Cancer Letters 527 (2022) 66–79

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Mini-review

# Unravelling glioblastoma heterogeneity by means of single-cell RNA sequencing

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ARTICLE INFO

*Keywords:*
*Glioblastoma*
*Single-cell analysis*
*scRNA-seq*
*Intra-tumoral heterogeneity*
*Tumor microenvironment (TME)*

ABSTRACT

Glioblastoma (GBM) is the most invasive and deadliest brain cancer in adults. Its inherent heterogeneity has been designated as the main cause of treatment failure. Thus, a deeper understanding of the diversity that shapes GBM pathobiology is of utmost importance. Single-cell RNA sequencing (scRNA-seq) technologies have begun to uncover the hidden composition of complex tumor ecosystems. Herein, a semi-systematic search of reference literature databases provided all existing publications using scRNA-seq for the investigation of human GBM. We compared and discussed findings from these works to build a more robust and unified knowledge base. All aspects ranging from inter-patient heterogeneity to intra-tumoral organization, cancer stem cell diversity, clonal mosaicism, and the tumor microenvironment (TME) are comprehensively covered in this report. Tumor composition not only differs across patients but also involves a great extent of heterogeneity within itself. Spatial and cellular heterogeneity can reveal tumor evolution dynamics. In addition, the discovery of distinct cell phenotypes might lead to the development of targeted treatment approaches. In conclusion, scRNA-seq expands our knowledge of GBM heterogeneity and helps to unravel putative therapeutic targets.

## 1. Background

Glioblastoma (GBM) is the most frequent and deadly primary neoplasm of the central nervous system (CNS) in adults [1]. This tumor is characterized by pronounced heterogeneity, high infiltrative power, and the presence of treatment-resistant GBM stem cells (GSCs). GSCs are thought to stand at the zenith of the tumor-cellular hierarchy and fuel tumor growth by generating a diverse differentiated progeny of cancer subclones that constitute the bulk of the tumoral mass [2]. Additionally, the TME plays a critical role in disease progression and requires further investigation [3].

An important milestone that paved the way to understanding the molecular landscape of GBM was The Cancer Genome Atlas (TCGA) project [4,5]; an undertaking that involves the comprehensive genomic and transcriptomic analysis of hundreds of tumors. Soon after, inter-patient heterogeneity was described in four-tier expression-based subtypes, namely, proneural (PN), classical (CL), mesenchymal (MES), and neural (NL) [6]. This classification has been demonstrated in other studies using bulk RNA sequencing [7,8]. Additionally, intra-tumoral heterogeneity was observed [9,10] by performing biopsies from non-adjacent localized areas from the same specimen to analyze the tumor core and tumor periphery separately. Although this research has led us to obtain relevant information regarding GBM biology, the underlying diversity of GBM tumors is misrepresented by bulk methods. The challenge posed by this complex disease is that tumor relapse after treatment is almost universal and standard-of-care therapies have barely evolved over the past few decades [11]. Fortunately, the increased resolution of single-cell approaches can be leveraged to unravel key features from highly heterogeneous tumors, such as GBM [12].

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https://doi.org/10.1016/j.canlet.2021.12.008
Received 5 October 2021; Received in revised form 6 December 2021; Accepted 7 December 2021
Available online 10 December 2021

**Abbreviations**

| | |
|---|---|
| AC | Astrocyte |
| BBB | Blood-Brain Barrier |
| CAF | Cancer-Associated Fibroblast |
| CL | Classical |
| CNS | Central Nervous System |
| CNV | Copy Number Variation |
| CSC | Cancer Stem Cell |
| DC | Dendritic Cells |
| DNA pol I | DNA polymerase I |
| FAP | Fibroblast Antigen Protein |
| GAM | Glioblastoma Associated Macrophage/Microglia |
| $GAM_B$ | Blood-derived Glioblastoma Associated Macrophage |
| $GAM_M$ | Microglial-derived Glioblastoma Associated Macrophage |
| GBM | Glioblastoma |
| GPC | Glial Progenitor Cell |
| GSC | Glioblastoma Stem Cell |
| IDHwt | IDH wild-type |
| IDHmt | IDH-mutant |
| IFC | Integrated Fluidic Circuit |
| IVT | *In Vitro* Transcription |
| MES | Mesenchymal |
| NK | Natural Killer |
| NL | Neural |
| NPC | Neural Progenitor Cell |
| NSC | Neural Stem Cell |
| OPC | Oligodendrocyte Progenitor Cell |
| oRG | Outer Radial Glia |
| PCA | Principal Component Analysis |
| PN | Proneural |
| QC | Quality Control |
| RG | Radial Glia |
| RNAase | Ribonuclease |
| scRNA-seq | Single-cell RNA sequencing |
| SNV | Single Nucleotide Variation |
| TCGA | The Cancer Genome Atlas |
| TME | Tumor Microenvironment |
| Tregs | Regulatory T cells |
| TSO | Template Switching Oligo |
| UMI | Unique Molecular Identifier |

## 2. Single-cell RNA sequencing

Population-based RNA studies measure the average expression level of each gene across all cells in a given sample. The relevant biological information corresponding to the minority cell compartments is omitted in this homogenization [13]. Gene expression insights can be gained at the single-cell level using scRNA-seq techniques [14], which are capable of overcoming traditional bulk RNA-seq limitations [15] and solve experimental questions that require single-cell resolution, such as identification of cell subpopulations responsible for treatment recurrence, profiling of novel or rare cell subtypes, surveying the TME composition, or studying tumor architecture and plasticity [16,17].

The existing scRNA-seq protocols function by similar underlying principles but differ at specific steps [18]; cell isolation, second cDNA strand synthesis, barcoding strategy, multiplexing, and sequencing library construction might be performed differently depending on the scRNA-seq protocol. Here, we present a typical scRNA-seq pipeline for GBM, which can be divided into nine steps (Fig. 1).

a. Sample harvesting

This step poses a non-trivial challenge in the clinical setting, as strong coordination among neurosurgeons and researchers is necessary to immediately transport biopsies from GBM patients undergoing surgical resection on ice. The sample manipulation time should be reduced to a minimum (within 2–3 hours of resection) to prevent the alteration of cellular states of interest and increase confidence in the results.

b. Single-cell suspension

Viable, fresh cells are typically required for optimal performance; however, some protocols can process cryopreserved or snap-frozen samples [19–21]. In this step, tumor dissection is followed by careful mechanical breakdown (cutting, chopping), enzymatic dissociation (papain, collagenase, and accutase), and several filtering and washing steps to remove myelin debris and RBCs. The exact protocol can be tailored by the researcher or standardized using available commercial kits (e.g., Tumor Dissociation Kit 130-095-929, Miltenyi Biotec). Once a single-cell suspension is obtained, precise enrichment is optional by cell-sorting the sample, previously stained with a fluorophore-conjugated antibody. Enrichment is common in GBM to isolate TME populations of interest (e.g., staining with CD45 for immune cells, CD3 for T cells, CD31 for endothelial cells). This purification process yields better results, as some cell identities are considerably less abundant than tumor cells. Additionally, some viability dyes can be used to distinguish live cells (e.g., calcein-AM, PI, or 7-AAD) as the presence of dead cells, free nucleic acids, and altered transcriptomes have been identified as the major sources of undesired technical variation [22].

