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REVIEW ARTICLE

The landscape of the mesenchymal signature in brain tumours

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The complexity of glioblastoma multiforme, the most common and lethal variant of gliomas, is reflected by cellular and molecular heterogeneity at both the inter- and intra-tumoural levels. Molecular subtyping has arisen in the past two decades as a promising strategy to give better predictions of glioblastoma multiforme evolution, common disease pathways, and rational treatment options. The Cancer Genome Atlas network initially identified four molecular subtypes of glioblastoma multiforme: proneural, neural, mesenchymal and classical. However, further studies, also investigated glioma stem cells, have only identified two to three subtypes: proneural, mesenchymal and classical. The proneural–mesenchymal transition upon tumour recurrence has been suggested as a mechanism of tumour resistance to radiation and chemotherapy treatment. Glioblastoma multiforme patients with the mesenchymal subtype tend to survive shorter than other subtypes when analysis is restricted to samples with low transcriptional heterogeneity. Although the mesenchymal signature in malignant glioma may seem at odds with the common idea of the ectodermal origin of neural-glial lineages, the presence of the mesenchymal signature in glioma is supported by several studies suggesting that it can result from: (i) intrinsic expression of tumour cells affected with accumulated genetic mutations and cell of origin; (ii) tumour micro-environments with recruited macrophages or microglia, mesenchymal stem cells or pericytes, and other progenitors; (iii) resistance to tumour treatment, including radiotherapy, antiangiogenic therapy and possibly chemotherapy. Genetic abnormalities, mainly *NF1* mutations, together with NF-κB transcriptional programs, are the main driver of acquiring mesenchymal-signature. This signature is far from being simply tissue artefacts, as it has been identified in single cell glioma, circulating tumour cells, and glioma stem cells that are released from the tumour micro-environment. All these together suggest that the mesenchymal signature in glioblastoma multiforme is induced and sustained via cell intrinsic mechanisms and tumour micro-environment factors. Although patients with the mesenchymal subtype tend to have poorer prognosis, they may have favourable response to immunotherapy and intensive radio- and chemotherapy.

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Abbreviations: CTC = circulating brain tumour cell; EMT = epithelial to mesenchymal transition; G-CIMP = glioma CpG island methylator phenotype; GBM = glioblastoma multiforme; GSC = glioma stem cell; MSC = mesenchymal stem cell; PMT = proneural to mesenchymal transition; TCGA = The Cancer Genome Atlas; TIL = tumour-infiltrating lymphocyte

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Glioma

Malignant primary brain tumours are the leading cause of death in children with cancers, and the third leading cause of death in adults aged 15–34 years (Surawicz *et al.*, 1999; Buckner *et al.*, 2007). Gliomas are tumours of the CNS that make up ~80% of all malignant brain tumours (Goodenberger and Jenkins, 2012). In the last century, the diagnosis of gliomas was based on microscopic analysis of histology features and the level of differentiation. The WHO grouped gliomas into four main groups (grades I–IV) depending on histological features (cellularity, mitotic figures, necrosis, and vascular proliferation) and astrocytic and oligodendroglial phenotype (Louis *et al.*, 2007). Astrocytoma grade IV, mostly known as glioblastoma multiforme (GBM), represents the most aggressive subtype of malignant brain tumour with a 12–16 month median overall survival (Stupp *et al.*, 2005; Wen and Kesari, 2008). However, this histological classification did not predict the clinical outcomes of gliomas well enough. Major revision was needed to update the century-old diagnostic principle by incorporating the microscopy analysis with the molecular parameters such as IDH status, ATRX loss, H3K27M mutation, TP53 mutation, and 1p/19q co-deletion (Bent, 2010; Louis *et al.*, 2016). The WHO revised classification of brain tumours (2016 CNS WHO) making some major changes. Diffuse astrocytic and oligodendroglial tumours are no longer separated at they were in the 2007 classification. Now, these tumours are grouped together according to their growth pattern and *IDH1*/*IDH2*-drived genetic mutations (Louis *et al.*, 2016).

Glioblastoma multiforme molecular subtypes

In 2006, Phillips *et al.* proposed subtyping of gliomas into three subtypes based on gene expression profiling: proneural, proliferative, and mesenchymal. They found a strong association between tumour grade and subtypes regardless of the oligodendroglial or astrocytic morphology (Phillips *et al.*, 2006). The Cancer Genome Atlas (TCGA) Research Network started a comprehensive genomic study targeting 33 different cancers. GBM and lower grade glioma were among their top targets. Their efforts resulted in deep genomic characterization and molecular subtypes of nearly 600 GBMs and 516 lower grade human gliomas (Wang *et al.*, 2017*b*).

The first TCGA pilot study identified new GBM genomic alterations such as homozygous deletions of *NF1* and *PARK2*, and amplifications of *AKT3*, in addition to previously known amplifications and deletions. GBM treated patients had higher mutation rates than untreated, ~5.8 versus 1.4 somatic silent mutations per sample, respectively. The *MGMT* promoter methylation, a predictive marker for alkylating agent treatment, induced a hypermutated GBM phenotype (Hegi *et al.*, 2005; Cancer Genome Atlas Research, 2008). Combining sequencing data with gene expression and DNA methylation identified the core biological pathways involved in GBM. RTK/RAS/PI3K was activated in 88% of the samples and p53-signalling was altered in 87% while RB signalling was altered in 78% of GBM samples (Cancer Genome Atlas Research, 2008). TCGA integrated gene expression data from three different platforms into a single unified dataset and identified 840 genes that classified GBM into four subtypes: proneural, mesenchymal, classical, and neural (Verhaak *et al.*, 2010). However, the human GBM tissues that were subcutaneously maintained and propagated in athymic null/null mice (Hodgson *et al.*, 2009), were classified into three subtypes only: proneural, mesenchymal, and classical (Verhaak *et al.*, 2010). The proneural subtype was associated with *IDH1* mutation, *TP53*, *PDGFRA* amplification and/or mutations (Noushmehr *et al.*, 2010; Verhaak *et al.*, 2010). The classical subtype showed the highest expression of *EGFR* amplification (95%) compared to other subclasses. Also, 95% of them exhibit *CDKN2A* (Ink4a/ARF) homozygous deletion. This class lacked *IDH1*, *TP53*, *PDGFRA* and *NF1* abnormalities that were common in the proneural and mesenchymal subtypes (Verhaak *et al.*, 2010). Interestingly, chr7 amplification and chr10 deletion, the most common GBM abnormalities, were low in the proneural subtype (20–54%), high in mesenchymal samples (>75%), and highest in the classical subtype of Verhaak *et al.* (2010) (93%). Phillips *et al.* (2006) and Verhaak *et al.* (2010) found that patients with the proneural subtype were younger than patients in other subtypes and tended to survive longer. However, Sturm *et al.* (2012) showed that the favourable outcome of the proneural GBM subtype was because patients were *IDH* mutant. When those patients are excluded from analysis, the proneural subtype has a worse prognosis than other subtypes (Sturm *et al.*, 2012). The proneural subtype was also different in terms of having normal *EGFR* expression, intact *PTEN*, *NOTCH* activation (Phillips *et al.*, 2006). Markers of oligodendrocytic development, *PDGFRA*, *NKX2–2*, and *OLIG2* were highly expressed in this subtype (Verhaak *et al.*, 2010). The mesenchymal subtype was associated with a high frequency of *NF1* abnormalities (Phillips *et al.*, 2006; Verhaak *et al.*, 2010), expressed high levels of *S100A1*, *CHI3L1*, *MET* and microglia markers *CD68*, *PTPRC*, and *TNF*, associated with inflammatory, wound healing, and NF-κB signalling pathways, in addition to a higher degree of necrosis (Phillips *et al.*, 2006; Verhaak *et al.*, 2010). Most secondary GBMs and >75% of low grade and grade III gliomas were classified as the proneural subtype (Phillips *et al.*, 2006; Verhaak *et al.*, 2010). Notably, comparing the Phillips classification with 35 genes, to the Verhaak classification with 840 genes, only three mesenchymal genes (*CHI3L1/YKL40*, *SERPINE1* and *TIMP1*) and five proneural genes (*DLL3*, *KLRC3*, *SCG3*, *C20orf42*, and *NCAM1*) were common (Phillips *et al.*, 2006; Verhaak *et al.*, 2010).

Using gene expression and DNA methylation profiles, Brennan *et al.* (2013) classified 396 GBMs into six methylation groups [clusters M1, M2, M3, M4, glioma CpG island methylator phenotype (G-CIMP), and M6]. The mesenchymal subtype was enriched in the M1 cluster (60%) and classical in the M3 cluster (58%), while the G-CIMP cluster contained mainly the proneural subtype and was associated with somatic mutations (*IDH1*, *TP53*, *ATRX*, *MYC*). The proneural subtype patients who were G-CIMP$^+$ were younger (41 versus 58 years of age) and survived longer than proneural patients who were G-CIMP−. All *IDH* mutant tumours were G-CIMP$^+$ (Brennan *et al.*, 2013). This in line with Turcan *et al.* who established the role of *IDH1*-mutation in inducing a distinct G-CIMP phenotype commonly seen in lower grade glioma and proneural subtype GBMs through altering the oncometabolite 2-hydroxyglutarate (2-HG) production and reorganizing the methylome and transcriptome landscape (Lu *et al.*, 2012*a*; Turcan *et al.*, 2012). Nevertheless, the G-CIMP$^+$ patients who lacked *IDH* mutations were not significantly different, in age and survival, from G-CIMP$^+$ patients who harboured *IDH* mutations, suggesting that their favourable survival in the proneural subtype is related to G-CIMP rather than *IDH* status. Although DNA methylation of the *MGMT* gene promotor (a gene that encodes O-6-methylguanine-DNA methyltransferase) has been associated with longer survival after temozolomide therapy in primary GBM, DNA methylation of this gene was correlated with a treatment response only in the classical subtype, but not proneural or mesenchymal subtypes (Hegi *et al.*, 2005; Brennan *et al.*, 2013). The TCGA proteomic profiling showed that *EGFR* amplification or mutation and phosphorylation were prominent in the classical subtype. In agreement with Phillips *et al.*, they confirmed that the mesenchymal subtype expressed higher levels of the endothelial markers CD31 and VEGFR2. Furthermore, inflammatory markers such as fibronectin and COX2 were elevated in mesenchymal subtype compared to other subtypes (Brennan *et al.*, 2013).

