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STATE-OF-THE-ART REVIEW

From sorting to sequencing in the molecular era: the evolution of the cancer stem cell model in medulloblastoma

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The cancer stem cell (CSC) model posits that tumors contain subpopulations that display defining features of normal stem cells including self-renewal capacity and differentiation. Tumor cells exhibiting these features are now considered to be responsible for tumor propagation and drug resistance in a wide variety of cancers. Therefore, the identification of robust CSC markers and characterization of CSC-specific molecular signatures may lead to the identification of novel therapeutics that selectively abolish this clinically relevant cell population while preserving normal tissue. Brain tumor researchers have been at the forefront of the CSC field. From initial *in vitro* cell sorting experiments to the sophisticated bioinformatic technologies that now exquisitely resolve primary brain tumors at a single-cell level, recent glioma and medulloblastoma (MB) studies have integrated developmental state with genomic and transcriptome data to identify the spectrum of cell types that may drive tumor progression. This review will examine the last two decades of CSC studies in the field. Seminal discoveries, emerging controversies, and outstanding questions will be covered with a particular focus on MB, the most common malignant primary brain tumor in children.

Introduction

The CSC model of tumor heterogeneity has undergone a significant evolution over the past two decades. Originally fuelled by studies in leukemia demonstrating that only a rare subpopulation of cells could re-establish tumor growth following transplantation into immunodeficient mice [1,2], current CSC theory posits that these 'stem-like' cells ultimately drive tumorigenesis. Operationally, these cells are defined by their self-renewal capacity, or ability to reconstitute a xenograft representing the original tumor in serial transplantation assays at clonal density [3]. In this model, only the CSCs are able to generate new tumors following long-term passage in immunodeficient animals. Daughter cells generated by CSCs may be highly proliferative but have limited self-renewal capacity and are ultimately unable to maintain tumor growth long-term. Thus, CSCs or 'cancer-initiating cells' were originally believed to sit atop of the tumor hierarchy. Indeed,

Abbreviations

Atoh1, atonal homolog 1; CSC, cancer stem cell; FACS, fluorescence-activated cell sorting; GBM, glioblastoma; GNPs, granule neuron progenitors; IDH, isocitrate dehydrogenase; MB, medulloblastoma; NES, neuroepithelial stem cells; NPCs, neural progenitor cells; NSCs, neural stem cells; OPC, oligodendrocyte precursor-like cell; *Ptch*, patched homologue 1; scRNA-seq, single-cell RNA sequencing; SHH, Sonic Hedgehog; TPCs, tumor-propagating cells; UBC, unipolar brush cell; WNT, wingless-related integration site.

