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Current Opinion in Neurobiology

Vascular regulation of glioma stem-like cells: a balancing act

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Glioblastoma (GBM) are aggressive and therapy-resistant brain tumours driven by glioma stem-like cells (GSCs). GSC behaviour is controlled by the microenvironment, or niche, in which the cells reside. It is well-established that the vasculature is a key component of the GSC niche, which drives maintenance in the tumour bulk and invasion at the margin. Emerging evidence now indicates that the specific properties of the vasculature within these two regions impose different functional states on resident GSCs, generating distinct subpopulations. Here, we review these recent findings, focusing on the mechanisms that underlie GSC/vascular communication. We further discuss how plasticity enables GSCs to respond to vascular changes by interconverting bidirectionally between states, and address the therapeutic implications of this dynamic response.

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Current Opinion in Neurobiology 2017, **47**:8–15

This review comes from a themed issue on **Glial biology**

Edited by **Alison Lloyd** and **Beth Stevens**

http://dx.doi.org/10.1016/j.conb.2017.06.008

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Introduction

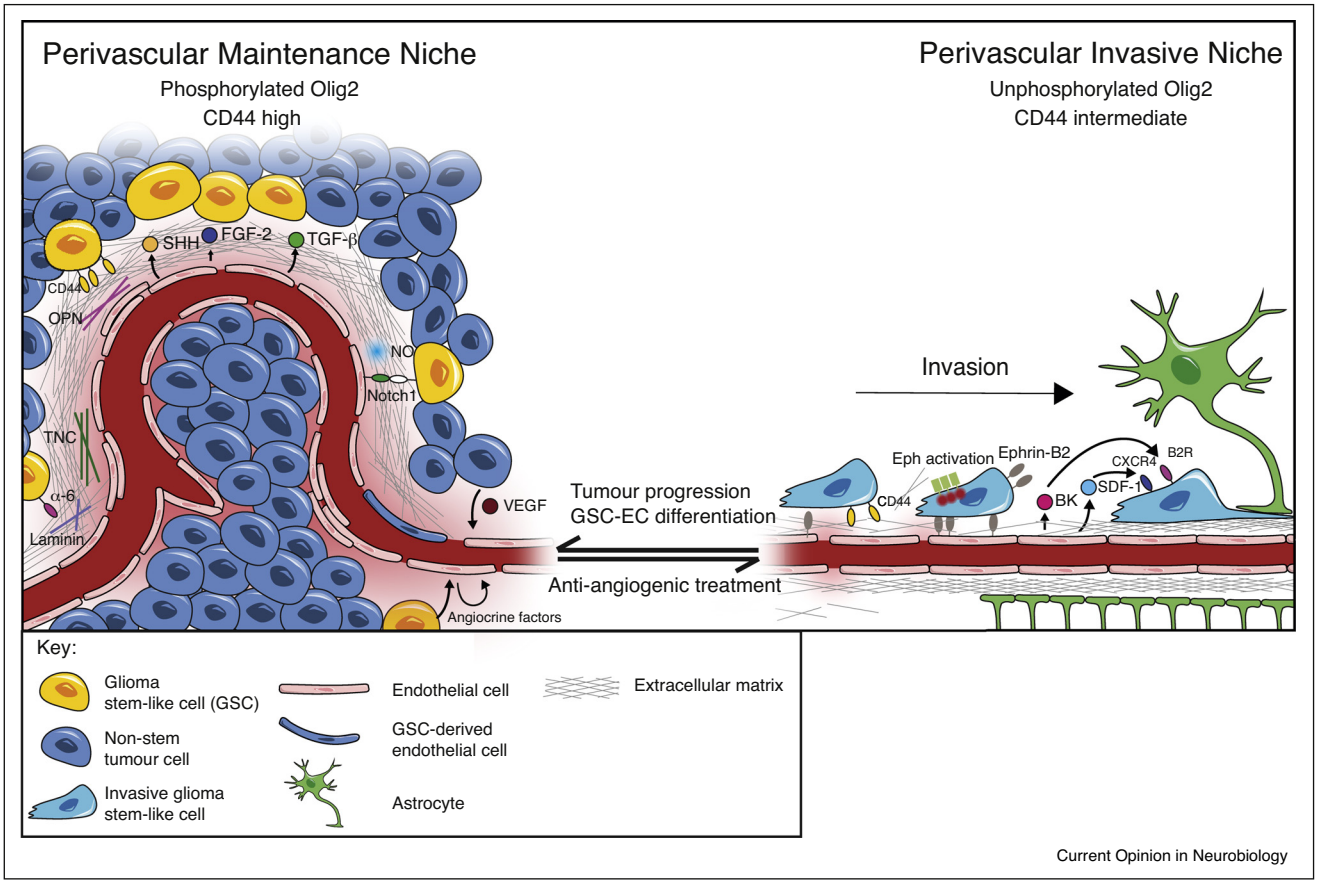
GBM is the most common and aggressive type of primary brain tumour. Despite available therapies, which include maximal surgical resection, radiation and chemotherapy, median survival of GBM patients remains at less than 15 months [1]. This extremely poor prognosis is due to the marked therapeutic resistance of these tumours, which rapidly leads to fatal recurrence following treatment [2]. Significant causes of the therapeutic resistance are the highly infiltrative and heterogenous nature of GBM [3–5]. Invasion into the brain parenchyma constitutes a major clinical problem because it precludes complete surgical resection and hinders radiotherapy [3,4]. Furthermore, extensive molecular and cellular heterogeneity lead to variability in responses to both standard and targeted therapies [5].

