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Review Article

Glioblastoma heterogeneity and the tumour microenvironment: implications for preclinical research and development of new treatments

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Glioblastoma is the deadliest form of brain cancer. Aside from inadequate treatment options, one of the main reasons glioblastoma is so lethal is the rapid growth of tumour cells coupled with continuous cell invasion into surrounding healthy brain tissue. Significant intra- and inter-tumour heterogeneity associated with differences in the corresponding tumour microenvironments contributes greatly to glioblastoma progression. Within this tumour microenvironment, the extracellular matrix profoundly influences the way cancer cells become invasive, and changes to extracellular (pH and oxygen levels) and metabolic (glucose and lactate) components support glioblastoma growth. Furthermore, studies on clinical samples have revealed that the tumour microenvironment is highly immunosuppressive which contributes to failure in immunotherapy treatments. Although technically possible, many components of the tumour microenvironment have not yet been the focus of glioblastoma therapies, despite growing evidence of its importance to glioblastoma malignancy. Here, we review recent progress in the characterisation of the glioblastoma tumour microenvironment and the sources of tumour heterogeneity in human clinical material. We also discuss the latest advances in technologies for personalised and *in vitro* preclinical studies using brain organoid models to better model glioblastoma and its interactions with the surrounding healthy brain tissue, which may play an essential role in developing new and more personalised treatments for this aggressive type of cancer.

Introduction

Glioblastoma is the most commonly diagnosed and aggressive type of brain cancer, accounting for 80% of primary malignant brain tumours of the central nervous system (CNS) and 60% of all brain tumours in adults [1]. There are 10 000 and 100 000 new cases of glioblastoma diagnosed each year in the U.S.A. and across the world, respectively [1,2], with the disease occurring 1.6-fold more frequently in men compared with women [3]. While rare relative to overall cancer incidence, glioblastoma accounts for 2.5% of total cancer-related deaths, having the highest rate of mortality in those aged between 15 and 34 years of age [1].

Glioblastoma is a form of glioma, a group of cancers that have long been thought to arise from glial cells of the CNS. However, recently strong evidence has emerged that glioblastoma arises from neural stem cells within the subventricular zone of the brain rather than mature glia [4]. Two broad classes of infiltrative gliomas are identified histologically which resemble normal glial populations; thus, astrocytomas and oligodendrogliomas have astrocytes and oligodendrocytes, respectively, as their normal morphological counterparts [5]. Gliomas are further graded and categorised according to the World

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Health Organisation (WHO) guidelines based on a combination of histologic and molecular features [6]. The most advanced (grade IV) astrocytomas are classified as glioblastoma. The essential diagnostic features of glioblastoma are atypical glial cells, brisk mitotic activity and evidence of microvascular proliferation (MVP) and/or significant necrosis. MVP typically appears as glomeruloid tufts of multilayered endothelial cells that are mitotically active along with smooth muscle cells or pericytes. This can be found around and directionally oriented towards necrosis. Because of extensive neo-angiogenesis, the vasculature is highly abnormal with leaky and hyperdilated vessels. Necrosis is a fundamental feature of glioblastoma and the strongest predictor of aggressiveness [7–9].

First-line therapy for newly diagnosed glioblastoma (often referred to as primary glioblastoma) is surgical intervention, or 'maximal safe resection', followed by concurrent chemo-radiation and maintenance chemotherapy. However, this treatment regimen extends the median survival time to only 15 months [10], and the disease remains incurable [11]. Despite these aggressive treatments, the disease almost inevitably returns as recurrent glioblastoma, for which there is no standard treatment approach [12]. Treatment options for recurrent glioblastoma include re-resection, treatment with the anti-angiogenesis agent bevacizumab and experimental approaches in the context of clinical trials but none of these approaches has been shown to prolong survival significantly. For recurrent glioblastoma patients, the 6-month progression-free survival is ∼15%, and overall survival generally less than 6 months [13].

Two key reasons for lack of progress in the treatment of glioblastoma are (i) extensive intra- and inter-tumour heterogeneity and (ii) the highly invasive and infiltrative nature of these tumours. This ability of glioblastoma cells to infiltrate surrounding healthy brain tissue is dependent upon complex interactions between tumour cells and their surrounding microenvironment, which are still poorly understood [14–17]. Therefore, we argue that there is a need to re-conceptualise the way that glioblastoma therapies are investigated, by studying the role of the tumour microenvironment in promoting tumour progression, invasion and resistance to therapy, while taking into account the heterogeneity that is inherent to human glioblastoma. This can be achieved by focusing on the use of patient-derived glioblastoma cells rather than long-term cell lines, coupled with advanced *in vitro* models that include elements of both the tumour and normal brain tissue. These advances have the potential to reveal new molecular signatures that, if appropriately targeted, can stop tumour growth and invasion without affecting the surrounding healthy brain tissue [17–24].

