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REVIEW

In vivo animal models for studying brain metastasis: value and limitations

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Abstract Brain metastasis is associated with a particular poor prognosis. Novel insight into the brain metastatic process is therefore warranted. Several preclinical models of brain tumor metastasis have been developed during the last 60 years, and they have in part revealed some of the mechanisms underlying the metastatic process. This review discusses mechanisms of brain metastasis with a key focus of the development of animal model systems. This includes the use of rodent, syngeneic brain metastasis models (spontaneous, chemically induced and genetically engineered models) and human xenotransplantation models (ectopic inoculation and orthotopic models). Current information indicates that none of these fully reflect tumor development seen in patients with metastatic disease. The various model systems used, however, have provided important insight into specific mechanisms of the metastatic process related to the brain. By combining the knowledge obtained from animal models, new important information on the molecular mechanisms behind metastasis will be obtained, leading to the future development of new therapeutic strategies.

Keywords Brain metastasis · Animal models · Orthotopic · Ectopic · GEMMs

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Introduction

Metastasis to distant organs is the major cause of morbidity and mortality in cancer patients [1, 2]. Patients with brain metastasis have a dismal prognosis with a median survival time of 4 months [3–5]. Brain metastases most commonly form highly circumscribed lesions with little single cell invasion into the normal brain [6, 7], although poorly defined borders and highly diffuse invasion patterns have sometimes been observed, as for instance in anaplastic small cell carcinomas metastasizing to the brain [8]. Brain metastases are most frequently derived from primary neoplasms localized either in the lung, breast, skin, kidney or colon [9, 10]. They occur 10 times more often than primary malignant brain tumors [1, 11]. Their incidence is increasing [2], which can be partially explained by improved local control of primary and secondary lesions and/or improved detection methodology [5]. However, smaller central nervous system (CNS) metastases are not readily detectable despite sensitive diagnostic imaging tools such as advanced computerized tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET).

The clinical problem of brain metastasis is well recognized [1, 2]. The exact mechanisms governing the formation of metastases are not known, partly because early

metastatic spread is hard to detect and visualize in humans [12]. To approach this problem, the metastatic process in the brain has been extensively modeled in animals, either by injecting cancer cells orthotopically or directly into the blood circulation of rodents.

In this review we will focus on animal models that have been specifically used to study brain metastasis. We will also provide an overview of several molecular mechanisms that may play a role in the metastasis process to the brain, and describe the various animal models that have been used, pointing out both their strengths and weaknesses.

The different steps of the brain metastasis process

The metastatic process includes a series of steps, which all have to be successfully completed in order to form metastatic lesions within the brain [10, 12–16]. The primary tumor has to establish a blood supply in the host organ to account for oxygenation and metabolic needs during tumor growth. This will later provide an escape route for the primary tumor cells, which through intravasation enter the circulatory system. Then the cells have to survive in the blood circulation until they reach the brain as the target organ, where they attach to the microvascular endothelial cells and penetrate the microvasculature (extravasation). The tumor cells then invade the brain parenchyma where they interact with the microenvironment to induce angiogenesis and proliferation. The final step is the metastatic colonization, which constitutes the transition from micrometastases to macrometastases. It is believed that the formation of solid brain metastases is a result of specific interactions between disseminated metastatic cells and the microenvironment of the brain parenchyma [14, 17–20]. This notion is consistent with the "seed" and "soil" theory of Paget [21], who proposed that metastasis is not a random process, but is caused by specific tumor cell clones (the "seed") that have a specific affinity for the microenvironment in certain organs (the "soil"). At present the mechanisms responsible for brain metastasis still remain elusive since information obtained from experimental model systems indicates that both metastatic competency genes as well as tissue-specific host cellular niches, in particular associated with the brain vasculature, play important roles in the metastatic process.

Genetic signatures associated with the metastatic process

Both clinical studies [20, 22–32] and animal experiments [22, 33–45] have provided some insight into the molecular mechanisms involved in the metastatic process (Fig. 1), where specific gene expression patterns have been shown to be associated with an organ-specific colonization [46–48]. In the brain, this process involves the expression of a number of growth factors and signaling molecules. However, in a search for common denominators that may reflect brain metastasis formation, it is evident that there is in general little overlap between information obtained in different animal model systems and the human situation (Fig. 1). Even so, a number of similarities have been observed between biopsies obtained from patients and human murine xenografts where one of the most interesting seems to be the sialyltransferase ST6GALNAC5 which enhances breast cancer cell adhesion to brain endothelial cells and plays a role in their passage through the blood brain barrier (BBB) [36]. Other molecules that may be of importance in breast cancer metastasis include cyclooxygenase-COX2 (also known as PTGS2) [36], the epidermal growth factor receptor (EGFR) ligand HB-EGF [36] and Her-2[2, 22]. It has been shown that breast cancer patients with Her-2 positive tumors frequently develop brain metastases after trastuzumab treatment. This can be explained by an efficient local tumor control, whereas poor penetration of trastuzumab through BBB prevents the targeting of putative tumor clones that have metastasized to the brain [23]. If Her-2 in itself plays a role in the metastatic process to the brain warrants further investigation. It should be emphasized that the studies mentioned above have focused on a characterization of the tumor cells ("seed") and neglect potential contribution from the microenvironment of the target organ ("soil"). At present, it is still unclear if specific gene expression signatures are associated with the establishment of brain metastases in humans.

The host vasculature: a niche for brain metastases

The CNS is regarded as a unique target organ for specific metastasis since it lacks lymphatic vessels and is surrounded by the BBB [49]. The CNS has been regarded to be an immunopriviliged site, where the BBB in part provides a controlled physiological environment separated from factors delivered by the systemic circulation [50].

It is generally acknowledged that the metastasizing cells arrive at the brain through the arterial blood supply [10], where they attach to the endothelial cells of the microvessels, followed by penetration of the BBB [51, 52]. In brain capillaries tumor cells arrest in areas of slow blood flow, as for instance at vascular branch points [53]. The neoplastic cells then need to attach to the endothelial cells and penetrate the BBB in order to form solid tumors.

