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Cancer cell heterogeneity and plasticity: A paradigm shift in glioblastoma

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Abstract

Phenotypic plasticity has emerged as a major contributor to intra-tumoral heterogeneity and treatment resistance in cancer. Increasing evidence shows that glioblastoma (GBM) cells display prominent intrinsic plasticity and reversibly adapt to dynamic microenvironmental conditions. Limited genetic evolution at recurrence further suggests that resistance mechanisms also largely operate at the phenotypic level. Here we review recent literature underpinning the role of GBM plasticity in creating gradients of heterogeneous cells including those that carry cancer stem cell (CSC) properties. A historical perspective from the hierarchical to the nonhierarchical concept of CSCs towards the recent appreciation of GBM plasticity is provided. Cellular states interact dynamically with each other and with the surrounding brain to shape a flexible tumor ecosystem, which enables swift adaptation to external pressure including treatment. We present the key components regulating intra-tumoral phenotypic heterogeneity and the equilibrium of phenotypic states, including genetic, epigenetic, and microenvironmental factors. We further discuss plasticity in the context of intrinsic tumor resistance, where a variable balance between preexisting resistant cells and adaptive persisters leads to reversible adaptation upon treatment. Innovative efforts targeting regulators of plasticity and mechanisms of state transitions towards treatment-resistant states are needed to restrict the adaptive capacities of GBM.

Keywords

glioblastoma | plasticity | treatment resistance | tumor heterogeneity | tumor microenvironment

Despite aggressive treatment available for glioblastoma (GBM) patients including surgical resection, radiation, and chemotherapy, tumor recurrence is unavoidable. According to the 2021 WHO classification of CNS tumors, GBMs are classified as *Isocitrate dehydrogenase* (*IDH*) wild-type (IDHwt) and represent the most aggressive form of diffuse gliomas.[1] Based on their diverse cellular organization and histological appearance, GBMs were historically considered among the most heterogeneous tumors and were referred to as "multiforme". GBMs commonly carry *TERT* promoter mutation and copy number changes at chromosomes 7 and 10 (+7/–10). Genetic alterations such as amplification of *EGFR*, *PDGFRA*, and *CDK4/6*, as well as deletions or inactivating mutations in *TP53*, *PTEN*, *NF1*, and *CDKN2A/B* are key determinants of inter-patient variability. GBMs corresponding to *IDH1/2* mutated (IDHmut) tumors are currently a separate grade IV entity within IDHmut astrocytomas.[1] Large-scale genetic and epigenetic profiling studies have uncovered molecular GBM subgroups characterized by distinct DNA methylation[2,3] and/or expression patterns,[4] highlighting the molecular heterogeneity of this disease. Although to date these subgroups have limited clinical relevance, it remains to be seen to what extent the different layers of inter-patient and intra-tumoral heterogeneity will inform future treatment decisions.

Accumulating evidence underscores the existence of extensive intra-tumoral phenotypic heterogeneity and plasticity in GBM (Table 1). Intrinsic plasticity adds another layer of tumor complexity, allowing flexible adaptation of tumor cells during tumor initiation, progression, and treatment escape. Here we

Table 1 A Summary of Recent Key Findings Showing Evidence of Tumor Cell Plasticity Shaping Phenotypic Heterogeneity in GBM

Highlights	Main Technology	Main Cellular Source	Reference
➢ GBM cells display variable expression of diverse programs related to oncogenesis ➢ GBM cells show a continuum of stemness signature	scRNA-seq	GBM patient material	Patel et al.[49]
➢ Developmental and transcriptomic signature genes are epigenetically primed for expression via bivalent domains at their promoters	ChIP-seq, RNA-seq	GBM patient material, GBM cell lines	Hall et al.[70]
➢ Phenotypic heterogeneity accelerates tumor growth ➢ Paracrine signaling from "differentiated" GBM cells supports CSC growth	FACS, functional assay	GBM stem-like cultures, GBM PDOXs	Wang et al.[93]
➢ GBM cells can transit between NG2 positive and negative states to establish phenotypic equilibrium ➢ Tumorigenic potential in vivo depends on the phenotypic equilibrium of implanted cells	FACS, functional assays, Microarray	GBM stem-like cultures	Al-Mayhani et al.[27]
➢ Phenotypic states in GBM are plastic and reversible ➢ Phenotypic heterogeneity arises from nonhierarchical, reversible stochastic state transitions ➢ Phenotypic equilibrium is dictated by TME ➢ Fast reconstitution of phenotypic heterogeneity provides growth advantage	FACS, functional assays, scRNA-seq	GBM PDOXs, GBM stem-like cultures	Dirkse et al.[29]
➢ Phenotypic heterogeneity is a result of nonhierarchical organization of proliferating phenotypic states ➢ Phenotypic states can undergo state transitions ➢ Equilibrium between cellular states is dictated by genetic background and TME cues	scRNA-seq, lineage barcoding, FACS, functional assays	GBM patient material	Neftel et al.[50]
➢ Proliferating GBM cells exist on the main axis of variation between mesenchymal and proneural states, representing tumor core and invasive cells respectively	scRNA-seq, snRNA-seq, scATAC-seq	GBM patient material	Wang et al.[52]
➢ GBM cells transition to a slow-cycling, persister-like state upon pressure from RTK inhibitors ➢ State transitions are reversible and depend on epigenetic remodeling ➢ Adaptive resistance can coexist with irreversible genetic evolution towards novel resistant clones	ChIP-seq, scRNA-seq, functional assays	GBM patient material, GBM stem-like cultures	Eyler et al.[141] Liau et al.[140]
➢ Developmental programs are reactivated in GBM ➢ Active stemness programs exist in different phenotypic states and are patient-specific ➢ Multiple GBM populations give rise to heterogeneous tumors with TME-specific composition ➢ PTPRZ1-positive GBM cells resembling outer radial glia promote invasion	scRNA-seq, FACS, functional assays	GBM patient material, GBM PDOXs	Bhaduri et al.[55]
➢ Transcriptomic gradient centered around proliferating glial progenitor-like cells based on fetal brain signatures ➢ Glial progenitor states are enriched in stem-like cultures and show strongest tumorigenicity	scRNA-seq, FACS	GBM patient material, GBM stem-like cultures, Normal fetal brain cells	Couturier et al.[28]
➢ Main axis of variation associated with neurodevelopmental programs ➢ The "naïve" CSC state, distinct from other phenotypic states, is maintained by YAP/TAZ	scRNA-seq, functional assays	GBM patient material, GBM stem-like cultures	Castellan et al.[51]
➢ GBM cells are distributed across neurodevelopmental and metabolic axes ➢ Metabolic states have divergent mitochondrial, glucose, glutamine, and lipid metabolism	scRNA-seq, bulk RNA-seq, Metabolic assays	GBM patient material, GBM stem-like cultures	Garofano et al.[53]
➢ GBM transcriptional states exist across the axis between neurodevelopmental and injury response programs ➢ Cultured stem-like cells display phenotypic heterogeneity similar to GBM tumors	scRNA-seq, snRNA-seq, Genome-wide CRISPR–Cas9 screens	GBM patient material, GBM stem-like cultures	Richards et al.[54]
➢ GBM cell-macrophage crosstalk induces GBM cell transition to mesenchymal-like cell state	scRNA-seq, FACS, MERFISH, functional assays	GBM patient material, Transgenic mouse model	Hara et al.[89]

