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REVIEW ARTICLE

Circulating MicroRNAs and Blood-Brain-Barrier Function in Breast Cancer Metastasis

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Abstract: Brain metastases are a major cause of death in breast cancer patients. A key event in the metastatic progression of breast cancer in the brain is the migration of cancer cells across the blood-brain barrier (BBB). The BBB is a natural barrier with specialized functions that protect the brain from harmful substances, including anti-tumor drugs. Extracellular vesicles (EVs) sequestered by cells are mediators of cell-cell communication. EVs carry cellular components, including microRNAs that affect the cellular processes of target cells. Here, we summarize the knowledge about microRNAs known to play a significant role in breast cancer and/or in the BBB function. In addition, we describe previously established *in vitro* BBB models, which are a useful tool for studying molecular mechanisms involved in the formation of brain metastases.

Keywords: Metastatic breast cancer, blood-brain barrier, *in vitro* models, microRNA, extracellular vesicles (EVs), brain metastases.

1. INTRODUCTION

Breast cancer is the most common malignant tumor that causes the highest cancer-associated death of women from industrial nations. Cancer metastases have a significant impact on mortality and overall survival of these patients. Importantly, the average mortality has been significantly reduced in recent years due to differential early detection and screening measures as well as advanced preventive examinations. However, despite all this, many patients die prematurely due to a pronounced tumor affliction.

MicroRNAs (miRs) are short, non-coding RNAs that are approximately 20 nucleotides in length. They regulate gene expression post-transcriptionally by degrading mRNA or blocking its translation [1]. Only 2% of the genome consists of protein-coding sequences, while the non-coding sequences predominate [2]. These are the least researched parts of the genome so far and their effects on biological processes in the human body, but also in tumorigenesis, are not yet well explained. In recent years, various research groups have demonstrated that miRs can not only be detected in tissue, but also circulate in cell-free body fluids such as plasma or serum [3]. More importantly, it has been demonstrated that miRs play a prognostic role in cancer of various entities [4-6].

Eukaryotic cells sequester extracellular vesicles (EVs) which, depending on their size, are divided into exosomes, activation or apoptosis-induced microvesicles and apoptotic bodies. Microvesicles are cell membrane vesicles with a diameter of 100-1000 nm, apoptotic bodies are vesicles with a diameter of 1-5 µm, while exosomes have a diameter of 30-100 nm [7-9]. Because of their small size, exosomes have emerged as a novel approach to drug delivery and biomarker research. Exosomes can transfer proteins and genetic material [10]. They circulate in body fluids carrying active molecules to distant cells in the body where they can incorporate and release their constituents. Exosomes are considered to be powerful non-invasive biomarkers because of their high stability and well-optimized methods for their isolation and characterization. Tumor cells (TCs) secrete more exosomes than normal cells [11, 12]. Due to this fact, expression profiles of exosomes isolated from serum/plasma of cancer patients showed different levels of numerous miRs as compared to healthy individuals [13]. In the advanced stage of the disease, the tumor can spread from the primary tissue and form metastases. Besides lung cancer (small cell and non-small cell), melanoma and renal cancer, the highest incidence to metastasize in the central nervous system (CNS) has been described for breast cancer. Exosomes isolated from patients with metastases show a different miR expression pattern compared to healthy individuals or patients with primary neoplasms [14]. An efficient characterization of these tumor signatures may enable the development of new classification criteria and novel therapies for these pathologies in the future. In particular, cerebral metastases of breast cancer have recently been dynamically studied [15]. TCs must pass through a highly selective and tight barrier, the blood-brain barrier (BBB), to form brain metastases. In the brain, TCs are "protected" by the BBB, as most effective anticancer drugs do not cross the BBB or are pumped out of endothelial cells (ECs) by active efflux transporters [16]. This review focuses on miRs differentially expressed in exosomes of breast cancer patients, and describes miRs that have been shown to affect the BBB. In this context, *in vitro* BBB models have become a powerful tool to study the molecular mechanisms involved in CNS disorders. We summarize recent developments in the modeling of the BBB, including a promising advancement in the use of human cell-based models.

2. BREAST CANCER

2.1. Molecular Characteristics

The common classification of the subtypes of breast cancer in the St. Gallen Classification divides it into four types. The classification is based on the analysis of biological markers in the primary tumor including estrogen receptor (ER), progesterone receptor

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(PR), human epidermal growth factor receptor 2 (HER2/neu) and proliferation marker Ki67, together with age, tumor size, histological grade and lymph node engagement [17, 18]. Four-type-classification divides breast cancer into luminal A (ER + and/or PR+, Ki67 low and HER2/neu-), luminal B (ER + and/or PR+, Ki67 high and/or HER2/neu+), HER2/neu-positive (ER+/-, PR+/- and HER2/neu+) and triple-negative (ER-, PR-and HER2/neu-). This classification gives a prediction of disease features, recurrence pattern and disease-free survival.