At present, the mechanisms favoring the binding of tumor cells to the brain endothelium are poorly understood [54], but it is believed to be mediated at least partly by interactions between receptors at the tumor cell surface and endothelial cell adhesion molecules. For instance, in an

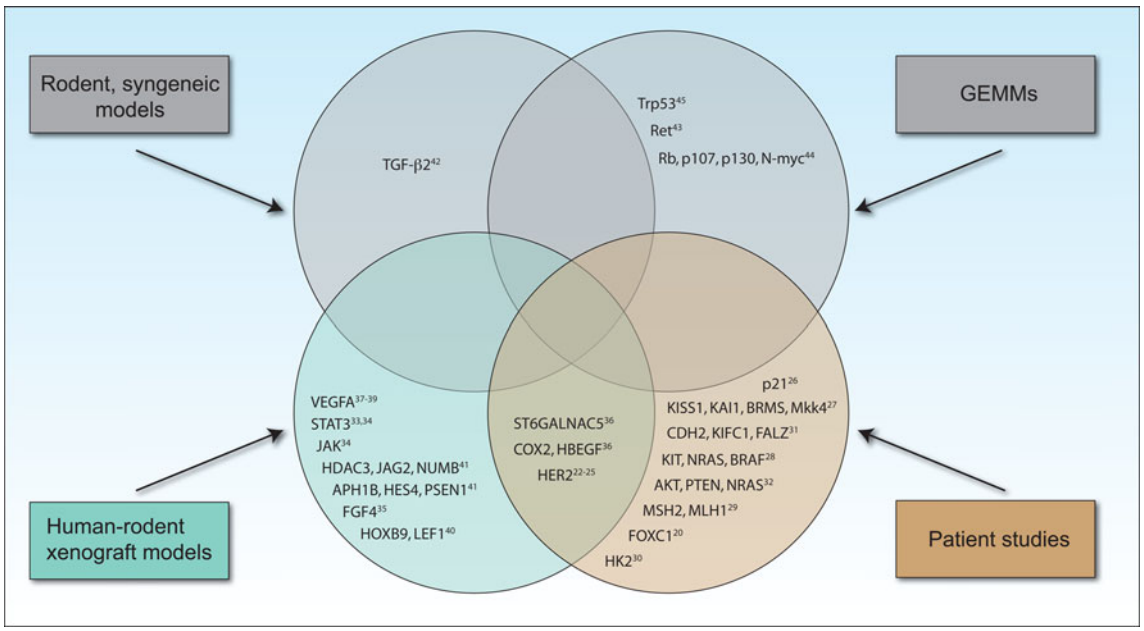

Fig. 1 Genes shown to be involved in the development of brain metastasis. These genes have been studied in preclinical animal models as well as in patient studies. The studies referred to in this review shows that there is relatively little overlap between brain metastatic genes found in animal model studies, and in the clinical setting

animal model of non-small cell lung cancer, the integrin α3β1 has been shown to be involved in metastasis formation [55]. Moreover, it has been demonstrated that chemokine/receptors like CXCR4 and its ligand CXCL12 can facilitate transendothelial breast cancer cell migration in the brain [56, 57]. However, CXCR4 is not specific to the CNS since it may also be expressed in extracranial metastases [58].

At present it is clear from several experimental and clinical studies on melanoma, that early metastatic tumor growth within the CNS appears along pre-existing brain vessels [37, 59–62]. This implies that the brain vasculature may constitute an important niche in the initiation of brain metastasis where the vascular basement membrane in particular, may represent an important substrate for tumor growth [63]. In this context the use of function-blocking antibodies has identified an important role of the ß1 integrin in metastatic tumor establishment from breast carcinoma and melanoma cell lines [63].

Moreover, several studies on brain metastasis from melanoma and breast cancer indicate that proteolytic enzymes, such as gelatinocytic serine proteases, plasminogen and matrix metalloproteinases, can facilitate transmigration through the endothelial layer [63–66]. After the neoplastic cells have entered the brain parenchyma, the formation of solid brain metastases depends on a rearrangement of the host vasculature, [67] where in particular VEGF expression levels directly correlate with angiogenesis and growth of brain metastases [38, 39].

It has been suggested that connections are established between normal brain cells and cancer cells where the normal cells create a pre-metastatic niche that is important for metastasis formation. In particular, it has been shown that bone marrow-derived hematopoietic progenitors that express VEGF, mobilize in response to a unique array of growth factors produced by the primary tumor [68]. Their arrival at distant sites may well change the local brain environment which dictates the pattern of metastatic spread [11]. At present, however, it is unclear if a premetastatic niche is established prior to the formation of a metastatic lesion in the CNS.

Animal models to study brain metastasis

Preclinical brain tumor metastasis models have been important in shedding light on some of the mechanisms behind the metastatic process. A brain metastasis model may be defined as a model where tumor cells repeatedly and successfully move to the animal brain and form solid tumors, after being injected ectopically or orthotopically into the animal. The models can be divided into 2 broad groups, rodent syngeneic models, and human-rodent xenotransplantation models (Fig. 2). Rodent syngeneic models involve murine derived cell lines, and can be divided into 2 groups, depending on whether the inoculation route is ectopic (commonly the injection of cells into the blood stream) or orthotopic (cells injected into the same

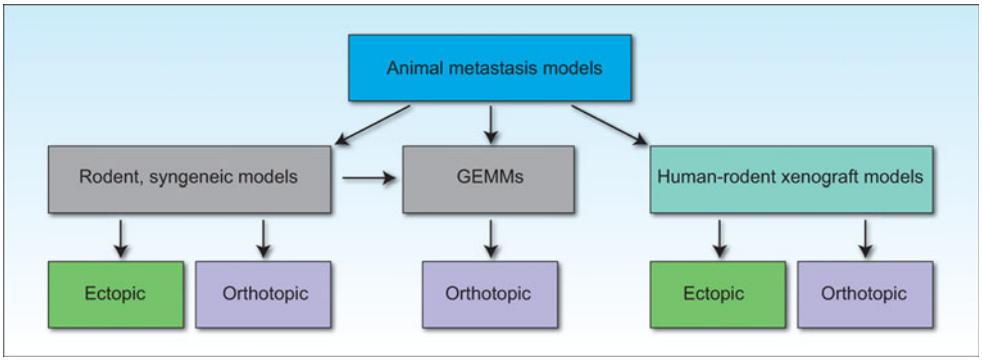

Fig. 2 Schematic grouping of the animal metastasis models that are available today. Animal metastasis models may be grouped based on which tumor material (rodent derived or human derived) is used or which genetic manipulations are performed to induce the brain metastasis, as well as the route of tumor tissue inoculation. In rodent, syngeneic models, murine derived cell lines are commonly injected either in the blood stream of the animal (ectopic models), or injected in the same organ as they originated from, for instance into mammary breast pad or skin (orthotopic models). Further, genetically engineered mouse models (GEMMs) may be regarded as a separate variant of the orthotopic rodent syngeneic models, as genetic manipulations in the mice results in formation of primary malignancies (in for instance lung or skin), with subsequent metastasis to other organs, including the brain. In the human-rodent xenograft models, either human derived tumor cell lines or patient tumor biopsies are xenografted into the animals, either into the blood circulation (ectopic), or in the corresponding animal organ as they originated from in the patient (orthotopic)

organ in the animal as the organ they originated from). Genetically engineered mouse models (GEMMs) may be regarded as a subclass of the orthotopic rodent syngeneic model, since genetic manipulations in mice results in development of primary malignancies, followed by metastasis to other organs, including the brain. In syngeneic models, the tumors develop in inbred animals with the same genetic background as the tumor cells. GEMM models use genetic techniques for genomic deletion of tumor suppressor genes or transgenic insertion of oncogenes in somatic cells.

