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Commentary

Emerging insights into the molecular biology of brain metastases

Guo Chen [a], Michael A. Davies [a,b,*]

[a] Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, United States
[b] Department of Systems Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, United States

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ABSTRACT

One of the foremost challenges in oncology is developing improved therapies for preventing and treating metastases to the brain. Recent research in this area is bringing about a shift in the understanding of brain metastases. Previously, the occurrence and poor outcomes associated with brain metastases have been largely attributed to the exclusion of anticancer drugs from the brain by the blood–brain barrier (BBB). However, studies in multiple tumor types have also demonstrated that brain metastases have significant molecular differences from primary tumors and extracranial metastases. These molecular differences may not only promote the formation of brain metastases, but they may also contribute to these tumors' poor responsiveness to therapies. Such changes may be intrinsic to the cancer cells or driven by unique interactions with the brain microenvironment. An improved understanding of the molecular characteristics of brain metastases that contribute to their aggressive behaviors will facilitate the development of rational, more effective treatments for these tumors.

1. Introduction

Brain metastasis is a significant and growing public health problem. It is estimated that more than 250,000 patients in the United States were diagnosed with brain metastases in 2009 [1], which is more than 10-fold greater than the incidence of primary brain tumors [2]. The prognosis for patients with brain metastases is dismal: most patients live only 4–6 months after diagnosis, and current treatment regimens provide marginal survival benefits [3]. With an increasing incidence [4], and a frequent occurrence in patients whose extracranial cancer has been controlled, brain metastasis is becoming a major limiting factor for cancer patient survival.

The most common causes of brain metastases are lung cancer, breast cancer, and melanoma, which account for 17–39%, 5–17%, and 8–11% of all brain metastasis cases [5]. Once these primary tumors become metastatic, they are associated with varied risks of brain metastasis: 30–40% for lung cancer [6,7], 18–36% for breast cancer [8,9], and 44–64% for melanoma [10,11]. Brain metastases originated from different tissues are associated with distinct temporal patterns. The brain is the only site of tumor relapse in 61.4% of lung cancer patients [12], 22.7% of breast cancer patients [12], and 54.1% of melanoma patients [13]. The diagnosis of brain metastasis occurs within 1 year of primary cancer diagnosis is relatively frequent in lung cancer (91%) and melanoma (50%), but is less common in breast cancer (19%) [5,14].

The most common pattern of brain metastasis is focal seeding of the brain parenchyma ("parenchymal brain metastases"). For the purpose of this review, findings about brain metastases not otherwise specified refer to these parenchymal lesions, as they have been the focus of the majority of research in this area. Parenchymal brain metastases may cause focal neurological deficits and seizures. Brain metastases that affect the central regions of the brain, or increase intracranial pressure due to cerebral edema, may also result in generalized confusion. The

Abbreviations: ADAM9, a disintegrin and metalloprotease 9; BBB, blood–brain barrier; BCL2-L1, B-cell leukemia 2-like 1; BIRC5, baculoviral IAP repeat-containing 5; cDNA, coding DNA; CNS, central nervous system; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERBB, avian erythroblastic leukemia viral oncogene homolog; FAK, focal adhesion kinase; FISH, fluorescence *in situ* hybridization; GSTA5, glutathione S-transferase alpha 5; HBEGF, heparin-binding EGF-like growth factor; HER, human epithelial growth factor receptor; HGF, hepatocyte growth factor; HK2, hexokiase 2; HSPG, heparan sulfate proteoglycans; IHC, immunohistochemistry; IL, interleukin; JAG2, Jagged 2; LEF1, lymphoid enhancer-binding factor 1; LMD, leptomeningeal disease; MAPK, mitogen-activated protein kinase; MCL1, myeloid cell leukemia-1; MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; pAKT, phosphorylated AKT; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PET, positron emission tomography; PI3K, phosphoinositol-3-kinase; PTEN, phosphatase and tensin homolog; RAS, rat sarcoma viral oncogene homolog; ROR2, receptor tyrosine kinase-like orphan receptor 2; SCID, severe combined immunodeficient; siRNA, small interfering RNA; SOCS1, suppressor of cytokine signaling 1; SYK, spleen tyrosine kinase; TCF, T-cell factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WBRT, whole-brain radiation therapy; WNT, wingless-type.

* Corresponding author at: Melanoma Medical Oncology and Systems Biology, University of Texas MD Anderson Cancer Center, 7455 Fannin Street, Unit 0904, Houston, TX 77054, United States. Tel.: +1 713 792 3454; fax: +1 713 563 3424.
E-mail address: mdavies@mdandersong.org (M.A. Davies).

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treatment of parenchymal brain metastases is generally determined by the extent of involvement at the time of diagnosis. Patients with a limited number of brain metastases may receive focal treatment such as surgical resection or stereotactic radiosurgery, which offer the possibility of durable disease control in some patients. Patients with more disseminated disease are usually treated with whole-brain radiation therapy (WBRT) or chemotherapy [3]. These treatments generally aim to slow disease progression, but are not thought to be curative in most cancer. Due to the limited efficacy of these approaches, supportive care alone is also an option for these patients.

Cancer that involves the leptomeninges, the tissue that lines the outside of the brain and the spinal cord, is termed leptomeningeal disease (LMD). Patients with LMD generally have a very poor prognosis, with even shorter survival than those patients with multiple parenchymal brain metastases [10,15]. LMD that involves the spinal cord may present as focal neurological changes. LMD that involves the brain may present as changes in mental status or other generalized changes. Because of the generally diffuse nature of LMD involvement, treatment is quite challenging and may include radiation therapy or the intrathecal administration of systemic therapies. However, neither of these interventions has demonstrated significant efficacy in LMD from solid tumors.

