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Brain malignancies: Glioblastoma and brain metastases

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ABSTRACT

Brain, the major organ of the central nervous system controls and processes most of body activities. Therefore, the most aggressive brain tumor – glioblastoma and metastases from other organs to the brain are lethal leaving the patients with very short time of survival. The brain tissue landscape is very different from any other tissues and the specific microenvironment, comprising stem cells niches and blood-brain barrier, significantly influences the low rate of glioblastoma metastasis out of the brain, but better accommodates brain-invading cancer. In contrast to low frequency (0.5%) of all glioblastoma metastases, 10%–45% of other primary cancers do metastasize to the brain. This review addresses general cellular and molecular pathways that are to some extent similar in both types of metastases, involving circulating tumor cells (CTCs) with cancer stem cells (CSCs) characteristics, and metastatic niches. The invasion is a dynamic process involving reversible epithelial-to-mesenchymal (EMT) cell process, creating a transient gradient state that is inter-connected with epigenetic plasticity of the metastasizing (m)CSCs. These cells can switch between stationary, low proliferating/dormant state to a migratory, mesenchymal-like state. Settling in their respective niches as dormant CSCs in the secondary organ is a common feature in all types of metastases. In glioblastoma metastasis, the malignant mGSC cells express markers of mesenchymal GSC subtype (MES-GSC), such as CD44 and YK-40 and their major obstacle seems to be propagating in the in various organs' microenvironments, different from the niches that home GSCs in the primary glioblastoma. Focusing on one stromal component in the glioblastoma niches, the mesenchymal stem cells (MSCs), we report herein on their differential effects on glioblastoma cells, highly depending on their genetic subtype. On the other hand, in brain metastases, the major hindrance to metastatic progression of mCSCs seem to be crossing the blood-brain-barrier. Novel therapeutic approaches for brain metastases from various cancer types are advancing slowly, and the general trends involve targeting metastatic sub-clones and selective determinants of their niches. The update on the four most common brain metastases from lung, breast, melanoma and colorectal carcinoma is presented.

1. Metastasis evolution

1.1. Characteristics of metastatic cells

Recently Welch and Hurst [1] redefined the "hallmarks of metastasis" after Hannahan and Weinberg [2] and the following four essential hallmarks of the metastatic cells were proposed: (a) motility and invasion, (b) ability to modulate local metastatic microenvironment(s), (c) high and reversible metastatic (stem) cell plasticity and (d) the ability to proliferate i.e. to colonize the secondary tissues. They also emphasize that the only characteristics that distinguishes malignant cancer from a benign tumor is invasion [2]. However, invasion is not sufficient to develop metastases that are the ultimate manifestation of neoplastic cells evolution toward autonomous activity. During growth of the primary tumor, high levels of genetic and genomic instability lead to the evolution of cells that acquire odd characteristics and sooner or later manifest as metastatic subpopulations with mostly, but not exclusively irreversible traits. These cells are progressively involved in sequential processes after the invasion, i.e. intravasation, survival as circulating tumor cells (CTCs) in body fluids, cell arrest and

Abbreviations: BBB, blood–brain-barrier; BM, brain metastases; CSF, cerebrospinal fluid; CNS, central nervous system; CAFs, cancer associated fibroblasts; CSCs, cancer stem cells; CR, colorectal carcinoma; CTCs, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; GSCs, glioblastoma (GB) stem cells; HSC, hematopoietic stem cell; HGG, high grade glioma; LGG, low grade glioma; MSCs, mesenchymal stem cells; MES, mesenchymal GB subtypes; mCSCs, metastatic CSCs; NSCLC, non-small cell lung carcinoma; PN, N, proneural, neural GB subtypes; TME, tumor microenvironment; VEGF, vascular endothelial growth factor

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extravasation, micro-metastasis formation and secondary organ colonization - altogether comprising a metastatic cascade [1,3,4]. Most frequent pattern of dissemination is *via* the bloodstream (i.e. hematogenous), but it could also occur through lymphatic vessels or across coelomic cavities, although the latter two are inter-connected with blood vessels. Some tumors can migrate along the basal side of endothelial cells [5], never entering the lumen. Alternatively, cancer cells might travel along the nerves and in brain along myelin fibers [6].

The perineural routes in various cancer are more frequent than suspected and associated with poor prognosis [7], most likely due to the fact that nerves are being spared by resection surgery and therefore cause recurrence.

The key process involved in regulating invasion and metastasis is epithelial-to-mesenchymal transition (EMT), where cells loose polarity, cell-cell adhesion and the expression of cell surface and cytoskeletal proteins, enabling the cells to acquire migratory properties [8,9]. In cancer, EMT is a dynamic process of interconversion of epithelial cells to mesenchymal-like cells that to some extent acquire stem cell-like properties, increased motility and invasive capacity, resistance to several treatment strategies as well as immune-evasive and immunosuppressive characteristics. EMT is induced by transient activation of several oncogenic signaling pathways, triggering reversible activation of transcription factors, e.g. Snail, Slug, Twist and ZEB family. The reverse process is called mesenchymal-to-epithelial transition (MET) that leads to re-appearance of slowly proliferating epithelial-like cells. These cells arrest in G0/G1 cycle phase and appear as dormant cells, such as were found in micro-metastases [10] and remain in specific microenvironment, so called “metastatic niches” for a longer time, until their proliferation is activated to colonize the secondary organ [10].

Thus, increase in the levels of genetic and genomic instabilities lead to evolution of cells that exhibit properties of metastatic subpopulations with mostly, but not exclusively irreversible traits. The metastatic cells are progressively selected as capable of sequential steps of the metastatic cascade, as described in details in recent reviews [1,3,4]. Briefly, these cells are progressively involved in sequential processes after the tissue and blood stream invasion i.e. intravasation. They must survive as CTCs in body fluids, arrest at the endothelial vessel walls, extravasate and form micro-metastasis, which may or may not colonize the secondary organ.

1.2. Evolution of metastatic cancer stem cells and metastases heterogeneity

Development of metastatic microenvironment may in fact begin long before primary tumors are detectable. Due to perpetual local heterotypic cell communication with stromal cells, or systemically *via* exosomes that carry mRNA and proteins to other parts of the body, so called “pre-metastatsic niches” are set-up prior to intravasation [11,12] (see also below). The metastasizing cells first appear as clumps of slow proliferating, and dormant cells that are highly resistant to therapy for a long duration of time [13]. The dormant metastatic cells that are speculated to possess the characteristic plasticity of cancer stem cells (CSCs), respond to activating signals from new metastatic microenvironment that turns them into highly proliferative and invasive cells. Research on targeting dormant metastatic cells is focused both (1) on specific characteristics of dormant metastatic cells that identified them as CSCs and (2) on the identification of the niches that promote CSC dormancy.

