The Role of Breast Cancer-Derived Extracellular Vesicles in Brain Metastasis

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Accessibility
The role of breast cancer-derived extracellular vesicles in brain metastasis

A dissertation presented

by

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to

The Department of Biological Sciences in Dental Medicine

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The role of breast cancer-derived extracellular vesicles in brain metastasis

Abstract

Breast cancer brain metastasis is a major clinical challenge that is often associated with an extremely poor prognosis. Current diagnostics lack the sensitivity to detect early stages of brain metastasis and available therapeutics have failed to improve the outcome of this disease. Elucidating the mechanisms underlying the early stages of brain metastasis development is, therefore, of high significance in informing the development of efficient diagnostics and therapeutics for breast cancer brain metastasis.

Tumor-derived extracellular vesicles (EVs) are known as intercellular communicators that can transfer a variety of proteins and genetic materials between cells within the tumor microenvironment and in distant pre-metastatic organs and promote tumor progression and metastasis. However, the current understanding of the role of EVs in breast cancer brain metastasis is limited. In my dissertation research, I investigated the role of breast cancer-derived EVs in the early stages of brain metastasis development. Comparing the EVs derived from a parental breast cancer cell line (P-EVs) and a brain-seeking variant of these cells (Br-EVs), I demonstrate that Br-EVs, but not P-EVs, promote brain metastasis incidence and growth in a mouse model of brain metastasis. I further demonstrate that Br-EVs have the ability to breach an intact blood brain barrier (BBB) and using state-of-the-art models of the BBB, we identify transcytosis as the mechanism underlying this transport. Moreover, I show that Br-EVs have the
ability to circumvent the low rate of transcytosis at the blood brain barrier, through down-regulating the expression of rab7 in brain endothelial cells.

Next, I demonstrate that following their transcytosis, Br-EVs are predominantly internalized by astrocytes. Astrocytes exhibit a preference to uptake Br-EVs when compared to P-EVs and this uptake occurs through a noncanonical clathrin-independent carriers/GPI-AP enriched compartments (CLIC/GEEC) pathway. Through quantitative proteomics and validation studies, we demonstrate that Br-EVs are highly enriched in GPI-interacting proteins which can drive their uptake by astrocytes. Next, through a series of *in vitro* and *in vivo* studies, I demonstrate that Br-EVs, but not P-EVs, decrease the expression of TIMP-2 in astrocytes. TIMP-2 is an endogenous inhibitor of matrix metalloproteinases and its down-regulation can be associated with the increased activity of these matrix-modulating enzymes, leading to extracellular matrix remodeling, a major component of pre-metastasis niche preparation. I further demonstrate that this down-regulation of TIMP-2 is driven by miR-301a-3p, transferred by Br-EVs to astrocytes.

Taken together, these studies identify, for the first time, the mechanisms by which breast cancer-derived EVs breach an intact blood brain barrier and elucidate some of the functional consequences associated with EV entry into the brain. These studies also identify novel targets that can guide the development of early diagnostics and efficient therapeutics for breast cancer brain metastasis.
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Chapter 1

Introduction

The contents of this chapter were published, in part, in:
1.1. Brain metastasis

1.1.1. Incidence and prognosis

Brain metastasis is the most common malignancy of the central nervous system. The reported incidence rates of brain metastasis vary largely (9-43%), most likely due to the limited sensitivity of diagnostic approaches for detection of metastatic lesions in the brain. Based on more recent analyses of the Surveillance, Epidemiology, and End Results (SEER) database released in 2016, it is estimated that more than 23,000 cancer patients in the United States will be diagnosed with brain metastasis per annum. Brain metastasis is often identified as a neurological complication associated with lung cancer, melanoma, and breast cancer and less frequently associated with renal, thyroid, head and neck, and colorectal cancer. Overall, among 217,687 cancer patients with de novo metastatic diseases, brain was the first site of metastasis in a total of 12.1% of patients (approximately 28, 25, and 7% in melanoma, lung adenocarcinoma, and breast cancer patients, respectively). Moreover, the incidence of brain metastasis has shown a rising trend, as a result of increased availability of diagnostics and longer overall survival of cancer patients.

This dissertation focuses on brain metastasis from breast cancer. Breast cancer, being the most common type of cancer in women worldwide, also serves as the most common cause of brain metastasis in women. Breast cancer brain metastasis is mostly associated with triple negative (30-47%) and HER2+ (28-34%) breast cancer subtypes. In breast cancer patients, brain metastasis often occurs later during the course of the disease most often subsequent to metastasis to two or more extracranial sites. While the overall survival rate of triple negative and HER2+ breast cancer patients is relatively high (78-94%), the prognosis of breast cancer patients diagnosed with brain metastasis is dismal, with a median survival of 10 months. The rising incidence and the poor prognosis of breast cancer brain metastasis has made this disease
an ongoing major clinical challenge and a subject of growing interest in basic and translational cancer research.

1.1.2. Diagnosis and treatment

The poor prognosis associated with breast cancer brain metastasis is attributed at least in part, to the lack of efficient diagnostics and therapeutics for this disease. The currently accepted methods for screening and detection of brain metastasis rely on imaging techniques. Magnetic Resonance Imaging (MRI) is often the method of choice due to its high sensitivity compared to other method (e.g., computed tomography)\(^1\). However, the current screening guidelines do not support routine assessment of breast cancer patients for development of brain metastasis. As a result, diagnosis of brain metastasis is often delayed until the patient presents with neurological symptoms\(^1\). Given that more than 60% of brain metastases are asymptomatic, the current diagnostic strategies can lead to late detection of breast cancer patients with brain metastasis\(^1\). While several studies have attempted to identify molecular biomarkers for early detection of brain metastasis\(^1\), biomarkers with proven clinical efficacy are yet to be reported.

Resection of lesions via surgery or stereotactic radiosurgery and whole brain radiotherapy of metastatic brain lesions are the most commonly used approaches for management of brain metastasis. Nevertheless, these approaches still do not lead to a significant increase in survival of patients with brain metastasis\(^1\). Penetrating the blood brain barrier (BBB) is a major hurdle in the management of brain metastasis being treated medical approaches\(^1\). Chemotherapy has shown initial response in brain metastasis patients especially in those with primary tumors that are responsive to chemotherapeutic agents. However, no improvement in survival has been observed with these agents. While targeted therapies have demonstrated a better response rate compared to chemotherapy, the majority of these agents still show limited rate of penetrance of
the blood brain barrier \(^{19}\). More recently, immunotherapy approaches have been tested in patients with brain metastasis. Multiple case studies have shown encouraging results in lung cancer, renal cancer and melanoma patients with brain metastases \(^{21-24}\). Initial response from an ongoing clinical trial demonstrated a durable response to immunotherapy in melanoma patients with brain metastasis \(^{25}\). However, randomized clinical trials are still required to determine the benefit of these immunotherapy approaches for patients with metastatic brain cancer.

1.1.3. Conclusion

A large number of patients with common cancers such as lung cancer, breast cancer, and melanoma are affected by brain metastasis throughout the course of their disease. Current diagnostic approaches lack the level of sensitivity required to screen and detect early metastasis development in the brain of cancer patients. Moreover, available approaches for management of brain metastasis have mostly failed to improve the survival rate of these patients. A better understanding of the mechanisms underlying brain metastasis development, particularly the early stages of this process, can provide novel opportunities to develop more efficient diagnostics and therapeutics for brain metastasis.

1.2. Extracellular vesicles

1.2.1. History

Extracellular vesicles (EVs) were initially introduced as vesicles through which cells can discard their components into the extracellular environment \(^{26,27}\). More than a decade after the initial discovery of these vesicles, Raposo and coworkers demonstrated, for the first time, the functional properties of EVs. This study showed that EVs from B lymphocytes contained MHCII and had the ability to activate T cells through antigen presentation \(^{28}\). This study was followed by other breakthrough studies a decade later that demonstrated the ability of EVs to carry and
transfer genetic materials including mRNAs and miRNAs between cells \(^{29,30}\). Since then, the field of EV research has evolved rapidly, leading to several discoveries that highlight the promise of this field to increase the current understanding of different pathologies and for developing novel diagnostics and therapeutics.

1.2.2. Definition and classification

EVs are defined as particles with bilayer membranes that are released from cells into the extracellular environment and that lack the ability to divide \(^{31}\). EVs contain a variety of different proteins and nucleic acids from the cells of origin. Once release into the extracellular environment, these vesicles can transfer their contents to other cells and change the behavior of the recipient cells. As such, EVs are recognized as contributors to intercellular communication. All normal and cancerous cells that have been tested thus far have shown the ability to release EVs. Moreover, EVs have been isolated from a variety of different biofluids including plasma, urine, cerebrospinal fluid, and milk \(^{32}\).

EVs are heterogeneous in nature and include a variety of different subtypes. Several classifications have been suggested to categorize the different subtypes of EVs, the widely accepted classification being based on the origin of EVs (Figure 1.1). EVs originating from the outward budding of the plasma membrane are generally known as microvesicles, microparticles, oncosomes, or large EVs. Microvesicles are generally > 200 nm in size and are enriched in plasma membrane proteins. A larger group of microvesicles, identified as large oncosomes, can be 1-10 μm in size \(^{33}\). These vesicles were originally isolated from prostate cancer cells but more recent studies have demonstrated the ability of other cancer types such as glioblastoma to release these large oncosomes \(^{34}\).
Another major class of EVs originates from the inward budding of the endosomal membrane and is commonly recognized as exosomes or small EVs. Exosomes are mostly within a 50-200 nm size range and are highly enriched in endosomal markers such as CD9, CD63, and Alix.

More recently, using an asymmetric flow field-flow fractionation method, a smaller class of cell-derived particles was identified as exomeres. Exomeres are generally <50 nm in size and due to the lack of a bilayer lipid membrane, they do not fall within the definition of EVs. Nevertheless, these small particles can have significant functions in physiologic and pathologic conditions and are subjects of active investigation.

Figure 1.1. Classification of EVs based on origin. Two major classes of EVs include microvesicles that originate from the plasma membrane and exosomes that originate from the endosomal membrane (Image credit: Kristin Johnston, Vascular Biology Program, Boston Children’s Hospital).
1.2.3. EV cargo

As described previously, EVs contain a variety of different lipids, proteins, and nucleic acids. The composition of EV contents highly depends on the cell type of origin as well as the conditions under which the cells grow. For instance, it has been shown that cells grown under hypoxic conditions can release EVs with a different protein and miRNA profile. Accordingly, it is critical that the cell culture conditions and EV isolation protocols are described in detail to allow for reliable reproduction of the results.

EVs contain a variety of internal and surface proteins. The internal proteins can be a combination of cytosolic proteins such as tubulin and actin and in case of exosomes, can be components of the endosomal sorting complexes required for transport (ESCRT) machinery such as Alix. The surface proteins of EVs have been studied more extensively and include a variety of transmembrane proteins and receptors such as tetraspanins, integrins, MHC molecules. These proteins have been shown to be important for the interaction of EVs with recipient cells and their subsequent function. Different mechanisms of protein sorting have been described for EVs. Post-translational modifications such as ubiquitination determine the interaction of proteins with the machinery involved in the biogenesis of EVs and can play in role in EV protein sorting. For instance, ubiquitin-like 3/membrane-anchored Ub-fold protein was shown to be critical for packaging of a variety of proteins into small EVs. Lipid raft domains also contribute to the protein composition of EVs through supporting the sorting of certain proteins such as MHC molecules into EVs.

Nucleic acids are another major group of cargos in EVs. Both coding and non-coding RNAs have been detected in EVs. mRNAs can be transferred via EVs in complete and fragmented forms and can be translated into proteins. For instance, an early study in the field of EVs
demonstrated for the first time that EV-driven transfer of mRNA from mouse cells to human cells can lead to the production of mouse proteins. Non-coding RNAs packaged into EVs have also been proven to be functional. While the majority of studies on EV non-coding RNAs focus on miRNAs, several studies have also demonstrated the presence and transfer of other types such as long non-coding RNAs, small nuclear RNAs, and piwi-interacting RNAs. More recently, EVs were also shown to contain single stranded and double stranded DNA in a cell type-dependent manner and the amount of internal EV DNA was shown to be higher in larger EVs compared to small EVs. A recent study demonstrated that mitochondrial DNA can also be packaged into exosomes, leading to escape from dormancy in breast cancer cells.

Elucidating the mechanisms underlying the packaging of RNA and DNA into EVs is a matter of active investigation. Certain RNA-binding proteins such as Y-box protein and sumoylated heterogeneous nuclear ribonucleoprotein A2B1 have been shown to play a role in loading of different RNAs into EVs.

Among the different types of EV cargo, lipids have been studied the least. Several studies have attempted to determine the lipidomic profile of EVs derived from different cell types. Whether lipids play active roles in the function of EVs is yet to be determined.

1.2.4. The function of EVs in cancer

Both tumor-derived EVs and EVs released from a variety of non-tumor cells can play major roles in the progression of cancer. This process was first demonstrated in a study showing that glioblastoma cells with an EGFRvIII mutation can transfer the mutant proteins to cells that lacked this mutation via EVs resulting in the induction of oncogenic phenotype. This study and several others show that tumor cells can communicate via EVs to induce an aggressive behavior to other tumor cells.
Tumor-derived EVs can also contribute to tumor progression through affecting the stromal cells in the tumor microenvironment. Tumor-derived EVs can contain a variety of angiogenic proteins such as vascular endothelial growth factor (VEGF), interleukin (IL)-6 and IL-8 as well as angiogenic miRNAs such as miR-210. Transfer of these contents to endothelial cells in the tumor microenvironment can promote an angiogenic phenotype in these cells and facilitate tumor angiogenesis and growth. Tumor-derived EVs can also change the behavior of fibroblasts in the tumor microenvironment to promote a cancer-associated fibroblast (CAF) phenotype. Moreover the interaction(s) of tumor-derived EVs with different immune cells such as lymphocytes, macrophages, and natural killer cells can create an immune-suppressive environment that promotes tumor growth and progression.

Similarly, EVs derived from stromal cells in the tumor microenvironment can also induce an aggressive behavior in the recipient tumor cells. For instance, CAF-derived EVs could change the tumor metabolic profile and also increased the migratory phenotype in tumor cells. Macrophage-derived exosomes containing miR-233 can promote chemoresistance in ovarian cancer. The role of endothelial-derived EVs in this context has been less studied. Our group has demonstrated a significant change in the proteomic profile of EVs derived from normal endothelial cells and VEGF-stimulated endothelial cells, suggesting a role for the EVs derived from tumor-associated endothelial cells in tumor progression (data not shown).

Interestingly, tumor-derived EVs released from the primary tumor can also enter the circulation and travel to distant pre-metastatic organs. The transfer of the contents of these EVs to normal cells in pre-metastatic organs can create an environment that is suitable for future metastasis growth, a concept commonly known as “pre-metastatic niche preparation.” Through a variety of mechanisms, tumor-derived EVs have been shown to induce a pre-metastatic niche
to promote liver metastasis from pancreatic cancer [69] and gastric cancer [70], lung metastasis [1] and bone metastasis from melanoma [71] and prostate cancer [72], as well as metastasis to lymph nodes [73]. The role of tumor-derived EVs in preparing a metastatic niche in the brain has also been studied but to a lesser extent [74-76]. These studies are discussed in depth in the following section.

1.2.5. Conclusions

Extracellular vesicles have emerged as novel mediators of cell communication in physiologic and pathologic conditions. Tumor-derived EVs have the ability to transfer major functional proteins and genetic materials from the primary tumor to other cells in the tumor microenvironment and in distant organs. These nanoscale vesicles therefore, serve as promising candidates to elucidate the mechanisms underlying the different steps of tumor progression and metastasis and to guide the development of novel diagnostic and therapeutic targets.

1.3. The role of EVs in metastatic and primary brain cancer

The survival and growth of cancerous cells in different organs highly depend on the ability of cells to adapt to and exploit the surrounding microenvironment. Cancer cells can develop a symbiotic relationship with different stromal compartments, such as endothelial cells, fibroblasts, and immune cells to reverse tissue homeostasis toward a tumor-supporting microenvironment [77]. This cooperation promotes an invasive phenotype in tumor cells and further supports tumor progression through enhancement of angiogenesis, alteration of the extracellular matrix (ECM), and facilitation of tumor immune evasion [78]. The supporting role of the microenvironment in the brain is defined by specialized stromal cells such as astrocytes, microglia, and neurons, the distinct composition of the ECM, and the presence of a blood-brain-barrier.

Neoplasms in the brain include primary brain malignancies and secondary (metastatic) brain tumors, most commonly originated from lung cancer, breast cancer, and melanoma. In primary
brain tumors, transfer of EVs between tumor cells and stromal cells can facilitate tumor-microenvironment interactions and enhance processes such as immune modulation and angiogenesis. Few studies have evaluated the role of EVs in the progression of metastatic brain tumors. A better understanding of the EV-mediated interactions between metastatic tumor cells and the brain microenvironment can shed light on novel mechanisms driving brain metastasis.

Despite the notable differences between the cells of origin in primary and metastatic brain tumors, these two types of intracranial neoplasms share a microenvironment that while not identical, could be comparable to some extent. Through interactions with the brain microenvironment, metastatic tumor cells develop a number of growth and survival mechanisms, which can be similar to the mechanisms that are characteristically adopted by the transformed brain cells in a primary brain tumor. For instance, neurotrophin 3, a neural growth factor involved in the differentiation of neural progenitor cells during neurogenesis, is highly expressed by primary brain tumors such as glioma to promote the survival and growth of brain tumor-initiating cells. Interestingly, it has been shown that breast cancer cells that metastasize to brain also hijack this mechanism to enhance their growth and survival. This and other comparable mechanisms raise the question as to whether a parallel assessment of the EV literature with respect to tumor-microenvironmental interactions in primary and metastatic brain tumors could inform and enhance our understanding of the potential mechanisms of the EV contribution in brain cancer, in particular in less-studied metastatic brain tumors.

1.3.1. Cellular components of the brain tumor microenvironment

1.3.1.1. Endothelial cells
One of the initial events associated with development of a malignancy is the establishment of a vascular network from the existing vasculature through the process of angiogenesis, which provides oxygen and nutrients critical for tumor cell survival and growth 88. Both primary and metastatic brain tumors depend on angiogenesis and are highly vascularized, making them potential targets for anti-angiogenic therapies, although their clinical efficacy has thus far been limited 89,90. In addition to angiogenesis, mechanisms such as vessel co-option and vascular mimicry also contribute to providing a blood supply for brain tumors and have been described both for primary and metastatic brain tumors 91,92. Angiogenic factors secreted from tumor cells such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) along with different pro-inflammatory cytokines, proteases and miRNAs play pivotal roles in tumor angiogenesis 88,93,94. Tumor-derived EVs are also involved in the process of tumor angiogenesis. This function has most commonly been attributed to EV contents including pro-angiogenic proteins and miRNAs, which can directly trigger the activation of VEGF-dependent and -independent angiogenic pathways in endothelial cells 95.