2.2. Current Therapies of Primary Breast Cancer

The different oncological societies worldwide regularly renew the recommended gold standard of care after the newest published state-of-art and clinical trials. There are active discussions about the best and valid treatments of breast cancer subtypes [19]. The treatment of breast cancer usually includes surgery, mostly radiation, and subtypes of higher risk systemic chemotherapy (adjuvant, neo-adjuvant, or even both) or anti-hormone therapy with subtypes of endocrine origin [20]. The main portion (approximately 75%) of the breast cancer types belong to estrogen receptor (ER) positive breast cancer and are classified into the luminal A and luminal B subtypes. The division into one or the other luminal-like group has major consequences for the treatment of the patient. The luminal A subtype is usually characterized by a favorable prognosis compared to the luminal B subtype. Systemic therapy is therefore limited to endocrine therapy for at least 5 years after the initial treatment (one or another kind of surgery and eventually radiation). In contrast, the luminal B subtype is distinguished by a high proliferation rate and/or a high histological grade, therefore it is suggested to treat those patients after surgery and mostly radiation with systemic chemotherapy followed by endocrine therapy for at least 5 years [21]. Endocrine therapy differs between the premenopausal and postmenopausal patients. For premenopausal patients, tamoxifen is recommended for about 5-10 years after the treatment, whereas postmenopausal patients should be treated with aromatase inhibitors [22]. Luminal B, HER2/neu and triple-negative tumor patients are recommended to get systemic chemotherapy. The regimes differ in the kind of applied chemotherapy or antibodies and the time when it is applied (adjuvant or neo-adjuvant) and in which dosing [22]. There is a wide range of different regimes. In general, it is recommended to treat luminal B tumors (tumors ER + with high risk) after surgery and mostly radiation with adjuvant chemotherapy based on anthracycline and taxane or a dose-intensified therapy [20]. The HER2/neu positive subtype of breast cancer is therefore mostly treated with a neo-adjuvant chemotherapy, which includes at least one systemic chemotherapy compound and either one anti-HER2/neu- antibody or even two (subtype with higher risk). After neo-adjuvant treatment and surgery plus radiation, the antibody (one or two) is given adjuvant for one year. Triple-negative breast cancers (abbreviated TNBC) are those cancer subtypes, which do not express ER, PR or HER2/neu [23]. TNBC is a molecularly very heterogeneous cancer, which is considered to be hard to treat, because of its aggressive characteristics. The risk of a secondary tumor spread and metastasis is higher in comparison to the other subtypes. The treatment in the curative situation of surgery and radiation plus systemic chemotherapy (neo-adjuvant or adjuvant treatment) with anthracyclines and/or taxanes are used in general as the first-line therapy [24]. Because of its heterogeneity and aggressiveness, major efforts were made in the past to subclassify the TNBC and find a targeted therapy [25].

2.3. Current Therapies of Metastatic Breast Cancer

The recurrence of TCs not locoregional but in other organs characterizes a systemic tumor disease. The consequence for patients is a life-long therapy of one or other kind. One of the first organs to suffer from a metastatic disease of breast cancer is the bones. A bone stabilization therapy with bisphosphonates or RANKL-antibody is recommended. There are different subtypes of therapy which are either applied intravenously, subcutaneously or orally. These have a bone stabilization effect, as they inhibit bones resorbing osteoclasts. Bone metastases with fracture risk, functional impairment, bone pain or neuropathic bone pain should be treated by radiotherapy [26]. Other organs, which are often affected by metastatic spread, are distant nodal metastasis, liver, bone, brain and lungs [27]. The metastatic breast cancer is again subdivided into the classification of luminal, HER2/neu and TNBC. Therefore it is necessary to regain a new histological sample, as there is always the possibility of a receptor switch. Afterwards, the decision of systemic therapy has to be made. The treatment of metastatic breast cancer is discussed intensively and regularly by the different oncological societies worldwide. New specific substances and medications regularly capture the market of oncological therapy [28]. Metastatic luminal-like tumors are treated with endocrine therapy or extended endocrine therapy (CDK4/6 inhibitors). At the state of a so-called “visceral crisis”, a systemic therapy with taxane, but also VEGF- antibodies is given. The so-calledd second or further chemotherapy lines include substances like anthracylines or microtubule-inhibitor. There is the possibility of mono- or poly-chemotherapy regime. It is always necessary to consider the pre-treated adjuvant therapies, the side-effects and conditions for choosing a therapy regime [24]. Patients who suffer from a breast cancer early onset (BRCA) gene mutation and a TNBC metastatic breast cancer can achieve an oral treatment with poly(ADP-ribose)-polymerase (PARP)-Inhibitor. Metastatic HER2/neu breast cancers are treated with two HER2/neu- antibody compounds and a systemic chemo-compound of the taxane group. After reducing tumor progression, the antibodies should be further applied until the progression of the disease. For the second-line therapy and further therapy lines, trastuzumab-emtansin (T-DM1) is often used, a combined chemotherapeutic molecule of HER2/neu-antibody and a systemic chemotherapy compound, capecitabine, a prodrug of 5-fluorouracil or tyrosin-kinase inhibitor (Lapatinib). It is very important to consider the pretreated adjuvant therapies and the side-effects of the therapy. The metastatic TNBC (mTNBC) tumor is a very challenging kind of cancer. Next to very innovative new therapies of phase-1 study trials, systemic chemotherapy compounds in mono- or poly-therapy regimes, there is a new way of medication with immune-checkpoint inhibitors given next to systemic chemotherapeutic compound [29]. Brain metastasis is associated with a poor prognosis, as treatment options are limited. Options of treatment involve multimodality approaches including surgery, radiotherapy, radiosurgery and sometimes systemic therapy [30]. Overall, upcoming therapies, especially targeted therapies and/or immune modulation, must show whether they suppress further metastatic progress, thus extending the overall survival of the patient.