First, regarding the general CSCs characteristics [14] the ability of asymmetric division, resulting in low proliferating, dormant stem cells and dedifferentiated progenitors with higher proliferating and invasive potential [15] should be emphasized. Secondly, high resistance to apoptosis, recognized by Mehlen et al. [16], as the key hallmark of metastatic progression, thirdly, high functional and molecular plasticity that is still keeping their characteristic stem cell phenotype [17] and lastly, homing to the niches to protect them from damaging effects of irradiation and chemotherapy [18].

CSC-mediated induction of EMT is associated with appearance of migratory, potentially metastatic CSCs (mCSCs) [19–21]. As mentioned above, acquisition of partial EMT can in turn promote different CSC-like traits during different steps in metastatic progression.

Although the similarity between primary CSCs, between CTC and metastatic stem cells (mCSC) as found by *Zhao et al.* [22], they pointed out on the differences in the transcriptomic regulation of key EMT-associated pathways in chemo-resistant stem cell-like subpopulations (displaying a typical invasive plasticity) as compared to the stem cells subpopulation from the parental cells. This suggests a partial link between primary CSC, and metastasis. Thus, an association between EMT and stem cell-like characteristics has been documented in various carcinomas, but the precise mechanisms that confer the ability to impart stemness to EMT, remain elusive. Moreover, due to EMT/MET reversibility, the transient EMT/MET gradient state determines whether dormant metastatic cells will remain dormant or emerge as metastatic outgrowth [23].

By manipulating dormancy-regulating processes it might be possible to suppress the outgrowth of disseminated metastatic tumors cells [22]. Moreover, the reversibility of these processes allow for co-existence of both types, the CSCs and mCSCs in the metastatic niches [9,24]. However, the following unresolved question remains: does one particular mCSC clone type seeks for a niche at the secondary site that is similar as in the primary tumor?

The heterogeneity of metastases cells is due the complexity of the progression process, but primarily to the fact that the original tumors are composed of multiple, genetically distinct sub-clones. However, metastatic cells, present within heterogeneous subsets of cells, are behaviourally, genetically, and biochemically distinct from the original cancer cells at the site of primary tumor [3], showing that metastases heterogeneity develops independently. We are only beginning to understand the complexity of metastatic routes and molecular mechanisms that regulate the spatial (organ-specificity) and temporal (start of metastatic growth) emergence of metastases [4]. Accordingly, Hunter [3] proposed basically two models of metastatic evolution, either the early dissemination model, or late dissemination model, leaving smaller or larger primary tumors, respectively. In each type of model, the dynamics of metastatic clones may be different, arising sequentially or in parallel and may evolve from one or multiple sub-clones, altogether comprising the four models. Furthermore, the dogma of monoclonal origin of metastases is challenged by the observed polyclonal origin, also demonstrating that heterogenous tumor cell clusters are much more efficient than metastatic lesions arising from monoclonal cells [25,26]. However, the clinical studies have suggested that no single model applies universally. Moreover, superimposed on genetic heterogeneity of the primary cancer, as well as on somatic mutations during metastases evolution, additional phenotypic heterogeneity is observed, due to the interactions with tumor microenvironment (TME) and similar as in primary tumors enhances metastases aggressiveness what lowers patient’s survival [27–29]. Genetic heterogeneity within metastases increase their “fitness” following the principles of Darwinian evolution-like selection at the metastatic site. Multiple metastases in a given patient are usually genetically more similar to each other than to the matched primary tumor [30], suggesting selection from genetically heterogeneous primary tumors for traits needed for successful metastasis. Furthermore, metastases within a given organ are genetically more similar than are to metastases in other organs in the same patient [31,32], which again suggests the relevance of adaptation of metastatic clones to the organ-specific microenvironment. Metastatic lesions can further metastasize to secondary metastases, again *via* novel environment-directed evolution, as has been shown in the *in vivo* experimental settings [33].

At molecular level, the key drivers are metastasis-specific genes, which are induced at the later stages of tumor progression and could be targeted by metastasis-specific therapies. Metastasis-specific loss of

heterozygosity and loss of gene expression [34] has been used to identify a class of genes known as “metastasis suppressors”, and since that finding, more metastatic genes have been discovered [35]. Notably, inherited genetic metastatic potential is not limited to the nuclear genome, as recent studies have demonstrated that polymorphisms in the mitochondrial genome also affects metastatic efficiency. This implicates an important role for mitochondria-mediated metabolism in tumor progression, as elaborated by Brinker et al. [36]. Finally, besides “hardwired” genetic events a flexible epigenome may also contribute to metastases plasticity [3]. These concepts have been confirmed in several animal experiments, demonstrating that exactly the same metastasis mechanism may affect the disease progression in different ways [6].

1.3. Homing to metastatic niches

Three types of niches have been proposed that are morphologically and functionally distinct and include perivascular, hypoxic and invasive niches [37]. The pre-metastatic niches and their selective cellular cross-talk with invading cells results in their accommodation, associated with epigenetic changes and cell phenotype in the process of MET [4,38]. Recent findings have provided information concerning specific TME and its interactions with CSCs, contributing to their survival, resistance to conventional therapy and metastasis [22]. Metastatic niches accommodate mCSCs by generating microenvironments in distant organs, composed of hematopoietic and stromal cells in a perpetual cross-talk mediated by cytokines, exosomes and gap-junctions, within an extracellular matrix. The dynamic architecture of this niche is able to support the disseminated tumor cells, similar to that seen in the normal stem cell niches. Lyden et al. [39] have proposed a model for the evolution of a metastatic niche. In his model, at secondary sites, the cells are first presented with a suitably conducive microenvironment (pre-metastatic niche) that then evolve into a metastatic niche, which allows engraftment and proliferation of the tumor cells. Accumulating evidence supports the classic ‘seed and soil’ theory of cancer dissemination, but as finding the “right soil” (metastatic niche) for the [40]. Therefore, successful therapeutic strategies may result from targeting the CSCs and their CSC niches as a whole [41]. Strong evidence in mice and humans revealed even a broader systemic genetic set up, comprised of measurable traits like body weight, resistance to infection, stress, etc. referred to as phenotypic noise or climate [53]. Finally, CSC and niche-targeted therapy can be applied in an adjuvant setting, after surgical resection and/or radiotherapy of the bulk tumor mass that may help eliminate residual CSCs that can be dormant in distant sites, and in this way effectively prevent distant metastasis [22].

2. Glioblastoma climate

2.1. Brain parenchyma as supportive environment for glioma progression

Brain, the central organ of the nervous system (CNS), controls most of our body activities and is thus highly protected; mechanically by the skull and chemically by the blood–brain-barrier (BBB) that selectively separates the brain from the blood circulation. The brain parenchyma is infiltrated by hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), differentiating into stromal cells that along with distinct resident host cells, e.g. neurons, astrocytes and microglial cells, comprise normal brain parenchyma. Abundant brain blood vasculature is importing nutrients and oxygen needed for brain functions and in such a compact environment [42], where extracellular space presents only 20% of the tissue volume.