With this perspective in mind, and focusing on studies of human clinical material, we outline sources of glioblastoma heterogeneity and review how glioblastoma tumour cells interact with their microenvironment. We also detail recent progress in the development of new preclinical models of glioblastoma based on newly developed human brain organoid approaches. These models show great promise for the development of precision therapies for glioblastoma patients.

Glioblastoma heterogeneity

Genetic and transcriptional heterogeneity

Several genetic drivers have been reported to contribute to the development of glioblastoma, including amplification of epidermal growth factor receptor (*EGFR*) gene and mutations in isocitrate dehydrogenase (*IDH*), telomerase reverse transcriptase (*TERT*), phosphatase and tensin homologue (*PTEN*), neurofibromatosis type 1 (*NF1*) gene and platelet-derived growth factor receptor alpha (*PDGFRα*) [1,22,25–28]. Since we focus here on the tumour microenvironment, we redirect readers interested in genetic drivers of this disease to dedicated reviews on the topic [29–33].

As well as varying in these individual genetic aberrations, transcriptomic analyses have revealed that glioblastoma tumours also vary dramatically in their global gene expression profiles. Early clustering analyses of microarray data generated by The Cancer Genome Atlas (TCGA) suggested the existence of four distinct glioblastoma subtypes: neural, pro-neural, classical and mesenchymal [34]. However, more recent analyses have proposed that the neural subtype arose from contamination by normal neuronal cells, refining the molecular subtypes into three instead of four [35]. Of note, the frequent transition between subsets has been noted in the progression from primary to recurrent glioblastoma [35,36]. Furthermore, even different areas of the same tumour can display distinct molecular profiles, highlighting the intra-tumour heterogeneity of glioblastoma [37,38]. Interestingly, Ross et al. [22] identified distinct signalling networks and potential druggable proteins specifically at tumour margins, as cells that belong to these regions are likely to escape surgery and lead to recurrence.

Heterogeneity at the cellular/tissue level: histopathology

Although no longer used in the current 2016 WHO classification of CNS tumours, the term glioblastoma multiforme (GBM) is still common in general use and highlights the wide variety of histological phenotypes that may be seen in this tumour, not only between patients but also within a single tumour [6,39]. The key features required for the histological diagnosis of glioblastoma include high cellularity, cytological atypia and mitotic activity together with MVP and/or necrosis [7–9,29,40]. However, few human neoplasms are as histologically heterogeneous in composition as glioblastoma and although there are usually some better-differentiated areas showing a clear astrocytic phenotype to help with diagnosis, a wide range of other phenotypes have been described [6–9,29].

Small cell glioblastoma is characterised by a monomorphic population of small round cells with scant cytoplasm, which, in ∼70% of cases, demonstrates *EGFR* amplification [41]. Glioblastoma with a primitive neuronal component is characterised by more solid-looking nodules, sharply demarcated from the surrounding tumour, with high cellularity and a high nuclear-to-cytoplasmic ratio reminiscent of medulloblastoma [42]. These frequently demonstrate the expression of neuronal markers such as synaptophysin and *MYC* or *MYCN* gene amplification [43]. The presence of oligodendroglioma-like components, characterised by cells with round, regular nuclei with a fine chromatin pattern, often with clear cytoplasm and with a fine capillary vasculature, has been a source of diagnostic difficulty in the past. However, with the incorporation of molecular testing, tumours with this phenotype that demonstrate loss of heterozygosity of chromosomes 1p and 19q and *IDH* mutation are now classified as anaplastic oligodendrogliomas, while tumours that are 1p and 19q intact and *IDH* wild type are classified as glioblastomas [44,45]. Giant cell glioblastoma is characterised by the presence of large numbers of multinucleated giant cells, although this is considered to probably represent a regressive change and the tumour does not demonstrate a characteristic genetic signature [46]. In contrast, BRAF V600E mutations occur in ∼50% of epithelioid glioblastoma tumours, which are composed of closely packed cells with abundant cytoplasm reminiscent of epithelium, often with a rhomboid cell component [47,48]. Gliosarcoma is probably the best-known phenotypic variant and is characterised by a biphasic pattern with areas demonstrating glial differentiation alternating with areas of densely packed bundles of spindle cells, which may show differentiation into mesenchymal tissues such as cartilage, bone, osteoid, muscle or even fat [49,50].

The most crucial difference between glioblastomas is the division between those that are wild type for *IDH* and those bearing *IDH* mutations, which has now been incorporated into the revised 4th edition of the WHO Classification of Tumours of the CNS published in 2016 [6]. The *IDH* wild-type glioblastoma makes up 90% of all glioblastomas and arises *de novo*, in contrast with the 10% of *IDH*-mutant glioblastomas which are believed to arise as a result of progression from lower-grade diffuse or anaplastic astrocytomas [51]. However, these two groups, which exhibit such a clear-cut genetic distinction, are morphologically indistinguishable by histology.

