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REVIEW

Cancer stem cell contribution to glioblastoma invasiveness

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Abstract

Glioblastoma (GBM) is the most aggressive and lethal brain tumor in adults. Its invasive nature currently represents the most challenging hurdle to surgical resection. The mechanism adopted by GBM cells to carry out their invasive strategy is an intricate program that recalls what takes place in embryonic cells during development and in carcinoma cells during metastasis formation, the so-called epithelial-to-mesenchymal transition. GBM cells undergo a series of molecular and conformational changes shifting the tumor toward mesenchymal traits, including extracellular matrix remodeling, cytoskeletal re-patterning, and stem-like trait acquisition. A deeper understanding of the mechanisms driving the whole infiltrative process represents the first step toward successful treatment of this pathology. Here, we review recent findings demonstrating the invasive nature of GBM cancer stem cells, together with novel candidate molecules associated with both cancer stem cell biology and GBM invasion, like doublecortin and microRNAs. These findings may affect the design of effective therapies currently not considered for GBM invasive progression.

Cancer stem cells and neural stem cells: common features with different purposes

Parallels between neurogenesis and the processes contributing to brain tumor formation exist. Neural stem cells (NSCs) are quiescent cells able to self-renew and generate partially committed, highly proliferative progenitors that subsequently undergo complete differentiation into one of the three lineages composing the brain. A recognized hallmark of neural stem/progenitor cells is their ability to migrate, an essential process for recovery after brain injury [1]. The same role exerted by

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NSCs in the physiological context has been proposed to be played in glioblastoma (GBM) by a rare fraction of self-renewing, multipotent tumor-initiating cells called cancer stem cells (CSCs), responsible for tumor progression, maintenance, and recurrence [2,3]. This subpopulation has shown intrinsic resistance to therapy, being able to repopulate the tumor after treatment [4]. Recently, many studies have ascribed to CSCs the infiltrative property of GBM.

Cancer stem cells and invasive cells: two sides of the same coin?

The clinically distinct feature of GBM lies within its infiltrative potential, rendering complete tumor resection nearly impossible. Tumor infiltration is an extremely complex program that requires the steady supply of extracellular cues, abrogation of cell-cell interactions, and extracellular matrix (ECM) remodeling. Invading GBM cells are particularly resistant to current therapies and are often localized within the neurovascular niche, two features in common with CSCs [5].

Recent experimental data started to suggest that CSCs are responsible for GBM invasiveness. Cells enriched for the putative stem cell marker CD133 display greater migratory and invasive potential *in vitro* and *in vivo* when compared with matched CD133-negative tumor cells derived from human primary GBMs, GBM xenografts [4], and brain tumor cell lines [6-9]. We and others reported a marked upregulation of proteins involved in the processes of migration and invasion in GBM CSCs, such as different types of matrix metalloproteinases, or different members of both ADAMs (a disintegrin and metalloproteinases) and ADAMTS (ADAM with thrombospondin motifs) families [4,6-8,10]. Therefore, the highly migrating and invasive ability of GBM CSCs may be due to increased expression of proinvasive genes. Based on the findings that the GBM CSCs are more infiltrative than their differentiated descendants, a novel strategy has also been proposed to isolate and enrich CSCs from the whole tumor population by exploiting the tumor cell heterogeneity of invasiveness [11].

Cells at the leading edge of the tumor have been found to be positive for putative stem cell markers such as

L1CAM [12], nucleostemin [13], and nestin [14-16], supporting the notion that CSCs are indeed responsible for GBM invasion. A recent paper provided new insights into the role of SOX2 as a novel determinant of the invasive and migration properties of GBM CSCs and glioma cell lines [17]. Recently, many groups have suggested that different CSCs can coexist in the same tumor. Both tumor margin and the corresponding tumor mass contain CSCs that are characterized by different stem cell marker expression, neurosphere formation ability, and *in vivo* tumorigenic potential [18-20]. Thus, the invasive edge of GBM may function as a new CSC niche [21]. In this scenario, the theory proposed by Brabletz and colleagues [22] about the plasticity of CSCs can also be applied to GBM: CSCs switch from a stationary and proliferative phenotype to a migratory one, and vice versa, ensuring the enlargement of the tumor core and the colonization of the neighboring normal brain tissue.

Epithelial-to-mesenchymal transition in glioblastoma?