Proteins of CNS extracellular matrix (ECM) belong mostly to lectins and tenascin, whereas collagen type I is only supporting blood vessels and basal lamina [43], the rest are collagen type IV, nidogen/entactin [44] with dominating laminin [45] and rather abundant proteoglycans, like hyaluronic acid and heparin sulphate. Nearly a mini-organ BBB is composed of a network of endothelial cells, joined by tight junctions composed of claudins and occludins network, and active transporter proteins to expel potentially harmful molecules that may penetrate the BBB barrier [46]. The brain-facing (abluminal) side of the BBB is surrounded by a thick basement membrane, which in turn is supported by pericytes, strengthening the brain blood vessels and capillaries. These are associated with protrusions of astrocytic end-feet and neurons, altogether known as the neurovascular unit [47]. Microglia, as immune-related cell component contributes to the integrity of BBB [48], helping to repair the damaged BBB. Only recently the lymphatic vessels have also been found in brain, called the glymphatic system, traversing sagittal sinus to end at denser base of the brain [49].

In progressing glioma, histologically classified as astroglial tumors, the unique brain ECM become disorganized, actually stiffening with increasing malignancy [50]. Normally dynamically regulating cerebral microvascular permeability, in cancerous tissue also BBB becomes irregular and leaky. BBB do not only act as barrier to cancer cells invasion, but is a biologically active entity, interfering with many processes of malignant progression [51]. Collectively, the brain tissue landscape is very different from any other tissues. Schneider [52] pointed on the importance of environmental factors specificity among organs’ tissue types that shape up the local progression of tumors and metastases, the concept that has long ago been recognized by Sir Stephen Paget In the 19th century, who observed the metastasis (“seed”) to grow in a specific, appropriate “soli” [1]. As brain are the major information processor, the malignant neoplasms growth in the brain are lethal to body functioning.

Gliomas account for 29% of all brain tumors, among which the least malignant are pilocytic astrocytomas (WHO grade I) with 5–10 years survival [55]. The most malignant glioma is glioblastoma (GB, WHO grade IV), with average survival of only 15 months after diagnosis [54]. Unfortunately, GB is also one of the most common brain tumors, occurring in 5–7 cases per 100.000 individuals per year [55]. In older population, GB mostly develops *de novo* with no recorded history of less malignant appearances, called primary GB, whereas the GB that develops from low-grade glioma (LGG, WHO grades I &II) is called secondary GB. The latter have distinct molecular pathways of progression, where the key determinant is isocitrate dehydrogenase 1 (IDH1) mutation. Clinically, all GB are similar, characterized by abundant necrosis, pleomorphism and vascularization. High resistance of GB to radio- and chemotherapy in addition to incomplete surgery due to diffuse invasion of single cells, called” guerrilla” cells [56], are the major reasons for early relapse. GB cells migrate along nerve tracts, meninges and blood vessels [43,56,57], as first described by Scherer [58]. However, they rarely leave the CNS despite of being closely attached to blood vessels and expression of a plethora of invasion-related proteases [42,59,60].

2.2. Glioblastoma stem cells and subtypes

GB stem cells (GSCs) have been first described by Singh et al. [61] and established by several studies [14,15,62–65]. GSC markers include surface proteins, such as CD133 (prominin 1), CD15, CD44, integrin-α6, L1CAM, CD9, as well as transcriptional factors, such as SOX2, NANOG, Olig2, NOTCH, SHH, WNT [66], all related to cell stemness features and signaling pathways, such as Akt and STAT3 [67].

Van Meir and co-workers [68] first proposed that GSC plasticity originates from neural stem cells, their progenitors or from MSCs, besides differentiated astrocytes. The authors then related the etiology of tumor initiating cells to the four GB subtypes, as deduced from The Cancer Genome Atlas (TCGA) data analyses [55,69,70]. Recently, these data have been revised by genomic profiling of GB cell components only and tumor-associated stromal cells’ genes were filtered out by Wang et al. [71]. They characterized the GB inter-patient heterogeneity at the molecular level by subtyping into three groups: proneural (PN), mesenchymal (MES) and classical (CL) that are characterized by PDGFRA/

IDH1, NF1 mutation and by EGFR amplification, respectively [70,71]. Only PN is also associated with IDH1 mutation, PDGFRA amplification and high expression of developmental/stemness genes *(SOX, DCX, DLL3, ASCL1 and TCF4*). On the other hand, loss or mutation of NF1, TP53 and PTEN, overexpression of mesenchymal markers (CD44, YKL-40, YMET, and CHI3L1) and activation of NF-κB pathways characterizes MES tumors that have poor survival.

Furthermore, Behnan et al. [72] identified among the 118 differentially expressed genes in GSCs and GB tissues, 12 genes that were common to the GB TCGA classification and can be used in GB subtype stratification. The final goal of GB subtype identification in a patient would be the development of a more effective personalized therapy. This has not yet been achieved, mainly due to the GB heterogeneity, i.e. the variability of cancer cells' subtypes across individual tumors [28], always resulting in shorter survival of GB patients [27,29]. However, more aggressive therapies are applied to MES and CL GB patients. PN GB subtypes reportedly recur as MES GB, suggesting an evolutionary shift from PN–MES phenotype, termed as PN to MES transition (PMT) [71]. This was demonstrated in humans and in animal models, presumably as response to therapy [71,73]. Recent data suggest that in some patients with heterogenous primary GB, the infiltrative sub-clones can arise during early tumor growth that after treatment may relocate and seed locally or to distant niches (that may also be extracranial) thus representing the "missing link" between the primary tumor, recurrent disease [29] and metastases [11].

2.3. Stromal microenvironment and glioblastoma niches

Similar to normal neural stem cells, dwelling in sub-ventricular niche, Hira et al. [18] and others found that GSCs reside in protective peri-arteriolar niches, where they localize close to abnormal blood vessels [20,74]. In the vascular-invasive niche GB cells migrate along blood vessels, enabling invasion deep into the brain parenchyma [37,57]. We pointed out on a similarities between GSC niches and HSC niches in bone marrow [18,73,75]. As shown in Fig. 1, we suggested proteolysis as the key regulator of the niche dynamics, for example by abundant levels of lysosomal cathepsins, B, K and X [60], possibly cleaving and inactivating chemoattractant SDF-1α. Thereby the chemoattraction and intercellular cross-talk, mediated by CXCR4 /SDF-1α in the niche would be interrupted and GSCs released from the niches [76,77], similar to the behavior of bone marrow niches-associated HSCs [78]. Dynamic interactions of GSCs with stromal cells and the ECM within niches create a supportive microenvironment for GSC survival, growth, and immune surveillance [37,20,79–81]. Standard irradiation and chemotherapy induce changes, both in GSCs and in the GB microenvironment, fostering cell-cell contacts that actually lead to more aggressive tumor growth [42,82,83]. Both, cancerous and stromal cells within the CSC niches promote stemness through direct and paracrine signaling. For example, cancer-associated fibroblasts (CAFs) with increased cytokine production activate GSC-linked Wnt and Notch signaling pathways, which are both implicated in stem cell maintenance [80]. On the other hand, CSCs themselves directly modulate the immune system cells to create an immune-suppressive environment within their niche [11].