3. CIRCULATING EXOSOMES AND MICRORNAS IN BREAST CANCER

3.1. Exosomes in Breast Cancer

Exosomes are nanovesicles, which are secreted and produced by almost all cell types. The approximate size is 30 to 100 nm in diameter and they play a role in the endosomal pathway by paracrine and autocrine cell communication [31]. Exosomes are loaded with a lot of different charges like proteins, lipids, mRNAs, miRs, long-noncoding RNA and DNA [9, 32]. Analyzing the content of exosomes might further affect the study of diseases and play a role in the understanding of those related to cancer research [33]. Exosomes are playing a role in tumor escape and also cancer immune surveillance through communication between immune cells and cancer cells [34]. Next to that, exosomes play a role in the signaling cascade from cancer cells to other cancer cells in order to induce cell growth, transformation, and survival signals. This could be shown through the use of exosomes from patients and breast cancer cell lines that induced transformation and tumor formation in non-tumorigenic mammary cells [35]. Exosomes travel in order to

fulfil their metastatic spread to healthy organic sites to prime the environment as future metastatic niche [36]. They interfere in the glucose metabolism of normal healthy cells in order to influence the precancerous environment [36]. Exosomes can fuse preferentially with resident cells at their predicted destination using specific integrins at their surface. Integrins in tumor exosomes differ and determine their organotropic metastasis and could be used to predict organ-specific metastasis. In addition, exosomes can activate specific signaling pathways in resident cells in order to establish a favorable microenvironment that promotes the growth of disseminated TCs [37]. Riches *et al.* could show in an *in vitro* model, that a breast cancer cell line secretes higher amounts of exosomes than non-tumorous cell lines and that exosomes from normal mammary epithelial cells also inhibit exosome secretion by breast cancer cells in a tissue specific manner [38]. Exosomes derived from highly metastatic breast cancer can transfer increased metastatic capacity to a poorly metastatic tumor [39].

Exosomes can cross the BBB and influence signaling in TCs as well as in brain microvascular ECs of brain vessels. Circulating cancer cells can traverse the BBB and colonialize the brain [40]. Brain metastatic cells express protocadherin 7 (PCDH7), which promotes the assembly of carcinoma–astrocyte gap junctions allowing for passage of cGAMP from cancer cells to astrocytes [41]. This activates signaling pathways in astrocytes leading to a production of inflammatory cytokines that support growth and chemoresistance in brain-metastatic cells [41]. Also brain microvascular ECs express multiple protocadherin genes, which might be involved in the interaction of ECs with TCs [42]. The mechanism of breaching the BBB by exosomes is mainly unknown. Morad *et al*. postulated that exosomes breach the intact BBB by transcytosis [43]. Exosomes circumvent the low physiological rate of transcytosis in the BBB by decreasing the expression of the endosomal GTPase Rab7 that controls endosomal trafficking [43]. Other authors published that exosomes can cross the BBB only under inflamed conditions but not under normal conditions [44]. Exosomes promote cancer cell colonization in brain metastasis by upregulation of pro-inflammatory cytokines, which promote brain vascular remodeling [45]. Circulating exosomes carry miRs with potential regulatory functions at the BBB. For example, cancer-derived EVs containing miR-181c promote the destruction of the BBB through the abnormal localization of actin. This is achieved by downregulating the miR-181c target gene PDPK1 and then downregulating phosphorylated cofilin, which leads to a modulation of actin dynamics induced by cofilin [46]. A number of different miRs have been implicated to directly regulate targets in brain ECs. Expression of miR-155 was strongly upregulated by inflammatory cytokines in brain microvascular ECs and led to an increase in permeability. Genes involved in cell contact organization such as claudin-1 (CLDN1), annexin-2 (ANXA-2), DOCK-1 and syntenin-1 have been identified as direct targets of miR-155 in brain microvascular ECs [47]. Similarly, in addition to CLDN1, miR-212/132 also targets other endothelial junction complex genes, such as junctional adhesion molecule 3 (JAM3) and tight junction-associated protein 1 (TJAP1) leading to increased endothelial permeability [48]. MiR-150 directly targets angiopoietin receptor Tie-2 at BBB and its overexpression leads to increased endothelial permeability, while its inhibition contributes to BBB protection [49]. BBB-stabilizing effects also inhibit miR-143, which targets p53 upregulated modulator of apoptosis (PUMA) [50]. Overexpression of miR-210 results in increased endothelial permeability by downregulation of miR-210 targets within the junctional complex, occludin (OCLN) and β-catenin (CTNB1) [51]. MiR-34a regulates BBB by targeting several mitochondria-associated genes such as cytochrome c [52]. All miRs that can target genes from brain ECs can be used by TCs to cross the BBB. However, more studies on tumor and endothelial miRs are required to use miRs profiles as prognostic markers.