During development, embryonic cells undergo a series of trans-differentiation programs collectively assembled under the name of epithelial-to-mesenchymal transition (EMT) [23]. Interestingly, carcinoma cells exploit the same principle to drive invasion and colonization to non-adjacent tissues. EMT is a process that allows a differentiated epithelial cell, entirely settled and patterned to establish stable contacts with neighbor cells, to assume a mesenchymal cell phenotype, characterized by loss of cell-cell interactions [24], reduced cellular adhesion [25], active production of ECM proteases [26], increased cytoskeletal dynamics [27], and changes in transcription factor expression [28,29]; all of these events eventually lead to increased migration and invasion ability. Moreover, the acquisition of mesenchymal traits by cancer cells undergoing EMT has been widely reported to be associated with the acquisition of a stem cell program [30]. Thus, the expression of both EMT factors and stem cell markers in selected epithelial tumor cells at the invasive tumor front produces the 'migrating CSCs' [23,31-33].

In GBMs, robust evidence for the existence of the EMT process is still lacking. Recently, Cheng and colleagues [34], analyzing data from The Cancer Genome Atlas (TCGA), demonstrated a strong association in GBM between an epithelial-mesenchymal expression signature, the expression of the putative stem cell marker CD44, and shortened time to recurrence following initial treatment.

The EMT program is regulated mainly by three major groups of transcription factors – the SNAI, TWIST, and Zinc-finger enhancer binding (ZEB) family members – whose upregulation promotes tumor invasiveness and has been associated with poor clinical prognosis in human cancers [28,29,35,36]. The activity of these transcription factors has been reported to be altered in GBMs. SNAI is overexpressed in GBMs [37] and is involved in the regulation of glioma cell proliferation and migration [10,38]. TWIST1 is also upregulated in GBMs, where it promotes cell invasion by the upregulation of genes such as matrix metalloproteinase-2 (MMP-2), hepatocyte growth factor (HGF), fibroblast activation protein (FAP), and SNAI [39]. The ZEB2 expression level is significantly increased in glioma tissues compared with normal brain tissues and is positively correlated with tumor grading. ZEB2 silencing reduces the expression of mesenchymal cell markers such as N-cadherin, Vimentin, and Snail and inhibits glioma cell migration and invasion [40,41]. The overexpression of all of these EMT transcription factors in GBM cell lines may occur following the activation of WNT/β-catenin pathway and results in increased *in vitro* cell migration and invasion [20,42].

The SNAI, TWIST, and ZEB transcription factors act by directly repressing the expression of the adhesion molecule E-cadherin, whose functional loss is considered a hallmark of EMT [43]. In parallel, there is an induction of mesenchymal markers (for example, N-cadherin and cadherin-11), reorganization of the cytoskeleton (for example, switch from cytocheratins to vimentin), and production of ECM components and metalloproteases [30]. In general, loss of E-cadherin correlates with high tumor grades and poor prognosis [44], whereas the induction of N-cadherin correlates with increased cell motility [45]. In the brain, E-cadherin expression is rare in both normal and tumoral tissues [46]. Only two recent studies argue that E-cadherin expression inversely correlates with brain tumor grade [40,47]. N-cadherin and cadherin-11 are expressed in different brain regions, including the cortex and the hippocampus, and their expression is upregulated in malignant gliomas [48]. Thus, no switch between cadherins has been described in malignant gliomas, except in some specific cases [49]. However, an alternative cadherin switch, different from the classic E-cadherin-to-N-cadherin switch described in epithelial cells, was recently described in GBMs. Lu and colleagues [50] showed Snail and N-cadherin upregulation in GBM cells, with the concomitant downregulation of T-cadherin. T-cadherin is an atypical member of the cadherin family, downregulated in different tumor types, and associated with a poorer prognosis [51]. T-cadherin expression is absent in invasive GBMs but higher in circumscribed, minimally invasive GBMs [50], and its overexpression in GBM cells inhibits proliferation and migration [52]. Consistent with these findings, Zeb-1 has been demonstrated to suppress T-cadherin expression and increase invasion in gallbladder cancer [53], thus reinforcing the role of T-cadherin in the acquisition of EMT phenotype in GBM.

