Preclinical Models of Brain Metastasis

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Models for Brain Metastasis Research: An Overview

The complexity of the multistep process of metastases cannot be fully recapitulated in vitro. Consequently, the use of mice as the experimental model of choice is broadly accepted. The study of brain metastasis in preclinical models includes several steps that, in principle, are common to metastases in other organs (e.g., the ability of cancer cells to migrate toward and intravasate into capillaries at the primary tumor as well as the survival of tumor cells while in circulation). Given the interest of this book, we will consider aspects of metastatic dissemination of particular interest in the brain. Preclinical models have been used to study these specific steps within the metastatic cascade that involve extravasation through the blood-brain barrier (BBB), survival of extravasated metastasis initiating cells, reactivation of proliferation to re-grow the tumor in the brain as well as the interaction with the surrounding microenvironment.

In order to study brain metastasis in the laboratory, researchers obtained cancer cells from patients, usually from pleural fluids or lymph node metastases (Fig. 3.1a). These cancer cells were engineered with different reporters, including those compatible with non-invasive imaging (e.g., luciferase, Luc, for bioluminescence) and/or histology (e.g., green fluorescence protein, GFP). Labeled cancer cells were then inoculated in mice using different routes such as intracardiac (IC) injections through the left ventricle, intracarotid, or intracranial approaches [1–3]. Intravascular injection is the preferred method since it incorporates the strong selective step of the extravasation through the BBB. The advantage of intracarotid injection is the reduction of the incidence of extracranial metastases. However, at the same time, this procedure requires surgery and thus increases the time to develop the experimental procedure. Consequently, intracardiac injection of human cancer cells has been the method of choice to induce experimental brain metastasis. Frequently, inoculation of metastatic cancer cells recovered from pleural fluids or lymph nodes, the so-called parental cell line (P), into mouse circulation does not yield a significant number of mice with brain metastases [1, 3]. This parental (P) cell line is highly heterogeneous and may or may not contain cellular clones that could have the ability to target the brain. In order to enrich those cancer cell clones with the ability to grow in the brain, parental cells are inoculated in mice IC and when metastases are detected in specific organs, the metastatic lesion is dissected out and grown in vitro. This process of positive selection has to be repeated between 3 and 5 times to enrich those variants

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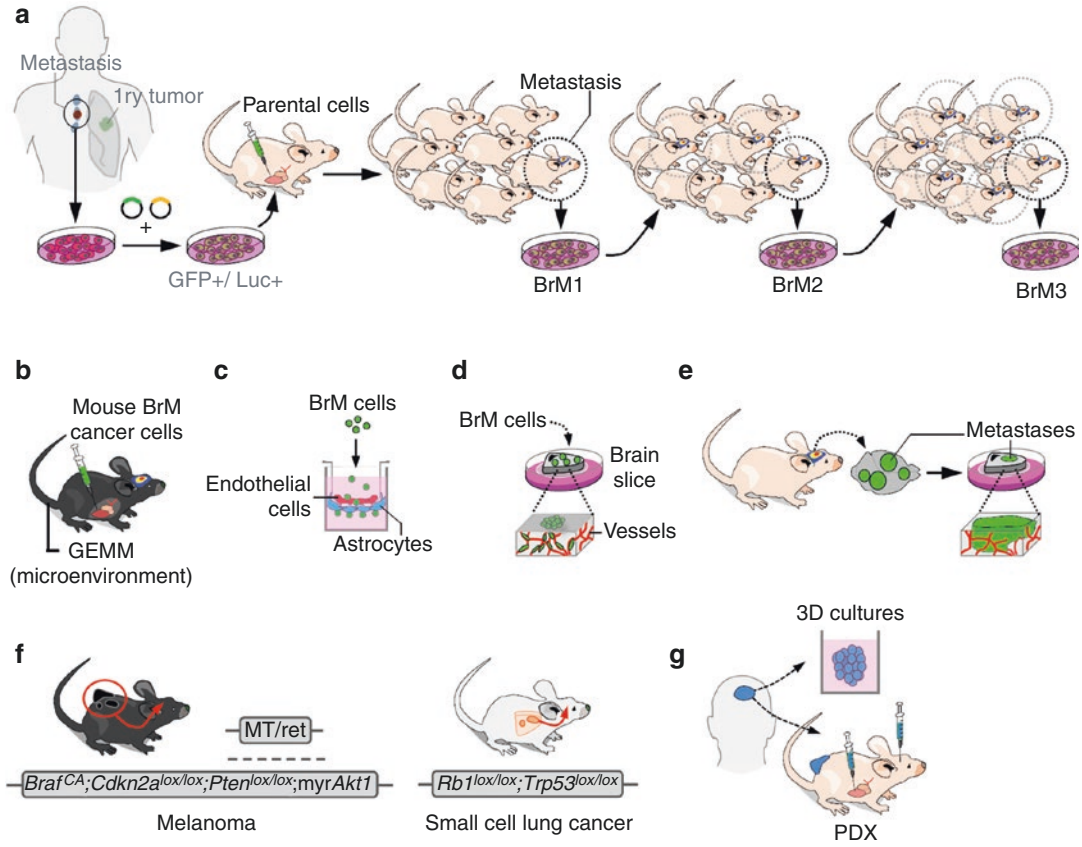