Natural killer cells (NK)- [84] and immunomodulatory MSC-secreted chemokines can also affect GSC phenotype [85], however by a different mechanisms in a complex cross-talk with GSCs. An example of how important are niche-associated stromal cells for GB/GSC propagation are MSCs, as an inherent part of GB microenvironment [24,86–89]. A number of studies [87,88,93,94] proved that MSCs affected GB cell growth, invasion, stemness and differentiation, by secreting a plethora of cytokines, and in direct cross talk (Fig. 2) [90,91]. Also, GB-MSC cell fusions [92] has been visualized. Notably, these processes are strongly dependent on the GB genetics that can for example switch the invasive cell response to MSCs into the opposite direction, as in genetically distinct U87 and U373 GB cells [93]. Radiotherapy even increases the tropism of MSCs towards GB cells in tumors [95] and enhances malignancy, as high frequency of MSCs in HGG correlated negatively with patients' survival [87].

3. Why leave the brain?

3.1. Plasticity and trans-differentiation of metastatic stem cells

The estimated incidence of extracranial GB metastasis is up to 0.5% of all patients. Despite their local aggressiveness, these tumors rarely leave the brain. Due to fast progression and damaging effects of the neural centers, patients die before the systemic metastases could be detected, and these usually are not investigated at autopsies, due to known cause of death and ethical reasons. Thus, two major questions are, first are spontaneous GB metastasis indeed rare, and secondly if so, which of the metastatic steps may represent the bottle neck to the overt colonization of the secondary organs? The notion that GB metastases mainly occur due to craniotomy releasing GB/GSC cells into blood vessels was suggested because GB metastatic features at soft tissue scar and skin were found near the original craniotomy site [96], and the ventriculo-peritoneal shunts were associated with extracranial metastasis [97,98]. This is challenged by the evidences that GB metastases appeared before the treatment and in non-treated patients; however, there are no clear experimental demonstrations about GB cells, spontaneously leaving the brain.

Suggested hindrances to GB metastases are (1): BBB protection, (2) lack of lymphatic metastasis, ascribed to the absence of lymphatic channels in the CNS system, (3) suppression of extracranial growth of GB cells by the immune system and (4) the speculation that inability of GB cells to invade/degrade ECM of other than brain tissues.

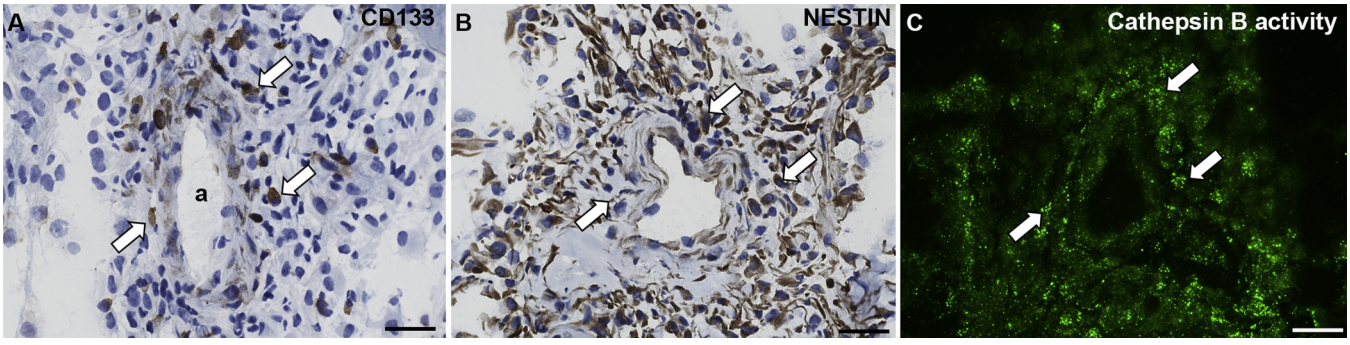

Fig. 1. Activity of pro-invasive cysteine peptidase cathepsin B is present in peri-arteriolar GSC niches.
Immunohistochemical staining of GSC marker CD133 (A) and nestin (B). Cathepsin B activity has been detected in cells around arteriolar walls (green dots, C). We found abundance of cathepsins B, K ad X present in peri-arteriolar niches [126] and suggest that cathepsins in GSC niches may be involved in remodelling of GSC microenvironment and processing cytokines [76]. Scale bar = 50 μm.