The angiogenic role of tumor-derived EVs in primary brain tumors has been investigated only in the context of glioma/glioblastoma, the most vascularized of all brain tumors (Figure 1.2). Early studies demonstrated that EVs released from the U87 glioblastoma cell line contain angiogenic factors such as Interleukin-6 (IL-6), IL-8, and angiogenin and can induce tube formation in human microvascular endothelial cells 29. Other studies have demonstrated the presence of functional angiogenic proteins such as tissue factor, coagulation factor VIIa 79, platelet-derived growth factor (PDGF) 36, transforming growth factor beta (TGF-β), and CXCR4 96 in glioblastoma-derived EVs. In addition, hypoxic glioblastoma EVs were demonstrated to be enriched in a number angiogenic proteins and promoted stronger angiogenic phenotype in
endothelial cells compared to normoxic glioblastoma EVs. Moreover, endothelial cells treated with hypoxic glioblastoma EVs were able to recruit more pericytes, *in vitro* and *in vivo* \(^{36}\).

Glioblastoma EVs contain a variety of proteases such as matrix metalloproteinase 2 (MMP-2), MMP-9, urokinase-type plasminogen activator (uPA), and tissue plasminogen activator (tPA) that increase the proliferation, migration, and capillary tube formation of human brain endothelial cells \(^{96}\). EVs isolated from the cerebrospinal fluid (CSF) of glioblastoma patients also induced angiogenesis in human umbilical vein endothelial cells \(^{97}\). This effect was associated with the activation of the Akt/ beta-catenin pathway, however further investigation is required to evaluate the causal effect of EVs on the activation of this pathway. In accordance with these studies on primary brain tumors, breast cancer derived-EVs were also shown to be involved in angiogenesis through the interaction of annexin AII on EVs with endothelial tPA \(^{98}\).

In addition to the pro-angiogenic protein cargo, a number of studies have indicated the importance of non-coding RNAs in the angiogenic function of glioblastoma-derived EVs (Figure 1). For instance, EVs isolated from the CD133\(^+\) glioma stem cells derived from the U251 human glioblastoma cell line contain miR-21 that can trigger VEGF signaling in human brain endothelial cells *in vitro* \(^{99}\). In contrast, miR-1 was demonstrated to be transferred between glioblastoma cells and tumor microenvironment through EVs and had a suppressive effect on angiogenesis, explaining its downregulation in glioblastoma patients \(^{82}\). While the majority of studies on non-protein contents of EVs have focused on miRNAs, a variety of other non-coding small RNA species have been shown to be enriched in EVs derived from U251 cells. These EVs were shown to modulate the gene expression of human brain endothelial cells, which was considered to be a function of their small RNA content \(^{100}\). It has also been reported that A172 glioma cell-derived EVs can transfer the long intergenic non-coding RNA, line-POU3F3, to
human brain endothelial cells, resulting in increased expression of FGF, FGFR, and VEGF and increased angiogenesis both in vitro and in vivo\textsuperscript{101}.

The potential contribution of non-coding RNAs in the angiogenic functions of EVs derived from metastatic brain tumors has yet to be evaluated. Comparative miRNA profile analysis of breast cancer cell lines demonstrated an overexpression of miR-210 in cells that specifically metastasize to brain versus the parental cell lines\textsuperscript{102}. miR-210 has been reported as a pro-angiogenic miRNA in normal adult mouse brain\textsuperscript{103}. The promising possibility of the involvement of EV miR-210 in breast cancer brain metastasis and angiogenesis requires further study.

In addition to tumor cells, several stromal cells in the tumor microenvironment such as bone marrow-derived tumor-associated macrophages and tissue resident microglial cells play substantive roles in tumor angiogenesis\textsuperscript{104}. While the presence of angiogenic factors in stromal cell-derived EVs has been reported previously\textsuperscript{105}, studies evaluating the importance of stromal cell-derived EVs in tumor angiogenesis are still lacking for both primary and metastatic brain tumors. Given the important role of the brain microenvironment in tumor angiogenesis, investigating the potential contribution of stromal cell-derived EVs in this process has the potential to uncover new opportunities for anti-angiogenic therapeutic approaches.

The interaction of EVs with the brain endothelium is not limited to increasing angiogenesis and vascularization. Breast cancer-derived EVs have been shown to facilitate brain metastasis through modulating the permeability of the brain endothelium (Figure 1). EVs derived from MDA-MB-231 breast cancer cells were shown to decrease the expression of the tight junction molecule, ZO-1, via their cargo of miR-105\textsuperscript{75}. Another study on brain-metastatic breast cancer cells also demonstrated an effect of EV miR-181c on the localization of actin filaments, resulting
in increased permeability of the brain endothelium \(^{76}\). The blood-brain-barrier is a unique obstacle faced by the disseminated tumor cells metastasizing to the brain, making the potential effects of tumor-derived EVs on the integrity of this barrier of utmost importance.

Figure 1.2. The effect of brain tumor-derived EVs on endothelial cells. EVs derived from primary and metastatic brain tumor cells can deliver angiogenic proteins and miRNAs and promote an angiogenic phenotype in endothelial cells (Image credit: Kristin Johnston, Vascular Biology Program, Boston Children’s Hospital).

1.3.1.2. Immune cells

A growing body of literature demonstrates that immune cells within the tumor microenvironment induce both stimulatory and inhibitory effects on tumor progression and resulting patient prognosis. The impact of these complicated tumor-immune cell interactions largely depends on the type and the location of the tumor \(^{106}\).

In the brain, resident immune cells including microglia and astrocytes along with infiltrating macrophages, neutrophils, and lymphocytes, form the immune component of the tumor
microenvironment. In glioma, tumor-associated myeloid cells, including both recruited macrophages and resident microglia, constitute up to 40% of cells in the tumor microenvironment. These cells facilitate the proliferation and invasion of glioma cells through secreting a variety of factors such as epidermal growth factor. In brain metastasis, recruitment of microglia/macrophages to the site of tumor has been shown to occur during early stages of brain metastasis. Immunohistochemistry analyses on human glioma samples have demonstrated significant infiltration of neutrophils in the majority of patients, particularly those with higher grade gliomas. While our understanding of the mechanisms by which tumor-infiltrating neutrophils contribute to tumor growth is incomplete, multiple studies have shown S100A proteins and elastase secreted by neutrophils can increase the proliferation and invasion of tumor cells, respectively. Tumor-infiltrating lymphocytes have also been observed in patients with primary and metastatic brain tumors. The balance between the pro- and anti-tumor effects of tumor-infiltrating lymphocytes relies on the composition of this cell population in the tumor microenvironment. For instance, infiltration of CD4+ CD25+ FOXP3+ regulatory T cells in the tumor microenvironment has been reported for glioblastoma and brain metastasis in patient samples but not in benign brain tumors.

The immune modulating role of EVs derived from primary brain tumors has been evaluated for glioblastoma (Figure 1.3). EVs derived from glioblastoma cell lines were reported to induce a tumor-supporting M2-like phenotype in microglia cells in vitro, as shown by upregulation of Arg-1. The expression of tumor promoting cytokines such as IL-6 was increased, while immunogenic cytokines such as IL-16 were downregulated. Intravital microscopy showed that glioblastoma-derived EVs were taken up by microglial cells in vivo. This transfer of EVs led to the transfer of miR-21 and the suppression of its target, c-Myc, demonstrating the functionality
of the EV transferred miRNA. This study supports the concept that the *in vivo* communication between glioblastoma and microglial cells can occur through tumor-derived EVs. The induction of the M2-like phenotype was also demonstrated in another study where patient-derived peripheral blood monocytes were treated with glioblastoma-derived EVs *in vitro*. The expression of M2 phenotype marker, CD163, as well as tumor-supporting cytokines such as IL-6, monocyte chemoattract protein-1, and VEGF were increased. In another study, transfer of cre recombinase mRNA through EVs derived from transplanted cre-expressing glioblastoma cells demonstrated the Gr1+CD11b+ myeloid-derived suppressor cell population to be the prominent recipients of glioblastoma EVs in the tumor microenvironment. This transfer was suggested to potentially lead to an immunosuppressive microenvironment but this was not directly demonstrated for glioblastoma EVs.

T cells can also be affected by glioma-derived EVs. Interestingly, glioma EVs were shown to have a dose-dependent effect on the T cells isolated from peripheral blood mononuclear cells, inducing an immunosuppressive phenotype only in high concentrations. In another study, incubation of EVs isolated from plasma of glioblastoma patients with peripheral blood monocytes from healthy donors led to an inhibitory effect on the proliferation of CD4+ T cells. This inhibitory effect was further demonstrated to be mediated through EVs inducing a suppressive phenotype in CD14+ monocytes. More recently, it was shown that a subpopulation of EVs isolated from glioblastoma stem-like cells contain functional PDL-1 and were able to suppress T cell activation through direct interaction with PD1. These authors also demonstrated that PDL-1 DNA was detected in plasma EVs from glioblastoma patients and correlated with tumor volume, suggestive of a potential biomarker.
Similar to primary brain tumors, metastatic brain tumors can communicate with macrophages/microglia in the brain through EVs (Figure 1.3). EVs derived from breast tumors could interact with the Toll-like receptor 2 (TLR2) on macrophages and could stimulate the NF-Kb signaling pathway, leading to an increase in pro-inflammatory cytokines such as IL-6 and TNF-α\textsuperscript{119}. Consistent with this study, EVs released from drug resistant MCF7 breast cancer cell line also stimulated the expression of IL-6 and decreased the chemotaxis ability of macrophages\textsuperscript{120}. The relationship between the EV-macrophage interactions and the development and

![Figure 1.3. The effect of brain tumor-derived EVs on immune cells.](Image credit: Kristin Johnston, Vascular Biology Program, Boston Children’s Hospital)
progression of breast cancer brain metastasis was not evaluated in either of these studies and requires further investigation.

Overall, these studies demonstrate that EVs derived from both metastatic and primary tumors can induce a tumor-promoting phenotype in resident microglial cells and infiltrating macrophages. Given the prominent role of macrophages in brain tumor progression, these findings can potentially contribute to development of actionable immunotherapeutic targets. The importance of the other components of the immune microenvironment in the brain cannot be neglected and studying the EV-based modulation of these components holds promise for novel discoveries regarding the role of the immune microenvironment in promoting primary and metastatic brain tumors.

1.3.1.3. Brain-specific cells

A variety of cell types including astrocytes, oligodendrocytes, neurons, and microglia are specific to the brain and can play a role in the brain tumor microenvironment. In the brain microenvironment, astrocytes are one of the major host cells that establish interactions with primary and metastatic tumor cells and play major roles in promoting tumor cell survival and growth both in primary and metastatic brain tumors is limited. EV transfer of alpha-crystallin B chain (CRYAB) from astrocytoma U373 cells was demonstrated to be involved in tumor cell resistance to apoptosis, a hallmark of cancer. Whether normal astrocytes in the tumor microenvironment can also promote tumor growth through transferring CRYAB-containing EVs to tumor cells is a potential mechanism that remains to be explored. Moreover, EVs released from astrocytes as well as the U87MG glioblastoma cell line contain mitochondrial DNA. A recent study demonstrated that cancer-associated fibroblasts could transfer their mitochondrial DNA to breast cancer cells via
EVs to induce an escape from metabolic dormancy in the cancer cells. Given these recent findings, it is of interest to determine whether the mitochondrial DNA found in astrocyte EVs can have any effect on the progression of primary and metastatic brain tumors.

In contrast to these potential promoting effects of astrocyte-derived EVs on tumor cells, tumor-derived EVs (oligodendroglioma G26/24 cell line) were shown to contain tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and could induce astrocyte apoptosis in vitro (Figure 3). This process was suggested to be a mechanism by which tumor cells create space for expansion. The significance of this finding has not been evaluated in vivo.

Studies on brain metastasis indicate that the EV-based interactions between astrocyte and metastatic tumor cells are mainly pro-tumorigenic (Figure 1.4). Astrocyte EVs were shown to transfer miR-19a to metastatic breast cancer cells, which downregulated PTEN and enhanced the proliferation of the recipient tumor cells. A series of elaborate in vitro and in vivo experiments demonstrated that EV transfer of this miRNA is critical for the downregulation of PTEN, a common feature of breast cancer brain metastasis. In line with these studies, breast cancer derived-EVs were also shown to affect the metabolic status of brain metastases. It was demonstrated that tumor EVs can transfer miR-122 to astrocytes and reduce glucose uptake by these cells. This effect increased the availability of glucose in the brain for the metastatic tumor cells, enabling their initial expansion in the brain.
Microglia and oligodendrocytes as well as neurons are among the other specialized cells in the brain that can contribute to tumor progression. The EV-based mechanisms of microglia-tumor interactions have been reported in multiple studies\textsuperscript{80,81,119,120} as discussed in detail in the immune section of this review. The role of oligodendrocytes and neurons in these EV-based interactions is yet to be understood. Neurons have been reported to lack the ability to take up breast cancer-derived EVs within a 48-h incubation time\textsuperscript{83}. The possibility exists that the uptake of EVs by neurons occurs through fusion with the plasma membrane, which would be difficult to detect through tracing the EV fluorescent labels. Moreover, it must be noted that the effect of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{brain-tumor-derived-EVs.png}
\caption{The effect of brain tumor-derived EVs on brain-specific cells.} Astrocytes can be affected by EVs from primary and metastatic brain tumors. Astrocyte-derived EVs can affect breast cancer brain metastasis through decreasing the PTEN expression \textit{(Image credit: Kristin Johnston, Vascular Biology Program, Boston Children’s Hospital).}
\end{figure}
EVs on recipient cells does not merely rely on the uptake of EVs and can occur through surface interactions with the receptors on the cell membrane.

1.3.2. Extracellular components of the brain tumor microenvironment

The extracellular matrix, predominantly composed of proteins and glycosaminoglycans (GAGs), not only provides a scaffold for the growth and expansion of tumor cells but also functions in various ways to enhance tumor development. The ECM serves as a reservoir for different growth factors, which can be released and presented to their receptors upon modulation of the ECM. ECM components can also directly trigger signaling pathways in tumor cells promoting their survival, proliferation, and invasion abilities.

Brain ECM is unique both in terms of its components as well as structure. Similar to many other organs, the ECM that forms the basement membrane surrounding the brain vasculature, i.e., the perivascular ECM, is mainly comprised of fibrous proteins such as collagen, fibronectin, and laminin, whereas the parenchymal ECM surrounding the neural and glial cells in the brain is abundant in hyaluronan and proteoglycans and has a very limited amount of fibrous proteins. Some of these components, such as brevicans, are specific to brain and nervous tissues, and have been reported to enhance tumor progression and angiogenesis in high-grade primary brain tumors. ECM receptors such as CD44, the main receptor for hyaluronan, are also upregulated in glioma and meningioma cells, indicating the importance of tumor-ECM interactions for tumor progression. Unlike the other aspects of the microenvironment discussed in this chapter, the ECM composition surrounding the tumor differs between primary and metastatic brain tumors. Primary brain tumors often demonstrate an infiltrative pattern of growth while brain metastases, except for late stage metastases, most frequently remain confined to the brain vasculature and grow along the vessels (i.e. vessel co-option). As a result, tumor-ECM interactions in primary
tumors involve the parenchymal ECM, whereas in brain metastases, these interactions prominently occur within the fibrous perivascular ECM. This difference was indeed highlighted in a comparison of the ECM associated with glioblastoma and lung cancer brain metastasis, where a distinct pattern of ECM alterations was demonstrated for each type. Proteoglycans such as brevican and neurocans were more abundant in glioblastoma. These inherent differences should guide the design of studies on tumor ECM modulation in primary and metastatic brain tumors.

Potential involvement of tumor-derived EVs in ECM modulation is supported by proteomics studies that indicate the presence of a variety of adhesion molecules as well as proteases and glycosidases on tumor-derived EVs. Whether these EV proteins are functionally active and whether they play a role in cancer progression is a matter of active investigation. A comprehensive analysis of the interactions of pancreatic adenocarcinoma EVs with different ECM molecules in vitro demonstrated that EVs can bind to ECM through their integrins and CD44. Moreover, these EVs were shown to contain active proteases such as MMPs and ADAMs, capable of degrading the ECM components and promoting tumor growth.

The role of EVs derived from primary and metastatic brain tumors in ECM modulation has been studied to a limited extent (Figure 1.5). In primary brain tumors, the direct role of tumor-derived EVs in modulation of ECM has yet to be elucidated. However, the current evidence suggests that EVs are capable of inducing ECM-modulating phenotypes in cells within the tumor microenvironment. EVs isolated from the glioblastoma U87 cell line increased the expression of MMP-14 in patient-derived microglia after 72 hours of incubation, suggesting a potential indirect role for these EVs in modulation of the ECM. In metastatic brain tumors, evidence of both a direct and indirect role of EVs in modulation of ECM in the brain microenvironment has been
reported. EVs derived from the breast cancer MDA-MB-231 cell line were shown to contain annexin AII, which induced an increase in plasmin activity and promoted angiogenesis. 

Annexin AII is a major co-receptor for plasminogen and tPA and is involved in the activation of plasmin that can degrade a variety of ECM component. Treatment of mice with Annexin AII+ EVs also led to an increase in the expression of MMP-9. The mechanism by which EVs induced this increase in expression has yet to be elucidated.

As evident by the limited number of studies on the role of EVs in ECM modulation in primary and metastatic brain tumors, many aspects of this process remain obscure. Further investigation of EV-based ECM modulation in the brain is necessary in order to understand the process involved in tumor progression. A comparison between the ability of EVs derived from primary and metastatic brain tumors to interact with and modulate the ECM can provide valuable

**Figure 1.5. The effect of brain tumor-derived EVs on extracellular matrix.** EVs derived from primary and metastatic brain tumor cells can increase the degradation of extracellular matrix directly through activation of tPA and indirectly via an increase in expression of MMPs. (Image credit: Kristin Johnston, Vascular Biology Program, Boston Children’s Hospital).
insights into the mechanisms driving the different infiltrative behavior of primary and metastatic brain tumors.