Fig. 3.1 Models for brain metastasis research. (**a**) Schema representing the generation of brain metastatic cell lines (BrM). (**b**) Syngeneic BrM cell lines could be used to evaluate brain metastasis in an immunocompetent host. This experimental model also allows interrogation of genetic modifications induced in specific components of the microenvironment by using genetically engineered mouse models (GEMMs). (**c**) Artificial blood–brain barrier (BBB) assay could be used to evaluate mediators of permeability as well as penetration of drugs. (**d**, **e**) Organotypic brain cultures allow modelling of initial (**d**) or advanced (**e**) stages of brain colonization. This preparation is a useful resource to analyze interactions with the microenvironment and it is compatible with genetic and pharmacologic manipulations. (**f**) Available GEMMs that have been described to generate spontaneous brain metastasis. (**g**) Human brain metastasis can be cultured in vitro or inoculated in immunosuppressed mice to establish brain metastasis patient-derived xenografts (PDX)

present in the P cell line with an increased ability to target the brain. These organotropic cell lines are termed brain metastatic (BrM) [1, 3–5] (Fig. 3.1a). This approach has been broadly applied to generate not only human BrM cell lines but also mouse BrM cell lines from the main sources of brain metastases, including breast, lung, renal cancer, melanoma and colorectal cancer among others, and representative of the most frequent oncogenomic profiles from each tumor type [3, 4, 6–10]. In addition, mouse BrM cell lines could be also used to study the contribution of the microenvironment by inoculating them into genetically engineered mouse models (GEMMs) (Fig. 3.1b). The use of these experimental models to study the last step of metastasis, brain colonization, has generated a significant amount of knowledge about the underlying biology by reporting multiple mediators of brain metastasis that have been validated in human samples [1, 3–5, 10–15]. Few of them have been translated into experimental therapeutic interventions with positive results, which later have been translated into clinical trials [3, 10].

In spite of the success of organotropic models, alternative and complementary approaches must be incorporated to preclinical research. For instance, models that generate spontaneous brain metastasis from orthotopic injections or from spontaneously developed primary tumors are highly needed. The significant inefficiency, the time required for detecting brain metastasis, and the limitation imposed by the faster growth of the primary tumor are all caveats that have prevented their use [16–18]. In addition, in order to incorporate the higher degree of genomic complexity in human cancer, it is mandatory to incorporate human brain metastasis through patient-derived xenografts (PDX) models [19–23]. However, their main caveats are that they require immunosuppressed hosts and they are not easy models to incorporate genetic manipulations.

In general, the field has been studying naive brain metastases when patients are usually heavily treated with neurosurgery, radiation, chemotherapy, targeted therapies, and immunotherapies. The next generation of brain metastasis preclinical models should include relevant therapies to validate the knowledge generated with naive models and to address critical questions including treatment resistance.

In addition, surrogates of the BBB have been studied not only to functionally validate molecular mediators required to cross the vascular barrier [1] but also to test drug permeability [3] (Fig. 3.1c). Brain organotypic cultures in which BrM cells are plated on the surface (Fig. 3.1d) or are already present after processing brains with established metastases (Fig. 3.1e) offer a good alternative to evaluate scientific hypothesis before testing them in vivo [3, 4, 10, 24, 25]. The main advantages of organotypic cultures are that they contain the brain microenvironment, which allows more in-depth studies, and that they are compatible with both human and mice tissues, in which both genetic and pharmacologic approaches could be tested.

Local Therapies in Experimental Models of Brain Metastasis

In spite of the broad use of neurosurgery to treat patients with brain metastasis, this approach has not been incorporated into experimental models. Given recent experimental protocols applied to other brain tumors [26], it is highly desirable that this clinically relevant model gets incorporated into brain metastasis research.

Recent clinical trials using whole brain radiation therapy (WBRT) have questioned the interest of this approach, given the limited benefit for patients and the negative impact on neurocognition [27–30].

Although limited scientific reports have addressed the efficacy of WBRT to challenge brain metastasis viability, their conclusions include the limited therapeutic benefit on established metastases.