Recently, GBMs have been classified by gene expression signatures in four distinct subtypes (proneural, neural,

classic, and mesenchymal) on the basis of a comprehensive analysis of The Cancer Genome Atlas (TCGA) data set [54], which are not dissimilar from the three subtypes (proneural, proliferative, and mesenchymal) described in the work of Phillips and colleagues [55]. Analysis of these data sets reveals that high expression of genes associated with mesenchyme-derived tissues is common in GBMs and associates with poor overall survival and treatment resistance [54-56]. Consistent with these findings, Joo and colleagues [57] demonstrated that CD133-negative GBM CSCs have a mesenchymal subtype genetic signature compared with CD133-positive GBM CSCs and give rise to tumors characterized by an invasive growth. Similarly, Lottaz and colleagues demonstrated that GBM CSCs positive for the putative stem cell marker CD44 [58] but negative for CD133 possess a mesenchymal gene signature and display enhanced invasive growth both *in vitro* and *in vivo* compared with CD44-negative CD133-positive cells, which instead display a proneural gene signature, limited invasiveness, and high proliferation rate [59]. In addition, several human glioma cell lines express genes associated with mesenchyme-derived tissues [60], which are reported to be involved also in migration, invasion, and EMT [61]. Notably, the ectopic coexpression of the two master regulators that define the mesenchymal subtype, CCAAT/enhancer binding protein (C/EBPβ) and signal transducer and activator of transcription 3 (STAT3), reprograms NSCs along the mesenchymal lineage, whereas their silencing in glioma cells abolishes the mesenchymal signature and reduces tumor invasiveness [62]. Similarly, the transcriptional coactivator with PDZ-binding motif (TAZ) drives the mesenchymal differentiation of GBMs: when it is overexpressed in proneural GBM CSCs as well as in NSCs, it induces mesenchymal marker expression and aberrant osteoblastic and chondrocytic differentiation, whereas its silencing in mesenchymal GBM CSCs reduces the expression of mesenchymal markers, invasion, and tumor formation [63]. It is likely that the high expression of mesenchymal genes in a subset of human GBMs can be considered to be reminiscent of the EMT program [54] or that the aberrant activation of EMT factors during gliomagenesis can trigger the mesenchymal shift in GBM [63]. All of these data suggest that GBMs share many features with EMT, but a deeper knowledge of the mechanisms driving this process is indispensable to better tackle the invasive nature of these tumors.

Unconventional molecular determinants of glioblastoma invasion

Cell motility is a complex biological process which is tightly regulated by a multitude of converging signals. Many signaling pathways regulating the stem cell compartment have been reported to be critical for triggering tumor cell migration and invasion. However, their specific involvement in glioma invasion remains poorly understood because of intricate feedback loop mechanisms and missing links between them. Given the large number of detailed reviews describing the role of different signaling pathways in tumor progression (NF-κB, notch, hedgehog-GLI, WNT/β-catenin, RTKs, and AKT are the most relevant), this review focuses on two unconventional molecular determinants of GBM invasion: doublecortin (DCX) and microRNAs.

Why should doublecortin garner cancer scientists' interest?

The identification of single cells responsible for GBM infiltration in normal brain parenchyma is of crucial relevance. Several molecules have been used to identify invading GBM cells (for example, nestin [15,16] and vimentin [64]), but their expression could be altered by the grade of differentiation of the tumors. NSC discovery in the adult brain and the strong relationship between NSCs and CSCs have led to the consideration that these two types of cells may share common markers as well. DCX is one of them.

DCX is a microtubule-associated protein: it promotes microtubule polymerization and stability [65,66], and it is crucial for controlled cell movement, since it promotes nucleus translocation and the maintenance of a bipolar cell shape, preventing branching and nucleokinesis defects [67,68]. In humans, *DCX* mutations cause type I lissencephaly syndrome and subcortical laminar heterotopia, severe cortical malformations associated with mental retardation and epilepsy [69]. Knockout mouse models display milder symptoms, which are probably due to genetic redundancy with doublecortin-like (*DCL*) and doublecortin-like kinase (*DCLK*) and which become more evident when combined with mutations in *LIS1*, another gene involved in lissencephaly [70].

DCX is a brain-specific gene expressed in differentiating neurons during brain development [69,71], in neuroblasts, in discrete niches along the wall of the lateral ventricles, in the subgranular zone of the hippocampus, along the rostral migration stream, and in the olfactory bulbs in the adult rodent brain [72-74]. *In vitro* studies on neurospheres derived from adult rodent SVZ have confirmed the essential function of DCX in promoting cell migration [75].