c. Cell isolation

Single-cell suspensions are derived to separate volumes for physical single-cell isolation. The so called "plate-based" methods (CEL-seq [23], MARS-seq [24], Quartz-seq2 [25], SCRB-seq [26], STRT-seq [27], and Smart-seq2 [28]) isolate cells into wells on a plate, while integrated microfluidic circuits (IFCs) [29] can isolate cells to microstructures, such as hydrodynamic traps (Fluidigm C1 [30]), microdroplets (Drop-seq [31], InDrops [32], and Chromium 10X Genomics Single Cell 3 Solution [33]), or nanowells (Seq-Well [34], CytoSeq [35], Microwell-seq [36], and STRT-seq-2i [37]). The use of microfluidics reduces cost and technical variability while scaling up to a massive parallelized processing of cells. Although not strictly based on single cells, spatial transcriptomics are also becoming highly popular in the field [38]. For example, the Visium Spatial Gene Expression platform from 10X Genomics enables the preservation of sample spatial information by capturing and labeling transcripts directly in tissue sections [39].

d. Cell lysis, mRNA capture, and barcoding

Every well or droplet contains reagents necessary for cell lysis and RNA release. Most techniques take advantage of the presence of polyadenylated tails on mature messenger RNAs (mRNAs) to avoid contamination from other abundant RNA species, although alternative protocols for profiling total RNA or micro RNAs are also available [40,41]. It is worth noting that scRNA-seq potency relies on barcoding strategies to identify transcripts of parental cells after pooling. At this step, 3′ or 5′ tag-based techniques use indexed poly(A) capture RT primers for both cellular and individual transcript barcoding by unique molecular identifier (UMI) sequences [42]. In contrast, full-length methods do not allow early barcoding, and cell identity is later assigned during library construction. In terms of mRNA capture efficiency, methods with higher throughput showed decreased sensitivity

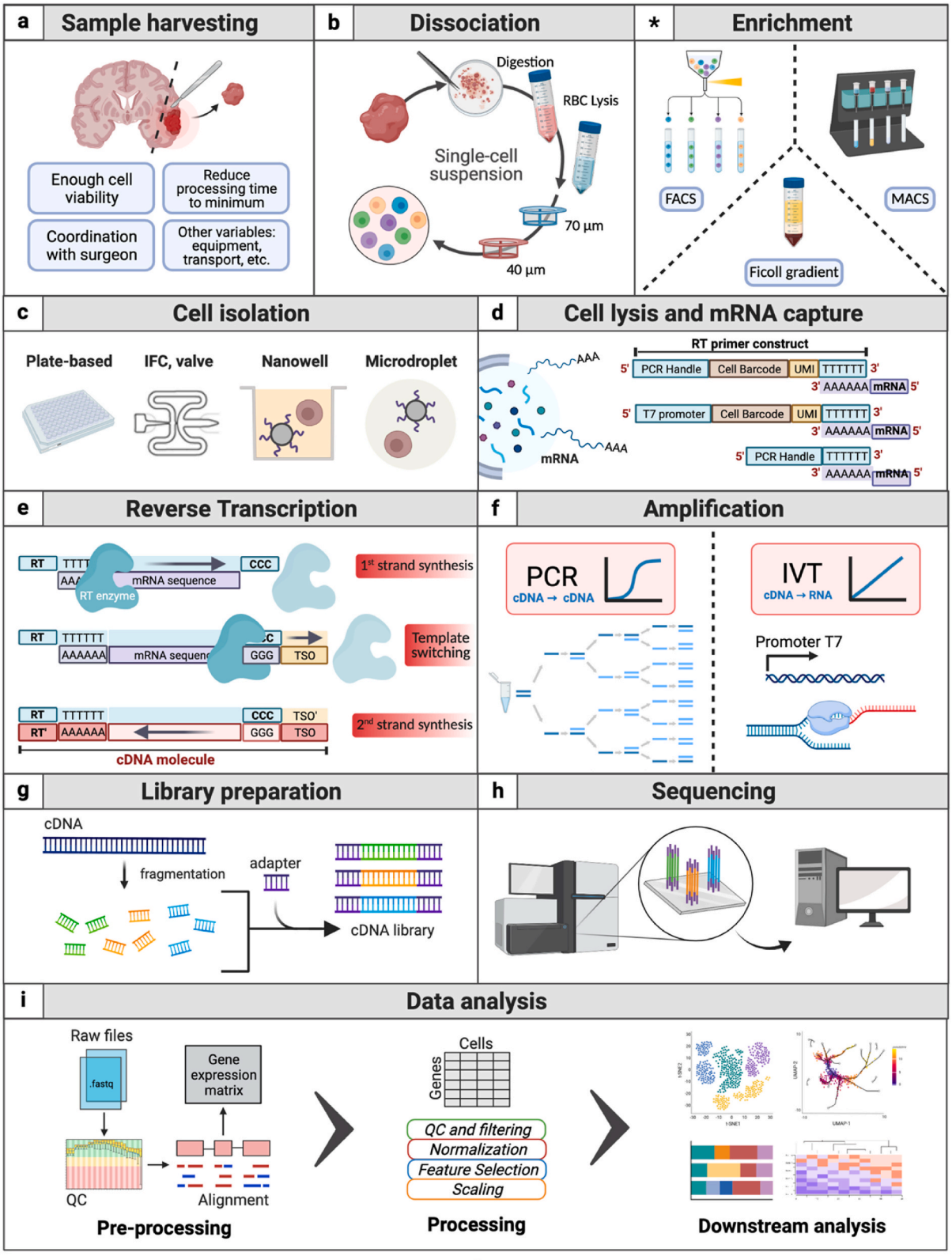


**Fig. 1.** General pipeline of a scRNA-seq experiment. After sample harvesting (a), specimen dissociation (b) is required to obtain a single cell suspension. Prior to single-cell capture, sample enrichment (*) is an optional step. Individual cells are then physically isolated from each other in microliter volumes (c), for cell lysis and mRNA capture through oligo(dT) RT capture primers (d). Most methods apply unique barcoding for each cell, allowing to identify cell-specific derivation for each transcript. Transcripts are then reverse transcribed into a first strand of complementary DNA (cDNA), followed by the second strand synthesis to generate a pool of double-stranded cDNA (e). The example on the figure depicts TSO chemistry, but other strategies exist. Finally, either PCR or IVT amplification is performed over pooled or non-pooled samples (f) for posterior library preparation (g) and high-throughput sequencing (h). Data analysis (i) can be summarized in three general steps, i.e., data pre-processing, data processing and downstream analysis. *RBC*: Red Blood Cell; *FACS*: Fluorescent Activated Cell Sorting; *MACS*: Magnetic-Activated Cell Sorting; *IFC*: Integrated Fluidic Circuit; *PCR:* Polymerase Chain Reaction; *IVT*: *In Vitro* Transcription; *QC*: Quality Control.

when compared to plate-based protocols [43].

e. Reverse transcription

Captured transcripts must be converted into cDNA molecules for amplification and sequencing. Different protocols use distinct methods such as homopolymer termination for poly(A)-based second strand synthesis [44], template switching oligo (TSO) strategy [45], or a combined action of ribonuclease (RNase) H and DNA polymerase (DNA pol I) activity [46,47].