The last study published by TCGA in 2017 looked at the intrinsic gene signature of brain tumour subtypes, correlating GBM subtypes with the immune micro-environment, and shedding light on treatment-induced phenotypic tumour changes in recurrent tumours (Wang *et al.*, 2017*b*). This study excluded *IDH*-mutant GBMs for its favourable survival effect on the proneural subtype (Sturm *et al.*, 2012). Unsupervized clustering, after several steps of gene filtering to exclude environmental factors, resulted in three subtypes: proneural, mesenchymal, and classical, while neural signature was not enriched in any cluster (Wang *et al.*, 2017*b*). Indeed, the neural subtype was not detected by us and others, and could be due to contamination with normal cells (Sturm *et al.*, 2012; Gill *et al.*, 2014; Behnan *et al.*, 2017*a*). The final gene signature of the three subtypes consists of 150 genes (50 genes/subtype). Around 42–54% of the classification genes were shared with the old TCGA subtype of 840 genes (Verhaak *et al.*, 2010; Wang *et al.*, 2017*b*). The subtypes showed a high concordance through different platforms, up to 93% concordance between RNA-seq and Affymetrix-U133A and 85% in a small sample size of 10 GBMs selected randomly. The intra-tumoural transcriptional heterogeneity on the single cell level was captured by the matching tumour bulk signature. The tumour bulks had the same subtype as the majority of their single cells in four of five samples (Wang *et al.*, 2017*b*). These subtypes were associated with different immune signatures (Bao *et al.*, 2006; Engler *et al.*, 2012; Ye *et al.*, 2012; Gabrusiewicz *et al.*, 2016; Wang *et al.*, 2017*b*). When subtype stability was compared in 91 wild-type IDH-paired samples of primary and recurrent gliomas, 55% kept the same subtype upon recurrence. The mesenchymal subtype was the most stable subtype (with only 35% changed to another subtype), while the proneural subtype recorded highest subtype shifting (59%). The recurrent tumours showed a decrease in immune signature compared to their primary tumours. Both primary and recurrent tumours with mesenchymal subtype tended to have worse survival compared to proneural and classical subtypes upon restricting sample analysis to those with low transcriptional heterogeneity that activate one subtype (samples with high simplicity score) (Wang *et al.*, 2017*b*). Behnan *et al*'s classification set was obtained by filtering differentially expressed genes between glioma stem cells (GSCs) of proneural and mesenchymal subtypes that grew adherently under neuro-sphere conditions and crossing them with the TCGA gene set to get the genes shared between GSCs and GBM tissues. Of the 118 differentially expressed genes in GSCs, only 12 genes were common with the GBM-TCGA classification set of 840 genes. Surprisingly, these 12 genes were able to classify all TCGA samples in good concordance with the 840-gene-dependent classification. The 12 genes classified GBM into three subtypes: proneural, identified by *P2RX7*, *STMN4*, *SOX10* and *ERBB3*; classical, by *ACSBG1* and *KCNF1*; and mesenchymal, by *S100A*, *DAB2*, *TGFB1*, *THBS1*, *COL1A2* and *COL1A1* (Behnan *et al.*, 2017*a*). The relevance of this 12-gene classification also relies in its intrinsic signature, being identified in cancer stem cells and tumour tissues. However, despite several interesting findings of TCGA consortium in the glioma field, its clinical implications remain debatable (Box 1 presents the main outcomes and weaknesses of large-scale gene expression studies).

The mesenchymal signature in glioblastoma multiforme

About 30–49% of GBM tissues have been classified as the mesenchymal subtype. Patients with this subtype, both with primary and recurrent tumours, tend to have the worst survival rates compared to other subtypes. Despite this signature having been identified in GBM tissues at the beginning, it is far from being a tissue artefact, as single cell

Box 1 The main outcomes and impact of TCGA large-scale gene expression studies

Main outcomes of TCGA consortium in glioma	Impact and weaknesses
In general	
• TCGA offered a huge amount of publically-available data for >1000 glioma samples characterizing the genetic and epigenetic background of these tumours, performing pathway analysis and suggesting biomarkers for future therapy.	• The inability to map genetic and protein changes to single cells or distinct cell populations within the tumour (Singh *et al.*, 2012).
• Several other studies used TCGA data to identify potential target therapy and group of patients with better response to specific treatment.	
Key findings	
• Characterizing the inter-patient heterogeneity in GBM at the molecular level through subtyping GBM patients into three subtypes: proneural, mesenchymal, and classical, characterized with *PDGFRA*/*IDH* mutation, *NF1* mutation and EGFR amplification respectively (Verhaak *et al.*, 2010; Wang *et al.*, 2017*b*).	• Most of the samples were taken from one single random spot of the tumour bulk, assuming that GBMs are homogenous tumours, and recently it was shown that GBM is highly heterogeneous, and anatomical location affects the subtype.
	• The single cell analysis showed that a GBM sample contain different cell populations that belong to three different subtypes (Patel *et al.*, 2014).
	• Around 8% of GBM samples score for more than one subtype.
	• In Phillips et al., EGFR is marker for proliferation and mesenchymal subtypes.
	• There was no significant association between subtypes and longer survival among patients. The tendency of better survival was observed only by restricting the analysis to patients with lowest simplicity score that represent 20% of the analysed GBM samples (Wang *et al.*, 2017).
• Characterization of G-CIMP+ tumours with younger age at the time of diagnosis, better overall survival, more common in lower grade than GBM, and most patients with G-CIMP+ belong to the proneural subtype and are IDH mutant. Identifying the correlation between genetic mutations and MGMT+ in recurrent GBM after temozolomide treatment. The recurrent GBM in these patients that lost MGMT methylation, harboured much higher genetic alteration compared to untreated patients, which might contribute to the aggressive and therapeutic resistant nature of the tumour (Brennan *et al.*, 2013; Noushmehr *et al.*, 2010).	• Abnormal methylation of MGMT and its favourable clinical outcomes in GBM after temozolomide treatment was discovered in 2000 (Esteller *et al.*, 2000).
• In lower grade glioma, three subtypes that correlated with patient survival were identified: IDH-mutant with 1p/19q co-deletion (which have the most favourable survival), IDH-mutant without 1p19q deletion, and IDH wild-type (with worst survival that tends to be similar to GBM) (Cancer Genome Atlas Research *et al.*, 2015).	• The favourable clinical outcome of IDH-mutant was discovered by others (Parsons *et al.*, 2008; Jiao *et al.*, 2012).
	• Oligodendroglioma, which often harbour 1p/19q deletion were known to have a better response to radio- and chemotherapy and have better survival (van den Bent *et al.*, 2013; Cairncross *et al.*, 2014).
	• The MGMT-methylation status was not considered here and this might have affected the results.
• Confirmed that RB, p53, and RTK/RAS/PI3K pathways are activated in all GBM (Cancer Genome Atlas Research, 2008).	• These were already known oncogenic pathways (Furnari *et al.*, 2007).
• Identifying EGFR-TACC fusion in a small subset of GBMs (3/97, 3.1%) (Singh *et al.*, 2012).	• The clinical significance of this finding needs to be described.
• NF1-deficiency drives macrophage/microglia recruitment.	• The role of NF1-deficiency in recruiting macrophages/microglia was reported in other studies (Daginakatte and Gutmann, 2007; Rutledge *et al.*, 2013; Solga *et al.*, 2015).
• TCGA suggested a biological meaning of the subtypes by correlating the GBM subtypes to three cell types in the CNS. The proneural subtype enriched for the oligodendrocytic signature, classical enriched for astrocytic signature, and mesenchymal enriched for genes expressed in cultured astrocytes (Cahoy *et al.*, 2008; Verhaak *et al.*, 2010).	• The cultured astrocytes were mouse derived astrocytes (Cahoy *et al.*, 2008).
	• The gene expression profile of the mesenchymal subtype showed similarity to human MSCs derived from bone marrow, adipose, and brain (epileptic patient), which weakened the belief of the astrocytic origin of the mesenchymal subtype depending on its similarity to cultured mouse astrocytes. (Behnan *et al.*, 2017).
	• In Phillips *et al.* (2006) the mesenchymal subtype samples were enriched for genes expressed in bone, synovium tissue, smooth muscle, endothelial, and dendritic cells, as well as cultured human foetal astrocytes.
• Differential activation of immune microenvironment by different subtypes.	• Limited sample size of hypermutated primary and recurrent GBMs, five to seven samples hypermutated compared to 238 non-hypermutated.
• MES subtype has lowest purity and simplicity score indicating the heterogeneity and complexity of this subtype comparing to non- mesenchymal tumours (Wang *et al.*, 2017).	
• Hypermutated primary and recurrent GBMs after temozolomide treatment were associated with increased CD8+ T-cell infiltration, suggesting that these patients might benefit from checkpoint inhibitors (Wang *et al.*, 2017).	
• Aggressively treated patients demonstrated a survival benefit in the classical and mesenchymal subtypes, but not in the proneuralsubtype (Verhaak *et al.*, 2010).	• Preliminary data need to be validated in clinical settings.
• Frequent subtype switch upon tumour recurrence (45% in IDH-wild-type samples). The mesenchymal subtype seemed to be the most stable subtype (65%) upon recurrence (Wang *et al.*, 2017).	• The change of the subtypes upon tumour recurrence, shift toward the mesenchymal subtype, and induced a shift in experimental set-up have been reported previously (Phillips *et al.*, 2006; Bhat *et al.*, 2013; Halliday *et al.*, 2014). Although the mesenchymal subtype is the most common subtype in recurrent GBM, the proneural?mesenchymal shift was not significant (Wang *et al.*, 2017).

GBM, GSCs, and circulating brain tumour cells expressed the mesenchymal signature as well.

The mesenchymal signature in glioblastoma multiforme tissue

The mesenchymal signature in GBM tissue was suggested by Phillips *et al.* (2006), where 49% of the study cohort samples were classified as mesenchymal subtype and were associated with poor survival compared to the proneural subtype. Later, TCGA classified GBM tissues into four molecular subtypes: proneural, neural, mesenchymal and classical, where mesenchymal patients constituted 29–30% of GBM samples in both the primary and validation set (200 and 246 GBMs, respectively) (Verhaak *et al.*, 2010). The mesenchymal subtype is one of the most consistent subtypes described in the literature, in both GBM tissues and culture-enriched GSCs (Verhaak *et al.*, 2010; Bhat *et al.*, 2013; Behnan *et al.*, 2017*a*; Wang *et al.*, 2017*b*). Upon integrating micro-environmental factors and using single cell analysis, the new TCGA classification confirmed the subtype segregation into three subtypes rather than four, with mesenchymal subtype constituting ~34% of the samples (Wang *et al.*, 2017*b*). Furthermore, these three subtypes were relevant to subtype even lower grade gliomas (Guan *et al.*, 2014; Ceccarelli *et al.*, 2016). The mesenchymal subtype samples express mesenchymal markers and are negative/downregulate proneural markers (Supplementary Table 1) (Phillips *et al.*, 2006; Verhaak *et al.*, 2010; Riddick and Fine, 2011; Bhat *et al.*, 2013; Jin *et al.*, 2017). However, some mesenchymal tumours express the proneural marker, *OLIG2*, and others do not express the mesenchymal marker, *CHI3L1* (Bhat *et al.*, 2013). Mesenchymal tumours are predominantly IDH wild-type and G-CIMP−, contrary to the proneural subtype (Noushmehr *et al.*, 2010; Verhaak *et al.*, 2010). GBMs that express high mesenchymal signature, even when the analysis was limited to wild-type IDH, were associated with poor radiation response and worse survival (Bhat *et al.*, 2013). Mesenchymal tumours expressed higher levels of angiogenic markers, in addition to higher levels of necrosis (Phillips *et al.*, 2006; Verhaak *et al.*, 2010). Genetic abnormalities and tumour micro-environment have been shown to drive mesenchymal subtype tumours (Verhaak *et al.*, 2010; Wang *et al.*, 2017*b*). Furthermore, the epigenetic chromatin modifiers *EZH2* and *BMI1* were shown to have differential expression between the mesenchymal and proneural subtypes, keeping in mind that 40% of GBMs harbour mutations in *BMI1* and *EZH2* (Brennan *et al.*, 2013; Jin *et al.*, 2017). The proneural subtype tumours expressed a higher level of *EZH2* compared to mesenchymal and classical subtypes. This expression was positively correlated with *OLIG2* and mature vascular signature expression (proneural marker). Mesenchymal tumours expressed a higher level of *BMI1* and its expression was positively correlated with *CD44* expression and micro-environment in mesenchymal and classical subtypes (Jin *et al.*, 2017). Correlating gene expression with the survival data showed that GBM patients with low *EZH2* and *BMI1* expression have the best survival, while those with upregulated expression of both genes have the worst survival, and those who express either *EZH2* or *BMI1* have intermediate survival (Jin *et al.*, 2017). This indicates that patients with more heterogeneous tumours have unfavourable survival. Also, recently it was shown that *ZBTB18* promotor methylation, which is strongly correlated with wild-type *IDH1*, acts as a negative regulator of the mesenchymal subtype, and *ZBTB18* loss is associated with poor prognosis and an aggressive tumour phenotype (Fedele *et al.*, 2017).