DCX has also been proposed as a marker for migrating neuroblasts in the adult human brain [76], where it plays a crucial role in pathologic conditions in which the provision of progenitors migrating out of neurogenic areas toward the lesion is needed to replace neurons [77]. In non-injured adult mammalian brain, DCX expression outside the neurogenic areas is very rare [72,78]. Recently, DCX-positive cells have been identified in

developing and mature neurons in the cerebellar cortex of guinea pig, cat, and primate [79,80] and, to a minor extent, in cells expressing the astrocytic marker glial fibrillary protein (GFAP) [81,82]; however, these DCX+ GFAP+ cells behave as neural progenitors [82].

Doublecortin: an appealing and promising marker for invasive glioblastoma cells

Microtubule-associated proteins play important roles in cellular functions involving cytoskeletal rearrangement, which is one of the main features of EMT in cancer, thereby allowing cells to detach and move. Interestingly, DCX expression has been found in invasive human brain tumors, with the highest intensity at the invasive front [83], and has been proposed as a specific marker for the identification of infiltrating glioma cells [84]. Furthermore, gene expression profiling of human GBMs has revealed *DCX*, together with osteonectin (also known as *SPARC*) and semaphorin3B, as useful genetic markers to predict patient survival: the higher the expression of each of these three genes, the poorer the survival [85]. It is relevant to note that all three of these genes are involved in the regulation of cell migration, thus suggesting a direct link between tumor invasion and patient survival.

Recently, we identified RAI (SHC3/SHCC/N-SHC), a member of the family of SHC-like adaptor proteins, as a novel regulator of migration of normal and GBM stem/progenitor cells, affecting DCX protein levels [10]. RAI silencing in cancer stem/progenitor cells isolated from human GBMs reduces cell migration and invasion, both *in vitro* and *in vivo*. The rare cells still expressing RAI are localized at the tumor invasion front and express markers for both immature and migratory cells, notably DCX and OLIG2 [86]. We always observed a significant reduction in the number of invading cells positive for DCX in all RAI-interfered GBM xenografts as well as a decreased amount of DCX in both RAI−/− normal stem/progenitor cells and RAI-interfered tumor stem/progenitor cells.

So far, only one group has reported an opposite effect of DCX on glioma cells, with exogenous DCX addition reducing CSC self-renewal [87] and tumor volume [88,89]. Such controversial results could well be related to the usage of GBM cells not expressing DCX. On the contrary, we and others reported endogenous DCX expression in both normal and cancer stem/progenitor cells [10,90].