Heterogeneity in tumour location

Sixty-one percent of glioblastoma incidence is reported within the four distinct regions of the cortex, with tumours showing a systematic preference for the frontal lobes (Figure 1) [11]. It is estimated that 25–43% of tumours are located in the frontal lobes (with ∼8% bifrontal), compared with 19–28% located in the temporal, 12–25% in the parietal and 3% in the occipital regions [11,52]. It has been reported that 80% of all recurrences stem from the initial tumour site. However, 13–45% of glioblastoma have been reported to be multifocal [11,53]. Laterality of glioblastoma appears non-biased with equal incidence rates in left and right hemispheres and also presents bilaterally and inter-regionally (i.e. frontoparietal). While it is not clear how this heterogeneity at the organ level can be used for therapeutic benefit, however, radio-genomics and imaging studies increasingly provide new information that advances current knowledge of glioblastoma [54–56]. It has been shown that brain regions are also differentially invaded by glioblastoma cells, highlighting the concept of varying susceptibility of different brain regions to glioblastoma progression, with the hippocampus being a particularly uncommon site for glioblastoma cell invasion [57].

Brain tumour microenvironment

Tumour extracellular matrix

The extracellular matrix (ECM) constitutes the non-cellular component of the microenvironment present in all tissues and functions as a physical scaffold as well as a source of biochemical signals [58]. It maintains a close relationship with intracellular biochemical and biomechanical processes and strongly influences the

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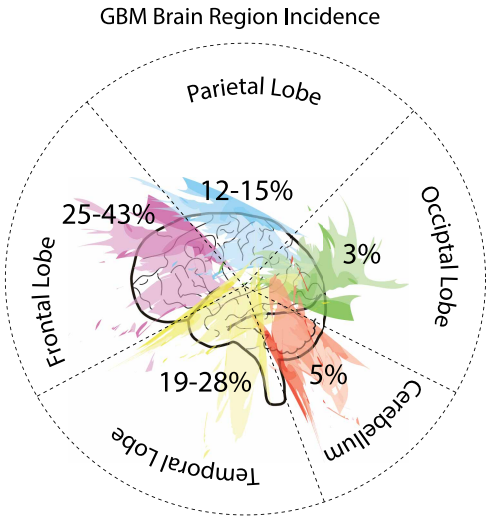

Figure 1. The incidence of glioblastoma by brain sub-regions.

Different colours divide the human brain into its five sub-regions: frontal (purple), parietal (blue), occipital (green) and temporal lobes (yellow), and the cerebellum (red). Percentages in each division are the glioblastoma incidence by sub-region, indicating the high topographical heterogeneity in the brain, with a predominance in the frontal lobes.

biochemistry and biomechanics of tissue. Cooperation between cellular and ECM factors regulates cellular fate, tissue morphology and organogenesis [59,60]. ECM makes up ∼20% of brain volume, varying in composition among different brain regions [14]. For recent comprehensive reviews of brain ECM, we refer the reader elsewhere [58,61].

The ECM is dynamic, changing with age and in response to biochemical, physical and mechanical signals. Furthermore, the ECM is unique to particular cell 'niches', with profound consequences for organogenesis and in human disease. During development, cell–ECM interactions regulate cell fate, differentiation and migration. Similarly, interactions between glioblastoma tumour cells and the ECM play a critical role in invasion and malignancy (Figure 2). For example, Rascher et al. [62] assessed changes in ECM in glioblastoma vasculature and its relationship with blood–brain barrier (BBB) integrity, revealing that under pathological conditions of glioblastoma, ECM agrin is partially lost from the basal lamina of blood vessels and replaced with tenascin. Conversely, analyses of protein expression showed that the ECM surrounding the tumour has the same components as healthy brain tissue (collagen IV, fibronectin and laminin), but contains increased levels of hyaluronic acid (HA), as a result of high levels of hyaluronan synthases 1, 2 and 3 (HAS 1/2/3) [63]. Other important ECM-binding proteins and/or modifying enzymes in glioblastoma cells are CD44 (HA receptor), matrix metalloproteinase-9 (MMP9) and hyaluronidases 1/2/3 (Hyal 1/2/3). All these have a direct impact on ECM remodelling, which facilitates the invasive and infiltrative phenotypes of glioblastoma.

Metabolic tumour microenvironment

Normal brain astrocytes are primarily glycolytic, while neurons rely on oxidative phosphorylation (OXPHOS) [18,64,65]. It is commonly reported that tumour cells rely heavily on elevated glycolytic rates when compared with normal cells, even in the presence of oxygen. Known as the Warburg effect, a high level of aerobic glycolysis denotes a metabolic switch in which cancer cells become reliant on glycolysis as their major energy source reportedly due to insufficient OXPHOS (Figure 2). Aerobic glycolysis is much less efficient at producing adenosine triphosphate (ATP) than OXPHOS, which has led researchers to speculate as to why cancer cells have evolved this method of energy production [65].