Abbreviations: GBM, glioblastoma; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single nuclei RNA sequencing; scATAC-seq, single-cell assay for transposase-accessible chromatin with high-throughput sequencing; FACS, fluorescence-activated cell sorting; PDOX, Patient-derived orthotopic xenograft; ChIP-seq, chromatin immunoprecipitation sequencing; RNA-seq, ribonucleic acid sequencing; TME, tumor microenvironment; RTK, receptor tyrosine kinase; CSC, cancer stem cell; NG2, neuron-glial antigen 2; PTPRZ1, protein tyrosine phosphatase receptor type Z1; YAP/TAZ, yes-associated protein/tafazzin; CRISPR, clustered regularly interspaced short palindromic repeats; MERFISH, multiplexed error-robust fluorescence in situ hybridization.

review the role of GBM plasticity in creating a heterogeneous and dynamic tumor ecosystem, where distinct GBM phenotypic states coexist, interacting with each other and with the evolving tumor microenvironment (TME). We consider how phenotypic heterogeneity and plasticity allow tumor cells to escape treatment and develop resistance mechanisms. Finally, we discuss the therapeutic potential of targeting molecular regulators determining GBM heterogeneity and plasticity.

Cancer Stem Cells and Intrinsic Developmental Plasticity – An Evolving Concept

Intra-tumoral heterogeneity in GBMs has been described at various molecular levels. Distinct genetic clones arise following Darwinian principles of hierarchical evolution where the selection of the fittest clones leads to a final genetic equilibrium.[5] Models explaining the creation and maintenance of phenotypic heterogeneity, defined as diversity in epigenetic, transcriptomic, proteomic, and metabolic profiles, are more complex. The initial cancer stem cell (CSC) hypothesis, established over 20 years ago posits that so-called CSCs or Tumor Initiating Cells (TICs) are solely responsible for tumor development and establishment of intra-tumoral phenotypic heterogeneity in a hierarchical manner. CSCs were postulated to display diverse stem cell properties and to be highly tumorigenic in experimental models *in vivo*. Identification of CSCs in GBMs was largely based on the expression of stemness-associated cell membrane antigens such as CD133, CD15/SSEA, CD44, or A2B5 or intracellular markers such as Sox2 and Nestin.[6–12] Recently, Glycerol-3-phosphate dehydrogenase 1 (GPD1) was proposed as a marker of dormant GBM CSCs with a distinct metabolic profile.[13] A hierarchical organization of GBM was also suggested based on cell clone tracing via genetic barcoding upon serial xenotransplantation[14] and lineage tracing in mouse models.[15] CSC phenotypes are maintained by a plethora of signaling pathways commonly active in healthy stem cells, such as WNT, Notch, TGFβ, and MET pathways.[16–18] In analogy to neural stem cells that terminally differentiate to neuronal and glial cells, GBM CSCs may give rise to more differentiated phenotypes with astrocytic or neuronal features (Figure 1).[6] A number of molecules regulating the switch between CSCs and non-CSCs have been described and include nitric oxide driving activation of Notch signaling and CSC phenotypes,[16] Bone morphogenetic protein 4 (Bmp4) driving astroglia-like differentiation and quiescence,[19,20] and retinoic acid driving the aberrant neuronal differentiation process.[21] These mechanisms are currently considered as potential therapeutic targets.

At the same time, a growing body of evidence emerged indicating that a unidirectional hierarchical CSC model is not entirely applicable to GBMs. Numerous studies showed that irrespective of CSC marker expression, cells were able to self-renew and proliferate indefinitely. Diverse GBM cells were tumorigenic in experimental models: consistently all

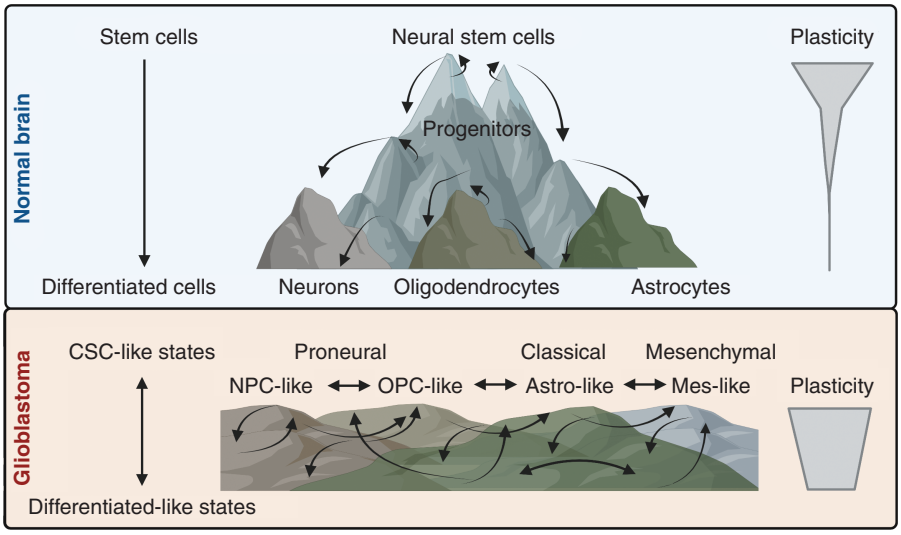

Fig. 1 **Dynamic organization of phenotypic heterogeneity in GBM**. The creation of phenotypic heterogeneity in GBM differs from the hierarchical differentiation process of normal stem cells. Neural stem cells create various committed progenitors and differentiated cells in a unidirectional hierarchical process. Reversibility of the differentiation process is very limited and can occur only between closely related progenitors and stem cell populations. In contrast, GBM constitutes dynamic and diverse tumor cell populations, where high plasticity is retained in all cells and differences between CSC-like and differentiated-like states are rather small. GBM cells exist in gradients of transcriptomic states, with multiple axes of variation. Interchanges have been documented between TCGA subtypes (Proneural, Classical, Mesenchymal), single-cell states (Neural progenitor cell (NPC)-like, Oligodendrocyte progenitor cell (OPC)-like, Astrocyte (Astro)-like and Mesenchymal (Mes)-like) as well as CSC-like and differentiated-like states. The phenotypic equilibrium at the population level is dictated by the genetic background, TME cues and treatment. Created with Biorender.com.