At the molecular level, GBMs display intertumoural and intra-tumoural heterogeneity, harbouring a range of mutations, transcriptional signatures and signalling alterations across and within tumours [6–10]. At the cellular level, GBMs comprise subpopulations of cancer cells with distinct therapy-resistance, proliferation, differentiation and tumourigenic potential [11,12]. GSCs, a subset of tumourigenic cancer stem cells, are thought to underpin this cellular heterogeneity. GSCs are able to self-renew, give rise to tumour-bulk cells of more restricted tumorigenic potential and reconstitute a phenocopy of the original tumour upon transplantation [13]. It is therefore believed that GSCs are the main drivers of GBM malignancy and progression. Importantly, GSCs are also intrinsically resistant to chemo and radiotherapy and more invasive than non-stem tumour cells [14,15]. This suggests that GSCs, which are spared by current therapies, may also drive recurrence and that targeting this cellular compartment should improve treatment outcome.

GSCs reside within specialised tumour microenvironments, or niches, which maintain their stemness and malignant properties [16]. In line with the heterogenous nature of GBM, the GSC microenvironment is also highly heterogenous and comprises at least three main structurally, spatially and functionally distinct niches: the perivascular, hypoxic and invasive niches [17]. The perivascular and hypoxic niches are found within the tumour bulk and consist of the angiogenic tumour vasculature and the necrotic regions of the tumour, respectively [16,17]. Both these niches maintain GSCs and support their stemness. The invasive niche comprises the tumour/brain interface at the tumour margin. Within the invasive niche, GSCs associate preferentially to normal pre-existing blood vessels, which they co-opt to migrate and invade into the healthy brain [3].

Increasing evidence indicates that, although both in intimate contact with blood vessels, GSCs in the perivascular maintenance and vascular-invasive niches may be in phenotypically and functionally distinct states. These states appear to be enforced by the specific structure, functional status and signalling properties of the vasculature in each niche and to retain the ability to interconvert bidirectionally as the vasculature evolves during tumour progression and in response to therapy (Figure 1).

Figure 1



The perivascular maintenance and invasive niches. The perivascular maintenance niche provides cues to neighbouring GSCs that promote self-renewal and maintain stemness. These comprise cell-ECM, diffusible and cell–cell signals. ECM molecules implicated to date include laminin alpha-2, which acts via integrin a6 on GSC, Osteopontin, which acts via CD44 expressed on GSC and Tenascin-C. Endothelial-derived diffusible signals include FGF-2, TGF-β, SHH and nitric oxide (NO). The Notch pathway is the best characterized mediator of direct cell–cell signalling in the perivascular niche. To sustain and expand the vascular maintenance niche, GSC can promote angiogenesis either directly via VEGF secretion and differentiation into endothelial cells, or indirectly by inducing endothelial cells to secrete angiocrine factors. In the perivascular invasive niche glioma cells use pre-existing blood vessels as invasion paths. Blood vessels attract tumour cells to the perivascular space via the chemoattractants SDF-1 and bradykinin (BK), which act through CXCR4 and BR2 receptors, respectively. The basal lamina serves as a migration-promoting substrate. GSC override inhibitory vascular ephrin-B2 signalling through ephrin-B2 overexpression and displace astrocyte endfeet to gain access to the perivascular space, resulting in BBB disruption. As cells invade perivascularly, this process ultimately leads to blood vessel regression and neo-angiogenesis, thus converting the invasive niche into a maintenance niche. Direct differentiation of GSC into endothelial cells also generates new maintenance niches at the invasive tumour margin, by recruiting normal endothelial cells and stimulating angiogenesis. Conversely, anti-angiogenic treatment can normalise vessels and promote invasion, thus transitioning the perivascular maintenance niche to an invasive niche.