1.3.3. Conclusions

While many efforts have been made to determine the pathological role and the translational potential of EVs in primary brain tumors, the role of EVs in metastatic brain cancer has been far less studied and is yet equally significant. In this section, I focused on the common microenvironment shared between primary and metastatic brain tumors to evaluate the effects of EVs on the development and progression of these types of cancer. The EV-based interactions of primary and metastatic tumor cells with the brain microenvironment supports the prominent effects of tumor microenvironment on the mechanisms by which brain tumor cells, regardless of their origin, undertake to support their growth and progression. This perspective can direct the future EV research in metastatic and primary brain tumors toward actionable translational endpoints. It is important to note that most EV studies on primary and metastatic brain tumors predominantly rely on in vitro experiments and in vivo models that might not provide a representative recapitulation of the disease. Determining the physiologic relevance of the reported findings is a prerequisite to reach translational goals in the EV field.
Chapter 2

Breast Cancer-Derived Extracellular Vesicles Promote Brain Metastasis

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2.1. Abstract

Breast cancer brain metastasis is a major clinical challenge associated with a dismal prognosis. Understanding the early mechanisms underlying brain metastasis development can guide the development of efficient diagnostics and therapeutics to improve the outcome of this diseases. Extracellular vesicles are known as intercellular communicators that contribute to tumor progress and metastasis. In this Chapter, we test the hypothesis that EVs derived from breast cancer cells can contribute to brain metastasis formation. First, we establish an *in vivo* model of brain metastasis based on parental, bone-seeking and brain-seeking triple negative breast cancer cell lines. We isolate and characterize EVs from these cells. Using the isolated EVs and the *in vivo* model of brain metastasis, we demonstrate that EVs derived from the brain-seeking breast cancer cell line but not parental or bone-seeking cell lines can promote brain metastasis, potentially through preparing a microenvironment that is supportive of tumor growth in the brain. The findings reported in this Chapter demonstrate that breast cancer-derived EVs play a role in brain metastasis formation through mechanism(s) unique to EVs derived from a brain-seeking population of breast cancer cells.
2.2. Introduction

Breast cancer is one of the leading causes of metastatic brain tumors \(^6\). The prognosis of breast cancer patients with brain metastasis is extremely poor, with a reported median survival of 10 months \(^12\). An urgent need exists, therefore, to develop early diagnostics and effective therapeutics for breast to brain metastasis, informed by an understanding of the early mechanisms involved in brain metastasis formation.

Tumor-derived extracellular vesicles (EVs) have been identified as early contributors to metastasis formation. Once released into the circulation, they transfer a variety of proteins and genetic material to stromal cells in distant organs \(^68\). These events lead to the modulation of the microenvironment in pre-metastatic organs in support of future metastatic growth \(^69,71,73\). The current understanding of the role of tumor-derived EVs in preparation of a pre-metastatic niche in the brain for promotion of brain metastasis remains limited. In this Chapter, we set out to test the hypothesis that breast cancer-derived EVs can promote brain metastasis formation and growth.

First, we established new experimental models to study this process. The rate of brain metastasis development is higher in triple negative and HER2 positive breast cancer subtypes \(^17\). These statistics along with the highly limited diagnostic and therapeutic options available for triple negative breast cancer patients \(^133-135\), led us to focus on triple negative breast cancer as our study model. We established a mouse model of breast cancer brain metastasis based on the intracardiac injection of brain-seeking triple negative breast cancer cells. We next, isolated and characterized a population of EVs defined as small EVs (size < 200 nm) or exosomes, from the brain-seeking and control cells.
Using these models, we demonstrated that EVs derived from brain-seeking cancer cells have the ability to promote brain metastasis formation and growth in mice. The findings in this chapter support our hypothesis and establish the foundation for investigating the mechanism(s) underlying the breast cancer EV-driven promotion of brain metastasis.

2.3. Materials and Methods

2.3.1. Cell lines and cell culture

Human breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC® HTB-26™, VA, USA). The brain-seeking (MDA-231Br) variant of the breast cancer cell line MDA-MB-231 was a gift from Dr. T. Yoneda, Indiana University. The bone-seeking variant of this line was obtained from the Massagué Lab, Memorial Sloan Kettering Cancer Institute. Breast cancer cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Cat # 11885084, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS, Cat # S11150, Atlanta BiologicalsTM, Atlanta, GA, USA) and 1% Penicillin-Streptomycin (10,000 U/mL) (Cat # 15140148, Thermo Fisher Scientific Inc.). All cells were maintained in a 37°C humidified incubator with 5% CO₂. All cultures were routinely monitored for mycoplasma contamination using the MycoAlert™ PLUS Mycoplasma Detection Kit (Cat # LT07-710, Lonza Inc.).

2.3.2. EV isolation

For EV isolation from cell culture flasks, breast cancer cells were cultured in Advanced DMEM (Gibco, Cat # 12491-015) supplemented with 10% EV-depleted FBS. EV-depleted FBS-containing medium was prepared by 18-h ultracentrifugation of media containing 40% FBS at 100,000 x g at 4°C. The EV-depleted media was then diluted to contain 10% EV-depleted FBS. Conditioned media was collected from breast cancer cell cultures after 24-48 hours of
incubation in EV-depleted media. Conditioned media was only used for EV collection if cell viability was >95%.

EVs were isolated using a sequential centrifugation technique. As depicted in Figure 2.1, conditioned media was centrifuged at 400 x g for 10 min, 2000 x g for 15 min, and 15000 x g for 30 min at 4°C (Sorvall® RC-5B centrifuge, Thermo Fisher Scientific Inc.) to remove dead cells, debris, and larger (>200 nm) microvesicles. The supernatant subsequently underwent a round of ultracentrifugation at 100k x g for 90 min at 4°C (Optima XE-90 Ultracentrifuge, Beckman Coulter Life Sciences) followed by a round of washes at 100k x g for another 90 min. The final pellet was resuspended in PBS for characterization and experiments.

![Figure 2.1. Schematic depicting the EV isolation protocol. (Image was created via www.biorender.com)](Image was created via www.biorender.com)

### 2.3.3. EV characterization

EV preparations were characterized according to the guidelines of the International Society for Extracellular Vesicles. EV size and concentration was measured by nanoparticle tracking analysis (NanoSight NS300, Malvern Instruments, UK). The presence of EV markers CD9, CD63, and Alix and the absence of a Golgi marker (GM130) as a negative control was evaluated by western blot. The shape of the EVs was evaluated by electron microscopy. To this end, EV
samples were adsorbed onto a formvar/carbon-coated grid and stained with uranyl formate. The grids were imaged using a JEOL 1200EX Transmission electron microscope and images were taken with an AMT 2k CCD camera. EV density was measured using an OptiPrep™ Gradient ultracentrifugation technique. Briefly, EVs were suspended in OptiPrep™ to prepare a 5% concentration. The EV-containing OptiPrep™ was then layered on top of a gradient consisting of 10%, 25%, and 30% OptiPrep™. Gradients were centrifuged at 100k x g for 4 hours at 4°C. All fractions were collected and were either used directly for luciferase assay or were further diluted in PBS (1:25) and centrifuged at 100k x g for 90 min at 4°C. The pellets were resuspended in PBS and were used for western blot analyses.

2.3.4. Establishment of luciferase-labeled cell lines for in vivo studies

All cells were transduced with a Gaussia Luciferase (Gluc) construct to allow for monitoring of metastasis development via in vivo bioluminescence imaging. A lentivirus vector encoding Gaussia luciferase fused to the transmembrane domain of platelet-derived growth factor receptor as well as GFP was used for this purpose. This construct was a gift from Dr. X. Breakefield, Massachusetts General Hospital (MGH) and the lentivirus vectors were made at the MGH Vector Core, Boston, MA. For transduction, 100 μl of virus combined with 900 μl of media and 1 μl of polybrene (10 mg/ml) was added to the cells. After 48-72 h cells were harvested and underwent fluorescence activated cell sorting (FACS) based on GFP expression to select for a pure population (>95% GFP positive) for future experiments. Expression of luciferase was evaluated via a luciferase assay. Briefly, a 20 μM concentration Gaussia luciferase substrate, native Coelenterazine (Prolume Ltd. Cat # 303), was prepared and incubated for 30 min at room temperature. The substrate (50 μl) was added to each well containing the samples and luminescence intensity was measured immediately using a SpectraMax M2 plate reader.
(Molecular Devices, Inc.). To confirm that the luciferase is bound to the membrane and not in its free form, we also conducted immunoblotting on cell lysates and conditioned media collected from the cells (anti Gluc Antibody, Prolume Ltd., Cat # 401M).

2.3.5. In vivo studies

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of Boston Children’s Hospital, Boston, MA. Six-eight-week-old female Nu/Nu nude mice were purchased from Massachusetts General Hospital. At least 4 days of acclimation was conducted prior to the start of the experiments.

2.3.5.1. Establishment of a breast cancer brain metastasis model

Intracardiac injections of the parental, bone-seeking, and brain-seeking MDA-MB-231 cells (2 x 10^5 cells in 100 µl HBSS) or 100 µl HBSS (sham) into the left ventricle were conducted to establish metastasis (n=6 per group), as described previously. Parental and bone-seeking cells were used as controls. The weight of the mice was measured every week to evaluate the overall health of the animals. Development of extracranial and intracranial metastases was evaluated via an in vivo bioluminescence imaging system (IVIS®, PerkinElmer), every week over a course of 12 months. For imaging, mice received retro-orbital injection of Guassia luciferase’s substrate, Coelenterazine (Inject-A-Lume, Prolume, 35 µl of 3.33 mg/ml solution for a 25-g mouse) and imaging was conducted within 1 min after injection, according to the manufacturer’s protocol. Signal detection threshold was set based on the background signal received from the HBSS-treatment mice and was kept constant throughout the experiment. Mice with deteriorating health conditions (lethargy, decreased appetite, hyperventilation, unsteady gait, signs of infection, extracranial tumor burden of > 1cm in diameter, signs of infection or ulceration, greater than 15% weight loss) were sacrificed earlier than at the endpoint according to the established IACUC
protocols. The presence of intracranial metastases was further confirmed via ex vivo bioluminescence imaging and histology. For histological analyses, brain tissues were collected and fixed in 4% paraformaldehyde. Each brain was cut into 5 coronal sections (bread-loafing technique), from which five 200-µm stepwise sections (10-µm-thick) were prepared, for a total of 25 sections for each brain. Following hematoxylin and eosin (H&E) staining, the presence of macrometastases and micrometastases was evaluated in brain sections in a blind manner by Dr. Roderick Bronson, Rodent Pathology Core, Harvard Medical School. The surface areas of metastasis foci were measured using the ImageJ software.

2.3.5.2. Brain metastasis studies

Mice were randomly divided into 4 groups to receive retro-orbital injections of EVs derived from parental, bone-seeking, and brain-seeking MDA-MB-231 cells (3 µg EVs in 100 µl HBSS per injection) or 100 µl of HBSS (n=8 mice per group). As depicted in Figure 2, injections were conducted every other day, for a total of 10 injections. Following this pretreatment with EVs, intracardiac injections of unlabeled parental or brain-seeking MDA-MB-231 cells (2 x 10^5 cells in 100 µl HBSS) into the left ventricle were conducted to establish brain metastasis. Four weeks

![Figure 2.2. Schematic depicting the in vivo brain metastasis study design](Image was created via www.biorender.com)
after intracardiac injection, mice were sacrificed and brain tissues were collected and fixed in 4% paraformaldehyde. Tissue preparation and histological analyses was conducted as described above.

2.3.6. Statistical analyses

All quantified data are presented as mean ± SD from 3 independent experiments. For animal experiments, the minimum number of animals required to obtain data amenable to statistical analysis was used. Animals were randomly divided into groups. Blinded analyses were only conducted to evaluate the presence of brain metastasis. All statistical analyses were performed using the GraphPad Prism software. Statistical significance was considered as $P$ values lower than 0.05. $P$ values were shown as * $P \leq 0.05$. All mouse experiments were evaluated using the Mann-Whitney test.

2.4. Results

2.4.1. Establishment of experimental models

To establish a brain metastasis model, stable clones of MDA-MB-231 cell lines expressing the membrane-bound Gluc were successfully created (Figure 2.3 A,B) and injected into mice via the intracardiac route. Within the 12-week period of monitoring via bioluminescence imaging, 16% and 66% of mice that received the parental MDA-MB-231 cells developed liver and bone metastases, respectively. Injection of the bone-seeking cells led to development of bone metastasis in 83% of mice. Brain metastases developed in 40% of mice treated with brain-seeking cells whereas no brain metastasis was observed in parental or bone-seeking groups. Figure 2.4A depicts representative images of metastasis formation in each group. Brain metastasis signal was confirmed through ex vivo bioluminescence imaging (Figure 2.4B) and via histology (Figure 2.4C).
Figure 2.3. Luciferase labeling of MDA-MB-231 cells. Cells were stably transduced with a Gluc lentiviral vector as demonstrated by staining (A) and immunoblotting (B).
These findings demonstrated that intracardiac injection of brain-seeking MDA-MB-231 cells provide an efficient model of triple negative breast cancer brain metastasis and that injection of parental and bone-seeking cells, which did not lead to brain metastasis, can be used as reliable controls.

Next, we isolated EVs from the parental, bone-seeking, and brain-seeking cells (P-EVs, Bo-EVs, and Br-EVs, respectively) using the sequential centrifugation technique. All EVs exhibited a lipid bilayer structure (Figure 2.5A) and a mode size of 170.2 +/- 2.8 nm, 151.5 +/- 6.2 nm, and 158.5 +/- 6.0 nm, respectively (Figure 2.5B). EVs were enriched in endosomal markers such as CD63, CD9, and Alix and our negative control marker, GM130, which is a Golgi marker, was not detectable in our samples (Figure 2.5C). Using OptiPrep™ density gradient ultracentrifugation, the density of EVs was found to be within a range of 1.105-1.184 g/ml, consistent with previous reports 19.

Figure 2.4. Pattern of metastasis in MDA-MB-231 cells, (A) In vivo bioluminescent imaging (representative images of n=6 mice per group), (B) Ex vivo bioluminescent imaging and (C) histological analysis of brain metastasis in a mouse treated with brain-seeking cells demonstrating a vessel co-option pattern of growth (representative image).
2.4.2. Breast cancer-derived EVs promoted brain metastasis

To address the hypothesis that breast cancer-derived EVs can promote brain metastasis, nude mice were pretreated with 3 μg of P- or Br-EVs (~3-4 x 10^9 particles; EVs from approximately 5 x 10^5 cells) every two days for a total of 10 retro-orbital injections. After the final EV injection, each mouse received an intracardiac injection of the brain-seeking MDA-MB-231 breast cancer cells. The weight of mice increased over time during this experiment in all groups, demonstrating that the procedures were well tolerated (Figure 2.6A). At week 4, histological analyses demonstrated that the incidence of brain metastasis was increased in the Br-
EV-treated group (Figure 2.6B). Moreover, pretreatment with Br-EVs significantly increased the size of brain metastases (Figure 2.6C). These findings indicate that Br-EVs but not P-EVs or Bo-EVs can facilitate brain metastasis growth. Interestingly, the total number of metastatic foci was not significantly different between the four groups (Figure 2.6D), suggesting that Br-EVs do not facilitate the extravasation and seeding of circulating tumor cells into the brain tissue but rather promote the growth of extravasated tumor cells into metastatic lesions. Consistent with previous reports, a vessel co-option pattern of growth was observed for all brain metastases (Figure 2.6E), supporting the role of BBB as an initial niche for tumor cell growth.

When we conducted this experiment with intracardiac injection of parental MDA-MB-231 cells, brain metastatic lesions could not be detected in any of the experimental groups. This finding suggested that within the setting of this experiment, Br-EV treatment was not sufficient to induce brain metastasis in breast cancer cells that have limited inherent ability to home to the brain. This observation is consistent with our earlier findings suggesting that Br-EVs promote the growth of extravasated tumor cells in the brain but have minimal effects on initial tumor cell homing to the brain.
Figure 2.6. EVs from brain-seeking breast cancer cells promote brain metastasis. (A) Average weight of mice in all experimental groups, measured weekly. (B) Incidence of brain metastasis development, 4 weeks post-intracardiac injection of tumor cells. (C) Average surface area per brain metastasis (mean ± SD; n= 8 mice in HBSS and brain-seeking group and 6 mice in parental and bone-seeking groups); Statistical analysis was performed using Mann-Whitney test. (D) (C) Total number of brain metastasis foci per animal (mean ± SD; n= 8 mice in HBSS and brain-seeking group and 6 mice in parental and bone-seeking groups); Statistical analysis was performed using Mann-Whitney test. No significance difference was found. (E) Representative H&E images of brain metastases. All metastases demonstrated a vessel co-option pattern of growth (black arrows). Scale bar, 50 µm.
2.5. Discussion

2.5.1. Breast cancer models of brain metastasis

The widely used orthotopic and transgenic breast cancer models exhibit an extremely low efficiency of metastasis to the brain. As a result, in vivo brain metastasis models predominantly rely on the injection of tumor cells into the circulation. Both intracarotid and intracardiac routes of tumor cell injection have been previously demonstrated to lead to brain metastasis formation. While the efficiency of intracarotid injection is relatively higher, intracardiac injection provides a better recapitulation of the homing process of circulating tumor cells to the brain. Accordingly, we used an intracardiac injection model of parental, bone-seeking, and brain-seeking variants of the MDA-MB-231 triple negative breast cancer cell lines. This model led to the formation of brain metastasis from the brain-seeking cells (with 33-50% efficiency) and the absence of brain metastasis in our control variants.

Brain-seeking variants have been established previously for only a limited number of triple negative and Her2+ breast cancer cell lines such as MDA-MB-231, SUM190, and JIMT-1. However, variants with the capacity to metastasize to other metastatic organs common for breast cancer (e.g., bone) are to date available only for the MDA-MB-231 cells. These variants could allow us to distinguish between the EV-driven effects that are specific to brain metastasis and those that are common to all metastatic sites. As a result, we chose MDA-MB-231 as our study model to understand the role of breast cancer-derived EVs in brain metastasis.

2.5.2. EV isolation and characterization

Tumor cells release a heterogenous population of EVs with different sizes and different origins (e.g., plasma membrane, endosomal membrane). EVs are commonly categorized based on their origin, with exosomes (<200 nm) of endosomal origin and microvesicles (>200nm) of
plasma membrane origin, being the two major types. Nevertheless, a large body of literature has demonstrated a significant overlap in size and proteins between exosomes and microvesicles. With current knowledge and technologies, it is not yet feasible to precisely distinguish between the different types of EVs. The International Society for Extracellular Vesicles has therefore recommended that this nomenclature be excluded from the literature. Instead, it has been suggested that a comprehensive description of isolation methods and EV characteristics be included in sample description along with the use of the general term “extracellular vesicles”.