f. Amplification

Amplification before sequencing is a key step in obtaining sufficient amount of cDNA. It can be performed by either PCR or *in vitro* transcription (IVT). Although the IVT yield is linear and introduces lesser amplification bias than PCR, it requires more time and additional steps, such as re-converting RNA to cDNA for sequencing [48].

g. Library preparation

By fragmenting, size-selecting, and ligating specific adapters to amplification products, a cDNA library is built. These steps are required because libraries are typically prepared for short-read sequencing. Different methodologies perform fragmentation, either enzymatically (e.g., Smart-seq2 [28], Drop-seq [31], and Seq-Well [34] use tagmentation), chemically (e.g., MARS-seq uses zinc [24], InDrops [32], and CEL-seq [23] use KOAc and MgOAc), or mechanically (e.g., Quartz-seq2 [25] uses ultrasound). This is followed by the ligation of sequencing adapters. Protocols that do not barcode RNA molecules during reverse transcription, typically full-length methods, add cell-specific barcodes at this step. While each sample leads to one library in RNA-seq, each sequencing library in scRNA-seq corresponds to a single cell.

h. Sequencing

The samples are multiplexed before sequencing to prevent unwanted technical biases through a balanced design [49]. There is a fixed amount of sequencing capacity even on high-throughput sequencing machines. The capacity of sequencing can be used in different ways: either by augmenting the sequencing target size (number of cells) or the sequencing depth, which is determined by the number of reads per cell, and it increases the sensitivity (number of genes detected per cell) [18].

i. Data analysis

High-throughput sequencing technologies generate a massive amount of raw material, which requires intense processing to provide biological significance. The computational challenges of single-cell data have led to the development of specific software and statistical methods [50]. However, the fast progress of this modality caused a lack of standardization regarding the computational aspect of studies, which motivated comprehensive manuscripts on the topic [51,52]. Briefly, demultiplexing and UMI deduplication are computed differently depending on each scRNA-seq platform. Thus, method-specific tools are used to pre-process the data and generate a count expression matrix (for example, Cell Ranger [33] for Chromium 10X Genomics data or scPipe [53] for CEL-seq2 data). Thereafter, the user can choose single-cell-specific computational tools from an extensive list to perform isolated tasks [54]. Alternatively, several packages can be applied as analysis toolboxes to go through all the steps of the data analysis pipeline. Some examples of these packages are Seurat [55,56], Scanpy [57], SCell [58], or scater [59]. In general, the processing of raw expression matrices consists of quality control (QC), normalization, feature selection, scaling, and dimensionality reduction. QC is typically based on filtering, considering biological and technical metrics to preserve only intact live cells for further analysis. Indeed, QC checkpoints at various levels of the analysis ensure information reliability and statistical robustness, preventing bias that could induce biological misinterpretation [60]. The result is a clean, statistically processed expression matrix that is ready for direct research querying. Finally, downstream analyses, such as clustering, cell type annotation, differential expression, or trajectory analysis, aim to extract biological insights from the data. Given the cancerous nature of the sample, concrete computational challenges and opportunities have been presented and insightfully reviewed by others [61].

## 3. Contribution of scRNA-seq to the understanding of GBM

A broader understanding of the various sources of heterogeneity shaping the aggressiveness of GBM tumors will clarify our understanding of previous therapeutic failures and help in improving treatment designs. As opposed to what has already been published [62–64], this manuscript comprehensively dissects all advances provided exclusively by scRNA-seq and delves into the different layers of GBM heterogeneity. Of note, a carefully selected set of articles resulting from a specific semi-systematic search (Supplementary Material) is provided. Given the recent WHO update on brain tumor classification [65], only studies featuring human GBM IDH wild-type (IDHwt) samples analyzed by scRNA-seq are discussed in this section. These studies are summarized in Table 1.

### 3.1. Inter-patient heterogeneity

Unique patient tumor characteristics might be key in developing more effective and personalized treatments. By averaging the data of all tumor samples, the distinctive features are partially ignored. Patel et al. applied single-cell transcriptomics to profile GBM tumors in five patients [66]. They found that each specimen contained cells matching all conventional TCGA subtypes, rather than conforming to a homogeneous population (Fig. 2.1). This finding advocated for updating from a 4 to 3-subtype classification with samples weighted by a simplicity score (ss) based on their purity for a unique subtype [67]. Furthermore, it was observed that samples with a higher proportion of PN cells correlated with improved survival [66]. Interestingly, Yu et al. identified a rare form of ciliated glioma in one patient, represented as 5% in TCGA, characterized by an unreported motile cilium-related phenotype (expressing *FOXJ1*, *FAM183A*, *HYDIN,* and *DNALI1*) [68]. This scenario suggests that several rare forms of GBM may be caused by small patient-specific subpopulations.

Different GBM patients harbor miscellaneous genomic aberrations despite a shared set of genetic hallmarks (Chr7 amplification or Chr10 deletion), which makes each tumor unique [66,69]. Patient-specific copy number variations (CNVs) have been shown to impact the dose effect of gene expression, which results in the fragmented clustering of cancerous cells by tumor origin [66,68,70–73]. This is in line with the vast genetic heterogeneity described between patients, as tumors might be composed of different clones. In agreement with this phenomenon, non-cancerous TME cells from different samples tend to cluster together to be devoid of CNVs, thus showing increased similarities across patients and potentially providing a better therapeutic target [72] (Fig. 2.1).

However, despite the uniqueness of individual tumors, generic expression patterns or meta-signatures can be defined from shared cellular backgrounds across patients (and even across different cancer types), including a set of stressed/hypoxia and cell cycle-related genes [69,73]. On the other hand, context-and disease-specific signatures awaken more interest and will be examined in subsequent sections.