In human-rodent xenotransplantation models, human cancer tissues or cell lines derived from human cancers are transplanted into immuno-compromised animals, most often mice or rats, either ectopically, or orthotopically.

Spontaneous brain metastatic models in rodents

In rare instances, mice have developed spontaneous melanomas. Although the formation of brain metastasis, which occurs frequently in melanoma patients, has not been observed in mice, several murine melanoma cell lines have been established from these spontaneous tumors [69] (Table 1). The B16-F1 cell line was derived from the original B16 melanoma cell line, established in 1954 from a spontaneous melanoma at the base of the ear of a C57BL/6 mouse [70, 71]. The B16-F1 cells showed strong tropism to the lung when injected intravenously in syngeneic mice [72]. These cells were then injected intracardially, and the resulting rare brain tumors were harvested to obtain the cell line B16-B1. After repeated intracardial (ICD) injections and subsequent selection for brain colonization, two neurotropic cell lines were established: The B16-B7b cells homing to the meninges, and the B16-B7n cell line metastasizing to the forebrain. After 3 additional passages, the lines B16-B10b and B16-B10-n were obtained, and almost every animal had brain metastases exclusively [47]. Several other derivatives of B16 cell lines have also been established, reviewed in detail by others [2, 69]. For instance, the highly invasive B16-BL6 melanoma cell line produced lesions in the meninges and the ventricles of the brain after intracarotid artery (ICA) injections in syngeneic mice [73].

Another spontaneous model is the KHT mouse sarcoma, which arose in a C3H mouse [74]. After ICD injections in female C3H/Bi mice, metastatic brain tumors occurred in 60–70 % of the animals. The tumors localized to the cerebrum, brainstem, cerebellum, and occasionally to the meninges [75]. McCutcheon and colleagues injected KHT sarcoma cells ICD in C3H mice, followed by adoptive immunotherapy treatment with IL-2 and lymphokine activated killer (LAK) cells. There was no reduction in the number of intracerebral metastases and no evidence of lymphocytic infiltration or cytolotic activity in the brain after treatment, suggesting that brain metastases in patients with systemic malignancies may not respond to intravenous (IV) treatment with LAK cells and IL-2 [76].

The 4T1 mammary carcinoma cell line is a thioguanine-resistant variant of the 410.4 cell line, which was isolated from a mammary tumor that spontaneously arose in a BALB/cfC3H breeding female [77, 78]. It was later shown that the 4T1 cell line was highly tumorigenic, and was able to spontaneously metastasize to distant organs, including lymph nodes, blood, liver, lung, bone and brain [79, 80]. A 4T1-derivative cell line 4T1-BR5 was injected ICA in female NuNu mice, by Lockman and coworkers. Texas

Table 1 Spontaneous brain metastatic models in rodents

Cell line	Origin	Phenotypes and examples of use	References
B16	Melanoma from a C57BL/6 mouse	Pigmented cells. Karyotyping of tumor cells and normal mouse. Low metastatic potential	[47, 70, 71]
B16-F1	Derived from B16 (one IV injection)	Mets to brain, lung, thoracic cavity, ovary, adrenals	[47, 72]
B16-B1	Derived from B16-F1 (one ICD injection)	Mets to brain, lung, thoracic cavity, ovary, lumbar spine	[47]
B16-B7b	Repeated injections of B16-B1 (3 times ICD, then 4 times IV)	Mets in meninges of the dorsal cerebrum	[47]
B16-B7n	Repeated injections of B16-B1 (3 times ICD, and 4 times IV)	Mets in forebrain (rhinal fissure)	[47]
B16-BL6	Derived from B16-F10 cell line	Mets in meninges and ventricles, but not in brain parenchyma (ICA injections). Used in studies of site specific metastasis	[73]
KHT	Sarcoma from a C3H mouse	Brain mets in 60–70 % of the mice, also lung, ovary, adrenal. Used in immunotherapy studies on brain metastasis	[74–76]
410.4	Mammary tumor from a BALB/cfCH_3H mouse	Aneuploid. Spontaneous mets in lung and liver after IV injections. Used in studies on mutations and metastatic potential	[77, 78]
4T1	Derived from the 410.4 cell line	Thioguanine resistant variant of 410.4 cell line. Spontaneous mets to lymph nodes, liver, lung, bone, brain. Used in studies of immunotherapy and BTB permeability	[79–81]

Red dextran (MW 3 or 70 kDa) was administered to tumor bearing mice, the brains were harvested, frozen and sectioned. Subsequent fluorescence studies on blood tumor barrier (BTB) permeability showed only minor leakage of tracer through the BTB, indicating that BTB remains a significant impediment to treatment [81].

An advantage of these models is that they were established from spontaneous tumors arising in mice, and as such the interaction between tumor cells and the immuno-competent host can be studied in detail. The short latency time between injection and metastatic spread is favorable for studying both molecular mechanisms related to metastatic spread as well as for assessing new therapeutic strategies. In particular, since the B16 derivative cell lines have shown clear preferences for different organ sites such as lung and brain, they may be well suited for studying mechanisms responsible for tumor cell homing to the brain. In this context, one of the B16 variants (the B16-B7n cell line) has shown an unusual spread to the mouse forebrain, which is somewhat different from the metastatic pattern seen in patients [69]. It should also be emphasized that spontaneous murine melanoma formation is rare [69], and, importantly, most likely does not reflect completely the oncogenic transformation events seen in humans. It is therefore still an open question as to what extent knowledge gained from such models can be translated into the human situation.

Induced brain metastatic models

Murine melanomas have also been induced chemically, or by exposure of healthy animals to ultraviolet (UV) radiation (Table 2). The K-1735 murine melanoma cell line (developed at the MD Anderson Cancer Center in 1979), and its derivatives have been extensively used to study intracerebral metastasis. The K-1735 cells were derived from a cutaneous melanoma in a C3H/HeN mouse, induced by UV exposure followed by painting with the carcinogenic compound croton oil which is extracted from the seed *Croton tiglium* [82]. *In vitro* studies have shown that the cells were phenotypically very heterogeneous, since the different clones isolated displayed variance in growth, melanin production and chromosome number [83]. The importance of the metastatic environment was also demonstrated, as subcutaneous (SC) implantantion of K-1375 cells led to amelanotic tumors, while IV injected cells formed melanotic metastasis, with expression of tumor melanocyte-stimulating hormone receptor [84, 85]. This model system has been used for the investigation of site specific metastasis, where the K-1735 cells produced tumors only in the brain parenchyma [86], and also to study BTB permeability [87].