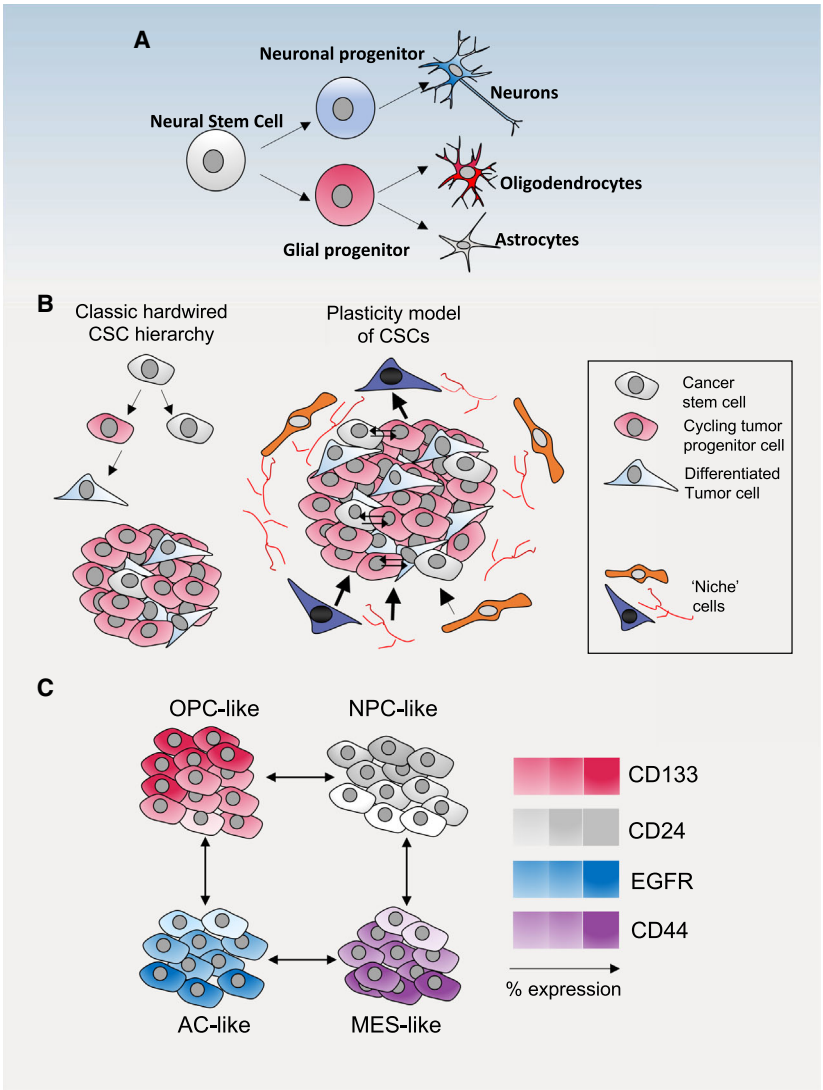

Fig. 1. Classical and updated models of cancer stem cell hierarchies in malignant tumors. (A) Classic neurodevelopmental hierarchical model in which neural stem cells give rise to gradually more restricted progeny including neurons, oligodendrocytes, and astrocytes. Emerging evidence has demonstrated additional complexity and plasticity within this hierarchical organization [114,115]. (B) Classic unidirectional, hardwired model of cancer stem cells (CSCs) (left) and updated plasticity model (right) in which CSCs are not necessarily quiescent or rare. Paracrine signals and interactions with the tumor microenvironment or niche contribute to neoplastic progression. (C) Updated view of 4 cellular states in glioblastoma (GBM) as described by Neftel *et al.* [9]. Cellular states were defined as oligodendrocyte precursor (OPC)-like, neural progenitor cell (NPC)-like, astrocyte (AC)-like, and mesenchymal (MES)-like, with different stem/progenitor cell markers exhibiting significant bias toward a particular state. Arrows depict cellular plasticity.

CSCs are a caricature of normal stem cell developmental paradigms (Fig. 1A), as they exhibit self-renewal capacity and differentiation capabilities, albeit in an aberrant fashion. In many cancer models, CSCs have been shown to be particularly aggressive and are major drivers of therapy resistance through mechanisms such as enhanced DNA repair and drug efflux [3,4]. Yet, despite the obvious clinical implications of these tumor-propagating cells (TPCs), some cancers, such as melanoma [5], do not adhere to the CSC model or exhibit a 'shallow' hierarchy in which a larger proportion of the cell population exhibits tumorigenic capacity. In fact, the CSC model has generated significant controversy attributed to the disparities in frequency, tumor-initiating capacity and proliferative potential of CSCs, the cell surface markers used to isolate putative CSC populations, and the choice of animal model for *in vivo* studies. While originally thought to be rare, quiescent, and adherent to a 'hardwired' unidirectional hierarchy, it has become increasingly clear that tumor stem and progenitor cells exhibit phenotypic plasticity and can transition in response to stimuli from the microenvironment (Fig. 1B).

This conceptual CSC shift has been driven by recent technological advancements in lineage tracing and single-cell RNA sequencing (scRNA-seq). Studies on brain tumors that integrate cellular states with underlying genotypes have been at the forefront of the single-cell biology movement [6–10]. For example, different classes of adult glioma exhibit unique cellular hierarchies, with highly aggressive isocitrate dehydrogenase (IDH)–wild-type glioblastomas (GBMs) exhibiting four malignant highly plastic cellular states, all of which can contribute to tumor growth [9]

(Fig. 1C). The most abundant cellular state within a given tumor dictates the results of bulk profiling and thus the previous classification of these GBMs into multiple subtypes [11]. However, this cellular composition does not apply to all gliomas, demonstrating that a 'one-size-fits-all approach' no longer works when considering the influence of putative CSCs across different classes of brain tumors.

More recent scRNA-seq studies in the malignant pediatric brain tumor MB elucidated the developmental origins of the different MB subgroups with some exhibiting a higher proportion of either undifferentiated or differentiated cells [12–15]. These studies have undoubtedly paved the way for additional work that will examine the cellular makeup at a single-cell level before and after treatment, as well as in matched primary and recurrent patient samples.

The last two decades have seen significant gains in the understanding of the extensive inter- and intratumoral heterogeneity in the most malignant and devastating brain tumors. This review will provide an overview of the initial studies that led to the isolation of putative brain tumor stem cells using traditional cell sorting-based technologies, the controversies involved with interpreting these data, and the evolution of the CSC model in the era of single-cell genomics. While the review will primarily focus on MB CSC studies, specific publications pertaining to glioma models are highlighted for context when appropriate, as the utilization of several techniques emerged in parallel for multiple types of brain cancers. For more detailed and comprehensive summaries of the CSC model in gliomas, I refer to the excellent reviews by Mitchell *et al.* [16], Gimple *et al.* [17], and Suva and Tirosh [10].