### 3.2. Intra-tumoral heterogeneity

#### 3.2.1. Spatial heterogeneity

Tumor geographical distribution is important for understanding the

**Table 1**
Semi-systematically selected manuscripts for comprehensive review (ordered by publication date).

| Reference | Samples | | | Platform | Transcript coverage | Heterogeneity addressed | Molecular level of analysis | Data accession |
|---|---|---|---|---|---|---|---|---|
| | IDHwt GBM | IDHmt GBM/NOS GBM | Other samples | | | | | |
| Patel et al., *2014* [66] | 5 Primary untreated | – | – | SMART-seq | Full length | Inter-patient; Cellular-neoplastic | Genetics and transcriptomics | GSE57872 |
| Müller et al., *2016* [79] | 3 Primary | – | – | Smart-seq2/ C1 | Full length | Inter-patient; Cellular-neoplastic; Tumor evolution | Genetics and transcriptomics | EGAS00001001900 |
| Lee et al., *2017* [74] | 2 Primary and recurrent | 1 Primary IDHmt | – | Smart-seq2/ C1 | Full length | Spatial; Tumor evolution | Genetics and transcriptomics | EGAS00001001880 |
| Darmanis et al., *2017* [71] | 3 Primary | 1 Primary IDH NOS | – | Smart-seq2/ FACS | Full length | Inter-patient; Spatial; Cellular-neoplastic; Cellular-TME | Genetics and transcriptomics | GSE84465 |
| Müller et al., *2017* [91] | 5 Primary | – | 1 Astrocytoma 1 Oligodendroglioma | Smart-seq2/ C1; 10X Chromium | Full length; 3′-tag | Cellular-TME | Genetics and transcriptomics | EGAS00001002185, |
| Yuan et al., *2018* [81] | 4 Primary 2 Recurrent | 1 Primary IDHmt | 1 Astrocytoma | Microwell-Based scRNA-seq platform | 3′ tag | Cellular-neoplastic; Cellular-TME | Transcriptomics | GSE103224 |
| Neftel et al., *2019* [69] | 20 Primary | – | 5 Primary Pediatric GBM 3 Recurrent Pediatric GBM | Smart-seq2/ FACS; 10X Chromium | Full length; 3′ tag | Inter-patient; Cellular-neoplastic; Tumor evolution; Plasticity | Genetics and transcriptomics | GSE131928 |
| Sankowski et al., *2019* [87] | 4 Primary | 6 Glioma NOS | 11 Epilepsy 2 Carcinoma | mCEL-Seq2 protocol | 3′ tag | Cellular-TME | Transcriptomics | GSE135437 |
| Wang et al., *2019* [110] | 22 Primary untreated | 1 Primary untreated IDHmt | 2 Astrocytoma 3 Oligodendroglioma | Smart-seq2/ C1; 10X Chromium | Full length; 3′ tag | Cellular-neoplastic | Genetics and transcriptomics | EGAS00001003845 |
| Bhaduri et al., *2020* [82] | 9 Primary | – | 1 Anaplastic astrocytoma 1 Gliosarcoma | Smart-seq2/ C1; 10X Chromium | 3′ tag | Cellular-neoplastic | Genetics and transcriptomics | PRJNA579593; SRP132816 |
| Jacob et al., *2020* [72] | 3 Primary | – | – | 10X Chromium | 3′ tag | Inter-patient; Cellular-neoplastic | Transcriptomics | GSE141946 |
| Wang et al., *2020* [105] | – | 3 Primary IDH NOS | – | 10X Chromium | 3′ tag | Cellular-neoplastic | Transcriptomics | GSE139448 |
| Yu et al., *2020* [68] | 6 Primary | 2 Primary IDHmt | 3 WHO grade II 1 WHO grade III 1 Gliosarcoma 1 Brain metastasis (NSCLC) | STRT-seq | 5′ tag | Inter-patient; Spatial; Cellular-neoplastic; Cellular-TME; Tumor evolution | Genetics and transcriptomics | GSE117891; HRA000179 |
| Couturier et al., *2020* [73] | 16 Primary | – | 4 Human fetuses (13–21 weeks of gestation) | 10X Chromium | 3′ tag | Inter-patient; Cellular-neoplastic; Tumor evolution | Transcriptomics | EGAS00001004422 |
| Zhai et al., *2020* [98] | – | 7 Primary IDH NOS | – | 10X Chromium | 3′ tag | Cellular-neoplastic; Cellular-TME | Transcriptomics | CGGA |
| Ebert et al., *2020* [100] | – | 3 Primary IDH NOS | – | 10X Chromium | 3′ tag | Cellular-TME | Transcriptomics | N/A |
| Liu et al., *2020* [90] | – | 1 Primary IDH NOS | – | SCOPE-seq2 | – | Cellular-neoplastic; Cellular-TME; Tumor evolution | Transcriptomics | GSE151137 |
| Goswami et al., *2020* [88] | 4 Primary | – | – | 10X Chromium | 3′ tag | Cellular-TME | Transcriptomics | PRJNA588461 |
| Richards et al., *2021* [83] | 10 Primary | 1 Primary IDHmt 4 Primary IDH NOS | – | 10X Chromium | 3′ tag | Cellular-neoplastic; Tumor evolution | Genetics and transcriptomics | EGAS00001004656 |

(*continued on next page*)

**Table 1** (*continued*)

| Reference | Samples | | | Platform | Transcript coverage | Heterogeneity addressed | Molecular level of analysis | Data accession |
|---|---|---|---|---|---|---|---|---|
| | IDHwt GBM | IDHmt GBM/NOS GBM | Other samples | | | | | |
| | | 2 Recurrent IDH NOS | | | | | | |
| Mathewson et al., *2021* [89] | 16 Primary | 15 Primary IDHmt | – | Smart-seq2; 10X Chromium | Full length; 5′ tag | Cellular-TME | Transcriptomics | GSE163108; DUOS000006 |
| Ravi et al., 2021 [[75], *preprint*] | 16 Primary | 1 Primary IDHmt | 1 Oligodendroglioma 6 Healthy cortex | Visium Platform 10X Genomics | – | Inter-patient; Spatial | Transcriptomics | N/A |
| Pombo et al., 2021 [92] | 7 Primary 4 Recurrent | – | – | 10X Chromium | 3′ and 5′ tag | Cellular-TME | Transcriptomics | GSE163120, EGAS00001004871 |
| Jain et al., 2021 [[103], *preprint*] | – | 1 Primary IDH NOS | – | 10X Chromium | 3′ tag | Cellular-TME | Transcriptomics | N/A |
| Chen et al., 2021 [93] | 7 Primary 1 Recurrent | 1 Primary | – | Microwell-Based scRNA-seq platform | 3′ tag | Cellular-TME | Transcriptomics | GSE141383 |
| Shaim et al., 2021 [99] | 7 Primary | – | 1 Low-grade oligodendroglioma 2 Diffuse astrocytoma | 10X Chromium | 3′ tag | Cellular-neoplastic; Cellular-TME | Transcriptomics | GSE147275 |
| Xie et al., 2021 [101] | 4 Primary | – | – | 10X Chromium | 3′ tag | Inter-patient; Spatial; Cellular-TME | Transcriptomics | GSE162631 |

From PubMed, Scopus and independent literature search (search strategy detailed in Supplementary Material) on September 22, 2021. *GBM* Glioblastoma *IDHwt* IDH wild-type *IDHmt* IDH mutant *NOS* Not Otherwise Specified *NSCLC* Non Small-Cell Lung Cancer.

progression, dissemination, and immune infiltration of a tumor. Lee et al. analyzed multifocal (from multiple locations) and longitudinal (from different time points) biopsies, aiming to characterize the spatiotemporal genomic architecture of GBM [74]. Multifocal tumors were more genetically diverse than locally adjacent tumors, displaying spatial genetic heterogeneity. Importantly, long-term recurrent tumors were seeded from distinct clones, rendering some subpopulations more prone to relapse than others (Fig. 2.2a). Yu et al. also claimed that a single biopsy is insufficient to represent intra-tumoral heterogeneity and proved that individual glioma cells resemble subtypes that change dramatically between different locations in the same tumor [68]. They were able to perform multi-sector biopsies by precision navigation surgery using a 3D-enhanced MRI model. Single-cell and CNV-driven analyses allowed them to spatially and temporally deconvolute the transcriptomic dynamics of GBM initiation, progression, and crosstalk with the TME. The number of somatic CNVs increased across different tumor regions, thus defining a clear pattern of tumor progression based on CNV accumulation [68]. Based on the hypothesis that environmental conditions shape transcriptomic states, a large cohort of different age groups and anatomic regions of control and GBM patients was spatially resolved using the 10X Visium platform [75]. The study revealed that local subclonal distribution was not random, with little or no overlap among CNV-bearing cells. Interestingly, a spatial overlap of chromosome 7 amplification and hypoxia-related signature was observed, and enrichment analysis of these cells revealed dysregulation of genes related to increased migratory capacity. On the other hand, the highly diffuse nature of GBM led researchers to hypothesize that disease recurrence, after tumor resection, is driven by the remaining long-distance migratory cells. Darmanis et al. separately biopsied the tumor core and periphery and performed scRNA-seq [71]. The authors confirmed that the presence of TME cells, mostly immune cells, usually contributes to a proportion of bulk results. In addition, the inner core of the tumor presents more hypoxia- and adhesion-related genes than the tumor margin. They also distinguished low percentages of proliferating cancer cells, which were more abundant in the tumor core than in the infiltrating fraction. This is in agreement with the results of previous studies, suggesting that hypoxia promotes GSC expansion *via* HIF-1α expression [76]. Interestingly, neoplastic infiltrating cells from peritumoral samples showed a converging mechanism of dissemination, summarized in a 22-gene signature, regardless of their parental tumor [71]. Lastly, distinct myeloid populations in the tumor core with a tumor-supporting role (pro-angiogenic and anti-inflammatory macrophages) were replaced by pro-inflammatory microglia in the peritumoral space [68,71].