The JB/MS melanoma cell line was developed in C57BL/6 mice by a single application of 7,12-dimethylbenz(a)anthracene to the scapular region of 4-day old mice, followed by twice-weekly painting with croton oil [88]. Metastatic spread of JB/MS cells to the mouse brain has been reported [89]. Immunologic studies have been performed on immunocompetent mice with SC tumors [90], however there have been no reports in the literature on brain metastatic development after using the JB/MS cell line.

The UV-2237 fibrosarcoma cell line was developed from a skin lesion in a female C3H/HeN mouse after

Table 2 Induced brain metastastic models in rodents

Cell line	Origin	Phenotypes and examples of use	References
K-1735	Melanoma (exposure with UV radiation and croton oil)	Melanotic brain metastasis in parenchyma after ICA injections. Heterogeneity in metastatic propensity, growth rate and chromosome number. Used to study site specific metastasis and BBB permeability	[82–87]
JB/MS	Melanoma (exposure with 7,12-dimethylbenz(a)anthracene and croton oil)	Brain mets have been reported, but no further studies done on brain metastasis	[88–90]
UV-2237 MM	Fibrosarcoma, derived from the UV-2237 cell line	Mets to all sites of the brain after ICA injections. Used in studies of site specific metastasis	[73, 91, 92]

chronic UV-B irradiation exposure [91]. From this cell line, highly metastatic UV-2237 MM cells were established [92] that, after injection into the internal carotid artery of syngeneic mice, induced brain metastasis in over 80 % of the animals, causing death after 2–3 weeks [73].

The models described above were all established in fully immunocompetent animals where they have been used to study interactions between metastatic tumor cells and the host microenvironment [84]. They therefore represent an important tool to obtain insight into mechanisms related to homing of tumor cells to the brain.

In studies using the B16 and the K-1735 murine melanoma cell lines, unique metastatic patterns to the mouse brain were found, including metastatic spread to meninges, parenchyma and ventricles. It has further been shown that after cell entrapment in the brain vasculature, the K-1735 cells failed to proliferate at this site, while the B16 grew rapidly. These data confirmed that initial entrapment in brain vasculature did not necessarily correlate with development of progressively growing tumors [93].

Based on current information, it is uncertain if chemically induced models have a clear-cut dissemination pattern. Another important point is that the genetic alterations that lead to transformation (induction of oncogenes, suppressor genes and cell signaling) have not been completely delineated. It is therefore not clear if these models actually reflect human disease.

Genetically engineered mouse models (GEMMs)

Significant contributions identifying the roles of oncogenes and tumor suppressor genes in tumor development have been derived from the use of GEMMs. In particular, inducible in vivo expression of oncogenes, as well as conditional, tissue specific deletion systems have provided important insight into the mechanisms of cancer initiation and early steps of metastatic dissemination [94]. A major problem with GEMM-induced tumors is the low incidence of metastatic spread [95], which in part may be explained by a rapid development of the primary lesions. Metastases from such models have mainly been restricted to the lymph nodes, lungs and abdominal organs [95, 96]. However, in a few GEMM models, genetic modifications have led to the formation of tumors with secondary spreading to the brain (Table 3). A mouse model for neuroendocrine lung tumors was developed by somatic inactivation of *Trp53* and *Rb1*, leading to lung tumors showing striking similarities to small cell lung carcinomas, with subsequent extrapulmonary metastasis, including the brain [45]. In a transgenic mouse model introducing the *ret* oncogene, melanocytic tumors developed in the skin, followed by metastasis to brain, liver, kidney, spleen, lung and lymph nodes [43]. These experiments indicate that GEMM models show considerable promise in the studies of brain metastasis formation. Further development and validation, however, is needed in order to find appropriate GEMM models that reflect human disease.

Human xenotransplantation models

From the 1970′s and onwards, several brain metastasis xenotransplantation models were developed using cancer cell lines established from breast carcinomas, lung adenocarcinomas, lung squamous cell carcinomas, renal cell carcinomas and melanomas (Table 4). After IV or IC administration in nude mice, brain metastases were observed at various take rates [38, 52, 97] where one of the most widely used cell line is MDA-MB-231 originally isolated in 1973 at the MD Anderson Cancer Center, from a human breast tumor pleural effusion [98]. Several other cancer cell lines of different origin as for instance MDA-MB-157 [99] and MDA-MB-435 [100] were also developed at the same institution. Lorger and Felding-Habermann captured the initial steps of brain metastasis, by showing that several Firefly luciferase tagged breast carcinoma cell lines (MDA-MB-231 and MDA-MB-231BR, MDA-MB-435, 4T1, MCF-7) were able to extravasate through the BBB between Day 3 and Day 5 after ICA

Table 3 Genetically engineered brain metastatic models (GEMMs) in rodents

Origin and genes involved	Phenotypes and examples of use	References
Neuroendocrine lung tumor (inactivation of *Trp53* and *Rb1)*	Lack of contact inhibition, rapid growth, epithelial morphology. Lung tumors, followed by extrapulmonary metastasis, including the brain	[45]
Melanocytic tumors (after inserting the *ret* oncogene)	Tumors in skin, followed by mets to brain, liver, kidney, spleen, lung and lymph nodes	[43]

injections in mice. Immediate astrocytic and microglial reactions were seen in the vicinity of tumor cells entrapped in the brain microvasculature [101]. Heyn and colleagues visualized single tumor cell spread to the brain, by performing MR imaging of the mouse brains 5 h after ICA injections of MDA-MB-231BR breast cancer cells prelabeled with iron oxide particles [102]. Further, a possible role for matrix metalloprotease 2 in breast cancer progression to the brain was demonstrated after ICD injection of MDA-MB-231 cells in nude mice [103].

During recent years there has been a controversy regarding the true origin of the MDA-MB-435 cell line. Rae and colleagues [104] have claimed that it originated from the M14 malignant melanoma but evidence from the literature now suggests that both these cell lines might be of MDA-MB-435 breast cancer origin [105].