3.2. MicroRNAs in Breast Cancer as Potential Biomarkers

MiRs are single-stranded short (about 19 to 25 nucleotides) RNA molecules, negatively regulating gene expression. They bind to the 3'-untranslated region (3'-UTR) of their specific target mRNA and repress the initiation of translation or destabilize the target mRNA leading to its degradation [53].

Breast cancer is a heterogeneous disease in which each patients' tumor has specific and different genetic characteristics [54, 55]. Those already known characteristics nowadays play a main role in the treatment, prognosis, and handling of the disease. In spite of all the existing knowledge and treatments, breast cancer becomes a life-threatening disease when cancer spreads from the origin breast tissue to other places and organs of the body (metastasis) [56]. At primary diagnosis, specific molecular characteristics are used to identify if the cancer has already spread and transformed, therefore in a systemic, mostly incurable disease. This raises the question, whether in addition to already established diagnostics and tumor characteristics (mammography, mammary Magnetic Resonance Imaging, tumor marker CA 15-3 / CEA, histological receptors and growth factors, gene analysis), if there are other markers for predicting the aggressiveness and metastasis potential of the tumor. MiRs might be a new and relevant tool to find a better way of prognosis prediction and understanding of metastasis development. In the following, we review the up to date published knowledge of miR peculiarities in different stages of breast cancer expansion.

3.2.1. Primary Tumors

Different studies show that various breast cancer subtypes present different kinds of miR expression [57-60]. Van Schooneveld and colleagues described various subtype-specific miRs in a study and meta-analyses. The miR-let-7c, miR-10a and let-7f were found to be associated with the luminal A type; miR-18a, miR-135b, miR-93 and miR-155 were shown to be associated with the basal type; miR-142-3p and miR-150 were associated with the HER2/neu type [58, 60]. Lowery and colleagues postulated in their study that with miR signatures ER, PR and HER2/neu, receptor status is predictable. Especially miR-342 should have a high expression in the ER-positive/HER2-positive tumors [3]. In *in vitro* cell culture investigations, Fkih M'hamed and colleagues showed that miR-10b, miR-26a, miR-146a and miR-153 could be possible TNBC biomarkers in the future [61]. In a very detailed overall systematic review, Adhami and colleagues postulated that especially two miRs (miR-21 and miR-210) were upregulated consistently. MiR-21 was upregulated in six profiling studies. In contrast, six miRs (miR-145, miR-139-5p, miR-195, miR-99a, miR-497 and miR-205) were downregulated consistently in at least three studies of the eleven regarded [62]. In a small clinical study, the authors used miR as a complementary tool in the diagnosis and prediction of treatment response under neoadjuvant chemotherapy and showed that especially before neoadjuvant therapy, exosomal miR-21 and miR-105 expression levels were higher in metastatic *versus* non-metastatic patients and healthy probands [63]. It could also be shown that higher levels of exosomal miR-21, miR-222, and miR-155 were significantly associated with the presence of circulating TCs [63]. There are also reports showing that a downregulation of miR-221/222 corresponds to an enhancement of tamoxifen sensitivity in TCs [64, 65].

3.2.2. Bone Metastatic Tumors

In advanced stages of breast cancer, patients suffer often from bone metastases, which also describe the incurability of the disease [66, 67]. Bone-only disease with bone as a single metastatic site has a better prognosis than those with visceral or both bone and visceral disease [68]. In the recent years, miR has become a subject of investigations analyzing the role of miR in the development of bone metastasis [69-72]. In *in vitro* studies, Cai *et al.* postulate that in mice models, especially miR-124 inhibits bone metastasis of breast

cancer by repressing interleukin-11 [73]. MiR-124 is described as a tumor suppressor in the development of bone metastasis [74, 75]. Next to miR-124, also miR-214 is strongly increased in the bone specimen of breast cancer patients with osteolytic bone metastases [76, 77]. In another study, five types of miRs (miR-33a, miR-133a, miR-141, miR-190, and miR-219) were shown to regulate tumor-induced osteoclast differentiation [78]. MiR-135 and miR-203 interact with the protein RUNX2, which plays a role in normal bone formation but is often dysregulated in bone-metastatic breast cancer cells [79, 80]. In another clinical trial, Zhao and colleagues showed that especially miR10b shows an overexpression in contrast to patients with no bone metastasis. They postulate therefore that miR-10b could be a useful biomarker in the future [81].

3.2.3. Visceral Metastatic Tumors

The dispersal of TCs in organs further manifests the systemic character of the disease. Also here, miR-10b is discussed as a potential marker to play a major role in the spread of metastasis, as it promotes metastasis in otherwise non-metastatic breast cancer cells [82]. This was shown by Ma *et al.* in *in vitro* models of metastatic breast cancer. He and his colleagues also postulate that the level of miR-10b expression in primary breast carcinomas correlates with the clinical progression of the disease. Ell and colleagues showed in *in vitro* models that the miR-23b/27b/24 cluster promotes breast cancer lung metastasis by targeting metastasis-suppressive gene prosaposin [83]. Huang *et al.* postulated that miR-373 and miR-520c are metastasis-promoting miRs, which are involved in tumor migration and invasion [84]. The infiltration of lymph nodes is often the first sign of metastatic spread. Chen and his colleagues investigated nodal positive patients and found a signature of four miRs, consisting of miR-191-5p, miR-214-3p, miR-451a, and miR-489, which seem to play a role in cell proliferation, migration, and invasion abilities [85]. MiR-21 is also discussed to be associated with advanced clinical stage, lymph node metastasis and patient poor prognosis [86].