cell populations gave rise to tumors either with equal[11,22,23] or with different potency.[24–29] Similarly, both CSCs and non-CSCs were found to be multipotent and able to regain the initial heterogeneity,[22–24,26,29,30] incongruent with the concept of hierarchical organization. In line with this, we have shown that cellular states arise via stochastic state transitions of existing populations, evolving towards a heterogeneous equilibrium instructed by the TME.[29] Mathematical modeling confirmed the lack of a hierarchical process, yet different subpopulations may differ in the time required to establish the equilibrium. Interestingly, most plastic subpopulations (ie, fast in regenerating heterogeneity) displayed accelerated tumor growth *in vivo*.[29] A similar effect was seen with $NG2^+$ and $NG2^-$ subpopulations that displayed differential tumorigenicity *in vivo* following direct implantation, while this effect was lost after the recreation of initial heterogeneity *in vitro* prior to implantation.[27] Thus, although certain GBM subpopulations may exhibit differences in functional assays, CSCs do not appear to constitute a defined cellular entity, but rather a flexible cellular state cooperating with other states and adapting to TME cues. Importantly, unlike neural stem cell differentiation, differentiation of CSCs is not terminal, and "differentiated" GBM cells (ie, GBM cells expressing differentiation markers) can revert to CSC phenotypes (Figure 1).[19,21,29] Extensive tumor cell plasticity has also been uncovered in other malignancies, which lead to an evolving concept of the classical CSC hypothesis.[31–34] Plasticity is defined as the inherent ability to interconvert from one cellular state to another in a stochastic nonhierarchical manner.[35–37] Current data point to a very limited differentiation axis in GBMs where CSCs appear as a context-dependent phenotype (Figure 1). Both CSCs and more differentiated GBM phenotypes represent different states that can flexibly interchange while subjected to various stimuli in TME or upon treatment. While CSC-like states retain a full plasticity potential, differentiated-like phenotypes may show a more stringent potential, requiring longer times for phenotypic interchange.[37] It remains to be seen, whether the axis of bidirectional conversions between CSCs and more differentiated phenotypes can be therapeutically exploited. Whilst we refer the readers to recent seminal reviews in the CSC field,[37–42] we will focus here on novel findings highlighting GBM heterogeneity and plasticity at different molecular levels and in the context of the complex GBM ecosystem.

Transcriptomic Heterogeneity and Inferred Plasticity

Initial attempts to study cellular heterogeneity in GBMs, including CSCs, were based on a limited number of markers and isolation of cells for *in vitro* studies, which carry several limitations. Marker-based purification methods are not 100% efficient and do not take into account the underlying genetic heterogeneity. Many stemness markers are also expressed by nonneoplastic cells in the brain, further obscuring cell purification.[4,11,43,44] Isolated CSC and non-CSC populations were often cultured under different conditions[6] leading to a divergence of molecular profiles that reflect culture conditions rather than intrinsic cellular properties.[45] While only a limited number of markers was generally assessed at the functional level, a defining set of CSC markers could not be established for GBMs. Furthermore, it has been shown that self-renewal *in vitro* may not predict *in vivo* tumorigenic potential[29,46] and cells subjected to brain TME show distinct growth dependencies.[47] Meanwhile the assessment of intra-tumoral heterogeneity in patient samples was long hampered by capturing data from bulk populations and limited deconvolution algorithms. Bulk transcriptomic profiles of patient tumors allowed to identify inter-patient differences, which led to the initial TCGA subtyping (neural, proneural, classical, and mesenchymal) based on TME-dependent signatures.[48] More refined studies revealed that the neural subtype represents samples with limited tumor content, retaining the tumor-intrinsic signatures of proneural, classical, and mesenchymal.[4]

Application of single-cell and single nuclei RNA sequencing (scRNA-seq and snRNA-seq respectively) to capture transcriptomic signatures within GBM patients revolutionized our understanding of the underlying molecular heterogeneity. The initial study by Patel et al.[49] showed that GBMs are composed of cells of different TCGA subtypes with multiple cells of intermediary signatures, suggestive of state transitions between phenotypes. Signatures linked to stemness, hypoxia, and quiescence revealed continuous gradients of expression, rather than distinct cellular subpopulations. Using scRNA-seq and cell lineage tracing combined with functional assays, Neftel et al.[50] ultimately demonstrated cellular transitions based on four single-cell transcriptomic signatures dictating the primary axis of variation: Astrocyte (AC)-like, Neural progenitor cell (NPC)-like, Oligodendrocyte progenitor cell (OPC)-like and Mesenchymal (MES)-like (Figure 1). While NPC, OPC, and AC-like expression signatures resemble neurodevelopmental programs, MES-like cells do not mirror any normal brain cells. Multiple cellular states are present in each GBM and all contain proliferating cells, incompatible with a hierarchical organization. These transcriptomic states are partially correlated with expression of cell membrane epitopes. Again, such marker-defined fractions (positive and negative) are tumorigenic *in vivo* and reconstitute the transcriptomic heterogeneity of the parental tumor.[50] It remains to be determined if different states reconstitute heterogeneity and *in vivo* tumor growth at the same speed. Recent studies revealed additional gradients based on various cellular properties including proliferation, stemness and neurodevelopmental programs,[51] proneural-to-mesenchymal axis,[52] cellular specialization, metabolism,[53] TME and injury responses (Table 1).[54] The continuous gradients of transcriptomic heterogeneity across tumor cells are in line with protein expression profiles commonly detected, for example, by flow cytometry. Though the interdependence between different gradients appears evident, the exact inter-correlations remain to be determined. Of note, proliferating cells are consistently found in multiple phenotypic states and common CSC markers are broadly expressed and patient-specific, suggesting a variety of active stemness programs across different phenotypes.[55]

Single-cell transcriptomic states in IDHwt GBMs differ from those identified in lower-grade IDHmut

astrocytomas,[56] oligodendrogliomas,[57] and H3K27 mutant pediatric gliomas.[58] Analyses of IDHmut and H3K27 mutant gliomas require different gene signatures and suggest a more hierarchical organization. Here proliferating cells reside mostly in stem-like states, whereas Astro-like and Oligo-like cells rarely contain cycling cells. The limited availability of patient-derived preclinical models of IDHmut lower-grade gliomas hampers the functional validation of multipotency of these states. The proportion of cycling stem-like cells increases in the most aggressive high-grade IDHmut gliomas and is high in H3K27 mutant gliomas,[56,58] suggesting that stemness and plasticity correlate with tumor aggressiveness. A recent analysis revealed common pan-glioma signatures, which combine previously described entities into differentiated-like (IDHwt AC/MES-like states and IDHmut Astro/Oligo-like states), stem-like, and proliferating stem-like states (IDHwt NPC/OPC-like states and IDHmut stem-like states).[59]

The identification of cellular states and reversible plasticity between states raises the question of the underlying factors that drive these phenomena. Below we review tumor-intrinsic and TME-driven factors that contribute to the complex and dynamic organization of the GBM ecosystem (Figure 2).