The origin of the CSC model in brain tumors

Our current definition of a CSC emerged from studies in the mid-late 1990s focusing on hematopoietic malignancies [1,2]. In what would soon become 'gold standard' assays in the CSC field, these experiments demonstrated that a reservoir of undifferentiated, low-cycling cells could recapitulate the entire tumor upon retransplantation into immunodeficient mouse models. Fluorescence-activated cell sorting (FACS) was based on a CD34+/CD38− cell surface marker profile enriched for tumor-initiating capacity [1,2]. This pioneering work was followed by a plethora of studies demonstrating the existence of cell subpopulations with similar characteristics in solid malignancies [18–24], including brain tumors [25,26]. While the idea that aggressive brain tumors such as MB arise from a more primitive precursor cell was originally proposed more than 100 years ago [27,28], the advancement of cell sorting-based technologies enabled this concept to be tested more rigorously both *in vitro* and *in vivo*. For example, Singh *et al.* [25] adopted neurosphere assays originally performed with neural stem cells (NSCs) [29–31] to demonstrate that MBs contained a subpopulation of cells that exhibited self-renewal capacity and differentiation, defining features of stem cells and proliferative potential. These putative brain CSCs were found exclusively in the fraction expressing the cell surface marker CD133 [25]. Subsequent studies provided further support for this model by demonstrating that only injection of CD133+ brain tumor cell fractions from MB and both adult and pediatric GBM patient samples were capable of initiating tumor growth in NOD SCID mice [26]. This subpopulation also had clinical relevance, as CD133+ glioma cells were more resistant to ionizing radiation [32] and chemotherapy [33]. Moreover, CD133 expression was also correlated with an overall poor survival [34,35].

While these initial studies certainly galvanized the brain tumor CSC field, issues with choice of cell surface markers, culture methods, and *in vivo* models created significant controversy. First, CD133 is expressed in a variety of cell types including normal stem cells and differentiated epithelial cells, and is not restricted to the putative CSC population [36]. In addition, the CD133 antibodies being utilized to isolate CSCs recognize two different epitopes (AC133/AC141), which were thought to be glycosylated [37,38]. This had many researchers questioning whether the CSC phenotype was associated with the glycosylation status of CD133 or the CD133 protein itself [37,38]. Interestingly, Kemper *et al.* [39] showed that CSC differentiation is accompanied by a loss in the AC133 epitope but not the CD133 protein, and this was attributed to differential glycosylation and epitope masking. Combined with the limited knowledge of the specific biological roles of CD133, these findings suggested that researchers should proceed with caution when interpreting CD133-based results, particularly for putative CD133-negative populations. This point is highly relevant, as several studies have challenged the notion that tumor-initiating capacity is limited to the CD133+ cell fraction in brain tumors [40–43]. For example, Chen *et al.* [41] showed that a primitive CD133- subset of GBM cells could generate highly aggressive GBM and could subsequently give rise to CD133+ cells. Further complicating FACS-based studies of brain tumor CSCs was the requirement for enzymatic dissociation of tumor cells and short or extended culture to assess self-renewal over subsequent passage. Tumorspheres

grown in serum-free conditions have been shown to best recapitulate the genotypic and phenotypic changes observed in primary tumors compared with cells grown in adherent serum-containing cultures [44]. However, the specific cell culture conditions vary between laboratories, and so, it is not surprising that published CSC studies often yield entirely different results. This is especially problematic for researchers working with MBs, as patient-derived tumor cells are notoriously difficult to adapt to cell culture.

CSC markers and mouse models of medulloblastoma

While CD133 has been used to isolate putative CSCs from primary human patient samples and cell lines, studies in mouse models, particularly for MB, have demonstrated that other markers enrich for tumorigenic cell subpopulations [45,46]. This has been most well-studied in Sonic Hedgehog (SHH) mouse models of the disease. For example, Read *et al.* [46] showed that CD133+ cells isolated from a patched homologue 1 (*Ptch*+/−) model of SHH MB did not form tumorspheres or generate tumors when transplanted *in vivo*. However, these tumors could be propagated by a progenitor cell fraction that expressed the cell surface marker CD15 and the transcription factor atonal homolog 1 (*Atoh1*) [46]. Similarly, Ward *et al.* [45] demonstrated that CD15+ stem-like cells, rather than progenitors, isolated from *Ptch*+/− SHH MB mouse models exhibit tumor-initiating capacity. In both cases, these fractions contained actively proliferating cells. In line with a stem or progenitor cell of origin, deletion of *Ptch1* in either granule neuron progenitors (GNPs) or NSCs leads to the development of SHH MB [47,48], demonstrating that mutations in distinct cells of origin can still converge to generate the same cancer phenotype.