A key hypothesis is that aerobic glycolysis yields precursors required by biosynthetic pathways for growth and invasion such as shuttling carbon from glucose into fatty acids, nucleic acids and some proteins [18,64,65]. While ATP is less efficiently produced in this mode of metabolism, it is reported that glioblastoma cells generate ATP through aerobic glycolysis at an abnormally high rate, as confirmed by lactate production, which is 20 times higher than lactate levels found in normal tissue [64]. Most importantly, lactate metabolism is strongly

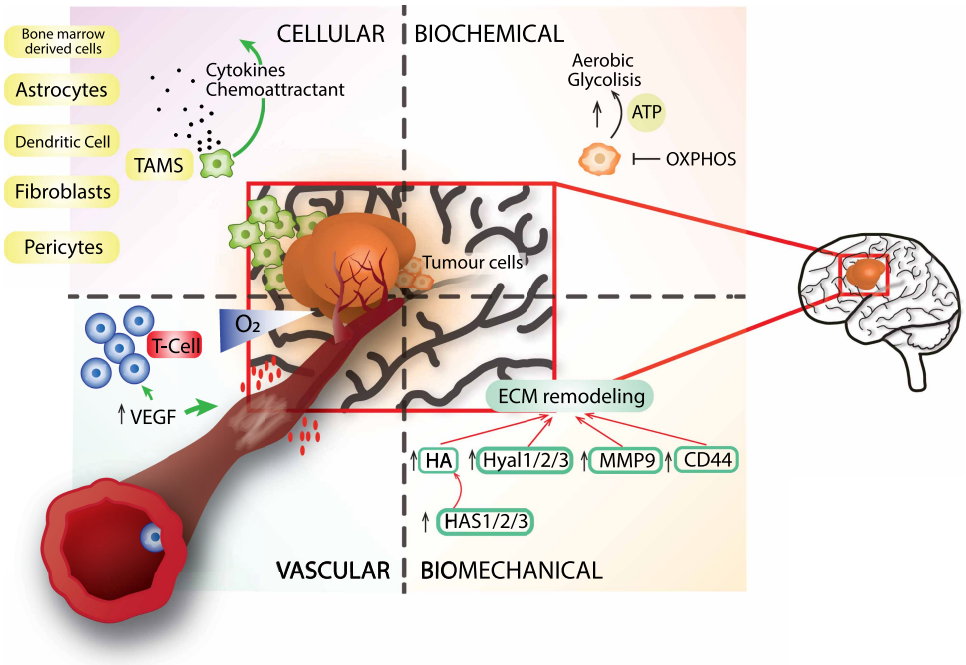

Figure 2. The brain tumour microenvironment.

Representation of the different cellular and extracellular contributions to the glioblastoma microenvironment by sector: cellular, biochemical, biomechanical and vascular. Highlighted cellular components of the tumour microenvironment include TAMs (indicated in green), which have a crucial role in tumour progression via the release of cytokines and chemoattractant that promote the recruitment of high numbers of TAMs to the tumour microenvironment. Tumour cells have altered metabolic pathways with elevated rates of aerobic glycolysis generating the main source of cellular energy, as outlined in the upper right quadrant (biochemical contribution to the tumour microenvironment). Specific changes in the ECM composition and biomechanical properties of the tumour microenvironment are characterised by an increase in HA, which contributes to glioblastoma invasiveness into surrounding healthy tissue. Finally, deformation of the vasculature (described in the lower left quadrant) arises because increased expression of VEGF induces the enlargement and permeabilisation of vessels, triggering brain oedema, interstitial pressure and inflammation. Reactive T cells (represented in blue) have an essential role in these processes. Abbreviations: ECM, extracellular matrix; HA, hyaluronic acid; HAS, hyaluronan synthases; Hyal, hyaluronidases; MMP9, matrix metalloproteinase-9; OXPHOS, oxidative phosphorylation; TAMs, tumour-associated macrophages/microglia; VEGF, vascular endothelial growth factor.

linked to extracellular pH, making the glioblastoma microenvironment more acidic than normal brain tissue [66]. To maintain the high levels of energy required for rapid proliferation, it is reported that glioblastoma cells can access fuel found in adjacent astrocytes [18,67,68]. The 'lactate shuttle hypothesis' takes advantage of astrocyte–neuronal coupling, in which exchanges of extracellular substrates result in OXPHOS. Here, the metabolites generated by astrocytes such as lactate are taken up by adjacent glioblastoma cells and oxidised as extra fuel [18,67,68].

Although tumour cells generally have a preference for glycolysis over mitochondria OXPHOS, results of recent studies performed in mice and *in vitro* models support the notion that mitochondrial energy production does occur in glioblastoma [18,67,69]. In this context, altered mitochondrial function in response to up-regulated glycolysis contributes to increased reactive oxygen species and lactate production, resulting in extracellular inflammatory signalling. Polson et al. [69] examined the clinical relevance of these findings by targeting the mitochondria with small molecules, demonstrating glioblastoma apoptosis without damage to healthy surrounding neuronal tissue.

Together, these changes in energy metabolism have consequences for the regulation of cell volume, synthesis of ECM and increased in cell motility [70,71] as well as epigenetic reprogramming [72] that is required for the invasion of glioblastoma tumour cells into the surrounding healthy tissue.