The mesenchymal signature in glioma stem cells

GSCs or glioma initiating cells, were first reported in 2003 (Singh *et al.*, 2003). GSCs are known as a small population of stem cells that exist in fresh tumours and are characterized by self-renewal and expression of stemness markers and multi-lineage differentiation properties that give rise to other types of cells in the tumour, in addition to *in vivo* tumour formation ability (Lathia *et al.*, 2015). While the origin and clear definition of GSCs are disputable, it is well established that these cells are responsible for maintaining tumour growth and invasion, and cause tumour recurrence because of their chemo- and radio-resistant properties (Bao *et al.*, 2006; Liu *et al.*, 2006; Chen *et al.*, 2012). Vascular niche in addition to other micro-environmental factors, such as hypoxia, nutrients, and acid, affect the distribution of these cells (Bao *et al.*, 2006; Calabrese *et al.*, 2007; Li *et al.*, 2009; Hjelmeland *et al.*, 2011; Flavahan *et al.*, 2013). Chemo- and radiotherapy are thought to enrich for GSCs (Bao *et al.*, 2006; Liu *et al.*, 2006). Because of the heterogeneity among patient tumours and within the same tumour, there is no unique marker that can distinguish GSCs from non-GSCs. We have recently suggested a panel of markers that distinguish GSCs from non-tumorigenic stromal cells and cover inter-tumoural heterogeneity: $CD56^+SOX2^+SOX9^+CD133^+CD15^+CD248^-CD105^-\alpha SMA^-$ (Behnan *et al.*, 2017*a*). Molecular subtyping has shown that GSCs have two to three subtypes, proneural, mesenchymal and classical, while the neural subtype is absent in GSCs. In fact, only normal oligodendrocytes were classified as neural (Huse *et al.*, 2011; Zheng *et al.*, 2012; Bhat *et al.*, 2013; Patel *et al.*, 2014; Behnan *et al.*, 2017*a*). Also, it was found that GSCs tend to express the transcriptional subtype of the dominant cell population in the parental fresh tumour (Joo *et al.*, 2013; Mao *et al.*, 2013). Furthermore, GSCs expressed proneural to mesenchymal transition (PMT) properties in response to chemo- and radiotherapy (Bhat *et al.*, 2013; Halliday *et al.*, 2014). GSCs subtype-specific markers have been suggested by several investigators

(Lottaz *et al.*, 2010; Bhat *et al.*, 2013; Behnan *et al.*, 2017*a*). The mesenchymal subtype GSCs were mainly negative for CD133 and highly positive for CD44, YKL40, and BMI1, while the proneural subtype GSCs were positive for CD133, OLIG2, SOX2 and EZH2 (Bhat *et al.*, 2013; Jin *et al.*, 2017). Lottaz *et al.* (2010) reported the subtyping of 17 GSCs into two groups: group I, which expresses the proneural signature, is $CD133^+$, while group II expresses the mesenchymal signature and is $CD133^-$ (Lottaz *et al.*, 2010). GSCs of the mesenchymal subtype expressed *ALDH1A3*, had aggressive *in vitro* and *in vivo* tumour phenotypes, and were more resistant to radiation treatment than the proneural subtype. Furthermore, the radiation treatment of proneural subtype induced PMT, while *ALDH1A3* inhibition blocked this process (Mao *et al.*, 2013). Cusulin *et al.* (2015) classified 20 GSCs according to their biological features including sphere formation, marker expression, asymmetric cell division, and label retention. They were divided into two groups: (i) a stem-like subtype that expressed the proneural signature and was associated with longer *in vivo* survival after transplantation; and (ii) a progenitor-like subtype that expressed mesenchymal-signature and was associated with shorter *in vivo* survival (Cusulin *et al.*, 2015). Bhat *et al.* (2013) subtyped 14 GSCs into two clusters. Cluster I enriched for the mesenchymal signature with ontology enrichment of genes that play a role in wound response, vasculature formation, and cell motility. Cluster 2 enriched for the proneural signature with ontology enrichment of genes that play a role in neural and glial function and homeostatic activity (Bhat *et al.*, 2013). Our subtyping also showed that the mesenchymal subtype upregulated genes related to epithelial to mesenchymal transition (EMT) such as *TWIST1*, *SNAI1*, *SNAI2*, *TGFB1*, *STAT3*, and *CD248*. Also, the *in vitro* and *in vivo* growth pattern and morphology seemed different in proneural and mesenchymal subtypes, where the mesenchymal subtype contained several GSCs that grow adherently in sphere culture (a serum and adherent free condition) (Behnan *et al.*, 2017*a*). Furthermore, GSCs with proneural and mesenchymal subtypes were found to have different distribution within the tumour. Proneural subtype GSCs were $SOX2^+OLIG2^+$ and were perivascular localized, whereas mesenchymal subtype GSCs were $CD44^+YKL40^+$ and were exclusively localized to the necrotic hypoxic regions (Jin *et al.*, 2017). Mimicking the necrotic region milieu through *in vitro* stress conditions such as hypoxia and nutrient deprivation revealed that mesenchymal subtype GSCs survived the stress, upregulated mesenchymal markers and maintained *BMI1* expression, while proneural subtype GSCs and normal neural precursors lost *BMI1* and *EZH2* expression and eventually died. Also, GSCs from proneural and mesenchymal subtypes responded differentially to BMI1 and EZH2 inhibitors (Jin *et al.*, 2017). Further, the epigenetic signature of mesenchymal and proneural subtype GSCs were different from their parental tumour tissue (Bhat *et al.*, 2013). Contrary to their parental tumour, proneural subtype GSCs became hypermethylated *in vitro* ($G\text{-}CIMP^+$) even in the absence of *IDH* mutation. However, mesenchymal subtype GSCs maintained the $G\text{-}CIMP^-$ profile and some mesenchymal tumours switched to the proneural subtype upon *in vivo* transplantation (Bhat *et al.*, 2013). This does not sound surprising as the selective pressure of cell culture conditions and growth factor supplements favour the enrichment of GSCs with the proneural subtype (Bhat *et al.*, 2013). Also, this phenomenon can be explained through the newly acquired understanding of intra-tumoural subtype heterogeneity generated by single cell analysis and multi-sampling techniques collected from different anatomical locations in the same patient (Patel *et al.*, 2014; Puchalski *et al.*, 2018). We expect that the subtype with the most proliferative GSCs that take best advantage of culture conditions, will dominate the cell culture, and other cell subtypes will gradually disappear.

The mesenchymal signature in single cell glioblastoma multiforme

Although the TCGA subtype approach has been useful to characterize the molecular diversity of tumour bulk among GBM patients (inter-patient heterogeneity), it has limited insight into the intra-tumoural heterogeneity within individual GBM patients (Cancer Genome Atlas Research, 2008; Verhaak *et al.*, 2010). Patel *et al.* performed the first study that investigated the DNA and RNA molecular diversity of neoplastic cells within individual GBM patients at the single cell resolution level, keeping in mind that both single cells and bulk were depleted of $CD45^+$ cells (Patel *et al.*, 2014). Using RNA-seq (96–192 single cells per patient from five GBM wild-type *IDH* patients) and comparing single cells to bulk tumours and their derived cultures, showed a broad correlation between neoplastic cells from single GBM biopsies (0.2–0.7 correlation coefficient) indicating intra-tumoural heterogeneity and that cells were more correlated to each other than were cells from different patients. Also, intra-tumoural heterogeneity was indicated by mosaic expression of RTK and their signalling molecules within the same tumour and across different patients. Interestingly, individual cells of single tumours have heterogeneous subtype expression. Although three of five tumour bulks were the mesenchymal subtype, all tumours contained a small number of cells from the proneural subtype and very few cells from the neural or classical subtypes. As the mesenchymal subtype was the dominant subtype in these tumour cells, this led to tumour bulks to be scored as mesenchymal subtype only. Importantly, the increased intra-tumoural subtype heterogeneity was associated with worse survival only in proneural subtype patients (Patel *et al.*, 2014). However, the most well-known genetic alterations of GBM, gain of chr7 and loss of chr10, were expressed in all single cells from different patients (Patel *et al.*, 2014; Darmanis *et al.*, 2017). Although neuro-sphere culture conditions are thought to enrich the culture for cells that mirror the

parental tumour cells (Lee *et al.*, 2006), cell diversity was highly reduced in cultured cells compared to parental tumour cells. Furthermore, only three subtypes were expressed *in vitro*, and none scored for the neural subtype (Patel *et al.*, 2014). Darmanis *et al.* (2017) profiled invasive glioma cells isolated from peri-tumoural areas at the single cell level and compared them to those of from the tumour core, in addition to profiling a variety of other normal cells within individual GBM patients. The single cell transcriptome analysis of 3589 cells from four paired samples of tumour cores and peri-tumoural samples revealed a large degree of diversity between cells within the same tumour and between different tumours. Their classification resulted in 12 clusters, only 3 of 12 were neoplastic cells, 3 of 12 contained vascular cells, 3 of 12 contained immune cells, one cluster were oligodendrocyte precursor cells, one cluster were oligodendrocytes, and one cluster were astrocytes. Notably, intra-tumoural subtype heterogeneity was seen in the three neoplastic clusters: neoplastic clusters 1 and 2 consisted of proneural and classical cells, while neoplastic cluster 3 consisted of mesenchymal and classical cells (Darmanis *et al.*, 2017). All this information together suggests that despite the intra-tumoural subtype heterogeneity, the mesenchymal subtype exists on a single cell transcriptomic level in neoplastic cells. This indicates that this signature is induced by an intrinsic driver of the mesenchymal signature within these cells rather than by environmental factors.