As demonstrated clinically with the use of preventive WBRT on small-cell lung cancer (SCLC) patients [31–33], experimental models have confirmed that treating micrometastasis is more effective than treating established metastases [34, 35]. In a triple-negative breast cancer (TNBC) model, 88% reduction of micrometastases was observed upon delivery of a fractionated dose consisting of ten sessions of 3 Gy each. In contrast, only 55% tumor reduction was observed in macrometastases [34]. Similarly, when a single dose of radiation was applied 5 days after cancer cell injection, a 70% reduction of brain metastases was reported [35]. However, if radiation was delayed 3 weeks and applied once brain metastases from the breast cancer cell line MDA-IBC3 were detected, responses were minimal [35]. Modelling responses to WBRT using in vitro approaches suggest that clonogenic growth (oncospheres) faithfully predict the low responses found in vivo [35]. In fact, c-Met is among the enriched genes in oncospheres [36]. When its expression is targeted, clonogenic growth, which is not sensitive to radiation, becomes affected. In vivo, targeting c-Met sensitizes MDA-435 to radiation not only in the brain but also in extracranial tumors, which are intrinsically more sensitive to the application of this therapy [36]. Results from these works suggest that the brain microenvironment might offer clues to the resistance of brain metastasis to radiation. Interestingly, when WBRT was applied to a naive brain before inoculation of cancer cells, tumor cells inoculated afterward experienced superior growth ability [37].

Similarly, breast cancer cells obtained from brains treated with radiation that were later cultured ex vivo did not reproduce their initial resistance in vivo [34]. Furthermore, upon reinjection into mice, the resistance of cancer cells to WBRT reappeared [34]. Mathematical models predicted that response of brain metastases to radiation could be improved by doses more than 20 Gy [35]. However, an experimental protocol of 30 Gy fractionated in ten doses of 3 Gy is enough to disrupt the generation of Dcx+ immature neurons from neural stem cells [34], discarding the possibility of providing higher doses, given the associated neurotoxicity. Alternative approaches to minimize the impact of radiation on neurocognition have been validated experimentally. Using metastasis-free mice subjected to WBRT or WBRT with hippocampal sparing (HSI, hippocampal sparing irradiation), radiation-induced toxicity was studied at both cellular and behavioral levels [38]. All mice (control, WBRT, WBRT + HSI) did well in non-specific neurocognitive tests, while differed in those involving the hippocampus. Specifically, an increased deficit in spatial memory was detected given that 40% of mice receiving WBRT failed the object placement task, while only 14% do so in the non-irradiated and HSI groups. If more time is given to perform the analysis, further challenging memory, 70% of the animals that received WBRT failed versus 45% of those receiving HSI and 33% of controls. Interestingly, hippocampal tests that do not involve neurogenesis were not altered upon WBRT [38]. Behavioral tests correlated with cellular findings, including increased cell death and absence of proliferation in the dentate gyrus, which has increased levels of microglia [38].

Experimental models recapitulate the lack of major benefit with WBRT reported by recent clinical studies and suggest that alternative approaches to deliver radiation could be better, as confirmed by the application of stereotactic radiosurgery (SRS) [39]. Nonetheless, identification of the molecular mediators of radio resistance associated with WBRT in vivo and the development of radio sensitizers will facilitate a more personalized approach to its application based on potential biomarkers.

Systemic Therapy in Experimental Models of Brain Metastasis: Chemotherapy and Targeted Therapies

The penetration of many systemic chemotherapeutic agents into the brain has been proved to be limited despite the assumption that the BBB is disrupted in brain metastasis and modified into a blood-tumor barrier (BTB). Paclitaxel and doxorubicin, two potent chemotherapies used in cancer, did not reach therapeutic levels in two experimental breast cancer brain metastasis models and were ineffective in treating brain metastases, despite higher accumulation of these two agents in the lesions compared to normal brain tissue [40]. This increased permeability of the BTB has been associated with alterations in pericyte subpopulations, specifically an increase of pericytes expressing desmin, as shown in different experimental brain metastases derived from breast cancer, including triple-negative, HER2+ and inflammatory breast cancer [41]. However, these drug concentrations remain insufficient to exert cytotoxic effects compared to that observed in peripheral metastases derived from the same model [40], proving that BBB-permeable agents are needed to target cancer cells in this secondary organ. In this regard, temozolomide, a well-known alkylating agent used for the treatment of primary brain tumors that penetrates the BBB, has been shown to be effective in preventing brain metastasis from a TNBC brain metastasis model expressing low levels of MGMT [42]; these results have not been successfully translated into patients [43]. However, these clinical studies have included temozolomide therapy for established macrometastases, so the use of this therapy as a preventive strategy has not been explored yet.

The BBB not only imposes a limitation to chemotherapeutic agents but also other drugs targeting specific molecular alterations from key oncogenic signaling pathways in cancer. Side-by-side assessment of drug efficacy of two PI3K/mTOR inhibitors (brain-permeable GNE-317 and nonpermeable GDC-0980) by in vivo two-photon microscopy in an experimen-

tal melanoma brain metastasis model showed effective targeting of brain metastases only by the brain-penetrating inhibitor [44]. BKM120, another selective PI3K inhibitor shown to be BBB-permeable, was effective in reducing brain metastasis incidence in 50% of the sample population when several HER2+ human breast cancer cell lines were implanted orthotopically or injected intravenously [45], suggesting that targeting the PI3K-AKT-mTOR pathway with brain-penetrating small molecules could be an effective treatment for brain metastasis (Table 3.1).