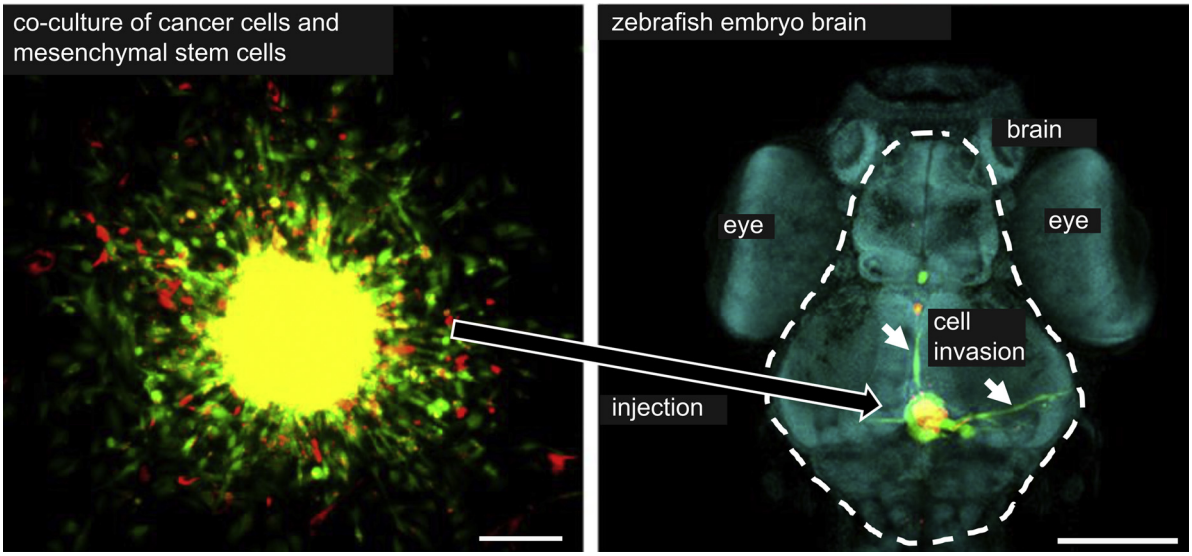

Fig. 2. Mesenchymal stem cells (MSCs) enhance the invasion of U373 glioblastoma (GB) cells *in vitro* and *in vivo*.
MSCs were labelled by red Vibrant dye, and GB cells were transfected to express GFP (green). They were co-cultured as spheroids *in vitro* (left) or co-injected into the brain of zebrafish embryos *in vivo* (right) as descried [93]. The invasion was monitored after 3 days. Enhanced invasion of GB cells was recorded in both systems. GB cells formed elongated pseudopodia and invaded along the ventricles and the central canal of the spinal cord in zebrafish embryo brain. Scale bar = 200 μm.

First, experimental evidences prove that BBB is not efficiently blocking the cellular trafficking beyond the intracranial space, and GB blood vessels are compromised by insertions of differentiated MSCs (pericytes), cancer associated fibroblasts (CAFs) and even cancer cells themselves, mimicking the endothelial cells [99]. Secondly, functional lymphatic vessels lining, composed of typical endothelial dural sinuses leading to the deep cervical lymph nodes were discovered in CNS of mice and humans [49]. This data corroborates previous reports on lymph nodal GB metastases in the absence of any pre-existing surgical procedure [16]. These appeared in about half of reported GB metastasis, others most common sites are pleural/lung (60%), followed by bone (31%), liver (12%) and even skin [96] metastases. Metastatic GB/GSC invasion *via* cerebrospinal fluid (CSF) is a potential alternative to lymphatics. In physiological conditions CSF fluid provides a “cushion” for the central nervous system, providing maintenance of extracellular ionic balance, and is carrying specific proteins, e.g. Sonic hedgehog and insulin growth factors 1 and 2 (Igf1 and Igf1), thereby contributing to the retention of neural stem cell properties in their subventricular zone niches [100].

As these factors are also upregulated in GSCs in some GB patients [54], they may also condition the metastatic mGSCs, thereby contributing to the retention of neural stem cell properties in their subventricular zone niches [100]. As these factors are also upregulated in some GSCs subtypes [54], they may also condition the metastatic GSCs (mGSCs). From CSF and lymphatics, GSCs can enter blood circulation, promoting hematogenous metastases. Mulller et al. [101] identified CTCs in peripheral blood in about 21% of GB patients by selective biomarker glial fibrillary acidic protein (GFAP), in addition to concomitant EGFR gene amplification/mutation, suggesting that the latter is crucial for hematogenous GB spread. CTC levels were not significantly enhanced by surgical treatment. Notably, metastases that do not manifest clinical symptoms within the limited life span of GB patients are easily overlooked, but checking for metastatic CTC might be beneficial in long-term GB survivors (see Sections below).

When in circulation, GSCs have the ability to evade immune response [102] through stimulation of release of myeloid-derived suppressor cells (MDSDs) that protect GSC by attack of natural killer (NK) cells [10,103]. Reportedly, the absence of Toll-like receptors on GSC surface play important role in this mechanism [104]. In immunocompetent patients, a large part of the CTCs ends up being detected and killed by natural killer cells (NK) [84]. Immunocompromised patients, such as those in post-radio-chemotherapy, are more prone to develop systemic metastases. These data support us a relationship between GB metastasis and the degree of the immune system competence.

3.2. Metastatic cells and their trafficking

Low proliferating GSCs residing in hypoxic peri-arteriolar niches in the GB tissues [75,77] over-express specific anti-angiogenic genes, like thrombospondin, angiomotin, and in particular ephrin (EphA5) [105]. Our recent study [106] was first to show that GSC niches are similar to HSC niches. Both types of niches are hypoxic, periarteriolar and contain MSC and the same functional chemo-attractive proteins and their receptors. We also localized MSCs associated with GSC niches corroborating the data of Hossain [86], showing that MSCs in GSC niches increase proliferation and self-renewal of GSCs *in vitro* and enhance GSC tumorigenicity, possibly via IL -6/STAT3 inducing GS-MES features *in vivo*. CD105-positive MSCs expressing high levels of SDF-1α are present around arterioles and produce high levels of the chemoattractant SDF-1α and OPN attracted CXCR4 and CD44- positive GSCs to niches and protect them from therapy, as GSCs in their niches are maintained in a quiescent state.

It seems that the angiogenic switch is needed for induction of a more proliferative GSCs to exit from a dormant state and metastasize. In recurrent GB, a shift to a more invasive GSC phenotype has been confirmed by many studies [107], presumably due to PMT. This transition was suggested to be triggered by the microenvironmental cues, such as stromal cells [80,107]. On the other hand, irradiation can trigger PMT accompanied by enhanced *CD44* and *YKL-40* expression, along with NF-κB, *STAT3* and CEBPB expression, as master transcription factors in PMT [73,107–109]. Activation of YAP/TAZ signaling, promoting GSC clonogenicity and resistance in the context of this transition was reported [107]. This notion corroborated with clinical study of Elena et al. [110], who described two metastatic GB cases that overexpress *YKL- 40* marker, featuring of invasive mesenchymal profile, which may favor this genotype in extracranial metastases. The analysis concluded that extracranial metastases occurred 8.5 months after first GB diagnosis with overall survival of average period of 12 months. When exploring GB CTCs, isolated from both patients and mouse PDX models,