In this study, we used a sequential centrifugation technique that has been described as a gold standard for EV isolation. The labor-intensive and costly nature of this technique have led to a rapidly growing interest in the development of efficient and cost-effective techniques for EV isolation. Several commercial kits have been introduced for EV isolation based on precipitation techniques and the use of water-excluding polymers. Co-precipitation of non-EV contaminants remains a major drawback associated with these kits. Other techniques such as ultrafiltration, size-exclusion chromatography (size-based selection) or immunopurification (marker-based selection) allow for isolation of EV samples with higher purity. Nevertheless, these techniques are only suitable for applications where a specific EV subpopulation of interest (e.g., CD63+ EVs or EV<100nm) is to be studied. More recently, microfluidic approaches have been introduced as promising techniques for EV isolation however these methods are yet to be thoroughly validated. Each of the currently available techniques are associated with certain advantages and disadvantages, and therefore, each technique can accommodate the requirements for certain applications defined by the purpose of each study. Sequential centrifugation continues to remain the gold standard for unbiased isolation of the whole population of EVs from cell conditioned media and was used in our study for isolation of EVs derived from breast cancer.
cell lines. We isolated a population of EVs that were below 200 nm in size (previously defined as exosomes). Enrichment of EVs in endosomal markers such as CD63, CD9, and Alix and the lack of detectable GM130, a Golgi marker, in EV samples indicated an endosomal origin of these samples.

2.5.3. Breast cancer-derived EVs in brain metastasis

Here, we demonstrated that EVs derived from a brain-seeking subpopulation of triple negative breast cancer cells have the ability to promote the formation and growth of brain metastases from brain-seeking breast cancer cells\textsuperscript{150}. In recent years, a number of studies have also evaluated the brain metastasis-promoting effect of breast cancer-derived EVs\textsuperscript{74-76,83,98}. Consistent with our findings, EVs from brain-seeking MDA-MB-231 cells were demonstrated to have a higher efficiency in promoting brain metastasis when compared to parental MDA-MB-231 cells\textsuperscript{76}. However, the effect of EVs was demonstrated on the metastasis of parental cells. Another study that compared EVs from brain-seeking cells to those from annexin II-knocked down brain-seeking cells, reported promotion of brain metastasis both for parental and brain-seeking MDA-MB-231 cells\textsuperscript{98}. The differences in the metastatic ability of parental cells in these studies compared to ours could be attributed to potential effects of luciferase labeling of tumor cells on their behavior\textsuperscript{151}.

It should be noted that various methodologies have been used in these studies for EV pre-treatment, with doses of injected EVs varying between 2\textsuperscript{75,83} to 100 µg\textsuperscript{98} per injection and the number of injections varying between one\textsuperscript{76} to eight\textsuperscript{98} times. Moreover, all of these studies were conducted with luciferase-expressing cells and evaluated metastasis formation via qPCR for luciferase. In our study we applied low doses of EVs per injection (an amount released from approximately 5 x 10\textsuperscript{5} cells) and repeated this injection every two days to recapitulate the
constant release of EVs into the circulation from tumor cells in the primary site. This dosage has been shown to be within the concentration range observed for circulating EVs in tumor-bearing mice \cite{71}. Our study is the first to compare the effect of EVs derived from parental and bone-seeking cells to those derived from brain-seeking cells in brain metastasis promotion and demonstrated a metastasis-promoting effect that was unique to Br-EVs. This comparison suggests that the observed effect was not a mere result of the high metastasis ability of tumor cells being mirrored in EVs as it was not observed in Bo-EV-treated mice.

Furthermore, our histological evaluation of brain metastatic lesions provides insights into the potential mechanisms underlying this EV-driven metastasis promotion. Our findings demonstrating the effect of Br-EVs on metastasis surface area but not the total number of metastatic foci, suggest that Br-EVs induce this effect by preparing a niche that promotes tumor growth following tumor cell seeding into the brain.
Chapter 3

Breast Cancer-Derived Extracellular Vesicles Breach the Intact Blood Brain Barrier via Transcytosis

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3.1. Abstract

Breast cancer-derived extracellular vesicles (EVs) have been shown to contribute to pre-metastatic niche preparation in the brain, however their ability to breach the blood brain barrier (BBB) and the mechanism(s) involved in this process remain unknown. Here, we demonstrate that brain metastasis-promoting breast cancer-derived EVs can breach an intact BBB \textit{in vivo} and using \textit{in vitro} and \textit{in vivo} models of the BBB, we have identified transcytosis as the mechanism by which breast cancer-derived EVs cross this barrier. High spatiotemporal resolution microscopy demonstrated that the endothelial recycling endocytic pathway is involved in this process. Moreover, our findings show that breast cancer-derived EVs decrease the brain endothelial expression of rab7, which can lead to an increase in the efficiency of their transport across the BBB. These findings reveal novel mechanisms by which breast cancer-derived EVs can contribute to pre-metastatic niche preparation at multiple levels to facilitate brain metastasis and provide potential early diagnostic and therapeutic targets for breast cancer brain metastasis.
3.2. Introduction

The unique vascular structure of the brain, the blood brain barrier has developed several protective mechanisms to eliminate unwanted molecules and pathogens from the brain parenchyma. This vascular barrier is primarily composed of brain endothelial cells, pericytes, and astrocyte end feet and tightly regulates the transportation of molecules to the brain. Brain endothelial cells form tight junction complexes that strengthen the attachments between adjacent endothelial cells. The endothelium is further reinforced through the crosstalk between endothelial cells and abluminal BBB cells such as astrocytes and pericytes. As a result, passive transport of molecules via the paracellular endothelial junctions is strictly limited to only those with a molecular weight of less than 400 Da. The restrictive nature of the BBB has created a major challenge in the understanding and treatment of a variety of brain disorders including primary and metastatic brain cancer.

Tumor-derived EVs that are released from the primary tumor into the circulation, can travel to distant organs, and modulate the microenvironment in pre-metastatic organs to facilitate future metastasis. However, in the context of brain metastasis, the mechanisms by which tumor-derived EVs of more than $10^3$ KDa in size, can interact with the restrictive BBB to promote a pre-metastatic niche in the brain is yet to be elucidated.

Here, we demonstrate for the first time that breast cancer-derived EVs can breach an intact blood brain barrier and we identify the mechanism driving this process. To overcome the challenges associated with studying the complex structure of the BBB, we investigated this process by using a combination of state-of-the-art in vitro and in vivo models of BBB. We demonstrate that these EVs cross the BBB through a transcellular transport mechanism. Moreover, we identify and characterize mechanisms by which tumor-derived EVs modulate the
endocytic pathway in brain endothelial cells to increase the efficiency of their transcellular transport. These findings are the first to elucidate the mechanistic events involved in the transport of EVs across the blood brain barrier and in doing so, provide novel insights that can inform and guide the design of successful BBB-crossing nanomedicines.

### 3.3. Materials and Methods

#### 3.3.1. Cell lines and cell culture

Human breast cancer cell line MDA-MB-231, and bone- and brain-seeking variants of these cells were obtained as described in Chapter 2. Primary human brain microvascular endothelial cells, human astrocytes, and human brain vascular pericytes were purchased from Cell Systems Co. (Cat # ACBRI 376, Kirkland, WA), Thermo Fisher Scientific Inc. (Cat # N7805100), and ScienCell Research Laboratories (Cat # 1200, Carlsbad, CA, USA), respectively. Breast cancer cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Cat # 11885084, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS, Cat # S11150, Atlanta Biologicals™, Atlanta, GA, USA) and 1% Penicillin-Streptomycin (10,000 U/mL) (Cat # 15140148, Thermo Fisher Scientific Inc.). Human astrocytes and brain pericytes were cultured according to the manufacturer’s instructions. All cells were maintained in a 37°C humidified incubator with 5% CO₂. All cultures were routinely monitored for mycoplasma contamination using the MycoAlert™ PLUS Mycoplasma Detection Kit (Cat # LT07-710, Lonza Inc.).

#### 3.3.2. EV isolation, characterization, and labeling

EV isolation and characterization was conducted as described in Chapter 2. For EV labeling, breast cancer cells were transduced with lentiviral vectors to express palmitoylated TdTomato (PalmtdTomato)¹⁵⁶ or membrane-bound Gaussia luciferase.¹⁴⁰ Both DNA constructs were gifts of Dr. X. Breakefield, Massachusetts General Hospital (MGH), and the lentivirus vectors were
made at the MGH Vector Core, Boston, MA. EVs were isolated from stable clones as described above. The presence of labels on EVs was confirmed via fluorescent microscopy for tdTomato and via luciferase assay for Gaussia luciferase.

3.3.3. In vitro EV uptake studies

To evaluate the uptake of EVs by brain ECs, astrocytes and pericytes, cells grown to confluence in 96-well plates were incubated with 2 x 10⁹ particle/well of tdTomato P- and Br-EVs. After 2 hours of incubation, cells were washed for 3 times and were fixed for imaging. Images from four different fields were taken using a Zeiss Fluorescent microscope and the level of fluorescence intensity was analyzed using the ImageJ software. To eliminate the confounding effect of cell size on the uptake level, fluorescent intensity for tdTom-EVs was measured per unit of cell surface area. For endocytosis inhibition studies, brain ECs cultured in 12-well plates were treated with chlorpromazine hydrochloride (Millipore Sigma, Cat # C8138, 20 µM), ML141 (100 µM, Millipore Sigma, Cat # 217708), 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) (100 µM, Tocris, Cat # 3378), cytochalasin D (500 nM, Tocris, Cat # 1233), and filipin III (10 µM, Millipore Sigma, Cat # F4767) for 30 min prior to addition of TdTom-Br-EVs (10¹⁰ particle/well). Following 3 hours of incubation with EVs, cells were washed and EV uptake was measured by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

3.3.4. In vitro transcytosis studies

3.3.4.1. Static BBB model

Transwell™ filters (0.4 µm pore polycarbonate membrane inserts, Cat # C3472, Corning™ Inc., MA) were coated with 50 µg/ml human plasma fibronectin (Cat # FC010, EMD Millipore) for 1 hour at 37°C. Brain ECs were cultured on filters (25 x 10³ cells per filter) and incubated for
48 hours to reach full confluency. At this time, the cells were fed with endothelial growth media supplemented with 8-(4-Chlorophenylthio) adenosine 3',5'-cyclic monophosphate (8-CPT-cAMP, 50 nM, Cat # ab120424, Abcam) and 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone Ro 20-1724 (17.5 nM, Cat # CAS 29925-17-5, Santa Cruz Biotechnology). To determine the integrity of the endothelial monolayer, 10 KDa Dextran, Alexa Fluor™ 647 (Cat 3 D22914, ThermoFisher Scientific) and 70 KDa Fluorescein isothiocyanate (FITC)–dextran (Cat# FD70S, Sigma Aldrich) were added to the upper chamber of the Transwell™ filters (100 µg/ml) and the fluorescence intensity in the media of the lower chamber was measured after 20 min using a SpectraMax M2 plate reader (Molecular Devices, Inc.). The apparent permeability coefficient was calculated for each tracer, as described previously.\textsuperscript{157}

To evaluate the transport of EVs using this model, Gaussia luciferase-labeled Br-EVs were added to the upper chamber (8 x 10\textsuperscript{9} particles in 100 µl of media). To evaluate the effect of temperature, filters were incubated at either 4°C or 37°C. To evaluate the effect of endocytosis, filters were pretreated with Dynasore hydrate (Millipore Sigma, Cat # D7693) for 30 min prior to adding the EVs and then incubated at 37°C. The media from the lower chamber was collected after 2 hours and luminescence intensity was measured as described before. To evaluate the intactness of EVs in the lower chamber media, the media collected from the lower chamber or Gaussia luciferase-labeled Br-EVs (as positive control) were run over an OptiPrep™ density gradient as described previously. After 4h of ultracentrifugation, different density fractions were isolated and luminescence intensity was measured for each fraction. To evaluate the effect of EVs on the integrity of the brain EC monolayer, filters were treated with either Br-EVs (8 x 10\textsuperscript{9} particles per filter) or with recombinant human vascular endothelial growth factor (R&D Systems Inc., Cat # 293-VE-010) as a positive control.\textsuperscript{88} After 2 hours of incubation, the
permeability of the filters to 10 KDa Alexa Fluor™ 647 Dextran and 70 KDa FITC–dextran was measured as described above.

3.3.4.2. Flow-based BBB Chip

Microfluidic BBB chips were prepared as reported previously.\textsuperscript{157} TdTom-Br-EVs with a concentration of $10^{11}$ particles/ml (for transcytosis studies) or a combination of unlabeled Br-EVs ($10^{11}$ particles/ml), 10 KDa Dextran, Alexa Fluor™ 647 (100 µg/ml) and 70 KDa FITC–dextran (100 µg/ml) (for permeability studies) were added to the lumen of the vascular channel at a flow rate of 100 µl/hour for 5 hours. Media from outlets of both the vascular and abluminal channels were collected separately at 3 hours and 5 hours and fluorescence intensity was evaluated using a BioTek plate reader and the Synergy Neo GEN5 2.09 software. Apparent permeability of the TdTom-Br-EVs and Dextran tracers under flow conditions were calculated using a previously reported formula.\textsuperscript{157} To measure the percentage of EVs that crossed the endothelial barrier, the number of particles in the media from the luminal and abluminal channels was calculated based on a standard curve created with TdTom-Br-EVs.

3.3.5. In vitro colocalization studies

Human brain endothelial cells were cultured on fibronectin-coated glass-bottom microslides (Ibidi, Cat # 80827). Confluent cells were used in these studies. Cells were co-incubated with TdTom-Br-EVs (8 x 109 particles/well) and 70 KDa FITC–dextran (0.5 mg/ml), Alexa Fluor™ 647-conjugated transferrin (50 µg/ml, Thermo Fisher Scientific, Cat # T23366), or DQ™ Ovalbumin (200 µg/ml, Thermo Fisher Scientific, Cat # D12053) for 30 min. Subsequently, cells were washed with PBS, 4 times and fixed with 4% formaldehyde for 10 min. For evaluation of co-localization with EEA1 and caveolin-1 (15 min incubation), or SNARE complexes (30 min incubation) cells were incubated with TdTom-Br-EVs (8 x 109 particles/well) and then washed
and fixed for staining with anti-EEA1 (1:100, Cell Signaling Technologies, Cat # 3288), anti-caveolin-1 (1:100, Cell Signaling Technologies, Cat # 3267), anti-VAMP-3 (1:100, Abcam, Cat # ab200657), anti-TGN46 (1:50, Abcam, Cat# ab2809), anti-VAMP-7 (1:100, Cell Signaling Technologies, Cat # 13786), anti-syntaxin4 (1:50, R&D Systems Inc., Cat # MAB7894), and anti-snap23 (1:100, R&D Systems Inc., Cat # AF6306) antibodies. For co-localization studies with Rab11, cells were initially transfected with GFP-rab11 plasmid using Lipofectamine 3000 reagent (Thermo Fisher Scientific). GFP-rab11 WT plasmid was a gift from Richard Pagano (Addgene plasmid # 12674 ; http://n2t.net/addgene:12674 ; RRID:Addgene_12674). Transfected cells were then cultured on microslides for incubation with TdTom-Br-EVs as described.

Epifluorescence microscopy was performed on a Leica microscope coupled to high-resolution objectives and a Hamamatsu Orca CCD (Japan). To quantify the colocalization of EVs with different markers, at least 10 different fields were evaluated for each experiment. Colocalization with rab11 and DQ™ Ovalbumin was quantified using a plugin for ImageJ developed by Jaskolski et al. Colocalization with VAMP-3 and VAMP-7 was quantified through manual counting of the percentage of the colocalized EV-containing endosomes.

3.3.6. Rab7 siRNA studies

Human brain endothelial cells (at 30% confluency) were treated with a pool of Rab7A siRNAs or non-targeting siRNAs (100 nM, Dharmaco, siGENO ME SMARTpool), using DharmaFECT 4 transfection reagent. Experiments were performed 72 hours after transfection. For imaging, cells cultured on microslides, were incubated with DQ™ Ovalbumin (200 µg/ml) or TdTom-Br-EVs (8 x 10⁹ particles/well) for 30 min. Subsequently, cells were washed with PBS, 4 times and fixed for staining with anti-Rab7 antibody (1:100, Abcam, Cat # 137029) and
anti-LAMP-1 antibody (1:100, Cell Signaling Technologies, Cat # 9091). Epifluorescence microscopy was performed as described previously. Using ImageJ, fluorescence intensity was measured in 6 fields for each condition and was normalized to autofluorescence intensity captured from empty microslides. For flow cytometry, transfected cells cultured in 12-well plates were incubated with TdTom-Br-EVs (10^10 particles/well) for 3 hours and cell uptake was quantified through flow cytometry as described above.

### 3.3.7. In vivo experiments

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of the Boston Children’s Hospital.

#### 3.3.7.1. Mouse studies

For all experiments, 6-8-week-old female Nu/Nu nude mice were purchased from Massachusetts General Hospital. At least 4 days of acclimation was conducted prior to the start of the experiments. For distribution studies, 3 µg of TdTom-Br-EVs in 100 µl of PBS were injected retro-orbitally. After 45 min, mice underwent transcardial perfusion with 25 ml of PBS. Brains were embedded in Tissue-Plus™ O.C.T. compound (Thermo Fisher Scientific) and frozen in liquid nitrogen. Frozen sections were immunostained with an anti-GFAP antibody (1:100, Abcam, Cat # 53554) and DAPI and evaluated for the uptake of Br-EVs by astrocytes, using a Zeiss Fluorescent microscope. To evaluate the integrity of the BBB during this experiment, a combination of 10 KDa Dextran, Alexa Fluor™ 647 (300 µg), and 70 KDa FITC Dextran (2 mg), with or without 3 µg of Br-EVs in 100 µl of PBS were injected retro-orbitally. Following 45 min, perfusion was performed with 25 ml of PBS. Collected brains were snap-frozen in liquid nitrogen for tissue lysate preparation. Brain tissue lysates were prepared in T-PER™ Tissue Protein Extraction Reagent supplemented with Halt™ protease inhibitor cocktail (Thermo
Scientific) using 0.9-2.0 mm stainless steel bead blend (Next Advance Inc.). Fluorescence intensity was measured using a SpectraMax M2 plate reader (Molecular Devices, Inc.) and was normalized to tissue weight. Homogenates form brain tissue of non-treated mice were used to measure the tissue autofluorescence.