Intrinsic Tumor Characteristics Defining Intra-tumoral Phenotypic Heterogeneity and Plasticity

Genetic Background

The underlying genetic background of the tumor directly contributes to plasticity and phenotypic heterogeneity. GBMs rarely contain equal proportions of single-cell transcriptomic states and the ratio is skewed by patient-specific genetic associations (eg, *PDGFRA*, *EGFR*, and *CDK4* amplification, *NF1* loss), where the most abundant phenotypic state defines the molecular subtype at the bulk level.[50] *PDGFRA* amplified tumors are generally enriched for OPC-like states, *CDK4* amplified tumors for NPC-like states, and *EGFR* amplified tumors for AC-like states, while GBMs with *NF1* loss contain higher proportions of MES-like states. The molecular mechanisms behind these differences are still unknown. Regardless of this genetic bias, scRNA-seq showed that genetic clones defined, for example, by different chromosomal aberrations present within the same tumor clearly recapitulate phenotypic heterogeneity and display multiple transcriptomic states.[49,50] At the functional level it was shown that genetic clones defined by different ploidy (ie, number of sets of chromosomes) display heterogeneous cell membrane marker expression and recreate phenotypic heterogeneity at the population level, suggesting that phenotypic heterogeneity provides a survival advantage to the tumor.[60] These heterogeneous profiles can adapt upon clonal selection, most likely in response to TME niches.[29] Genetic clones are also spatially distributed across different niches,[61] further impacting phenotypic heterogeneity in the spatial context. This implies that phenotypic heterogeneity and plasticity of GBM cells represent a general phenomenon also in the framework of underlying genetic heterogeneity.

In contrast to the hierarchical process of conventional genetic evolution, extrachromosomal DNA (ecDNA) carrying oncogenes shows a more plastic behavior. As ecDNA structures do not contain centromeres, they are randomly distributed to daughter cells, creating an additional level of heterogeneity.[62] Although the mechanisms regulating ecDNA maintenance are not fully understood, it was found that the generation of ecDNA can be flexibly regulated following therapeutic stress. *EGFRvIII*-containing ecDNAs are lost upon treatment with erlotinib and reemerge rapidly in surviving GBM cells.[63] Genetic heterogeneity based on ecDNA carrying *EGFRvIII* is also rapidly reconstituted from purified cells with or without *EGFRvIII* amplification, further demonstrating the plastic behavior of ecDNA emergence. Whether other sub-clonal ecDNA events such as *EGFR* or *PDGFRA* amplifications display similar plasticity remains to be seen. Gene amplifications carried on ecDNAs are often dynamic during glioma treatment,[64] and it is currently unclear if this is due to clonal selection, plasticity of ecDNA production, or both.

Permissive Epigenome

Epigenetic mechanisms such as DNA methylation, histone modification, and chromatin remodeling are essential in shaping dynamic gene expression. The phenotypic equilibrium in GBM is dictated in part by the cell of origin,[65] implicating a key role of the subjacent epigenome in shaping phenotypic heterogeneity. DNA methylation further magnifies the outcome of chromosomal aberrations, as amplified genomic regions (eg, chromosome 7) show low DNA methylation, whereas regions of copy number loss appear highly methylated.[66] Copy number alterations also associate with DNA methylation disorder, that is, discordant DNA methylation status comprising methylated and unmethylated CpGs in regulatory sequences, which mark epigenetically dynamic regions.[59] DNA methylation profiles enable the stratification of GBMs into several subclasses that largely correlate with transcriptomic subtypes.[2,3] Similar to transcriptomic states, disparate DNA methylation and chromatin accessibility profiles are found not only in spatially separated tumor zones but also at the single-cell level in different phenotypic states.[52,66–68] The recent analysis by Chaligne et al.[66] showed that IDHmut gliomas contain cells with LGm1-LGm3 DNA methylation subtypes, while IDHwt GBMs show gradients of LGm4-LGm5 subtypes. LGm4 cells represent AC-like and MES-like states and LGm5 cells are mostly NPC-like and OPC-like, highlighting a closer resemblance of these state pairs at the DNA methylation level.

Still, the epigenetic regulation of flexible GBM cellular states remains poorly understood. Globally, DNA of GBM cells is hypomethylated, creating open and active chromatic areas similar to pluripotent states. Analysis of phylogenetic trees based on DNA methylation revealed that most differences between phenotypic states arise from stochastic passenger changes, rather than encoded cell state differentiation events.[66] Promoter regions regulated by Polycomb repressive complex 2 (PRC2, responsible

