrane, in close contact with endothelial cells. Even gap junctions have been described between the two cell types (Cuevas et al, 1984). Pericytes are contractile cells able to synthesize a plethora of biologically active substances. Although the exact function of pericytes in the formation and function of the BBB is insufficiently understood, they can participate in the regulation of blood flow, endothelial proliferation, angiogenesis or inflammatory processes. Absence of pericytes leads to endothelial hyperplasia, abnormal vasculogenesis (Hellström et al, 2001) and increased BBB permeability (Armulik et al, 2010). Pericyte-endothelial cell interactions were found to be critical in the regulation of the BBB during development (Daneman et al, 2010).

Astrocytes play a crucial role in the induction of barrier properties of CECs (Abbott et al, 2006; Haseloff et al, 2005; Krizbai et al, 2012). Astrocytic endfeet nearly completely ensheath the capillary walls, thereby covering not only endothelial cells, but also the intimately associated pericytes (Kacem et al, 1998). The coverage is not complete, allowing a direct contact of nerve endings with the basal membrane (Cohen et al, 1997; Paspalas & Papadopoulos, 1996). Astrocytic endfeet express a high level of several specific proteins at their capillary side, like glucose transporter 1, P-glycoprotein (P-gp), aquaporin-4, connexin-43 and Kir 4.1 K+ channel. Astrocytes have an indispensable role in the maintenance of BBB properties of CECs.

On the other hand, astrocytes have a protective role for brain metastases. Reactive astrocytes immediately localize to individual breast cancer cells even before extravasation and continue to associate with metastatic cells during the transmigration process and throughout the growth of the lesions (Lorger & Felding-Habermann, 2010). Moreover, astrocytes secrete soluble factors that stimulate the proliferation of tumor cells in the brain microenvironment. In this respect, neurotrophins have a special importance in supporting the growth of melanoma cells (Menter et al, 1995). Astrocytes have been shown to protect tumor cells through gap junctional communication (Lin et al, 2010), upregulation of survival genes (Kim et al, 2011) and secretion of soluble factors like inflammatory cytokines (Seike et al, 2011).

In the brain distinct immune cells interact with the BBB. The most frequent CNS innate immune cells are microglia cells (Spindler & Hsu, 2012). They are localised in physical association with the brain capillaries and participate in angiogenic processes. Microglia cells influence BBB function in health and disease, but it is not completely understood how. Some in vitro co-culture experiments demonstrated influence of microglia

activation on barrier disturbances though modifications of TJ proteins (Nishioku et al, 2010; Sumi et al, 2010).

1.2.2 Functions of the blood-brain barrier

The main function of the BBB is to maintain the homeostasis of the CNS and to protect the brain from harmful compounds coming from the systemic circulation. The BBB has dual role, operates both as a barrier and as a carrier too. This latter function refers to the transport of nutrients to and removal of metabolites from the brain, and is mainly assured by solute-like carrier (SLC) transporters.

Transport across the brain endothelium is strictly limited through a four-fold defense line (Wilhelm et al, 2011): the paracellular barrier (represented by interendothelial junctions); the transcellular barrier (assured by the low level of endocytosis and transcytosis); the enzymatic barrier (including acetylcholinesterase, alkaline phosphatase, γ-glutamyl transpeptidase, monoamine oxidases and drug metabolizing enzymes); and the efflux transporters (ABC-B1, -C1, -C4, -C5 and -G2). Small gaseous molecules, such as O_2 and CO_2, can freely diffuse through the lipid membranes, and this is also a route of entry for small lipophilic agents, including barbiturates, nicotine and ethanol. However, specific blood-to-brain influx transport systems exist to supply nutrients, like glucose, amino acids and nucleotides, which cannot freely diffuse to the brain. Endothelial cells also express several ABC (ATP-binding cassette) transporters, which are mainly efflux transpoters (Figure 1C).

The main role of the ABC transporters in the CNS is to work as active pumps consuming ATP and transporting a wide range of lipid-soluble compounds out of the brain capillary endothelium and the CNS. These transporters remove from the brain potentially neurotoxic endogenous or xenobiotic molecules and carry out a vital neuroprotective and detoxifying function (Abbott et al, 2010). In addition, certain efflux transporters are able to pump out chemotherapeutic agents as well; therefore, they decrease the effectiveness of chemotherapy. As a consequence, ABC transporters play a crucial role in the development of multidrug resistance (Löscher & Potschka, 2005).

Integrity of the BBB has been reported to be disturbed in several disorders including cerebral ischemia, inflammation and brain tumors. Moreover, during inflammation and metastasis formation, the BBB is actively involved in the transmigration of leukocytes and tumor cells.

1.2.3 Molecular structure of the tight junctions (TJs)

The paracellular permeability is mainly regulated by the TJs between endothelial cells (Figure 1B). Key components of the TJs are the transmembrane proteins, which form three protein families. These are the four transmembrane proteins (occludin, claudins, tricellulin/marvelD2, marvelD3), which are perhaps the most important from the point of view of paracellular permeability; molecules belonging to the immunoglobulin superfamily (JAM-Junctional adhesion molecule, CAR-Coxsackie and adenovirus receptor, ESAM-Endothelial cell-selective adhesion molecule); and non-immunoglobulin-like molecules with a single transmembrane domain (CRB3-Crumbs homolog 3; Bves-Blood vessel epicardial substance). Best characterized in CECs are occludin, claudins and JAMs (Bauer et al, 2011).

Occludin, the first identified transmembrane TJ protein (Furuse et al, 1993), is a 65 kDa molecule. It is characterized by four transmembrane regions, two extracellular loops, a shorter N-terminal and a longer C-terminal cytoplasmic domain. The two extracellular loops are rich in tyrosine and glycine, playing a role in sealing the junctions (Lacaz-Vieira et al, 1999; Wong & Gumbiner, 1997), while the C-terminal region is important in the interaction with other junctional proteins.

Claudins, first described by Furuse et al. (Furuse et al, 1998), are small proteins (20-27 kDa), which show a similar membrane topology to occludin; however, there is no sequence homology between them. Interactions of claudins are largely determined by the C-terminal intracellular region, which contains PDZ (Psd95/Discs large 1/Zonula Occludens) binding domains. Furthermore, claudins are able to form homophylic interactions as well needed for the formation of TJ strands (Piontek et al, 2008). The principal claudin in brain endothelial cells is claudin-5, but other claudins (especially claudin-1, -3 and -12) have also been detected (Ohtsuki et al, 2008). The exact role of individual claudins is not known; absence of claudin-5 leads to a selective opening of the BBB to molecules smaller than 800 Da (Nitta et al, 2003).

Junctional adhesion molecules (JAMs) are single-span molecules belonging to the immunoglobulin superfamily, characterized by homophilic binding and two extracellular loops, first described by Martin-Padura et al. (Martìn-Padura et al, 1998). Brain endothelial cells express mainly JAM-1 (JAM-A) and JAM-3 (JAM-B) (Aurrand-Lions et al, 2001), but also JAM-C. They are involved in the extravasation of leukocytes. Endothelial cells also express ESAM, another immunoglobulin-like molecule localized to the TJs. JAM-C and

ESAM have been shown to promote melanoma lung metastasis formation (Cangara et al, 2010; Langer et al, 2011).

Zonula Occludens (ZO) proteins are peripheral proteins of TJs. There are three members of the zonula occludens family: ZO-1 (Stevenson et al, 1986), ZO-2 (Gumbiner et al, 1991) and ZO-3 (Haskins et al, 1998). Common structural features of the ZO family include three PDZ domains in the N-terminal region, a SH3 (Src homology 3) domain and an enzymatically inactive GUK (guanylate kinase) domain. ZO proteins are important scaffold proteins, but are essential in signaling processes as well (Balda & Matter, 2000; Bauer et al, 2011; Traweger et al, 2003).

1.3 Interaction of tumor cells with the blood-brain barrier

1.3.1 Transmigration routes

Great amount of data is available regarding motility and migration of cancer cells, but information about the mechanisms involved in the migration of cancer cells across endothelial barriers is limited. Even less is known about the transmigration of tumor cells through the BBB. The process of transendothelial migration has been intensively studied using leukocytes. Although the steps of transmigration (rolling, adhesion and transmigration/diapedesis) may show some similarities, due to different physiological, molecular and mechanical characteristics of immune and metastatic cells, there may be significant differences (Strell & Entschladen, 2008).

Transendothelial migration of cells can occur by two routes: the paracellular pathway (through the interendothelial junctions between endothelial cells) and the transcellular one (through single endothelial cells). Leukocytes are able to use both routes during their migration through peripheral and brain endothelia as well (Carman, 2009; Dejana, 2006; Reijerkerk et al, 2006; Wolburg et al, 2005). Paracellular transmigration of metastatic cells is possible only with the involvement of endothelial TJs and junctional proteins. Using an in vitro system, we have previously observed that melanoma cells damaged the integrity of the brain endothelial monolayer and decreased the transendothelial electrical resistance (TEER) which is a widely used indicator of junctional integrity (Fazakas et al, 2011). The mechanisms by which metastatic cells are able to disrupt TJs are incompletely understood; however, proteolytic processes probably play an important role. It is less known, whether breast cancer

cells are able to disrupt the TJs or migrate preferentially transcellularly. So far, transcellular migration of tumor cells has only been described in the case of intravasation of breast cancer cells into an artificial vascular network prepared from calf pulmonary artery endothelial cells (Khuon et al, 2010) and migration through umbilical cord endothelial cells (Arvanitis et al, 2014). However, no data on the transmigration pathway of breast cancer cells through BBB endothelial cells exist.

1.3.2 Molecular mechanisms involved in the extravasation of tumor cells through the blood-brain barrier

Mechanisms of extravasation of tumor cells through the BBB are largely uncharacterized. According to our current knowledge, both metastatic and endothelial cells actively participate in this process by modulating the expression of surface molecules, secretion of soluble factors and activation of diverse signaling pathways.

Attachment of tumor cells to the endothelium depends on the expression of adhesion molecules. Cancer cells, similar to leukocytes, express selectin ligands, which may play an important role in their adhesion to CECs. Selectin-dependent mechanisms are also important in the interaction of tumor cells with platelets and leukocytes, which facilitates attachment of tumor cells to the vessel wall. Moreover, heparin – which inhibits not only coagulation, but selectin-mediated interactions as well – was shown to inhibit adhesion of melanoma cells to brain endothelial cells (Fazakas et al, 2011) and to delay melanoma brain metastasis formation (Maraveyas et al, 2010). In addition, several integrins were shown to be involved in cancer progression, metastasis formation, transendothelial migration of tumor cells and angiogenesis in different metastatic sites. Activation of integrin αvβ3 was observed to support efficient brain metastatic growth of breast cancer cells through continuous upregulation of VEGF (vascular endothelial growth factor), without influencing the growth of primary lesions (Lorger et al, 2009). Cadherin dysfunction may also be involved in tumor progression and metastasis formation, e.g. metastatic brain tumors were shown to express high levels of E-cadherin (Shabani et al, 2003). Transendothelial migration of melanoma cells through human lung microvascular endothelial cells has been shown to involve N-cadherin-mediated adhesion (Qi et al, 2005). A similar mechanism is possible in the case of brain endothelial cells as well.

Different proteolytic enzymes have also been implicated in brain metastasis formation and migration of tumor cells through the BBB, including matrix metalloproteinases (MMPs),

serine proteases and heparanase. Tumor cell-secreted MMPs might have special importance, because TJ proteins can be targets of MMP degradation. According to literature data, MMP-2, MMP-3 and MMP-9 are implicated in the development of breast cancer brain metastases (Mendes et al, 2005). In addition, heparanase is considered a critical molecular determinant of brain metastasis in melanoma (Roy & Marchetti, 2009) and breast cancer (Zhang et al, 2010) as well. According to our previous results, during transmigration through the brain endothelium melanoma cells produce and release large amounts of gelatinolytic serine proteases, including seprase (Fazakas et al, 2011). These proteases facilitate the transendothelial migration of tumor cells.

1.3.3 Signaling pathways involved in the extravasation

Several signaling pathways (Rho and Rac signaling, the PI3K-Akt-PTEN pathway, MAPK signaling, Src signaling), alone or in combination, can underlie the extravasation process. Understanding the role of pathways activated either in tumor cells or in endothelial cells will help to identify molecular targets for cancer therapy. In our investigations we focused on the role of Rac, Rho/ROCK and PI3K signaling in the migration of tumor cells through the BBB.