Additional studies provided evidence for rare subsets of either unipotent nestin + progenitors [49] or Sox2+ stem cells [50] as the SHH MB cell of origin. Elegant work by Vanner *et al.* [50] further refined these studies using a combination of transplantation experiments and fate mapping to demonstrate that a smaller proportion of the larger CD15+ cell fraction, namely a rare, quiescent, SOX2+ cell subset, drives tumor propagation and is resistant to both chemotherapy and SHH pathway inhibition. However, *SOX2* is highly expressed in human SHH MB patient samples relative to the other MB subgroups, particularly in adolescent and adult cases, suggesting that it may play specific roles in tumor development for these age-groups [51]. The high levels also suggest that in human SHH MB tumors, the SOX2+ cell population is likely heterogeneous and can be further dissected. Indeed, our laboratory has shown that cells exhibiting another marker, the low-affinity CD271/p75 neurotrophin receptor (p75NTR), are nearly exclusive to primary SHH MB patient tumors, are associated with a stem/progenitor cell phenotype in human cell models, and are sensitive to MAPK pathway inhibition [52–54]. Interrogation of additional primitive cell markers and stem cell regulators will likely yield a combination of factors enabling further deconstruction of these heterogeneous, yet highly clinically relevant, cell subpopulations across both mouse and human model systems. Some evidence exists for the enrichment of putative CSCs in aldehyde dehydrogenase (ALDH+) cells [55] or the side population [56] (based on efflux properties of ATP-binding cassette transporters and delineated by Hoechst 33342 exclusion using flow cytometry) in highly aggressive brain tumor models. However, the results are controversial, particularly for the side population [57,58], and limited studies exist on the specific relevance to MB CSCs. Collectively, these studies underscore the issues surrounding CSC identification, frequency, and quiescence in solid tumors. While rarity and quiescence are not prerequisites for defining CSCs, the cellular composition and the cell cycle parameters of each subpopulation must be considered in the design of drug screens and combination therapy studies both *in vitro* and *in vivo*. For example, a high-throughput compound screen designed to target proliferative populations would fail to eradicate a putative quiescent CSC subset. Moreover, assessments of drug synergy are complicated by the fact that combinations of drugs could be abrogating the same or different cell populations in heterogeneous cell cultures grown in stem cell-enriched conditions. Recent conceptual evolution of the CSC model to incorporate cellular plasticity and bidirectional cell state transitions [3] would require consideration of both concurrent and sequential combinatory therapy to accommodate initial debulking followed by targeting of the residual drug-resistant population in animal models. Some of these challenges have been overcome by the advancements in sequencing technologies that facilitate comprehensive evaluation of both inter- and intratumoral heterogeneity in primary human tumor samples.

Medulloblastoma heterogeneity in the molecular era

While MB CSC characterization studies using traditional sorting strategies and lineage tracing were ongoing, intensive molecular profiling of large MB patient

sample cohorts was being conducted in parallel and gaining significant momentum. Indeed, early gene expression array profiling studies [59–62] led to the stratification of MB into 4 consensus molecular subgroups [63] that exhibit different genomic alterations, gene expression profiles, and response to treatment: Wingless-related integration site (WNT), SHH, Group 3, and Group 4 [59,63,64]. These initial studies paved the way for large-scale next-generation sequencing (NGS) on primary MB samples that provided deep insight into their biological complexity [65–67]. WNT and SHH MB tumors are aptly named for the signaling pathways that are constitutively activated in these subgroups. With an excellent > 95% 5-year survival rate, WNT MBs have the best prognosis, very rarely metastasize, and are characterized by somatic mutations in *CTTNB1* encoding β-catenin [63]. SHH tumors account for 30% of all MBs, have an intermediate prognosis, and are commonly associated with infants (< 3 years) and adults. However, *TP53* mutations confer a poor prognosis in older children with SHH MB tumors and constitute a very-high-risk form of this molecular group [68]. Group 3 tumors account for a quarter of MB cases, are associated with *MYC* amplification, and exhibit the worst prognosis, while Group 4 MBs represent the most common subgroup and exhibit cyclin-dependent kinase (*CDK6*) and *MYCN* amplifications [63]. Further analysis of molecular features and clinical trends among these groups has resulted in additional substructure and classification of the MB subgroups into multiple subtypes [69–71]. These studies underscore the highly heterogeneous nature of MB that was once considered a single disease entity. For a full, comprehensive overview of the extensive multi-omic bulk tumor analyses on MB patient samples, see the excellent review by Hovestadt *et al*. [72].

From a clinical perspective, molecular subgrouping/subtyping has improved risk stratification, thus providing opportunities to intensify therapy for the very-high-risk Group 3 MB patients and reduce therapy for lower-risk groups such as WNT [73]. Sequencing SHH MBs has revealed mechanisms of resistance to SHH pathway inhibitors that are not predicted to work in younger patients, as their tumors frequently harbor mutations in downstream SHH pathway genes such as *SUFU* or *MYCN* [74–77]. Despite concerted efforts to improve therapy, over 30% of MB patients die, while survivors are left with the long-term physical and cognitive side effects associated with chemotherapy and radiation [64,78].