The challenges in diagnosing and treating LMD likely play a large factor in the extremely poor outcomes in these patients. However, there is evidence that leptomeningeal metastases may also differ molecularly from parenchymal brain metastases. Murine melanoma cells that exclusively metastasized to the leptomeninges expressed high level of transforming growth factor β2 (TGF-β2) and were growth-stimulated by TGF-β; in contrast, the cells that showed preference for parenchyma expressed low level of TGF-β2 and were growth-inhibited when treated with TGF-β [16,17]. Furthermore, manipulating TGF-β2 expression in these cells changed the distribution patterns of their brain metastases [18], indicating that TGF-β signaling is a key determining factor in forming parenchymal vs. leptomeningeal metastases.

2. Brain metastases and the BBB

The BBB is a network composed of both endothelial cells and supporting components (i.e. pericytes, glial cells) that protects the CNS microenvironment. The endothelium of brain microvessels is characterized by continuous tight junctions, decreased pinocytosis activity, and overexpressed efflux pumps [19]. With the reinforcement of the surrounding extracellular matrix (ECM), basal membrane, pericytes, and the end-feet of astrocytes, the BBB effectively prevents the free exchange of substances between the blood and the interstitial fluid of the brain [20]. Only lipophilic molecules with molecular weights of $<$400 Da can permeate normal brain microvessels [21]. As a result, the ions, neurotrophic factors, and neurotransmitters that maintain neuron function are contained in the brain, and neurotoxic substances in the blood are kept out. However, the permeability of the BBB can be regulated, and the BBB may be disrupted by pathologic conditions [20].

The BBB's exclusion of anticancer agents from the brain parenchyma may contribute to brain metastases' resistance to chemotherapy. However, there is growing evidence that brain metastases disrupt the BBB. Zagzag et al. measured the leakage of Evans blue dye (68,500 Da) from the brain vasculature in a rabbit model of brain metastasis [22]. They detected leakage of dye at sites of brain metastases as soon as 14 days after tumor cell implantation when the average volume of tumor was 13.2 mm^3. The permeability of the BBB surrounding the implanted tumor increased in a time-dependent manner and positively correlated with tumor size [22]. Similar findings were also observed in murine models of brain metastasis. In one study, the BBB adjacent to tumor deposits became permeable to sodium fluorescein dye (376 Da) once brain metastases exceeded 0.5 mm in diameter [23]. Thus, the BBB may no longer be intact in patients with radiographically detectable brain metastases. Several clinical studies also support the disruption of the BBB by brain metastases. Electron microscopy has revealed that brain metastases form leaky blood vessels [24], and positron emission tomography (PET) has detected increased blood vessel permeability in brain metastases [25]. Furthermore, brain metastases have responded to certain chemotherapies at a rate similar to that of the primary tumor [26], suggesting that these therapeutics were able to penetrate the BBB.

Despite the potential compromise of the BBB, the delivery of systemic therapies to brain metastases remains an area for further research and development. Stemmler et al. found detectable levels of the monoclonal antibody trastuzumab, which would not be expected to cross the intact BBB, in the central nervous system (CNS) of breast cancer patients with brain metastases receiving this treatment. However, the level of trastuzumab in cerebrospinal fluid was 421 times lower than that in serum [27]. Although small compounds such as paclitaxel (~850 Da) and doxorubicin (~580 Da) have shown enhanced delivery in brain metastases compared to the normal brain tissue, their concentrations in the brain metastases may be too low to elicit a therapeutic response [28]. Complicating the issue of effectively delivering drugs to the brain lesions, the BBB surrounding brain metastases of different tissue origins may present distinct ultrastructures and varied permeabilities [29,30]. Even brain metastases from the same progenitor cells have shown remarkable variation in their BBB permeability [28]. The molecular determinants of the permeability of the BBB surrounding brain metastases remain unknown. Therefore, it is not possible to predict the degree of drug penetration to the brain lesions in patients with metastatic brain tumors.

Numerous techniques to improve the delivery of therapeutics across the BBB are in various stages of development [31–33]. These techniques include chemical modification of the drug [34,35], liposome-mediated delivery of the drug [36,37], implantation of drug-containing capsules [38], inhibition of BBB efflux pumps [39,40], intra-arterial or intra-cerebral injection of therapeutics [41,42], and temporary disruption of the BBB [43]. Some of these methods have shown promising results in clinical trials [36,37,41,43]. Therefore, if a therapeutic agent effective against brain metastases can be identified, it is probable that the method to deliver this agent across the BBB will be available.

3. Molecular characteristics of brain metastases

The selection for, or induction of, specific molecular characteristics in cancer cells that metastasize to the brain may play an important role in the aggressive biology and therapeutic resistance of these tumors [44–46]. The unique molecular profile of brain metastases may be derived through multiple mechanisms (Fig. 1). Multiple lines of evidence suggest that clonal selection of a subpopulation of primary tumor cells with the *de novo* capacity to metastasize to the brain drives at least some of these differences. For example, Fidler et al. demonstrated that single cell clones isolated from the B16 mouse melanoma cell line harbor distinct metastatic activity, and 1 of 21 clones examined gave rise to a brain metastasis [47]. Alternatively, the tumor cells in the primary tumor may not initially have the ability to metastasize to the brain, but then acquire the necessary molecular traits after forming regional or distant non-CNS metastases. For example, breast cancer cells are thought to disseminate early from the primary tumor and reside in