1.3.3.1 Role of Rho/ROCK and Rac signaling in the interaction of tumor cells with the BBB

During invasion of tissues and migration through vessel walls and ECM components, metastasizing tumor cells require increased motility, which is dependent on the remodeling of the cytoskeleton. In this respect, members of the Rho family small GTPases were shown to have an indispensable role by regulating the two major modes of tumor cell movement, characterized by mesenchymal and amoeboid phenotype. The mesenchymal type of tumor cell movement requires elevated Rac1 activation and reduced Rho/ROCK signaling and is characterized by elongated cell morphology, formation of large membrane protrusions and dependence on integrins and extracellular proteolysis (Figure 2A). On the other hand, the amoeboid migration type mimics movement of leukocytes, with a rounded morphology and generation of Rho/ROCK-dependent actomyosin contractile forces (Sahai & Marshall, 2003; Sanz-Moreno et al, 2008; Wolf et al, 2003). These two types of movement are interconvertible and depend on the environment the cancer cells are move in (Symons & Segall, 2009).

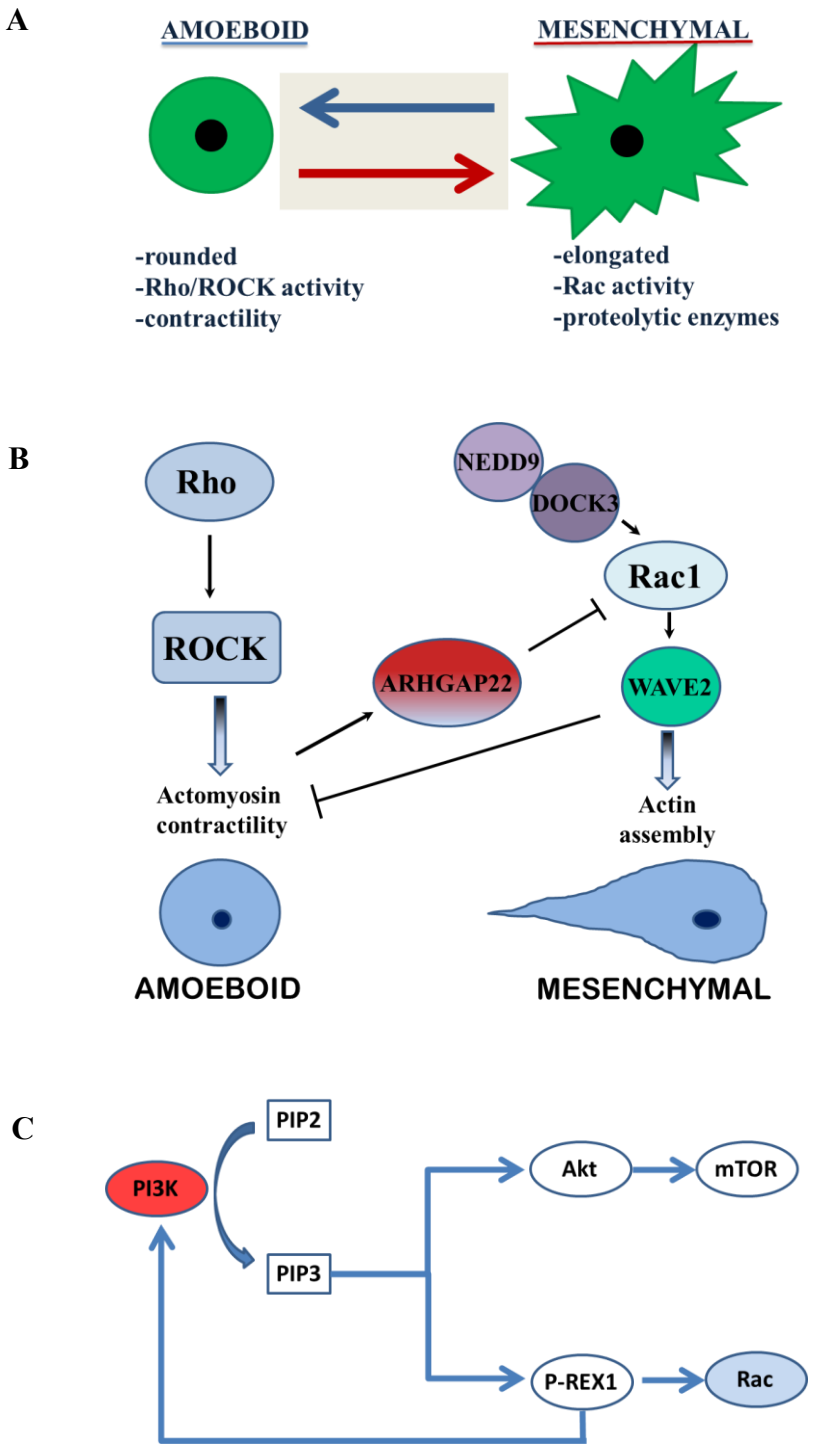


Figure 2. Schematic representation of the two types of movement of cancer cells and signaling pathways implicated. Characterization of the amoeboid and mesenchymal types of cancer cell movement (A). Rho/ROCK and Rac signaling in the amoeboid and mesenchymal phenotypes (B). PI3K/Akt/mTor signaling and PI3K/P-Rex1/Rac signaling in breast cancer cells (C).

The activity of Rho family GTPases is controlled by activators, guanine nucleotide

exchange factors (GEFS) and inactivators, GTPase accelerating proteins (GAPs). Rac activation is mediated through the adaptor protein NEDD9 and the Rac GEF DOCK3, which drive mesenchymal movement and suppress amoeboid movement through the Rac effector WAVE2. In amoeboid movement a Rac GAP, ARHGAP22 suppresses the mesenchymal movement through inactivation of Rac (Sanz-Moreno & Marshall, 2009) (Figure 2B).

Our previous results indicated that during transmigration through the brain endothelium, melanoma cells favor the mesenchymal type of cell movement. By inhibiting Rho/ROCK signaling, and therefore triggering the mesenchymal phenotype, we observed a significant increase in the number of transmigrated melanoma cells through brain endothelial monolayers. The question whether tumor cells prefer Rho/ROCK or Rac-dependent transendothelial migration is of clinical importance, since inhibitors of both Rho/ROCK (e.g. fasudil) and Rac pathways (Wertheimer et al, 2012) are emerging as potential therapeutic agents.

1.3.3.2 Role of the PI3K signaling in the interaction of tumor cells with the BBB

The pathway most frequently altered in human cancer is the PI3K signaling pathway. Genes encoding proteins in this pathway are mutated in more than 70% of breast cancers. Therefore, several potential therapeutic agents targeting nearly every aspect of this pathway are under development, clinical or preclinical assessment (http://am.asco.org/exploring-pathway-despite-lukewarm-clinical-benefit-pi3k-inhibitors-optimism-remains-regarding).

Class IA PI3Ks phosphorylate phosphatidylinositol (4,5)-bisphosphate (PIP2) in the inner side of the plasma membrane and produce phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This activity is directly opposed by the tumor suppressor PTEN (phosphatase and tensin homolog). Akt/mTOR has canonically been regarded as the primary downstream pathway of PI3K, regulating cell growth, proliferation, survival and motility of cancer cells.

However, PI3K regulates other pathways as well, e.g. the Rac activator PIP3-dependent Rac exchange factor 1 (P-Rex1) (Figure 2C). Levels of P-Rex1 correlate with PI3K activation. P-Rex1 creates a positive feedback loop to activate PI3K/Akt and promotes viability of breast cancer cells (Dillon et al, 2015). Therefore, PI3K signaling may also be involved in the regulation of the amoeboid vs. mesenchymal type of cancer cell movement.

In a recent study, a novel inhibitor of downstream PI3K was found to effectively control metastatic growth of HER2 positive breast cancer cells in multiple organs and

resulting in a significant proportion of mice free from brain and bone metastases (Nanni et al, 2012). In addition, brain metastases of melanoma were shown to have significantly higher levels of phosphorylated Akt and lower PTEN than lung or liver metastases (Davies et al, 2009). This pathway was shown to be activated not only in brain metastatic melanoma cells, but also in brain endothelial cells coming in contact with melanoma cell-conditioned media, inducing increased endothelial cell proliferation and motility (Anfuso et al, 2009). Moreover, the PI3K inhibitor LY294002 was shown to reduce the number of ECM-invading breast cancer cells in the presence of pulmonary microvascular endothelial cells (Mierke, 2011). It was also shown that melanoma cell-associated VE-cadherin breakdown in human umbilical vein endothelial cells (HUVECs) was not sensitive to LY294002, whereas transendothelial migration of melanoma cells was reduced in the presence of the PI3K inhibitor (Peng et al, 2005). However, inhibition of PI3K had no effect on the transmigration of small cell lung cancer cells through brain endothelial cells (Li et al, 2006).

1.3.3.3 Other pathways involved in the interaction of tumor cells with the BBB

Several other signaling pathways have been shown to influence transendothelial migration of metastatic cells. Src signaling is known to participate in many aspects of tumor progression and metastasis. It plays an important role in the promotion of mesenchymal and inhibition of amoeboid motility (Ahn et al, 2012) and in the phosphorylation of N-cadherin and dissociation of β-catenin during transendothelial migration (Qi et al, 2005).

In melanoma, transforming growth factor-beta 2 (TGF-β2) was found to be crucial, since its expression was found indispensable for the formation of parenchymal brain metastases (Zhang et al, 2010). Stat3 activation was also found to play an important role in angiogenesis, invasion and brain metastasis formation of melanoma cells through dysregulated expression of bFGF (basic fibroblast growth factor), VEGF and MMP-2 (Xie et al, 2006). VEGF and its receptors may also be involved in the transmigration process. In breast cancer cells HER2 increases VEGF protein production, which induces the disruption of interendothelial junctions (Fan et al, 2011). In addition, VEGF was shown to increase the adhesion of highly metastatic MDA-MB-231 breast cancer cells to brain endothelial monolayers and to enhance their transmigration through an in vitro BBB model (Li et al, 2013).

2 Aims

In our studies we aimed at understanding the mechanisms of transmigration of tumor cells through the BBB. We focused on two tumor types with high incidence of brain metastasis formation, i.e. melanoma and breast cancer. Breast cancer is the second most common cancer type giving cerebral metastases; however, in comparison to melanoma it has a much lower propensity to metastasize to the brain. Comparison of the interaction of these two different tumor cell types with the cerebral endothelium might help in understanding whether the transendothelial migration step of metastasis formation has any role in the higher tropism of melanoma cells towards the CNS. In addition, we investigated the impact of Rac, Rho-ROCK and PI3K signaling on the diapedesis of melanoma and breast cancer cells into the brain. Understanding the role of these signaling pathways might lead to the elaboration of new preventive and treatment strategies in metastatic diseases of the brain.

Our investigations had five specific aims:

1. to compare the transmigration properties of melanoma and breast cancer cells through the brain endothelial monolayer under static and dynamic conditions,
2. to compare the effects of melanoma and breast cancer cells on the tight junctions of confluent cerebral endothelial cells,
3. to understand the role of Rac signaling and of amoeboid vs. mesenchymal phenotype in the transmigration of melanoma and breast cancer cells through the BBB,
4. to investigate the impact of PI3K inhibition on the transmigration of tumor cells (melanoma and breast cancer cells) through the cerebral endothelium during brain metastasis formation,
5. to observe the effect of Rac and PI3K inhibitors on the barrier integrity of the brain endothelium.