Around 18% of patients diagnosed with brain metastasis are eligible for targeted therapies, specifically those harboring molecular alterations in their primary tumor: HER2+ breast cancer, EGFR-mutant and ALK-translocated lung cancer, and BRAF-mutant melanomas, all of which have shown positive intracranial response to different targeted agents that are both under clinical development or Food and Drug Administration (FDA)-approved [46]. Preclinically, these results have been recapitulated with different experimental mouse models. Lapatinib has been shown to delay brain metastases growth in some HER2+ breast cancer models in a preventive scenario [47]; however, established intracranial lesions from other models are resistant to trastuzumab and lapatinib treatment while orthotopic implantation (i.e. fat pad) of the same cells does respond to both treatments [48]. Efforts to overcome this resistance have resulted in combination therapies of anti-VEGFR2 antibody DC101 together with trastuzumab and/or lapatinib, resulting in more than fourfold survival benefit of the triple combination treatment compared to untreated control mice [48]. In this same line, targeting of other tyrosine kinases related to the pathway like HER3 with the monoclonal antibody LJM716 reduces brain metastases and increases survival significantly in a HER2+ breast cancer model compared to treatment with trastuzumab or pertuzumab alone, which do not give any benefit compared to the untreated control group [49], reflecting the need of targeting oncogenic pathways through several mediators for overcoming treatment-derived drug resistance (Table 3.1).

The use of EGFR tyrosine kinase inhibitors (TKIs) for patients with advanced EGFR-mutant non-small-cell lung cancer (NSCLC) has resulted in positive intracranial response apart from inhib-

Table 3.1 Use of preclinical models to test targeted therapies

Compound	Target	BBB permeability	Preclinical model	Setting	Result	Ref
GNE317/GDC-0980	PI3K/mTOR	Yes/No	Melanoma (A2058)	Interventive	+/–	[44]
BKM120	PI3K	Yes	HER2+ breast cancer (MDA-MB-453/BT474)	Preventive	+	[45]
Lapatinib	HER2	Yes	HER2+ breast cancer (MDA-MB-231-BR-HER2)	Preventive	+	[47]
Lapatinib + trastuzumab	HER2	Yes/?	HER2+ breast cancer (BT474)	Interventive	–/–	[48]
Trastuzumab/pertuzumab	HER2	?/?	HER2+ breast cancer (BT474)	Interventive	–	[48]
Lapatinib/trastuzumab + DC101	HER2/ VEGFR2	Yes/?/?	HER2+ breast cancer (BT474)	Interventive	+	[48]
Trastuzumab/pertuzumab + LJM716	HER2/HER3	Yes/?/?	HER2+ breast cancer (BT474)	Interventive	+	[49]
Rociletinib/osimertinib	$EGFR^{MUT}$	No/Yes	$EGFR^{MUT}$ lung cancer (PC9)	Interventive	–/+	[51]
Crizotinib/alectinib	ALK	No/yes	EML4-ALK variant 5a lung cancer (A925LPE3)	Interventive	–/+	[55]
Entrectinib	ALK/ROS1/ TRK	Yes	EML4-ALK (NCI-H2228)	Interventive	+	[56]

iting extracranial disease thus increasing overall survival [50]. However, preclinical studies, including therapies for brain metastases from this particular primary tumor, are scarce. Osimertinib, a third-generation EGFR TKI selective for EGFR-TKI-sensitizing mutation (EGFRm) and T790M resistance mutations approved in 2017 for clinical use, showed greater penetration of the BBB than gefitinib, rociletinib, or afatinib [51]. It induced sustained tumor regression in an EGFRm-NSCLC brain metastasis experimental model at clinically relevant therapeutic doses while rociletinib did not (Table 3.1), and could potentially overcome resistance to previous treatment with EGFR-TKIs as shown by patients included in the AURA phase I/II study (NCT01802632) [51].

ALK-translocated lung cancer patients have shown positive responses to the first-generation TKI crizotinib, although intracranial response was only achieved with BBB-permeable next-generation TKIs like ceritinib, brigatinib, and alectinib due to suboptimal accumulation of crizotinib in the brain [52–54]. These responses have been faithfully recapitulated preclinically with an EML4-ALK variant 5a lung adenocarcinoma brain metastasis model sensitive to both crizotinib and alectinib at the primary tumor site, but resistant to crizotinib and sensitive to alectinib in the brain [55] (Table 3.1). In spite of these advances, progression-free survival (PFS) of patients receiving these TKIs does not exceed 15 months. Drug resistance developed through prolonged treatment thus remains as an unmet need and novel small molecule inhibitors targeting resistant ALK-dependent brain metastases are necessary. Studies with next-generation ALK inhibitors such as lorlatinib and brigatinib are promising. Entrectinib, an orally bioavailable potent inhibitor of ALK, ROS1 and TRK family kinases, has been reported to induce significant reduction of intracranially implanted tumors from EML4-ALK rearranged NSCLC increasing mice survival in more than 70% [56] (Table 3.1). Future clinical trials could open the way to a drug potentially suited to treat brain metastases from several molecularly defined primary tumors.