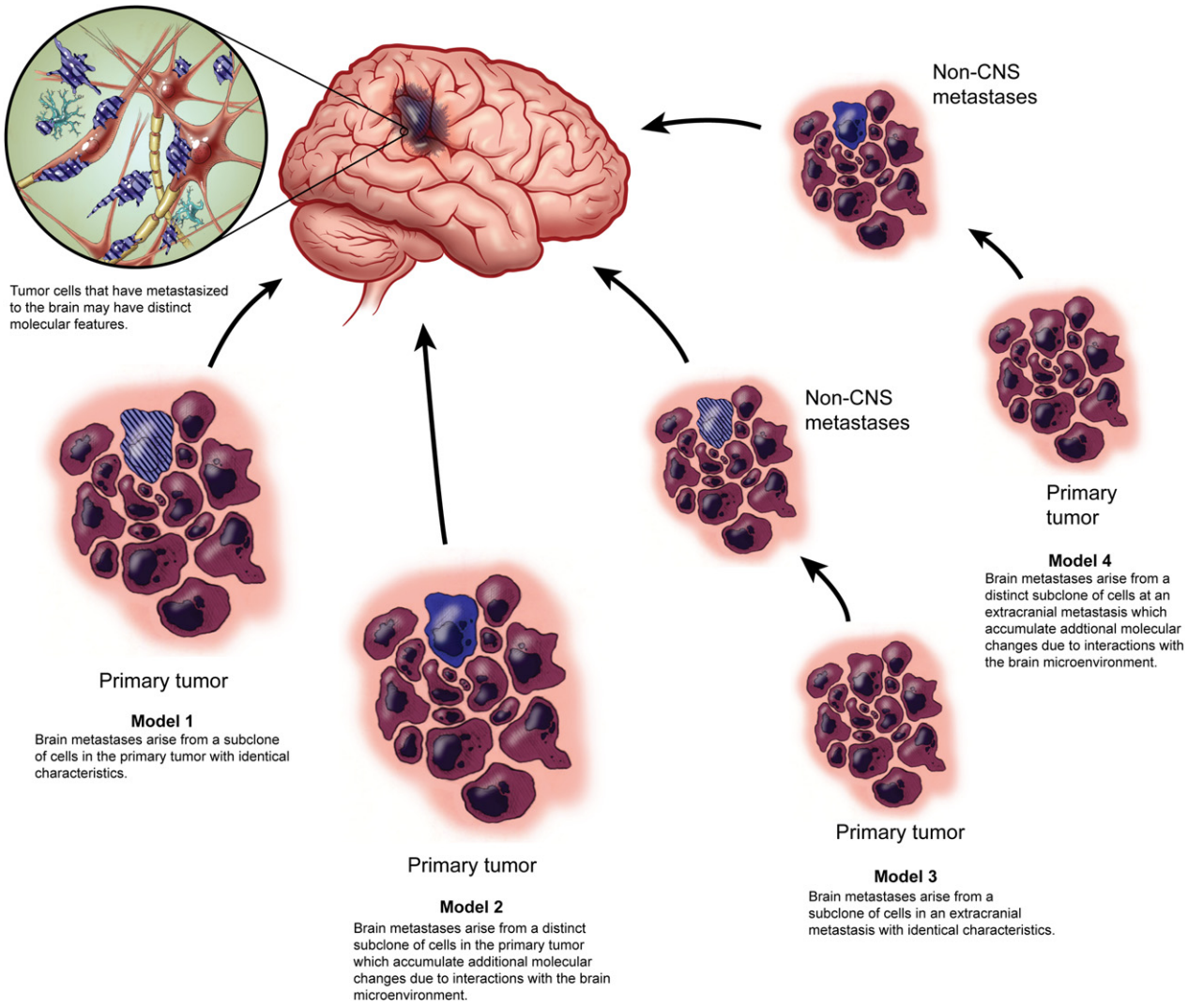

Fig. 1. Mechanisms underlying molecular changes in brain metastases. Molecular differences between brain metastases and extracranial tumors may arise through multiple mechanisms. Brain metastases may originate from a molecularly distinct subgroup of cells in the primary tumor that have *de novo* brain metastatic activity (Models 1 and 2). These cells may spread to and grow in the brain without further molecular changes (Model 1), or they may accumulate further changes after coming in contact with the brain microenvironment (Model 2). Alternatively, cells from the primary tumor may initially metastasize to other extracranial sites prior to spreading to the brain (Models 3 and 4). Such cells may acquire all of the molecular changes that characterize brain metastases in the process of forming such extracranial metastases (Model 3), or these cells may acquire additional molecular characteristics upon tumor establishment in the brain (Model 4).

a second tissue in a dormant state, sometimes for years, before colonizing distant organs like the brain [48,49].

In addition to the clonal selection of cells with distinct phenotypes to metastasize to the brain, molecular changes may be induced by interactions of the tumor cells with the brain microenvironment. Such molecular changes can occur in tumor cells that originated from either the primary tumor or a non-CNS metastasis. A recent report demonstrated that co-culture of human cancer cells with non-transformed astrocytes, which are cells that provide support and nutrients to neurons in the brain, but not with stromal cells from other organs, induced the expression of multiple pro-survival genes, including *glutathione S-transferase alpha 5 (GSTA5)*, *B-cell leukemia 2-Like 1 (BCL2L1)*, and *TWIST1* [50]. The induction of genes required physical contact between tumor cells and astrocytes, and thus was not mediated by a secreted factor. The tumor cells became resistant to chemotherapies when co-cultured with astrocytes, indicating that interacting with brain tissue may enhance the chemoresistance of brain-metastatic tumor cells. The induction of molecular changes due to interactions with the brain microenvironment is not mutually exclusive with the clonal selection model. Instead, these inductions are likely epigenetic, and may bring further changes to the molecular landscape shaped by the clonal selection process. In addition, such findings illustrate the need for epigenetic analyses of brain metastases, in addition to analyses of point mutations and/or copy number variations [50].

An expanding body of research demonstrates that regardless of the causative mechanism, brain metastases are characterized by changes in a broad spectrum of cellular pathways. The following is a summary of some of the pathways and molecules that have been implicated in clinical specimens and/or preclinical models of brain metastasis (Table 1).