3 Materials and methods

3.1 Cell culture and treatments

MDA-MB-231 and MCF-7 human breast cancer cells were kept in DMEM medium (Sigma) supplemented with 5% FBS (Lonza). A2058 human melanoma cells (obtained from the European Collection of Cell Cultures) were maintained in EMEM (Sigma) supplemented with 5% FBS (Sigma). A375 human melanoma cells were kept in DMEM medium (Sigma) supplemented with 10% FBS (Lonza). 4T1 mouse breast cancer cells were kept in RPMI medium (Lonza) supplemented with 5% FBS (Lonza). The hCMEC/D3 human microvascular cerebral endothelial cells (abbreviated as D3) (Weksler et al, 2005) were grown on rat tail collagen-coated dishes in EBM-2 medium (Lonza) supplemented with EGM-2 Bullet Kit (Lonza) and 2.5% FBS (Sigma). Rat brain endothelial cells (RBECs) were used for immunofluorescence experiments because of their superior barrier characteristics. Primary rat brain endothelial cells (RBECs) were isolated from 2-week old rats. Briefly, after removal of meninges cerebral cortices were cut into small pieces and digested with 1 mg/ml collagenase type 2 (Sigma). After separation of myelin by centrifugation in 20% bovine serum albumin (BSA), a second digestion was performed with 1 mg/ml collagenase/dispase (Roche). Microvessel fragments were collected after 10 min 1000•g centrifugation on Percoll (Sigma) gradient, and plated onto fibronectin/collagen-coated dishes. Endothelial cells growing out of the microvessels were cultured in DMEM/F12 (Life Technologies), 10% plasma-derived serum (First Link) and growth factors. In the first two days, 4 μg/ml puromycin was added to remove contaminating cells.

ROCK inhibitors (Y27632, Tocris and fasudil, Santa Cruz) were used in a final concentration of 10 μM. EHT1864 (Tocris), an inhibitor of the Rac family GTPases, was applied in a 20 μM concentration. LY294002 (Cell Signaling Technology) – a reversible and highly selective inhibitor of phosphatidylinositol 3 kinase (PI3K) – was used in a concentration of 25 μM.

3.2 Adhesion experiments

Brain endothelial cells (D3) were grown until confluence in 24-well plates. Tumor cells (MDA-MB-231, MCF-7, A2058 or A375 cells) were fluorescently labeled using Oregon Green 488 carboxylic acid diacetate succinimidyl ester (Life Technologies) using the protocol supplied by the manufacturer. $5 \cdot 10^4$ tumor cells/well were plated onto the endothelial monolayer in serum-free medium and incubated for 90 min. Non-attached cells were washed and the remaining cells were fixed using ethanol/acetic acid (95/5) at -20°C for 5 min. Tumor cells adhered to endothelial cells were photographed and counted using the Image-Pro Plus software (Media Cybernetics).

3.3 Static transmigration experiments using time-lapse video imaging

Human cerebral endothelial cells (D3) were cultured until confluence in 12-well plates. $2 \cdot 10^4$ tumor cells/well were plated onto the endothelial monolayer in Leibovitz's L-15 medium (Sigma). Cells were monitored for 6 h using an Andor NEO sCMOS camera connected to a Nikon Eclipse Ti-E inverted microscope, equipped with a home built incubator set to 37°C. Phase-contrast images were made every 5 min from 5 optical fields/well and time-lapse videos were constructed. The movement of each tumor cell was evaluated and transmigrated cells were counted.

3.4 Dynamic transmigration experiments using microfluidics

3.4.1 Design and fabrication of microdevices

To investigate the transmigration of tumor cells under low shear stress conditions we designed and constructed a biocompatible artificial capillary network. The schematic representation of the microfluidic setup is shown in Figure 3.

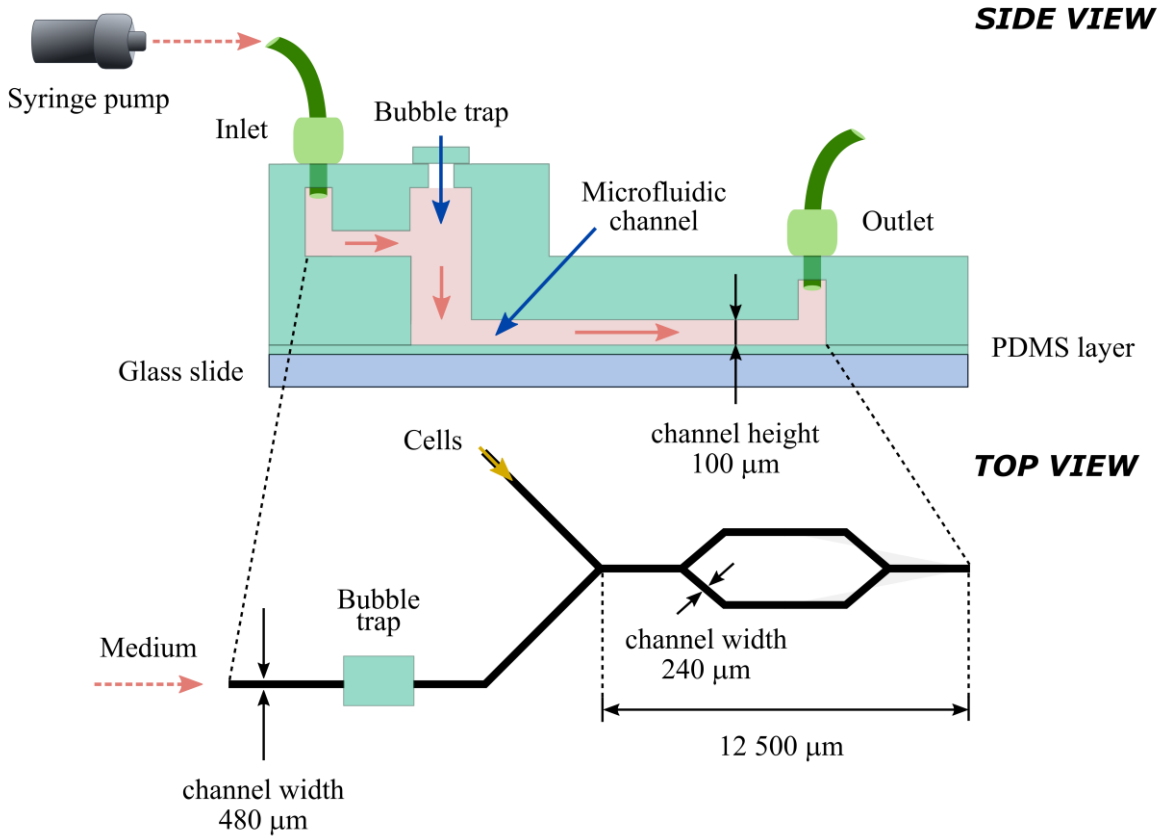


Figure 3. Dynamic transmigration experimental setup: schematic representation of the microfluidic device.

The microfluidic capillary device was fabricated from poly-dimethylsiloxane (PDMS, Sylgard 184, Dow Corning) using standard photolithography and soft lithography techniques. (Qin et al, 2010) Imprints of the microdevices were built by creating 100 µm high SU8-2050 negative photoresist (MicroChem) layers on silicon wafers. The photoresist layers were exposed to UV light through a chromium mask (JD Photo-Tools), using a flood exposure source with mask aligner (500W Hg lamp, i-line, model 97435, Newport & Digital Exposure Controller model 68945, Newport). In order to prevent the attachment of PDMS to the SU8 molds, the molds were treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-trichlorosilane (Gelest) under vacuum overnight. Positive replicas were fabricated by PDMS molding. The PDMS replicas were cured, inlet holes and bubble traps were punched and the devices were bound to PDMS-covered microscope glass slides using oxygen plasma treatment.

3.4.2 Cell seeding and microfluidic cell culture

Prior to seeding of brain endothelial cells, the inner surface of the channels was coated with rat tail collagen. 10^6 D3 cells were collected in 100 µl Leibovitz's L-15 media completed with 2.5% FBS (Sigma), growth factor mix (Lonza), hydrocortisone and gentamicin-amphotericin-B and injected into the microchannels. The microfluidic devices were placed in a home built incubator installed on a microscope stage set to 37°C. Cells were kept in "static conditions" for 24-36 h to reach a confluent layer. During this static state the medium was refreshed every 8 h. When the confluent endothelial layer fully developed, a continuous flow of 300 µl/h rate was started and maintained for 24 h to mimic the blood circulation.

During transmigration experiments, $3•10^5$ tumor cells (A2058 or MDA-MB-231) were collected in 100 µl media and injected manually (with syringe). After the injection, a continuous flow with 100 µl/h rate was established and maintained for 6 h. Considering the physical parameters of the device (channel height of ~100 µm, channel width of ~240-480 µm) and the used fluid flow rates (100-300 µl/h), we can estimate the shear stress (based on Song et al, 2005) acting on the endothelial cells in the microchannels. The applied fluid flow generated a low stress regime in our device, in which the shear stress was around ~0.3-2 dyn/cm^2.

3.4.3 Microscopy

Phase contrast microscopy images were taken during cell growth and transmigration phase, using an Andor NEO sCMOS camera and a Nikon Eclipse Ti-E microscope (Nikon), equipped with a 20× Plan Fluor phase contrast objective and a Proscan II motorized microscope stage (Prior Scientific). We used the Nikon NIS Elements AR software (Nikon) to control the microscope setup during the recordings. Microscopy images were taken every 30 min during the endothelial cell attachment phase and every 5 min during the transmigration experiments (representative images are shown in Figure 4).

3.5 Immunofluorescence studies

RBECs were cultured until confluence on collagen/fibronectin-coated filter inserts. Tumor cells (MDA-MB-231, A2058 or 4T1) were fluorescently labeled using CellTracker™ Red (Life Technologies) and plated onto the endothelial monolayer. After 5 h or 8 h cells were

washed and fixed with ethanol/acetic acid. After blocking with 3% BSA, filter inserts were incubated with anti-claudin-5 primary antibody (Life Technologies). The staining was visualized using Alexa488-conjugated secondary antibody (Jakson Immunoresearch). Nuclei were stained with Hoechst 33342 (Sigma). Samples were mounted in FluoroMount-G (SouthernBiotech) and studied with a Nikon Eclipse TE2000U microscope connected to a digital camera (Spot RT KE, Diagnostic Instruments).

3.6 Cell viability assay

Viability of tumor cells and endothelial cells was quantified with the EZ4U non-radioactive cell proliferation and cytotoxicity assay (Biomedica). D3, A2058, A375, MDA-MB-231 and MCF-7 cells were seeded in 96-well plates. Next day cells were treated for 5 h with 20 μM EHT1864 or 25 μM LY294002 in serum-free, phenol red-free DMEM (Life Technologies). After incubation with the EZ4U substrate for 45 min, the absorbance (OD at 450 nm) was detected using a BMG FLUOstar OPTIMA microplate reader.

3.7 Wound healing assay

Tumor cells (MDA-MB-231, MCF-7 or A2058) were seeded into 24-well plates. After attachment the cell layer was wounded by scratching with a pipette tip, washed with PBS, and exposed to treatments with 20 μM EHT1864 or 25 μM LY294002 in serum-free Leibovitz's L-15 medium. Cells were monitored over 24 h, and phase contrast images were taken every 30 min with an Andor NEO sCMOS camera connected to the Nikon Eclipse Ti-E inverted microscope equipped with a home-built incubator set to 37°C and a 20× Nikon Plan Fluor objective, all placed onto a Prior Proscan II motorized stage (Prior Scientific Instruments). The wound healing effect was quantified by averaging the number of migrating cells counted in five wounded areas.

3.8 Real-time impedance monitoring

To monitor the effects of EHT1864 and LY294002 on D3 cells in real-time, we measured the electrical impedance using the xCELLigence system following the manufacturer's instructions (Acea Biosciences). Briefly, cells were seeded at a density of

10^4 cells/well into 100 µl of media in an E-Plate® (i.e., 96-well tissue culture plates having micro-electrodes integrated on the bottom) and allowed to attach onto the electrode surface over time. The electrical impedance was recorded every 15 min. When the impedance reached plateau (i.e. confluent monolayer with well-formed junctions), the cells were treated with 20 µM EHT1864 or 25 µM LY294002 for an additional 10 h. The cell impedance (which depends on cell number, degree of adhesion, spreading and proliferation of the cells and also on the tightness of the junctions), expressed in arbitrary units (cell index), was automatically calculated by the software of the instrument.

3.9 Western-blot analysis

Confluent D3 brain endothelial cells were treated with 20 µM EHT1864 or 25 µM LY294002 for 5 h. Cells were washed with PBS and scraped into ice-cold RIPA buffer (20 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM EDTA/ethylenediaminetetraacetic acid, 1 mM Pefabloc®) and incubated on ice for 30 min. Lysates were clarified by centrifugation at 10,000•g for 10 min at 4°C. Proteins were electrophoresed and blotted onto nitrocellulose (Bio-Rad) membranes. Blocking was carried out at room temperature for 30 min in TBS-T containing 3% BSA. Anti-claudin-5 (Life Technologies), primary antibody was used. After washing the membranes in TBS-T (Tris-buffered saline and Tween 20), blots were incubated with the HRP (horseradish peroxidase)-conjugated secondary antibody (BD Transduction Laboratories) diluted in TBS-T. The immunoreaction was visualized using Clarity ECL Western-Blot Substrate kit (Bio-Rad) in a Bio-Rad ChemiDoc MP Imaging System.