The mesenchymal signature in circulating glioblastoma multiforme cells

Despite the aggressive nature and highly invasive cells of GBM, this tumour rarely develops extracranial metastasis, with an incidence of $<0.4\%$ (Smith *et al.*, 1969). This disease property, supported by the seed-soil hypothesis, made it unreasonable to question the existence of circulating brain tumour cells (CTCs) in GBM until recently (Sullivan *et al.*, 2014; Gao *et al.*, 2016). CTCs were identified in blood samples of GBM patients and a patient-derived xenograft GBM mouse model. CTCs were isolated using a microfluidic platform (CTC-iChip). These assays deplete blood cells and excessive magnetic beads, then sort CTC cells positive for SOX2, TUBB3, EGFR, A2B5, and c-MET. Thirty-nine per cent of GBM patients scored positively for CTCs (13 of 33 GBM patients). Patients with progressive GBM had a median of 11.8 cells/ml while patients with stable GBM had 2.1 cells/ml. Although CTCs expressed a similar pattern of *EFGR* amplification as tumour cells from the tumour core biopsy, the CTCs were less proliferative. To compare the molecular subtype of CTCs to tumour core cells, a 25-gene panel was used: *ASCL1*, *SOX2*, *OLIG2*, and *DLL3* for proneural subtype; *GFAP*, *AKT2*, and *EGFR* for classical; *SYT1* and SLC12A5 for neural; *SERPINE1*, *TGFB1*, and *RELB* for mesenchymal subtype, in addition to other embryonic stem cells and self-renewing markers (Sullivan *et al.*, 2014). Comparing the single cell analysis of CTCs with their parental tumours showed that all patient-derived CTCs and xenograft-derived CTCs expressed an elevated mesenchymal transcriptional profile (of *SERPINE1*, *TGFB1*, *TGFBR2*, and *VIM*), and downregulated neural and oligodendrocyte markers (*ASCL1*, *GFAP*, *NCAM1*, and *SOX9*). However, CTCs that were positive for *PROM1/CD133* (a proneural marker) also retained expression of this stem cell marker transcriptionally, similar to parental tumours. Cells with similar CTC mesenchymal signature were detected mainly around the necrotic foci and palisading cells in GBM tissues. A GBM patient with recurrent brain lesions and rare condition of extracranial metastases to the pulmonary nodules and hilar lymphadenopathy, had high CTCs after 12 months of initial diagnosis (48 cells/ml) that were similar to his primary brain GBM regarding *EGFR* amplification. Although his recurrent brain tumours had cells from different subtypes (neural: 19.0%, neural/mesenchymal: 65.3%, and mesenchymal: 15.7%), his metastatic lesions were mainly the mesenchymal subtype ($\sim$62% and $\sim$54% of the cells of the metastatic left hilar lymph node and the pulmonary metastases, respectively, were mesenchymal). Furthermore, *PDGFRB* mutation increased from 3.5% in primary GBM to 50% in all metastatic lesions. Also, the metastatic lesions acquired mutations in *EGFR*, *RB1*, and *SETD2* that were not detected in the primary tumour (Sullivan *et al.*, 2014).

The shift towards mesenchymal subtype upon glioma recurrence

High grade gliomas, especially GBMs, tend almost always to recur, often at the site of the initial tumour, but sometimes recur away from this site or even in the other hemisphere (Wen and Kesari, 2008; Kim *et al.*, 2015). The subtype of a primary and recurrent tumour might be different. In total, two-thirds of primary GBMs switch transcriptional subtypes at tumour recurrence, while secondary GBM seems more stable (2/7 switched subtype) (Wang *et al.*, 2016). In another study, 45% of wild-type IDH tumours switched subtype upon recurrence (Wang *et al.*, 2017*b*). Phillips and colleagues reported that $\sim$30% of proneural tumours shifted to mesenchymal-signature upon recurrence. This shift appears to reflect tumour progression and was marked by losing *OLIG2* expression and the upregulation of *YKL40* expression. Furthermore, these tumours upregulated *CD44*, *STAT3*, and vimentin (*VIM*) expression upon recurrence (Phillips *et al.*, 2006). It is thought that the unidirectional shift proneural$\rightarrow$mesenchymal might happen through accumulating genetic and epigenetic abnormalities with the time. This hypothesis is supported by the fact that mesenchymal subtype patients are older than proneural subtype patients. It is also supported by another hypothesis that suggests the

proneural subtype as an origin for all subtypes (Phillips *et al.*, 2006; Ozawa *et al.*, 2014). *In vitro* proneural→mesenchymal reprogramming of GSCs and neural stem cells was achieved by overexpression of master transcription factors such as *TAZ* or *STAT3* and *CEBPB* (Carro *et al.*, 2010; Bhat *et al.*, 2011). Later, it was found that these master transcription factors of mesenchymal transition were under the control of NF-κB (Bhat *et al.*, 2013). Furthermore, *TAZ* overexpression in the proneural mouse model of *RCAS-PDGFB* transformed the tumours into more aggressive mesenchymal-like tumours (Bhat *et al.*, 2011). Bhat *et al.* suggested that the mechanism of PMT upon radiation treatment was through NF-κB activation. Macrophages and microglia were suggested as the potential driver of this transition *in vivo*. Also, when TNF-α treatment induced mesenchymal differentiation in proneural subtype GSCs and was followed by radiation treatment, it resulted in the development of radioresistant GSCs with stronger mesenchymal signature. However, some proneural subtype GSCs were resistant to TNF-α treatment and retained the proneural signature, indicating that not all proneural cultures have a similar response (Bhat *et al.*, 2013). The PMT could be induced in proneural mouse model (*PDGFA/shP53*) and GBM cell lines with *PDGFRA* amplification through subsequent knockdown of *NF1*. The *NF1* deletion resulted in significantly shorter survival in the triple infected mice with mesenchymal-like subtype compared to the double infected proneural-like subtype. This PMT was converted by mTOR inhibitor rapamycin *in vitro* (Ozawa *et al.*, 2014).

Although later studies showed that the mesenchymal subtype was the most stable subtype (55–65% kept the same subtype), the shift towards the mesenchymal subtype was not significant upon tumour recurrence with some tumours shifted toward proneural and others toward classical (Verhaak *et al.*, 2010; Wang *et al.*, 2016, 2017*b*). Interestingly, the mesenchymal subtype at tumour recurrence tended to have a worse overall survival (Wang *et al.*, 2016). However, the frequency of mesenchymal and proneural subtypes was highest among recurrent tumours, confirming previous findings about classical being more sensitive to treatment (Wang *et al.*, 2017*b*). Also, the loss of *EGFR* expression in ∼80% of tumours upon recurrence supports the loss of the classical signature upon recurrence (van den Bent *et al.*, 2015). Another study showed that the loss of *EGFRvIII* was associated with the transition from classical to other subtypes (Wang *et al.*, 2016). Additionally, the increased transcriptional heterogeneity was associated with a higher subtype switch upon tumour recurrence, suggesting an important role of tumour heterogeneity in this phenomenon (Wang *et al.*, 2017*b*). Using multi-cancer computational analysis, 64 genes were identified as a signature of mesenchymal transition. These genes were markers of aggressive and invasive stage tumour expressed in several solid tumours, including neuroblastoma and glioma (Kim *et al.*, 2010; Cheng *et al.*, 2012). The low expression of the 64 mesenchymal metagene signature was associated with preferable survival and longer time to recurrence in GBM with an extremely low expression level in GBM patients with exceptionally long time to recurrence. Seven of the 64 genes (*POSTN*, *COLA1A1*, *COLA3A1*, *COLA1A2*, *LOX*, *COLA6A2*, and *LUM*) were among the top differentially expressed genes in GBM versus low grade gliomas. The metagene was strongly associated with *CD44* expression (Cheng *et al.*, 2012).

The tumour evolution theory in response to treatment, adapted a Darwinian model to explain the clonal shift in recurrent tumour after treatment. This theory suggests that the tumorigenic clones that are vulnerable to treatment will disappear, while positive selection will favour resistant clones to expand and dominate the recurrent tumour (Nordling, 1953; Nowell, 1976). Despite having >45% of the mutation pool shared between primary and recurrent tumours, the dominant clones at recurrence are not lineal ancestors of the dominant clone at first disease diagnosis. Rather both types of clones seem to have been branched from a common ancestor a decade or more before diagnosis. The whole-exome and transcriptome analyses of untreated and recurrent GBM from 114 patients, in addition to their matched normal tissue, found some genetic markers and tumour evolutionary trajectories in recurrent tumours. The primary tumours had 60 mutations on average, whereas the hypermutated recurrent tumours (six primary GBM and 11 secondary GBM) were all temozolomide-treated and harboured >500 mutant genes per tumour, whereas none of temozolomide-untreated tumours were hypermutants (0/14). The mutational average of non-hypermutant tumours was <50 mutations/tumour. Also, hypermutated tumours were associated with *MGMT* promoter methylation (Wang *et al.*, 2016). The tumour evolution mutational dynamic seems to have a similar alteration rate for a given time, for both primary and recurrent tumours after treatment (∼0.03 substitutions/megabase/year), except for those with a hypermutated profile. The replacement of dominant clones before treatment with new clones at relapse is a frequent event in GBM. The main GBM-driver mutations including *TP53*, *PTEN*, *EGFR*, *PIK3CA*, *ATRX*, *IDH1*, *PIK3R1*, and *PDGFRA* were shared between both primary and recurrent tumours. Interestingly, *EGFR*, *EGFRvIII*, *TP53*, *PDGFRA*, *PTEN*, *ATRX*, *NF1*, and *RB1* seem to express differentially mutated versions of the same gene, indicating their important roles in both primary and recurrent tumours. Also, significant associations were observed between co-deletion of *RB1* and *PTEN*, co-mutation of *NF1* and *TP53*, and *MGMT* promoter methylation and hypermutation (Wang *et al.*, 2016).

Origin and role of the mesenchymal signature in brain tumours

From neurosurgical and neuropathological point of view, gliomas are thought to arise from glial cells, and preliminary