These findings suggest that the spatial molecular information of GBM might help in understanding tumor initiation, progression, cell populations driving recurrence, and TME roles in disease homeostasis. The molecular features of long-distance migrating neoplastic cells might share vulnerabilities for putative therapeutic targets, as current knowledge is primarily based on tumor core samples. Therefore, the dissemination of subclones resistant to therapies should be considered for targeted treatments. Finally, the spatial organization of infiltrating immune cells is of high interest in targeting appropriate cell compartments.

### 3.2.2. *Cellular heterogeneity*

scRNA-seq cancer research has enormously contributed to deciphering the exact cellular composition of tumor specimens [77]. The different cell types coexisting within the tumor, along with the molecular diversity in individual cells, conform to a complex ecosystem that hampers treatment efficacy. The identification of clinically relevant (e.g., drug resistant, aggressively migrating, highly proliferative, immunosuppressive, etc.) subpopulations may act as new biomarkers and therapeutic targets.

*3.2.2.1. Cancerous cell fraction.* Cells in a scRNA-seq dataset are commonly classified as either malignant or normal based on the presence or absence of CNVs, respectively [78]. However, aneuploidy does not completely explain intra-tumoral heterogeneity. Point mutations and altered transcriptional programs may largely contribute to the existence of diverse subpopulations [66]. Through the use of scRNA-seq, it was shown that tumor-specific somatic variants, occurring in specific neoplastic clusters, produce unique gene expression patterns [71]. In accordance with previous studies [66], Müller et al. found multiple genetic variants of receptor tyrosine kinases (e.g., *EGFR*) linked to a

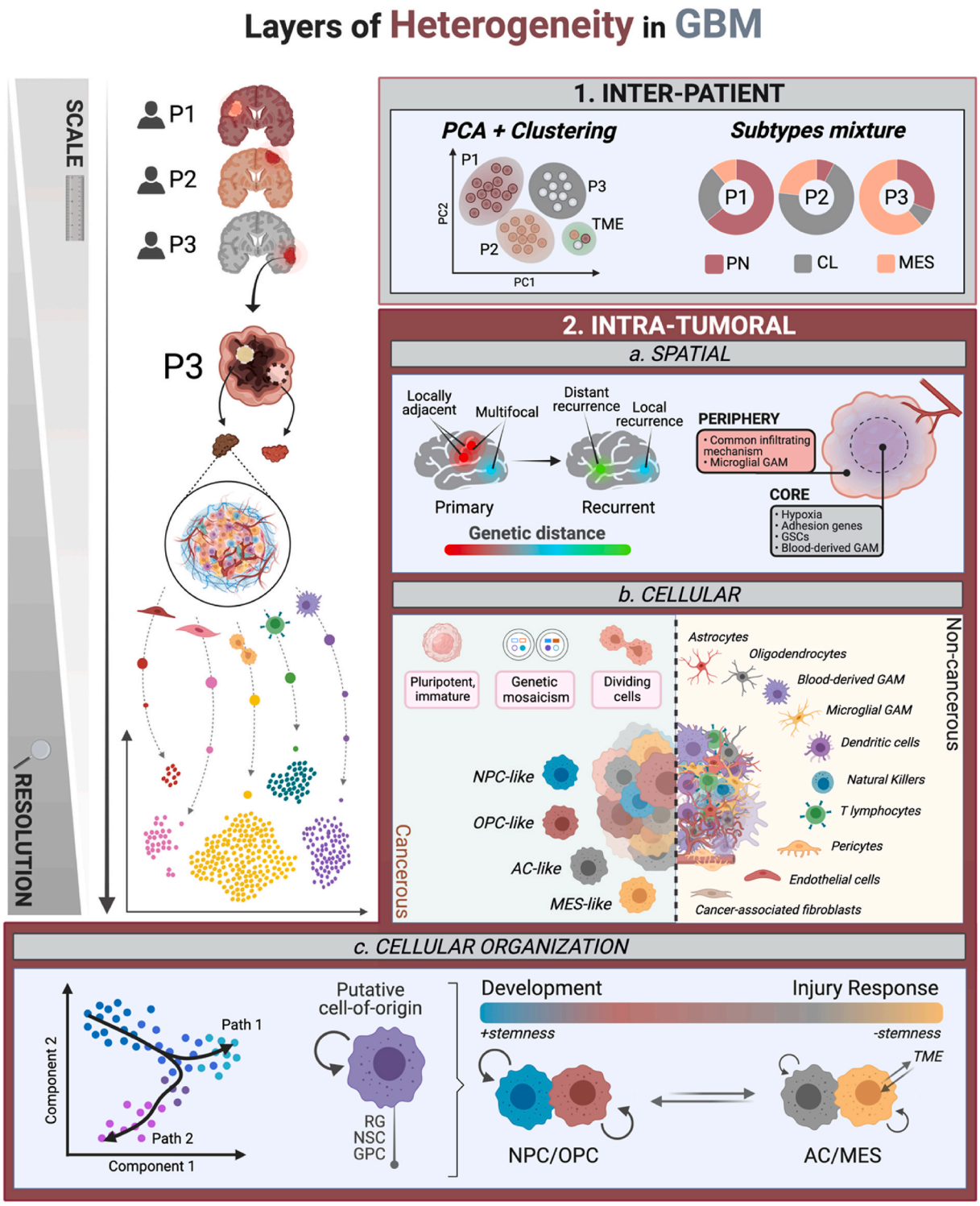


**Fig. 2.** Zooming into the different levels that depict the complexity of GBM by means of single-cell RNA sequencing. (1) Inter-patient heterogeneity is mainly reflected by fragmented clustering and subtypes mixture. (2) Intra-tumoral heterogeneity can be studied in terms of (a) spatial distribution, (b) cellular diversity and (c) cellular organization. *PCA*: Principal Component Analysis; *GAM*: Glioblastoma-Associated Macrophage/Microglia; *NPC*: Neural Progenitor Cell; *OPC*: Oligodendrocyte Progenitor Cell; *AC*: Astrocyte; *MES*: Mesenchymal; *RG*: Radial Glia; *NSC*: Neural Stem Cell; *GPC*: Glial Progenitor Cell; *TME*: Tumor Microenvironment.

mosaic pattern of receptor expression [79]. Indeed, they discovered an in-frame deletion in the *PDGFRA* gene as a potential therapeutic target. The effect of clonal heterogeneity on gene expression was assessed by reconstructing clonal lineages in tumor phylogenies. Mutational profiles were found to be associated with distinct transcriptional programs during tumor evolution. Specifically, PDGF-driven GBM tumors showed a milder phenotype, progressively inducing OPC-like cells by aberrantly activating oligodendrogenesis developmental programs. Conversely, more aggressive EGFR-driven GBM tumors have been linked to invasive transcriptional programs. However, in other single-cell studies, it has been reported that *EGFR* alterations are late-stage events, while *PIK3CA* alterations occur early during tumor evolution [74]. In addition, other studies have shown that important therapeutic targets such as immune checkpoints PD1 and CTLA ligands are inconsistently expressed in GBM

cells [66,71], suggesting the existence of alternative inhibitory mechanisms. These results demonstrate that genetic and transcriptional intra-tumoral diversity at important therapeutic targets can seriously compromise treatment efficacy [80].