Melanoma brain metastasis patients also benefit from targeted therapies, mainly BRAF V600E TKIs dabrafenib and vemurafenib [57–59]. Preclinical models of brain metastasis, including these therapies, are limited. Several BRAF V600E mutated melanoma human melanoma cells have been shown to generate experimental brain metastases [60]. Vemurafenib-resistant melanoma cells generated in vitro show distinct expression profile to vemurafenib-sensitive cells but do not change their ability to colonize the brain despite their increased ability to metastasize to the lung and the liver [60]. Since 50% of melanoma brain metastasis results from BRAF-V600E-mutated primaries, new experimental models incorporating this molecular alteration and targeted therapies are needed to study metastatic spread to the brain in the skin cancer with highest death rates.

Unbiased Screens for Brain Metastasis Mediators

In Vitro Transcriptomics

Metastatic colonization is a multistep process that enriches disseminated cancer cells through positive and negative selection from an initial cellular pool derived from the primary tumor. Consequently, metastatic lesions will be richer in cancer cells with all the attributes required to reach and colonize the target organ. This has been the rationale for the development of organotropic metastatic derivatives that are established through multiple rounds of in vivo selection (Fig. 3.1a).

In order to dissect brain tropism at the molecular level, comparisons between P cell lines, without the ability to target the brain, and BrM cells were performed. This approach has been applied to breast cancer [1] and lung cancer models of brain metastasis [5] (Fig. 3.2a). Transcriptomic analysis of P versus BrM cells growing in vitro reflects significant differences between them. Although the overlap of differentially expressed genes among different models is more limited [4], upregulated genes, potential mediators of brain metastasis tro-

pism, or downregulated genes, potential brain metastasis suppressors, were successfully validated in functional experiments using in vivo brain metastasis assays and in human samples, where their increased levels at the primary tumor correlate with a higher risk of brain metastasis incidence. Many of the genes found with this approach mediated the ability to cross the BBB [1, 12, 13, 15] or interactions with the brain microenvironment [4, 10, 11] (Fig. 3.2a).

For instance, a 17-gene signature named brain metastasis signature (BrMS), obtained by comparing two different ER⁻/HER2⁻ breast adenocarcinoma models tropic to the brain (MDA231-BrM and CN34-BrM) respect to their parental cell lines, was sufficient to predict brain relapse when applied to three independent patient cohorts [1]. Among BrMS genes present in cancer cells, the α2,6-sialyltransferase encoded by *ST6GALNAC5* was selected. Mechanistic studies