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Cellular tumour microenvironment

Like that of most solid tumours, the glioblastoma microenvironment contains other cell types in addition to neoplastic tumour cells, in particular, vascular cells and immune cells (Figure 2). These cells secrete inflammatory signalling molecules and ECM components in response to the presence of tumour cells and the evolving biochemical and biomechanical conditions near the tumour. They also actively engage with the ECM, which is dynamically modified due to the presence of cancer cells [16].

Abnormal vasculature in glioblastoma is a consequence of up-regulated angiogenic factors, specifically, vascular endothelial growth factor (VEGF). Increased VEGF causes new blood vessels to form within the tumour via angiogenesis and the associated proliferation of endothelial cells. The resulting vascular networks display increased vessel permeability and enlarged vessel size that result in plasma leakage into tumour tissue and disruption to the BBB. Together, these abnormalities induce cerebral oedema, increased interstitial pressure and inflammation [73]. Oxygen delivery is also compromised, resulting in subsequent hypoxia and pseudopallisading necrosis [74,75]. Therefore, anti-angiogenic treatments have been extensively investigated for the treatment of glioblastoma, including monoclonal antibodies such as bevacizumab that inhibit VEGF function, or small molecules that target its receptor (VEGFR) [74,76,77]. Nevertheless, these treatments have proved much less effective than had been hoped [76–78]. Problematically, the combination of temozolomide (standard treatment) with bevacizumab has been associated with high toxicity and intracranial haemorrhage, highlighting treatment complications with multidrug therapies [78].

Astrocytes vastly outnumber neurons in the CNS, playing essential roles in glutamate, ion and water homeostasis, as well as defence against oxidative stress, energy storage, and synaptic formation and remodelling [18,79,80]. Astrocyte endfeet cover more than 99% of the cerebrovascular surface of the brain, where they contribute to the formation of the BBB via astrocyte–neuronal coupling to regulate solute passage through contact with pericytes and endothelial cells [79–81]. Astrocytes are present within the glioblastoma microenvironment, as well as in the surrounding brain tissue, and are thought to play important roles in regulating glioblastoma progression via displacement/degradation of astrocytic endfeet, decoupling the protective function of neurovascular homeostasis [79,81,82]. Invasion and migration routes of glioblastoma are reported to occur along these perivascular spaces, highlighting an essential role this restructuring has on proliferation [79,80].

Myeloid lineage cells, including brain-resident microglia and infiltrating macrophages, play a critical signalling role between the cellular microenvironment and tumour cells [16,83]. Remarkably, tumour-associated macrophages/microglia (TAMs) have been reported to constitute 30–50% of the glioblastoma tumour mass [40]. A recent flow cytometric analysis of human tumour tissue estimated that ∼40% of these cells are infiltrating macrophages, 20% are brain-resident microglia, and the remaining 40% are myeloid-derived suppressor cells [84]. Accumulating evidence suggests multiple mechanisms by which such TAMs can promote glioblastoma growth and invasion [7,40,84]. T cells are also present in the glioblastoma microenvironment, although at lower frequencies than TAMs [85]. They have a profoundly exhausted phenotype, characterised by expression of LAG3, TIGIT, CD39 and especially programmed cell death 1 (PD1) [86], likely accounting for their inability to control tumour growth. The lack of effective T-cell response is also highlighted by the ineffectiveness of checkpoint blockade immunotherapy in glioblastoma, with the striking exception of tumours with germline mismatch repair deficiency [87].

Neuronal activity has also been implicated in glioblastoma tumour growth and progression [20,23]. In the normal brain microenvironment, neurons are strong mitogenic signallers, stimulating the growth of neural and oligodendrocyte precursor cells, an important consideration in the role of stem/progenitor cells in glioblastoma [88,89]. Elegant studies of neuronal activity conducted by Venkatesh et al. [23,24] in xenograft glioma mouse models show that presynaptic and postsynaptic function is disrupted in the presence of glioma, with neuroligin-3 (NLGN3) being hijacked to induce signalling through the PI3K/PTEN/AKT/mTOR pathway, an important cell-cycle regulator. Under normal conditions, this pathway is essential to promote growth and proliferation over the differentiation of neural stem cells.

Disruption to this intracellular pathway not only increased tumour cell proliferation, but also up-regulated NLGN3 production in a feed-forward manner [23,24]. Correlating data from human glioblastoma, lower levels of NLGN3 were found to be associated with increased survival [23]. Moreover, there may be therapeutic applications in targeting neuronal activity, with ADAM10 inhibitors preventing the release of NLGN3 into the tumour microenvironment, disabling tumour cell proliferation. ADAM10 inhibition is currently being trialled clinically in the treatment of other cancers [24].