Conversely, other authors have clearly stated that genetics does not explain GBM heterogeneity. Instead, somatic mutations shape the proportion of cellular states within a tumor by favoring one of them [69,73]. Neftel et al. performed an elegant computational analysis that revealed six meta-signatures of genes converging into four highly recurring cellular states across multiple tumors [69]. The authors defined astrocyte-like (AC-like), mesenchymal-like (MES-like), oligodendrocyte progenitor-like (OPC-like), and neural progenitor-like (NPC-like) cells present in every patient, in combinations of two to four states. OPC-like, NPC-like, and AC-like were found to be enriched in, but not specific to, tumors comprising *PDGFRA*, *CDK4*, and *EGFR* alterations, respectively, whereas MES states were characterized by *NF1* alterations, vast immune infiltration, and hypoxia [69]. These states matched TCGA subtypes and are consistent with other studies suggesting that genomic aberrations do not perfectly explain GBM subtypes [5,73]. Despite the fact that pioneering work in this field associated GSCs with either a quiescent signature [66] or low percentages of proliferating cells resembling OPCs [71], this has since been revoked. GBM states resembling distorted developmental cell types (NPC and OPC) harbor higher fractions of neural and oligo-lineage cycling cells and form more aggressive tumors than the two other states (AC and MES), comprising more differentiated reactive astrocyte cancer cells and mesenchymal cancer cells, respectively [69,73]. In addition, Yuan et al. observed a strong correlation between the proliferative state of GBM and OPC-like populations, suggesting that GSCs were preferentially associated with proliferation instead of quiescence [81]. Indeed, Bhaduri et al. and others [69,81] demonstrated that every putatively transformed cell expresses high GSC marker levels, suggesting that all cancer cells in GBM display a pluripotent, immature, and plastic state [82] (Fig. 2.2b). In another study [83], high transcriptional diversity in the GSC fraction was verified. Two core transcriptional programs have been shown to be related to the main states observed in GSCs. One program contained cells resembling gliogenesis/neural development. The other group of cells showed a mesenchymal and inflammatory-related signature. These two fractions formed a transcriptional gradient that, together with a second axis of variation along differentiation, might underpin inter-GSC heterogeneity [83]. These results might explain why fluctuating GSC markers have been proposed, but no consensus has been reached [84,85].

*3.2.2.2. TME cell fraction.* The interaction between tumor cells and non-malignant cells from the microenvironment influences the progression and outcome of several cancers [86]. However, there is scarce information about the cell identities conforming to the TME of GBM. Pre-enrichment based on specific cell markers has helped to uncover the complexity of non-cancerous cells [87–89]. In brief, normal astrocytes, oligodendrocytes, glioma-associated macrophages/microglia (GAM), several T cell phenotypes, dendritic cells (DCs), natural killer (NK) cells, pericytes, and endothelial cells (ECs) have been unmasked by scRNA-seq in the GBM TME [90] (Fig. 2.2b).

The myeloid compartment accounts for the greatest proportion of GBM TME and is the most common query in scRNA-seq because of its diversity. Müller et al. defined two types of GAMs based on their ontogeny: blood-derived macrophages ($GAM_B$) and brain-resident microglia ($GAM_M$) [91]. Consequently, a signature of 66 differentially expressed genes between $GAM_B$ and $GAM_M$ was identified [91]. It was shown that $GAM_B$ significantly infiltrates the GBM core, upregulates the expression of immunosuppressive cytokines, and has an altered metabolism when compared to that of $GAM_M$ [91]. This myeloid dual ontogeny was also recently noted by Pombo et al. [92], who explored the evolution of the GAM compartment across disease stages by sequencing both newly diagnosed and recurrent tumors. Tumor-associated microglia in treatment-naïve patients switched toward monocyte-derived GAMs in recurrent ones, which were characterized by a more heterogeneous immune compartment [92]. According to these results, Goswami et al. identified a unique $CD73^{hi}$ immunotherapy-resistant population in GBM, which is more similar to $GAM_B$ than to resident microglia, and it negatively correlated with survival [88]. By merging new and published scRNA-seq data, Chen et al. recently discovered a previously unreported pro-tumor myeloid subpopulation of $GAM_B$, marked by the expression of MARCO receptors [93]. Higher counts of $MARCO^+$ macrophages were observed at the hypoxic tumor core and were associated with anti-inflammatory traits. Of note, GBM IDHwt samples, but not IDH-mutant (IDHmt) or lower-grade glioma specimens, harbored significantly greater amounts of these GAMs, which also correlated with poorer prognosis and the MES subtype. Indeed, it has been shown that MARCO GAMs are recruited by tumor cells through the secretion of a set of factors (e.g., CSF1, TGF-β) [93]. Similarly, the crosstalk between myeloid and GBM cells was partially deconvoluted by Yu et al. through a ligand/receptor interaction map [68]. The role of several actors of the CXCL family was highlighted in the interaction; a mechanism of immune recruitment also suggested by others [81,88].

Although $GAM_B$ are often classified as pro-tumor cells, the yolk sac-derived $GAM_M$ phenotype does not perfectly resemble homeostatic microglia, and their role is still uncertain [94]. Another scRNA-seq study showed that $GAM_M$ gene signatures substantially differ from microglia in healthy brain by upregulating the expression of inflammatory, metabolic, and hypoxia-associated molecules and downregulating the expression of microglia core genes [87]. In fact, the *P2RY12* gene was more specific for characterizing microglial GAMs than the commonly used microglial marker CX3CR1. Furthermore, three transcriptional states were uncovered in the GAM compartment of GBM by lineage trajectory analysis [68]. The microglial phenotype turned into polarized blood-derived macrophages and converged into M2 macrophages with activated expression of angiogenesis-signaling molecules [68]. This transitional state potentially explains the fact that most $GAM_B$ simultaneously co-express canonical markers of M1 and M2 activation phenotypes [91], dimming the controversy surrounding this topic [95,96].