3.1. Vascular endothelial growth factor (VEGF) signaling

VEGF (also known as VEGFA) signaling is a critical regulator of angiogenesis and vascular permeability [51–53]. The biological effects of the VEGF ligand are mainly mediated by the VEGFR2 receptor [51], which is a classical receptor tyrosine kinase. Ligand-bound VEGFR2 phosphorylates and activates a series of targets including phosphatidylinositol 3-kinase (PI3K), phospholipase C γ, and Src family kinases, which further propagate downstream signaling [54,55].

Table 1
Summary of molecular changes in brain metastases.

Molecule	Changes in brain metastases	Function in brain metastasis	Tumor types	Experimental models	Refs.
VEGF	Increase in secreted protein	Increase incidence, growth and angiogenesis	Lung cancer, breast cancer, melanoma, prostate cancer, etc.	Mouse model of brain metastasis, tissue culture	[56–59]
HBEGF	Increase in mRNA	Cross the BBB	Breast cancer	Mouse model of brain metastasis	[68]
EGFR	Increase in mRNA and the active form of protein	Cross the BBB	Breast cancer, lung caner	Clinical trials, clinical samples, mouse model of brain metastasis	[69,71]
HER2	Increase in mRNA. Higher percentage of samples with DNA amplification and protein overexpression	Promote the outgrowth and possibly micrometastases	Breast cancer	Clinical trials, clinical samples	[69]
HER3	Increase in mRNA, total and active form of protein	NA	Breast cancer, lung cancer	Clinical samples	[70,71]
HER4	Decrease in mRNA	NA	Breast cancer	Clinical samples	[69]
LEF1	Increase in mRNA and protein		Lung cancer	Mouse model of brain metastasis	[77]
WNT5A	Increase in mRNA	NA	Breast cancer	Clinical samples	[78]
WNT5B	Increase in mRNA	NA	Breast cancer	Clinical samples	[78]
STAT3	Increase in p-STAT3	Up-regulates invasive and angiogenesis genes	Melanoma	Clinical samples, mouse model of brain metastasis	[80]
SOCS1	Decrease in protein	Down-regulates STAT3 signaling	Melanoma	Mouse model of brain metastasis, tissue culture	[81]
Integrin $\alpha3\beta1$	Increase in protein	Migration and invasion	Lung cancer	Mouse model of brain metastasis, tissue culture	[86]
ST6GALNAC5	Increase in mRNA	Cross the BBB	Breast cancer	Mouse model of brain metastasis	[68]
Heparanase	Increase in activity	Invasion	Melanoma	Brain slice model, tissue culture	[89–92]
microRNA-1258	Decrease in expression	Down-regulates heparanase	Breast cancer	Clinical samples, mouse model of brain metastasis, tissue culture	[93]
C-MET	Increase in total and active form of protein	NA	Lung cancer	Clinical samples	[94]
ADAM9	Increase in mRNA	Increase integrin $\alpha3\beta1$	Lung cancer	Mouse model of brain metastasis, tissue culture	[95]
JAG2	Increase in mRNA	Migration and invasion	Breast cancer	Mouse model of brain metastasis, tissue culture	[96]

VEGF signaling and function in brain metastasis has been extensively characterized in preclinical models. Measurement of VEGF levels in the culturing media of cells growing *in vitro* has shown that VEGF is secreted by tumor cells with high brain metastatic activity, regardless of the tumor origin [56,57]. Increased VEGF secretion has also been detected in brain metastasis xenografts in nude mouse models [57]. Inhibiting VEGF expression in KM12SM colon cancer cells and PC14PE6 lung adenocarcinoma cells decreased the incidence and size of brain metastases, suggesting that VEGF is necessary for brain tumor initiation and growth [57]. Transfection of SKMEL2 melanoma cells with antisense VEGF coding DNA (cDNA) reduced the size of brain metastases formed by these cells [58]. In turn, overexpression of VEGF in SKMEL2 and Mel57 melanoma cells increased the size of the resultant brain metastases. However, up-regulating VEGF in H226 small cell lung cancer cells failed to increase the incidence of brain metastasis [58,59].

A variety of therapies targeting the VEGF pathway have been evaluated in preclinical models, with mixed results. The VEGFR kinase inhibitor PTK787, VEGFR and platelet-derived growth factor receptor (PDGFR) dual inhibitor AZD2171, and VEGF antibody bevacizumab have shown efficacy in slowing the growth of brain metastasis cells from breast cancer, prostate cancer, and lung cancer, respectively [56,60,61]. On the other hand, although the VEGFR and epidermal growth factor receptor (EGFR) dual inhibitor ZD6474 inhibited neoangiogenesis, the brain tumors adapted by co-opting pre-existing blood vessels and continued to grow [62]. Despite the negative results for ZD6474, the clinical activity, and the resultant FDA approval of bevacizumab in treating glioblastoma (a primary brain tumor) supports the examination of bevacizumab's effect in patients with metastatic brain tumors [63,64].

3.2. Human epithelial growth factor receptor (HER) family receptors

Four members constitute the HER family of receptor tyrosine kinases: EGFR (HER1 or ERBB1), HER2 (Neu or ERBB2), HER3 (ERBB3), and HER4 (ERBB4) [65,66]. Upon ligand binding, HER monomers undergo conformational changes that initiate dimerization by unmasking the extracellular dimerization motifs. Dimerization of HER receptors activates their tyrosine kinase activity, and results in autophosphorylation of residues that attract key second messengers, which in turn activate intracellular signaling pathways. HER receptors can form homo- and heterodimers, with potentially different impact on signaling output. For example, because HER3 lacks kinase activity [67], it must form heterodimers to initiate downstream signaling events. Of the four HER receptors, HER2 is constitutively active for dimerization because its dimerization motif is constantly exposed. Due to this unique conformation, HER2 does not bind to a ligand. In contrast, ten ligands have been described for EGFR, HER3, and HER4. Of these ligands, EGF, TGF-α, amphiregulin, and β-cellulin are specific for EGFR; epiregulin and heparin-binding EGF-like growth factor (HBEGF) interact with both EGFR and HER4; and neuregulins 1–4 are ligands for HER3 and HER4 [65]. Activation of HER receptors promotes proliferation and survival via signaling pathways like PI3K-AKT and rat sarcoma viral oncogene homolog (RAS)-mitogen-activated protein kinase (MAPK) [65].