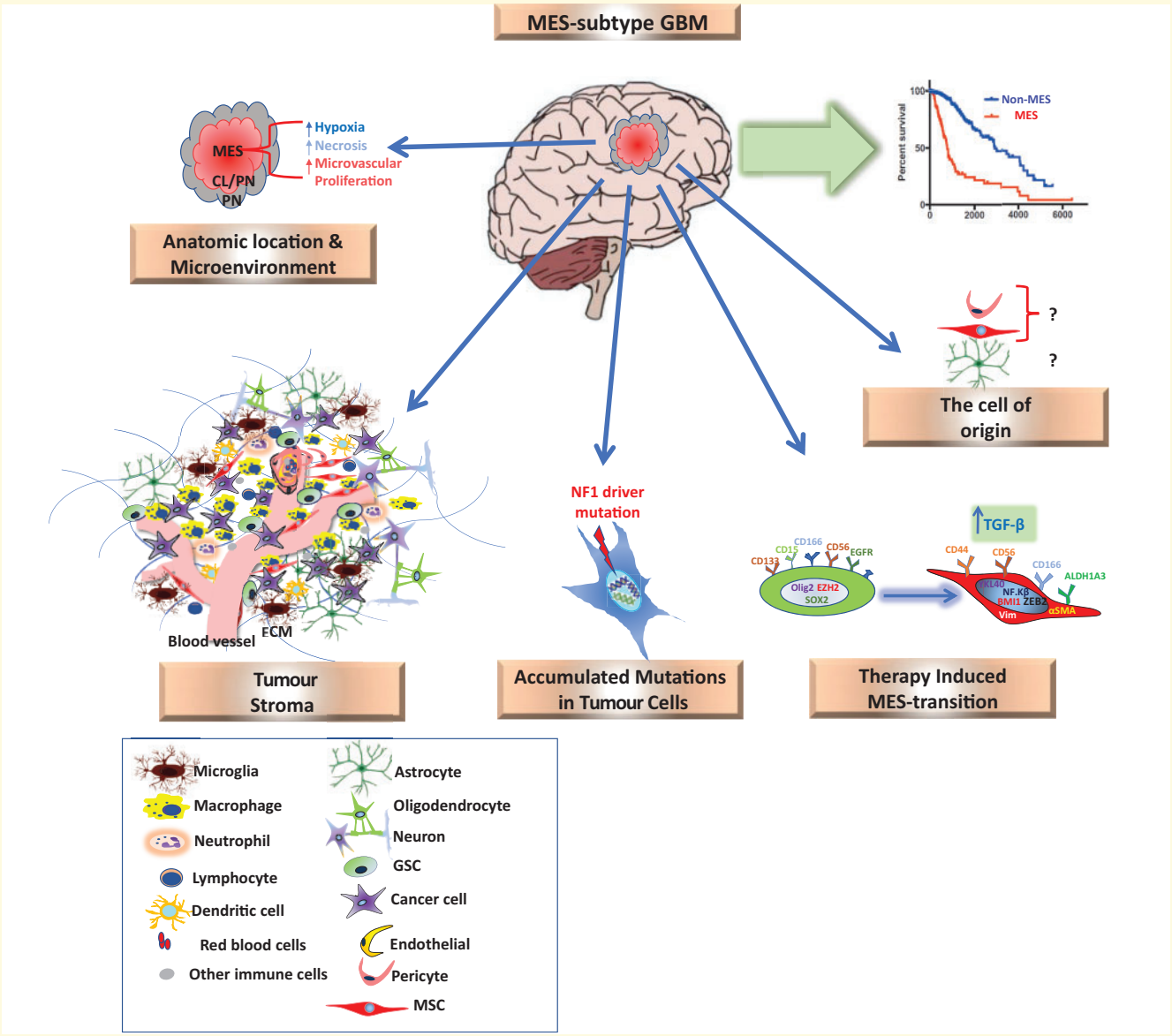

Figure 1 The origin of the mesenchymal signature in malignant glioma. GBM patients with the mesenchymal (MES) subtype tend to have worse survival than non-mesenchymal subtype patients. The mesenchymal signature in glioma can be induced by several factors: (i) stromal cells of recruited macrophages/microglia, MSCs/pericytes, and other progenitors; (ii) intrinsic expression of tumour cells, *NF1* as main driver mutation; (iii) the cell of origin; (iv) anatomical location and tumour micorenviroments; and (v) therapy-induced mesenchymal-signature. Radiotherapy, antiangiogenic therapy and chemotherapy might induce the mesenchymal signature. ECM = extracellular matrix.

understanding of embryonic development suggests that the brain and spinal cord are developed from the ectoderm. These concepts made researchers hesitant to address the mesenchymal components in malignant brain tumours until recently. So, what is the origin of mesenchymal signature in brain tumours and what is its role? (Fig. 1 illustrates the factors that induce the mesenchymal signature in GBM).

Mesenchymal stem cells/pericytes and other cell progenitors in glioma

The current understanding of brain tumour biology suggests that the tumour stroma of non-neoplastic origin make up ~50% of GBM tumour mass (Darmanis *et al.*, 2017). Several stromal factors including vasculature cells such as endothelial cells, perictyes/mesenchymal stem cell (MSCs), immune cells, and glial cells are recruited into and play a role in tumour formation and progression (Fig. 2) (Charles *et al.*, 2011).

It is thought that the expression of mesenchymal lineage markers of smooth muscle, endothelial cells, and cartilage by mesenchymal subtype brain tumours is related to the reported multipotentiality of NSCs from adult brain (Bani-Yaghoub *et al.*, 2004; Phillips *et al.*, 2006). NSCs are suggested as the common cell of origin for brain tumours and tumour subtypes could be driven by the plasticity and multi-

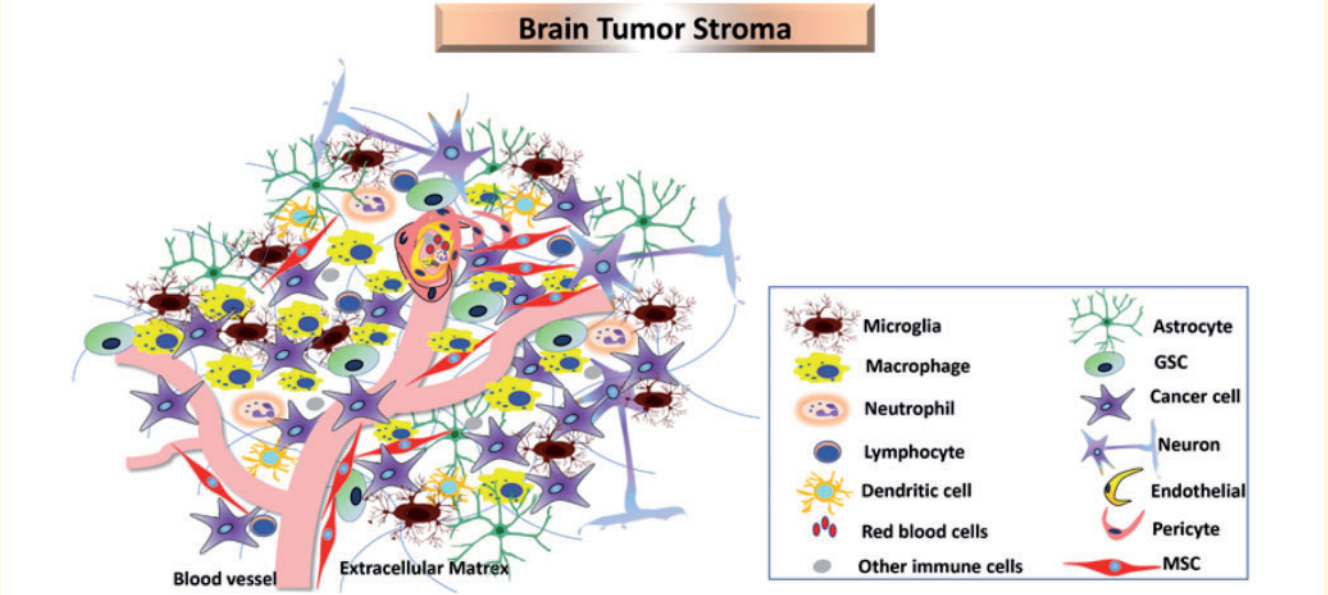

Figure 2 Cellular heterogeneity in brain tumours. GBM consists of heterogeneous cell populations including cancer cells, GSCs, macrophages, microglia, neutrophils, lymphocytes, dendritic cells, red blood cells, astrocytes, neurons, endothelial cells, pericytes and MSCs.

differentiation pathways of these cells (Galli *et al.*, 2004; Verhaak *et al.*, 2010). Another source for the mesenchymal signature in glioma could be the recruited stromal cells including endogenous MSCs and other stem cell-like populations that accumulate in the tumour during disease progression (Behnan *et al.*, 2014). It was also speculated that the growth of mesenchymal subtype tumours might be supported by the increased level of VEGF and other endothelial derived factors in brain tumours (Jin *et al.*, 2002; Phillips *et al.*, 2006). It is thought that tumour-associated stromal cells might be one of the main drivers in acquiring the mesenchymal signature (Behnan *et al.*, 2017*a*; Wang *et al.*, 2017*b*). This has been seen to be the case in colorectal cancer, but no definitive proof of these phenomena yet exists in glioma (Isella *et al.*, 2015). However, the stromal signature has been correlated to reduced tumour purity and is associated with the mesenchymal signature in different solid tumours including GBM (Martinez *et al.*, 2015). We have previously reported that endogenous MSCs are recruited into glioma and contribute to tumour progression in the GL261 glioma model, which was defined as mesenchymal-like subtype. Furthermore, MSC-like cells were identified in both fresh human GBM tissues and their derived cell cultures (Behnan *et al.*, 2014). Svensson *et al.* (2015) using the same GL261 model, confirmed that cells expressing pericyte markers infiltrated into the tumour and mainly localized around the blood vessels (Svensson *et al.*, 2015). However, perivascular localization of MSCs was described in several tissues indicating an MSC/pericyte dualism (Crisan *et al.*, 2008). So far, there is no single unique marker that characterizes MSCs or pericytes, which makes it difficult to distinguish between them. Furthermore, perivascular MSCs which were isolated from different tissue types and expanded in culture showed similarity to each other and to bone marrow MSCs more than to their original tissue. This suggests a common developmental origin for the perivascular MSCs (Crisan *et al.*, 2008).

The question presented here is that if the tumour stroma and recruited cells are behind the acquisition of the mesenchymal signature in brain tumours, how do the tumour cells *per se*, even at a single cell level, express the mesenchymal signature? And how do propagated GSCs under neurosphere culture conditions express the mesenchymal signature, keeping in mind that such culture conditions do not favour the expansion of stromal cell components. Additionally, macrophages/microglia markers (*ITGAM* and *AIF1*) that were highly expressed in GBM tissues were absent in GSCs (Behnan *et al.*, 2017*a*; Wang *et al.*, 2017*b*). One proposed explanation is that the tumour cells acquire the mesenchymal signature in response to radiotherapy (Bhat *et al.*, 2013). Bhat *et al.* showed that cultured GSCs with the proneural subtype acquired the mesenchymal signature through activation of the NF-κB pathway. However, the general practice treatment of GBM is surgery followed by radio- and chemotherapy. Thus, most tissue samples in the subtype's dataset of primary GBM are left over from biopsies dissected before being exposed to radio- or chemotherapy stress. This means that the mesenchymal signature was already present before the either radio- or chemotherapy stress.

The mesenchymal subtype can be driven by genetic mutation

The main known driver mutation of mesenchymal subtype is *NF1* deletion or mutation (Bhat *et al.*, 2013; Herting *et al.*, 2017; Wang *et al.*, 2017*b*). NF1 is best known to also be

expressed in WHO grade I pilocytic astrocytoma. These patients have a higher risk of progression to higher grade infiltrating gliomas (Rodriguez *et al.*, 2008). *NF1* encodes for neurofibromin protein, which plays a role as a GTPase activating protein that negatively regulates Ras and Ras-associated downstream signalling pathways (Ratner and Miller, 2015). However, *NF1* deletion or mutation was detected in 24–37% of mesenchymal samples in GBM (Bhat *et al.*, 2013; Herting *et al.*, 2017; Wang *et al.*, 2017*b*). Also, GSCs that had normal *NF1* expression, released from the influence of macrophage/microglia micro-environment, maintained the mesenchymal expression profile indicating that these cells have an intrinsic and *NF1*-independent mesenchymal signature (Bhat *et al.*, 2013). This means that approximately two-thirds of mesenchymal subtype samples cannot be explained by *NF1* abnormalities. Our subtyping with 12 genes showed that mesenchymal subtype samples were separated into two clusters. Cluster I was named tissue-mesenchymal and was characterized by overexpression of *COL1A1*, *COL1A2* and *THBS1*, which are known as matrix and connective tissue factors. Cluster II was named cell-mesenchymal. GBM tissues in this second cluster showed similarity to cultured MSCs derived from bone marrow, adipose tissue, and brain, and expressed high levels of *TGFBI*, *S100A4*, and *DAB2* (Behnan *et al.*, 2017*a*). Recently, single cell analysis of immune cells in GBM showed that *TGFBI* and other *S100* family members including *S100A8/9* (macrophages markers) were highly expressed in immune cells in the tumour core (Darmanis *et al.*, 2017). This finding supports our hypothesis that there are at least two subclasses within the mesenchymal subtype, one subclass induced by recruited cells and the other by tumour cells. Furthermore, in our experience, the mesenchymal subtype cells from GBM patients, enriched under neuro-sphere culture conditions, also have two subclasses depending on their *in vitro* growth morphology (Behnan *et al.*, 2016, 2017*a*). One type grows as floating spheres and the other grows adherently in serum-free non-adherent conditions (Behnan *et al.*, 2017*a*).