A deeper knowledge of the molecular mechanisms regulating DCX expression could help in the identification of new targets for therapy. While there is a large amount of literature about DCX regulation in the developing brain, little is known about its regulation in adult normal and cancer brain. DCX activity is regulated by several serine-threonine kinases and phosphatases; upon phosphorylation on Ser297 residue by cyclin-dependent kinase 5,
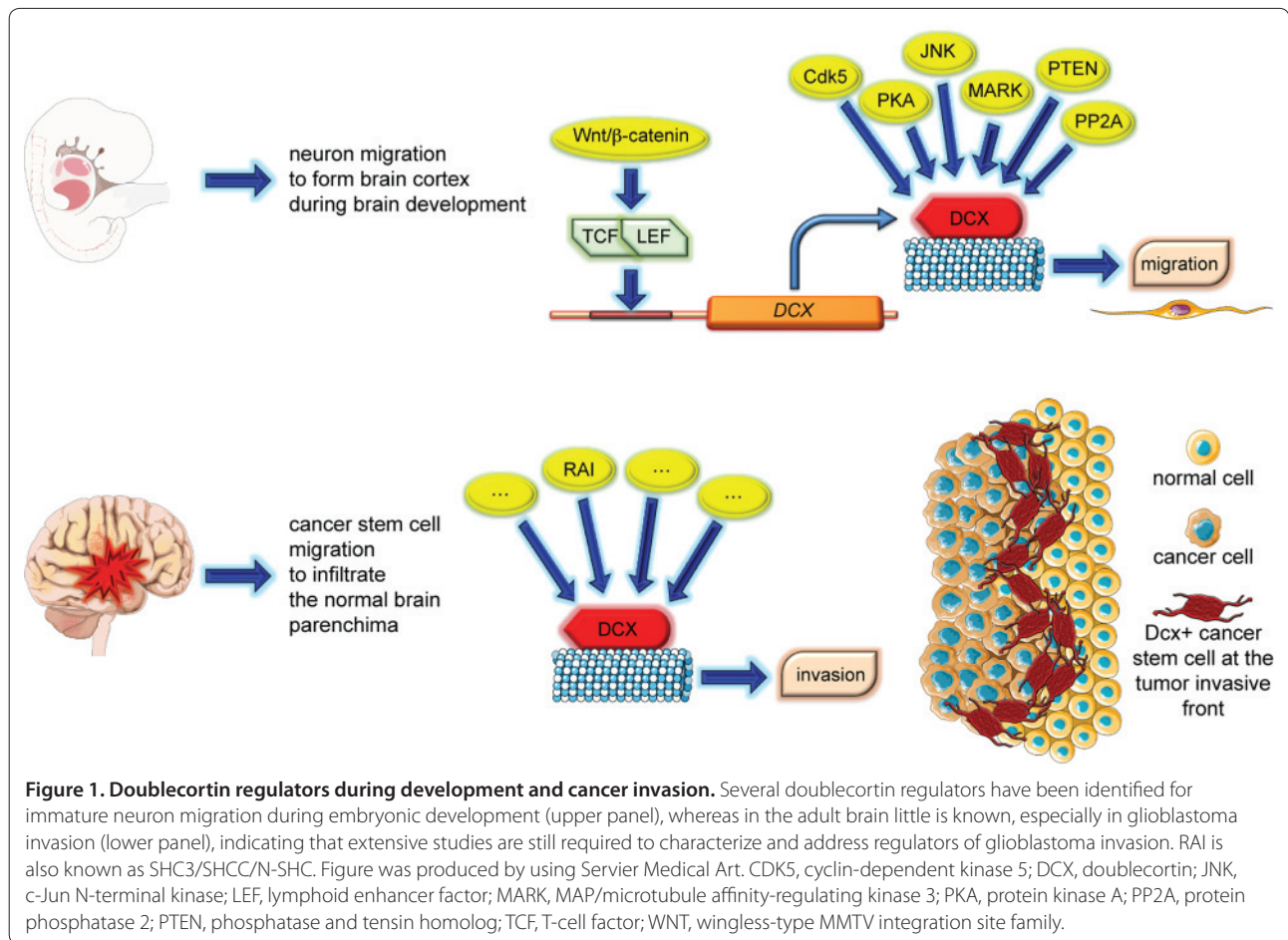
DCX affinity for microtubules decreases, promoting cell migration [91]. c-Jun N-terminal kinase (JNK), which regulates neurite extension, can phosphorylate DCX on Ser332, decreasing its affinity for tubulin and promoting cell migration [92]. Protein kinase A (PKA) and MAP/microtubule affinity-regulating kinase (MARK) regulate DCX binding affinity for microtubules, phosphorylating it on Ser47 [93]. DCX is also spatially regulated: when phosphorylated, it is expressed in the cell soma; when de-phosphorylated (likely by protein phosphatase 2A, or PP2A), it shifts to the tips of the neurites [93]. Another phosphatase acting on DCX is the tumor suppressor phosphatase and tensin homolog (PTEN) [94], which is the major inhibitor of the PI3K/AKT pathway and is frequently deleted in GBMs. A de-regulation of kinases and phosphatases is frequently observed in GBMs, therefore suggesting a possible mechanism enhancing GBM invasion. Another possible mechanism of DCX de-regulation in GBM could be linked to its transcriptional regulation. Recent studies have identified an upstream promoter region of *DCX* containing binding sites for LEF/TCF (lymphoid enhancer factor/T-cell factor) transcription factors [95], which are central effectors of the WNT/β-catenin pathway. This pathway is frequently altered in glioma: nuclear translocation and, therefore, activation of β-catenin have a direct impact on GBM invasion [96]. In both adult normal NSCs and CSCs isolated from human GBMs, DCX is a target of the adaptor protein RAI, which mediates multiple signaling pathways, eventually leading to metalloproteinase upregulation and GBM invasion [10] (Figure 1).

MicroRNAs: a specific role in glioblastoma invasion

MicroRNAs (miRNAs/miRs) are a class of small non-coding RNAs, 21 to 24 nucleotides long, which post-transcriptionally regulate gene expression by either inhibiting mRNA translation or inducing mRNA degradation, binding, via imperfect base pairing, to the 3' untranslated region of mRNAs, allowing the targeting of a high number of mRNAs.

miRNAs play important roles in physiologic conditions as well as in various diseases, including cancer, in which they could be downregulated, acting as tumor suppressors, or upregulated, acting as oncogenes. miRNAs are involved in many aspects of gliomagenesis and GBM CSC biology: proliferation, survival, migration, invasion, and angiogenesis [97]. Moreover, recently, bioinformatics analysis of 82 gliomas indicated the association between some miRNAs and the expression of mesenchymal markers [98].