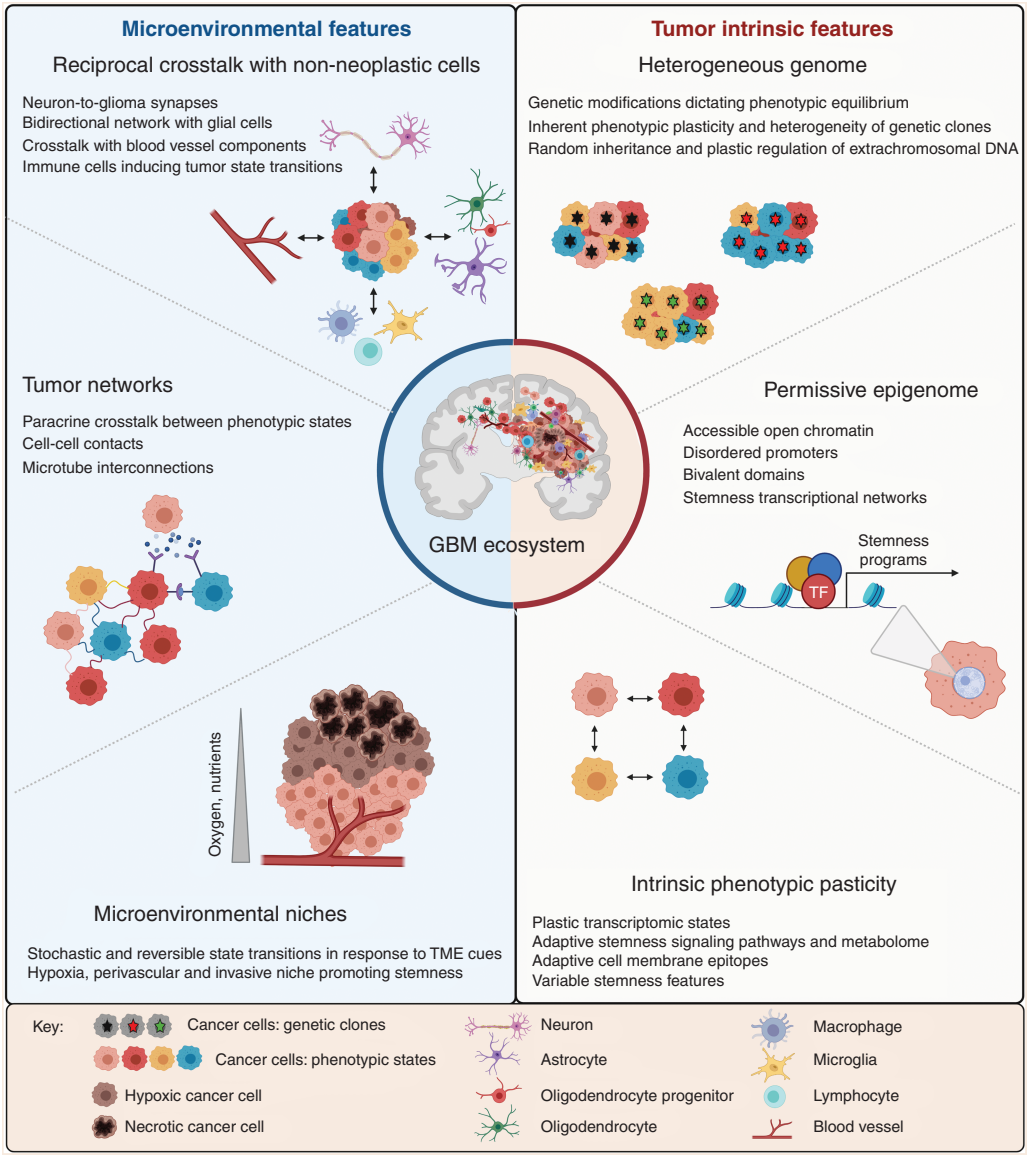

Fig. 2 **Intrinsic and microenvironmental features of the GBM ecosystem defining plasticity and intra-tumoral heterogeneity.** The GBM cellular ecosystem comprises of diverse tumor cells residing in different TME niches. Tumor cell plasticity and the equilibrium of phenotypic states at the population level is defined by multiple tumor-intrinsic features and extrinsic cues from the TME. Created with Biorender.com.

for depositing H3K27me3 repressive mark) linked to stemness pathways show strong hypomethylation and low DNA methylation disorder (HOX and Homeobox genes, lineage-specific transcription and growth factors).[59,66] Interestingly, NPC/OPC-like states show modestly higher DNA hypomethylation of PRC2 targets than AC/MES states, which correlates with enhanced chromatin accessibility and increased active histone marks such as H3K4me3 and H3K27ac.[66] On the other hand, regulatory elements of AC/MES-like signature genes are highly hypomethylated and accessible in these states, suggesting a combined role of DNA and histone methylation with DNA accessibility in state transitions. Intermediate states at the proneural–mesenchymal axis show heterogeneous profiles with partial overlap of proneural (NPC/OPC-like) and mesenchymal accessibility features, and appear largely associated with AC-like states.[52,69] Again, stemness-associated chromatin profiles were present across different phenotypic states and heterogeneous CSC-like states. The relative difference in DNA methylation levels between phenotypic states are minor and certain gene promoters (eg, *Prominin-1/CD133* (*PROM1*), *MutL Homolog 1* (*MLH1*)) show heterogeneous accessibility profiles without detectable DNA methylation. Promoters of many developmental and signature-specific genes were also identified as bivalent in GBM, defined by the presence of active H3K4me3 and repressive H3K27me3 histone marks combined with low DNA methylation levels.[70] Bivalent domains, initially described in pluripotent

stem cells, are indicative of plasticity as they allow for temporal suppression of transcription while protecting the genes from irreversible silencing by DNA methylation and keeping them "poised" for action.[71] Such domains were reported on PRC2 target gene promoters and were enriched in NPC/OPC-like states.[66] Although the simultaneous presence of active and repressive histone marks remains unproven at the single-cell level, current data suggest a "primed" status of nonexpressed signatures and epigenetically-encoded plasticity between transcriptomic states. On the other hand, higher DNA methylation levels and DNA methylation disorder are present at the promoters of genes associated with cell differentiation processes, leading to reduced gene expression. High DNA methylation disorder is also present at DNA elements regulated typically by transcription factors associated with extracellular stress stimuli such as hypoxia (eg, *HIF1A*), most probably facilitating plasticity during stress.[59] Continuous pressure via hypoxia or radiotherapy leads to accumulation of additional DNA methylation disorder, further enhancing GBM plasticity.

Stemness Transcriptional Network

GBMs highjack core transcriptional networks of reprogramming reminiscent of pluripotent stem cells. The pluripotency reprogramming transcription factors Sox2 and c-Myc are widely active in GBM cells; Oct3/4, Nanog, and Klf4 were also reported though at lower levels.[72] Genetic activation of pluripotency or of neural-specific transcription factors (Brn2, Sox2, Sall2, Olig2) induces tumorigenic CSC-like states in GBM via modulation of epigenetic regulators (eg, Rcor2/Lsd1 histone demethylase, DNA methyl transferase Dnmt1) and noncoding RNAs (eg, HOTAIR, MALAT-1).[73–76] Such reprogramming can be triggered by oncogenic pathways, such as HGF/cMET signaling,[77] or TME cues, such as hypoxia.[78] Of note, Sox2 expression appears rather ubiquitous in GBM cells, regulating distinct downstream gene networks in stem-like and differentiated-like cells.[79] A set of active enhancers and transcription factors were also found to be subtype-specific, where Sox10 repression led to chromatin remodeling and transition towards the mesenchymal state.[80] Other factors, such as Ascl1 can activate a switch towards more differentiated states.[81]