The glioma subtype driving mutations can be explained in glioma transgenic mouse models. While *RCAS-PDGFB*-amplification can drive a proneural-like transcriptome pattern, *RCAS-NF1* silencing in a paediatric or adult glioma mouse model drive a mesenchymal-like subtype (Hambardzumyan *et al.*, 2009; Liu *et al.*, 2011; Halliday *et al.*, 2014; Ozawa *et al.*, 2014; Herting *et al.*, 2017). The *PDGFB* model exhibits some features of oligodendroglioma such as *OLIG2* and *DCX* expression, while *NF1*-silenced models exhibit more astrocytoma-like features represented by high expression of *GFAP* and *CD44*. Similar to human mesenchymal subtype tumours, the *NF1* tumour model contains significantly higher macrophage numbers compared to the proneural *PDGFB* model. Interestingly, the *NF1* model had significantly longer survival than the proneural *PDGFB* model. However, by reanalysing the survival of proneural versus mesenchymal subtypes in the TCGA dataset, and after limiting the mesenchymal subtype to only *NF1*-deleted or mutant samples, no adverse survival was seen for mesenchymal subtype. Also, the *NF1* model had less proliferating cells, smaller vessel size, and less permeability than the proneural model. All these together suggest that genetic driver mutations may impact tumour vasculature and structure (Herting *et al.*, 2017).

The cell of origin dictates the tumour subtype

The cell of origin might play an important role in determining GBM phenotype. The proneural subtype *PDGFB* overexpressing model has 100% tumour formation with GBM characteristics when *RCAS-PDGFB* is injected into the left or right hemisphere or subventricular zone. However, the *TP53/NF1* model requires co-delivery of *RCAS shNF1*, *shTp53*, and Cre-*Pten* specifically to the subventricular zone of adult mice to get GBM-like tumour formation (Hambardzumyan *et al.*, 2009; Herting *et al.*, 2017). This suggests that each tumour subtype might have a different cell of origin (Alcantara Llaguno *et al.*, 2009; Liu *et al.*, 2011). The oligodendrocyte precursor cells (OPCs) were identified as the cell of origin for the *Tp53/Nf1* tumour model that have dramatic growth expansion prior to malignancy (Liu *et al.*, 2011). Alcantara Llaguno *et al.* (2015) showed that two populations of adult CNS progenitors, with different cell of origin, gave rise to molecularly and biological distinct gliomas despite being hit with the same tumour suppressor mutation in *Nf1*, *Trp53*, and *Pten*. The two transformed progenitor populations aligned either with normal OPCs or astrocytes, where this last one enriched for mesenchymal signature (Alcantara Llaguno *et al.*, 2015). These data suggest an astrocytic lineage and OPCs as origin of the mesenchymal and proneural subtypes, respectively (Alcantara Llaguno *et al.*, 2015; Herting *et al.*, 2017).

Contrary to this, Mazor *et al.* (2015) showed that the differences between primary and recurrent tumours that progressed from low grade to GBM, were more likely attributed to tumour progression rather than cell of origin. The malignant progression was associated with strong promoter hypomethylation (1953 CpG sites) leading to alteration in gene transcription that enriched for cell cycle genes. The authors suggested an interdependency between genetics and epigenetics during the tumour evolution process and that phyloepigenetic tree recapitulated the early divergence between primary and recurrent tumours similar to somatic phylogenetic tree (Mazor *et al.*, 2015).

The mesenchymal subtype can be shaped by immune cells

The main characteristic of the mesenchymal subtype is its association with immune-related genes and pathway analysis showed enrichment of immunological processes and inflammation. This subtype scored the lowest purity score compared to proneural and classical subtypes indicating the infiltration

of non-neoplastic cells into this subtype (Wang *et al.*, 2017*b*). Macrophages/microglial cells that constituted the largest stromal cell populations in GBMs and their infiltration was associated with the mesenchymal subtype, were suggested to induce PMT (Engler *et al.*, 2012; Li and Graeber, 2012; Bhat *et al.*, 2013; Gabrusiewicz *et al.*, 2016; Wang *et al.*, 2017*b*). The mesenchymal subtype enriched for macrophage/microglia of M1 and M2 signatures, in addition to neutrophil cell signature, while the classical subtype included activated dendritic cells (Bao *et al.*, 2006; Engler *et al.*, 2012; Ye *et al.*, 2012; Gabrusiewicz *et al.*, 2016; Wang *et al.*, 2017*b*). Also, activated natural killer cells were less expressed in mesenchymal subtype samples than in classical and proneural samples (Wang *et al.*, 2017*b*). Furthermore, tumour-infiltrating lymphocytes (TILs) were enriched in mesenchymal tumours versus non-mesenchymal tumours (Engler *et al.*, 2012; Li and Graeber, 2012; Bhat *et al.*, 2013; Gabrusiewicz *et al.*, 2016; Wang *et al.*, 2017*b*).

Although the mesenchymal subtype has a significantly higher level of necrosis compared to other subtypes (Verhaak *et al.*, 2010; Cooper *et al.*, 2012), the mesenchymal tumours with abundant TILs did not have significantly higher necrosis than those with no TILs. This indicates that TILs might represent an adaptive immune response against the tumour rather than a response against necrosis. TILs were also associated with specific histopathological features including sarcomatous regions, giant cell, epithelioid, and gemistocytic components (Rutledge *et al.*, 2013). Not surprising to us, tumours that were enriched with sarcomatic regions, pathologically known as gliosarcoma, were also classified as the mesenchymal subtype recently (Brain tumor conference, Warsaw, 2018).

Forty-two per cent of mesenchymal tumours in TCGA set have TILs, indicating its immunogenic nature. Interestingly, classical subtype tumours were depleted of TILs. TIL infiltration was associated with *NF1* and *RB1* mutations, a common alteration in the mesenchymal subtype (Rutledge *et al.*, 2013). It was shown recently that *NF1* abnormalities were associated with less purity and higher stromal signature indicated by the infiltration of macrophages/microglia, which were supported by higher expression of M1 and M2 macrophages in the mesenchymal subtype. Further, *NF1* knockdown increased the *in vitro* recruitment of human macrophages isolated from GBM patients (Wang *et al.*, 2017*b*). Additionally, TILs were strongly associated with *NF1* deletion or mutation, and absent in both *EGFR*-amplified tumours and those with homozygous *PTEN* deletion (Rutledge *et al.*, 2013). Recurrent glioma tumours showed a decreased monocyte gene signature compared to primary tumours; the recurrent tumours with mesenchymal transition exhibited an increased immune cell infiltration. M2 macrophages were higher in recurrent tumours that underwent mesenchymal transition. Non-polarized M0-macrophages were also increased in recurrent tumours with the mesenchymal profile, compared to primary tumours from the same subtype (Wang *et al.*, 2017*b*).

While tumour cells with *NF1* deletion exert their effect by recruiting macrophages/microglial cells to the tumour, the macrophage/microglia cells also affect the tumour and create a micro-environment that shapes mesenchymal subtype tumour cells (Wang *et al.*, 2017*b*). This predicts that a two-way interaction might exist between immune cells and tumour cells, represented by macrophages/microglia cells within the tumour expressing a unique transcription profile that demonstrates a direct effect of the tumour milieu on these cells. On the other hand, the macrophages/microglia cells express anti-inflammatory, pro-angiogenic, and extracellular matrix remodelling factors that are known to enhance tumour growth, survival, and invasion (Szulzewsky *et al.*, 2016; Darmanis *et al.*, 2017; Wang *et al.*, 2017*b*). Furthermore, the stroma heterogeneity investigated on single cell level has yield a precise quantification of cellular compartments within the tumour core and invasive edges of GBM patients. This study showed that only 44% of the core cells were tumour cells, ~50% were infiltrative immune cells ($CD45^+$ cells), ~1.5% clustered with oligodendrocytes ($GalC^+$ cells), 2% clustered with oligodendrocyte precursors ($O4^+$ cells), 2% clustered with endothelial cells (sorted as $BSL1^+$ cells), and $<0.05\%$ with neurons (sorted as $CD90^+$ cells). Macrophages and microglia were the main cell populations in the immune cell cluster, ~95%, whereas dendritic cells made up 4.5% (Darmanis *et al.*, 2017).

The mesenchymal subtype can be shaped by anatomical location and micro-environment

One important criticism for the TCGA subtyping is that each sample was dissected from only one single random spot of the tumour bulk, assuming that GBMs are homogenous tumours and intra-tumoural heterogeneity is not big issue. This was shown not to be the case when a fluorescence-guided multiple sampling (FGMS) approach was used to dissect four to six tumour fragments from spatially distinct regions within an individual tumours from each GBM patient. In 6 of 10 patients analysed, samples from the same patient were classified into two to three different subtypes revealing intra-tumoural heterogeneity (Sottoriva *et al.*, 2013). Recently, Puchalski *et al.* correlated the inter- and intra-tumour heterogeneity with GBM anatomical regions. Their data showed that samples from the same anatomical region, regardless of whether they were from the same patient or different patients, were more similar to each other than to samples from different anatomical regions from the same patient. While the mesenchymal signature was expressed by perinecrotic/hypoxic regions and microvascular proliferative areas, the vascular areas and invasive edges were proneural. Patients who expressed both vascular and hypoxic signatures had worse survival than those did not (Jin *et al.*, 2017; Puchalski *et al.*, 2018).

Surprisingly, the three or four TCGA subtypes can exist within the same tumour. The leading edges were subtyped as neural, the infiltrative tumours were subtyped as neural/proneural, the central tumour regions were either the classical

or neural/proneural subtype, and the pseudopalisading cells around necrosis and microvascular proliferation were almost exclusively mesenchymal subtype (Puchalski *et al.*, 2018). Also, single cell analysis revealed that the tumour bulk consists of individual tumour cells that belong to at least three subtypes and that cell diversity impacted patient survival. This study concluded that a subtype label of glioma sample is the same subtype signature of the dominant cell population within the tumour bulk (Patel *et al.*, 2014; Darmanis *et al.*, 2017). Furthermore, the vascular and hypoxic signatures associated with tumour grade and histology were also associated with different GBM subtypes. Including the gene expression profiles of human microvascular endothelial cells (HMVEC), endothelial cells (HUVEC), and GBM cells that underwent hypoxia and normoxia showed that proneural subtype expressed markers of mature vessels, while mesenchymal subtype expressed markers of hypoxia and microvascularity. Patients that expressed high hypoxic and vasculature signature simultaneously, exhibited the worst survival (Jin *et al.*, 2017).