In this review we will summarise current understanding of the molecular pathways that underlie GSC/vascular interactions in the perivascular maintenance and invasive niches, with a focus on the role of endothelial cells. Furthermore, we will discuss how the interplay of extrinsic factors and intrinsic plasticity modulates the balance between GSC invasion and self-renewal and how alterations in this balance may affect malignancy.

The perivascular maintenance niche

GBMs are characterized by abnormal angiogenesis that produces leaky and dysfunctional blood vessels and microvascular proliferating structures, a histological hallmark of these tumours [18]. Within the tumour bulk, GSCs often reside adjacent to these aberrant vascular structures, an area known as the perivascular niche [16,17]. This preferential localisation was first described in the seminal work of Calabrese *et al.* [19••]. The authors demonstrated that vascular endothelial cells maintain the GSC pool by secreting soluble factors that promote self-renewal and stemness character. Whilst the identity of these factors is still incompletely understood, Sonic Hedgehog, TGFβ and FGF2 have all been implicated [20–22].

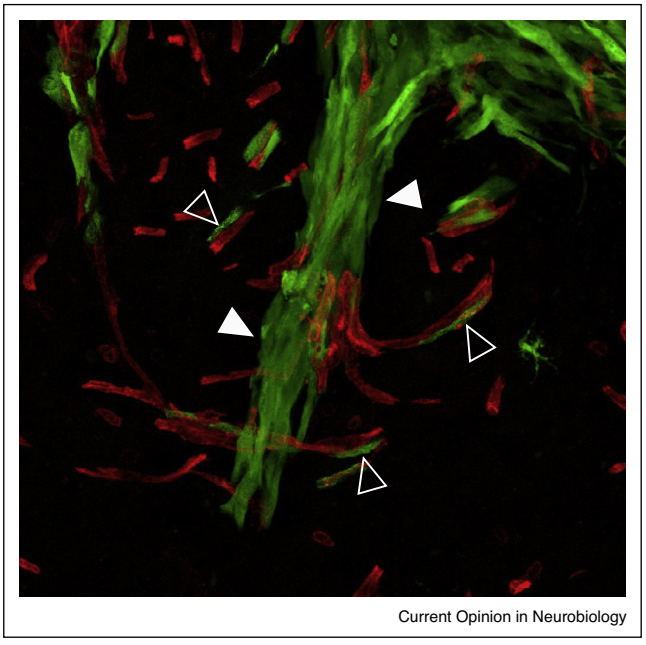
Subsequent work also highlighted the importance of cell–cell contact-dependent and cell-extracellular matrix (ECM) signalling. One of the best-described mediators of perivascular GSC maintenance through direct cell–cell interactions is the Notch pathway. In xenograft models, activation of Notch signalling by endothelial cells promoted GSC self-renewal, as demonstrated by the lower GSC content and smaller size of tumours formed upon co-injection of DLL4-depleted or Jag1-depleted ECs compared to wildtype ECs [23,24]. Furthermore, nitric oxide produced by the tumour endothelium promoted stem-like characteristics in glioma cells through activation of Notch signalling [25]. The extracellular matrix (ECM) is a major component of the perivascular niche and GSCs express a variety of ECM receptors, including several integrins [26,27]. Activation of integrin-dependent signalling by the perivascular ECM has been shown to maintain GSCs. Lathia *et al.* reported that laminin α2 is enriched around tumour blood vessels and promotes GSC self-renewal [28]. This likely occurs through activation of integrin α6 on GSCs, as this receptor was shown to specifically mark GSCs and drive their proliferation [29]. In addition, the matricellular protein Osteopontin also plays an important role. Pietras *et al.* recently demonstrated that Osteopontin and its CD44 receptor are selectively expressed in the perivascular ECM and in GSCs, respectively, and that CD44 maintains GSC stemness through activation of HIF2α signalling [30].