The MCF-7 breast carcinoma cell line, originally established in 1973 from a pleural effusion in a patient with metastatic breast carcinoma [106], has also been widely used in brain metastasis research. For instance, MCF-7 cells transfected to overexpress Her-2 were transplanted into the cerebrum of athymic rats. Treatment by intracerebral microinfusion (ICM) with trastuzumab increased median suvival by 96 % to 52 days, showing that ICM was superior to systemic delivery of chemotherapy in this model [107]. The list of brain metastasis models has been extended to include other breast carcinoma cell lines (such as ZR75-1 [108, 109], MDA-MB-361 [110], MDA-MB-468 [100] and MA11 [111]) [109–111] as well as lung carcinoma cell lines (such as A549 [112, 113]) and melanoma [114].

Ectopic models: the inoculation route: an important factor in brain metastasis formation

An important issue for ectopic brain metastases models relates to the route of inoculation (Fig. 3). Tumor cells can be inoculated IV (into the tail vein), resulting in dissemination primarily to the lungs with further metastasis to the CNS (Fig. 3a) [69, 115].

Table 4 Human xenotransplantation models in rodents

Cell line	Origin	Phenotypes and examples of use	References
MDA-MB-157	Pleural effusion of a patient with stage III breast carcinoma	Lack of contact inhibition, rapid growth, epithelial morphology	[99]
MDA-MB-231	Pleural effusion of a patient with stage III breast carcinoma	ER negative. Mets mostly to bone, also to brain, adrenal, ovary. Increased number of brain mets after transfection of pro-MMP-2. Used in studies on the initial steps of brain metastasis	[98, 101, 103]
MDA-MB-231BR	Repeated ICD injections of MDA-MB-231 cells (6 times)	Brain mets in 100 % of animals, no other mets detected. Used in studies on the initial steps of brain metastasis, and MRI of single tumor cell spread to the mouse brain	[101, 102]
MDA-MB-361	Breast cancer brain metastases	ER positive. Multiple brain mets after ICA inj., some showing angiogenesis	[110]
MDA-MB-435	Repeated injections of B16-B1 (3 times ICD, and 4 times IV)	ER negative. Multifocal, circumscribed brain mets. Used in studies of BBB permeability, and activation of $\alpha v\beta 3$ promotes brain mets	[100, 101]
MDA-MB-468	Pleural effusion of a patient with breast carcinoma	Brain mets in 9/15 mice after intraarterial injections	
MCF-7	Pleural effusion of a patient with breast carcinoma	Epithelial-like, polygonal morphology in cell culture. Intracerebral microinfusion of trastuzumab in Her-2+ MCF-7 cells	[106, 107]
ZR75-1	Ascitic effusion of a patient with breast carcinoma.	Hematologous spread to brain not reported. Used in studies of angiogenesis in brain mets through cranial chamber.	[108, 109]
MA11	Human breast cancer isolated from bone marrow.	Brain mets in 87 % of the animals after ICD injections. Mets were not found in other organs.	[111]
H1_DL2	Brain metastasis from malignant melanoma.	Brain mets in 100 % of animals, also to ovaries, bone, adrenals. Single cell tracking by MRI using USPIO prelabeling.	[114]

To avoid the passage of tumor cells into the pulmonary circulation with subsequent lung metastases, ICD injection of various tumor cell lines into the left ventricle of immuno-compromised mice has been performed [36, 75, 116]. This leads to a systemic distribution of cells to most organs, including the brain (Fig. 3b). In this context, we have recently developed a novel melanoma-derived model, by injecting a cell line from a human melanoma derived brain metastasis (expressing Green Fluorescent Protein (GFP) and Firefly luciferase) ICD into NOD/SCID mice [117]. Intracerebral tumors were detected 2–4 weeks after injection by bioluminescence imaging (BLI), and subsequent MRI verified multiple metastatic CNS lesions. Although tumor dissemination was also seen in other organs, there was a clear propensity for tumor dissemination to the CNS, where the melanocytic properties of the original tumor were maintained.

Tumor cells have also been delivered through the carotid artery, sometimes involving a permanent ligation of the artery, where collateral blood circulation through the circle of Willis ensures adequate blood supply to the brain (Fig. 3c). This procedure minimizes the spread of tumor cells to other sites than the brain [101]. An assessment of cellular dissemination by this injection technique has been performed, using multiphoton laser scanning microscopy through cranial windows, where individual metastatic tumor cells have been monitored over prolonged time periods in vivo. Using this method tumor cell arrest at vascular branch points was elegantly demonstrated, being followed by extravasation and perivascular growth [53].

Ideally, tumor cell inoculation and metastatic spread should mimic progress of clinical disease, but at present none of the inoculation methods fully reflect disseminated disease in humans. For instance IV injection methods almost inevitably lead to lung metastases, followed by tumor development in other organs, including the brain. As mentioned before a potentially better approach is the injection of tumor cells into the arterial blood stream, thereby avoiding the filtering of tumor cells in the lungs.

ICD injections are technically relatively simple to perform. However the difficulty lies in the ability to control the exact number of cells injected, as the needle tip has to be positioned accurately into a beating heart during the whole injection process. Thus, inoculation of cells into the heart wall can lead to unwanted tumor formation inside the ventricular walls, and/or injection of cells into the right cardiac ventricle with subsequent spread to the lungs [16]. With our melanoma brain metastasis model, we were able to perform BLI within 15 min of IC inoculation. Obvious mistakes during injection were observed in around 10 % of the animals, characterized by a BLI signal in the lungs and/or in the heart wall. It is also recognized that the IC procedure sometimes may cause a relatively high degree of procedural mortality [69].

ICA inoculation targets to a large extent the tumor cells directly to the brain, which is a disadvantage in homing studies. The technique usually also involves permanent ligation of the carotid artery after injection, which changes the normal blood supply to the brain. A high level of microsurgical skill is also needed, and the technique is