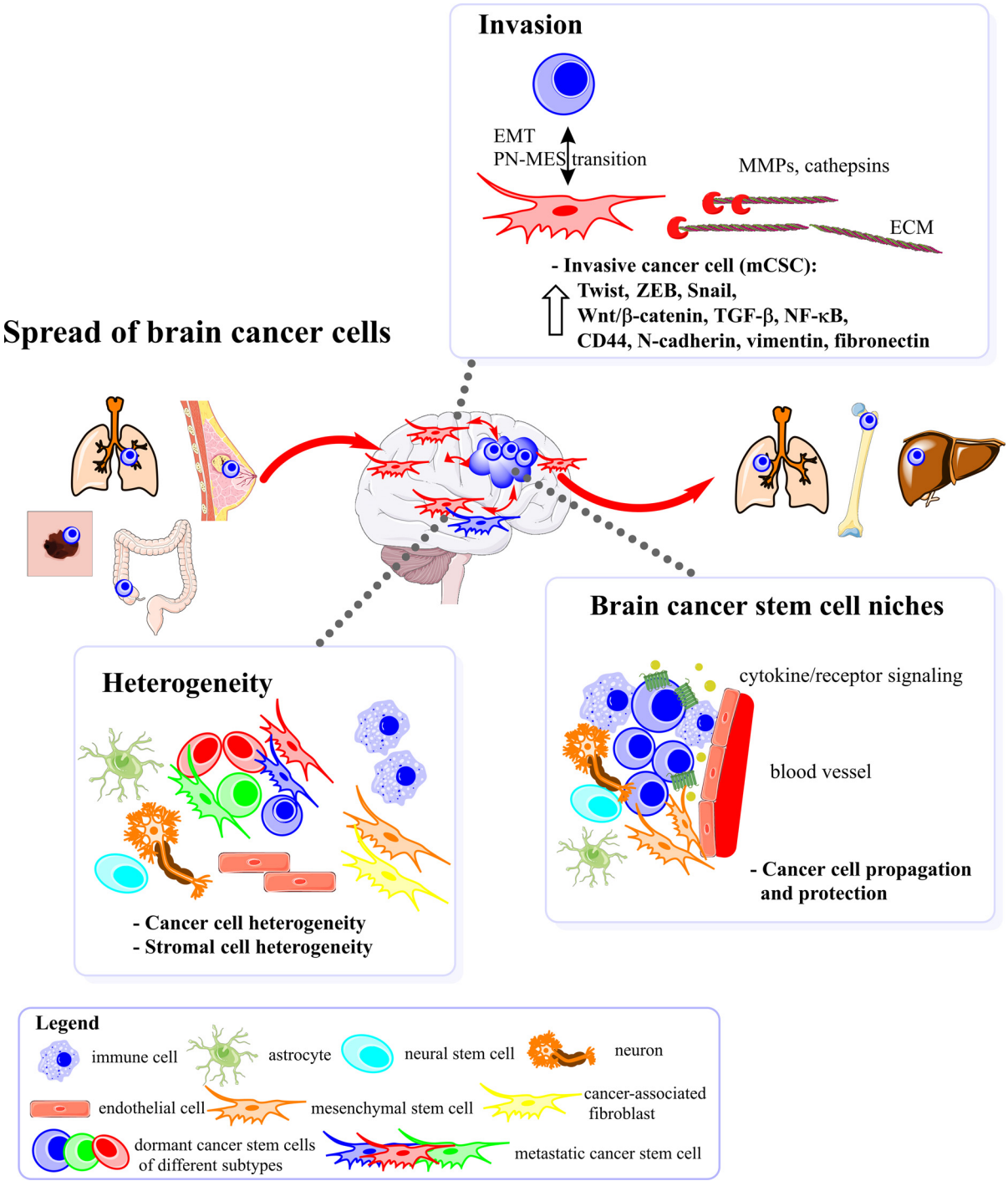

Fig. 3. Malignancies in brain – two way of cancer cells trafficking.
Brain tumor microenvironment comprises different subtypes of cancer cells as well as various types of non-cancerous stromal cells, which are normally present in the brain (astrocytes, neurons, microglia) or infiltrate during tumor progression (lymphocytes, mesenchymal stem cells, fibroblasts, macrophages). Dormant cancer stem cells (dCSCs) of melanoma, colorectal, breast and lung cancer undergo transformation into metastatic cancer stem cells (mCSCs) that colonize the brain and create there their niches. On the other hand, dormant glioblastoma stem cells (dGSCs) home in their protected niches, but propagate and invade upon exogenous events such as irradiation, chemotherapy, hypoxia and endogenous microenvironmental cues. Cytokine signaling trigger the EMT associated with transition of dGSCs to mGSCs.

Sullivan et al. [111] demonstrated enrichment for MES over N/PN markers in metastasis, compared with primary GB.

However, within these primary GB, RNA *in situ* hybridization identified a subpopulation of highly migratory mesenchymal subtypes tumor cells. In few patients with disseminated GB, systemic lesions were exclusively mesenchymal with additional mutations. The bottle neck in metastases progression seem to be homing to metastatic site niches, after which, as in the primary niche, the angiogenic switch would activate dormant cells [13,105] and their detachment from the niche [77,112], subsequently stimulating proliferation and colonization of secondary organ by GSCs. There the degradome of the metastatic GSC must adjust to novel kind of matrix for successful extracranial metastases.

In conclusion, although still not completely clear, the metastatic niches, associated with the disseminated GSCs, are promising target for treatment of distant GB metastasis (Fig. 3). Zhao et al., [22] listed

examples of the clinical translation of CSC niche-targeting approaches, that promise therapy enhancement, however based on the application of more sophisticated animal models and organoid systems. Finally, these strategies can be applied in an adjuvant setting that after surgical resection and/or radiotherapy of the bulk tumor mass that may also eliminate residual CSC.

3.3. Clinical evidence of glioblastoma metastases

Several systematic reviews have summarized the GB metastases data, revealing certain relevant differences regarding the patients number, age the time of observations. In Lun's study [113] of 88 patients, the median overall survival period of only about 10 months from diagnosis, and 8.5 months after detection of metastases has been determined. Lung metastases were recognized as extremely poor prognostic factor. Notably, patients treated with intense therapy, even CSF shunting, had the longer survival interval from metastasis to death, arguing against the notion that therapy may induce metastasis and worse prognosis and detection. Pietschman's meta-analyses reported on 150 patients with 6 months survival after the metastases [114]. The comparison of the cohort with 84 metastatic GB *vs.* non-metastatic GB suggested that in metastatic GB, younger patients exhibited more favorable outcome, pointing on beneficial systemic immuno-protective response. Again, surgical excision was associated with longer interval to extracranial metastasis than biopsy, which was even longer if followed by radiotherapy and chemotherapy, suggesting that GB metastases are associated with prolonged patient survival. The most recent meta–analysis, comprised a total of 115 younger patients, showed that the time frame between the identification of the metastasis and death was significantly prolonged in patients undergoing surgery, and when receiving radiation and chemotherapy [115]. Here, the liver was the metastatic site with the shortest survival period. Finally, anti-angiogenic therapy with bevacizumab, known to actually elicit malignant progression due to induction of hypoxia, correlated with earlier metastasis [116].

Collectively, these clinical observations indicated that spontaneous trafficking of GB cells out of brain may occur *via* CSF and lymph drainage that is ending in lymph nodes or in blood. Hematogenous metastases are not exclusively induced by surgery and may provide a more favorable prognosis due to activating systemic immune response.

4. Metastases to brain

4.1. Incidence and genetics determinants of brain metastases

Brain metastases (BM) from other primary tumors are more frequent than are brain tumors. These include mostly those from the lung, breast and melanoma, of total BM accounting for average of 45%, 15%, 10%, respectively, and a less than 2%, although increasing in colorectal carcinomas [46].