Interestingly, the relationship between GSCs and the vasculature is not unidirectional. Rather, as endothelial cells influence GSC phenotype, GSCs also promote angiogenesis in an intricate bidirectional crosstalk. This is partly mediated by secretion of angiogenic factors such as VEGF, which GSCs produce at much higher concentrations than non-stem tumour cells [31]. In addition, GSCs can induce angiogenesis indirectly, by promoting endothelial production of angiogenic factors [33]. Interestingly, the pro-angiogenic effects of GSCs appear to be context-dependent. A recent study demonstrated that Tenascin-C, which accumulates in tumour vessels, modulates the GSC secretome to increase expression of a range of angiogenic factors, including ephrin-B2 [32]. Finally, GSCs can also help form the perivascular niche directly by differentiating into endothelial cells and pericytes, though the extent to which this process occurs in human GBM remains controversial [33–36]. Thus, a self-sustaining positive feedback mechanism exists whereby endothelial cells maintain GSCs that, in turn, stimulate further angiogenesis to expand the perivascular maintenance niche.

The perivascular invasive niche

In addition to providing a niche for GSC maintenance within the tumour bulk, the vasculature serves as substrate for invasion at the tumour margin [3,17,37,38] (Figure 2). Indeed, perivascular invasion is one of the main routes of GBM infiltration originally described by Hans Joachim Scherer in 1938 as 'secondary structures,' due to their close interaction with pre-existing brain structures [39]. More recent studies also revealed that among the tumour cell populations, GSCs have a particularly strong propensity to home to and migrate along pre-existing blood vessels [3,40••,41••,42]. Though many chemoattractants likely cooperate to drive GSC vascular homing, two main pathways have been implicated to date. The first involves chemokine stromal-derived factor-1 (SDF-1 or CXCL12) and its receptor CXCR4, a pathway that also regulates stem/progenitor cell trafficking to the vasculature in the normal neurogenic niche [36,43,44]. SDF-1 and CXCR4 expression are elevated in blood vessels and tumour cells, respectively, with GSCs displaying highest CXCR4 expression [44,45]. Furthermore, CXCR4 knockdown impaired GSC vascular homing in xenograft models [36]. The second homing factor is Bradykinin, a chemotactic peptide produced by vascular endothelial cells that activates the G-protein coupled receptor Bradykinin receptor 2 (B2R) on tumour cells [46]. B2R exhibits increased immunoreactivity in GBM, most notably in perivascular regions, and its pharmacological inhibition prevented the association of GSCs to

Figure 2



Glioma cells invade along blood vessels. Glioma cells, particularly GSC, use blood vessels as paths for invasion into the healthy brain parenchyma. Immunofluorescence image of perivascularly invading glioma cells in a GSC-derived mouse orthotopic glioma model. The majority of GSC that migrate out of the tumour bulk co-opt pre-existing blood vessels, either forming multicellular cuffs around the vessels (filled arrowheads) or aligning as singles cells along them (open arrowheads). Glioma cells are labelled with GFP (green) and blood vessels with antibodies against the endothelial marker CD31 (red).

the vasculature, resulting in blunted invasion and reduced tumour growth.

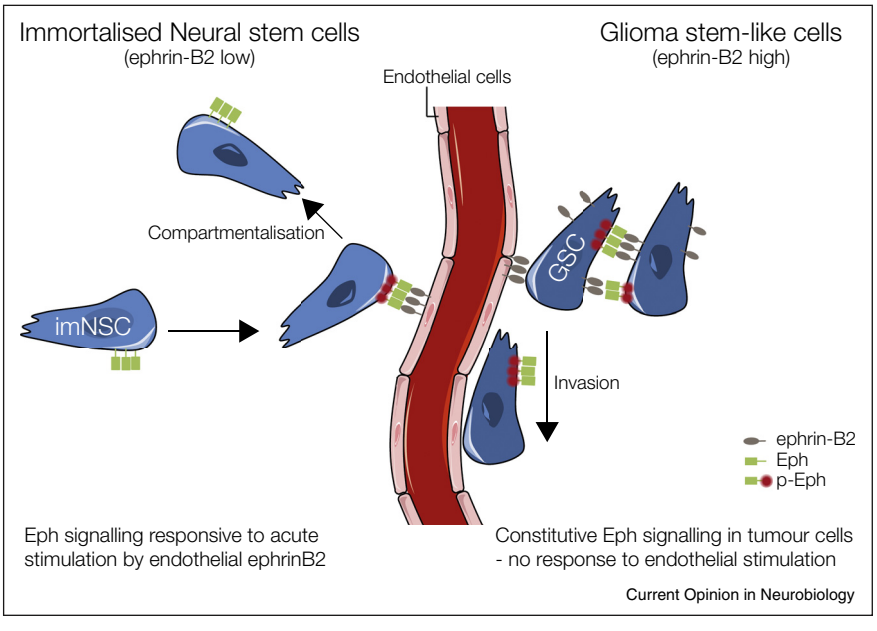
Upon homing to the vasculature, GSCs gain exposure to a particularly favourable environment for invasion because the basal lamina that surrounds blood vessels is enriched in ECM components, such as laminin and fibronectin, that stimulate cell migration [3,26,47]. In addition, the perivascular space is fluid-filled, and therefore opposes lower physical resistance to invading cells than other brain regions [3,48]. By invading along blood vessels, GSCs also obtain ready access to the high levels of oxygen and nutrients they require to meet the metabolic demands of cell migration [3,42].