Table 1 depicts the most intriguing ones found to be directly involved in the GBM invasion process, suggesting interesting putative targets for GBM therapy. Of note, the majority of the literature analyzes the role of miRNAs in GBM infiltration through experiments using glioma

Figure 1. Doublecortin regulators during development and cancer invasion. Several doublecortin regulators have been identified for immature neuron migration during embryonic development (upper panel), whereas in the adult brain little is known, especially in glioblastoma invasion (lower panel), indicating that extensive studies are still required to characterize and address regulators of glioblastoma invasion. RAI is also known as SHC3/SHCC/N-SHC. Figure was produced by using Servier Medical Art. CDK5, cyclin-dependent kinase 5; DCX, doublecortin; JNK, c-Jun N-terminal kinase; LEF, lymphoid enhancer factor; MARK, MAP/microtubule affinity-regulating kinase 3; PKA, protein kinase A; PP2A, protein phosphatase 2; PTEN, phosphatase and tensin homolog; TCF, T-cell factor; WNT, wingless-type MMTV integration site family.

cell lines, and very little experimental evidence on the role of miRNAs in migration and invasion of GBM CSCs is available. Given the contribution of CSCs to GBM invasiveness (highlighted in the previous section), we believe that further validation of the properties of specific miRNAs regulating migration and invasion in CSCs is essential.

MicroRNAs acting as oncogenes in glioblastoma

TWIST1, a transcription factor involved in EMT, has been shown to bind the upstream portion of the miR-10 hairpin region, modulating its expression [99]. MiR-10b, as well as TWIST [100], is overexpressed in glioma samples, with the highest peaks in GBM as compared with low-grade astrocytomas, and induces glioma cell invasion *in vitro* by decreasing HOXD10 (homeobox D10) protein levels, which in turn control *MMP-14* and *uPAR* (urokinase receptor) expression, resulting in their upregulation [101]. Other metalloproteinases, such as MMP-2, are upregulated in cancer as a consequence of the repression of MMP inhibitors, such as TIMP3 (metalloproteinase inhibitor 3) and RECK, which are themselves direct targets of miR-21, found to be

overexpressed in GBM, thus promoting glioma invasion and angiogenesis [102]. Moreover, analysis of miRNAs in gliomas belonging to different grades associated high miR-21 with poor patient survival [103].

Overexpression of the miR-221/222 cluster in malignant glioma cells results in the reduced expression of the tumor suppressor connexin 43 (Cx43), leading to the reduction of gap junction intercellular communication and, as a result, to an increase in proliferation and invasion of glioma cells [104]. In a highly intricate regulatory system, other important transcription factors, involved in cancer development when de-regulated, have been linked to miRNAs, such as NF-κB and c-Jun. These factors can induce glioma cell proliferation and invasion by promoting overexpression of miR-221/222 [105], resulting in the repression of the cell cycle inhibitor p27kip1 and in the activation of the AKT pathway leading to, among several targets, MMP activation [106,107].

MicroRNAs acting as tumor suppressors in glioblastoma

Mir-7 blocks cancer invasion, proliferation, and survival. It has been found strongly downregulated in GBM

Table 1. Biological functions of the different microRNAs associated with glioblastoma invasion