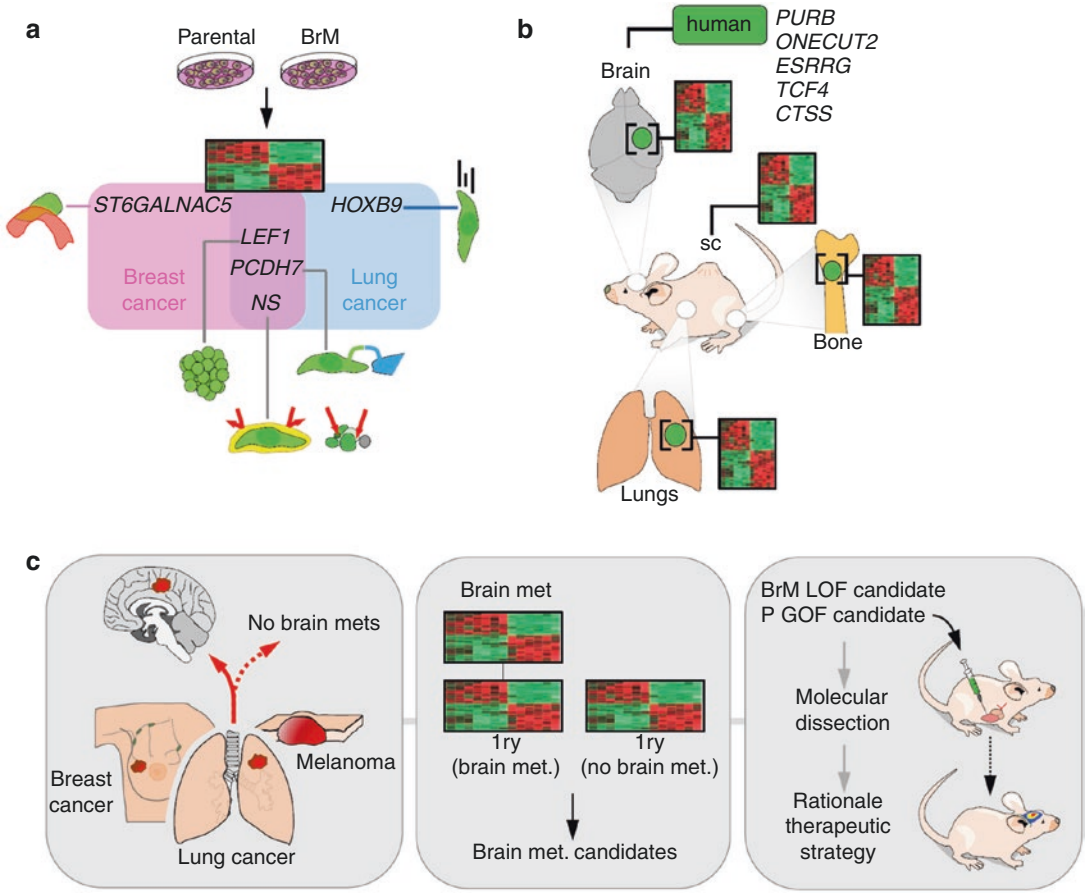

Fig. 3.2 Use of preclinical models to dissect the molecular regulation of brain metastasis. (**a**) Parental and brain metastatic derivatives (BrM) have been interrogated in vitro. Analysis of differentially expressed genes shows not only cancer-type specific but also commonly deregulated mediators of the disease (LEF1, PCDH7, NS) involved in a variety of mechanisms required for brain colonization. (**b**) Brain metastases have been interrogated in situ and compared with orthotopic and subcutaneous tumors and metastases growing in other organs. These studies not only identified potential mediators of brain metastases when human cancer cells were analyzed but also allowed evaluating the tumor microenvironment by analyzing mouse genes. (**c**) Evaluation of human samples, including primary tumors and brain metastasis, can allow identification of candidate genes that may contribute to brain metastasis formation. In order to functionally validate candidate genes, loss of function (LOF) and gain of function (GOF) approaches can be applied using preclinical models. These mechanistic assays in experimental models will help improve therapeutic strategies

proved that cancer cell surface decoration with 2–6 sialyl groups was required to increase the ability to cross the BBB [1].

In contrast to breast cancer, lung cancer usually disseminates fast. A Wnt-dependent program is responsible for facilitating the aggressive dissemination of lung cancer to multiple organs including the brain [5]. Two lung adenocarcinoma models (H2030-BrM and PC9-BrM) tropic to the brain were used to identify key components of the Wnt pathway. LEF1 increases the ability of BrM cells to grow in spheres, which is a surrogate of metastasis-initiating capabilities, while HOXB9 is required for a superior migratory behavior that is necessary for brain colonization [5] (Fig. 3.2a). Although both requirements are critical for brain metastasis, they are equally important for bone metastasis [5].

In Situ Transcriptomics

In vitro unbiased screens to identify mediators of brain metastasis have been complemented with the analysis of transcriptomes in situ [15, 61–63] (Fig. 3.2b). The rationale of this alternative approach is that there may be important mediators of brain metastasis not permanently but transiently induced in cancer cells tropic to the brain. In fact, these analyses confirm that there are transcriptomic modifications only manifested when the cancer cell is studied in a given organ. Breast, lung, melanoma, and colon cancer cells were grown as either subcutaneous tumors, at the orthotopic location according to the origin of the cancer cell, or in the brain after intracarotid injection [61]. Differentially expressed genes show that the transcriptome of cancer cells does not change significantly when grown at the subcutaneous location or in the orthotopic location. However, when the same cancer cells are grown in the brain, their transcriptomic profile diverges from those obtained at other locations (in vitro, subcutaneous, orthotopic) and become more similar to other cancer cell lines from different tumor types also obtained from the brain. Changes in gene expression correlate with altered methylome patterns. Since the methylome obtained from cancer cells growing in the brain also differs from the one obtained from orthotopic tumors [61], epigenetic mechanisms may play a critical role in reprogramming cancer cells during the adaptation to the brain microenvironment. Reprogramming of cancer cells growing in the brain involves the upregulation of neuronal genes [61]. This emerging expression pattern was suggested to be regulated by various transcription factors, including *PURB*, *ONECUT2*, *ESRRG*, and *TCF4*, that show reduced promoter methylation in brain metastatic lesions.

A similar approach comparing different organotropic cell lines including a lung metastatic (LM) derivative, a bone metastatic derivative (BoM), and a BrM one derived from the same parental $ER^{-}/HER2^{-}$ breast cancer cell line (MDA231) was used to evaluate in situ differential expression patterns of proteases and their inhibitors specifically [15]. Transcriptomic differences among metastatic cells in different organs are amplified along the process of organ colonization, suggesting that the transcriptome of cancer cells reflects organ adaptation [15]. These approaches also allow scoring the microenvironment by excluding human genes derived from human cancer cells. Attending to the expression of mouse genes, the three organs evaluated (brain, bones, and lungs) cluster independently. However, the brain differs significantly more from lungs or bones than these two organs among themselves. When cancer cells initiate organ colonization to form micrometastases, they do not significantly alter the expression pattern of the organ compared to the naive one without metastasis. In contrast, at late stages (macrometastases), the organ transcriptome is significantly altered in the lungs, bones, and brain. Again, the degree of transcriptomic changes in lungs and bones is more discrete than that in the brain [15]. This could reflect the abundance of specific barriers in the brain that limit the growth of incoming metastatic cells compared to other secondary organs more similar to the primary tumor that may only require a limited adaptation of cancer cells to thrive.