However, following initial vascular contact, in order to invade perivascularly GSCs must first overcome several hurdles, highlighting the advantageous nature of this infiltrative mode. First, the perivascular space is filled with astrocyte endfeet that wrap around endothelial cells and their surrounding basal lamina to form the blood–brain-barrier (BBB) [3,49]. To circumvent these physical obstacles, invading glioma cells lift up astrocyte endfeet and pericytes, remodel the basal lamina and migrate along the abluminal surface of endothelial cells [40••,41••]. Second, the extracellular space surrounding blood vessels is extremely narrow [50]. To squeeze through this tight environment, invading cells actively decrease their size by up to 35%, by shedding cytosplamic water [40••]. This remarkable hydrodynamic process is mediated by ion channels and Cl^- cotransporters that become constitutively expressed in glioma cells [51]. Third, endothelial cells express repulsive signals that inhibit the migration of normal cells along blood vessels. This process is mediated by endothelial ephrin-B2 ligands, which activate Eph receptors on vascular-associated cells [41••]. Therefore, to enter the perivascular space for invasion, glioma cells must also override vascular repulsion. In GSCs of mesenchymal subtype, this is achieved through up-regulation of ephrin-B2 in the tumour cells themselves. Increased ephrin-B2 saturates Eph forward signalling in neighbouring GSCs through homotypic cell–cell interactions, thereby desensitising the cells to heterotypic repulsion by vascular ephrin-B2. An additional effect of Eph activation is increased repulsion among tumour cells, thus promoting dispersion of single cells away from the tumour bulk (Figure 3) [41••].

Striking the right balance

Increasing evidence suggests that perivascular GSCs of the tumour bulk and the invasive margin may represent phenotypically and functionally distinct subpopulations. Piccirillo *et al.* showed that GSCs isolated from the invasive margin are less proliferative and clonogenic than bulk GSCs, are prone to adherent growth *in vitro* and fail to form tumours when orthotopically transplanted [52].

Figure 3



Schematic representation of the regulation of perivascular invasion by Eph/ephrin signalling. ephrin-B2 levels and Eph forward signalling are low in normal cells. As a result, contact with ephrin-B2 on vascular endothelial cells results in cellular repulsion and compartmentalisation (left). In contrast, transformation overrides this tumour suppressive mechanism, enabling perivascular invasion. In GSC of mesenchymal subtype this is achieved through upregulation of ephrin-B2 in the tumour cells themselves. High ephrin-B2 levels saturate Eph forward signalling in GSC, thereby desensitising the cells to further stimulation by vascular ephrin-B2 and overcoming compartmentalisation (right).

These findings are echoed by other studies that reported decreased proliferation and increased expression of mesenchymal markers at the tumour periphery relative to the core in both primary tumours and xenograft models [53–56].

These distinct GSC phenotypes are likely imposed by their distinct microenvironments. Though at first glance counterintuitive for two populations that share a perivascular microenvironment, it is important to keep in mind that the molecular composition and functional states of the vasculature in the two regions are markedly different. Vessels in the tumour bulk are dysfunctional leading to regions of hypoxia, express high levels of angiogenic factors (e.g. Notch ligands) and are surrounded by atypical inflammatory ECM components (e.g. Osteopontin) [17]. As described above, all these signals enforce stemness and promote proliferation. In contrast, vessels of the tumour margin are functional and thus normoxic, relatively normal in structure and provide an environment that promotes invasion, an effect that may also actively suppress proliferation [17]. A recent elegant study in *C. elegans* has indeed provided a first mechanistic explanation for the long-standing observation in many cancers, including GBM, that invasion and proliferation are mutually exclusive [57••]. The authors identified the transcription factor Nhr67 as a critical mediator of cell invasion and showed that it functions by enforcing a G1 cell-cycle arrest, which in turn is required for acquisition of invasive characteristics. Although the relevance of these findings to mammalian systems is still to be confirmed, it is tempting to speculate that a similar mechanism takes place at the invasive perivascular niche. Consistent with this idea, live imaging of glioma cells invading along blood vessels were found to be largely quiescent and to stop migrating in order to divide [38].