The knowledge gleaned from over a decade of genomic, epigenomic, transcriptome, and even proteomic [79,80] MB studies has certainly paved the way for functional studies that will fully characterize the mechanistic role of newly identified genes/pathways both *in vitro* and *in vivo*. Yet, putative rare CSC fractions are inevitably missed in bulk sequencing data. Aberrant stem/progenitor cell signatures are associated with tumor growth, drug resistance, metastasis, and/or poor prognosis in MB [81], as well as a variety of other cancers [4,82]. However, these profiles are less likely to be captured when sequencing the highly heterogeneous tumor in its entirety. This would require a single-cell approach.

Convergence of the CSC hierarchical model and functional genomics: lessons learned from gliomas and emerging concepts in medulloblastoma

Traditional sorting-based CSC technologies and bulk sequencing studies evolved separately, but their convergence in the last 5 years has provided unprecedented insight into the complexity and intratumoral heterogeneity across a variety of brain tumors. Indeed, the recent surge of large-scale functional genomics studies has renewed interest in delineating the cell of origin for adult and pediatric cancers, including embryonal brain tumors such as MB. In particular, scRNA-seq has enabled a much deeper understanding of the transcriptomes representing early neurodevelopmental hierarchies and how the signaling pathways driving these hierarchies may be hijacked in brain tumors [10,13–15].

Studies on malignant gliomas have been at the forefront of single-cell analyses in brain tumors and will be discussed first for context. Initial characterization of a glioma cellular hierarchy was published in 2016 for IDH mutant oligodendrogliomas, which are associated with 1p/19q co-deletion [6]. This was quickly followed by work with another class of IDH mutant gliomas, astrocytomas (*TP53* and *ATRX* mutant) [7], which together demonstrated that IDH mutant gliomas are hierarchically driven by a cycling population of neural progenitor cells (NPCs) at the apex, which constitute a small portion of the overall cell population. The proportion of undifferentiated cells is even higher in the aggressive histone H3 lysine 27-to-methionine mutant (H3K27M) midline pediatric gliomas, as these tumors are driven by a cycling oligodendrocyte precursor-like cell (OPC) that accounts for approximately 80% of the tumor [8]. In contrast, IDH–wild-type adult GBMs are characterized by four plastic and highly malignant cellular states including

an NPC-like, OPC-like, astrocyte-like (AC-like), and a mesenchymal-like state (MES-like) [9]. Unlike other gliomas, proliferative compartments were observed in all four states, and multiple states were able to generate tumors in mice. In these elegant studies, the authors also examined the expression of previously defined brain CSC markers across the four cellular states, with each marker showing significant bias toward one particular state (Fig. 1C) [9]. For example, CD133 expression is highest in the OPC-like cell state. This provides an explanation for the wide variation in CD133 levels observed across GBMs depending on the proportion of that particular cellular state in a given tumor. Several additional scRNA-seq studies in GBM have further interrogated the CSC phenotypes [83,84] and their reversible state transitions [85] and have unveiled additional complexity within the stem cell compartments including identification of an invasive cell phenotype [86,87].

Following in the footsteps of the comprehensive glioma studies, several research groups recently applied scRNA-seq technologies to primary patient MBs [13–15]. Vladoiu *et al.* [13] analyzed eight patient samples representing SHH, Group 3 and Group 4 MBs, and the other posterior fossa tumors, ependymoma, and pilocytic astrocytoma, by scRNA-seq. Not surprisingly, SHH MBs contained tumor cells in different states along the GNP lineage spectrum and were in agreement with the heterogeneity observed in mouse models of the disease (Fig. 2) [50]. Consistent with experimental evidence [88–90] and the more aggressive nature of Group 3 MBs, Vladoiu *et al.* [13] also demonstrated that this subgroup contains a mixed population of malignant cells with divergent differentiation along the cerebellar lineages, thus suggesting a more undifferentiated cerebellar stem cell of origin (Fig. 2). Group 4 MB tumors were most closely aligned with the unipolar brush cell (UBC) lineage, a glutamatergic neuronal cell population that arises from the upper rhombic lip [91] (Fig. 2). Similarly, Hovestadt *et al.* [15] employed scRNA-seq to characterize 25 patient samples representing all 4 MB subgroups in addition to patient-derived MB xenografts. The authors also determined that SHH MBs exhibit various cell phenotypes along the GNP lineage. However, they also defined a continuum of transcriptional states or 'metaprograms' for Group 3 and Group 4 MBs that account for overlapping signatures traditionally complicating classification based on bulk profiling (Fig. 2) [69,70,92]. Approximately 90% of Group 3 MB tumor cells expressed an undifferentiated cell metaprogram characterized by translation/elongation factor and ribosome genes [15]. In contrast, Group 4 MBs were associated with a neuronal differentiation metaprogram and the UBC lineage [15]. Finally, Jessa *et al.* [14] demonstrated that WNT MBs match to the lower rhombic lip pontine mossy fiber lineage in the brainstem consistent with previous experimental evidence from mouse models (Fig. 2) [93]. Collectively, these recent MB scRNA-seq studies support the notion that embryonal brain tumors are diseases of dysregulated early brain development. However, we have yet to translate the results into targeted therapies that specifically abrogate CSCs/progenitors. As such, clinicians remain limited to cytotoxic chemotherapy and radiation that can lead to extensive cognitive and physical delays. To identify more nuanced treatment options, it is important to assay the effects of targeted therapies beyond cell growth and survival.