Although the main findings of unbiased transcriptomic screens applied to brain metastasis

experimental models have been validated in patient samples [1, 5, 15], the inverse approach has not been equally investigated. Evaluating candidates obtained from unbiased screens in human samples using experimental models would allow testing their functional contribution to brain colonization as well as to dissect the underlying molecular regulation (Fig. 3.2c). Both considerations are key to rationalize more specific and effective therapies. Although the limited number of studies that have compared human and experimental transcriptomic screens found reduced overlap in terms of specific genes, pathways were partially conserved. This suggests that experimental brain metastasis models are valuable platforms for the identification of novel mediators of the disease and to test them functionally.

Noncoding RNA

In parallel to transcriptomic analyses, expression profiles of small noncoding RNAs, mainly miRNAs, have been developed to identify mediators of brain metastasis. Unbiased screens comparing organotropic cell lines in vitro [64, 65], their exosome content [66], and human samples have been performed [67–70]. Differentially regulated miRNAs between primary tumors with or without brain relapse or directly at brain metastases [67, 68], as well as liquid biopsies from the cerebrospinal fluid (CSF) [70], have been evaluated to validate the importance of selected candidates.

miRNAs functionally validated in experimental models include modulators of extravasation through the BBB. High levels of miR-181c contained in extracellular vesicles (EVs) from breast cancer cell lines metastatic to the brain are responsible for downregulating the expression of *PDPK1*, which is an essential factor for actin dynamics by mediating the phosphorylation of cofilin. Defective actin dynamics impairs intracellular trafficking of multiple proteins required for the maintenance of brain endothelial cell intercellular junctions such as tight junction proteins and N-cadherin [66]. This finding confirms that miRNA enriched in EVs secreted from primary tumors could influence vascular barriers to facilitate extravasation of cancer cells [71]. In addition, miR-509 downregulation in human brain metastasis as well as experimental brain organotropic breast cancer cell lines allows maintenance of high expression levels of RhoC, which is required to produce MMP9, an enzyme targeting endothelial cell-junctions of the BBB, and TNFα [72], which plays an important role for increased BBB-permeability in sepsis [73].

miRNAs continue to be required once metastatic cells have crossed the BBB. Re-initiation of the secondary tumor requires stem cell-like properties [74], which could be provided by the expression of pluripotency factors. Among them KLF4 is required for the initiation of breast cancer brain metastasis. To maintain high expression levels of *KLF4*, $CD24^{-}/CD44^{+}/ESA^{+}$ brain metastasis cancer stem cells downregulate miR-7 [64]. In addition, miRNAs from the microenvironment also play an important role in colonization. Reactive astrocytes, which closely interact with cancer cells, are highly secretory cells known to produce EVs [75]. miR-19a-containing EVs produced by astrocytes are transferred to cancer cells. miR-19a downregulates *PTEN* expression leading to the attraction of $CCR2^{+}$ macrophages/microglia as a consequence of the increased production of CCL2 from $PTEN^{low}$ cancer cells [76].

The brain microenvironment could be also modulated by cancer cells residing at the primary tumor through the production of miR-122-contained EVs. miR-122 targets enzymes involved in glucose metabolism. Decreased levels of *PKM2* and *GLUT1* induced by miR-122 lead to the reduction of glucose uptake and consumption by brain astrocytes, which increases the available extracellular pool of this nutrient, thus benefiting incoming cancer cells [77].

Although mesenchymal traits are required at various steps of the metastatic process, some experimental models show an additional step that takes place upon organ colonization. The process of mesenchymal to epithelial transition (MET) is regulated by miR-200s family [78]. Liquid biop-

sies from the CSF of patients with parenchymal or leptomeningeal metastases could be separated from noncancerous biopsies by a combination of several miRNAs contained in this family, including miR-10b, miR-21, miR-200a/c, and miR-141 [70]. miR-141 is required to mediate MET in breast cancer brain metastasis [65].

These transcriptomic screens should be complemented with others that have interrogated the epigenome [79–81] and the proteome [82–90]. Comparative analysis of omic approaches will offer a more accurate view of the regulatory mechanisms and pathways that are key in experimental models, where investigational therapies can be tested, and in humans.

Advanced Modeling of Brain Metastasis in Mice

Preclinical models extensively used for studying brain metastasis include cell line-derived xenotransplants, generally based on organotropic human cell lines that preferentially target the brain and are implanted intracardiac or intracranially in immunodeficient mice. Syngeneic mouse cell lines with brain tropism have been used to address the interaction of cancer cells with the brain microenvironment or the immune system [1, 4–7, 10, 15, 41, 76, 91, 92]. However, these models of induced brain metastasis have limitations when recapitulating the course of the human disease, where brain metastases are spontaneously generated in the presence of a primary tumor and generally other extracranial metastases.