Brain metastases display abnormalities in the expression of several HER family ligands and/or receptors. Genome-wide gene expression analysis identified HBEGF as one of 17 genes that were overexpressed in breast cancer brain metastases and were predictive of brain relapse in estrogen receptor negative (ER^-) breast cancer patients [68]. HER2 overexpression is the hallmark of a subgroup of aggressive primary breast cancers, and evidence

suggests that an even greater proportion of breast cancer brain metastases are associated with up-regulated HER2 [69]. The enrichment of $HER2^+$ tumors in brain metastases may be due to HER2's role in promoting brain metastasis. Alternatively, it may be caused by a better control of systemic cancer in $HER2^+$ patients, which gives more time for brain metastases development. The average messenger RNA (mRNA) level of HER2 is also higher (5-fold increase) in brain metastases when compared to unmatched primary tumors. The mRNA levels for other HER receptors have also been determined: EGFR is higher in brain metastases (9-fold increase), HER3 showed no change, and HER4 mRNA is lower in brain metastases [69]. The difference in the expression pattern of HER receptors between brain metastases and primary breast tumors suggest that HER receptors may play different roles in brain metastasis development. Moreover, conflicting results have been reported for HER3: more HER3 transcripts were found in brain metastases when primary breast tumors were compared to matched brain metastases from patients with both samples available [70]. Considering that tumors are heterogeneous, the difference in results supports that comparing brain metastases to matched primary tumors of the same background may be a more sensitive way to uncover brain metastasis–specific changes.

Brain metastasis-specific changes in HER family proteins have also been observed in matched lung cancer specimens. Sun et al. examined the status of HER family receptors using fluorescence *in situ* hybridization (FISH), DNA sequencing, and immunohistochemistry (IHC) in a set of 55 matched brain metastases and primary lung tumors [71]. FISH and DNA sequencing revealed that there were no *EGFR* amplification and/or mutation in brain metastases when they were compared to the matched primary tumors. However, IHC analysis demonstrated a significant increase in the levels of phosphorylated (active) EGFR and HER3 proteins in the brain metastases. In contrast, the total protein levels of these receptors were unchanged. Mutational analysis has ruled out that the hyperactivation of EGFR in brain metastases is due to activating mutations acquired by these tumors. Furthermore, IHC has detected markedly increased levels of EGFR ligands EGF and amphiregulin in lung cancer brain metastases, raising the possibility that HER family receptors are activated in brain metastases through an autocrine mechanism [68,71].

HER family proteins may promote several steps of brain metastasis formation. EGFR and its ligands may facilitate metastatic cells' infiltration of the brain, as the EGFR blocking antibody cetuximab impaired the transmigration activity of brain metastatic breast cancer cells in a tissue culture model of the BBB [68]. In contrast, HER2 promotes the growth of tumor deposits that have colonized the brain: overexpression of HER2 increased the number of large brain metastases [69,72]. Inhibition of HER2 reduced cell migration *in vitro* and decreased the number of brain micrometastases formed *in vivo* by breast cancer cells [72].

Targeting HER receptor signaling has shown promises in treating brain metastases. Cetuximab prolonged brain-metastasis-free survival in immunodeficient mice injected with metastatic breast cancer cells [68]. The HER2 inhibitor lapatinib impaired the formation and progression of brain metastasis in a similar model [72]. Moreover, a phase II clinical trial revealed that lapatinib had modest activity (6% objective response) in treating HER2+ breast cancer brain metastases [73]. Finally, intrathecally injected trastuzumab produced complete response in leptomeningeal metastasis and over 30% shrinkage in the parenchymal brain metastases of one breast cancer patient [74].

3.3. Wingless-type (WNT) pathway

The WNT signaling pathway consists of WNT ligands, their receptors and signaling effectors. WNT ligands can signal through the canonical WNT pathway as well as through a number of non-canonical WNT pathways. In the canonical WNT pathway, ligand binding to the receptor frizzled leads to the stabilization and nuclear translocation of β-catenin, which then interacts with the transcription factor T-cell factor (TCF) to regulate gene expression [75]. In the non-canonical pathways, either frizzled or an alternative receptor such as receptor tyrosine kinase-like orphan receptor 2 (ROR2) is involved, and signaling is propagated through molecules such as dishevelled, c-Jun N-terminal kinase, and phospholipase C [76].

Alterations in canonical and non-canonical WNT pathways have been associated with brain metastasis. Lymphoid enhancer-binding factor 1 (LEF1), a transcriptional target of the canonical WNT pathway, is overexpressed in lung adenocarcinoma cells with high brain metastatic-activity comparing to their parental cells [77]. In contrast, the mRNA of the non-canonical WNT ligands WNT5A and WNT5B, but not the canonical ligand WNT3A, was enriched in brain-metastatic breast cancer cell lines and breast cancer brain metastases comparing to a control breast cancer cell line [78]. Moreover, inhibiting canonical WNT signaling reduced the invasiveness of metastatic tumor cells and impaired brain metastasis formation in lung cancer cells *in vivo* [77]. At this time the functional effect of non-canonical WNT signaling on brain metastasis remains unknown.