4 Results

4.1 Comparison of the adhesion and transmigration properties of breast cancer cells and melanoma cells in vitro

The first step in the process of brain metastasis formation is the adhesion of tumor cells to cerebral CECs. To compare metastatic mammary carcinoma and melanoma cells' adhesion ability we applied an in vitro model of the blood-brain barrier. Since melanoma cells have higher propensity to metastasize to the brain than breast cancer cells, we aimed to understand whether there is any difference in the interaction of melanoma cells or breast cancer cells with the brain endothelium.

We first aimed to compare the adhesion properties of breast cancer and melanoma cells to the brain endothelium. 90 min after plating the tumor cells upon brain endothelial cells, significantly more melanoma cells than breast cancer cells were able to attach to the endothelium (Table 1).

	D3+MDA-MB-231	D3+MCF-7	D3+A2058	D3+A375
average	18.44%	18.15%	34.64%	35.05%
st dev	8.66%	8.92%	7.41%	0.75%
p	<0.05 vs. A2058 or A375	<0.05 vs. A2058 or A375	<0.05 vs. MDA or MCF	<0.05 vs. MDA or MCF

Table 1. Comparison of the adhesion of breast cancer (MDA-MB-231, MCF-7), and melanoma cells (A2058, A375) to D3 brain endothelial monolayers. % of plated cells is represented (mean and SD); P value was assessed using ANOVA and Bonferroni's post-hoc test.

Therefore, we wanted to test whether the increased adhesion of melanoma cells in comparison to breast cancer cells results in an increased transmigration as well. First, under static conditions we studied the transmigration properties of melanoma and breast cancer cells and we used a novel in vitro approach based on a time-lapse video setup described in the Materials and Methods section. This innovative assay developed in our laboratory makes possible to follow the fate of each individual cell in time (adhesion, migration, division, etc.). This approach eliminates the drawbacks of assays using filter inserts, where cells migrating through the endothelial monolayer but not moving through the pores of the filter cannot be considered. Moreover, several cell types (including D3 cells) cannot be properly grown on

large pore-size filters due to the formation of a double monolayer on both sides of the membrane.

Comparing the transmigration properties of breast cancer and melanoma cells using the static transmigration assay, we observed that fewer breast cancer cells than melanoma cells were able to migrate through the brain endothelium (Table 2). The difference was significant: 27-28% of plated melanoma cells completed the transmigration process, while only 16% of breast cancer cells migrated through.

	D3+MDA-MB-231	D3+A2058	D3+A375
average	15.78%	26.99%	28.28%
st dev	0.82%	2.54%	4.72%
p	<0.05 vs. A2058 or A375	<0.05 vs. MDA-MB-231	<0.05 vs. MDA-MB-231

Table 2. Comparison of transmigration of MDA-MB-231, A2058 and A375 cells through D3 brain endothelial monolayers under static conditions. % of plated cells is represented (mean and SD); P value was assessed using ANOVA and Bonferroni's post test.

To confirm our results in physiologically more relevant conditions, we constructed a dynamic transmigration model. Brain endothelial cells were cultured in a microfluidic device (Figure 3; described in details in the Material and Methods section) until confluence (Suppl. video 1). After reaching confluence, a slow, physiologically relevant flow of the culture medium was initiated (300 µl/h, 1-2 dyn/cm^2) for 24 h. Shear stress induced elongation of endothelial cells (Figure 4A). This morphological change to fluid shear stress has been observed in earlyer studies with different endothelial cells (Dewey et al, 1981).

After injection of MDA-MB-231 or A2058 cells, the flow was re-started with a rate of 100 µl/h, phase-contrast images were taken and time-lapse videos were constructed to analyze the movements of the tumor cells (Figure 4B, Suppl. video 2 and 3).

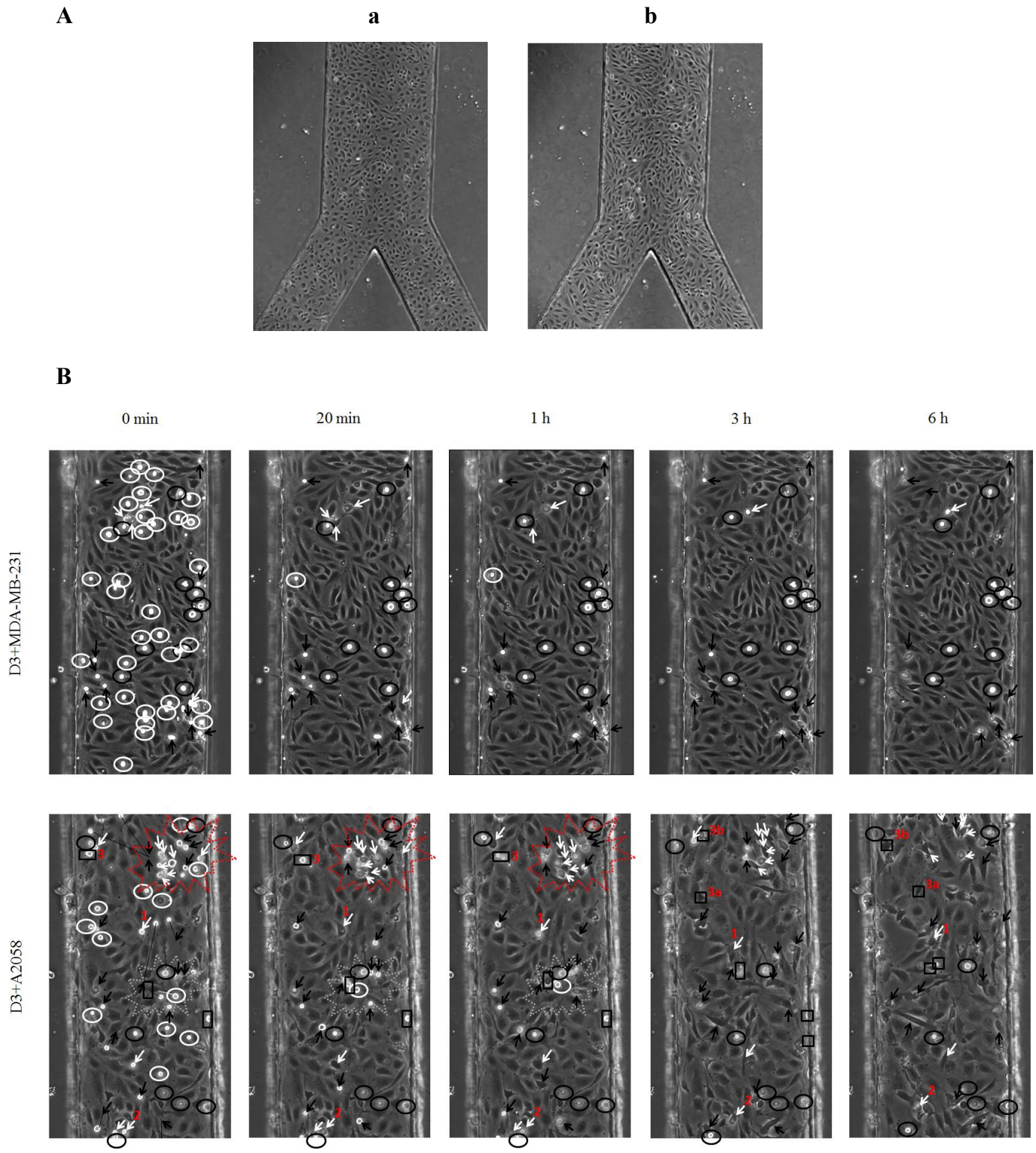


Figure 4. Confluent D3 monolayer before (a) and after (b) exposed to continuous flow (A). Dynamic transmigration experimental setup: images used for constructing the time lapse videos. D3 cells were cultured until confluence in the microfluidic device, exposed to medium flow, then tumor cells were injected and left under a continuous flow of 100 µl/h for 6 h. Phase contrast images were taken every 5 min. Individual tumor cells are marked as follows: white circle = no attachment, black circle = no transmigration, white arrow = transmigration in 20 min, black arrow = transmigration in >20 min, black box = division. The red dotted stars delineate clusters of transmigrating melanoma cells (B).

In these conditions approximately 50% of breast cancer cells and 42.5% of melanoma cells showed no attachment and were washed away by the flow of the culture medium in early stages of the experiment (Figure 4: white circles, Table 3: no attachment). The percentage of tumor cells not washed away from the optical fields studied, but not able to transmigrate in 6 h, was significantly more in case of breast cancer cells than in case of melanoma cells (15% vs. 4.5%, respectively) (Figure 4: black circles, Table 3: no transmigration). Taken together, 53% of melanoma and 35% of breast cancer cells transmigrated through the brain endothelial monolayer in these conditions. The difference in the transmigration between the two cell types was the most pronounced in early time points: 23.5% of the total number of melanoma cells migrated through the brain endothelium in the first 20 min, while the percentage of breast cancer cells transmigrating in this time frame was only 3% (Figure 4: white arrows, Table 3: transmigr. in 20 min).

	D3 + MDA-MB-231			
	disappearance	no transmigration	transmigr. in 20 min	transmigr. in >20 min
average	50.00%	**15.00%**	**2.80%**	32.20%
st dev	4.24%	4.04%	1.73%	3.54%
	D3 + A2058			
	disappearance	no transmigration	transmigr. in 20 min	transmigr. in >20 min
average	42.50%	**4.50%**	**23.50%**	29.50%
st dev	7.21%	1.00%	4.95%	5.12%
p		<0.05	<0.05	

Table 3. Comparison of transmigration of A2058 and MDA-MB-231 cells through D3 brain endothelial monolayers under dynamic conditions. MDA-MB-231 breast cancer cells or A2058 melanoma cells were injected into the microchannels already containing confluent D3 monolayers. Tumor cells were monitored for 6 h under a continuous medium flow of 100 µl/h. Tumor cells were divided into four groups: cells washed away by the flow of the culture medium (no attachment), cells not transmigrating in 6 h (no transmigration), cells migrating through the brain endothelium in the first 20 min (transmigr. in 20 min) and cells transmigrating after 20 min (transmigr. in >20 min). % of plated cells is represented (mean and SD); P value (comparing D3+MDA-MB-231 and D3+A2058) was assessed with ANOVA and Bonferroni's post-hoc test.

Melanoma cells tended to attach and transmigrate in small groups (Figure 4: red dotted star), as we have previously observed in static conditions (Fazakas et al, 2011). After transmigration, several melanoma cells continued to move beneath and between endothelial cells (as previously seen in static conditions (Fazakas et al, 2011)), sometimes rounding up

and flattening again (Figure 4: cells 1 and 2). A few divisions were also observed (Figure 4: black boxes) and the daughter cells usually transmigrated rapidly after division (Figure 4: cell 3). Taken together, these results show that melanoma cells are able to adhere to and to migrate through the brain endothelium more effectively than breast cancer cells. This might be partly responsible for the higher propensity of melanoma cells to metastasize to the brain.

4.2 Differences in the effects of breast cancer cells and melanoma cells on the tight junctions of brain endothelial cells

We have previously observed that during transmigration melanoma cells are able to disrupt the TJs of CECs and use (at least partly) the paracellular way of migration (Fazakas et al, 2011). We were interested to understand whether breast cancer cells are also able to impair the junctional integrity of the cerebral endothelium. Therefore, we performed claudin-5 immunostaining on primary rat brain endothelial cell (RBEC) monolayers challenged with MDA-MB-231 breast cancer, A2058 melanoma cells, or 4T1 mouse breast cancer cells. We have left the tumor cells on the RBEC monolayers for different time intervals (5 h and 8 h). As shown in Figure 5, melanoma cells could breach the junctions of RBECs as indicated by focal loss of claudin-5 staining. This was not observed in case of breast cancer cells.

These data suggest that differences in the transendothelial migration of mammary carcinoma and melanoma cells might be partly due to differences in their ability to impair interendothelial junctions.