In glioma tissue, neurons are often closely apposed to tumour areas, suggesting an interaction between healthy brain tissue and the tumour [20], with neuronal programmed death ligand 1 (PD-L1) possibly playing a key role. PD-L1 is not normally highly expressed in the brain other than in BBB endothelial cells. However, it is up-regulated in glia in response to viral perturbations and inflammatory responses [20]. Low PD-L1 expression in the bulk tumour and high expression by neurons in the tumour microenvironment were strongly associated with favourable prognosis for GBM patients. In contrast, high PD-L1 expression in tumour cells and low neuronal expression in the microenvironment were associated with poor prognosis [20]. Accordingly, the expression of PD-L1 by neurons surrounding glioblastoma tissue increased neuronal killing of tumour cells, suggesting that up-regulation of PD-L1 in native brain neurons was a negative feedback signal for down-regulation of PD-L1 expression by tumour cells, and that PD-L1 expression by glioblastoma cells limits T-cell activation and helps cells escape immune surveillance [20].

In contrast with most solid tumours, classical fibroblasts are not a major component of the glioblastoma microenvironment and historically have been largely ignored. However, recent studies have identified several fibroblast-like cell types within the glioblastoma microenvironment. For example, glioblastoma-associated stromal cells (GASCs), which closely resemble cancer-associated fibroblasts in epithelial tumours, are particularly prevalent at the invasive periphery of tumours and enhance tumour growth [90], while a population of mesenchymal stromal/stem cells (MSCs) are enriched in the perivascular niche and appear to enhance the self-renewal capacity of glioblastoma stem cells [91].

Taken together, these studies highlight a clear role for non-tumour components of the tumour microenvironment in supporting tumour cell proliferation and invasion. Furthermore, these studies implicate neuronal activity and transmembrane receptor ligands in the invasion process, but also highlight therapeutic opportunities to protect surrounding native brain tissue.

New experimental models for glioblastoma

Currently, most clinical trials for the treatment for glioblastoma do not target the non-cellular tumour microenvironment, with a majority targeting genetic drivers within tumour cells. Problematically for this approach, heterogeneity both within and between tumours has limited the development of a single drug that 'cures' glioblastoma, or consistently prevents invasion and spread. Although the non-cellular tumour microenvironment displays dynamic changes throughout the invasion process, it may represent a valid and more genetically stable target for new therapeutic approaches.

Fundamental preclinical models that have been used to develop and assess the utility of new treatments for glioblastoma include the implantation into mice of patient-derived glioblastoma stem cell-like cultures [92,93] and organotypic brain slices [14,94]. Unfortunately, animal models do not accurately replicate native human brain tissue or serve as a physiologically relevant platform to assess dynamic changes in the microenvironment, exposing a fundamental flaw in modelling disease using human cells and tissues in mice. In contrast, cells cultured on tissue culture plastic do not recapitulate the dimensionality, complex cellular composition or structurally and physiologically relevant components of human brain tissue.

Platforms such as 3D bio-scaffolds, microfluidic devices and high-throughput systems aim to improve on current brain tumour stem cell-like culture models [58,95–97]. However, *in vitro* models that better reflect human brain tissue structure and functionality are necessary. In this regard, recent advances in stem cell biotechnologies have seen the creation of cerebral or 'brain organoids' [98–100]. Brain organoids are 'organ-like' structures grown *in vitro*, which can recreate in a 3D environment some of the characteristics of a human brain. These structures provide a mechanism for studying human brain disease, including cancer, in a physiologically relevant context and form a foundation for the development of personalised therapies [99]. Brain organoid technology can also be complemented by newly developed autochthonous models of glioblastoma in mice generated using CRISPR–Cas9 approaches [101], which have yielded important new insights into genetic drivers of the disease and are likely to prove useful in studying the glioblastoma microenvironment. Moreover, these technological advances, together with, for example, the recent insights into metabolic reprogramming using synthetic and natural compounds in targeting glioblastoma mitochondria without perturbation to surrounding healthy brain tissue will be further advanced by glioblastoma-brain organoid models [69,102,103].

Preclinical models in assessing brain tumours using organoid approaches have the potential to be transformative in the development of new therapies. In this sense, the first organoid models developed to study glioblastoma were created from tumour cells. For example, Hubert et al. [104] generated long-lived and complex

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organoids from human glioblastoma biopsies that maintained key aspects of the source tumour, including the maintenance of regional heterogeneity and hypoxia gradients, as well as a high tumorigenic capacity after implantation into the frontal lobes of mice. These aspects were not observed when the cells were cultured as simple spheroids. In a different approach, da Silva et al. [105] co-cultured glioblastoma spheroids with early-stage brain organoids and demonstrated an efficient and rapid fusion between the sphere and the organoid, followed by spontaneous infiltration of tumour cells into the organoid. Of note, although the primitive neuro-epithelial structures used in this study are precursors of mature brain organoids, the former have an inverted topology (i.e. apical junctions are exposed to the medium and reversion happens when the early-stage organoids, i.e. embryoid bodies, are embedded within Matrigel [100]).