3.4. Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway

The JAK-STAT pathway transduces signals from the cell surface to the nucleus. The pathway is activated by the interaction between a variety of ligands and their receptors, including interleukin-6 (IL-6)/IL6R, TGF-α/EGFR, insulin-like growth factor 1 (IGF-1)/IGF-1R, PDGF/PDGFR, hepatocyte growth factor (HGF)/C-MET, and VEGF/VEGFR. Upon ligand binding, the receptor tyrosine kinases activate the cytoplasmic JAK, which in turn phosphorylate STAT proteins. Phosphorylated STAT proteins form dimers, which then translocate into the nucleus, interact with the promoter region of their target genes, and activate transcription [79].

STAT3 activity regulates pro-survival genes [*BCL2-L1*, *Myeloid cell leukemia-1* (*MCL1*), and *Baculoviral IAP repeat-containing 5* (*BIRC5*)], growth-promoting genes [*c-MYC* and *Cyclin D1* (*CCND1*)], angiogenic factors [VEGF and basic fibroblast growth factor (bFGF)], invasive genes [*matrix metalloproteinase 2* (*MMP2*) and *MMP9*], and immune suppressing factors (IL-10 and VEGF). Given that STAT3 function is intimately involved in so many aspects of tumor growth, it is not surprising that a vast variety of tumors have constitutively active STAT3 [79]. A role for STAT3 has also been implicated in melanoma brain metastasis. Xie et al. found that over 57% of melanoma brain metastases showed strong staining for phosphorylated (active) STAT3, as compared to 16% of unmatched primary tumors [80]. The investigators further demonstrated that the expression of constitutively active STAT3 in the non-metastatic A375P human melanoma cell line increased its brain metastatic potential. A375P cells expressing the constitutively active STAT3 formed brain tumors in 14 of 15 mice following intracardiac injection, compared to 0 of 5 mice injected with the parental cell line. Reciprocally, expression of a dominant negative construct of STAT3 significantly impaired the brain-metastatic potential of the TXM-18 melanoma cell line, which was established from a resected metastatic brain tumor [80]. How active STAT3 affect brain metastasis formation remains unclear, but STAT3 induced the transcription of *MMP2*, *VEGF*, and *bFGF* in A375P cells, and increased cell invasion in an *in vitro* assay. While these results support a role for STAT3 in brain metastasis, functional experiments support that this may not be a brain-specific phenomenon. Manipulation of STAT3 did not affect primary tumor formation by

either cell line, but activation of STAT3 also promoted lung metastasis formation by the A375P, and inhibition of the gene reduced lung metastasis formation by the TXM-18.

Suppressor of cytokine signaling 1 (SOCS1), which is a negative regulator of JAK-STAT signaling, has also been implicated in melanoma brain metastasis formation [81]. SOCS1 expression inversely correlated with brain metastatic potential in human melanoma cell lines. Enforced expression of SOCS1 in cells with high brain-metastatic potential decreased the incidence of brain metastasis. Mechanistically, SOCS1 inhibits STAT3 activity and decreases the transcription of *MMP2* and *VEGF*, thereby reducing invasiveness and angiogenesis [81]. Manipulation of SOCS1 did not significantly affect the formation or size of subcutaneous tumors. However, whether SOCS1 also plays a role in the metastasis to another distant organ (lung, bone, etc.) has not been determined.

3.5. PI3K-AKT pathway

The PI3K-AKT pathway, which promotes cell survival and proliferation, is often hyperactivated during tumorigenesis. Davies et al. quantitatively analyzed the levels of proteins and phosphoproteins in the PI3K-AKT pathway in clinical specimens of melanoma metastases [44]. The brain metastases were compared to other distant and regional metastases. The brain metastases had significantly higher levels of phosphorylated (active) AKT (pAKT), and lower levels of the pathway negative regulator phosphotase and tensin homolog (PTEN), compared to unmatched lung and liver metastases. Moreover, patients who demonstrated high levels of PI3K/AKT activity in their regional metastases showed a trend for a shorter time to brain metastasis formation, although this difference did not reach statistical significance in the relatively small cohort. Analysis of one patient with matched distant metastases available demonstrated markedly increased expression of pAKT in the patient's brain metastasis compared to the lung metastasis, suggesting an additional possible role for activation by interactions with the local microenvironment.

3.6. Hexokinase (HK)

Hexokinase 2 (HK2) is implicated in metabolic control and apoptosis regulation. Palmieri et al. found that when compared with histologically matched primary tumor samples, breast cancer brain metastases have higher levels of *HK2* mRNA. Breast cancer cells (231-BR) with high brain metastasis activity also overexpressed HK2, and depleting HK2 in these cells impaired cell survival when glucose was limited [82]. Interestingly, Chen et al. compared the protein expression profile of a breast cancer brain metastasis variant (BCM2 BrainG2) to its parental line (BCM2), and found an increased expression of metabolic enzymes in the brain metastasis cells overall. These enzymes are associated with glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation, suggesting a global remodeling of metabolic pathways during brain metastasis [83].

3.7. ECM proteins

ECM consists of structural proteins and secreted factors. To form a brain metastasis, metastatic tumor cells have to migrate through the ECM surrounding the brain capillaries and survive in the ECM of brain tissue. Therefore, the interactions between metastatic tumor cells and ECM proteins are essential for the development of brain metastases.

3.7.1. Integrins

Integrins are a family of cell surface receptors that mediate cell adhesion and signal transduction. Integrins interact with ECM components such as collagen, laminin, and fibronectin and play crucial roles in cell migration. Integrins can also activate signaling cascades through focal adhesion kinase (FAK) and spleen tyrosine kinase (SYK) to mediate cell survival [84]. Integrins function as heterodimers of α and β subunits. The expression of integrin α3β1 has been associated with lung cancer brain metastases. Compared with their parental cell line and bone-metastasizing counterparts, tumor cells that preferably metastasize to the brain highly expressed α3β1 integrin [85]. Moreover, inhibiting α3β1 integrin function decreased brain metastases formation in nude mice: 1 of 6 mice developed brain metastases when a blocking antibody was used, while 6 of 6 mice developed brain metastases in the presence of a control antibody [85]. It has been posited that the interaction of the α3β1 integrin with laminin, which promotes tumor cell migration and invasion, may be critical to this effect [85].