3.2.4. Cerebral Metastatic Tumors

At least 10-30% of the breast cancer patients develop brain metastases, which is associated with a very poor prognosis with a one-year survival of 20% [87, 88]. Therefore, the need for specific biomarkers like miRs to identify potentially metastatic tumors in the early stage of the disease is very high, especially for high-risk patients such as HER2/neu positive and triple-negative patients. As a result of improved therapy opportunities, the development of brain metastases has become the most limiting factor in relation to time and quality [89]. During the formation of cerebral metastasis, there is an exceptional situation where single circulating tumor cells have to pass the BBB. There are many recent studies on up- or downregulated miRs in the metastatic tissues because of the importance of early detection and development of new therapies for cerebral metastasis. Analysis of a data-set with carcinoma patients and patients with cerebral metastasis showed that miR-17-5p and miR-16-5p have the highest association with targeted mRNAs (such as B-cell lymphoma 2 (BLC-2), SMAD3 and SDOSCS1) and regulate processes of metastatic progression [88]. Moreover, there was a negative correlation between miR-17-5p and the total observed viability of patients [88]. Heparanase (HPSE), which is considered to be an important enzyme in tumor-formation and metastatic progression, is overexpressed in brain metastatic tissue. It was shown that miR-1258 inhibits HPSE and therefore, a low amount of it correlates with highly aggressive brain metastatic breast cancer [90].

Furthermore, in a report about the miR profile of cancer stem cells, which are considered to be a key player in metastatic tumors, a significantly lower level of miR-7 was shown. MiR-7 modulates the activity of KLF4, which is an important stem-cell gene. In addition to that, a high level of miR-7 inhibits the development of brain metastasis in animal models [91]. Plasma level of specific miRs could be a predictive biomarker of chemotherapy resistance in metastatic breast cancer. As shown by Shao *et al.,* plasma levels of miR-200a and miR-210 showed high diagnostic accuracy for distinguishing chemotherapy-sensitive from chemotherapy-resistant patients [92]. MiR-20b showed increased expression in brain metastases of breast cancer patients, compared to primary breast tumors and patients without brain metastasis [93]. More research is needed to identify more potential biomarkers of brain metastatic breast cancer as well as therapeutic targets.

4. BLOOD-BRAIN-BARRIER

4.1. BBB Characteristics

The BBB is a physical and metabolic barrier formed by specialized brain microvascular ECs, together with other components of the neurovascular unit (NVU) such as pericytes, astrocytes, microglia, neurons and extracellular matrix (ECM). Brain microvascular ECs express high levels of tight junction (TJ) proteins, efflux and influx transporters that selectively regulate the movement of molecules through the BBB [94]. TCs interact with brain ECs affecting them in different ways [95, 96]. For *example*, breast cancer cells expressing low levels of claudin-3, -4 and -7 metastasize with a high probability to the brain [97]. Other molecules, such as heparin-binding EGF-like growth factor (HB-EGF) and cyclooxygenase 2 highly expressed by TCs facilitate brain metastasis process [40]. The key players of metastasis forming can be identified at a molecular level in animal models or *in vitro* by using isolated cells of NVU.

4.2. *In vitro* Models for Studying Mechanisms of Breast Cancer Metastasis

During the last two decades, many *in vitro* BBB models have been developed due to the increasing need to facilitate cerebrovascular research and drug development [98]. However, none of these cell culture models fully reflect the complexity of the BBB structure and its dynamics due to the limitations of an *in vitro* system [99]. Therefore, data acquired in studies where such models have been applied might be considered with respect to the chosen model. The most important cell type of all BBB *in vitro* models is brain microvascular ECs building a highly selective monolayer. Besides brain microvascular ECs, other cell types associated or interacting with the BBB can be involved in such models. The advantage of co-cultures comprising two or three different cell types allows breaking down the complexity of the BBB into the cell types of interest and enables the analysis of cellular processes with regard to a limited number of parameters. Here, we outline the most widely used *in vitro* BBB models with a special focus on opportunities to study the interaction of brain microvascular ECs with TCs.

BBB *in vitro* models can be subdivided into two categories, static and dynamic, as schematically depicted in Fig. (**1A** & **B**).

4.2.1. Static In Vitro Blood-Brain Barrier Models

Depending on the number of cell types involved, static BBB models can be subcategorized into monocultures or co-cultures. Moreover, there are primary cell cultures and immortalized cell lines used to study the BBB. Besides rodents, which are still the most widely used animals in the BBB research, brain ECs have been isolated from larger animals, such as bovine, porcine and non-human primate in order to increase the EC yield [100-104]. However, problem appearing with the use of brain microvascular ECs derived from larger animals is that there are no or only a few respective antibodies available for these species and further, there are no transgenic animals available.