GBM Plasticity and the Tumor Microenvironment

Tumor Microenvironmental Niches

TME conditions have a strong impact on the phenotypic equilibrium of spatial and temporal heterogeneity in GBM. While initial reports suggested a preselection of CSCs in TME niches, such as hypoxia, perivascular area, or invasive zone, it has become clear that GBM cells undergo dynamic and reversible transitions in response to TME changes. Barcoding technology confirmed the lack of cellular selection during invasion into the surrounding brain, highlighting phenotypic adaptation as the main mode of action.[14] Invasive cells activate diverse molecular mechanisms, for example, reminiscent of radial glia or proneural features, allowing for digestion of extracellular matrix and migration.[55,82,83] TGF-β driven mesenchymal phenotypes were also reported to be highly invasive.[84] Similarly, hypoxia and associated *pH* and glucose levels are potent inducers of phenotypic adaptation[85,86] leading to quiescence, activation of survival mechanisms such as autophagy,[87] and mesenchymal features.[52,88,89] A phenotypic switch towards CSC-like states can be induced, for example, by HIF1α-driven activation of VEGFA and CD133 in severe hypoxia (<1% O_2),[90,91] by HIF2α-driven activation of a pluripotent transcriptional network in modest hypoxia (2–5% O_2),[78] or by endothelial cell-derived nitric oxide in the perivascular niche.[16] Hypoxia may decrease global DNA methylation by reducing the availability of methionine and induction of nicotinamide N-methyltransferase (NNMT), leading to a mesenchymal switch and increased tumorigenicity.[92] TME-driven gradients depend on variable chromatin regulators such as Polycomb repressive complexes: while proneural states are driven by EZH2 in vascular niches, hypoxic mesenchymal states depend on BMI1, depositing H3K27me3 or H2AK119Ub histone marks respectively.[88] Niche adaptation follows a stochastic state transition model, where GBM cells create patchworks encompassing the most favorable phenotypic states.[29] Interestingly, although TME drives distinct phenotypic states towards TME-specific equilibria, the transition speed may not be equal across all tumor cells.[29] Analysis at the single-cell epigenetic level is needed to understand why certain GBM cells can create TME-specific equilibria faster than others.

Molecular Crosstalk and Tumor Networks

Paracrine crosstalk between phenotypic states plays a key role in shaping the overall GBM ecosystem. Wang et al. showed that reciprocal crosstalk between tumor cells of different phenotypes creates supportive growth stimuli via BDNF-NTRK2-VGF paracrine signaling.[93] Cells with more differentiated phenotypes stimulate stem-like states, promoting tumor initiation and growth.[93,94] Such paracrine mechanisms could explain the increased tumor growth capacity of those GBM subpopulations, that are more efficient in recreating heterogeneity.[29] Paracrine crosstalk via soluble CD109 was reported between cells of the tumor core and invasive edge.[83] Apoptotic GBM cells in the necrotic zone release extracellular vesicles that transport components of spliceosomes to neighboring viable cells, which subsequently modulate RNA splicing and promote survival in the recipient cells.[95] Phenotypic crosstalk also exists between different genetic clones, for example, *EGFRvIII*-amplified cells release cytokines such as Il-6 and LIF, which directly activate gp130 and *EGFR* in surrounding *EGFRwt*-amplified cells, leading to sustained tumor growth.[96]

In addition to paracrine signaling, GBM cells communicate with each other via direct cell-cell contacts, via exosomes or microtubes. IDHwt GBM and IDHmut high-grade astrocytoma cells interconnect via ultra-long tumor microtubes protruding from the cell membrane, which enhances survival and resistance to radio- and

chemotherapy.[97,98] Recent data show that connected cells possess enhanced stem-like features[99] and compensate for the loss of cells in the perivascular niche following Notch1 inhibition.[100] It remains to be seen to what extent this functional network plays an active role in state transitions upon tumor expansion and treatment escape.

Crosstalk with TME Subpopulations

Direct interactions between tumor cells and nonneoplastic cells play a vital role in the maintenance of cellular plasticity in GBM. Recent data demonstrate a critical role for physical contacts between tumor cells and neurons, where crosstalk occurs via molecular and electrochemical signaling through a neuron-to-glioma cell synapse. Some glioma cells (10–30%) can thus hijack the neuronal network to receive electrochemical and paracrine signals promoting growth and invasion.[101,102] Membrane depolarization further enhances cellular communication, where depolarization-induced nonsynaptic calcium currents are amplified via gap junctions of the tumor network itself. Tumor cells are also impacted by the bidirectional crosstalk with nonneuronal cells, including glial cells, endothelial cells, pericytes, resident microglia and infiltrating immune cells. These interactions involve cell-cell contact and paracrine mechanisms, leading to phenotypic adaptation of both tumor and TME subpopulations in different tumor niches. This complex reciprocal interplay has been thoroughly reviewed elsewhere.[37,39,41,103,104]

The genetic and phenotypic status of tumor cells is important in the bidirectional crosstalk and in shaping the TME, although the "what comes first" question remains unresolved. For example, the immune component is influenced by the IDH status in gliomas and differs significantly from brain metastases.[105] IDHmut gliomas display an increased proportion of microglia-derived macrophages, whereas IDHwt GBMs show enhanced infiltration of monocyte-derived macrophages and lymphocytes. The TME varies across different transcriptional GBM subtypes: while mesenchymal tumors contain lower tumor content and a higher proportion of macrophages, neutrophils, and neuroglial cells, classical tumors have increased dendritic cell signatures.[4] Transitions towards mesenchymal/injury response-like GBM states may occur via inflammatory cytokines released by mesenchymal-specific macrophages[54,106,107] such as TNFα, CCL5, CCL12, and G-CSF, further underlining the reciprocal crosstalk between the TME and tumor cell phenotypic states. A recent study by Hara et al.[89] shows that Oncostatin M (OSM) released by macrophages induces GBM transitions towards the mesenchymal state through activation of OSMR/LIFR-GP130 receptors and STAT3 signaling in GBM cells.

The Role of Plasticity in Treatment Resistance and GBM Recurrence

GBM at Recurrence

GBMs relapse quickly independent of treatment, indicating strong intrinsic resistance mechanisms. DNA lesions induced by ionizing radiation and chemotherapy can be repaired by a plethora of DNA damage response mechanisms.[108] Standard-of-care temozolomide (TMZ) treatment confers a narrow survival advantage only to the subset of patients with a silenced *O-6-methylguanine-DNA methyltransferase* (*MGMT*) promoter.[109] Distinct genetic clones may confer variable responses to TMZ and other drugs.[110,111] Still, unlike other solid tumors, only limited genetic changes are detected upon recurrence, indicating a restricted role of genetic evolution in GBM resistance.[112–114] No common treatment-induced genetic trajectories were identified and loss or emergence of mutations is generally patient-specific.[114] Such genetic differences may arise from a different genetic make-up of cells remaining after surgery, rather than treatment-induced changes.[115] Likewise, hypermutation[116] and DNA methylation changes[61,117] are rare in IDHwt GBMs, indicating less pronounced (epi)genetic evolution compared to IDHmut gliomas. Of note, *MGMT* promoter methylation status can differ not only between patients but also within the same tumor, resulting in cells of varying sensitivity to TMZ.[110]