The αvβ3 isoform of integrin has been implicated in the metastasis of breast cancer cells to the brain. Tumor cells expressing the constitutively active αvβ3 integrin showed elevated tumor growth as a brain implant but not as a xenograft in the mammary fat pad [86]. αvβ3 integrin may promote the growth of brain metastases by inducing VEGF expression, as increased VEGF was observed following the expression of active αvβ3 integrin. Mechanistically, active αvβ3 integrin led to the phosphorylation and inactivation of 4E binding protein 1 (4EBP1), which increases VEGF mRNA translation [86]. However, the direct effectors of αvβ3 integrin in this process, and its status in clinical brain metastases, are unknown.

3.7.2. (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide-alpha-2,6-sialyltransferase 5 (ST6GALNAC5)

ST6GALNAC5 is a 2,6-sialyltransferase that transfers sialyl groups to cell surface glycoproteins and gangliosides [87]. ST6GALNAC5 activity regulates cell-to-cell and cell-to-ECM interactions. A comparative genome-wide transcriptional analysis of highly brain metastatic breast cancer cells and the control cells revealed that increases in ST6GALNAC5 mRNA were correlated with brain metastatic activity [68]. Small interfering RNA (siRNA) directed against ST6GALNAC5 decreased the tumor cells' adhesion to brain endothelial cells and their ability to transmigrate the BBB *in vitro*. Moreover, loss of ST6GALNAC5 inhibited *in vivo* brain metastasis development. Targeting ST6GALNAC5 does not affect tumor cells' proliferation in culture, growth in mammary fat pad, or lung colonization, which strongly suggests that ST6GALNAC5 specifically promotes brain metastasis development [68].

3.7.3. Heparanase

Heparanase degrades the heparan sulfate chains of heparan sulfate proteoglycans (HSPGs) [88], which are located on the outside surface of cells and in the ECM. The functions of HSPGs include providing structural support to capillaries; retaining biomolecules such as growth factors, chemokines, and lipoproteins; and acting as co-receptors for a variety of ligands. Therefore, the degradation of HSPG by heparanase not only affects a number of physiological processes but also profoundly impacts invasive tumor growth and angiogenesis [88]. Elevated heparanase activity has been found in melanoma brain metastases [89], as well as in astrocytes co-cultured with the tumor cells [90]. In a brain slice model, pretreating a melanoma cell line with heparanase increased the number of brain-invading cells and the depth of brain invasion; this increase was mitigated by the addition of a heparanase inhibitor [91]. In addition to its enzymatic activity, heparanase may also regulate cell invasion by modulating syndecan and guanosine triphosphatase signaling [92].

Heparanase production is down-regulated by microRNA-1258 [93]. While primary tumors express microRNA-1258, this expression is lost in breast cancer brain metastases [89]. The amount of

microRNA-1258 negatively correlates with the brain metastatic capacity of breast cancer cells. In a mouse xenograft model, restoring microRNA-1258 expression in breast cancer brain metastasis cells reduced the number of brain metastases formed in mouse by 75% [93].

3.8. Additional molecules and pathways

A study of matched lung cancer brain metastases and primary tumors from the same patients identified increased expression of total and phosphorylated (active) C-MET, the receptor tyrosine kinase for HGF, in the brain metastases. The expression and activation of C-MET in the primary lung tumors also correlated with the development of brain metastases [94].

A disintegrin and metalloprotease 9 (ADAM9), a membrane-tethered protease, is overexpressed in brain metastatic lung cancer cells. Shintani et al. found that the expression of ADAM9 up-regulated integrin $\alpha 3\beta 1$ and facilitated brain metastasis formation [95].

Nam et al. found that Jagged 2 (JAG2), a ligand for Notch signaling, was up-regulated at the mRNA level in brain metastatic breast cancer cells (MDA-MB-435 Br4). Hyperactivation of the Notch signaling pathway in these cells was confirmed, and inhibiting Notch activity decreased the migration and invasion of brain metastasis cells [96].

4. Opportunities and challenges in brain metastasis research

Brain metastasis is becoming one of the main factors limiting cancer patients' survival. New, more effective therapies for brain metastases are urgently needed. A growing body of literature supports that brain metastases harbor a number of unique molecular features as compared to extracranial tumors of the same cancer type. This understanding has resulted in the improved understanding of the molecular pathogenesis of these tumors, which will facilitate the development of rational therapeutic approaches. However, a number of critical challenges still need to be overcome to improve outcomes in patients with brain metastases.

A powerful tool that will assist in both the development of new insights and effective therapies will be the development of improved preclinical models of brain metastases. The existing models for brain metastases have recently been reviewed by Cruz-Munoz et al. [97]. Currently, one of the most commonly used models involves the injection of highly metastatic tumor cells into the cardiac ventricle or the internal carotid artery of immunodeficient mice [50,77]. This model enables researchers to rapidly test the effects of molecular alterations on the ability of cells to invade through the BBB and establish a viable brain tumor. However, it clearly does not recapitulate several of the other steps that are significant to brain metastasis in patients. Models that also incorporate the steps required for a tumor cell to migrate from primary tumor or extracranial metastasis sites, and travel in the bloodstream before depositing in the brain will be important for the development of therapeutic strategies to prevent the development of brain metastases. Recently, a spontaneous brain metastasis model has been described that addresses some of these deficiencies. Melanoma cells with high brain metastatic activity (131/4-5B1 and 131/4-5B2) were generated by isolating metastatic tumor cells from the brain of severe combined immunodeficient (SCID) mice that were injected with the parental cells (131/6-4L, a lung metastatic derivative of WM239A cells). Subsequently, 131/4-5B1 and 131/4-5B2 cells were injected into SCID mice, and they produced spontaneous brain metastases following the removal of established subcutaneous tumors [98]. This new model is an advance in that it recapitulates the early steps of brain metastasis such as dissemination and intravasation. However, it is limited by the use of a SCID mouse model, which precludes meaningful examination of the interaction between metastatic tumor cells and the immune system. Establishing models in immunocompetent mice would allow for improved interrogation of the therapeutic potential of activating the immune system in the treatment and prevention of brain metastasis. Recent clinical trials with the immunotherapy ipilimumab have reported activity in brain metastases, this supports the development of such models and therapeutic approaches [99].