3.3.7.2. Zebrafish studies

Tg(kdrl:GFP) zebrafish were used in this study. Embryos were incubated in E3 medium at 28.5°C and experiments were performed at 6-7 days post-fertilization (dpf). Embryos were anesthetized using tricane (160µg/ml, Sigma) and were mounted laterally in 0.8% low melting point agarose (ThermoFisher Scientific, Cat # 16520050). For transcytosis experiments, intracardiac injection of TdTom-Br-EVs (5 nL of a 400 µg/ml suspension per injection) was performed using the Narishige Injection System. One hour post-injection, live imaging of embryos was conducted using a Nikon Eclipse Ti inverted microscope with a Yokogawa spinning disk scan head and an Andor iXon EM-CCD camera. To evaluate the integrity of the BBB, intracardiac injection of 5 nL of a combination of unlabeled Br-EVs (400 µg/ml), 10 KDa Dextran, Alexa Fluor™ 647 (60 µg/ml) and 70 KDa Rhodamin B-Dextran (60 µg/ml, Thermo Fisher Scientific) was performed (n=3-6 zebrafish, 3 independent experiments) and z-stack images of the brain region were taken 1 hour post-injection. To quantify the permeability of the BBB, the mean fluorescence intensity of an intravascular area and the adjacent extravascular area were measured in 5 different locations in the brain of each zebrafish using the ImageJ software. The ratio of intravascular/ extravascular fluorescence intensity was calculated as a measure of BBB permeability.
3.3.8. Western blot analyses and ELISA

Cells were lysed with lysis buffer (Cell Signaling Technology, Danvers, MA), supplemented with Phenylmethylsulfonyl fluoride protease inhibitor. Following centrifugation of the lysates at 14,000 g for 10 min at 4°C, the supernatant was collected for western blot (30 μg total protein/lane). EV samples resuspended in PBS were directly used for western blot (15 μg total protein/lane). Protein concentration was measured using the Bradford method (Biorad laboratories, CA). Immunoblotting was conducted as reported previously. Antibodies against the following proteins were used for immunoblotting: CD63 (1:500, Abcam, Cat # 59479), CD9 (1:500, Cell Signaling Technologies, Cat # 13174), Alix (1:1000, Cell Signaling Technology, Cat # 2171S), and GM130 (1:1000, Cell Signaling Technologies, Cat # 12480), Rab 7 (1:1000, Abcam, Cat # 137029), Rab 11 (1:250, Cell Signaling Technology, Cat # 5589S).

3.3.9. Immunocyto/histochemistry

For immunocytochemistry, cells were fixed with 10% formalin for 10 min and then permeabilized with triton 0.1% triton X-100 for 5 min. For immunohistochemical staining, frozen sections were fixed with ice-cold acetone for 10 min. Blocking was performed using 3% bovine serum albumin for 30 min. Cells or tissue sections were incubated with the primary antibody for 1 hour at room temperature or overnight at 4°C, respectively. Following washes, cells or tissue sections were incubated with the relevant secondary antibody (1:200) for 1 hour. Sections were washed and mounted with Fluoro-gel mounting medium (Electron Microscopy Sciences). Images were taken using a Zeiss Axiocam fluorescent microscope.

3.3.10. Statistical analyses

All quantified data are presented as mean ± SD from 3 independent experiments. For animal experiments, the minimum number of animals required to obtain data amenable to statistical
analysis was used. Animals were randomly divided into groups. All statistical analyses were performed using the GraphPad Prism software. Statistical significance was considered at P values lower than 0.05. P values were shown as * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001. The methods of statistical analyses have been indicated in figure legends. All comparisons between two experimental groups were performed by unpaired two-tailed Student’s t-test. Comparisons between more than 2 groups were performed by one-way ANOVA with Tukey’s correction for multiple comparisons. Groups of data involving more than one variable were analyzed by two-way ANOVA with Sidak’s correction for multiple comparisons. All mouse experiments were evaluated using the Mann-Whitney test.

3.4. Results

3.4.1. Brain metastasis-promoting breast cancer EVs breach the BBB in vivo

As demonstrated in Chapter 2, Br-EVs exhibited the ability to promote brain metastasis formation in vivo. Brain metastases tend to grow along the brain vasculature, the BBB, suggesting that the microenvironment at the BBB serves as an initial niche for tumor cell growth. To study the interaction of breast cancer-derived EVs with the BBB for subsequent preparation of niche at this barrier, we first evaluated the uptake of P-, Bo-, and Br-EVs by the major components of the BBB. TdTomato-labeled EVs (TdTom-P-EVs, -Bo-EVs, and -Br-EVs) were incubated with primary human brain microvascular endothelial cells, brain pericytes, and astrocytes. Interestingly, astrocytes demonstrated a preferential uptake of the Br-EVs compared to P-EVs (Figure 3.1A,B). The ability of astrocytes to effectively take up Br-EVs suggested a prominent role for these cells in the Br-EV-driven facilitation of brain metastasis. Given the restrictive characteristics of the BBB, we next examined the ability of Br-EVs to breach the BBB in a mouse model. We performed retro-orbital injections of the TdTom-Br-EVs (3 μg per
mouse) and evaluated the distribution of EVs to the brain. Histological analyses demonstrated that Br-EVs were taken up by GFAP+ astrocytes (Figure 3.1C), confirming their ability to cross the BBB in vivo. The integrity of the BBB remained unaffected throughout the course of this experiment (Figure 3.1D).

Figure 3.1. Brain metastasis-promoting breast cancer EVs breach the BBB. (A) Representative fluorescent microscopy images (x200) and (B) quantification of the *in vitro* uptake of TdTom-EVs by the components of the BBB (mean ± SD; 3 independent experiments). Statistical analysis was performed using two-way ANOVA with Sidak’s multiple comparison tests. (C) Two representative fluorescence images of anti-GFAP immunostaining (green) of brain sections of mice that received retro-orbital injection of TdTom-Br-EVs (red). Arrows demonstrate Br-EVs taken up by astrocytes (x400, n=3 mice). (D) Average fluorescence intensity in perfused brain tissue homogenates collected 45 min following injection of a combination of PBS or Br-EV with 10 KDa Alexa647 dextran and 70 KDa FITC dextran (mean ± SD; n=3 mice per group). Statistical analysis was performed using Mann-Whitney test. In all panels, ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
3.4.2. Br-EVs cross the brain endothelium via transcytosis

To gain insight into the mechanism(s) by which Br-EVs breach the brain endothelium, we developed an in vitro static BBB model. Primary human brain endothelial cells rapidly lose their junctional features in culture and therefore cannot recapitulate the integrity of the in vivo BBB. It has been shown that with an increase in internal cAMP, these cells can regain their barrier characteristics. Accordingly, we developed a platform in which human brain endothelial cells cultured on transwell filters were treated with a combination of CPT-cAMP and Ro 20-1724, an inhibitor of cAMP degradation, to enhance the expression of junctional proteins such as ZO-1 (Figure 3.2A,B). This treatment resulted in an approximately 50% and 80% reduction in the permeability coefficient of the endothelial monolayer to 10 KDa Alexa 647 (post-treatment Papp $2.15E-6 \pm 4.964E-07$ cm/s) and 70 KDa fluorescein isothiocyanate (FITC) dextran (post-treatment Papp $1.933E-07 \pm 6.26E-08$ cm/s), respectively (Figure 3.2C). To determine whether the transfer of EVs across the brain endothelial monolayer is through an active or passive mechanism, Gaussia luciferase-labeled Br-EVs were incubated with brain endothelial cells in the luminal (top) chamber of the transwell filters for 2 hours at 37°C or 4°C. The amount of luminescent signal detected in the abluminal (lower) chamber was significantly lower when the filters were incubated at 4°C (Figure 3.3A), suggesting that a mechanism that is active in physiological conditions is involved in the transport of Br-EVs across the brain endothelial monolayer. Moreover, treatment of cells with Dynasore, an inhibitor of endocytosis, also resulted in a dose-dependent decrease in the abluminal signal (Figure 3.3B). The permeability of the barrier to 10 KDa and 70 KDa dextran was not changed by Br-EVs during this incubation (Figure 3.3C). To verify that the source of the detected signal in the abluminal chamber is the luciferase associated with intact EVs as opposed to free luciferase, we ultracentrifuged the media.
from the lower chamber on an iodixanol OptiPrep™ density gradient. As a positive control, Gaussia luciferase-labeled Br-EVs were added directly to the top of a gradient for ultracentrifugation. Consistent with our previous findings, in the positive control group, luminescent signal was detected at low- and high-density fractions, corresponding to EV density of 1.105-1.184 g/ml (Figure 3.3D). In the fractions collected from the media in the lower chamber, luminescent signal was detected in the high-density fraction, corresponding to EV density of 1.184 g/ml (Figure 3.3E), confirming the EV source of the signal. It should be noted that some signal was also detected in the supernatant, indicative of free luciferase that could have been released during the processing and degradation of a subpopulation of EVs. Interestingly,

**Figure 3.2. Characterization of the in vitro BBB model and the transcytosed EVs.** (A) Schematic showing static BBB model preparation and transcytosis experiments. (B) Representative images of brain endothelial cells immunostained with anti-ZO-1 antibody following treatment with cAMP and Ro 20-1724 (3 independent experiments). (C) Fold change in permeability coefficient of brain endothelial monolayer to 10 KDa (upper graph) and 70 KDa (lower graph) dextran following treatment with cAMP and Ro 20-1724 (mean ± SD; 3 independent experiments). Statistical analysis was performed using one-way ANOVA with Tukey’s correction for multiple comparisons. In all panels, ns, not significant; * \( P \leq 0.05 \); ** \( P \leq 0.01 \); *** \( P \leq 0.001 \).
electron microscopy analysis of the low- and high-density fractions of EVs showed that high-density Br-EVs generally had a smaller size with 68% being below 70 nm, whereas this percentage was 34% in the low-density EVs (Figure 3.3F,G). This finding suggests that a high-density subpopulation of EVs that are smaller in size can undergo a transcellular transport. Taken together, these findings in a static in vitro BBB model, suggested that the transport of Br-EVs across the brain endothelial monolayer relies on an active endocytic mechanism, indicative of transcytosis.

Figure 3.3. Br-EVs cross the brain endothelium via transcytosis in an in vitro static model of the BBB. (A) Fold change in luminescent signal in the media from abluminal chamber of a Transwell BBB model under the effect of temperature and (B) endocytosis inhibition (mean ± SD; 3 independent experiments). Statistical analyses were performed using (A) unpaired two-tailed Student’s t-test and (B) one-way ANOVA with Tukey’s multiple comparison test. (C) The effect of Br-EVs and VEGF (positive control) on the permeability coefficient of the endothelial monolayer to 10KDa and 70KDa dextran (mean ± SD; 3 independent experiments). Statistical analysis was performed using two-way ANOVA with Sidak’s multiple comparison tests. (D) Luminescent intensity of in density fractions following density gradient fractionation of luciferase-labeled Br-EVs (positive control). (E) Fold change in luminescence intensity of the density gradient fractions of the media from the abluminal chamber. Luminescent signal was normalized to that of the 30% fraction, which does not contain EVs. Fifteen, and 25% fractions correspond to EV density (mean ± SD; 3 independent experiments). (F) Electron microscopy images and (G) quantification of the size of EVs isolated from the low density (15% Optiprep) and high density (25% Optiprep) fractions. Statistical analysis was performed using Student’s t-test. In all panels, ns, not significant; * \( P \leq 0.05; ** \( P \leq 0.01; *** \( P \leq 0.001.

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To confirm that the static incubation of EVs with endothelial cells as well as the increase in internal cAMP in endothelial cells does not act as a confounding factor on the mechanism of EV transport, we verified these findings in a microfluidic organ-on-a-chip model of the BBB (BBB chip)\(^{157}\). The BBB chip is a 2-channel microfluidic culture device that contains a vascular channel lined by induced pluripotent stem cell-derived human microvascular endothelial cells, which is separated by a porous extracellular matrix-coated membrane from an abluminal channel containing primary human astrocytes and pericytes\(^{157}\). TdTom-Br-EVs were flowed through the lumen of the vascular channel for 5 hours. Fluorescent signal was detected in the abluminal chamber at 3 hours and increased significantly over time (Figure 3.4A), under conditions in which permeability of the barrier to 10 KDa and 70 KDa dextran did not change (Figure 3.4B). Moreover, fluorescence microscopic analysis revealed the presence of Br-EVs that were taken up by astrocytes in the abluminal chamber (Figure 3.4C). Overall, these findings demonstrated that Br-EVs can interact with endothelial cells under flow conditions and continuously cross the endothelial monolayer through transcytosis.

We next explored the transcytosis of Br-EVs \textit{in vivo}, using a zebrafish model. Zebrafish develop a mature BBB at 3 days post-fertilization (dpf) and serve as a suitable model for BBB studies\(^{165}\). We conducted intracardiac injection of TdTom-Br-EVs in 6-7-dpf Tg(kdrl:GFP) zebrafish embryos and monitored the distribution of Br-EVs in the brain through live imaging. At the time of imaging, Br-EVs were taken up by a number of cells within the brain parenchyma, demonstrating their ability to go beyond the BBB \textit{in vivo} (Figure 3.5A). Moreover, with time-lapse imaging, movement of EV-containing endocytic vesicles within endothelial cells could be observed. As shown in Figure 3.5A, a number of these vesicles moved toward the plasma membrane and fused with the membrane, suggestive of a transcytosis process. The integrity of
the BBB remained intact throughout the duration of these experiments, as treatment with Br-EVs did not increase the permeability of the BBB to 10 KDa and 70 KDa dextran in zebrafish (Figure 3.5B,C).

Figure 3.4. Br-EVs cross the brain endothelium via transcytosis in an in vitro microfluidic model of the BBB. (A) Time-dependent increase in fluorescent signal in the abluminal channel of an in vitro BBB chip (mean ± SD; 3 independent experiments). Statistical analyses were performed using unpaired t-test with Welch’s correction. (B) The effect of Br-EVs on the permeability of the BBB model to 10KDa and 70KDa dextran (mean ± SD; 3 independent experiments). Statistical analysis was performed using two-way ANOVA with Sidak’s multiple comparison tests. (C) Fluorescent microscopy images of TdTom-Br-EVs taken up by endothelial cells (left panel) and astrocytes (right panel) in the BBB-on-a-chip model. In all panels, ns, not significant; * P ≤ 0.05.
Figure 3.5. Br-EVs cross the brain endothelium via transcytosis in vivo. (A, upper panels) Representative fluorescent images of the zebrafish brain (area selected by black square), 1 hour after EV injection. White arrows demonstrate EVs in brain parenchyma. (A, lower panels) Time-lapse images of the interaction of Br-EV-containing endocytic vesicles (white arrows) with the endothelial abluminal plasma membrane (3 independent experiments). (B) Representative fluorescent images of dextran distribution in zebrafish brain vasculature. (C) Intravascular to extravascular ratio of fluorescence intensity in zebrafish brain following injection of dextran (mean ± SD; 10 KDa Dextran, 11 fish per group; 70 KDa Dextran, 14 fish per group; 3 independent experiments combined). Statistical analysis was performed using two-way ANOVA with Sidak’s multiple comparison tests. In all panels, ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. 
Together, these *in vitro* and *in vivo* findings demonstrate that a subpopulation of Br-EVs can breach the brain endothelial barrier through transcytosis, in a manner that does not compromise junctional permeability. This finding provides an opportunity to develop novel approaches to address the long-standing clinical challenge of delivering therapeutics to the brain. The previous attempts for brain delivery of a variety of antibody-based drugs have been associated with delivery rates of \(~ 0.1\%\).\textsuperscript{166,167} Comparable to these reports, in our BBB chip model, we found the efficiency of EV transfer across the endothelial barrier to be 0.16 \pm 0.0003\%, including the technical loss of EVs that usually occurs due to the entrapment of vesicles on the membrane between chambers or their uptake and processing by cells in the barrier. In the field of drug delivery, several advantages have been suggested for EVs as drug delivery vehicles compared to other currently available nanomedicines (e.g. lack of immunogenicity and enhanced distribution)\textsuperscript{168}. Our discovery of the inherent ability of tumor-derived EVs to breach an intact BBB via transcytosis provides an additional advantage to EV-based drug delivery to the brain and can potentially be generalized to a variety of EV types for such applications. Moreover, the observed association between the physical characteristics of EVs and their ability to breach the BBB underscores an important factor that should be taken into consideration in the development of future brain drug delivery approaches.

### 3.4.3. Br-EV transcytosis involves caveolin-independent endocytosis, recycling endosomes and basolateral SNAREs

We further explored the mechanistic details of Br-EV transport through brain endothelial cells by focusing on the three major steps of transcytosis: 1) endocytosis through the apical (luminal) membrane of brain endothelial cells, 2) transfer through the endocytic pathway, and 3) release into the extracellular environment from the basolateral (abluminal) membrane. To
evaluate the mechanism(s) of uptake, we pretreated brain endothelial cells with chemical inhibitors of the different endocytosis pathways and measured the uptake of TdTom-Br-EVs via flow cytometry. Inhibition of clathrin-dependent endocytosis by chlorpromazine and Cdc42/Rac1 GTPase inhibitor, ML141, significantly decreased the uptake of Br-EVs (Figure 3.6A). Inhibition of macropinocytosis by 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) and cytochalasin D also lead to a significant decrease in the uptake of Br-EVs. Further confirming these findings, Br-EVs partially colocalized with transferrin and 70 KDa dextran, markers of clathrin-dependent endocytosis and macropinocytosis, respectively (Figure 3.6B). Filipin, an inhibitor of caveolin-dependent endocytosis, showed no effect on Br-EVs uptake by endothelial cells (Figure 3.6A). A lack of co-localization of Br-EVs with caveolin also indicated that caveolin-dependent endocytosis is not involved in the uptake of Br-EVs by brain endothelial cells (Figure 3.6B). At the BBB, both clathrin- and caveolin-dependent endocytosis pathways have been shown to initiate the transcytosis of different macromolecules. Consistent with these well-defined mechanisms, our findings suggest the involvement of the clathrin-dependent pathway but not the caveolin-dependent pathway in the transcytosis of Br-EV across the BBB. Our findings also implicate the involvement of macropinocytosis in the uptake of Br-EVs by brain endothelial cells. However, the association of macropinocytosis and transcytosis of macromolecules is yet to be elucidated.