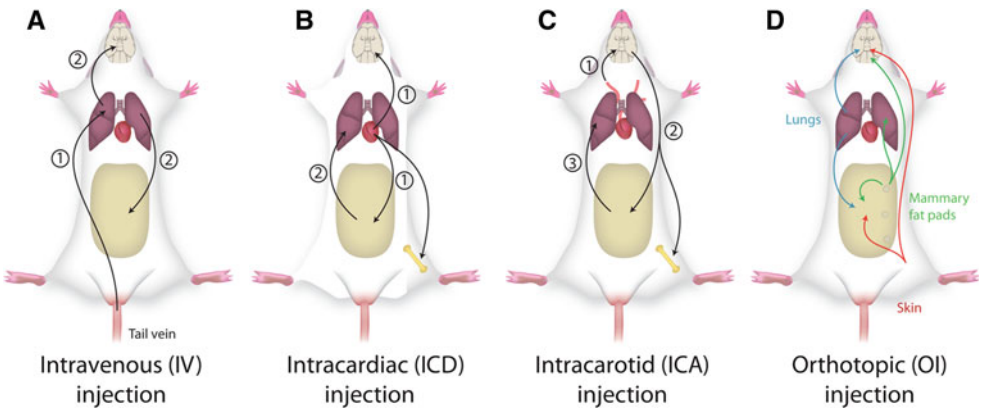

Fig. 3 Inoculation routes used in brain metastasis models. The primary (*1*), secondary (*2*) and tertiary (*3*) routes of metastatic tumor cell dissemination are indicated. **a** When ectopic, intravenous (IV) inoculations are performed, the primary route of tumor cell dissemination is to the lungs (*1*), followed by a secondary spread to brain and other organs of the body (*2*). **b** When an ectopic intracardiac (ICD) inoculation route is chosen, the tumor cells will primarily spread to the brain and the abdominal organs including bone (*1*), followed by a secondary spread to the lungs (*2*). **c** When an ectopic, intracarotid (ICA) inoculation is performed, the tumor cells will first disseminate to the brain (*1*), followed by spread abdominal organs including bone (*2*), and finally the lungs (*3*). **d** An orthotopic, organ specific (OI) inoculation of tumor cells will result in tumor growth at the primary site, followed by a metastatic spread of tumor cells to abdominal organs and brain. Orthotopic brain metastasis models have been developed for lung carcinomas (*blue arrows*), melanomas (*red arrows*) and breast carcinomas (*green arrows*). (Color figure online)

hampered because of a relatively high mortality. Even so, the ICA technique may be more reproducible, compared to the ICD technique.

Ectopic models: the importance of the inoculation route when studying epithelial to mesenchymal transition (EMT)

The precise mechanisms involved in the transition of non-invasive tumor cells into cells with metastatic potential are not known [118]. However, epithelial to mesenchymal transition (EMT) has been suggested as one of the major mechanisms in metastatic progression [119]. Similar to the epithelial cells during embryonic development [120], the tumor cells in the primary tumor lose their epithelial characteristics and cell-to-cell contacts, and acquire a mesenchymal gene expression [121, 122]. Genes encoding epithelial junctional complexes (e.g. E-cadherin and β-catenin) and cytokeratins may be repressed, while expression of mesenchymal markers (e.g. vimentin and N-cadherin) is induced [123]. The cells then detach from the primary tumor, intravasate into the circulation and reach the distant organ where they form solid metastases. The opposite of EMT i.e. mesenchymal to epithelial transition (MET), is also likely to play a role in the formation of metastatic tumors [118].

The inoculation method is of importance when studying EMT in experimental metastasis models, and injecting tumor cells into the bloodstream of animals is not likely to reflect the changes in epithelial and mesenchymal gene expression seen in primary tumors prior to the metastatic process. The orthotopic injection technique has previously been established as a relevant method, and in a xenograft model of breast cancer, MDA-MB-468 human breast carcinoma cells were injected into the fat pad of mice. EMT occurred in the primary tumors, which was associated with an enhanced ability to intravasate and generate circulating tumor cells, as shown by increased expression of vimentin and loss of E-cadherin. The changes in vimentin and E-cadherin expression in lung macrometastases also suggested that MET-phenomena occurred in secondary organs, facilitating metastatic growth [124].

Orthotopic models metastasizing to the brain

Transplantation of tumor cell lines or dissociated patient-derived tumor tissue into orthotopic locations frequently show spontaneous metastatic potential after a certain lag time (Fig. 3d). Orthotopic brain metastasis models have been developed for several cancer types, such as lung carcinomas [113], melanomas [69, 125] and breast carcinomas [35, 36, 109, 110] (Table 5). Mathieu and colleagues developed an orthotopic model of human non-small cell lung carcinomas (NSCLC) by injecting the NSCLC A549 cell line into the left lung of nude mice. A 100 % tumor take was observed, with metastatic spread to brain (61 %) and liver (40 %). The histopathology showed poorly differentiated tumors, which were CK7+ and CK20− [113].

A highly metastatic variant of the WM239A human melanoma cell line, named 113/6-4L was obtained after SC injection in SCID mice. After receiving metronomic cyclophosphamide and vinblastine therapy, prolonged survival of animals was obtained, yet the animals eventually developed brain metastases. From these metastases, two new cell lines 131/4-5B1 and 131/4-5B2 were generated, which, after SC injection, led to formation of brain metastases 97–180 days after primary tumor resection [125].

Also MCF-7 FGF transfected human breast carcinoma cells injected into the upper right mammary fat pad of athymic female nude mice developed micrometastases in several organs including the brain after 3 weeks, and macroscopic metastasis were detected in the brains in 35 % of the mice after 12 weeks [35]. All these models show that metastasis formation in various organs can occur from orthotopic sites and they represent, therefore, valuable experimental tools to study mechanistic aspects related to the formation of secondary brain metastases.

Several models of direct orthotopic placement of patient tumor biopsies in animals were introduced in the early 1990′s, by suturing fresh tumor specimens from patients into the corresponding organs of immuno-compromised mice [126]. Histologically intact human colon cancer specimens were implanted into the cecum or colon of nude mice [127]. Orthotopic growth was observed in 13 of 20 cases, with regional metastases, as well as metastases to lymph nodes and liver. An orthotopic breast cancer model was also developed, by implanting breast cancer tissue from a patient into the mammary fat pad of nude mice [128]. Extensive growth and lung metastases were seen. However, none of these experiments resulted in brain metastases.

At present it is not clear if mechanisms related to the formation of brain metastases actually reflect the biological events that occur in humans. Current limitations include a lack of validation against clinical brain metastases from biopsies or autopsies. It has been recognized for a long time that established cell lines have undergone a clonal selection and have become adapted to tissue culture. This has resulted in different genotypic and phenotypic profiles compared to the tumor of origin. Conclusions from such experiments should thus be handled with caution where a detailed validation to corresponding human tumors is important. Such models have revealed, however, novel insights into the ability of circulating, metastatic tumor