Macrophages and microglia that constitute the largest stromal population in GBM, had a variable distribution between the tumour core and the invasive edge. While macrophages were dominant within the tumour core (813 single cell macrophages versus 365 microglial), microglia were the main population in the invasive edge (85 macrophages versus 574 microglia). This distribution was associated with a specific cytokine profile with myeloid cells in the tumour core express anti-inflammatory cytokines and pro-angiogenic markers suggesting an essential role in tumour growth, survival, extracellular matrix remodelling and promoting angiogenesis. On the other hand, immune cells with pro-inflammatory cytokine profile were dominant in the tumour periphery (Darmanis *et al.*, 2017). However, this study has major limitations as the analysis of stromal cells depended on single cells sorted after labelling with known but unspecific markers and most importantly, the leftover stromal cells were discarded. Addressing cells like neurons, endothelial cells, and astrocytes with single marker does not mean that these cells are what they are thought to be, because many other cell types can express these markers. This can be seen when a single marker picked three different cell types clustered under what was supposed to be an endothelial cluster. Also, CD90, which was used as marker for neurons, is highly expressed by brain derived-MSCs/pericytes (Behnan *et al.*, 2017*a*, *b*).

All these together highlight the complexity of GBM heterogeneity and subtypes and suggest that *NF1* abnormality and tumour micro-environment with its recruited cells, vasculature and anatomic location induce the mesenchymal criteria.

Therapy-induced mesenchymal transition

The EMT, a critical biological process in embryonic development and wound healing, has been shown to play essential roles in tumour cell migration, metastasis and therapy resistance (Lee *et al.*, 2014). In brain tumours, this process could have another name such as glial-mesenchymal transition (GMT) or PMT, because of the believed glial origin of these tumours. Antiangiogenic treatments, DNA damaging agents and ionizing radiation might affect cancer cell behaviour through EMT/GMT/PMT and, eventually, patient prognosis (Fig. 1). The mesenchymal subtype GBM patients, of both primary and recurrent tumours, tended to have worse survival than other subtypes (Phillips *et al.*, 2006; Verhaak *et al.*, 2010; Wang *et al.*, 2017*b*). As the mesenchymal subtype was associated with higher expression of angiogenesis and endothelial markers, the mesenchymal subtype patients were predicted to benefit from the antiangiogenic treatments. However, antiangiogenic drugs, DNA damaging agents and ionizing radiation impact the biology of the tumour and its subsequent behaviour (Phillips *et al.*, 2006; Piao *et al.*, 2012, 2013).

In combination with radiotherapy and chemotherapy, the antiangiogenic drugs, including the U.S. Food and Drug Administration approved anti-VEGF (bevacizumab) for recurrent GBM and many other solid tumours, have been shown to decrease vascular permeability resulting in preliminary radiographic response associated with prolongation of progression free survival, but not overall survival (Batchelor *et al.*, 2007; Friedman *et al.*, 2009; Port *et al.*, 2010; Lai *et al.*, 2011; Chinot *et al.*, 2014; Taal *et al.*, 2014). The preclinical studies showed that a combined bevacizumab and sunitinib (anti-VEGFR) treatment improved animal survival, in addition to delayed hypoxia and decreased the recruited myeloid cells compared to bevacizumab single treatment (Piao *et al.*, 2012, 2013). However, the clinical outcomes of both anti-VEGF and/or anti-VEGFR or the combined treatments with chemo- and radiotherapy, did not achieve significant overall survival despite the first one showed better tumour oxygenation at early stage associated with improvement of progression free survival, while the anti-VEGFR induced rapid hypoxia and tumour progression (Friedman *et al.*, 2009; Galanis *et al.*, 2013). Upon disease progression after anti-VEGF failure or combined anti-VEGF and anti-VEGFR, treated tumours became even more aggressive and treatment-resistant (Quant *et al.*, 2009; de Groot *et al.*, 2010; Scott *et al.*, 2010; Piao *et al.*, 2012, 2013). The preclinical data showed a significant increase in recruited macrophages/microglia and aggressive mesenchymal features at the time of tumour progression in treated groups (Piao *et al.*, 2012, 2013). EMT or PMT has been suggested as one of the main mechanisms behind the development of resistant phenotype tumours after antiangiogenic treatments (Lu *et al.*, 2012*b*; Piao *et al.*, 2012, 2013). Comparing the recurrent tumours in GBM patients who developed bevacizumab resistance to their original untreated tumours, the recurrent tumours showed increased MET activation and elevation in mesenchymal markers including VIM, CD44, YKL-40, N-cadherin and downregulation of T-cadherin (Lu *et al.*, 2012*b*). Piao *et al.* (2013) showed an increase in cell

migration or invasion, elevation in pro-inflammatory cytokine secretion, and enrichment of genes associated with mesenchymal signature and EMT transcription factors in tumour cells that developed resistance to bevacizumab. The histology analysis of these specimens revealed regions containing sarcoma and spindle-shaped cells (Piao *et al.*, 2013). Bevacizumab treatment (in a GBM xenograft model derived from patient tumour spheroids) resulted in significant reduction in vascularity and blood flow revealed by dynamic contrast enhanced MRI, and reduced blood vessel size by histology analysis. This resulted in a glycolytic hypoxic micro-environment that enhanced tumour cell invasion (Keunen *et al.*, 2011). Lu *et al.* (2012b) showed that knockout of *VEGF* enhanced *MET* activation (phospho-*MET*), which induced highly aggressive and invasive mesenchymal-like GBM. Importantly, combining the ablation of VEGF with knocking down *MET* expression reduced vascularity and invasion, converted cell phenotype into epithelial-like cells, and extended animal survival 3-fold compared to the control group (Lu *et al.*, 2012*b*).

The current standard of care for GBM treatment includes maximal tumour resection followed by radiotherapy (whole brain radiation or stereotactic radiosurgery) plus adjuvant temozolomide chemotherapy (Stupp *et al.*, 2005). Preclinical data has shown that GBM samples contain radioresistant GSCs (specifically $CD133^+$ cells) that activate DNA damage checkpoint signals and increase DNA repair capacity upon radiation treatment. This cell population expanded after radiation treatment, both *in vitro* and *in vivo*. Using checkpoint inhibitors (Chk1 and Chk2) sensitized $CD133^+$ cells to radiation treatment, consequently, impaired tumour growth (Bao *et al.*, 2006). Bhat *et al.* (2013) and others showed that GSCs with mesenchymal subtype are more radio-resistant than proneural subtype (Segerman *et al.*, 2016; Pencheva *et al.*, 2017). Upon radiation treatment, proneural subtype cells pretreated with TNF-α displayed an upregulation of CD44 expression and activation of NF-κB pathways to undergo PMT (Bhat *et al.*, 2013). PMT was detected as early as 6 h post radiation treatment. NF-κB, STAT3, Snail, P53, E2F, and ALDH1A3 were defined as the main mediators and regulators of PMT and their inhibition blocked this phenomenon. Radiation treatment upregulated CEBPB and mesenchymal markers (CD44, α-SMA/ACTA2, VIM, FN1, COL1A1 and COL1A2, MMP2, MMP9, and YKKL-40), while downregulating proneural markers (SOX10 and PDGFR-α) (Mao *et al.*, 2013; Halliday *et al.*, 2014; Lau *et al.*, 2015). Murine and human GBM cells that underwent PMT post-radiation treatment increased cell motility, invasion, reduced cellular stiffness, and significantly increased temozolomide-resistance compared to non-irradiated cells (Lau *et al.*, 2015). Clinical data showed that GBM patients with high CD44 expression and NF-κB activation were associated with poor response rate to radiation treatment and have lower survival than those with low mesenchymal metagene expression (Bhat *et al.*, 2013). It is thought that the proneural tumours contain a small population of mesenchymal subtype cells, marked as $SOX2^-CD44^+$ cells, which are radioresistant and they dominate the culture after radiation inducing the mesenchymal signature (Mao *et al.*, 2013).

Despite alkylating agents such as temozolomide has been considered as first-line treatment for GBM patients since 2005, it offers limited survival benefit for those patients (Stupp *et al.*, 2005; Wick *et al.*, 2012). In fact, it has been recommended to avoid temozolomide in elderly GBM patients, and retain it for patients harbouring a methylated *MGMT* promoter gene that has a good response to this treatment (Wick *et al.*, 2012). However, the outcomes of a recent clinical trial confirmed some survival benefit in elderly patients when hypofractionated radiotherapy was combined with temozolomide (Perry *et al.*, 2017). *MGMT* has been associated with developing resistance to the standard of care treatment with temozolomide. Direct inhibition of *MGMT* expression or increasing the promotor methylation of this gene decreased glioma chemoresistance (Hegi *et al.*, 2005; Siebzehnrubl *et al.*, 2013). Chen *et al.* (2012) suggested that temozolomide targets the active proliferating progenitors, not the quiescent chemoresistant GSCs that recapitulate the tumour and no survival benefit was achieved. Even after combining temozolomide with ablation of quiescent GSCs, a less invasive circumscribed tumour recurs with different histology from the primary tumour expressing high level of S100B and PDGFR-α, and very low GFAP, resembling oligodendroglioma (Chen *et al.*, 2012). Temozolomide-resistant glioma cell lines were established by treating cultured cells with temozolomide for 6 months. The temozolomide-resistant cells expressed some mesenchymal properties such as: reduced cell polarity, increased pseudopodia formation, cell motility and invasion, upregulation of EMT markers (VIM, N-cadherin, and Slug), and CDC20. Inhibition of CDC20 abolished the EMT features and enhanced temozolomide sensitivity (Wang *et al.*, 2017*a*). *ZEB1*—a tumour formation regulator, and EMT- and stemness-inducer—is expressed in 45% of GBM patients with 50% overlapping with *MGMT* expression. Its expression is correlated with glioma grade, patient survival, and tumour invasion. Surprisingly, *ZEB1*, and not *MGMT*, correlated with shorter temozolomide response and reduced GBM patient survival. *ZEB1* knock-down not only decreased GSC marker expression (SOX2, OLIG2 and CD133), it also reduced tumour invasion and chemo-resistance. On the other hand, over expression of *ZEB1* induced c-*MYB*, miR-200c and *MGMT* elevation, which made GBM cells more chemo-resistant. However, even in the absence of *ZEB1*, overexpression of c-*MYB* increased *MGMT* expression and chemo-resistance (Siebzehnrubl *et al.*, 2013). Also, upregulation of *TWIST1* and loss of *CDH1* was associated with poor temozolomide response and short survival in GBM patients, indicating the role of EMT in tumour progression (Velpula *et al.*, 2011; Siebzehnrubl *et al.*, 2013). Further, recent outcomes from drug screening on clonal levels showed that the *in vitro* expanded clones with

mesenchymal characteristics are more radio- and chemo-resistant than those with proneural characteristics and changed the methylation pattern of the mesenchymal subtype master regulators upon treatment. Interestingly, the clones that showed resistance to one drug, were mostly resistant to all other drugs in the panel, and this multidrug resistance was correlated with radio-resistance (Segerman *et al.*, 2016). Finally, it is speculated that tumour surgery might activate EMT as a response to wound healing signals triggered by the injured tissue. However, only few reports from breast cancer, prostate, and oral squamous cell carcinoma addressed this issue (Kast *et al.*, 2017). To our knowledge, such studies have not been performed in glioma. However, more research is needed to know whether PMT/EMT/GMT is caused by accumulated genetic alterations upon disease progression or by stromal cells and accelerated by treatment regimes.