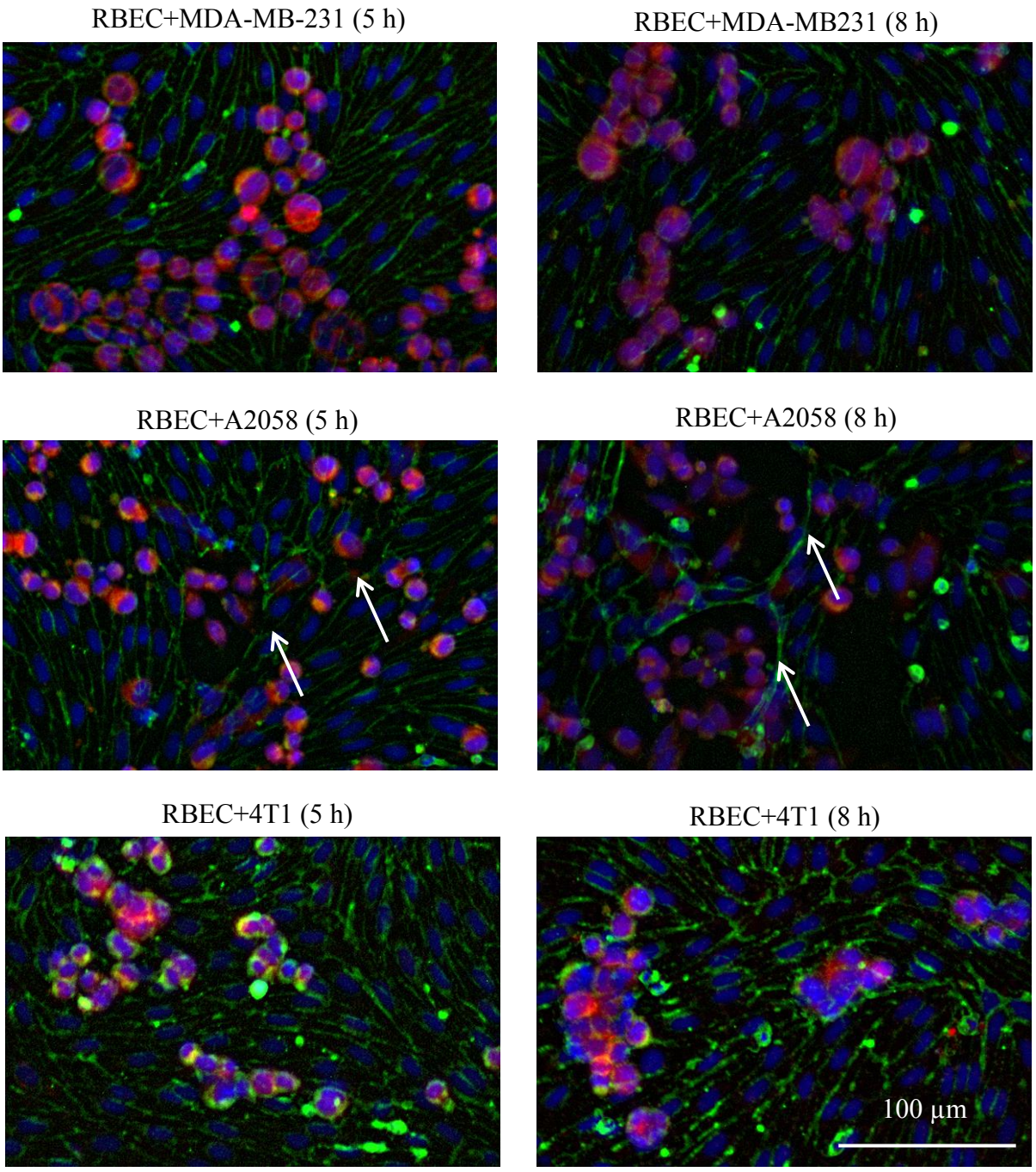


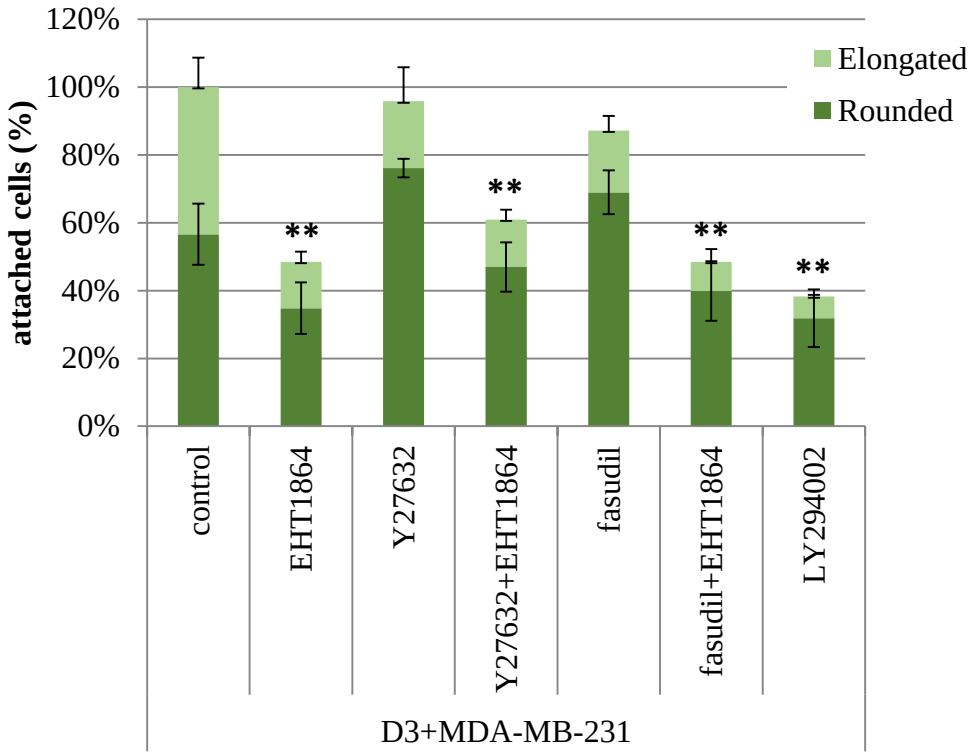
Figure 5. Effect of breast cancer and melanoma cells on the tight junctions of brain endotheial cells. MDA-MB-231 human breast cancer cells, A2058 human melanoma cells or 4T1 mouse breast cancer cells (labeled in red) were plated onto confluent RBEC monolayers and left for 5 h or 8 h. Tight junctions of endothelial cells were stained in green using anti-claudin-5 antibodies. Arrows indicate disappearance of claudin-5 staining.

4.3 Effect of Rac or PI3K inhibition on the adhesion of breast cancer cells and melanoma cells to the brain endothelium

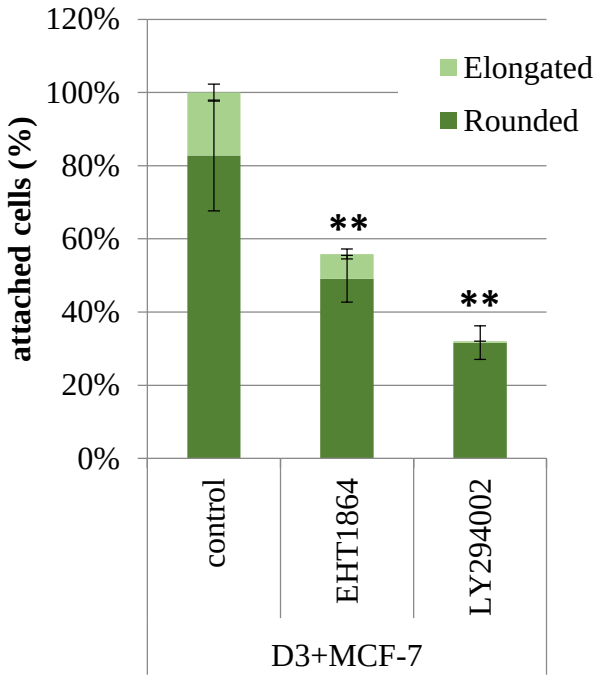
The small GTPases Rho and Rac play important role in the movement of tumor cells. Rho/ROCK signaling was shown to be involved in the ameboid invasion phenotype of cancer cells, which is characterized by rounded morphology and increased actomyosin contractility. During mesenchymal type of cell movement, tumor cells gain an elongated morphology, and activate extracellular proteolytic mechanisms, and the small GTPase Rac. Tumor cells are able to switch between the ameboid and mesenchymal type of cell movement (Symons & Segall, 2009). We aimed to understand which mechanism is applied by breast cancer cells during transmigration through CECs. We have previously shown that inhibition of the ROCK in melanoma cells increases their adhesion to brain endothelial cells. In case of breast cancer cells ROCK inhibitors (10 μM Y27632 or 10 μM fasudil) were not able to influence the number of breast cancer cells adhering to the brain endothelium (Figure 6A and B). On the other hand, the Rac inhibitor EHT1864 (20 μM) hampered the adhesion of both MDA-MB-231 and MCF-7 breast cancer cells (Figure 6A and B). As expected, mainly the number of elongated adherent cells was decreased, which have a mesenchymal phenotype. When both ROCK and Rac inhibitors were applied, a similar reduction in the adhesion of tumor cells was seen as with the Rac inhibitor alone (Figure 6A). We also observered that the Rac inhibitor EHT1864 decreased the number of adherent melanoma cells (Figure 6C).

In addition, the PI3K inhibitor LY294002 in a concentration of 25 μM significantly reduced the number of breast cancer and melanoma cells attaching to the brain endothelium. The reduction was approximately 40% compared to control in case of both breast cancer and melanoma cells, and mainly affected tumor cells with elongated (flattened, mesenchymal) phenotype (Figure 6A,B and D,E). In case of A375 melanoma cells, which presented a rounded morphology during adhesion, LY294022 could also significantly reduce the number of adherent cells (Figure 6E).

A


120%
100%
80%
60%
40%
20%
0%
attached cells (%)
Elongated
Rounded
**
**
**
**
control
EHT1864
Y27632
Y27632+EHT1864
fasudil
fasudil+EHT1864
LY294002
D3+MDA-MB-231