Table 5 Orthotopic models in rodents, metastasizing to the brain

Cell line	Origin	Phenotypes and examples of use	References
A549	Human non-small cell lung carcinoma cells, grafted into left lung of nu/nu mice	Properties of type II alveolar epithelial cells. Cells synthesize lecithin. Poorly differentiated brain mets developed in 61 % of the mice. CK7+, CK20−	[112, 113]
B16-B10n	Murine B16 melanoma. Implanted SC into syngeneic mice	Mets were found in lymph nodes after 2 weeks, and brain mets after 4 weeks	[47]
113/6-4L	Lung tumor from mouse injected SC with WM239A human melanoma cells	Metronomic therapy (cyclophosphamide + vinblastine) lead to prolonged animal survival, and the mice developed brain mets. These mets were used to develop 131/4-5B1 and 131/4-5B2 cell lines	[125]
131/4-5B1	Cell line based on brain mets from 113/6-4L cell line. Mice were injected subdermally	Following subdermal injections, 54 % of the mice developed spontaneous brain mets. Effective spread to lungs were also seen	[125]
131/4-5B2	Similar procedure as for 131/4-5B1	Spontaneous spread to brain and liver	[125]
MCF-7	Human breast carcinoma, transfected with FGF. Injected into mammary fat pad	Micromets within the brain after 3 weeks, macroscopic brain mets in 35 % of the animals after 12 weeks	[35]

cells (CTCs) to colonize their own tumor of origin, in a process called "tumor self-seeding". For instance it has been shown that self-seeding of cell lines from human breast cancer, colon cancer and melanomas in immunodeficient mice is preferentially mediated by aggressive CTCs expressing MMP1, collagenase-1 and fascin-1. In this context, a brain-derived metastatic cell line (CN34-BrM2) originating from a pleural effusion cell line expressing GFP/luciferase was able to seed the MDA231 recipient tumor [129].

Interestingly, the tumors arising from orthotopic cell injections show relatively low metastatic rates, compared to models where tumor fragments taken from patients are surgically implanted into orthotopic sites in animals [126]. The reason for this difference is not known.

A major limitation of using orthotopic patient xenografts, is that metastasis to the CNS is rare. At present it is not clear if this is due to an inability of circulating tumor cells to cross the BBB, or if this is due to a rapid local tumor growth, causing a lethal tumor burden before metastases in the CNS become evident. By the use of advanced imaging techniques (MRI, CT, PET, BLI) it may in the future be possible to address this important question [130].

Direct implantation of metastatic tumor cells and tissue into the rodent brain

Orthotopic brain metastasis models have also been established by direct implantation of brain metastases, either as cell lines or tumor biopsies, into the animal brain. This should be regarded as local growth models rather than metastatic models, since they only reflect the final step of the metastatic process. The injection of tumor cells is usually performed by stereotactic guidance [131, 132], but specialized inoculation techniques have also involved the use of a subarachnoid catheter placed into the cisterna magna along the spinal cord [133, 134]. This technique has enabled studies of leptomeningeal melanoma metastases, and has also allowed for studies of therapeutic drug delivery [135].

To avoid using cell lines [136], we recently developed a clinically more relevant model, based on the implantion of patient biopsies obtained from brain metastases directly into the brains of immunodeficient rats, thus minimizing a putative clonal selection process in vitro [117]. Nine different brain metastases from four different primary cancers (colon, lung, skin, ovary) were xenografted into the brain, and tumor growth was achieved in seven out of nine biopsies (Fig. 4). The brain metastases that developed in the animal brains had similar radiological appearances as seen clinically, showing expansive growth, contrast enhancement, necrotic areas and edema (Fig. 4a, c). Histological and immunohistochemical evaluations were performed by two independent neuropathologists. Histological comparisons between the primary tumors from the patients, patient brain metastases and the derived xenografts, showed similarities in histology and growth patterns, with mitotic figures, necrotic areas, and cytological signs of malignancy, such as pleomorphic and hyperchromatic nuclei with prominent nucleoli (Fig. 4b, d). In addition, immunohistochemistry of 20 commonly used clinical tumor markers showed a strong similarity in expression between the patient tumors and the corresponding xenografts. By comparing DNA copy number expression of patient brain metastases and animal brain xenografts, striking similarities in chromosomal aberrations were also seen, indicating that xenografting did not result in a clonal selection.

The models of brain metastasis described above can make significant contributions to our understanding of the

molecular mechanisms that occur during the final stages of metastastic growth in the brain and they should therefore also be well suited for the study of new treatment modalities, since the tumor characteristics originally present in the biopsies from patients are to a large extent preserved. Histological evaluations presented in the literature are in general usually very superficial, and we would thus like to stress the importance of using experienced neuropathologists with the aim of model validation. Importantly, the tumor stroma, which may play a major role in metastatic disease, [95, 137] is partially preserved when biopsies from patients are implanted directly. However these models are less suited to the study of mechanisms related to the metastatic process in the CNS.

From mouse to man: what can brain metastatic animal models teach us?

In the clinic, controlling metastatic disease within the CNS represents a formidable problem. Based on the various animal brain metastasis models developed, it is envisaged that this important question can be answered in the near future [16]. In this context, a recent cell-line model of