microRNA	Biological function	Reference
miR-7	Downregulated in GBM. Acts as a tumor suppressor in GBM, preventing GBM CSC invasion, proliferation, and survival by repressing EGFR and its downstream pathway AKT on one side and FAK and the production of MMP-2 and MMP-9 on the other side.	[108,109]
miR-10b	Acts as oncogene: it is overexpressed in glioma samples. Induces glioma cell invasion *in vitro* by decreasing HOXD10 protein levels, which in turn control *MMP-14* and *uPAR* expression, resulting in their upregulation.	[101]
miR-21	Acts as oncogene: it is overexpressed in GBM, where it represses the MMP inhibitors TIMP3 and RECK, thus resulting in metalloproteinase MMP-2 upregulation, promoting glioma invasion and angiogenesis. High levels of this miR are associated with poor patient survival.	[102,103]
miR-23b	Acts as a tumor suppressor and is downregulated at the invasive edge in GBM. Pyk2, whose expression is higher in high-grade gliomas, is one of its direct targets.	[110]
miR-34a	Acts as a tumor suppressor and is downregulated in human glioma tumors as compared with normal brain. Its overexpression exerts a suppressive effect in GBM CSCs by inhibiting proliferation and migration while inducing differentiation (Guessous et al. [111]).	[111]
miR-101	Acts as a tumor suppressor and is downregulated in GBM. Its target EZH2, a histone metyl transferase belonging to the Polycomb Group of proteins, is therefore overexpressed, promoting cell migration, neo-angiogenesis, and cell growth.	[112]
miR-124a	Acts as a tumor suppressor and is downregulated in GBM, in association with a shorter patient survival. *In vitro,* it negatively regulates cell migration and invasion through the negative regulation of its direct targets IQGAP1, laminin 1, and integrin β1. *In vivo*, it inhibits glioma invasion by targeting SNAI2. It inhibits glioma stem cell traits through SNAI2 and promotes neuronal differentiation, antagonizing the transcriptional repressor RE1 silencing transcription factor (REST).	[113,114]
miR-137	It is downregulated in GBM and acts as a tumor suppressor. *In vitro*, it negatively regulates glioma cell migration, invasion, and proliferation, reducing MMP-9 levels and directly targeting Cox-2 expression.	[116]
miR-145	Not clear yet. Suggested as pro-invasive oncogene by Koo et al. [124] but as a tumor suppressor inhibiting glioma cell invasion by Lee et al. [123].	[123,124]
miR-146b	Acts as a tumor suppressor. Represses both *EGFR* and *MMP* expression, reducing glioma cell migration and invasion.	[117,118]
miR-195	Acts as a tumor suppressor. It is downregulated in GBM, where it promotes proliferation and invasion through deregulation of its targets E2F3 and cyclin D3, respectively.	[119]
miR- 221/222	Act as oncogenes: their overexpression in glioma cells causes downregulation of connexin 43, a major component of gap junctions, inducing cell proliferation and invasion. Their overexpression is induced by de-regulated NF-κB and c-Jun transcription factors, resulting in the repression of the cell cycle inhibitor p27kip1 and in the activation of the Akt pathway leading to, among several targets, MMP activation and thus promoting glioma cell proliferation and invasion.	[104-107]
miR-302/367	Act as a tumor suppressor: when expressed, they suppress stemness gene signature, CSC self-renewal, infiltration, and tumorigenic capacity through a drastic inhibition of the CXCR4 pathway (Fareh et al. [120]).	[120]
miR-410	Acts as tumor suppressor in GBM, decreasing cell proliferation and invasion by targeting MET and consequently AKT signaling and MMP-9 levels.	[122]

AKT, serine/threonine-specific protein kinase; CSC, cancer stem cell; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; GBM, glioblastoma; HOXD10, homeobox D10; miRNA/miR, microRNA; MMP, matrix metalloproteinase; NF-κB, nuclear factor-kappa-B; RECK, Reversion-inducing cysteine-rich protein with Kazal motifs precursor; TIMP, tissue inhibitors of metalloproteinase; uPAR, urokinase-type plasminogen activator receptor.

samples compared with normal brain, and *in vitro* experiments showed that it inhibits GBM CSC proliferation and invasion by signaling on different targets: it directly inhibits the EGFR expression and therefore the downstream AKT pathway [108] and directly inhibits focal adhesion kinase (FAK), which has a role in the production of metalloproteinases such as MMP-2 and MMP-9 [109]. MiR-23b is downregulated at the invasive edge of human GBM samples as opposed to the tumor core and suppresses glioma cell migration and invasion in *in vitro* and *ex vivo* experiments. It directly targets Pyk2

(proline-rich tyrosine kinase 2), the expression of which correlates positively with tumor grade and is known to promote GBM cell migration and invasion [110]. MiR-34a is downregulated in human glioma tumors as compared with normal brain. When overexpressed in GBM CSCs, it exerts a suppressive effect by inhibiting proliferation and migration while inducing differentiation [111].

MiR-101, when downregulated in GBM, causes not only cell migration and invasion but also neo-angiogenesis and cell growth [112]. MiR-124a expression was