Accumulating evidence suggests the prevalence of resistance mechanisms linked to phenotypic adaptation of tumor cells and TME. At bulk level, GBMs may manifest transcriptomic subtype transitions upon recurrence, although the majority of tumors retain the same subtype.[4] It is plausible that transcriptional subtyping at the bulk level may not have the granularity to understand cellular resistance. Further deconvolution of transcriptomic signals revealed differences in TME composition upon treatment. While an overall tendency towards decreased blood-derived monocytes is observed, mesenchymal transitioning correlates with increased M2-like macrophages, whereas proneural transitions lack immune infiltration.[4] In contrast, recent single-cell data from unpaired patient tumors noted an increase in the proportion of monocyte-derived macrophages in recurrent tumors, while hypermutated GBMs had more CD8+ T cells.[118] Moreover, TME subpopulations were shown to adapt towards resistance-promoting phenotypes, for example, radiotherapy induced dynamic resistance-specific macrophages that can be reverted by Colony-stimulating factor-1 receptor (CSF-1R) inhibition.[119]

Tumor Plasticity as a Mechanism of Resistance

Plasticity allows for the creation of a plethora of cellular states with different sensitivity to the treatment.[120] Treatment-related phenotypic changes can generally be attributed to two scenarios: (1) increased proliferation and selection of preexisting resistant cellular states over time or (2) adaptation of tumor cells towards resistant phenotypes (Figure 3). Such plastic tumor cells, so-called drug-tolerant persisters, can survive therapeutic pressure by adapting towards treatment-resistant states with a faster response than Darwinian selection.[120] Although quiescence was proposed as a main feature of adaptation, proliferating persisters have also been reported.[121] Preexisting resistance may involve different genetic clones, different cellular states or both. While the selected treatment-resistant genetic clones and/or preexisting resistant phenotypic states retain their genotype and phenotype over time,

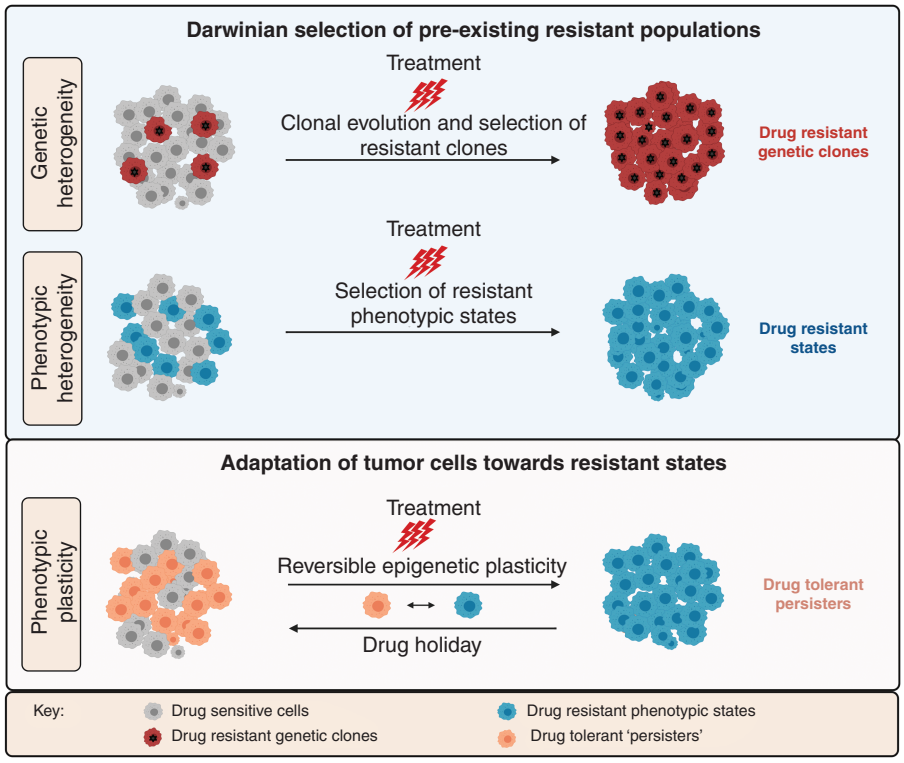

Fig. 3 Tumor heterogeneity and plasticity as resistance mechanisms. Tumors contain cells with varying sensitivity to treatment. Treatment leads to the eradication of drug-sensitive cells. Resistance can be driven by Darwinian selection of preexisting resistant cells with advantageous genetic or phenotypic tumor characteristics. Highly resistant genetic clones may also be acquired upon treatment (ie, clonal evolution and selection). Adaptive resistance is driven by drug-tolerant persisters that survive treatment and adapt towards resistant phenotypic states. Persisters can revert to their initial phenotypic states and recreate phenotypic heterogeneity when released from the treatment (ie, drug holiday). Drug resistance may thus be a result of reversible epigenetic plasticity combined with irreversible clonal expansion. Created with Biorender.com.

drug persisters can revert back to the initial states upon a drug holiday period. Recent reports from breast cancer,[122] lung cancer,[121,123] and melanoma[124] point to multifactorial resistance, indicating that heterogeneous subpopulations can undergo diverse state transitions and activate concurrent genetic and nongenetic resistance mechanisms upon treatment. By following so-called Lamarckian adaptation, that is, inheritance of acquired characteristics, cells surviving drug treatment first undergo plastic and reversible changes at the phenotypic level, some of which may become permanent over time.

To what extent GBM cells with different sensitivity to treatment are reflected by various phenotypic states remains to be determined. The initial CSC studies describe preexisting GBM CSCs to be highly resistant to radio- and chemotherapy through, for example, enhanced activation of DNA repair mechanisms (eg, via MGMT, Chk1, and Chk2), and inhibitors of apoptosis (eg, FLIP, BCL-2, and BCL-XL).[9,15,125] Mouse models show that GBM cells can also escape chemotherapy via ABCG2-driven drug efflux,[126] which is however not reflected in human GBM.[44,127,128] On the other hand, a preexisting proneural-to-mesenchymal gradient was shown to correlate with resistance to radiation and multi-drug treatment, without a direct link to CSC-like phenotypes.[129] Other studies also do not find convincing evidence for CSC selection and describe resistance in non-CSCs.[130–132] This controversy may in part be explained by diverse definitions of CSCs (eg, quiescent vs proliferative) and variable proliferative properties of the studied populations across patient tumors and preclinical models. Tumors containing quiescent CSC-like cells may show increased stemness upon treatment due to their lower susceptibility to radio- and chemotherapy, whereas tumors driven by proliferative CSC-like cells may not show such selection.[19] The recent insight into GBM plasticity proposes additional scenarios. While some GBM cells may preexist in highly treatment-resistant states, persister cells can activate various adaptive mechanisms upon treatment, such as quiescence,[13] induction of regulatory loops of mRNAs, small and long noncoding RNAs,[133] and transition to stem-like states.[25,130,134] Stemness pathways can then act as protectors against treatment, for example, Notch signaling attenuates resistance to radiotherapy via upregulation of PI3K/Akt and Bcl-2 survival pathways.[135] Ionizing radiation activates a switch from $CD133^+$ to $CD109^+$ stem-like phenotypes in invasive cells, concomitant with CCAAT/enhancer binding protein β (C/EBPβ)-mediated transition from proneural to YAP/TAZ-dependent mesenchymal signatures.[51,136] Similar plasticity has been described in the

context of anti-angiogenic treatment, where tumor cells adapt to TME changes by upregulating glycolysis, invasion, and mesenchymal features via ZEB1-regulated mechanisms.[137,138]