Next, we evaluated the intracellular trafficking of Br-EVs in brain endothelial cells. Upon endocytosis, the majority of molecules are transferred to early endosomes, where they are sorted to different routes. Molecules sorted into late endosomes are eventually transferred to lysosomes for degradation, whereas molecules sorted into recycling endosomes will be transferred to the plasma membrane. Rab11 recycling endosomes have been shown to be involved in the
transcytosis of macromolecules.\textsuperscript{175,176} As expected, following endocytosis, Br-EVs colocalized with EEA1, a marker of early endosomes\textsuperscript{177} (Figure 3.6C). To examine whether Br-EVs can proceed through the recycling route in the endocytic pathway for transcytosis, we evaluated the co-localization of Br-EVs with rab11, a marker of recycling endosomes.\textsuperscript{177} We found that Br-EV-containing vesicles colocalized with rab11 in the perinuclear region (62.9 ± 1.27\% colocalization of red and green fluorescent signal, Figure 3.6D,F). We also evaluated the trafficking of Br-EVs to the degradation route using BODIPY\textsuperscript{®}-conjugated DQ-Ovalbumin as a marker of endo-lysosomal structures.\textsuperscript{178,179} DQ-Ovalbumin is a self-quenched marker that only fluoresces upon the release of quenching following degradation in late endosomal structures and lysosomes.\textsuperscript{179} As expected, colocalization of a subset of Br-EVs with DQ-Ovalbumin was also observed in the perinuclear region (61.1 ± 6.4\% colocalization of red and green fluorescent signal, Figure 3.6E,F). The trans-Golgi network can also be involved in the transfer of molecules back to the plasma membrane.\textsuperscript{180} We found no colocalization of Br-EVs with TGN46, a marker of the trans-Golgi network, suggesting that this network is not involved in the intracellular trafficking of Br-EVs in brain endothelial cells (Figure 3.6G). These findings demonstrate that different subpopulations of Br-EVs can be sorted into different endocytic pathways that would lead to their recycling/transcytosis or degradation.
Figure 3.6 (Continued). Br-EV transcytosis involves caveolin-independent endocytosis, recycling endosomes and basolateral SNAREs. (A) Flow cytometry quantification of TdTom-Br-EV uptake by brain endothelial cells in the presence of chemical inhibitors of different pathways of endocytosis (mean ± SD; 3 independent experiments). Statistical analysis was performed using unpaired two-tailed Student’s t-test. (B) Representative fluorescence microscopy images of the colocalization of TdTom-Br-EVs with 70 KDa FITC Dextran (marker of macropinocytosis, left panel) and Alexa647 transferrin (marker of clathrin-independent endocytosis, middle panel), and caveolin 1 (right panel) from 3 independent experiments. The bottom panels show magnification of the area selected by white square. White arrows indicate colocalization. Scale bar, 25 µm. Representative fluorescence microscopy images of the colocalization of TdTom-Br-EVs with (C) eea1, (D) rab 11, (E) DQ-Ovalbumin, (G)TGN46, (H) VAMP-3, and (I) VAMP-7. The right panels show magnification of the area selected by white square. White arrows indicate colocalization. Scale bar, 25 µm. Quantification of the percentage of colocalized Br-EV-containing vesicles with rab11, DQ-Ovalbumin (F) and VAMP-3 and VAMP-7 (J) (mean ± SD; 3 independent experiments). Statistical analyses were performed using unpaired two-tailed Student’s t-test. (K,L) Representative fluorescence microscopy images of the colocalization of TdTom-Br-EVs with Syntaxin 4 (K) and Snap23 (L) from 3 independent experiments. The right panels show magnification of the area selected by white square. White arrows indicate colocalization. Scale bar, 25 µm. In all panels, ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
Rab11 recycling endosomes can transfer their contents both to the apical membrane for recycling/exocytosis as well as to the basolateral membrane for recycling/transcytosis of molecules. To evaluate the last step of EV transcytosis (i.e., the release of EVs on the abluminal side), we studied the factors involved in the interaction of EV-containing endosomes with the basolateral membrane. Soluble NSF Attachment Protein Receptors (SNARE) are known to be involved in vesicle fusion with the target membrane and include vesicle SNAREs (v-SNAREs) and target SNAREs (t-SNAREs). Among the different types of v-SNAREs, vesicle-associated membrane protein (VAMP)-3 is associated with recycling endosomes and is involved in exocytosis, whereas VAMP-7 is involved in the fusion of late endosomes with lysosomes. Our microscopy analyses demonstrated that Br-EV-containing vesicles colocalized with both VAMP-3 and VAMP-7 (Figure 3.6H-J). However, colocalization with VAMP-3 was significantly higher than VAMP-7, suggesting that recycling of Br-EVs was a prominent event in this case. The fusion of recycling endosomes with the basolateral plasma membrane, required for transcytosis of macromolecules, can occur through the interaction of VAMP-3 with SNAP23/Syntaxin 4, a t-SNARE complex localized on the basolateral membrane. Here, we found that Br-EV-containing endosomes colocalized with both SNAP23 and Syntaxin 4 (Figure 3.6K,L), demonstrating the involvement of these SNARE complexes in the fusion of these vesicles with the basolateral membrane. It is important to note that our findings do not exclude the possibility of the transfer of Br-EV-containing recycling endosomes to the apical membrane through other mechanisms, a matter of further investigation.

Taken together, these findings demonstrate that while a subpopulation of Br-EVs are sorted into late endosomes for degradation, a large subset of these EVs can be sorted into rab11+
recycling endosomes, which could lead to the VAMP3/Snap23/Syntaxin4-dependent release of these EVs at the basolateral membrane.

3.4.4. Br-EVs downregulate endothelial Rab7 to facilitate their transport

Under physiologic conditions, the rate of transcytosis in the BBB is maintained at a low level through different regulatory mechanisms. This characteristic of the BBB raised the question as to whether Br-EVs can facilitate their transcellular transport. We hypothesized that Br-EVs can specifically modulate the endocytic pathway in brain endothelial cells to increase their transport efficiency. We evaluated the effect of EV treatment on the two major routes in the endocytic pathway, degradation and recycling. Interestingly, we found that Br-EV treatment of brain endothelial cells significantly decreased the expression of the late endosomal marker, rab7, whereas the expression of rab11, marker of recycling endosomes, was not changed (Figure 3.7A-C). This finding demonstrated that Br-EVs exhibit a unique ability to modulate the degradation pathway in brain endothelial cells.

Rab7 is involved in the transfer of early endosomes to late endosomes and late endosomes to lysosomes. To determine whether the decrease in endothelial rab7 can lead to a decrease in the transfer of molecules to lysosomes, we used siRNA to knockdown the expression of rab7 in brain endothelial cells and treated the cells with DQ-Ovalbumin, which fluoresces upon being processed in late endo-lysosomal structures. Rab7 KD decreased the fluorescent signal from DQ-Ovalbumin, suggesting a decrease in the transfer of this molecule to late endosomes and lysosomes (Figure 3.7D,E). The total number of lysosomes as measured by the lysosomal marker, LAMP1, was not changed by Rab7 KD.

Rab7 can also interact with, and increase, the activity of rac1, a small GTPase protein that acts as a central regulator of actin remodeling. The activation level of Rac1 can control the
rate of endocytosis and rab7 can be indirectly involved in this process\(^{189,190}\). Accordingly, we hypothesized that the Br-EV-driven decrease in rab7 can indirectly affect the rate of EV endocytosis. Our flow cytometry studies demonstrated that rab7 KD significantly increased the uptake of Br-EVs by brain endothelial cells (Figure 3.7F,G). Fluorescent microscopy of Br-EV uptake by endothelial cells demonstrated that the pattern and the size of Br-EVs-containing endosomes were not different between Rab7 and control siRNA-treated cells (Figure 3.7H,I). This result confirms that increased signal detected by flow cytometry is due to increased uptake of Br-EVs rather than the accumulation of Br-EVs in lysosomal structures.

Taken together, these findings suggest that Br-EVs can increase their transport efficiency across the brain endothelial cells by decreasing the expression of rab7 in brain endothelial cells\(^{191}\). This decrease in rab7 expression can eventually increase the uptake of Br-EVs and disrupt the endocytic trafficking into the degradation path.
Figure 3.7. Br-EVs downregulate the endothelial Rab7 to facilitate their transport. (A-C) Western blot images and quantification of rab7 and rab11 expression in brain endothelial cells following treatment with EVs in vitro (mean ± SD; duplicates in 3 independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey’s multiple comparison test. (D,E) Representative fluorescent microscopy images and quantification of the effect of rab7 KD in brain endothelial cells (upper panel) on the transfer of DQ-Ovalbumin to lysosomes for degradation (middle panel) and the expression of LAMP1 lysosomal marker (lower panel) (mean ± SD; 3 independent experiments). Scale bar, 25 µm. Statistical analyses were performed using unpaired two-tailed Student’s t-test. (F) Western blot images of rab7 knockdown in brain endothelial cells. (G) Flow cytometry quantification of TdTom-Br-EV uptake by brain endothelial cells with or without rab7 KD (mean ± SD; 3 independent experiments). Statistical analyses were performed using unpaired two-tailed Student’s t-test. (H) Representative fluorescent microscopy images of TdTom-Br-EV uptake by rab7 KD brain endothelial cells (I) and quantification of the size of Br-EV-containing endosomal vesicles (mean ± SD; 3 independent experiments). Scale bar, 25 µm. Statistical analyses were performed using unpaired two-tailed Student’s t-test. In all panels, ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
3.5. Discussion

Homing of tumor-derived EVs to pre-metastatic organs has been described as an early event that leads to the preparation of a pre-metastatic niche for future metastasis\textsuperscript{68}. Here, we demonstrate for the first time that breast cancer-derived EVs can cross an intact BBB through transcytosis. Importantly, we have identified the mechanistic events that lead to tumor-derived EV transcytosis across the brain endothelium and the functional consequences of this transcellular transport on astrocytes. These findings expand our understanding of the early events in the process of pre-metastatic niche preparation prior to brain metastasis and provide opportunities for development of early diagnostics and therapeutics for brain metastasis.

Using static and flow-based \textit{in vitro} as well as \textit{in vivo} models of the BBB, we demonstrated that Br-EVs undergo a transcellular transport to enter the brain parenchyma, without disrupting the BBB. A leaky BBB, \textit{i.e.}, the blood-tumor-barrier, is one of the hallmarks of brain metastases\textsuperscript{192}. It has been reported that the integrity of the BBB is only disrupted following metastases growth, remaining unaffected even during the early micrometastasis stage\textsuperscript{192-194}. These reports along with our findings suggest that at least during the early stages of pre-metastatic niche preparation, the BBB remains intact. The timing of BBB disruption however, remains a matter of controversy. Recent studies have demonstrated that treatment with breast cancer-derived EVs can increase the permeability of BBB through downregulating ZO-1 expression and modulating actin localization\textsuperscript{75,76}. Variability in the methodology of EV treatment and evaluation of the permeability partly accounts for such contrasting results. Here, we have focused on the early stages of pre-metastatic niche preparation and demonstrated the ability of EVs to breach the BBB \textit{via} transcytosis prior to a disruption in the BBB integrity.
Interestingly, our density gradient fractionation studies suggested that a high-density subpopulation of EVs that are smaller in size have the ability to undergo transcytosis in brain endothelial cells. Previous studies have attempted to isolate EV subpopulations with different densities\(^{195,196}\). Consistent with our findings, one study found two distinct subpopulations of EVs with low and high density and showed that the high-density EVs were smaller in size\(^{195}\). This study also demonstrated that the two subpopulations had distinct protein and RNA profiles. More recently, using an asymmetric flow field-flow fractionation method a subpopulation of extracellular vesicles smaller than 50 nm were isolated and were introduced as exomeres\(^{35}\). Whether the subpopulation of EVs, for which transcytosis was observed in our studies, have similar characteristics to those of exomeres remains unknown.

We demonstrated that through downregulating rab7, Br-EVs can modulate the endocytic pathway in brain endothelial cells to increase the efficiency of their transport. This process occurred through two separate mechanisms. Downregulation of rab7 in brain endothelial cells disrupted the degradation route in the endocytic pathway through decreasing the transfer of molecules to lysosomes. This process might also enable endosomes to switch tracks to the recycling route. A supporting mechanism was described recently in a study that showed that knockdown of the NBEAL2 gene in megakaryocytes can disrupt the transport of fibrinogen to rab7 late endosomes and lysosomes\(^{197}\). This disruption of degradation increased the transfer of fibrinogen to rab11 recycling endosomes. Rab7 has also been shown to increase the activity of rac1\(^{189}\). Increased rac1 activity has been associated with an increase in the rate of macropinocytosis\(^{189}\) and a decrease in clathrin-mediated uptake of molecules such as epidermal growth factor and transferrin\(^{190}\). Interestingly, in our study, downregulation of rab7 in brain endothelial cells significantly increased the uptake of Br-EVs. Whether the observed increase in
uptake of Br-EVs is specific to the subpopulation that are taken up through clathrin-dependent endocytosis and can be subject to future transcytosis is a possibility that requires further investigation.

In summary, we have identified transeytosis as the mechanism by which breast cancer-derived EVs can breach the BBB. Our mechanistic studies indicate that EVs derived from a brain-seeking subpopulation of breast cancer cells can exclusively modify the physiological regulation of the BBB at multiple levels to promote metastasis development in the brain microenvironment. These findings provide new opportunities for early detection and therapeutic intervention in brain metastasis. Moreover, this pathological process can be further exploited to develop efficient drug delivery approaches for a variety of brain disorders including, but not limited to, brain malignancies and neurodegenerative diseases.
The functional consequences of breast cancer-derived extracellular vesicle transcytosis across the blood brain barrier

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4.1. Abstract

Breast cancer brain metastasis is a major clinical challenge and is associated with a dismal prognosis. Understanding the mechanisms underlying the early stages of brain metastasis can provide opportunities to develop more efficient diagnostics and therapeutics for this significant clinical challenge. We have previously reported that breast cancer-derived extracellular vesicles (EVs) breach the blood brain barrier (BBB) via transcytosis and can promote brain metastasis. Here, we elucidate the functional consequences of EV transport across the BBB. We demonstrate that brain metastasis-promoting EVs can be internalized by astrocytes and modulate the behavior of these cells to promote extracellular matrix remodeling \textit{in vivo}. We have identified protein and miRNA signatures in these EVs that can lead to the interaction of EVs with astrocytes and as such, have the potential to serve as novel targets for development of diagnostics and therapeutics for early detection and therapeutic intervention in breast cancer brain metastasis.
4.2. Introduction

During the early stages, brain metastases follow a vessel co-option pattern of growth and remain confined to the brain vasculature, the blood brain barrier (BBB)\textsuperscript{198}. As such, the microenvironment surrounding the BBB serves as an initial niche for metastatic tumor cells\textsuperscript{142}. Identifying the dynamic changes that occur in the microenvironment around the BBB prior to brain metastasis is essential to our understanding of the early mechanisms of brain metastasis.

It is widely acknowledged that tumor-derived extracellular vesicles (EVs) can promote tumor progression and metastasis formation. Once released into the circulation, theses nanoscale vesicles can transfer their contents including proteins, Lipids, DNA, and coding and non-coding RNA to stromal cells in distant organs\textsuperscript{68}. The resulting alterations in the behavior of these cells change the microenvironment in pre-metastatic organs in a manner that promotes future metastatic growth\textsuperscript{69,71,73}. In the brain, the role of tumor-derived EVs in the progression of primary brain tumors has been studied extensively, however the knowledge on their role in metastatic brain tumors is still limited\textsuperscript{199}. In Chapter 3, we demonstrated that EVs derived from brain-seeking breast cancers can breach an intact BBB via a transcytosis process and can be taken up by astrocytes in vivo. Importantly, we found that among the different cells in the BBB, astrocytes exhibit a preferential uptake of Br-EVs. This preferential uptake of Br-EVs by astrocyte is of potential mechanistic significance for the observed Br-EV-driven promotion of brain metastasis. Accordingly, in this Chapter, we sought to elucidate the mechanisms underlying this preferential uptake and the functional consequences associated with it.

We demonstrate that astrocyte uptake of breast cancer-derived EVs relies on the cdc42-dependent clathrin-independent carriers/GPI-AP enriched compartments (CLIC/GEEC) endocytic pathway. Using quantitative proteomics analysis, we demonstrate the enrichment of a
protein signature with the potential to interact with the CLIC/GEEC cargo. We next demonstrate that the uptake of Br-EVs by astrocytes changes the expression profile of astrocytes to prepare a tumor-supporting microenvironment at the BBB. We present data suggesting that at least one mechanism by which this process occurs is through alterations in the expression of extracellular matrix (ECM)-remodeling proteins by astrocytes. We further present data supportive of the role of miR-301a in EV-driven down-regulation of TIMP-2.

4.3. Materials and Methods

4.3.1. Cell lines and cell culture

Human breast cancer cell line MDA-MB-231 and their brain-seeking variants were obtained as described in Chapter 2. Primary human brain microvascular endothelial cells, astrocytes, and human brain vascular pericytes were purchased from Cell Systems Co. (Cat # ACBRI 376, Kirkland, WA), Thermo Fisher Scientific Inc. (Cat # N7805100), and ScienCell Research Laboratories (Cat # 1200, Carlsbad, CA), respectively.

Breast cancer cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Cat # 11885084, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS, Cat # S11150, Atlanta Biologicals™, Atlanta, GA, USA) and 1% Penicillin-Streptomycin (10,000 U/mL) (Cat # 15140148, Thermo Fisher Scientific Inc.). For extracellular vesicle (EV) isolation, breast cancer cells were cultured in Advanced DMEM supplemented with 10% EV-depleted FBS. EV-depleted FBS-containing medium was prepared as described previously. Human brain endothelial cells were cultured with endothelial cell growth medium (EGM™-2MV, Cat # CC-3202, Lonza Inc., ME, USA). Human astrocytes and brain pericytes were cultured according to the manufacturer’s instructions. All cells were maintained in a 37°C humidified incubator with
5% CO₂. All cultures were routinely monitored for mycoplasma contamination using the MycoAlert™ PLUS Mycoplasma Detection Kit (Cat # LT07-710, Lonza Inc.).

4.3.2. EV isolation and characterization

EVs were isolated and characterized as described in Chapter 2 and 3.

4.3.3. In vitro EV uptake studies

To evaluate the uptake of EVs by astrocytes for endocytosis inhibition studies, astrocytes were treated with chlorpromazine hydrochloride (Millipore Sigma, Cat # C8138, 20 µM), 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) (50 µM, Tocris, Cat # 3378), and filipin III (10 µM, Millipore Sigma, Cat # F4767), CDC42/Rac1 inhibitor, ML141 (100 µM, Millipore Sigma, Cat # 217708), and rac1 inhibitor, CAS 1177865-17-6 (10 µM, Millipore Sigma, Cat # 553502) for 30 min. Subsequently, TdTom-Br-EVs (10¹⁰ particle/ well in a 12-well plate) were incubated with astrocytes for 3 hours, following which, cells were washed and EV uptake was measured by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

To evaluate the colocalization of EVs with GPI-APs in astrocytes, cells were initially transfected with GFP-GPI plasmid using Lipofectamine 3000 reagent (Thermo Fisher Scientific). GFP-GPI WT plasmid was a gift of Dr. Anna-Katerina Hadjantonakis (Addgene plasmid # 32601; http://n2t.net/addgene:32601; RRID:Addgene_32601)²⁰⁰. Transfected cells were cultured on glass-bottom microslides (Ibidi, Cat # 80827) and were incubation with TdTom-Br-EVs (8 x 10⁹ particles/well) for 30 min. Subsequently, cells were washed 4 times with PBS and fixed with 4% formaldehyde for 10 min. Epifluorescence microscopy was performed on a Leica microscope coupled to high-resolution objectives and a Hamamatsu Orca CCD (Japan).
4.3.4. *In vitro* EV functional studies

To evaluate the direct effect of EVs on the expression profile of BBB cells, primary human brain ECs, astrocytes, and pericytes cultured in 12-well plates were treated with P- or Br-EVs for 48 h (25 µg EVs per treatment). Conditioned media were collected for downstream protein analyses. The amount of TIMP-2 was measured in conditioned media using a human TIMP-2 ELISA (R&D Systems Inc. Cat # DTM200) according to the manufacturer’s protocol. Cell lysates were used for RNA analyses.