Integrating genomics and stem cell biology in search of novel targeted therapies

In the last 5 years, we have gained tremendous insight into the heterogeneity and complexity of malignant brain tumors. Further advancements in single-cell-based technologies for assessment of the epigenome, transcriptome, and proteome will enable even more comprehensive analyses of rare or quiescent cell populations that may drive disease progression. Validation of identified cellular states and hierarchies using gold standard *in vitro* and *in vivo* stem cell assays combined with comparisons of these profiles before and after treatment will be essential. For example, in a multidisciplinary tour de force by Zhang *et al.* [12], the authors identified an OLIG2+ cell population in mouse models of SHH MB that drives tumor initiation and also re-emerges at relapse. They further demonstrated that this population is associated with HIPPO-YAP and AURORA-A/MYCN signaling, and co-targeting these pathways significantly improved survival *in vivo* [12]. The hierarchical relationship between OLIG2+ cells and the SOX2+ stem cells previously identified as SHH MB drivers [50] will need to be further explored along with a deeper assessment of the clinical relevance of these subpopulations in human models of the disease. Of particular importance will be the analyses of these cell populations in relapsed models of MB. While SHH MB subtype classification typically holds following treatment, divergence of dominant clones after therapy suggests that novel agents will be needed to abrogate this emerging cell population [94]. This is underscored by the fact that specific subsets of stem-like cells within the SOX2+ cell population are resistant to treatment with vismodegib, a SHH pathway

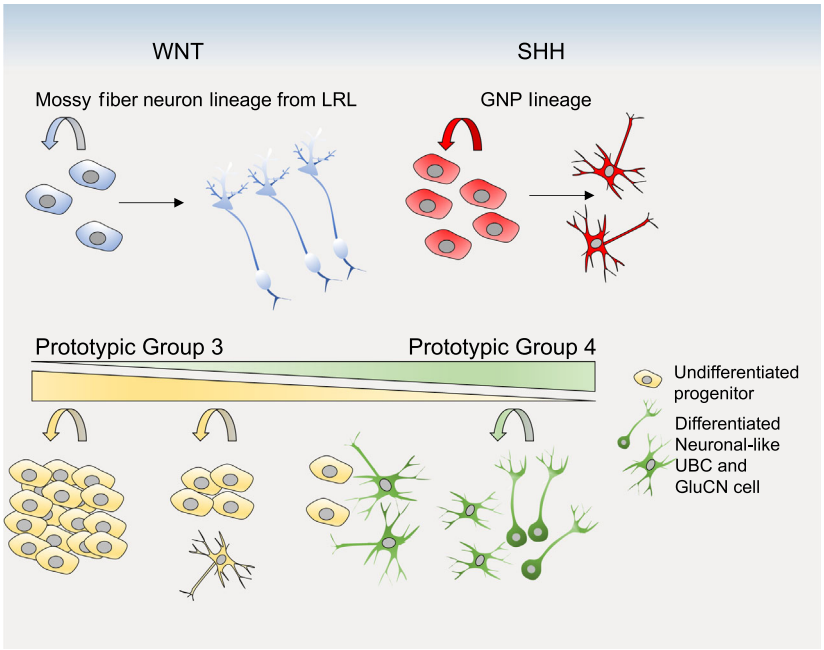

Fig. 2. Proposed cell of origin for each medulloblastoma subgroup based on scRNA sequencing. Schematic representations of putative cells of origin for each of the 4 medulloblastoma (MB) subgroups based on compiled evidence from Hovestadt *et al.* [15], Vladoiu *et al.* [13], and Jessa *et al.* [14]. WNT MBs are most closely aligned with the mossy fiber neuron lineage derived from the lower rhombic lip (LRL). SHH MBs exhibit features of progenitors and more differentiated cells along the granule neuron progenitor (GNP) lineage of the cerebellum. Group 3 and Group 4 MBs exhibit a continuum of phenotypes in which prototypical *MYC*-amplified Group 3 MBs are predominantly associated with an undifferentiated neuronal progenitor or cerebellar stem cell state. Both Vladoiu *et al.* [13] and Hovestadt *et al.* [15] have demonstrated that Group 4 MBs are most closely associated with a unipolar brush cell (UBC) of origin and have a more differentiated phenotype. Various intermediate states exist in between.

antagonist [95]. Recent advances demonstrating the utilization of cerebrospinal fluid-derived circulating tumor DNA for characterization and monitoring of MB [96,97] and detection of residual disease may open up new opportunities for real-time tracking of CSC populations during treatment.