B


120%
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LY294002
D3+MCF-7

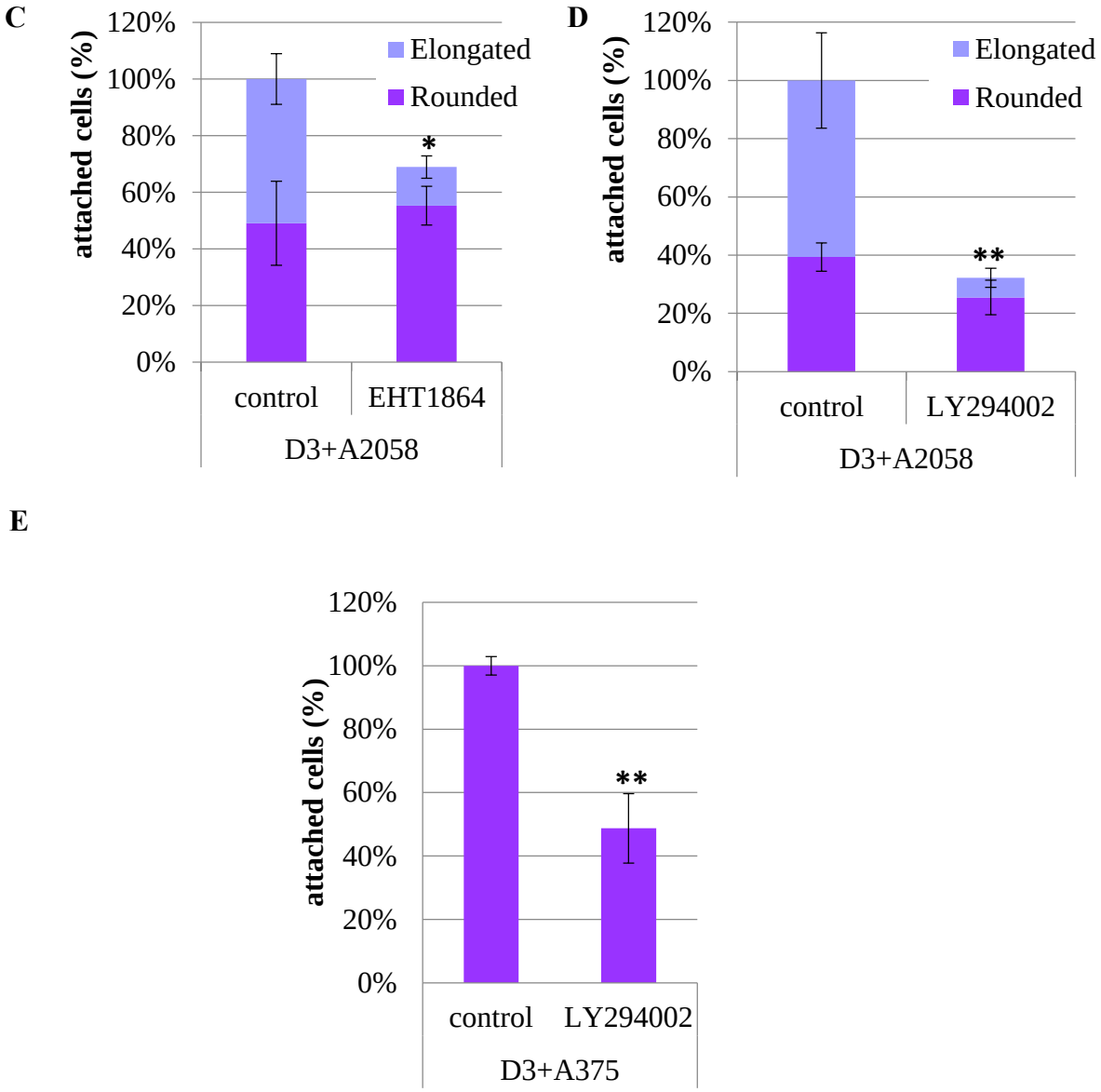
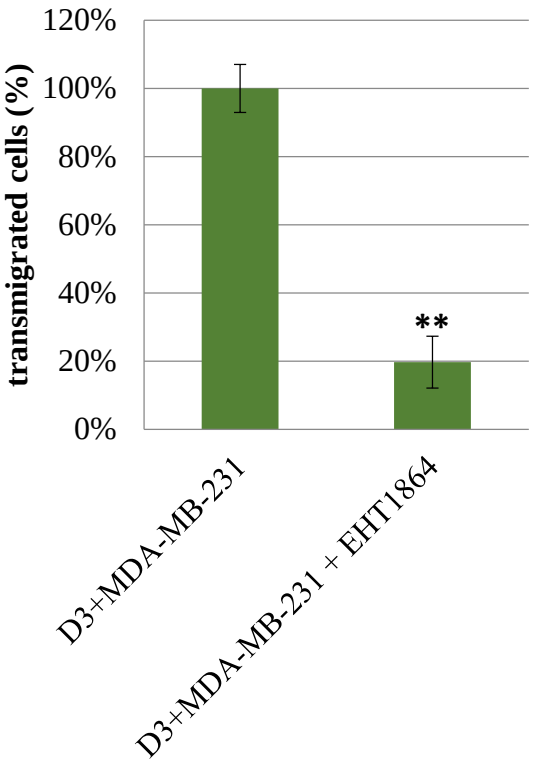


Figure 6. Effect of Rac- or PI3K-inhibition on the adhesion of breast cancer cells or melanoma cells onto the brain endothelium. Fluorescently labeled MDA-MB-231 (A) or MCF-7 (B) breast cancer cells, A2058 (C, D) or A375 (E) melanoma cells were plated onto confluent D3 monolayers and left for 90 min. Results are expressed as % control and given as mean ± SD. N = 3, ** = P<0.01, * = P<0.05, as assessed by ANOVA and Bonferroni's post-hoc test or Student's t-test.

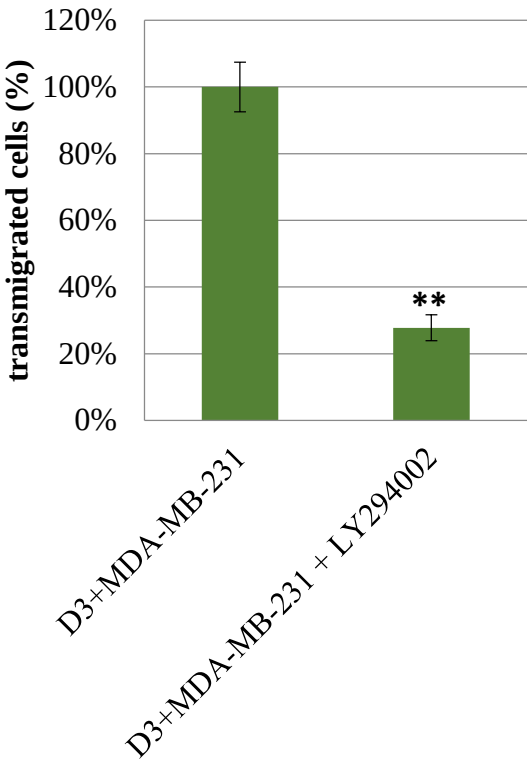
4.4 Effect of Rac or PI3K inhibition on the transendothelial migration of breast cancer cells and melanoma cells in static conditions

Since inhibition of Rac or PI3K decreased the adhesion of both melanoma and breast cancer cells, we tested the effect of Rac or PI3K inhibitors on the transmigration of melanoma and breast cancer cells through brain endothelial monolayers as well (Figure 7). In this assay we used two invasive melanoma cell lines (A2058 and A375) and the MDA-MB-231 breast cancer cell line, which is more invasive than the MCF-7 cell line. Inhibition of Rac with EHT1864 significantly reduced the transmigration of both tumor cell types to approximately 20% in case of breast cancer cells (Figure 7A) and to 15% in case of melanoma cells (Figure 7C). LY294002 had similar effects: the transmigration percentage was 30% and 40%, respectively, compared to control (Figure 7B,D,E).

A



B



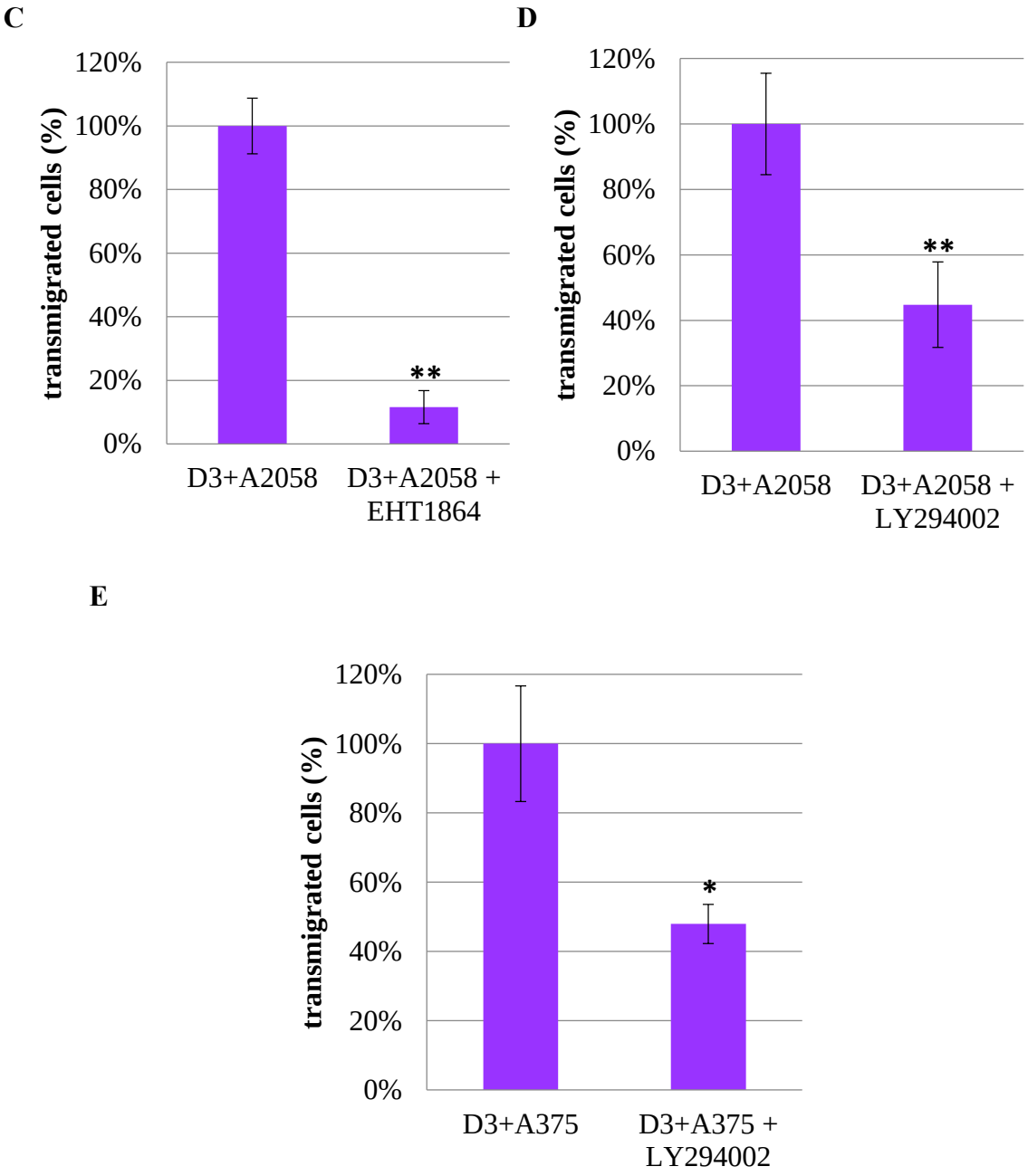


Figure 7. Effect of Rac or PI3K inhibition on the transmigration of breast cancer and melanoma cells through the brain endothelium in static conditions. MDA-MB-231 breast cancer cells (A, B), A2058 (C, D) or A375 (E) melanoma cells were plated onto confluent D3 monolayers and left for 6 h. Phase-contrast images were made every 5 min and transmigrating tumor cells were counted. Results are expressed as % control and given as mean ± SD. N = 3, ** = P<0.01, * = P<0.05 as assessed by Student's t-test.

4.5 Effect of Rac or PI3K inhibition on the transendothelial migration of breast cancer cells and melanoma cells in dynamic conditions

Using our dynamic model, we explored which step of transmigration of breast cancer or melanoma cells was inhibited by EHT1864 or LY294002. As previously discussed (Table 3), only 8% of total transmigrating breast cancer cells completed transmigration in the first 20 min (2.8% of 35%), while this was significantly higher in case of melanoma cells (44.34%, i.e. 23.5% of 53%). This suggests that melanoma cells can transmigrate more rapidly through the brain endothelium than breast cancer cells (Figure 8). Nevertheless, EHT1864 and LY294002 inhibited the rapid transmigration of melanoma cells. However, in case of breast cancer cells the number of cells transmigrating after 20 min was reduced by inhibitors of Rac and PI3K (Figure 8).