There are some important requirements that generally applicable BBB *in vitro* models have to incorporate [105, 106]. The polarized localization of transporters, receptors and enzymes is necessarily required in brain ECs used for BBB studies, but the suitable

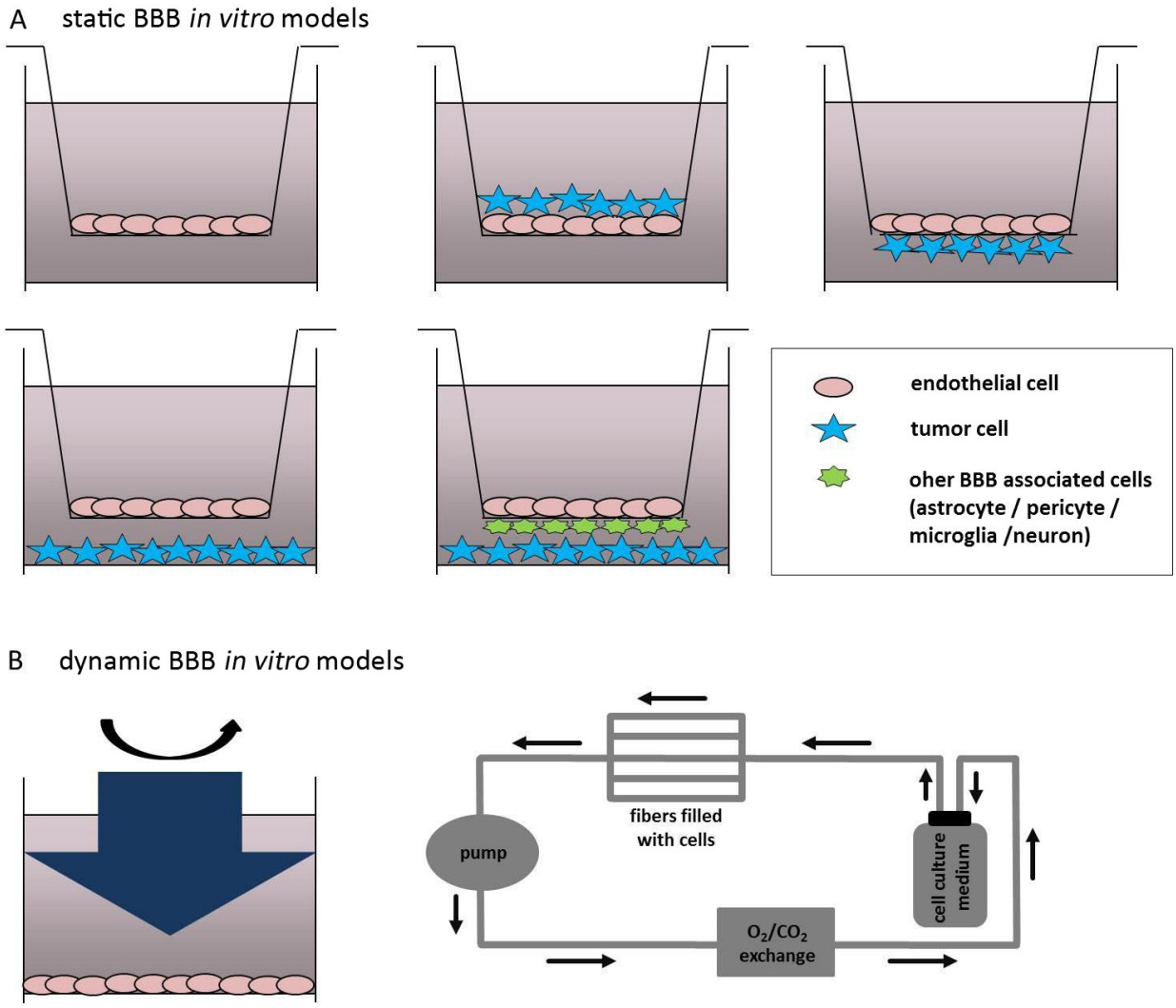

Fig. (1). *In vitro* models to study the blood-brain barrier-tumor cell interactions
(**A**) Static Blood-Brain Barrier (BBB) *in vitro* models involving a single monoculture of brain microvascular ECs in a transwell insert (upper left transwell insert); a co-culture system of brain endothelial cells (ECs) grown in the presence of tumor cells (TCs) at the luminal side of the insert; co-culture of brain ECs on the luminal indirectly contacting TCs on the abluminal side of the transwell insert (upper right transwell insert); brain ECs grown in the upper compartment of the transwell insert and TCs seeded on the bottom of the cell culture vessel in which the insert is placed (lower left transwell insert); luminally grown cerebrovascular ECs indirectly contacting other BBB associated cell types (astrocytes, pericytes, neurons or microglia cells) and TCs seeded on the bottom of the cell culture vessel. (**B**) Dynamic BBB *in vitro* models represented by the cone-plate apparatus generating shear stress to brain ECs (left picture) and by the microporous hollow fibers connected with a speed pump generating shear stress and with a gas-permeable tubing system allowing O_2/CO_2 exchange of the system (right picture).

expression of BBB-specific tight junction (TJ) proteins responsible for the characteristically high TEER of brain ECs displays a serious problem in the development of suitable BBB *in vitro* systems [105, 107-109]. Primarily, low passage brain microvascular ECs were identified to display a differentiated phenotype and physiological and biochemical properties, that are characteristic for the BBB in vivo [102, 110]. Nevertheless, there are some disadvantages of the usage of primary brain ECs, which have to be mastered. First, isolation and cultivation of primary brain ECs cultures are limited due to the cell number within the brain. Brain microvascular ECs account only for 0.1% (v/v) of the brain, meaning that a large number of animals might be necessary to generate enough cells for the respective experiment. Moreover, there is a high probability to contaminate the brain microvascular EC culture with other cell types. A temporary application of puromycin into the cell culture medium has been reported to improve the purity of primary brain ECs [111].