Spontaneous Models of Brain Metastasis

Genetically engineered mouse models (GEMMs) that result in spontaneous brain metastases are limited. Two genetic mouse models of melanoma based on different oncogenic drivers have been reported (Fig. 3.1f). The MT/ret transgenic mouse model resembles the process of malignant transformation in human melanoma, resulting in metastases to distant organs including the brain. This process is accompanied by a progressive increase in expression and activity of the *ret* transgene, leading to hyperactivation of the MAPK-related pathway [16]. The PI3K-AKT-mTOR pathway has been shown as a viable therapeutic target in several brain metastasis preclinical models pharmacologically [44, 45]. Genetically, a melanoma mouse model with activated AKT1 in the context of BRAF V600E and silenced *INK4A-ARF*, generated spontaneous brain metastases recapitulating the human disease, and this metastatic capacity was augmented by additional *PTEN* silencing [17] (Fig. 3.1f). This model allows functional validation and characterization of PI3K-AKT-mTOR pathway as key in brain metastasis biology. Although lung cancer is the most common source of brain metastases, GEMMs of lung cancer scoring incidence of metastatic spread to this secondary organ are scarce. A GEMM of small-cell lung cancer (SCLC), a subtype of lung cancer with high incidence of brain metastasis, has been reported to generate spontaneous intracranial lesions from neuroendocrine lung tumors that were engineered by conditional somatic inactivation of *Rb1* and *Trp53* in lung epithelial cells [18]. These tumors gave rise to extrapulmonary metastases including the brain and resembled human SCLC both morphologically and immunophenotypically [18] (Fig. 3.1f), which allows more reliable translation of preclinical results into clinical approaches. GEMMs that faithfully recapitulate the human disease will open new scenarios for brain metastasis research such as the study of prevention. Mouse models representing primary tumors with high incidence of brain metastasis like non-small-cell lung cancer, HER2+ and triple-negative breast cancer are urgently needed.

Patient-Derived Xenografts

The use of patient-derived xenografts (PDXs) for modeling brain metastasis during the past few years [19, 20, 23, 93] has opened new possibilities for personalized medicine to be applied to patients with cancer dissemination to the brain

(Fig. 3.1g). PDXs from patients' brain metastases from different primary sources (non-small-cell lung cancer (NSCLC) [20], several subtypes of breast cancer [19, 23], and melanoma [93]) have been used to establish preclinical mouse models by engraftment of cells derived from fresh surgical samples in immunodeficient mice. In all studies, PDXs show highly similar histopathological features, genetic or functional properties when compared to the parental human brain metastasis, thus proving that PDXs are a reliable resource for recapitulating the human disease. Based on these similarities, PDXs have been used for evaluating the efficacy of targeted therapies or to perform low-throughput drug screenings. In vitro tumor spheres from PDXs from NSCLC brain metastases that maintain their in vivo brain metastatic potential have been established for this purpose [20]. Five PDX-derived tumor spheres were screened for 20 agents targeting commonly altered oncogenic pathways in NSCLC such as EGFR, MET, Mtor, and VEGFR. Efficacy of these agents varied among the different samples, indicating that each one relies on different oncogenic alterations and that personalized approaches based on PDXs will improve current therapies by predicting drug responses. In vivo, inhibition of the PI3K/mTOR pathway using a combined treatment with the PI3K inhibitor BKM120 and the mTOR inhibitor RAD001 (both able to penetrate the brain) resulted in durable tumor regressions in 3/5 PDXs of HER2+ breast cancer brain metastases [23], suggesting the potential efficacy of this combined therapy in the respective donor patients. In this same study, whole-exome sequencing of the PDXs and matched tumor samples from the donor patients showed that each PDX and its matched patient sample shared almost identical genetic alterations regarding copy-number variations and somatic mutation rate. Interestingly, the two non-responding PDXs and their matched patient specimens showed hypermutated genomes with enriched mutation frequencies in DNA-repair genes, suggesting that genomic instability is correlated with therapy resistance. Based on these observations, PDXs are not only a useful tool for drug testing, but also a valuable resource for evaluating biomarkers that predict response to therapy in the context of brain metastasis.

Future Challenges

Despite the efforts in improving currently available experimental models for brain metastasis, whether these models faithfully recapitulate the human disease is a matter of continuous debate. Intracardiac, intracarotid, or intracranial injection of brain tropic human or syngeneic cell lines are still the most commonly used preclinical models for studying the biology of the disease and developing novel therapeutic strategies for brain metastasis patients. Spontaneous brain metastases from GEMMs are still limited. Available GEMMs [16–18] generate aggressive primary or extracranial metastases, thus imposing an additional limitation since brain macrometastases are rare and clinically relevant stages of the disease cannot be easily observed in these models. Most PDXs maintain pathological features of the parental tumor—their increased heterogeneity clinically allows more personalized approaches. CRISPR/Cas9 technology will improve available models by introducing specific genomic alterations detected in human brain metastasis to dissect their functional contribution and test their importance as a therapeutic target. On the other hand, since most patients have been treated with multiple lines of therapy before brain metastases occur, experimental models that incorporate them will allow developing more realistic experimental studies, which will be further improved by the addition of local therapies such as neurosurgery and radiotherapy.

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