In addition to appropriate mouse models, it will also be important to validate the findings of large-scale scRNA-seq studies in biologically relevant human brain tumor models including cell lines, low passage cultures from primary tumors, patient-derived xenografts, organoids [98], or even humanized stem cell models of MB [99,100]. Recent work by Ballabio *et al.* demonstrated that cerebellar organoids can be used to model Group 3 MB and to test the effect of specific genes in modulating tumor growth [98]. Our laboratory recently employed scRNA-seq to assess heterogeneity in stem cell-enriched tumorsphere cultures from several newly established Group 3 MB cell lines [101]. These tumorspheres mirrored the specific undifferentiated 'metaprogram' associated with Group 3 MB patient samples [15] (Fig. 3) and were characterized by translation/protein synthesis genes, thus underscoring the strength of stem cell-enriched *in vitro* cultures in modeling primary patient tumor phenotypes. This is particularly important for molecular dissection of rare, but heterogeneous, fractions of brain tumor CSCs. For example, comprehensive analyses of > 69 000 enriched CSCs derived from GBM patient samples revealed a high degree of inter- and intratumoral heterogeneity along with characterization of a new cellular CSC subset exhibiting an inflammatory wound response signature [102]. However, given that matched tumors, cell cultures, and xenografts in brain tumors such as GBM exhibit divergent methylation and gene expression profiles [103], future MB studies will continue to benefit from integrative approaches that combine bioinformatic data from primary samples and both *in vitro* and *in vivo* models. Alternatively, transformed human embryonic stem cell-derived neural precursors [100], as previously demonstrated by my group, as well as various induced pluripotent stem cell-derived neuroepithelial stem cell (NES) models [99,104], have emerged as powerful resources for studying the genetic and molecular mechanisms driving tumorigenesis. This includes NES models from Gorlin

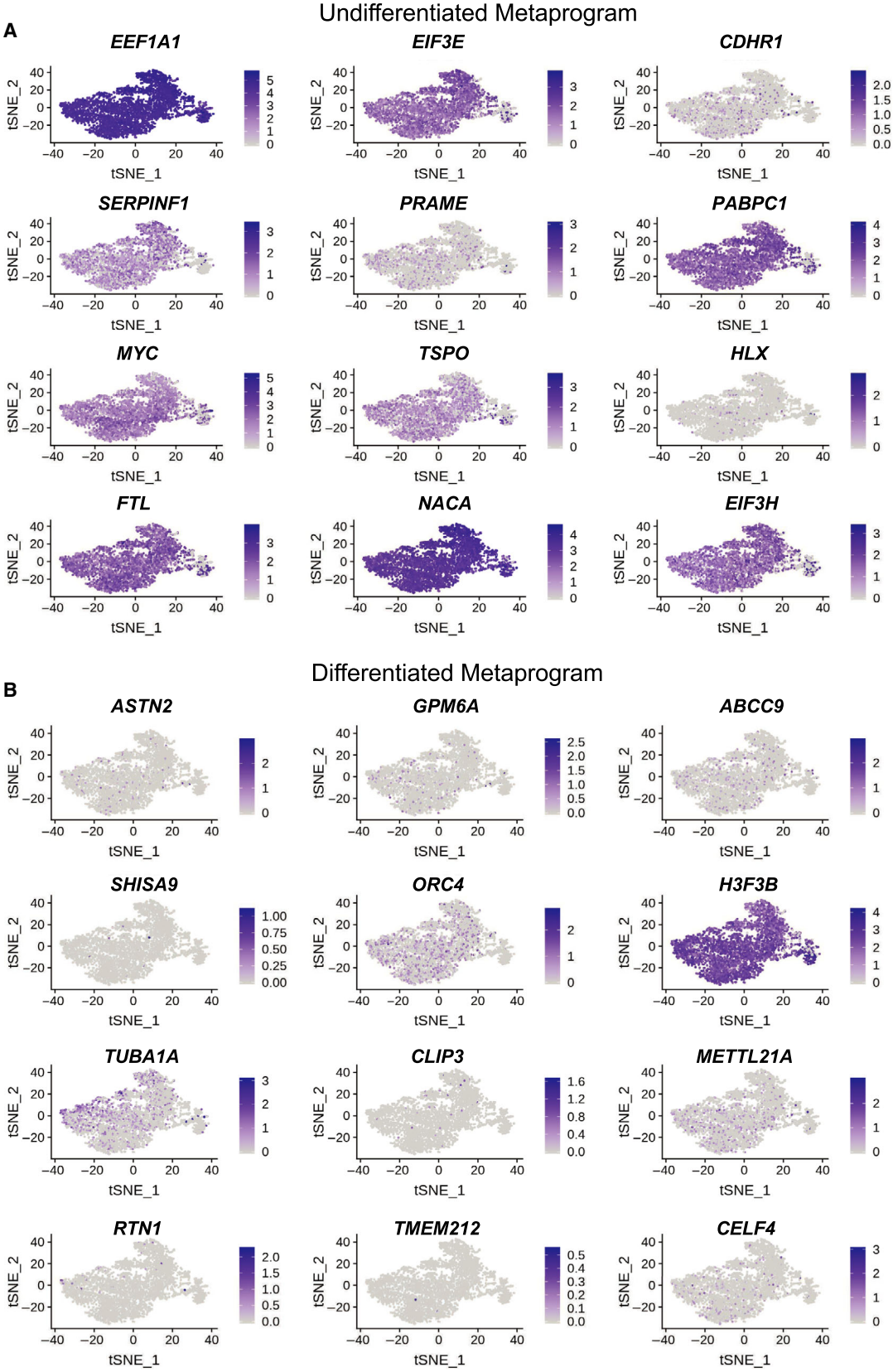
A
Undifferentiated Metaprogram
EEF1A1
EIF3E
CDHR1
SERPINF1
PRAME
PABPC1
MYC
TSPO
HLX
FTL
NACA
EIF3H
B
Differentiated Metaprogram
ASTN2
GPM6A
ABCC9
SHISA9
ORC4
H3F3B
TUBA1A
CLIP3
METTL21A
RTN1
TMEM212
CELF4
tSNE_1
tSNE_2