In order to solve the problem of yield and purity, the immortalized brain microvascular EC cell cultures were developed, which however, often resulted in a more de-differentiated phenotype [98]. Moreover, not all immortalized systems achieve the high TEER of brain microvascular ECs in vivo due to the minor expression of TJ proteins [112]. However, some immortalized BBB systems reflect BBB properties known from primary brain EC cultures, and because of the lower effort in cultivation, they are widely used in the research of cerebrovascular diseases [113-118].

A transwell insert mimicking the blood (luminal) and the well in which the insert is placed, simulating the parenchymal (abluminal) side of a vessel can be used as a simple BBB *in vitro* system (Fig. **1A**). The pore size of the transwell insert should be 0.4 µm in order to allow the exchange of small molecules and growth factors secreted by the cells. Furthermore, the relatively small pore size functions as a migration barrier for the cells from one compartment to the other. The application of transwell inserts allows the co-

cultivation of two or more different cell types together with brain microvascular ECs in one system, thereby allowing a more realistic simulation of the BBB [119-121]. Besides the interaction of brain ECs with other types of the NVU, which has in part been described in this section, transwell insert can be used for co-culturing ECs in the presence of tumor-secreted factors or brain TCs/TCs isolated from brain metastases, which allows the interaction between both cell types and investigation of the signaling pathways. As depicted in Fig. **1A**, TCs can be grown on the top of the brain EC culture at the luminal side of the transwell. This co-culture system might be used in order to study the interaction of TCs with ECs during the period prior to extravasation from the bloodstream into the CNS. The disadvantage of this culture is that there is no possibility to separate the cultures from each other. Therefore gene or protein expression analysis cannot be performed. However, electrophysiological TEER measurements are still feasible applying a respective control culture of brain ECs grown alone on the transwell insert. Comparing with that model, the interaction of TCs with the cerebrovascular endothelium on molecular level would be possible by seeding ECs luminally and TCs abluminally, directly on the transwell (Fig. **1A**). The advantage of these models is that either electrophysiological measurements or gene and protein expression analyses of each culture are feasible. This co-culture model might mimic the co-option phase of TCs with brain ECs after invasion of the brain parenchyma, since the TCs are in indirect contact with the abluminal side of the EC culture. A report where this kind of co-culture system of immortalized human brain ECs (hCMEC/D3 cells) with human medulloblastoma cell line (VC312R cells) has been applied to study cellular interactions between EC and TCs revealed an impact of TCs on permeability and transport properties of brain ECs [122]. Similarly to this model, TCs might also be seeded at the bottom of the cell culture vessel, as it is demonstrated in the left lower transwell Fig. **1A**. This technique is even easily applicable when compared to the co-culture system described above. Following this co-culture system, the impact of mouse glioma cell line GL261 on the expression of relevant genes involved in Hedgehog pathway, as well as on the proliferation and migration of the brain EC (b.END3 cell line) has been tested [123]. Finally, a co-culture model is prepared with luminally grown brain ECs, other BBB associated cells, *i.e.* astrocytes or pericytes seeded at the abluminal side of the transwell insert, and TCs seeded on the bottom of the cell culture vessel where the transwell insert is placed. This co-culture system might allow analyzing a relatively physiological BBB *in vitro* model that interacts with TCs. All cell types involved in this model can be analyzed exclusively on a molecular level. A recent study of Anfuso and colleagues established a similar triple-culture model applying primary ECs with abluminal cultured pericytes and C6 glioma cells grown on the bottom of the culture plate [124]. As controls, brain ECs were also cultured with either C6 cells or pericytes alone (double co-cultures). Following this technique, the authors of the study investigated the role of pericytes in the interaction with brain microvascular ECs and glioma cells, the influence of TCs in the presence of pericytes on EC permeability, TJ expression, and prostaglandin production. Moreover, pericytes used in this culture system exhibited an important modulating role in the initial stages of angiogenesis driven by brain TCs [124]. Brain microvascular ECs in co-cultures with TCs and pericytes or astrocytes are characterized by a different permeability and are therefore ideal for cell-cell interaction through growth factors released by the cell types that are involved in the system and have an impact on the identification and optimization of drugs. The most significant disadvantage of these co-culture models is the lack of shear stress, being critical for the induction and maintenance of the BBB phenotype [125, 126].

4.2.2. Dynamic In Vitro Blood-Brain Barrier Models

A further parameter reflecting the BBB under physiological conditions and having an important impact on brain microvascular EC properties is the presence of shear stress. Shear stress affects the expression of transporters and cellular contacts directly influencing the brain microvascular EC monolayer permeability [127]. There are different kinds of dynamic BBB models *in vitro*, including cone-plates and microfluidic *in vitro* models. Cone-plates transmit shear forces on ECs through rotating inside of the cell culture medium (Fig. **1B**). The limiting factor in this dynamic model is that besides brain microvascular ECs, no other cell types can be applied to the experiment, therefore diminishing the significance of data it generates and the application for brain metastasis research *in vitro*.