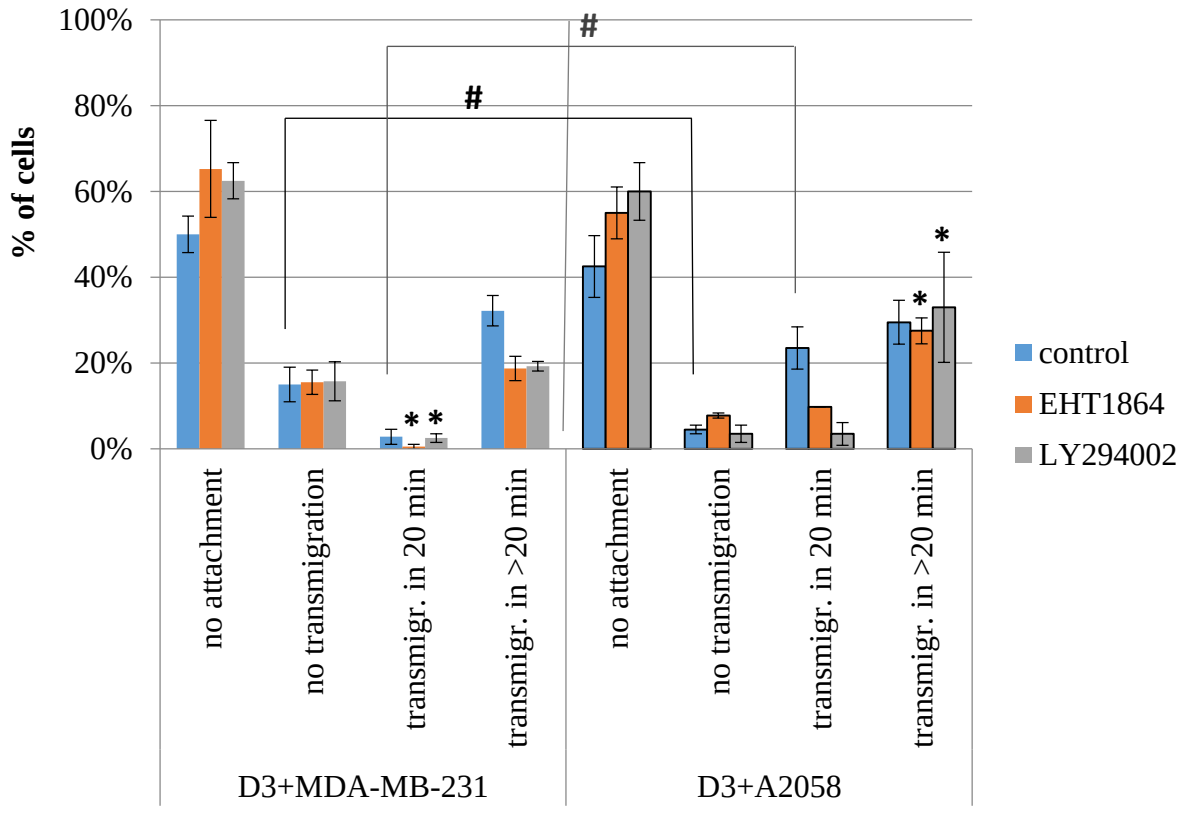


Figure 8. Effect of Rac or PI3K inhibition on the transmigration of breast cancer cells or melanoma cells through the brain endothelium in dynamic conditions. Results are expressed as % control and given as mean ± SD. N = 3, * = P<0.05 compared to control, # = P<0.05 A2058 cells compared to MDA-MB-231 cells, as assessed by ANOVA and Bonferroni's post-hoc test.

Taken together, our results indicate that inhibition of Rac or PI3K impairs the ability of both breast cancer and melanoma cells to adhere to and to migrate through the brain endothelium. Differences exist however, between the velocities of the transmigration of the two tumor cell types.

4.6 Effects of Rac and PI3K inhibitors on the viability, proliferation and migration of tumor cells and brain endothelial cells

We wanted to exclude that the inhibitory effect of EHT1864 and LY294002 on the adhesion and transmigration of breast cancer and melanoma cells was due to toxicity on tumor cells. Using the EZ4U assay no toxic effect of either EHT1864 or LY294002 on A2058, A375, MDA-MB-231 and MCF-7 cells was observed. Moreover, the EZ4U assay did not show any toxicity of EHT1864 or LY294002 on D3 brain endothelial cells (Figure 9). In addition, as assessed during the time-lapse video experiments, the number of dividing cells was approximately 2.5% in case of MDA-MB-231 cells and 1% in case of A2058 cells. Therefore, the observed changes in the adhesion and transmigration are unlikely to be the result of an anti-proliferative effect of EHT1864 or LY294002. Moreover, the wound healing assay indicated no change in the migratory properties of melanoma or breast cancer cells in response to EHT1864 or LY294002 (not shown).

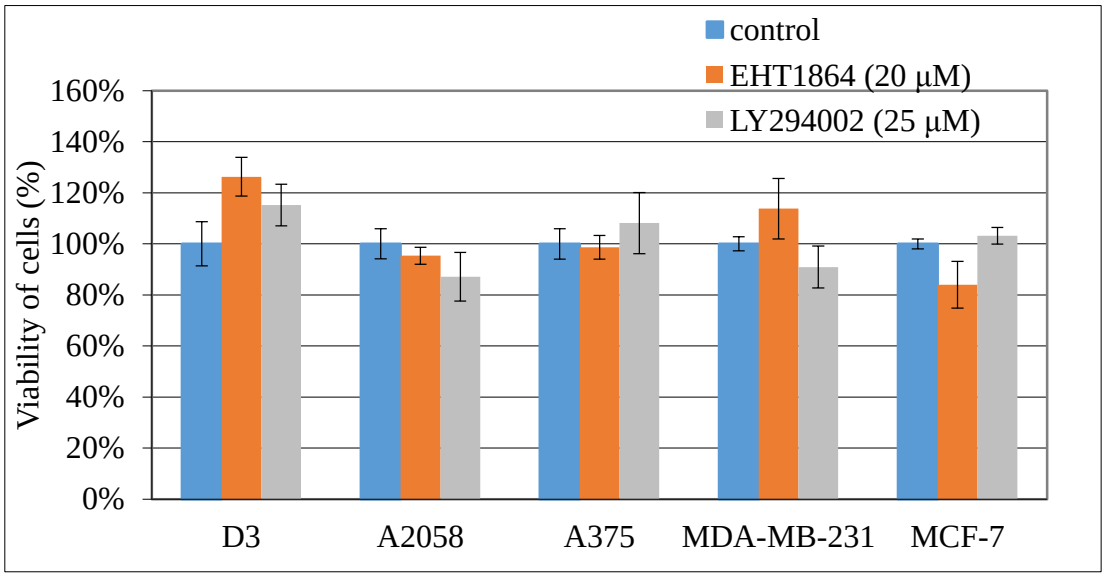
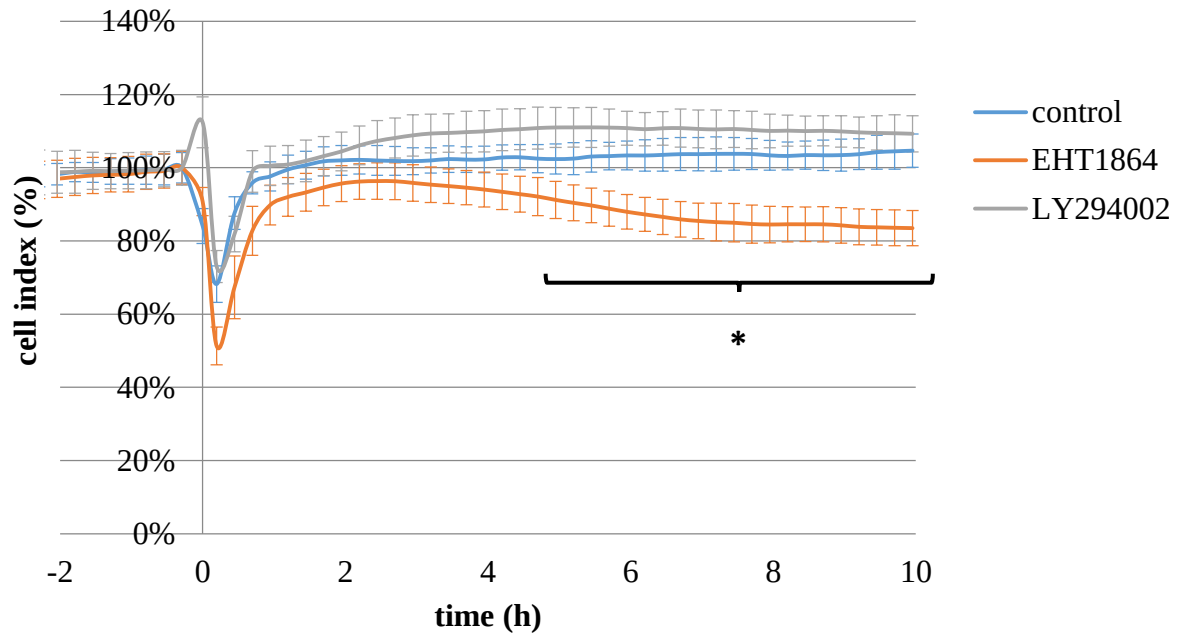


Figure 9. Effect of the Rac and PI3K inhibitors on the viability of endothelial and tumor cell lines. Viability of the cells was measured using the EZ4U kit. Data are expressed as mean ± SD.

4.7 Effect of Rac or PI3K inhibition on the junctional integrity of the brain endothelium

We observed that EHT1864 induced a decrease in the impedance of D3 cells, as reflected by the cell index (Figure 10A). After an initial drop induced by the medium change, the impedance of control and LY294002-treated D3 cells recovered rapidly. However, in case of EHT1864-treated cells the recovery was not complete, and after 5 h a significant drop in the impedance was seen. The cell impedance reflects changes in the cell number, viability and tightness of the junctions. Since no change in the viability of D3 cells was observed using the EZ4U assay, we next investigated the possible damaging effect of the Rac inhibitor on the TJs. We observed a significant down-regulation of claudin-5 protein in D3 cells with Western-blot, in response to EHT1864 (Figure 10B). LY294002 did not significantly affect the amount of claudin-5 protein in D3 cells.

A



B

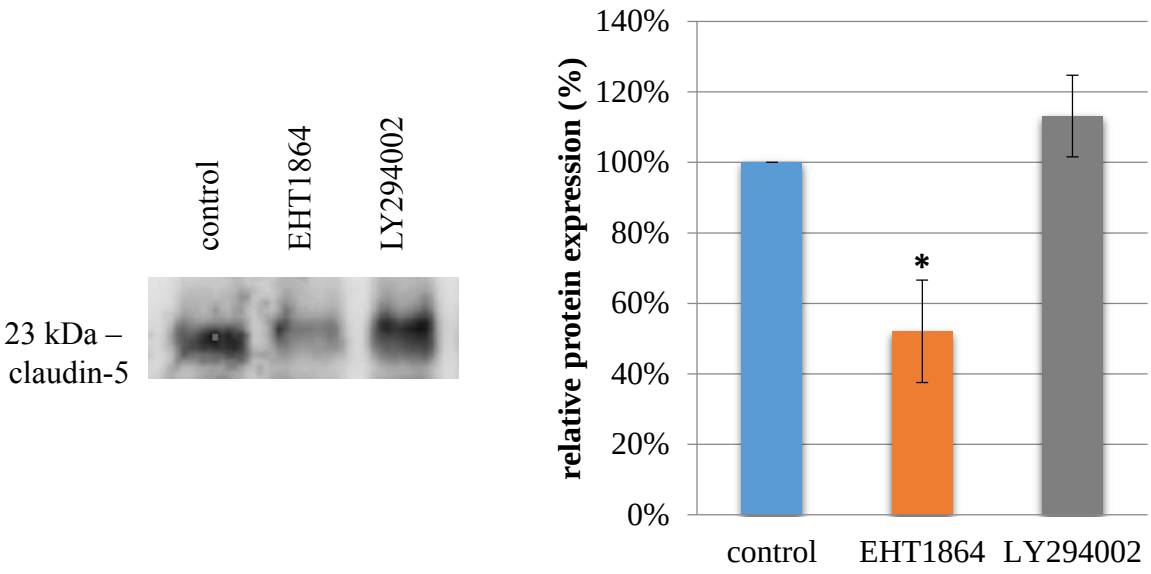


Figure 10. Effect of the Rac inhibitor EHT1864 and of the PI3K inhibitor LY294002 on the junctional integrity of the brain endothelium. Impedance of D3 brain endothelial cells (represented by the cell index) was assessed by the ACEA xCELLigence system. Results are expressed as % control and given as mean ± SD. N = 3, * = P<0.05 compared to control, as assessed by ANOVA and Bonferroni's post-hoc test (A). D3 cells were treated with 20 µM EHT1864 or 25 µM LY294002 for 5 h. Claudin-5 Western-blot was performed from the RIPA-soluble fractions. One representative blot and densitometry based on three independent experiments is shown. * = P<0.05 compared to control, as assessed by ANOVA and Bonferroni's post-hoc test (B).

5 Discussion

Brain metastases are devastating complications of lung cancer, breast cancer, melanoma and other malignancies. Among all solid tumors, melanoma has the highest affinity to the CNS. This has been explained by the specific brain environment which supports the growth of cells of ectodermal origin (Denkins et al, 2004; Fidler et al, 1999). However, besides soluble and cellular elements of the central nervous system, other factors might also contribute to the neurotropism of melanoma cells. One of the most important steps in the process of brain metastasis formation is the diapedesis of metastatic cells through the barriers of the CNS, mainly the BBB-forming microvascular endothelium. The role of the BBB in the formation of cerebral metastases is largely unexplored and probably very complex. Being the tightest endothelial barrier in the organism, it hinders the transmigration of tumor cells into the brain. On the other hand, unique brain endothelial properties might differentially affect the diapedesis of different cancer cell types. In order to understand the mechanisms of melanoma and breast cancer brain metastasis formation we applied two different in vitro models to study the interaction between tumor cells and brain endothelial cells.