Fig. 3. Expression of top genes associated with 'metaprograms' defined by Hovestadt *et al.* [15], using scRNA sequencing. (A, B) Expression of 12 of the top genes associated with the undifferentiated (A) and differentiated (B) metaprograms as defined by Hovestadt *et al.* [15] in single cells derived from HDMB03 Group 3 MB tumorspheres. Tumorspheres were previously analyzed by scRNA-seq in Zagozewski *et al.* [101]. The undifferentiated metaprogram is associated with an undifferentiated progenitor state and is characterized by ribosomal and translational initiation/elongation factor genes.

syndrome (*PTCH1*+/−) patients who are predisposed to MB [104]. Application of barcoding technology to assess clonal evolution in xenotransplantation assays of GBM [105] and MB will inevitably shed further light on CSC dynamics in the most biologically relevant human model systems. Moving forward, we also have to consider the co-dependent relationship between CSCs and the tumor microenvironment including the vasculature and immune system [106] with further assessments of how future targeted therapies can disrupt the tumor-promoting ecosystem as a whole. Exploiting the immune system to specifically attack CSCs based on cell surface receptor profiles (i.e., CD133) has gained momentum, particularly in GBM models [107–110], and could also be applied in the most relevant MB models.

Conclusions

The last decade has witnessed an explosion of multi-omics publications comprehensively characterizing the lineage hierarchies in malignant brain tumors. Following the path of gliomas in adults and children, recent studies in MB and other brain tumors [111] have added to the ever-growing list of cancers to be resolved at the single-cell level. These studies may predict the efficacy of novel targeted therapies, as in principle, elimination of a CSC population that initiates tumor progression would have immense clinical benefit. That being said, validations *in vitro* and *in vivo* are still essential. This is underscored by recent work demonstrating that functional screens can identify more treatment options than sequencing alone [112]. Moreover, not all brain tumor CSCs are created equal and must therefore be approached in a context-dependent manner. The unique challenges associated with the CSC plasticity model would also suggest that eradicating one CSC-like cell state would merely be compensated for by another exhibiting unique molecular dependencies within the tumor mosaic. Thus, future treatment regimens must consider how to eradicate or even differentiate all CSC-like cell states, and whether combination therapies would be administered in a concurrent or sequential fashion to minimize relapse. With recent advances in digital spatial profiling applications, we can now utilize fixed tissue to assess protein/RNA abundance *in situ* [113] creating more opportunities to determine the impact of treatments on putative CSCs within the complex cellular ecosystem *in vivo*. In this molecular and multi-omics era of brain tumor CSC biology, we are now much closer to the implementation of targeted therapies that will ultimately improve survival and enhance the quality of life for brain tumor patients.

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Conflict of interest

The authors declare no conflict of interest.

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