Other immune cells coexist in the GBM environment in fewer numbers than myeloid cells [88]. In a comprehensive single-cell study, Mathewson et al. depicted the gene expression landscape of GBM-infiltrating T cells and identified four major T cell clusters composed of cytotoxic T cells, helper T cells, regulatory T cells (Tregs), and cycling T cells [89]. By focusing on clonally expanded tumor-reactive T cells, they demonstrated a correlation between cytotoxicity gene expression and NK cell signature. The inhibitory NK receptor CD161 was expressed in tumor-infiltrating lymphocytes but absent in Tregs or patient-matched peripheral lymphocytes. Interestingly, CLEC2D (CD161 ligand) was primarily expressed by malignant and myeloid cells, revealing similarities with the PD-1/PDL-1 system [89]. CD161/CLEC2D and other mechanisms render GBM-infiltrating T lymphocytes dysfunctional, correlating them with poor prognosis [97]. The scRNA-seq study conducted by Zhai et al. revealed an evolutionary trajectory of T cells in GBM, indicating functional differences at the initial and end stages of disease progression by gradually expressing suppressive immune checkpoints [98]. Analogously, Shaim et al. demonstrated that GBM-infiltrating NK cells display a reduced cytolytic function, marked by inhibitory receptors upregulation and activating receptors downregulation when compared with NKs from healthy individuals [99]. By analyzing scRNA-seq data from seven adult GBM tumors, they hypothesized that GSCs specifically alter NK phenotypes to evade NK-mediated killing. The authors experimentally demonstrated that this mechanism was based on the direct contact of GSCs with infiltrating NK cells *via* the v-integrin/TGF-β axis, which releases the immunosuppressive cytokine TGF-β and subsequently activates other pathways [99].

Despite most studies focusing on the immune composition of the

GBM TME, some studies have also examined the heterogeneity of non-immune cell types. Ebert et al. [100] identified a putative tumor-antigen expressed in ECs, pericytes, and tumor cells, which was absent from healthy cells. Fibroblast antigen protein (FAP) appeared to be a unique feature of angiogenic tumor blood vessels, correlated with GSC markers and poor prognosis. More recently, EC heterogeneity was investigated by Xie et al. to elucidate their role in blood-brain barrier (BBB) impairment, which commonly causes cerebral edema and neurologic damage [101]. Canonical marker-based annotation of $CD31^{+}$ sorted cells from core and peripheral tumor samples revealed the presence of vascular, glial/neuronal, and immune cells. Re-clustering over ECs revealed five phenotypically distinct groups. Two of them reflected exclusive specialization from individual patients. The other three clusters showed anatomical functional differences, with vascular leakage of ECs preferentially occurring by transcellular transport (vesicular transcytosis) in the GBM core, whereas in peripheral tumor regions it was mediated by BBB transporters [101]. Exploring the role of ECs could provide useful knowledge for improving treatment administration and responses.

Another population of interest in the TME of several tumors is cancer-associated fibroblasts (CAFs) [102]. Although CAFs have been barely studied, CAF-like cells have been recently identified in GBM after enriching a patient sample through serial trypsinization [[103], *preprint*]. Single-cell data from the isolated population confirmed the identity of CAF-like cells, although non-uniform expression of CAF markers suggested the existence of subtypes. Additionally, lineage trajectory analysis indicated that bone marrow-derived mesenchymal stem cells (MSCs) are the shared source of pericytes and CAFs in GBM, which greatly overlap in transcriptomic profiles. Further experiments demonstrated a pro-tumoral impact of GBM CAFs over the TME and tumor microvasculature, as well as their prevalence in GSC-rich areas (e.g., perivascular and subventricular zones). A larger cohort and further studies will be needed to support these results and elucidate the extent to which GBM patients could benefit from CAF-targeted therapy. Finally, although not comprehensively analyzed by scRNA-seq in humans, other cell compartments, such as astrocytes, neurons, and oligodendrocytes, might play a central role in maintaining GBM homeostasis [104].

### 3.3. Cellular organization and plasticity

The variety of neural lineages in GBM led researchers to hypothesize that tumor organization is governed by the abnormal recapitulation of brain developmental programs, as was observed in low-grade glioma and other cancer types [70,78,106,107]. To test this hypothesis, dynamic analyses helped decrypt cellular hierarchies by ordering single-cell transcriptomes on a virtual timeline [108,109]. An *in silico* lineage tracing analysis performed on GSCs described a single axis of variation from proliferating PN cells to quiescent MES cells, resembling gliogenesis [110]. Accordingly, Richards et al. showed that GSCs were distributed across a gradient of transient states defined by two core transcriptional programs, one comprising development-related genes and the other comprising injury response genes [83]. Similar conclusions were reached by others, who described astrocyte-like cells converging to mesenchymal-like cells and OPC/NPC cells branch into cycling cells [90]. A recent study has also supported the idea that a portion of GBM cells mirror brain development lineages, with the transformation to reactive states associated with hypoxia and inflammation underpinning GBM heterogeneity. Interestingly, this reactive transformation of GBM cells was found to be age-related [[75], *preprint*]. Furthermore, Yuan et al. examined the structural organization of malignant cells using force-directed graphs [81]. GBM cells resembling glial and neuronal lineages formed ordered, branched structures with close similarities to development, whereas mesenchymal transformation was associated with the loss of neural signatures and chaotic unstructured conformations [81].

A different strategy to identify the origin of a tumor cell is to use external datasets as reference data. Bhaduri et al. annotated a cell atlas of primary GBM tumors based on the adult human cortex and developing brain datasets [82]. GBM cells resembling outer radial glia (oRG) phenotypes were identified and placed at the apex of the tumor organization. Radial glia (RG) are primary progenitors of uncertain presence in adulthood, with the potential to differentiate into distinct lineages, namely neurons, astrocytes, and oligodendrocytes. These GBM oRG-like cells were described to disseminate through the characteristic behavior of their developmental counterparts (i.e., mitotic somal translocation) [82]. Another study supported this finding by identifying $GLAST^{+}$ RG-like cells in human GBM tumors. Remarkably, they described both cycling and non-cycling RG-like cells, which were driven from dormancy into the cell cycle upon IL-1β exposure [105]. In their study, Couturier et al. used a fetal single-cell dataset to generate a roadmap for GBM cells and confirmed a trilineage hierarchy [73]. Neural, astrocytic, and oligodendrocyte cells were derived from a unique population of glial progenitor cells (GPCs). In contrast with these findings, other studies have shown that malignant astrocytes are derived from GSCs, but they do not share an evolutionary relationship with oligodendrocytes [98].

Building upon the GSC hierarchical model, it is becoming increasingly accepted that transcriptional plasticity exists among stem-like and differentiated GBM cells, which poses another therapeutic challenge [111,112]. For example, Neftel et al. demonstrated that a xenotransplant injection of cells enriched in a single GBM state could recapitulate all states and proportions in the parental tumor [69]. Thus, previous studies suggest that most GBM cells can adopt fluctuating phenotypes with flexibility and interconvert between known states [83]. In fact, it has been claimed that phenotypic heterogeneity follows a non-hierarchical organization, characterized by reversible state transitions induced by driver mutations and microenvironment perturbations [113]. For example, several authors have suggested that mesenchymal-like cells are induced by microenvironment transcriptional reprogramming, as there is a good correlation with the frequency of immune infiltration [69,83]. Importantly, the CSF1-CSF1R axis was putatively assigned as a recruitment mechanism of myeloid cells by mesenchymal tumor cells [81,93]. Therefore, immune cells might secrete molecules that trigger the transition of cancer cells to mesenchymal phenotypes.