Natural, synthetic and semi-synthetic mediums that better reflect native tissue and tumour microenvironments are critical for the advancement of preclinical models [58,59,106–108]. Both varying synthetic scaffolds and mediums/hydrogels will allow researchers to optimise organoid culture, reduce variability between organoids and reproduce brain regions systematically [59,108–110]. To clarify, increasing knowledge of the biochemical and biomechanical properties of organoid models that better reflect brain and inter-regional brain tissue can be incorporated into these models in the foreseeable future [109–111]. Personalised preclinical models can then be constructed to reflect the heterogeneity of both the cellular microenvironment and brain sub-regions. Precise characterisation of organoid models is already possible, as evidenced by cellular mapping of whole organoids by Quadrato et al. [109] and regionally by Bagley et al. [111]. To illustrate, not only did Quadrato et al. [109] show functional connectivity in brain organoids but also described how cell lines and batch to batch culture either increased or decreased organoid variability at a genetic, cellular and regional level. Ultimately, increased understanding of the cellular composition in both organoid and glioblastoma tumour microenvironments, and superior designs of scaffolds and hydrogels for optimal organoid growth [58,108,112], will surpass current modelling platforms.

Taking this technology further, combining 3D organoid models with CRISPR genetic engineering can effectively model glioblastoma initiation, growth and invasion in a microenvironment mimicking the human brain. Bian et al. [113] showed the development of what they refer to as a 'neoplastic cerebral organoid' (neoCOR), which can reproduce many elements of brain tumourigenesis, such as overgrowth, cell identity and invasiveness. An essential characteristic in the neoCOR is the mixture of structures, with the presence of normal and tumour tissue within the same culture, making this model an excellent way to test the influence of genome aberrations on tumour cell invasion. In a related study, Ogawa et al. [114] targeted the human TP53 locus to integrate a RAS expression cassette to block TP53 activity within established human brain organoids. The tumour cells that formed invaded throughout the brain organoid, and injection of organoid-derived tumour cells into mice generated tumours typical of glioblastoma, with a low rate of animal survival, cellular heterogeneity, necrotic areas and angiogenesis.

Sophisticated models such as these, complemented by next-generation (genome-wide tumour/normal exome and tumour RNA) sequencing [115], to offer personalised treatment in a clinically useful timeframe (<35 days) hold enormous potential for the treatment of glioblastoma (Figure 3), and especially of the complex interactions between tumour cells and their microenvironment [18,79,81,82,107,110]. Such models will also be valuable for the development of preclinical testing platforms for personalised medicine approaches. However, it is important to mention that brain organoid-glioblastoma models are under development and still limited by low to medium throughput as well as requiring a significant amount of time, equal to, or more than, the clinically relevant time frame, to develop (see also Oksdath et al. [58] for further discussion on this topic). Importantly, the incorporation of high-throughput and robotic platforms are expected to reduce organoid variability and are required to distinguish significant challenges associated with significant tumour and microenvironment heterogeneity. Moreover, key discrepancies between human brain tissue and these models are still present. One particular issue is the lack of vascularisation, although this may be overcome by transplanting organoids into the brains of mice [116]. *In vitro* approaches to vascularisation of organoids are also developing, with encouraging results observed for a single patient specimen demonstrating that induced pluripotent stem cell (iPSC)-derived endothelial cells can be incorporated into brain organoids as blood vessel-like structures [117]. The additional incorporation of further components into organoid models, including immune cells and BBB function (see, e.g., [118,119]), will remain a focus for ongoing studies.

Taken together, the development of patient-derived glioblastoma organoids shows significant promise for transformative preclinical models and pharmaceutical testing. These can be expedited by using high-throughput and robotic culture systems, effectively reducing batch variability. In conjunction with continuously developing