Similar results were reported by Achrol [117], who stated that lung carcinoma BM are prevalent in men (see Section 4.2.1), while metastases from breast carcinoma are most common type in women new options of brain metastases treatment metastasizing cancers also develop BM. At that time of first clinical detection of metastatic lump in the brain, more than 80% of patients already possess multiple BM [31]. Metastases often occur and grow synchronously with the primary tumor, indicating an early dissemination. Once BM appeared, the outcomes are dismal. If left untreated, the average survival is less than 2 months, although palliative therapies including corticosteroids, chemotherapy, and radiotherapy can extend survival. Studying branched evolution of metastases, these authors had sequenced 86 "trios" of patient-matched BM, primary tumors, and normal samples, all of which were collected during the course of clinical care. They identified additional oncogenic alterations in BM, that were in up to 53% of cases not detected with primary tumors [31].

In current clinical practice, therapeutic decisions are often made after molecular analysis [55,56] of only a single biopsy sample from the primary tumors, but not of metastasis that may reveal branched dissemination to brain [31]. For example, efficacies of ALK and EGFR inhibitors, used in primary lung cancer therapy, are being investigated in BM from lung cancer, and BRAF inhibitors are used in treatment in BRAF-mutant primary melanoma, whereas in HER2-positve breast cancer, HER2 inhibitors are used, although these drivers may not be the key modulators in BM growth. In future studies, analysis of CTCs or cell free DNA (from either blood or CSF) is suggested to be determined in the context of the presumed BM. However, brain specific metastatic oncogenes have been recognized and comprise *DSC2, ST7, PIK3R1* and *SMC5* mutations, besides primary cancer related *TP53, NRAS*, and *KRAS* mutations [117,118]. In general, BMs do not resemble primary tumor tissue, nor regional lymph nodes and other organs' metastases. A more comprehensive characterization of the primary lesions might even disclose the sub-clones that more closely feature intracranial disease that is crucial to the development of metastasis–tailored therapies [81,118–120].

Although brain metastases (BM) are not prevented by active BBB, this still represents a barrier to systemic therapies [38]. BM development depends on colonization of a distinct perivascular niche in the brain, where multiple interactions of metastatic cancer cells with the brain microenvironment occur [11,38]. The interactions of metastatic cancer cells with host tissue in the brain may overlap with those in other organs, whereas others are unique to the brain, due to an adaptation to brain-specific microenvironment (see Section 2.1). Specifically, restricted blood flow regulated oxygen and nutrients are directing brain specific metabolic environment. This determines critical concentration of neurotransmitter precursor glutamine [121] and its utilization may be an important functional constraint on successful BM outgrowth. To reveal the common denominators of brain colonization by widely different types of carcinomas, the key brain metastatic hallmarks still need to be revealed [1] by well-designed informative animal and clinical studies.

In animal experiments Steeg et al. [35] observed that tumors cells crawl outside the blood vessels and interact with an inflamed neural microenvironment to colonize the brain. Metastatic cells traverse the vascular system and use the outside of vessels as first a site of adhesion and migration and the inflamed brain microenvironment as a type of the niche. Tumor cells interact with activated microglia and astrocytes and damage neuronal axons, cause edema and vascular changes, *e.g.* disruption of the BBB. Both vessel co-option and angiogenesis have been observed in BM, although only a small number of CTCs could be detected to intravasate, indicating a highly inefficient process with 95% of cells failing to successfully grow micro-metastasis in brain [122].

When in brain microenvironment, activated astrocytes congregate around the metastatic cells, secreting IL-1β, augmenting their autocrine expression of Notch stem cell marker signaling via its ligand JAGGED1 (JAG1) The astrocytes may stimulate mCSCs cells proliferation [123,124] by secreting growth factors and enhance expression of cytokine receptors [125]. A plethora of cathepsins and other proteolytic enzymes play specific roles in various metastasis processes [59]. For example, protease signaling may be coupled to cytokine signaling in the metastatic peri-arteriolar niche [126,127], similar as in the primary GSC niche (18). In the brain microenvironment BM mCSCs protect themselves from secreted reactive oxygen species (ROS) by anti-apoptotic response. They secrete serpins to inhibit plasmin that could degrade FAS-L ligand [128]. At the end of these processes, dormant solitary mCSCs were found accommodated in brain niches and reside there, leading to further spread of BM metastasis and possibly metastases from metastases [4].

4.2. Short overview on brain metastases and their treatment

4.2.1. Brain metastasis from lung cancer

Lung cancer is a prime cause of cancer related deaths worldwide and in 57% of diagnoses it will present with metastasis, among them in 20% patients present with brain metastasis (LC-BM) at diagnosis and 50% at the relapse [129,130]. The rate by which a lung carcinoma metastasizes to the brain after being diagnosed, may vary. Treatment of patients with BM includes whole-brain radiotherapy alone or in combinations with surgical resection [130–132].

In Non Small Cell Lung Cancer (NSCLC), systemic targeting EGFR mutations and (anaplastic lymphoma kinase) *ALK* gene rearrangements, are the most common [133]. First generation EGFR-tyrosine kinase inhibitors (TKI), gefitinib and erlotinib [134,135], and Afatinib, as a second- generation, have shown increased activity compared to first line of chemotherapy [131,136]. All irreversible EGFR-TKI, exhibited a significant overall survival benefits compared to chemotherapy [137,138]. Combination of radiotherapy with EGFR-TKIs has significantly prolonged overall survival, but also increased skin damaging effects, and despite the high initial effects, all the patients developed resistance after prolonged follow-up time [139]. A third-generation of EGFR-TKIs, osimertinib, targets specifically activating and resistance EGFR mutations and has shown very BM cell toxicity and better BBB penetration [140,141]. To our knowledge no established therapy for resistant patients has been developed [142].

ALK rearrangements can be targeted in only 5% of NSCLC patients and are also maintained in BMs [142]. First-line ALK-TKI crizotinib was associated with moderate response rate, and even better than first-line pemetrexed-plus-platinum chemotherapy [143,144]. Ceritinib and alectinib, as a second-generation of *ALK TKIs*, have indicated efficacy among patients developing resistance to crizotinib [145–147]. Most patients that are sensitive towards *ALK-TKIs* develop resistance and third-line *ALK-TKIs*, such as brigatinib and loratinib showed efficacy against BM only during first initial application [148–150].

Immunotherapy has also been adopted for treatment of LC-BMs. Four monoclonal antibodies (pembrolizumab, nivolumab, atezolizumab and MEDI4736) that target PD-1 receptor pathway are being well tolerated and approved for first-line or following therapy [131] and are being evaluated in several clinical trials alone or in combination with radiotherapy [131,151,152].