found to be downregulated in a wide range of GBM samples, in association with shorter patient survival. *In vitro* studies showed that it negatively regulates cell migration and invasion, through the negative regulation of its direct targets, IQ motif-containing GTPase-activating protein 1 (IQGAP1), laminin 1 (LAMC1), and integrin β1 (ITGB1) [113]. Recently, miR-124 was shown to inhibit glioma cell invasion and stem cell traits through SNAI2, which is often upregulated in glioma and enriched in the stem cell population [114]. The fact that miR-124a also promotes neuronal differentiation, antagonizing the transcriptional repressor RE1 silencing transcription factor (REST) [115], is even more promising for the therapeutic use of miR-124a, which could block GBM invasion and induce tumor cell differentiation at the same time. MiR-137 expression inversely correlates with glioma grade. *In vitro* experiments on glioma cell lines showed that it inhibits migration and invasion, decreasing MMP-9 levels, and proliferation, directly targeting Cox-2, which is more expressed in high-grade tumors and correlates with poorer prognosis [116]. MiR-146b represses both *EGFR* and *MMP* expression, reducing glioma cell migration and invasion [117,118]. MiR-195 has been found to be strongly downregulated in human GBM cells, causing upregulation of its targets E2F3 and cyclin D3, thus promoting glioma cell proliferation and invasion, respectively [119].

The cluster miR-302/367 is induced during serum-mediated stemness suppression in GBM CSCs; when expressed, it suppresses stemness gene signature, CSC self-renewal, infiltration, and tumorigenic capacity through a drastic inhibition of the CXCR4 pathway [120]. The blockage of CXCR4 signaling leads to a drastic repression of the sonic hedgehog (SHH)-GLI-NANOG network, which has been demonstrated to regulate self-renewal and expression of the embryonic stem cell-like signature [121].

MiR-410 was recently characterized as a direct regulator of MET (the receptor for hepatocyte growth factor receptor, or HGF), downregulating MET-induced AKT signal transduction, with a negative effect on cell proliferation and invasion, decreasing P-AKT and MMP-9 levels [122].

Contrasting pieces of evidence have recently been reported for miR-145. MiR-145 has been characterized as a possible tumor suppressor gene in GBM, negatively acting on the expression of several metastasis-related genes, such as *PLAUR* (plasminogen activator urokinase receptor), *SPOCK3*, *ADAM22*, *SLC7A5* (solute carrier family 7 member 5), and *FASCN1*, and inhibiting glioma cell invasion *in vitro* [123]. On the other hand, miR-143 and -145 (which are expressed by the same genetic locus) have been suggested as possible oncogenes and, in particular, have been shown to exert pro-invasive function in *in vitro* experiments [124].

Conclusions and perspectives

Highly diffuse and infiltrative growth is an important effector of GBM pathogenesis and represents the main limitation in the feasibility of current treatments. In recent years, the molecular players and the signaling cascades triggering the motility and invasion of glioma cells have been widely investigated in order to increase our understanding of the mechanism of glioma spreading and eventually improve the survival of patients with glioma. Following the classification of gliomas in subtypes based upon molecular signatures [54,55], a similar approach based upon microRNA expression has been proposed. Gene expression profile analysis of gliomas revealed a capacity to effectively distinguish GBMs from low-grade gliomas, which overexpressed different classes of microRNAs [125], but also to establish a signature able to predict the survival of patients with GBM on the basis of the expression of 10 microRNAs [126]. In this scenario, the analysis of both GBM molecular signatures and miRNA expression signatures may contribute to cancer diagnosis, prognosis, patient stratification, and a more personalized treatment.

This review lends further weight to these findings, highlighting the role of GBM CSCs as novel determinants of the infiltrative behavior of GBMs. Of further interest, specific effectors implicated in the process of migration and invasion (that is, EMT-associated factors, DCX, and microRNAs) are known to be associated with CSC biology. Given the resistance of CSCs to traditional therapies, approaches designed to target CSCs and their effectors will represent exciting therapeutic targets effective in suppressing the CSC-mediated GBM invasion.

Recently, GBMs have been shown to contain microvesicles carrying angiogenic proteins and inhibitors of MMPs, of *EGFRvIII* mRNA, and of miRNA-21, responsible for GBM invasion [102,127]. These microvesicles have been used as biomarkers since they carry brain tumor-specific antigens and cytokines and are present in the serum of patients with high-grade glioma [128]. Furthermore, these microvesicles could be exploited as possible vehicles for innovative anti-miR therapy of GBM since short-hairpin RNAs have been efficiently delivered to the brain by systemic injection [129]. The possibility to limit the GBM infiltrative component is likely to have a large impact on therapies designed to block tumor progression.

Abbreviations
CSC, cancer stem cell; DCX, doublecortin; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma; miRNA/miR, microRNA; MMP, matrix metalloproteinase; NSC, neural stem cell.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
All authors contributed equally to the manuscript. BO prepared the figure and the table. All authors read and approved the final manuscript.

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