The concept of the proneural origin of all glioblastoma multiforme subtypes

Ozawa *et al.* (2014) suggested that all non-G-CIMP GBMs, including both proneural and mesenchymal subtypes, arise from common proneural-like precursors and that disease lethality is increased by accumulated mutations such as *TP53*, *PTEN*, or *CDKN2A* in *PDGFA*-driven gliomas. Their computational model identified cells harbouring both chr7-gain, driven by *PDGFA* amplification, and chr10-loss, driven by *PTEN* loss, as the most likely cells of origin for gliomas. The mesenchymal subtype might evolve from the proneural subtype and be driven by accumulated mutations (*NF1* loss in this case) in tumour cells that overexpress *PDGFA*. These tumours have been shown to upregulate mesenchymal-related markers including *CD44*, *pSTAT3*, *STAT5*, *STAT6*, *C/EBPb.IRF1* and *RUNX1* (Ozawa *et al.*, 2014). However, the hypothesis that one subtype, such as the proneural subtype, is the origin of all other subtypes, and it is just matter of disease progression that shifts tumours from one subtype to another, can be also criticized. The mesenchymal subtype does exist in lower grade gliomas. Furthermore, upon tumour recurrence, ~50% of the tumours keep the same subtype and some tumours shift in the opposite direction, from mesenchymal→proneural upon disease progression (Murat *et al.*, 2008; Verhaak *et al.*, 2010; Wang *et al.*, 2016, 2017*b*). Also, simultaneous deletion of *NF1* and *Tp53* without *PDGFA* amplification background induced GBM with the mesenchymal profile in an *RCAS/tv-a* model (Ozawa *et al.*, 2014). Altogether, these results suggest that the mesenchymal subtype might have a different origin than the proneural subtype. Despite having clear proof of the effect of macrophages/microglia, inflammatory cytokines, and radiation on subtype plasticity, it is still difficult to confirm the hypothesis that suggests all GBM subtypes evolve from a proneural origin through the exposure to different micro-environmental factors (Bhat *et al.*, 2013). Also, current understanding of GBM indicates that all primary GBMs (not secondary), arise *de novo* without previous history of low grade tumour. Therefore, it is challenging to draw tumour evolution maps based on this hypothesis and to predict when the subtype shift happened in response to micro-environmental factors.

In spite of Ozawa *et al*'s suggestion that the proneural-like precursors are the origin of all subtypes, the clonal evolution of GBM does not support this conclusion (Gerstung *et al.*, 2011; Ozawa *et al.*, 2014; Kim *et al.*, 2015; Wang *et al.*, 2016). However, the clonal evolution concept is indeed controversial and does not have clear agreement on the early GBM-driving mutational events. Sottoriva and colleagues (2013) have suggested that mutations in *EGFR* and *CDKN2A/B/p14ARF* are early event drivers, while *PDGFRA* and *PTEN* mutations are later events (Sottoriva *et al.*, 2013). A study by Wang *et al.* (2016) described mutations in *IDH1*, *PIK3CA*, and *ATRX* as early events while mutations in *TP53*, *NF1*, and *PTEN* occur later during tumour evolution (Wang *et al.*, 2016). Ozawa *et al.* (2014) suggested that *PDGFA* amplification and *PTEN* deletion are the common and leading events in all subtypes of non-GCIMP GBMs. *NF1* loss is a late event based on the subsequent targeting of *NF1* in the proneural (*PDGFA/shP53*) mouse model that results in mesenchymal-like tumour formation (Ozawa *et al.*, 2014). However, these studies have several limitations. First, *IDH1* mutation, which is a common mutation in the proneural subtype, is detected in fewer than 10% of GBM patients. Thus, it would be difficult to make the assertion that this mutation is the driver for all tumour subtypes. Second, the *NF1* mutation, which is associated with the mesenchymal subtype, and *IDH1*, which is the common driver mutation of the proneural subtype, are expressed in different groups explicitly. Third, a mesenchymal-like subtype has a different cell of origin in other mouse model and can arise *de novo* by *Nf1/Tp53* loss. Finally, *NF1* is a driver mutation in primary and recurrent tumours, even among a subgroup of low grade tumours (Rodriguez *et al.*, 2008; Ozawa *et al.*, 2014; Cancer Genome Atlas Research *et al.*, 2015; Eckel-Passow *et al.*, 2015; Wang *et al.*, 2016). All these together suggest that it is unlikely that the proneural subtype is the origin of all other subtypes.

The polyclonal hypothesis of glioblastoma multiforme origin

The concept of a single clone derived from one type of cell that gives rise to cancer can be accepted in some haematological cancers, but it is highly unlikely to stand for the origin of highly heterogeneous tumours such as GBM

(McGranahan and Swanton, 2017). Although the cancer stem cell model and the stochastic clonal evolution model might explain the intra-tumoural heterogeneity, it can't ascertain the clonal origin of the tumour (Parsons, 2018). Furthermore, recent *in vitro* outcomes of GBM clonal analysis showed a high clonal heterogeneity among GBM patients, even within the same tumour. These clones vary in their chemo- and radio-sensitivity. Contrary to previous studies, the therapy resistance was not associated with increasing GSC marker expression (Auffinger *et al.*, 2014; Segerman *et al.*, 2016). This clonal heterogeneity might be behind therapeutic failure where minor untargeted clones can drive treatment resistance upon GBM recurrence. However, we should keep in mind that such an *in vitro* system is influenced by clonal selection dynamics during cell culture and xenografting (Patel *et al.*, 2014; deCarvalho *et al.*, 2018). The multiple cell of origin hypothesis was suggested a long time ago in hereditary neurofibromas, a disease related to *NF1* mutation/deletion (Fialkow *et al.*, 1971). In medulloblastoma, genetic analysis of dissected primary and recurrent tumours, from both transgenic mice and patient samples, revealed a high degree of genetic divergence with $<5\%$ overlap. The conventional treatment of medulloblastoma including surgery and radiation treatment, imposed an evolutionary pressure that selected highly divergent clones at recurrence, while the sensitive clones that were dominant in the primary tumour tended to disappear (Morrissy *et al.*, 2016). The GBM heterogeneity at the mutational, clonal, and transcriptional subtype level, in addition to the cell of origin, suggests a polyclonal evolution of GBM origin rather than monoclonal one. The polyclonal hypothesis of GBM origin can be supported by several arguments: (i) the glioma animal models of the mesenchymal and proneural subtypes suggest that different cell populations are the cell of origin of these two different subtypes (Liu *et al.*, 2011; Alcantara Llaguno *et al.*, 2015; Herting *et al.*, 2017); (ii) the clonal divergence between primary and recurrent glioma, especially in multifocal tumours, long-term recurrence and distant recurrence (Wang *et al.*, 2016; Lee *et al.*, 2017); (iii) $<45\%$ of the mutations are shared between primary and recurrent glioma (Johnson *et al.*, 2014; Wang *et al.*, 2016); (iv) switching the transcriptional subtype in recurrent GBM (Wang *et al.*, 2016); and (v) the intra-tumoural heterogeneity among geographically-distinct tumour parts obtained from a single patient, in addition to the outcomes of single cell analysis, showed that an individual GBM patient has cells that belong to the three different subtypes (Sottoriva *et al.*, 2013; Patel *et al.*, 2014). Altogether, these results suggest a polyclonal origin of GBM rather than a monoclonal one. However, more research is needed to confirm this hypothesis in glioma field.

Our hypothesis is that specific genetic mutations that hit specific cell types give rise either to proneural, classical or mesenchymal subtypes, and the polyclonal evolution of GBM, which is influenced by accumulated genetic and epigenetic changes, the cell of origin, recruited stromal cells with their secreted factors, and the dynamic interactions between these variable clones, in addition to their interaction with micro-environment, drive molecular subtype heterogeneity that leads to intra- and inter-tumoural heterogeneity.

The clinical implication of subtypes

The subtype driving mutations exhibit differential therapy responses in clinical and preclinical tests. The proneural-*PDGFB* overexpressing model was significantly sensitive to both temozolomide and radiation treatment treatments with preferable survival achieved with radiation treatment over temozolomide. On the other hand, the *NF1*-silencing model was in general less sensitive than the proneural-*PDGFB* model and significant survival was achieved with radiation treatment treatment alone but not temozolomide treatment. And recurrent tumours displayed a trend toward more aggressive tumours (Herting *et al.*, 2017). It has been suggested that GBM patients with pure proneural subtype that have minimum transcriptional signal of other subtypes have significantly better survival rates than patients with mesenchymal subtype (Patel *et al.*, 2014). However, we want to keep in mind that patients with mesenchymal subtype tumours are older than proneural patients, and age by itself is negative predictor in GBM survival (Wick *et al.*, 2012). It is speculated that the tumour micro-environment with its immune cell and vasculature components plays an essential role in the differential treatment response between different subtypes (Herting *et al.*, 2017). Another reason for treatment sensitivity could be the intrinsic nature of tumour cells in each subtype. The oligodendroglioma characters observed in the proneural subtype model could explain the preferable response to temozolomide treatment (Schindelin *et al.*, 2012; Schmid *et al.*, 2016). The sensitivity of proneural subtype tumours to chemo and radiation treatment could be the reason for the induced PMT upon tumour recurrence (Segerman *et al.*, 2016; Pencheva *et al.*, 2017). It is expected that tumour cells with proneural subtype die in response to treatment, while cells with the mesenchymal subtype proliferate and dominate the recurrent tumour. It is worthy to note that patients from the classical and mesenchymal subtypes, but not those from the proneural subtype, get a survival benefit from aggressive treatment protocols with chemo- and radiotherapy or more than three repeated cycles of chemotherapy (Verhaak *et al.*, 2010). Preliminary data from some immunotherapy targets showed promising outcomes in mesenchymal subtype tumours. Animal data of adoptive transfer of engineered T-cells expressing IL13-zetakine. T-cells displayed potent anti-tumour activity in the mesenchymal subtype (Brown *et al.*, 2012). Recently it was shown that $CD4^+$ chimeric antigen receptor (CAR) T cells targeting IL13Rα2 antigen induced a long term anti-oncogenic effect and outperformed $CD8^+$ CAR T cells or a combination of $CD4^+$ and $CD8^+$ CAR T cells (Wang *et al.*, 2018).

Further, the proneural and mesenchymal subtypes showed a differentiational response to epigenetic therapeutic targets, such as EZH2 and BMI1 inhibitors, with proneural being more sensitive to EZH2 inhibitor and mesenchymal more sensitive to BMI1. However, a heterogeneous tumour showed a better response to a combination therapy of EZH2 and BMI inhibition (Jin *et al.*, 2017). These agents are suggested to reverse the radio and chemo-resistance properties of cancer cells (Facchino *et al.*, 2010; Fan *et al.*, 2014). However, although tumour subtype might give a better prediction for treatment response, intra-tumoural heterogeneity remains one of the main obstacles that contributes to GBM treatment resistance and recurrence (Patel *et al.*, 2014).

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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