For astrocyte migration studies following continuous EV treatment, astrocytes were trypsinized and were plated in Transwell filters (8 µm pores, Costar Transwell Assay; Corning Inc., Corning NY) in astrocyte serum-free media (2 x 10^4 cells in 100 µl media per filter). Filters were placed in 24-well plates containing 600 µl of complete astrocyte media. After 16 h, cells were fixed and stained with DAPI. Cells attached to the top of the filter were removed. Membranes were separated from the filters and were mounted on glass slides. The number of cells on the bottom of the filters were counted using a Zeiss Axiocam fluorescent microscope at 200X magnification (4 fields/filter, n=2 filters per treatment).

To assess any indirect effects of EVs on astrocytes, brain ECs were initially treated with P- or Br-EVs for 3 consecutive days (5 µg EVs per treatment), as previously described^{150}. After treatment, EC conditioned media was collected. Astrocytes cultured in 6-well plates were incubated with EC conditioned media for 48 hours, following which the cells were serum starved overnight and the astrocyte conditioned media was collected for analysis. TIMP-2 levels were measured using a human TIMP-2 ELISA as described above.
4.3.5. Validation of EV surface proteins

Tdtomato P-EVs and Br-EVs (10^{10} particles in 100 µl PBS) were incubated with 5 µg/ml of fluorescent-conjugated antibodies: FITC anti-human CD73 antibody (BioLegend, Cat # 344015), FITC anti-human uPAR antibody (Sino Biological, Cat # 10925-MM09-F), Alexa Flour® 488 anti-human ICAM1/CD54 antibody (BioLegend, Cat # 322713), Alexa Flour® 488 anti-human CD29 antibody (BioLegend, Cat # 303015), FITC anti-human CD49b antibody (BioLegend, Cat # 359305), and Alexa Flour® 488 anti-human CD63 antibody (BioLegend, Cat # 353037), Alexa Flour® 488 mouse IgG1, κ isotype control (BioLegend, Cat # 400132), and FITC mouse IgG1, κ isotype control (BioLegend, Cat # 400107). Following a 2-h incubation at room temperature, EVs were washed through ultracentrifugation to remove the free antibodies. Pellets were resuspended in PBS and fluorescence intensity was measured using a SpectraMax M2 plate reader. The fluorescence intensity of FITC or Alexa Flour® 488 was normalized to that of TdTomato for both antibodies and isotype controls. The normalized measurements of antibodies were then subtracted from those of isotype controls.

4.3.6. Proteomics analysis

Quantitative proteomics analysis was performed using the isobaric tags for relative and absolute quantitation (iTRAQ) technique as our Lab has described previously. For protein identification, the peak list was searched against the Swiss-Prot database including all human proteins. The confidence score of >1.3, which corresponds to 95% peptide confidence level, was used for analysis. Hierarchical clustering of samples and features was done using the Unweighted Pair Group Method with Arithmetic-mean (UPGMA) method with Pearson’s correlation as the distance measure. The expression data matrix was row-normalized prior to
the application of average linkage clustering. Functional enrichment analysis of the proteins that were enriched in Br-EVs was performed using the FunRich software.

4.3.7. In vivo experiments

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of the Boston Children’s Hospital.

Six to eight-week-old female Nu/Nu nude mice were purchased from Massachusetts General Hospital. Following 4-7 days of acclimation, mice were randomly divided into 3 groups and received retro-orbital injections of PBS, P-EVs or Br-EVs (3 µg in 100 µl PBS per injection). Injections were repeated every two days for a total of 10 injections, following which the mice were sacrificed and brain tissue was collected for analysis. For each brain, the left hemisphere was snap-frozen in liquid nitrogen. Tissue homogenates were prepared as described above. The expression of MMPs and TIMPs were evaluated using ELISA for MMP-2 (R&D Systems Inc. Cat # MMP200), MMP-9 (R&D Systems Inc. Cat # MMPT90), MMP-14 (Lifespan Biosciences Inc., Cat # LS-F7353), TIMP-1 (R&D Systems Inc. Cat # MTM100), and TIMP-2 (Abcam, Cat # ab100746). All kits were mouse-specific, except for the MMP-2 ELISA kit that could recognize both human and mouse MMP-2. All assays were conducted according to manufacturer’s protocol. The right hemisphere was fixed in 10% formalin. Formalin-fixed and paraffin-embedded tissue sections were analyzed using anti-TIMP-2 antibody (1:1000, Servicebio, Cat # GB11523) and anti-GFAP antibody (1:1000, Servicebio, Cat # GB11096). Immunohistochemistry was conducted as previously described.

To evaluate the integrity of the BBB during this experiment, the experiment was conducted as described above. Following the 3-week EV treatment, at the time of sacrifice, mice received a retro-orbital injection of a combination of 10 KDa Dextran, Alexa Fluor™ 647 (300 µg), and 70
KDa FITC Dextran (2 mg), in 100 µl of PBS. Following 45 min, perfusion was performed with 25 ml of PBS. Collected brains were snap-frozen in liquid nitrogen for tissue lysate preparation. Brain tissue lysates were prepared in T-PER™ Tissue Protein Extraction Reagent supplemented with Halt™ protease inhibitor cocktail (Thermo Scientific) using 0.9-2.0 mm stainless steel bead blend (Next Advance Inc.). Fluorescence intensity was measured using a SpectraMax M2 plate reader and was normalized to tissue weight. Homogenates from brain tissue of non-treated mice were used to measure the tissue autofluorescence.

4.3.8. miRNA target validation

For target validation, astrocytes were transfected with dual luciferase reporters (TIMP-2 and control clones, GeneCopoeia™, Cat # HmiT018093-MT06, and CmiT000001-MT06, respectively). Following 48 hours, cells were transfected with miRNA-301a-3p mimics (50 nM miRIDIAN, Dharmacon Inc.) using Dharmafect 4 transfection reagent. Luciferase assays were conducted 48 h after mimic treatment, with the Luc-Pair™ Duo-Luciferase HS Assay Kit (GeneCopoeia™), according to manufacturer’s instructions. For functional evaluation of miR-301a-3p, astrocytes were treated with 50 nM miRNA-301a-3p mimics for 48 h after which RNA was isolated for analysis.

4.3.9. RNA isolation and analysis

RNA isolation from EV samples, astrocytes, and brain tissue was conducted using the miRNeasy kit (Qiagen), according to manufacturer’s protocol. Brain tissues were homogenized in Qiazol reagent using the stainless-steel bead blend, as described in Chapter 3. For analysis of TIMP-2 mRNA expression, mature miRNA expression and miRNA precursor analyses, we used the SuperScript™ VILO™ cDNA Synthesis Kit and the SYBR™ Green PCR Master Mix (ThermoFisher Scientific), miRCURY LNA RT Kit and SYBR Green PCR kit (Qiagen), and the
miScript II RT kit and SYBR Green PCR kit (Qiagen), respectively. The following primers were used for these studies: PrimePCR™ SYBR® Green Assay: TIMP2, Human (Bio-Rad, assay ID qHsaCID0022953); miRCury LNA miRNA PCR assays (U6 snRNA-hsa, hsa-miR-301a-3p, hsa-miR-301b-3p) and Hs_miR-301a_1_PR miScript precursor assay, and Hs_miR-301a_1 and Hs_RNU6-2_11 miScript primer assays (Qiagen).

4.3.10. Statistical analyses

Statistical analyses were performed using the GraphPad Prism software. All quantified data are presented as mean ± SD from 3 independent experiments. Statistical significance was considered at $P$ values lower than 0.05. $P$ values were shown as * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. The methods of statistical analyses have been indicated in figure legends. All comparisons between two experimental groups were performed by unpaired two-tailed Student’s t-test. Comparisons between more than 2 groups were performed by one-way ANOVA with Tukey’s correction for multiple comparisons. Groups of data involving more than one variable were analyzed by two-way ANOVA with Sidak’s correction for multiple comparisons. For in vivo experiments, the minimum number of animals required to conduct statistical analysis were included in the study and were randomly assigned into experimental groups. All in vivo experiments were evaluated using the Mann-Whitney test. Correlation analyses were conducted via Pearson’s correlation test.

4.4. Results

4.4.1. Uptake of breast cancer-derived EVs is mediated through CLIC/GEEC pathway

Breast cancer-derived EVs were isolated from parental MDA-MB-231 breast cancer cell line (P-EVs) as well as brain-seeking variants of these cells (Br-EVs). Breast cancer-derived EVs were characterized according to the guidelines suggested by the International Society for
Extracellular Vesicles, as described in Chapter 2 (Figure 4.1A)\textsuperscript{31}. We have previously demonstrated that, following their breaching of the BBB, Br-EVs are taken up by astrocytes \textit{in vivo}\textsuperscript{150}. Br-EVs, but not P-EVs, had the ability to promote brain metastasis. Moreover, our \textit{in vitro} studies demonstrated that astrocytes exhibited a preferential uptake of Br-EVs as compared to P-EVs\textsuperscript{150}.

To study the mechanisms underlying the specific uptake of Br-EVs by astrocytes, we first explored the possibility of the involvement of specific endocytosis mechanism(s) in the uptake of Br-EVs. Endocytic pathways such as macropinocytosis, clathrin-dependent and caveolin-dependent endocytosis, have been commonly reported to be involved in the uptake of EVs by different cell types\textsuperscript{204}. Interestingly, using different chemical inhibitors of endocytosis pathways, we found that none of these common pathways were involved in the uptake of EVs by astrocytes (Figure 4.1B). This finding was also in contrast to our previous findings demonstrating the involvement of macropinocytosis and clathrin-dependent endocytosis in the uptake of Br-EVs by brain endothelial cells\textsuperscript{150} and emphasizes on the cell-type dependency of EV uptake mechanisms.

We next, explored the role of clathrin-caveolin-independent pathways in EV uptake, focusing on rac1 and CDC42, two major players in this process\textsuperscript{170}. We found that a Cdc42/Rac1 GTPase Inhibitor, ML141, significantly decreased the uptake of both types of EVs by astrocytes, whereas a specific Rac1 inhibitor, CAS 1177865-17-6, had no effect on their uptake, suggesting that CDC42, but not Rac1, is involved in the uptake of breast cancer-derived EVs (Figure 4.1B).
CDC42 is known to be involved in the endocytosis of glycosylphosphatidylinositol-anchored proteins (GPI-APs), via the clathrin-independent carriers/GPI-AP enriched compartments (CLIC/GEEC) pathway. To evaluate whether EV uptake by astrocytes occurs through the CDC42-dependent CLIC/GEEC pathway, we transfected astrocytes with a GFP-fused GPI construct. High spatiotemporal resolution microscopy demonstrated a large colocalization of TdTomato-labeled EVs with GPI (Figure 4.1C), confirming that the breast cancer-derived EVs share the endocytic pathway with GPI-APs.

**Figure 4.1.** Astrocyte internalize breast cancer-derived EVs through the CLIC/GEEC pathway. (A) Electron microscopy images of EVs isolated from parental and brain-seeking MDA-MB-231 breast cancer cells (P-EV and Br-EV, respectively). (B) Flow cytometry quantification of TdTom-EV uptake by astrocytes treated with chemical inhibitors of endocytosis pathways (mean ± SD; 3 independent experiments). Statistical analysis was performed using unpaired two-tailed Student’s t-test (** P ≤ 0.01; *** P ≤ 0.001). (C) Representative fluorescence microscopy images of the colocalization of TdTom-EVs with GFP-fused GPI in astrocytes from 3 independent experiments. Scale bar, 25 µm.
Taken together, these findings demonstrated that the uptake of breast cancer-derived EVs by astrocytes is mediated through the CDC42-dependent CLIC/GEEC endocytosis pathway. Moreover, the observed similarity in the mechanism of uptake of P-EVs and Br-EVs suggests the mechanism of endocytosis is not the underlying reason for the observed preferential uptake of Br-EVs by astrocytes.

4.4.2. **Br-EVs are enriched in interacting partners of the CLIC/GEEC cargo**

We hypothesized that a particular combination of proteins highly enriched in Br-EVs could drive the specific uptake of Br-EVs by astrocytes. To identify the proteins with differential expression in Br-EVs compared to P-EVs, we performed quantitative mass spectrometry using the isobaric tag for relative and absolute quantitation (iTRAQ) technique on the two types of breast cancer-derived EVs. Among a total of 126 proteins detected with >95% confidence, 14 proteins were significantly enriched \( (P \leq 0.05) \) in Br-EVs compared to P-EVs (Figure 4.2A). Enrichment analysis using the FunRich software\(^{203}\) demonstrated that the majority of these proteins belonged to receptor activity and cell adhesion categories (Figure 4.2B), supporting our hypothesis of their involvement in the specific interaction between Br-EVs and astrocytes. We validated and quantified the surface localization of these proteins on P- and Br-EVs through staining of the intact EVs (Figure 4.2C).

Interestingly, a number of the surface proteins enriched in Br-EVs, have been identified previously as cargoes associated with the CLIC/GEEC pathway. While GPI-APs are the most studied cargo of the CLIC/GEEC pathway, a variety of other proteins, predominantly adhesion factors, have also been identified as the cargo of this endocytosis route. These include integrin \( \beta 1, \) galectin 3, CD44, and CD98\(^ {205-207}\). Moreover, it has been shown that ICAM1 binding to integrins can induce nucleation and colocalization of integrin clusters and GPI-APs\(^ {207}\). We
demonstrated that Br-EVs were enriched in Ecto-5'-nucleotidase or CD73 and urokinase plasminogen activator receptor (uPAR), well-known GPI-interacting proteins\(^{208,209}\), as well as, integrin \(\beta 1\) and integrin \(\alpha 2\). Both types of EVs had similar expression of ICAM1 on their surface (Figure 4.2C). Together, these findings identify a combination of surface proteins enriched in Br-EVs that have the potential to interact with GPI-AP clusters and therefore, can be responsible for the preferential uptake of these EVs by astrocytes.

**Figure 4.2. Br-EVs are enriched in interacting partners of the CLIC/GEEC cargo.** (A) Heatmap visualization of quantitative proteomics analyses, demonstrating the significantly enriched proteins \((P \leq 0.05)\) in Br-EVs vs. P-EVs (red demonstrates enrichment in Br-EVs). (B) Functional enrichment analysis of proteins enriched in P-EVs (blue) and Br-EVs (orange). (C) Quantification of surface localization of membrane-associated proteins enriched in Br-EVs, CD63 serves as positive control (mean ± SD; 3 independent experiments). Statistical analysis was performed using unpaired two-tailed Student’s t-test \((*P \leq 0.05)\).
4.4.3. Br-EVs decrease the astrocyte expression of TIMP-2

We next studied the functional consequences of Br-EV uptake by astrocytes in vivo. We hypothesized that upon transcytosis through the brain endothelium, Br-EVs can change the behavior of astrocytes to prepare a microenvironment supportive of tumor cell growth. It is widely acknowledged that matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) can contribute to tumor progression and metastasis \(^{88,93,210,211}\). Through modulating the ECM, these enzymes can trigger different signaling pathways and promote the tumor-supporting microenvironment \(^{68,212}\). Several studies have demonstrated a prominent role for MMPs and TIMPs in preparation of a niche for tumor cell growth in the brain \(^{213-217}\). While these studies predominantly focus on tumor cell-derived MMPs and TIMPs, astrocyte conditioned media was shown to modulate the tumor cell expression of MMPs. Moreover, astrocyte-derived MMP-2 and MMP-9, have also been shown to promote tumor cell invasion in breast cancer brain metastasis \(^{218}\). Accordingly, we postulated that Br-EVs can alter the expression of MMPs and TIMPs in astrocytes to facilitate brain metastasis. To address this hypothesis, we performed retro-orbital injections of P-EVs and Br-EVs (3 µg in 100 µl PBS per injection) in mice, every two days for a total of 10 injections, following which the mice were sacrificed to analyze the brain tissue (Figure 4.3A). Using mouse-specific enzyme-linked immunosorbent assays (ELISA), we analyzed the expression of a number of MMPs and TIMPs that are known to be involved in ECM remodeling in brain tissue \(^{219}\), including MMP-2, MMP-9, MMP-14, TIMP-1, and TIMP-2 in mouse brain tissue homogenates (Figure 4.3B-F). Interestingly, we found that TIMP-2, the endogenous inhibitor of MMP-2 activity \(^{220}\), was exclusively and significantly decreased by brain metastasis-promoting Br-EVs but not P-EVs (Figure 4.3C).
We investigated whether astrocytes could be the source of the Br-EV-driven decrease in brain TIMP-2. We treated human BBB cells, endothelial cells, pericytes, and astrocytes, with P-EVs and Br-EVs in vitro and evaluated TIMP-2 expression using a human-specific TIMP-2 ELISA. EV treatment did not affect the expression of TIMP-2 in brain endothelial cells (Figure 4.3G). Both P-EVs and Br-EVs significantly decreased the expression of TIMP-2 in astrocytes (Figure 4.3G). Moreover, Br-EVs were able to increase the migration of astrocytes, which is consistent with the observed decrease in TIMP-2 expression and a subsequent increase in ECM modulation (Figure 4.3H). This finding is consistent with our hypothesis that Br-EVs can change the behavior of astrocytes. To rule out the possibility that the decreased astrocyte TIMP-2 might be an indirect effect of Br-EVs acting through brain endothelial cells, we prepared conditioned media by treating brain endothelial cells with EVs or PBS. Astrocytes were incubated with the endothelial cell conditioned media for 48 h, followed by the media exchange and subsequent analysis of the astrocyte conditioned media. No difference in TIMP-2 levels was found in conditioned media from astrocytes that were incubated with PBS-, P-EV-, or Br-EV-treated endothelial cell conditioned media (Figure 4.3I). In addition, we stained consecutive brain tissue sections for TIMP-2 and the astrocyte marker, GFAP, and found that areas that were rich in astrocytes also had a high expression of TIMP-2, further supporting astrocytes as the major source of TIMP-2 (Figure 4.3J). We then evaluated whether the observed decrease in astrocyte TIMP-2 levels following Br-EV treatment were accompanied by alterations in the permeability of the BBB. No increase in permeability of brain endothelium to 10 KDa and 70 KDa-dextran was observed following P- or Br-EV treatment (Figure 4.3K). This observation suggested that the BBB remained intact during this experiment, supporting the conclusion that the EV-induced decrease in TIMP-2 was a direct effect of transcytosed Br-EVs on astrocytes.
Overall, these findings indicate that the transcytosis of Br-EVs and their subsequent uptake by astrocytes can have functional consequences, such as suppressed TIMP-2 expression that can lead to the preparation of a microenvironment at the BBB suitable for metastases growth.
Figure 4.3 (Continued). Br-EVs decrease the astrocyte expression of TIMP-2. (A) Schematic showing the EV functional study design. Average concentration of (B) TIMP-1, (C) TIMP-2, (D) MMP-2, (E) MMP-9, and (F) MMP-14 in brain tissue homogenates measured by ELISA (mean ± SD; n= 6 mice per group). Statistical analysis was performed using Mann-Whitney test. (G) Average fold change in concentration of TIMP-2 in conditioned media of brain endothelial cells, pericytes and astrocytes treated with PBS, P-, and Br-EVs (mean ± SD; 3 independent experiments). Statistical analysis was performed using two-way ANOVA with Sidak’s multiple comparison tests. (H) Fold change in the number of migrated astrocytes in a transwell migration assay following pre-treatment with PBS, P- or Br-EVs (mean ± SD; 3 independent experiments). Statistical analysis was performed using one-way ANOVA with Tukey’s test for multiple comparison. (I) Fold change in the concentration of TIMP-2 in astrocyte conditioned media following treatment with conditioned media from PBS-, P-EV, and Br-EV-treated endothelial cells (mean ± SD; 3 independent experiments). Statistical analysis was performed using one-way ANOVA. (J) Representative images of mouse brain sections immunostained with anti-GFAP (upper panels) and anti-TIMP-2 (lower panels). Middle panels represent a colormap of areas of protein enrichment (3 independent experiments). Scale bar, 200 µm. (K) Average fluorescence intensity in perfused brain tissue homogenates collected 45 min following injection of a combination of 10 KDa Alexa647 dextran and 70 KDa FITC dextran (mean ± SD; n=3 mice per group). Statistical analysis was performed using Mann-Whitney test. In all panels, ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
4.4.4. miR-301a-3p transferred by breast cancer-derived EVs downregulate TIMP-2 in astrocytes