Emerging studies lend further mechanistic support to the dichotomous relationship between GSC of the two perivascular niches. For example, Klank and colleagues, recently reported that CD44 expression and GSC motility are correlated in a biphasic manner, where only intermediate CD44 expression supports migration [58•]. As CD44 expression is highest in the perivascular maintenance niche, it is conceivable that these high CD44 levels would suppress GSC invasion whilst strongly activating maintenance pathways. Thus, high CD44 would shift the balance towards self-renewal in the tumour bulk, whereas intermediate CD44 at the invasive front would favour infiltration. In a similar vein, the phosphorylation status of the transcription factor Olig2 has been proposed as a molecular switch controlling the balance between GBM invasion and proliferation, with unphosphorylated Olig2 driving invasion at the tumour margin [59•]. This suggests that phosphorylation/dephosphorylation of Olig2 downstream of niche signals may also fine-tune the balance in GSC states.

Despite their distinct roles, the perivascular maintenance and invasive niches are not comparmentalised entities, but rather continuous environments that interconvert during tumour evolution and following therapy (Figure 1). As invading GSCs intercalate between astrocyte endfeet and endothelial cells, they disrupt the BBB, leading to vascular remodelling and allowing blood-borne cytokines and immune cells to enter the brain. As the tumour grows, this process, together with the increase in tumour mass, eventually leads to regression of coopted vessels and induction of angiogenesis [60]. Thus, as a result of tumour progression, the perivascular invasive niche evolves into a maintenance niche. Recent findings suggest that a similar niche conversion can also occur through direct differentiation of GSC into endothelial cells. GSC-derived endothelial cells were shown to support seeding of satellite tumours by recruiting normal endothelial cells to the peritumoural region and creating new perivascular maintenance niches [61]. The reverse conversion also occurs following anti-angiogenic therapies and has been best characterized following Bevacizumab treatment, a VEGF blocking antibody in clinical use. Several studies demonstrated that by normalising the tumour vasculature, Bevacizumab treatment stimulates perivascular invasion, thus transitioning the perivascular maintenance niche into an invasive niche [62,63]. Mechanistically, this effect is at least partially mediated by Met activity in glioma cells, which, in the absence of VEGF, complexes with VEGFR2 to drive a mesenchymal-like transition and trigger invasion [64].

Together, this evidence suggests that the perivascular invasive niche may induce GSC to undergo mesenchymal-like differentiation, at the expense of self-renewal and tumorigenicity. This raises the important question of how, then, can invasive GSC regenerate the tumour following surgical resection. The answer likely lies in the remarkable plasticity of GBM. Indeed, recent studies indicate that non-stem tumour cells retain the ability to de-differentiate to GSC in response to a plethora of niche signals. An intriguing report demonstrated that GSC only undergo partial differentiation towards the astroglial lineage and remain vulnerable to de-differentiation by mitogenic stimuli [65••]. Similarly, lineage tracing of non-stem tumour cells indicated reversal to a GSC state following exposure to chemotherapeutics [66]. Consistent with this rampant plasticity, single-cell RNA-sequencing revealed a continuum of stemness across tumour cells, rather than the existence of a well-defined, rare stem cell population [8]. It appears therefore that perivascular GSC phenotypes are not fixed, but rather plastic and niche-dependent.

Conclusions

Perivascular GSCs exist in a dynamic balance between self-renewing and invasive states, imposed, at least partially, by the vasculature. As GSC plasticity enables these

states to interconvert bidirectionally, unilateral therapeutic targeting of either perivascular niche will likely prove insufficient for tumour eradication and may lead to resistance by favouring the alternate state. Rather, the development of improved anti-GBM therapies is bound to require concomitant targeting of both perivascular subpopulations. Increased understanding of the molecular mechanisms that underlie GSC/vascular interactions in both niches will be essential to achieve this goal.

Competing interests

The authors have no competing interests.

Acknowledgements

We thank Benjamin Krusche for preparing Figure 3, Cristina Ottone for image acquisition and Samuel Marguerat for critical reading of the manuscript. Research in the laboratory is supported by the Medical Research Council (MC-A652-5PZ10) and by Cancer Research UK (WCMA_P58950).

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