5.1 Model systems used. Comparison of the adhesion and transmigration properties of breast cancer and melanoma cells

The static and dynamic in vitro BBB models used in our experiments are based on the culture of hCMEC/D3 (D3) human cerebral endothelial cells. This cell line has been widely used as a human BBB model (Carl et al, 2010; Förster et al, 2008), thus this is the most well characterized human brain endothelial cell line (Weksler et al, 2013).

To study the effect of the tumor cells on brain endothelial TJs we used cultures of primary rat brain endothelial cells (RBECs). Primary cells in culture maintain the main in vivo characteristics of the brain endothelium, i.e. expression of von Willebrand factor, presence of a continuous line of TJs, high TEER and low permeability values and high activity of P-gp, preserving superior permeability characteristics to cell lines.

The time-lapse videos indicated that melanoma cells have increased ability to attach to the brain endothelium than breast cancer cells under static and dynamic conditions as well. In order to exclude the possibility that this is due to differences in the invasive and metastatic capacities between the melanoma and breast cancer cell lines used, we used two different

human breast cancer cell lines: the less invasive MCF-7 and the highly migratory and metastatic MDA-MB-231 (Wertheimer et al, 2012). We also used two different human melanoma cell lines, A2058 and A375, both invasive BRAF V600E mutants, having similar propensity to metastasize to different organs (Rozenberg et al, 2010), A2058 being vemurafenib resistant, while A375 vemurafenib sensitive (Boussemart et al, 2014).

The difference between the adhesive properties of melanoma cells and either of the breast cancer cell lines was significant. Moreover, such a difference was not observed when other endothelial cell types (HUVECs, dermal microvascular cells, lymphatic endothelial cells) were used: melanoma cells had similar adhesion properties to non-cerebral endothelial cells as breast cancer cells (Safuan et al, 2012). We have also observed a significant difference in the number of transmigrating melanoma and breast cancer cells under static and dynamic conditions as well. The number of cells not able to migrate through the brain endothelium was much higher in case of breast cancer cells than in case of melanoma cells. Moreover, invasive melanoma cells tended to complete the transmigration process much more rapidly than invasive breast cancer cells.

The fact that melanoma cells can more easily overcome the BBB may be one of the factors leading to the high tropism of melanoma cells towards the CNS. The high proportion of brain metastases in melanoma patients has been reported to be a consequence of the so-called “homing” influence (Denkins et al, 2004), i.e. the ectodermal origin of both melanocytes and the CNS. According to our results, the higher ability of melanoma cells to impair brain endothelial TJs might also play a role in the higher ability of melanoma cells to form brain metastasis in comparison to breast cancer cells.

5.2 Effect of tumor cells on interendothelial junctions

Differences between the ability of the two tumor cell types to migrate through the brain endothelium might be partly due to their different ability to impair the TJs.

Our data indicate that melanoma cells are more effective in breaking down the paracellular barrier than breast cancer cells. In order to exclude species-specific effects, we used both human and mouse breast cancer cell lines. Neither human, nor mouse mammary carcinoma cells were able to disrupt the junctions of CECs. On the other hand, human melanoma cells induced a time-dependent disappearance of junctional proteins from the membranes of brain endothelial cells. Moreover, we have previously shown that mouse

melanoma cells (the B16/F10 cell line) had similar effects (Fazakas et al, 2011). Therefore, we conclude that melanoma cells are more effective in disrupting the paracellular barrier of the brain endothelium than breast cancer cells, independent of species.

We have previously shown that both breast cancer and melanoma cells are able to induce endothelial-mesenchymal transition in CECs (Krizbai et al, 2015), which results in loss of junctional integrity. On the other hand, activation of the proteolytic cascade during transmigration through the cerebral endothelium seems to be more effective in melanoma cells than in breast cancer cells (our unpublished results). Since junctional proteins are targets of proteolytic degradation, this difference may be partly responsible for the differences in the induction of junctional breakdown.

On the other hand, breast cancer cells were previously shown to be able to use not only the paracellular pathway (through interendothelial junctions), but also the transcellular pathway (through the endothelial cell body) during migration through non-cerebral endothelia (Arvanitis et al, 2014; Khuon et al, 2010). In this process endothelial myosin light chain kinase was activated at the invasion site.

Our results suggest that breast cancer cells might be more effective in the transcellular type of migration than melanoma cells, these latter having increased ability to use the paracellular pathway than mammary carcinoma cells. Further analyses will clarify this possible difference between the two tumor cell types.

5.3 Role of Rac and PI3K in the extravasation of tumor cells

We have also assessed the role of two signaling molecules (Rac and PI3K) in the transmigration of melanoma and breast cancer cells through the BBB. In a recent study we showed that melanoma cells prefer the Rac-dependent mesenchymal type of cell movement to the Rho/ROCK-dependent amoeboid one during transmigration through the BBB. We also showed that inhibition of Rac not only impedes the adhesion and transmigration of melanoma cells, but of breast cancer cells as well. Interestingly, the ROCK inhibitor Y27632 – which significantly facilitated the adhesion of melanoma cells – did not affect the adhesive properties of breast cancer cells. This may be the consequence of the very high basal activity of the P-Rex1/Rac signaling pathway, which is highly overexpressed in human luminal mammary tumors, particularly those expressing HER2 and ER (Wertheimer et al, 2012).

The effect of Rac inhibition was similar in case of both melanoma and breast cancer cells: a significant decrease in the number of tumor cells attaching to and transmigrating through CECs was observed. This supports the idea that inhibition of the mesenchymal movement of tumor cells might be beneficial in reducing the diapedesis of different metastatic cells through the BBB. Unfortunately, Rac inhibitors – in contrast to ROCK inhibitors, which prevent the disruption of the TJs of CECs – might impair the integrity of the BBB.

The effect of induction of the mesenchymal phenotype on tumor cell motility and invasion are contradictory and seem to depend on the tumor cell type. Inhibition of ROCKs (i.e. induction of the mesenchymal phenotype) has been shown to decrease the invasion and migration of lung (Yang et al, 2012; Yang et al, 2010; Zhu et al, 2011), breast (Wyckoff et al, 2006), and hepatocellular carcinoma cells (Chen et al, 2011; Itoh et al, 1999; Ying et al, 2006). In contrast, in case of osteosarcoma (Yui et al, 2010), pancreatic carcinoma (Fujita et al, 2011) and colon carcinoma (Adachi et al, 2011; Vishnubhotla et al, 2012), ROCK inhibitors improved invasive and migratory properties of the cells. In general, the ameboid type of movement appears to more favourable movement for the tumor cells, but this depends on the surrounding tissues, properties of the extracellular matrix or the endothelial barrier they need to cross (Symons & Segall, 2009). According to our results the mesenchymal phenotype seems more effective in case of overcoming the BBB.

Besides Rac, we tested the role of the PI3K/Akt/PTEN pathway, which is a key regulator of tumorigenesis and metastasis formation. BRAF-mutant melanoma cells have been shown to have higher levels of pAkt-Ser473, pAkt-Thr308 and decreased expression of PTEN (Davies et al, 2009). The A2058 cell line used in our experiments is a V600E BRAF mutant expressing high amounts of phosphorylated Akt and low levels of PTEN (Xing et al, 2012). It has been shown that inhibition of PI3K results in a reduction of melanoma cell transmigration through HUVECs (Peng et al, 2005). Since brain metastases of melanoma have been shown to have significantly higher pAkt and lower PTEN levels than extracerebral metastases (Davies et al, 2009; Niessner et al, 2013), we aimed to test whether inhibition of this pathway impedes the transmigration of melanoma cells through CECs. We observed a marked inhibition of melanoma cells able to attach to and to migrate through the brain endothelium in response to PI3K inhibition. According to our results, breast cancer cell transendothelial migration could be partly blocked using the PI3K inhibitor LY294002 – similarly to melanoma cells.

The morphology of PI3K-inhibited tumor cells was similar to that of Rac-inhibited cells, i.e. we could see a reduction in the number of elongated, flattened cells. This suggests

that PI3K inhibition – similar to Rac inhibition – induces an amoeboid-like phenotype in both melanoma and breast cancer cells. This is not surprising because PI3K has been shown to regulate Rac through P-Rex1 in breast cancer cells (Ebi et al, 2013) and several PI3K lipid products have been shown to interact with different RacGEFs (Campa et al, 2015). Moreover, a positive feed-back loop exists between P-Rex1 and PI3K (Dillon et al, 2015).

5.4 Effect of Rac and PI3K inhibitors on the barrier integrity

Unfortunately, inhibition of Rac impaired the integrity of brain endothelial junctions. TJ proteins – which are one of the main elements of barrier properties of CECs – are linked to the actin-cytoskeleton and are influenced by small G-proteins. Inhibition of Rho/ROCK signaling was shown to prevent disruption of epithelial and endothelial TJs in different pathological conditions, e.g. Ca^{2+}-depletion (Samarin et al, 2007; Wilhelm et al, 2007) or HIV infection (Xu et al, 2012). We have also observed that ROCK inhibition induces increase of TEER in CECs. Although interactions between the two pathways is complex, activation of Rho usually leads to inactivation of Rac and vice versa (Burridge & Wennerberg, 2004). Therefore, it is not suprising that while inhibition of the Rho/ROCK pathway strengthens the junctions, Rac inhibition has an opposite effect.

In contrast to the Rac inhibitor (EHT1864), the PI3K inhibitor (LY294002) did not affect the integrity of the BBB. We observed that EHT1864 decreased the impedance of endothelial cells, and we also demostrated a significant down-regulation of claudin-5 protein in D3 cells in response to EHT1864, but not in response to LY294002. Many PI3K inhibiting agents are in different phases of clinical trials for the treatment of different cancer types (Rodon et al, 2013). Based on our results, PI3K inhibitors might turn out to have clinical benefits not only in the treatment of primary tumors, but also in preventing brain metastasis formation of breast cancer and melanoma.

In **conclusion**, we have shown that invasive melanoma cells have an increased capacity to:

(1) attach to,

(2) migrate through and

(3) impair the tight junctions of

the brain endothelium than breast cancer cells.

In addition, inhibition of Rac or PI3K decreases the number of both melanoma and breast cancer cells able to transmigrate through cerebral endothelial cells; however, Rac inhibition (but not PI3K inhibition) impairs the junctional integrity of the blood-brain barrier. Since inhibitors of the PI3K/Akt pathways are emerging as candidates for anti-cancer therapy, the mechanism described here might be of clinical relevance.

6 Summary

Metastatic cells invading the CNS parenchyma have to overcome the blood-brain barrier. Tumor cells meet a supportive environment in the brain, protected from chemotherapeutics and antitumoral immune response. It is not surprising, therefore, that brain metastases of malignant tumors have limited therapeutic options. Hence, it would be of crucial importance to prevent the formation of brain metastases. One of the possible strategies is to target the step of migration of metastatic cells through the blood-brain barrier. The mechanisms of this process are largely uncharacterized. In order to understand these mechanisms involved in this process we have used in vitro experimental setups based on the culture of cerebral endothelial cells and tumor cell lines.

We have demonstrated that melanoma cells have enhanced ability of adhesion to and transmigration through the brain endothelium than breast cancer cells under static and dynamic conditions as well. Moreover, melanoma cells tend to complete the transmigration process more rapidly than invasive breast cancer cells.

Our experiments revealed that melanoma cells are more effective in breaking down the tight junctions of cerebral endothelial cells than breast cancer cells.

Our data indicated that inhibition of Rac impedes the adhesion and transmigration of melanoma and breast cancer cells as well.

We observed a reduction of melanoma and breast cancer cells able to attach to and to migrate through the brain endothelium in response to PI3K inhibition.

In addition, considering their potential therapeutic effects, we have investigated the effect of Rac and PI3K inhibitors on the barrier integrity of cerebral endothelial cells. We have shown that the Rac inhibitor EHT1864 decreases the amount of junctional protein claudin-5, while the PI3K inhibitor LY294002 does not affect the integrity of the BBB.

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Appendix