To determine the EV factors driving the decrease in TIMP-2, we examined the role of EV miRNAs in this process. Previous reports have identified a number of miRNAs with the ability to target the 3’UTR of TIMP-2 mRNA in tumor cells, including miR-106a, miR-761, and miR-301a. Interestingly, in a whole miRNome analysis conducted by our group (data not shown), treatment of brain endothelial cells by breast cancer-derived EVs increased the miR-301a-3p levels, suggesting the ability of breast cancer-derived EVs to transfer this miRNA into recipient cells. This observation prompted us to investigate the potential role of miR-301a-3p in the observed EV-driven down-regulation of TIMP-2 in astrocytes. Computational target prediction tools (www.miroRNA.org) demonstrated perfect complementarity between the miR-301a-3p seeding sequence and the TIMP-2 3’ UTR (Figure 4.4A). To examine the ability of miR-301a-3p to physically interact with the 3’ UTR of TIMP-2 in astrocytes, we transfected the cells with a dual luciferase reporter vector of TIMP-2 3’ UTR or a control vector. miR-301a-3p mimic significantly decreased the luminescence activity in the TIMP-2 3’UTR-transfected cells, validating TIMP-2 as a target for miR-301a-3p (Figure 4.4B). Treatment of astrocytes with miR-301a-3p mimic also led to a decrease in endogenous TIMP-2 mRNA levels (Figure 4.4C), demonstrating the functionality of this miRNA in astrocytes.

To examine whether breast cancer-derived EVs carry this miRNA, we measured the miR-301a-3p levels in P- and Br-EVs and found that both types of EVs carried similar amounts of this miRNA (Figure 4.4D). To determine the ability of breast cancer-derived EVs to transfer this miRNA to astrocytes we treated astrocytes with EVs and measured the alterations in the levels of miR-301a-3p in astrocytes. Treatment of astrocytes with P- and Br-EVs led to an increase in
miR-301a-3p, demonstrating the transfer from EVs to astrocytes (Figure 4.4E). Furthermore, the levels of primary and precursor miR-301a was not changed following EV treatment, confirming that the observed increase in mature miRNA was not due to upregulation of endogenous miRNA in astrocytes and was a result of direct transfer from EVs. As expected, this increase in miR-301a-3p was associated with a down-regulation of TIMP-2 mRNA (Figure 4.4F). Together, these findings demonstrated that breast cancer-derived EVs transfer miR-301a-3p to astrocytes, which can then directly target and downregulate TIMP-2 in these cells.

To evaluate the ability of breast cancer-derived EVs to transfer this miRNA to the brain \textit{in vivo}, we analyzed the level of miR-301a-3p in brain tissues collected from the \textit{in vivo} experiment described above. It is important to note that the conserved and identical sequence of miR-301a-3p in mouse and human limited our ability to detect and analyze the direct transfer of miR-301a-3p by human breast cancer-derived EVs. Nevertheless, an increasing trend in the levels of miR-301a-3p was observed in mice that were treated with Br-EVs (Figure 4.4G). Importantly, the level of miR-301a-3p was significantly and negatively correlated with the level of TIMP-2 in Br-EV-treated mice, whereas this correlation was not observed in P-EV-treated mice (Figure 4.4H, I). These studies, demonstrated the role of miR-301a-3p in the observed down-regulation of TIMP-2 \textit{in vivo}. Given that Br-EV-driven down-regulation of astrocyte TIMP-2 can occur prior to brain metastasis formation, miR-301a-3p has the potential to serve as a diagnostic marker for early stages of brain metastasis. Interestingly, analysis of 1262 patients in the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium \textsuperscript{224}) dataset, demonstrated that higher levels of miR-301a-3p were significantly associated with decreased survival (www.kmplot.com, Figure 4.4J).
Figure 4.4. miR-301a-3p in breast cancer-derived EVs downregulates astrocyte TIMP-2. (A) Complementarity between the seeding sequence of miR-301a-3p and the 3’ UTR of TIMP-2. (B) Dual luciferase reporter assay to determine the physical interaction between miR-301a-3p and TIMP-2 3’ UTR (normalized to Renilla luciferase activity, mean ± SD; 3 independent experiments). Statistical analysis was performed using unpaired two-tailed Student’s t-test. (C) TIMP-2 mRNA levels in astrocytes following treatment with miR-301a-3p mimic (normalized to GAPDH, mean ± SD; 3 independent experiments). Statistical analysis was performed using unpaired two-tailed Student’s t-test. (D) Levels of miR-301a-3p in P-EVs and Br-EVs, measured against a standard curve created by miR-301a-3p mimic (mean ± SD; 3 independent experiments). Statistical analysis was performed using unpaired two-tailed Student’s t-test. (E) Level of pri/pre or mature miR-301a in astrocytes following treatment with EVs (normalized to U6 expression, mean ± SD; 3 independent experiments). Statistical analysis was performed using two-way ANOVA with Sidak’s multiple comparison tests. (F) TIMP-2 level in astrocytes following treatment with EVs (normalized to GAPDH, mean ± SD; 3 independent experiments). Statistical analysis was performed using two-way ANOVA with Sidak’s multiple comparison tests. (G) Level of miR-301a-3p in brain tissue lysates (normalized to U6 levels, mean ± SD; n= 6 mice per group). Statistical analysis was performed using Mann-Whitney test. (H) and (I) Correlation analysis between miR-301a-3p and TIMP-2 levels in brain tissue lysates in mice treated with P-EVs (H) and Br-EVs (I) (n= 6 mice per group). Correlation coefficient was measure using Pearson’s correlation analysis. (J) Kaplan Meier curve demonstrating the association of miR-301a-3p levels with survival in breast cancer patients from the METABRIC dataset. In all panels, ns, not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
4.5. Discussion

In this study, we have elucidated the functional consequences of transcellular transport of breast cancer-derived EVs across the BBB, with a focus on the interaction of these EVs with astrocytes. We identified a series of mechanisms through which EVs are internalized by and modulate the behavior of astrocytes to promote a microenvironment supportive of metastasis growth.

We demonstrated that astrocytes internalize breast cancer-derived EVs through the specific cdc42-dependent CLIC/GEEC pathway. To the best of our knowledge, this study is the first to report the uptake of EVs through this endocytosis pathway\textsuperscript{204,225}. Interestingly, it has been shown that adeno-associated viruses can hijack the CLIC/GEEC pathway to gain entry into the cells\textsuperscript{226}. These findings are consistent with a previous report of EVs using the virus entry machinery to enter the cells\textsuperscript{227}. Cells can internalize EVs through a variety of pathways including non-specific pathways (fusion, macropinocytosis) and receptor-mediated pathways. The uptake of EVs through receptor-mediated pathways is attributed to the interaction of EV surface proteins with ligands/receptors on the cell membrane\textsuperscript{204}. However, the significant heterogeneity of EV populations suggests that multiple EV surface proteins are likely involved in the uptake of EVs by a particular cell type. Through a combination of proteomics analyses and localization studies, we identified a group of proteins, enriched on the surface of brain metastasis-promoting breast cancer-derived EVs. These proteins were recognized as interacting counterparts of several CLIC/GEEC pathway cargoes and therefore, can play significant roles in the uptake of Br-EVs by astrocytes. Future studies incorporating these proteins into synthetic nanoparticles can evaluate the necessity and significance of each of these proteins for internalization by astrocytes. Collectively, the identified protein signature can define a
subpopulation of breast cancer-derived EVs that have the ability to interact with astrocytes and, in doing so, provide novel opportunities to address the longstanding challenge of dismantling the heterogeneity of EVs for identification of functional subpopulations.

Through *in vitro* and *in vivo* functional studies, we further demonstrated that brain metastasis-promoting breast cancer-derived EVs can promote ECM remodeling via down-regulation of TIMP-2 in astrocytes. While the role of matrix metalloproteinases and their endogenous inhibitors in progression of metastasis has been studied extensively\(^\text{211}\), this study is the first to demonstrate that EVs derived from the primary tumor can initiate this process in the brain and provides insight on the early mechanisms involved in the development of brain metastasis. We identified miR-301a-3p as the causal factor that can be transferred by breast cancer-derived EVs to astrocytes and down-regulate TIMP-2. Interestingly, we found that both P-EVs and Br-EVs carried similar amounts of this miRNA and were able to induce similar effects on TIMP-2 *in vitro*. This is in contrast to our *in vivo* findings where down-regulation of TIMP-2 was only observed following treatment with Br-EVs but not P-EVs. The specificity observed *in vivo* can potentially be explained by differences in the efficiency of P-EVs and Br-EVs to breach the BBB *via* transcytosis. Importantly, and in support of this hypothesis, we have previously shown that Br-EVs but not P-EVs have the ability to modulate brain endothelial cells to facilitate their transcellular transport\(^\text{150}\). miR-301a-3p was also found to be associated with shorter survival, suggestive of its clinical significance.

Taken together, our studies uncover novel mechanisms by which breast cancer-derived EVs prime the microenvironment in the brain following their transcytosis across the BBB. These mechanisms provide novel insights into the early events that occur during the process of brain metastasis development and can be extended to studies on brain metastases arising from other
primary tumors such as lung cancer or melanoma. Moreover, the identified protein and miRNA signatures in this study have the potential to guide the development of diagnostics and therapeutics that would enable early interventions in breast cancer brain metastasis. Future longitudinal preclinical studies and prospective clinical studies are required to validate the clinical implications of these findings.
Parts of the materials described in this chapter have been published in:


5.1. Summary of Findings

In my dissertation research, I investigated the role of breast cancer-derived EVs in early stages of brain metastasis development, through determining (1) whether breast cancer-derived EVs promote brain metastasis; (2) whether breast cancer-derived EVs breach the BBB and the mechanisms underlying this process; and (3) whether breast cancer-derived EVs modulate the environment at the BBB and the mechanisms underlying this process.

In Chapter 1, I canvassed the literature to provide an overview of the clinical features of brain metastasis and to highlight the gaps in the pathophysiological mechanisms of this disease. Through a comprehensive review of literature on the role of EVs in primary and metastatic brain tumors, I demonstrated the potential of EVs for elucidating the mechanisms underlying brain metastasis.

In Chapter 2, I demonstrated that pre-treatment of mice with breast cancer-derived EVs facilitated the development of brain metastasis from brain-seeking breast cancer cells. This effect was exclusive to EVs from brain-seeking breast cancer cells (Br-EVs) and was not observed in those from parental or bone-seeking cancer cells. Through histological analyses of brain metastatic lesions, I demonstrated that the Br-EVs did not affect the total number of metastases in the brain, suggesting that these EVs play a minimal role in the facilitation of circulating tumor cell dissemination and extravasation into the brain. However, Br-EVs promoted the growth of the extravasated tumor cells in the brain, leading to an increase in the surface area of metastatic lesions. The findings of this Chapter suggest that a brain-seeking subpopulation of breast cancer cells releases EVs that have the capacity to promote a suitable microenvironment in the brain that can facilitate metastasis development.

In Chapter 3, I demonstrated that Br-EVs have the ability to breach an intact BBB in vivo.
I developed a platform to study the mechanisms underlying this process and demonstrated, for the first time, that EV transport across the BBB occurs via a transcytosis process. We further confirmed this finding in an in vitro microfluidic model of the BBB and in vivo in a zebrafish model. Through a variety of endocytosis assays and high spatiotemporal resolution microscopy, we demonstrated that the transcellular transport of EVs through brain endothelial cells depends on a rab11- and the basolateral SNARE-dependent pathway. Moreover, I demonstrated that Br-EVs, but not those from parental or bone-seeking cells, had the ability to downregulate the expression of rab7 in brain endothelial cells. I showed that this downregulation, disrupts the degradation pathway in brain endothelial cells and further increases the uptake of EVs by these cells, which could lead to facilitation of EV transcytosis across the brain endothelium.

In Chapter 4, I studied the functional consequences of EV transcytosis into the brain. I demonstrated that Br-EVs were preferentially taken up by astrocytes, and that this uptake relied on a CDC42-, GPI-AP-dependent process, not previously known to be involved in the uptake of EVs by recipient cells. Through proteomics analyses of breast cancer-derived EVs, we demonstrated the Br-EVs were highly enriched in GPI-interacting proteins, which could lead to their preferential uptake. Next, through a series of in vitro and in vivo studies, I demonstrated that Br-EVs were able to decrease the expression of TIMP-2 in astrocytes. TIMP-2 is an endogenous inhibitor of matrix metalloproteinases and its downregulation can be associated with increased activity of these matrix-modulating enzymes, leading to extracellular matrix remodeling, a major component of pre-metastasis niche preparation.

To identify the EV factors responsible for the downregulation of TIMP-2 in astrocytes, I studied the role of miRNAs in this process. I demonstrated that breast cancer-derived EVs carry and transfer miR-301a-3p that can downregulate the TIMP-2 expression in astrocytes.
Figure 5.1. Schematic summarizing the main findings of this dissertation (Image was created via www.biorender.com)
5.2. Future directions and clinical implications

Along with their role as mediators of cancer progression, EVs have shown great promise in diagnostic and therapeutic applications. The potential of EVs as biomarkers for brain cancer detection was initially demonstrated when EVs isolated from the CSF of glioblastoma patients were shown to contain mutant EGFRvIII RNA \(^{29}\). Interestingly, when compared to matched tissue samples, CSF EVs could identify two patients with EGFRvIII mutations that were previously identified to be negative for this mutation based on tissue analyses \(^{29}\). Since then, EVs containing a variety of proteins and mRNAs/miRNAs have been correlated to the prognosis of patients with glioblastoma \(^{29,36,228,229}\) suggesting their potential as biomarkers of disease progression. EVs have also been used as drug delivery vehicles to transfer therapeutic mRNA \(^{230}\), miRNA \(^{231}\), and siRNA \(^{232}\) to primary brain cancer in preclinical studies. The absence of studies on the application of EVs as diagnostic and therapeutic agents for metastatic brain tumors reflects the limited number of mechanistic EV studies of metastatic brain tumors. Dissecting the mechanisms underlying the transport of breast cancer-derived EVs across the blood brain barrier and the functional consequences of this transcellular transport in this dissertation, have uncovered potential targets that can be leveraged for development of early diagnostics and efficient therapeutics for breast cancer brain metastasis.

In this dissertation, I identified a protein signature present on the surface of brain metastasis-promoting breast cancer-derived EVs that can potentially lead their internalization by astrocytes in the brain. Moreover, I identified a miRNA signature that can be transferred by these EVs to astrocytes, leading to modulation of the behavior of these cells. Multiplexing these protein and miRNA signatures has the potential to facilitate the detection of the subpopulation of circulating breast cancer-derived EVs that have the ability to prime the brain microenvironment in
preparation for future metastasis formation. We envision using this approach to identify breast cancer patients that are prone to developing brain metastasis or those in the early stages of brain metastasis formation.

Our findings on the ability of breast cancer-derived EVs to breach an intact blood brain barrier via transcytosis has significant applications for the development of EV-based drug delivery approaches 233, with clinical applications that are not limited to brain metastasis and can be extended to other brain disorders such as neurological and neurodegenerative diseases. Moreover, this study identified rab7 as a target in brain endothelial cells through which breast cancer-derived EVs can promote their internalization and disrupt their degradation in these cells, potentially facilitating their transcellular transfer. Targeted approaches to downregulate rab7 in brain endothelial cells have the potential to increase the efficiency of transcellular drug delivery to the brain 191.

The current study focused on the mechanisms underlying brain metastasis from triple negative breast cancer. Given the high incidence of brain metastasis in both triple negative and HER2 positive breast cancer subtypes 10, future studies are required to examine the importance of the identified mechanisms and targets in brain metastasis from HER2 positive breast cancer. Moreover, along with breast cancer, other solid tumors such as lung cancer and melanoma also frequently metastasize to the brain 6. The clinical behavior of brain metastasis is very different among these cancers. For instance, lung cancer brain metastasis is often diagnosed early during the course of disease whereas breast cancer brain metastasis usually occurs years after the initial diagnosis 234, suggesting a longer dormancy period. Future investigations of the differences in EV-driven mechanisms of pre-metastatic niche preparation in the brain in these tumor types have the potential to provide mechanistic explanations for the clinical behavior of brain metastasis.
It is also important to note that this study was conducted in immunedeficient mouse models of brain metastasis and as a result, does not provide information on the potential interactions between breast cancer-derived EVs and the brain-infiltrating immune cells. The role of the immune system in different steps of brain metastasis has been acknowledged by several studies and is a matter of growing interest in the field. The recent preliminary success of immune checkpoint inhibitors in the management of brain metastasis further highlights the importance of the immune system in development and progression of brain metastasis. Future investigations using syngeneic models of brain metastasis are therefore needed to elucidate the mechanisms by which EVs derived from the primary breast cancer cells can interact with the immune system to promote brain metastasis.
References