All these findings suggest a model in which a pool of GSCs gives rise to more lineage-restricted cells with proliferating potential, thus resembling development. A neural progenitor signature transits into an inflammatory injury response, possibly due to genetic events and microenvironmental cues (Fig. 2.2c). However, it seems that a GSC plasticity model explains tumor biology better, where GBM resembles a dynamic ecosystem governed by reversible state transitions that occur to fulfill tumor needs and improve its adaptive capacity.

## 4. Clinical significance

In recent years, cancer therapy programs have slowly shifted from traditional radio- and chemotherapy to more personalized approaches [114]. Precision medicine builds upon identifying diagnostic, druggable, or prognostic molecular features in tumors, which is a significant challenge in GBM treatment due to its diversity. As cancer biomarkers are typically only partially expressed in tumors, combinatorial therapeutic approaches are highly recommended in these settings. Therefore, it can be concluded that scRNA-seq can successfully guide strategies for the treatment of GBM. In this report, information provided by scRNA-seq analysis is summarized to provide a resource for promoting the development of patient-specific medicinal regimens. The potential clinical significance of scRNA-seq contributions to GBM pathobiology is summarized in Table 2. Although most findings from these studies are not yet clinically applicable, it is expected that further research will set new clinical guidelines for the diagnosis, treatment, and prognosis of GBM patients to increase their survival and quality of life.

**Table 2**
Clinical significance of scRNA-seq contributions to GBM.

| Level | scRNA-seq contribution | Clinical significance |
|---|---|---|
| Inter-patient | Coexistence of GBM subtypes | • Improve prognosis accuracy<br>• Upgrade patient segregation to optimize response to treatment |
| | Discovery of rare GBM subtypes | • Associate clinical information to new molecular information |
| | Miscellaneous genetic alterations | • Non-cancerous TME cells as better inter-patient targets |
| Spatial | Genetic diversity of multifocal tumors | • Required characterization of more than one biopsy |
| | Similarities of GBM infiltrating cells across patients | • Routinely analyze peritumoral biopsies<br>• Target GBM infiltrating cells |
| | GBM subtypes spatially segregated | • Combinatorial therapeutic strategies to target all tumor areas |
| Cellular-neoplastic | Study of tumor phylogenies | • Target truncal alterations |
| | Mosaicism at therapeutic targets | • Withdrawal of ineffective treatments to prevent side effects and toxicity |
| | GSCs associated to proliferation | • Explore the use of metabolic cancer therapy in combination with other regimes |
| | Novel tumor antigens | • New immunotherapeutic targets |
| | scRNA-seq in recurrent GBM | • Identify and target drivers of tumor recurrence |
| Cellular-TME | Characterization of immunosuppressive cell populations | • Target pro-tumoral GAMs |
| | Immunotherapy resistant GAM subsets | • Design second-line immunotherapy drugs |
| | GAM recruitment and transformation | • Target $GAM_B$ recruitment<br>• Reprogramming GAM polarization to increase anti-tumor GAMs |
| | Novel tumor mechanisms of immune escape | • Reactivate natural immune responses by reverting inhibitory mechanisms |
| | Pro-tumoral role of CAF-like cells | • Non-immune target for combinatorial therapy.<br>• Use of anti-angiogenic therapy to control CAF tumor-promoting role in microvasculature proliferation |
| | Altered transcriptome of ECs | • Modulate drug delivery based on BBB phenotype |
| Cellular hierarchies | Identification of cell-of-origin | • Target cells at the apex of tumor organization responsible for fueling tumor growth |
| | IL-1β driving RG-like cells from dormancy into cycle | • Test the therapeutic effect of blocking IL-1β production in GBM |
| Plasticity | GBM cells display a pluripotent, immature and plastic state | • Use differentiation therapy towards stem-like cells to arrest tumor growth |

*GBM* Glioblastoma *TME* Tumor Microenvironment *scRNA-seq* single-cell RNA sequencing *GAM* Glioma-Associated Macrophages/Microglia *CAF* Cancer-Associated Fibroblasts *ECs* Endothelial Cells *BBB* Blood Brain Barrier *RG* Radial Glia.

## 5. Conclusion

GBM is one of the most lethal and aggressive cancers. Its extensive heterogeneity renders standard treatments ineffective; hence, a deeper understanding of the mechanisms driving its pathogenicity is required. scRNA-seq methods, contrary to transcriptome averaging bulk approaches, can be leveraged to uncover the cellular landscapes of GBM and the underlying architecture of this tumor.

In studies on cancer cells, scRNA-seq data have shown that genetic events are not sufficient to explain GBM heterogeneity. Cancer cells span a spectrum of stem-like and proliferative phenotypes, resembling GSCs with varying degrees of differentiation. Therefore, GBM aggressiveness partially resides in a heterogeneous GSC compartment that largely recapitulates neurodevelopmental programs, as tumor organization displays a similar cellular hierarchy. Indeed, most trajectory inference studies describe axes of transcriptional variation ranging from progenitor-like OPC/NPC phenotypes to inflammatory-related AC/MES cells. However, phenotypic transitions occur among GBM cellular states, conforming to an evolving target. Therefore, therapies that prevent plasticity or skew it toward a single subtype should be considered.

Conversely, scRNA-seq technologies have been used to perform a detailed dissection of GBM micro-environment, which is known to be corrupted by the tumor. GAM recruiting or transforming factors, as well as tumor-supporting cell population markers, are promising targets for designing immunotherapy combination regimens. Novel inhibitory systems of natural anti-tumor immune responses could be therapeutically reversed to improve treatment effectiveness. In summary, non-cancerous cell subpopulations have been proposed as promising therapeutic targets for GBM immunotherapy.

Further studies to accurately characterize molecular programming of GSCs and TME will pave the way for designing more effective therapies to control tumor differentiation and disease progression and to stratify patients based on unique features that pose them as candidates to benefit from specific treatments. Importantly, the fact that most of this data is available for re-analysis exposes previously unreported aspects of GBM biology [113,115], which enhances the relevance of the present report, where all previously published datasets are covered.

Notwithstanding the above, the current value of scRNA-seq in clinical and translational medicine still mostly resides in its potential to fathom GBM pathobiology. The academic knowledge gathered by these studies needs to be experimentally validated before being translated into clinical care. On the other hand, the time of maturation, standardization, and development still needs further investigation before scRNA-seq can be incorporated into clinical practice. The most important aspect that needs to be elucidated for the success of scRNA-seq translational process is the extent of its usefulness in decision-making and disease monitoring in a cost-effective manner.

## Funding

This work was supported by grants from the "Fondo de Investigaciones Sanitarias" (FIS) (PI17-01489) and the Ministerio de Economía y Competitividad–FEDERER (RTC-2019-6918-1).

## CRediT authorship contribution statement

**Ana Hernández Martínez:** Methodology, Conceptualization, Search, Data curation, Writing – original draft, Review and editing. **Rodrigo Madurga:** Methodology, Conceptualization, Review. **Noemí García-Romero:** Writing, review and editing. **Ángel Ayuso-Sacido:** Funding acquisition. The authors read and approved the final manuscript.

## Declaration of competing interest

The authors declare that they have no competing interests.

## Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing.

We also thank Tomás Duraj for helpful comments. Josefa Carrión-Navarro provided insightful

suggestions regarding figure composition. Figures created with BioR

ender.com.

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