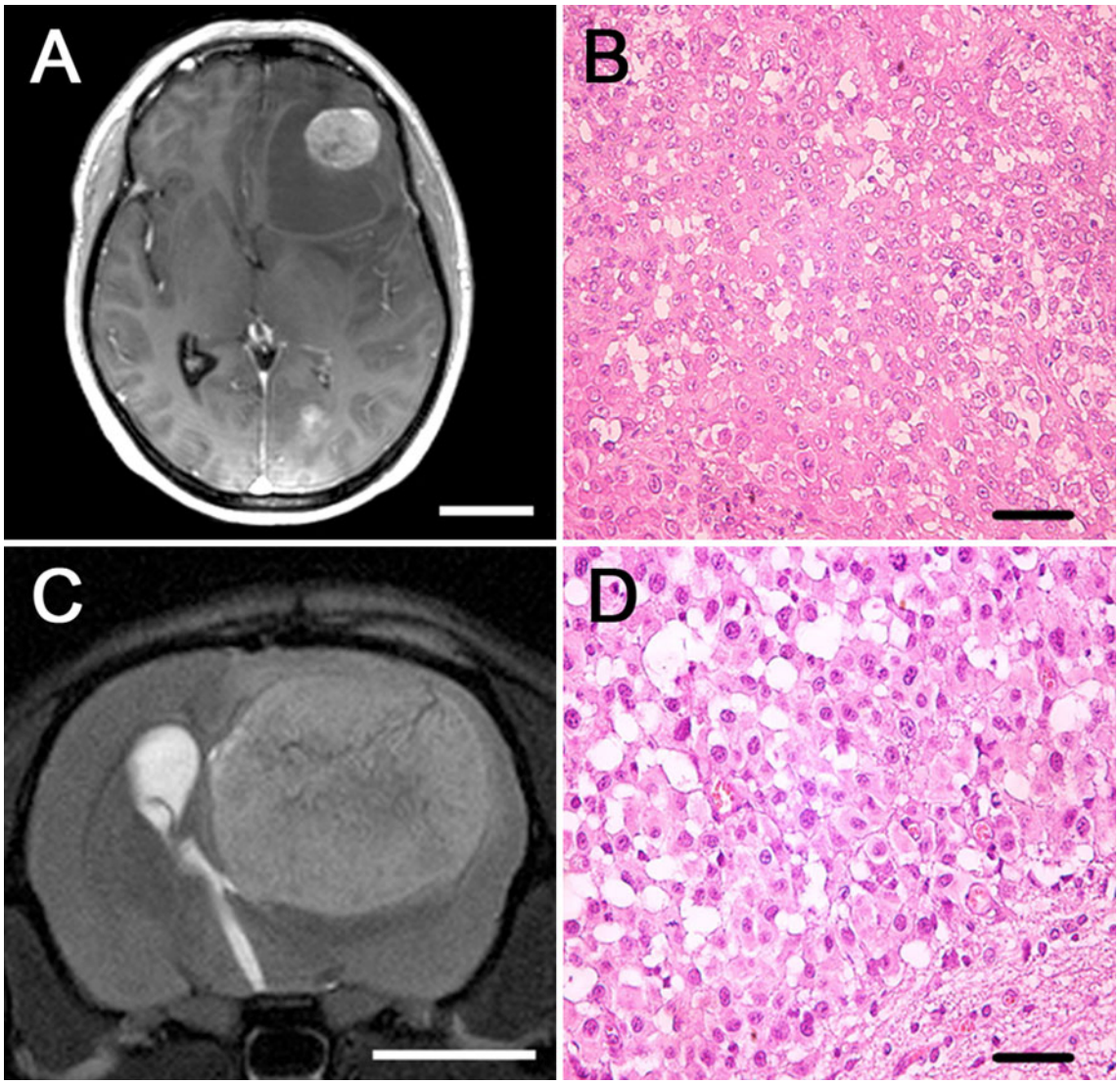

Fig. 4 MR and histological comparison of brain metastases from patient with malignant melanoma and the corresponding animal brain metastases. **a** Clinical T1 weighted MR image of the brain metastases after contrast injection. This tumor was then harvested, put in culture and implanted into nude rats. *Scale bar* 3 cm. **b** Histological section of the patient brain metastases. *Scale bar* 40 μm. **c** Preclinical T2 weighted MR image, obtained 3 weeks after implanting biopsy pieces of the melanoma brain metastasis seen in (**a**), intracranially into immunodeficient rats. *Scale bar* 5 mm. **d** Histological section of the animal brain metastatic tumor seen in (**c**). The histological features of the animal brain metastases were similar to the corresponding patient brain metastases. *Scale bar* 40 μm

spontaneous CNS metastasis has been generated from the 113/6 subline derived from the WM-239 human melanoma. The treatment of established 113/6-4L tumors using metronomic therapy (vinblastine and CTX), led to prolonged animal survival with eventual emergence of brain metastases [138]. As this model mimics the complete cascade of metastatic events, it is likely to provide important insight into the mechanisms of the formation of metastasis in the brain.

The animal brain metastasis models to be used should obviously be chosen based on the scientific questions sought to be answered. Syngeneic brain metastasis models enable the study of interaction processes between tumor cells and the host microenvironment, but limits the researcher to study only mouse metastatic tumor cells. Thus, results from such studies need validation in human samples. In xenograft models there is a wide range of human samples as well as metastatic sites, including the brain. Despite the lack of adaptive immune interactions with the tumor tissue, such models can be regarded as a better choice to study human metastasis in vivo. In this context it is important to emphasize that the injection site should be adjusted according to the question in focus. For instance, can effects of treatment with new therapeutic drugs be studied in a clinically relevant model where human tumor material is implanted directly into the animal brain? Direct implantation of tumor biopsies from patients also ensures that tumor stromal elements from the patients are transferred into the model. The success of colonization of relevant organs including the brain, should be studied by ICA or ICD injections in the animals.

Further, GEMM models are used primarily as tools to study the biological function of genes during neoplastic transformation and tumorigenesis. However, specific strains are now being used in selective chemo-prevention and chemotherapy trials [139].

The animal models currently available give relatively little insight into some of the important steps of the metastatic process. For instance, an issue still to be resolved, is whether the metastatic process is an early or late event in cancer progression [140]. Although it has been generally accepted that metastasis occurs late in tumor progression, there is now clinical and experimental evidence indicating that metastasis may occur also early during tumor development [16, 141]. As most animal models exhibit relatively rapid tumor growth in vivo, there may not be a time window large enough to assess late metastatic growth.

Tumor dormancy can be defined clinically as the disease-free period between cancer treatment and late recurrence [142]. Tumor dormancy mechanisms are usually categorized into two groups, single dormant tumor cells and dormant micrometastasis [143]. This phenomenon has been observed in the clinic for decades. For instance prostate and breast cancers show metastatic spread years after primary diagnosis, suggesting that tumor cells may lie dormant in distant organs for long periods of time [2], or that the metastatic process is initiated by treatment resistant genes at the primary site.

The mechanisms causing tumor dormancy are poorly characterized, and it is currently not known if dormant metastatic cells are a subpopulation of tumor cells which are programmed to stay in a dormant state or if the tumor cells simply are unable to grow effectively in a new microenvironment, or both [144]. With the exception of a few murine models, such as for instance for mammary carcinomas [145], there are at present very few animal models available to address these issues, due to the rapid growth of the commonly used metastatic cell lines in vivo, as well as problems related to detecting the dormant tumor cells by in vivo imaging [95, 144]. A novel method to study tumor cell dormancy in the brain from breast cancer cell lines has previously been reported. This involves performing MR imaging after prelabeling the cells with micro-sized iron oxide particles (MPIOs) [102]. We developed the prelabeling technology further, by ICD injection of human melanoma brain metastatic cells after prelabeling them with ultrasmall superparamagnetic iron oxide particles (USPIOs). By T2* weighted MR imaging with subsequent automated cell detection using our own software developed in MatLab, and verification by histology and immunohistology, we could quantify single tumor cells after a few hours as well as 8 weeks after injection [114].

In conclusion, considerable efforts have been put into developing representative animal models of brain metastasis. These models have provided new insight into mechanisms of metastatic spread where physical as well as molecular mechanisms have partly been delineated. Even though it has been difficult to establish animal models that reflect all the steps during brain metastasis formation in humans, there are a number of models that show considerable promise. In particular orthotopic models, where the tumor cells are placed in the organ of origin, may be regarded as the model of choice when studying metastasis of human cancer cells in vivo [95]. There is, however, an urgent need for a thorough validation of such models where in depth comparisons to the patient material is important. Current knowledge from animal models indicates that the mechanisms responsible for the metastatic process occurring in the brain involve important interactions with the brain vasculature but the actual contribution of normal brain tissue is at present not clear. Future work should focus on the challenging task of finding the right animal models that reflect human disease. With the establishment of such models there is hope that the mechanisms behind the metastatic process can be further delineated.

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