Cellular plasticity is also involved in the resistance to targeted drugs. Receptor tyrosine kinase inhibitors, a major class of targeted therapeutics, generally lead to plastic escape mechanisms via activation of alternative signaling pathways.[139] Dasatinib, a PDGFRA inhibitor, was shown to activate reversible GBM transitions towards quiescent Notch and KDM6-dependent persister states via remodeling of H3K27 modifications from H3K27me3 to H3K27ac and activation of neurodevelopmental programs.[140] These states can also preexist in treatment-naïve GBM and are high at baseline in certain stem-like cultures, suggesting a variable balance between preexisting and adaptive resistance in different tumors. Moreover, scRNA-seq combined with lineage tracing showed that this adaptive resistance coexists with irreversible genetic evolution towards novel resistant clones.[141] Adaptive resistance was also observed via single-cell phosphoproteomic analysis upon mTOR inhibition, where GBM cells shift from mTORC1/C2 to ERK and Src signaling.[142] Further studies are needed to reveal the molecular mechanisms and epigenetic regulators underlying treatment-induced GBM plasticity in the context of standard-of-care and targeted therapies. We speculate that GBMs may differ with regards to the ratio of preexisting resistant cells versus adaptive persisters. Based on the vast plasticity described in GBM, resistance most likely originates in large part from adaptive changes of drug-tolerant persister states. Moreover, the signatures of resistance are likely to be treatment-specific rather than universal.

Perspectives

The concept of CSCs at the apex of a hierarchical organization in GBM brought major hopes for straightforward therapies that could eradicate the entire tumor by specifically targeting CSCs at their roots. Over the years numerous promising targets have been proposed including cell membrane markers and stemness signaling pathways.[38,40] The evidence of powerful intrinsic cellular plasticity dampens these expectations as at the therapeutic level, tumor plasticity represents a conceptual departure from the classical CSC hypothesis. Indeed, so far none of the identified targets passed the preclinical efficacy. For example, $CD133^+$ CSCs with anti-CD133 antibodies or CD133-specific CAR-T cells did not result in complete elimination of GBM in preclinical models, only temporary effects are observed and tumors regrow as soon as the treatment is halted.[143] Similarly, cell differentiation protocols are largely unsuccessful in eliminating proliferating GBM cells. Thus, GBM eradication will require targeting the dynamic states rather than single entities. To achieve this, further studies are needed to reveal the drivers of plasticity and treatment escape. The molecular signatures of preexisting treatment-resistant and plastic persister GBM cells in the context of standard-of-care and targeted therapies remain largely unknown. Future studies should address which of the phenotypic changes are fast and reversible, and which are retained in tumors long after treatment. The assessment of the ratio between preexisting treatment-resistant and persister cells may allow patient stratification according to different treatments. Initiatives such as the GLASS consortium[112] will reveal long-term changes in longitudinal patient samples prior and after treatment. While scRNA-seq is still limited to fresh samples, adaptation of the technology to single nuclei extracted from frozen or fixed tissue samples opens new opportunities. Tumor multisampling, spatial-omics and emerging technologies permitting simultaneous assessment of genetic, epigenetic, and transcriptomic information will foster an integrative analysis of dynamic states in a spatio-temporal context.

On the other hand, identifying short-term reversible drug-induced adaptations will require experimental models. These changes may be masked in recurrent patient samples because of the drug holiday phenomenon and/or long-term evolution of the tumor post-treatment. Combining drug exposures directly with single-cell multi-omics[144] and functional analyses in clinically-relevant models will accelerate the functional characterization of preexisting and adaptive resistant states. In this context, patient-derived organoids[145–147] and orthotopic xenografts (PDOXs),[43,45,148,149] which recapitulate tumor heterogeneity and TME niches, should be preferred over *in vitro* cell lines. Barcoding lineage tracing strategies[50,121] will allow the tracking of single cells in a spatio-temporal manner. This may overcome the limitation of (sc)RNA-seq that captures gene expression at a specific snapshot in time and does not reveal the relationship between treatment-naive cells and their resistant progeny. Inclusion of (sc)RNA-seq analysis of tumor dynamics as part of clinical trials may be key to investigating resistance mechanisms towards targeted treatment and discriminate responders from nonresponders.

The pressing question remains on how to design therapies against a dynamic target. Gene regulatory networks, master regulators, and epigenetic modifiers dictating tumor plasticity may represent more powerful targets than signature molecules of resistant subpopulations per se.[88,150] Noncoding regulators, such as miRNAs or long noncoding RNAs are additional emerging therapeutic targets.[76] Reversible feedback loops in signaling pathways and selective translation of mRNAs marked by N6-methyladenosine (m^6A) modification are emerging examples of other molecular layers of plastic regulation of state transitions. Interestingly, Shen et al. showed that mRNAs selected for translation in melanoma persister cells largely comprise chromatin regulators and stress-response kinases.[151] Blocking cellular state transitions in melanoma[124] and other cancers[152,153] effectively decreased heterogeneity and delayed the onset of resistance. Targeting of Retinoid X receptor-γ (RXRG), a master regulator responsible for the reversible shift towards treatment-resistant melanoma, successfully inhibited transitions towards drug-resistant states.[124] Regulators of mesenchymal states, such as NF-kB, STAT3, YAP/TAZ, or C/EBPβ might represent therapeutic targets for GBM resisting standard-of-care therapy. On the other hand, mesenchymal states were recently linked to a higher abundance of cytotoxic T cells,[89] creating novel opportunities for immunotherapies. Lastly, the synergistic effects of genetic evolution and nongenetic state transitions upon treatment will have to be considered,[35] as new genetic modifications may influence the capacity of state transitions and the

population equilibrium of phenotypic states. Relying solely on hierarchical Darwinian selection (genetic or nongenetic) or tumor plasticity may not be sufficient.[154] Models developed by evolutionary ecology, which simultaneously take into account selective and adaptive factors, may bring novel understanding of the dynamic processes in tumors.[155] Novel modalities such as the use of nonlethal doses to control state transitions and retain sufficiently less aggressive drug-sensitive/permissive states in the tumor ecosystem merits experimental validation. In conclusion, major research efforts are needed to unravel the molecular mechanisms and regulators of GBM plasticity and generate effective drugs against a moving target.

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