4.2.2. Brain metastasis from breast cancer

Among women with metastatic breast cancer, from 15% to 20% of patients develop BMs [46] and among those 50% that have human epidermal growth factor 2 (HER-2)-positive and 25% to 40% triple-negative disease [153]. Number of BMs is not significant prognosis factor, unlike Karnofsky performance status (KPS), age and tumors subtype (HER2, estrogen and progesterone status) [132,153]. The median overall survival time for breast cancer patients with BMs ranges from 2 to 25 months from diagnoses [154]. There has been no proven metastases prevention protocol developed by now, [153], except standard radiation therapy, and various types of systemic therapies [153] as well as new therapies, overcoming the BBB restriction to large scale molecules that play an important role in immune response [133] and would interact efflux transporters [155].

The major targeted therapies in clinics for breast cancer BM are focusing on HER2 pathway, VEGF pathway, PI3K/Akt/mTOR pathway, EGFR pathway and CDK-4/6 inhibitors [133]. For HER2- positive breast cancer, no specific treatment is design specifically for BM [153]. Several targets may reach and get activated in the brain but always in much lower concentrations and thus exhibit questionable effects [156]. More than 100 clinical trials at different stages for solo therapies and their combinations with radiotherapy or immune checkpoint blockade, are now ongoing to explore their efficacy in breast cancer BM (http://clinicaltrials.gov). The trastuzumab, antibody-drug conjugate (T-DM1) is already approved in clinics and has been shown to pass through BBB and is active in the brain as a single agent or in combination with irradiation, extending progression-free and overall survival [157,158]. Other HER2 antibodies such as pertuzumab [159], neratinib [160] and afatinib [161] as well as dual EGFR and HER2 tyrosine kinase inhibitor lapatinib [162], alone or in different combinations have demonstrated a response in HER2-positive cancers, but the metastases eventually relapse [46,132,153,163]. The role of VEGF inhibitors like bevacizumab is not that clear, since bevacizumab acts in peritumoral vascular system, and in long term treatment induces invasion of tumor cells [133]. For hormone receptor-positive breast cancer that express from 28% to 47% PIK3CA mutation [164,165] everolimus, an mTOR inhibitor has been approved for use in combination with an aromatase inhibitor or vinorelbine and trastuzumab, and is currently in clinical trials [166,167]. Relatively novel small-molecule CDK-4/6 inhibitors like abemaciclib, ribociclib and palbociclib, all impairing cell proliferation, are being evaluated in several clinical trials for breast cancer BM [132,133].

4.2.3. Brain metastases from melanoma

Up to 40% of stage IV melanoma patients will develop brain metastases, which makes melanoma the third most prevalent cancer to metastasize to the brain [168], limiting the overall survival to approximately 4 months [169]. Most of the melanoma metastases are located in frontal lobe and less than 1% in the hippocampus [170,171]. Systemic treatment prior the immunotherapy included the cytotoxic chemotherapy dacarbazine (DTIC) as alkylating agent and the high-dose interleukin-2 (HD-IL2) cytokine with poor transient affect [172]. Since the FDA approval of immunotherapy in 2011, with an inhibitors of the immune checkpoints targeting surface proteins CTLA4 (with ipilimumab), and PD-1 (with nivolumab and pembrolizumab) the survival improved [173].

Hotspot for treatment are also targeted therapies, such as targeting mutation (V600E) in the BRAF protein (with dabrafenib, or vemurafenib and encorafenib) responsible for melanoma proliferation and progression due to altered downstream signaling pathway of MAPK. In the clinic, V600E BRAF inhibitors are combined with MEK inhibitors such as trametinib, cobimetinib and binimetinib, respectively [174–176]. Combinatorial treatment with radiotherapy and immunotherapy, led to further improvement in clinical outcome [176]. Besides *BRAF*, mutations, there are other driver mutations involving *CDKN2A*, *NRAS* and *KIT* oncogenes, found in melanoma [177]. Due to the hyperactivation of AKT pathway, inhibition with PI3K inhibitors shows additional potential therapeutical use [178]. Having more treatment options improves survival and longevity of cancer patients but also patients with brain metastases, including melanoma. New options of brain metastases treatment dwells on better understanding metastases processes [179].

4.2.4. Brain metastases from colorectal cancer

Brain metastasis of colorectal carcinoma (BM CRC) is still a rare event (less than 4% of all metastatic CRCs) but with very poor patient prognosis of 3–6 months [180,181]. BM CRC are diagnosed 20–50 months after initial CRC diagnosis [181]. Metastatic seeding of CRC to brain and liver occurs early in CRC progression, in 80% of cases even before CRC is clinically detectable and years before diagnosis and surgical resection. Several driver gene mutations have been identified in the process of early CRC cell dissemination that disrupt key signaling pathways, such as WNT, TP53, TGF-β and EGFR, as well as cellular adhesion, enabling cancer cell invasion and growth in secondary organs [180]. Mutations in *RAS* and *PIK3CA* genes are found in CRC BM [182]. Almost two thirds of BM CRC originate from mutant *RAS* CRC. The reason for increased frequency of BM in RAS mutants may be in RAS-mediated activation of focal adhesion kinase (FAK) that promotes cell migration as well as cell BBB invasion [183]. Moreover, mutations in *RAS* oncogene family have been also associated with resistance to anti-EGFR CRC therapies [182]. Recently, DNA damage response deficiencies have been identified in BM CRC. Compared with primary CRC,

BM exhibits elevated mutational signatures of homologous recombination deficiency (HRD) and mismatch repair deficiency (MMRD) and elevated microsatellite instability levels. Similar to other studies, researchers found that DNA damage response signatures could emerge early in CRC development as well as that these signatures are enhanced in BM, but are later eventually eliminated in matched primary CRC [184].

There are limited therapeutic options for BM CRC besides radiotherapy, chemotherapy and surgical resection. However, identification of novel BM-specific drivers might provide promising targets for improved therapy.

In conclusion, the following three therapeutic approaches should be explored: (1) preventing an initial metastatic cancer cell invasion, (2) affecting bidirectional interactions of metastatic cells with their microenvironments, in particular in the metastatic niches, and (3) preventing *de novo* niche formation. Several studies have emphasized that the invasive mCSC subpopulations are critical for successful metastatic colonization, whereas stromal niche signals are crucial for CSC expansion process [185]. Finally, increased molecular understanding of the disease leads to development of novel immunotherapies and BM-targeted therapies. Advances in radiotherapies and minimally invasive surgical techniques are also promising tools to treat BM [117] although selective metastasis irradiation is preferable [4].

Further issues on targeting metastases is beyond the scope of this review and we recommend certain excellent reviews on targeting metastases in general [1,3,4] brain metastasis [35], and stem cells [89] and their niches [81].

Declaration of Competing Interest

The authors declare no conflict of interest.

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