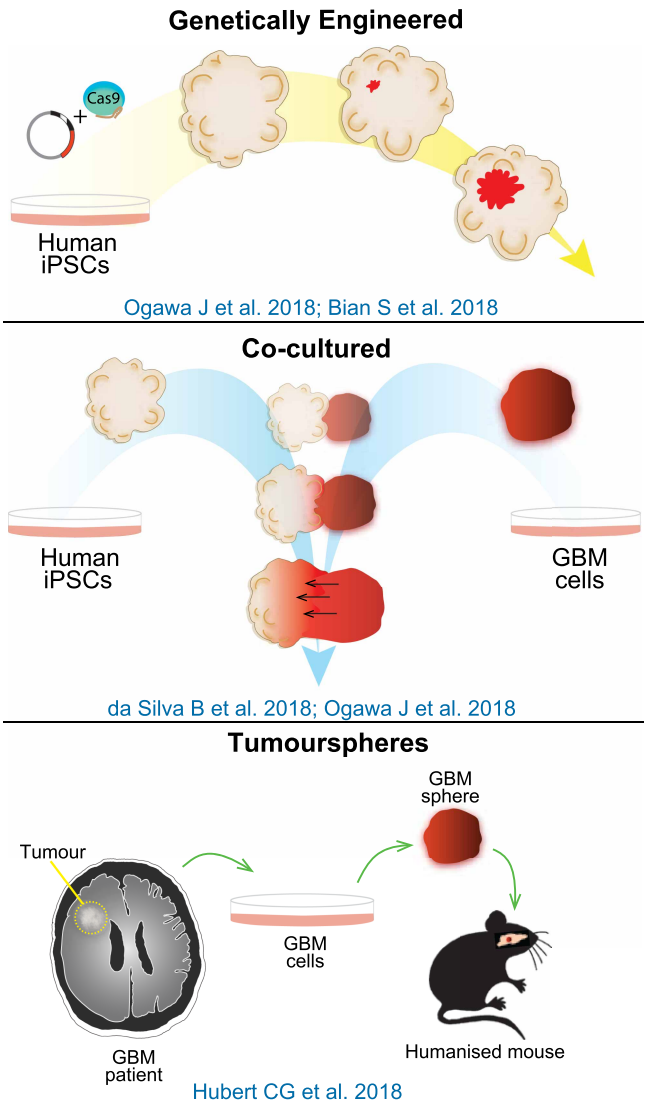

Figure 3. Human brain organoid models for glioblastoma studies.

Summary of the current 'brain organoid' strategies to study glioblastoma: Top, genetically engineered iPSC-derived brain organoids allow modelling of glioblastoma initiation, growth and invasion. An advantage of this system is the presence of healthy and tumour tissue within the same organoid, a condition that reflects the clinical context of the disease. Middle, Co-culture methods address tumour cell invasion in a short period but require the use of brain organoids at very early stages before they fully develop. Bottom, glioblastoma spheres (organoids) derived from human biopsies recapitulate many features of the disease, including regional heterogeneity and hypoxia gradients but lack the surrounding healthy brain tissue microenvironment, which could be partially mimicked by orthotopic xenotransplantation of organoids within the mouse brain.

biotechnologies such as synthetic scaffolds and various hydrogels, these approaches to study glioblastoma and its interaction with the surrounding healthy brain tissue microenvironment are tantalisingly imminent.

Perspectives

- Glioblastoma is a highly heterogeneous disease, not only because of the different signalling pathways that drive it but also differences in tumour location, phenotype and the cellular and non-cellular and metabolic tumour microenvironments. Current treatment options for glioblastoma extend survival rates to a median of only 15 months, with the last 30 years showing no significant progress in its treatment. Two key reasons

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for lack of progress in the treatment of glioblastoma are (i) extensive intra- and inter-tumour heterogeneity and (ii) the highly invasive and infiltrative nature of these tumours.

- Although much of the progress to date in developing new therapies for glioblastoma have been achieved in cell lines and animal models, these do not accurately reflect the actual heterogeneity present in the clinic, in particular, the relationship between the genetics of the of tumours and the complexity of their surrounding microenvironment. This has limited our capacity to understand (i) the origin of glioblastoma heterogeneity, (ii) what makes these tumours more aggressive and (iii) the underlying causes of resistance to therapies. Thus, research now aims to better characterise these factors and find new molecular targets and treatments for glioblastoma.
- The development and clinical validation of new preclinical models based on patient-derived samples that allow better reproduction of the complexity of the tumour in the patient will facilitate a more accurate assessment of whether patients will respond to a particular treatment. In this regard, organoid technologies are particularly promising and provide a clinically relevant time frame for personalised medicine approaches. Finally, as our knowledge grows in relation to the tumour microenvironment, and our capacity to better mimic this in preclinical *in vitro* models improves (i.e. using organ on a chip and high-throughput approaches with clinical validation), we anticipate the future discovery of novel therapeutic targets for glioblastoma treatment.

Abbreviations

ATP, adenosine triphosphate; BBB, blood–brain barrier; CNS, central nervous system; ECM, extracellular matrix; *EGFR*, epidermal growth factor receptor; GASCs, glioblastoma-associated stromal cells; GBM, glioblastoma multiforme; HA, hyaluronic acid; HAS, hyaluronan synthases; Hyal, hyaluronidases; *IDH*, isocitrate dehydrogenase; iPSC, induced pluripotent stem cell; MMP, matrix metalloproteinase; MSC, mesenchymal stromal/stem cells; MVP, microvascular proliferation; neoCOR, neoplastic cerebral organoid; NLGN3, neuroligin-3; OXPHOS, oxidative phosphorylation; PD1, programmed cell death 1; PD-L1, programmed death ligand 1; *PTEN*, phosphatase and tensin homologue; TAMs, tumour-associated macrophages/microglia; VEGF, vascular endothelial growth factor; WHO, World Health Organisation.

Author Contribution

S.L.P. and G.A.G. conceived the review with conceptual inputs from L.M.E. and M.O. S.L.P. contributed the first entire draft of the manuscript, which was further edited by S.L.P., L.M.E., M.O. and G.A.G with inputs from M.S. S., B.K. and M.P.B. M.O. contributed to figures with inputs from S.L.P. and G.A.G.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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