Another challenge for research in this area is the development of significant repositories of clinical specimens for well-powered hypothesis testing. One of the key questions in the study of molecular features of brain metastases is the appropriate comparator group. Many studies have been performed in which brain metastases have been compared to primary tumors. While the identification of differences is interesting, such studies do not address whether significant differences are actually brain metastasis-specific, or simply represent changes associated with distant metastasis to any organ site. Thus, comparison to other extracranial metastases will be critical to the development of brain metastasis-specific therapeutic strategies. The evidence of broad molecular changes induced specifically by the brain microenvironment suggests that such studies will be critical to our understanding of these tumors. Similarly, the comparison of brain metastases to matched tumors from the same patient is a powerful and precise way to assess specific molecular hallmarks of these tumors. These samples are generally quite rare, and likely will require dedicated clinical protocols for their collection to establish significant collections for research. One possible solution is the expanded implementation of warm autopsy programs for patients with brain metastases [100]. Such programs are clearly labor intensive, and require the commitment of multidisciplinary team to succeed. Most critically, the collection of such idealized specimens for research ultimately depends upon the generosity of patients who are willing to undergo tissue collections that are unlikely to benefit them directly. While such generosity is sometimes offered spontaneously, it often instead requires the involvement of motivated clinicians who are willing and able to spend the time discussing how such samples will be critical to the development of new treatments for this increasingly important disease entity.

The large and diverse collection of brain metastasis-related changes underscores the degree of complexity and heterogeneity of brain metastasis. The molecular features of brain metastases are likely the end product of a series of intrinsic and extrinsic events, in which multiple interconnected signaling pathways are capable of being affected. The intricacy of those signaling networks raise the concern that targeting one signaling molecule may have limited impact on the growth and survival of brain metastasis. One way to overcome this obstacle is to identify signaling nodes that are shared by various essential pathways, and hitting these targets to bring down multiple pathways simultaneously. In this aspect, components of the PI3K-AKT pathway are attractive targets because they transduce signals from HER family receptors, VEGF receptors, and heparanase. Bolstering this rationale, Zhao et al. recently showed that using a small molecule inhibitor against the mammalian target of rapamycin (mTOR) to inhibit the signaling downstream of PI3K and AKT reduced the number and size of breast cancer brain metastases in a rodent model [101]. Another option is to inhibit multiple pathways concurrently. Zhao et al. demonstrated that the concurrent inhibition of mTOR and MAPK/ERK kinase (MEK) suppressed brain metastasis more effectively than inhibiting the two molecules individually [101]. Better molecular targets and more effective therapy combinations may

emerge as improvements being made in the understanding of the molecular mechanisms in brain metastasis.

Ultimately, the development of new, more effective therapies for brain metastases will depend upon a change in the clinical development and evaluation of new agents. Many of the molecules and pathways implicated in the development and/or maintenance of brain metastases have been implicated in the growth of both primary tumors and extracranial metastases as well (i.e. VEGF signaling). However, others (i.e. *ST6GALNAC5*, *GSTA5*, *BCL2L1*, *TWIST1*) may be specific to the pathogenesis of brain metastasis. Traditionally, clinical trials in oncology with exciting new agents have often excluded patients with brain involvement. This policy has been due in part to concerns about penetration of agents into the CNS by the BBB, but there is strong evidence both clinically and in preclinical models that this blockade is significantly compromised by tumors that are visible on standard imaging techniques. A second factor that may contribute to the reluctance to include these patients in clinical trials is the fact that patients with brain metastases have a very poor prognosis. Thus, a lack of activity in these patients may underestimate the overall therapeutic potential of an agent. This factor could be addressed by the development of defined cohorts within larger trials that specifically consist of patients with brain metastases. By separating their evaluation from the other patients, this generalization can be avoided. In fact, the threshold for the demonstration of potential clinical benefit is generally much lower in these patients due to their poor prognosis. Therefore, a positive signal suggesting further development for an agent may actually be achieved with much smaller cohorts than is required in the overall cancer population, and perhaps could provide an incentive for pharmaceutical companies for such trials. The detection of such signals will be facilitated by the development of new imaging techniques for brain metastases that, similar to PET scans, can allow for early detection of tumor responses. The distinct molecular characteristics of brain metastases reviewed here suggest that there could be treatments that are effective in these tumors that may not be effective in extracranial metastases. Thus, including patients with brain metastases early in the evaluation of new agents may potentially provide benefits not only to patients, but also to companies that are trying to identify active drugs.

5. Conclusion

Brain metastasis is a complicated process that can be regulated by heterogeneous signaling pathways. The need for the development of brain metastasis–specific therapeutic approaches is supported by a growing literature demonstrating many unique molecular features of these tumors. The development of such approaches is a critical need in oncology, and will likely require the expansion of both preclinical resources and access in clinical trials for affected patients. Overcoming this critical challenge will be essential for making lasting impact on survival in patients with advanced cancer.

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