To allow the application of shear stress and incorporate two different cell types, brain microvascular ECs and TCs (or other BBB-associated cell types), the microporous hollow fibers are used [128-131]. In this system, brain microvascular ECs are grown in the luminal, the inner porous of the hollow fiber, whereas the TCs are seeded on the abluminal, the outer side of the fiber. A variable-speed pump pumps the culture medium into this culture system and generates shear stress that can vary from 5-23 dynes/cm^2 [132, 133]. Further, a gas-permeable tubing system maintains a stable microenvironment by exchanging O_2 and CO_2. Cucullo and colleagues used human aortic ECs and the C6 glioma cell line in order to determine the impact of TCs on ECs in the presence of shear stress [134].

4.2.3 Human Stem-cell Based In Vitro BBB Models

The generation of human brain capillary-like EC cultures from pluripotent stem cells constitutes the new approach allowing purification of a large number of cells of interest as well as characterization of BBB function in the direct context of different pathologies including brain tumors or metastasis compared to normal individuals. So far, there are varying protocols for isolation of brain capillary-like ECs from induced pluripotent stem cells (iPSCs) that relied on non-defined (*i.e.* containing serum) [135-139] or chemically defined media without prior purification of endothelial progenitor cells (EPCs) [140, 141]. These methodologies focus on selective isolation of brain capillary-like ECs from a heterogeneous cell mixture containing neural progenitor cells using selective media and ECM components. Due to the heterogeneity of the cell suspensions, serum or ECM mixtures, specification of iPSCs into brain capillary-like ECs and the cost efficiency remained unsatisfactory and insufficient. Moreover, the yield and the phenotypic features of the brain capillary-like ECs were strongly dependent on the original iPSC line applied [135]. In contrast, chemically defined media allow better reproducibility, control of the basic experimental conditions and the framework of the cell purification but remain cost-intensive.

Qian *et al.* described the derivation of brain capillary-like ECs using an intermediary EPC population and chemically defined media [142]. The differentiation of iPSCs into EPCs was defined by the expression of VEGFR2 and CD31. The specification of EPCs into brain capillary-like ECs was assured by retinoic acid. The influencing role of other soluble or non-soluble signaling and small molecules, and growth factors involved in the development of specific BBB characteristics was however neglected.

A recent work of Praça *et al.* described a complex multifactorial experimental setup to derive brain capillary-like ECs followed by a molecular and functional characterization of differential BBB properties [143]. The initial isolation was performed by differentiating brain capillary-like ECs from an intermediary EPC population followed by modulation of three different signaling pathways: VEGF, retinoic acid and WNT and applying chemically defined endothelial cell medium. Compared with purification protocols shown by others, Praça and colleagues described a method to isolate brain capillary-like ECs with relatively high TEER. Moreover, co-culture experiments with pericytes showed a substantial maturation of the brain capillary-like ECs through an increase of TEER values and

monolayer organization by improving the expression of cell-cell contact molecules.

As experiments of Praça, Lippmann and Katt have demonstrated, the control of the Wnt3a, VEGF and retinoic acid signaling pathways plays a decisive role in the differentiation of EPCs into brain capillary-like ECs and their maturation. Moreover, a defined ECM is important to provide physical stabilization and provides molecules essential to develop BBB characteristics. Platforms using channels structured in thick three-dimensional hydrogels could provide a useful tool for multicellular approaches *in vitro* by mimicking tissue structures that play a role in differentiating EPCs [144]. On one hand, defined ECM can be achieved by chemically applied substrates or by cultivating brain capillary-like ECs with other types of cells building the NVU. The decellularized ECM components are derived from different animal sources. The impact of cell-free ECMs of human origin should be investigated in future studies. In this context, iPSCs also play an important role. Patient-derived brain capillary-like ECs differentiated from iPSCs of breast cancer patients versus healthy individuals would be a useful tool for studying cellular mechanisms of metastasis forming.

Another useful human brain capillary-like ECs model has been established from human cord blood-derived hematopoietic stem cells [145-147]. The cells were first differentiated into ECs, then the BBB properties were induced by co-culture with pericytes. Such a model appeared to be very reproducible and easy to establish.

In summary, BBB *in vitro* models play an important role in data collection regarding the BBB function under physiological and pathological conditions. In addition, the use of *in vitro* models allows the study of drugs used in various neurological disorders, including brain tumors. Knowing the advantages and disadvantages of the BBB models presented here, the appropriate cell culture system relevant to the study must be selected and the generated data might be adequately interpreted.

CONCLUSION AND FUTURE PERSPECTIVES

Treatment for brain metastasis is the most challenging. Finding novel therapies to prevent the metastatic progression of breast cancer and treat brain metastases require a better understanding of the biology and molecular mechanisms behind this process. The *in vitro* models, especially those based on cells from human origin, may contribute to gaining more knowledge and finding novel biomarkers of metastatic brain disease.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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