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REVIEW

Overview and recent advances in the targeting of medulloblastoma cancer stem cells

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ABSTRACT

Introduction: Medulloblastoma, an embryonal small round blue cell tumor primarily arising in the posterior fossa, is the most common malignancy of the central nervous system in children and requires intensive multi-modality therapy for cure. Overall 5-year survival is approximately 75% in children with primary disease, but outcomes for relapsed disease are very poor. Recent advances have identified molecular subgroups with excellent prognosis, with 5-year overall survival rates >90%, and subgroups with very poor prognosis with overall survival rates <50%. Molecular subtyping has allowed for more sophisticated risk stratification of patients, but new treatments for the highest risk patients have not yet improved outcomes. Targeting cancer stem cells may improve outcomes, and several candidate targets and novel drugs are under investigation.

Areas covered: We discuss medulloblastoma epidemiology, biology, treatment modalities, risk stratification, and molecular subgroup analysis, links between subgroup and developmental biology, cancer stem cell biology in medulloblastoma including previously described cancer stem cell markers and proposed targeted treatments in the current literature.

Expert opinion: The understanding of cancer stem cells in medulloblastoma will advance therapies targeting the most treatment-resistant cells within the tumor and therefore reduce the incidence of treatment refractory and relapsed disease.

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KEYWORDS
Medulloblastoma; cancer stem cells; pediatric oncology; personalized medicine; cd133; cd15; cd114; sox2; olig2; targeted therapeutics

1. Introduction

Medulloblastoma is the most common malignant childhood brain tumor, with approximately 4 cases per million children (Figure 1)[1]. The treatment of medulloblastoma includes surgical resection, craniospinal radiation, and systemic chemotherapy. While the 5-year overall survival of patients is approximately 75%, outcomes are widely variable. Recent advances in molecular profiling have clarified heterogeneity within medulloblastoma and helped explain differences in outcomes. Applying this clinically, new clinical trials have begun to prospectively risk stratify patients by molecular subgroup. However, even in patients with low-risk tumors, treatment has high morbidity. Furthermore, outcomes remain poor in the worst subgroups, with less than 50% 5-year overall survival despite aggressive, multi-modal therapy [2]. Therefore, a better understanding of medulloblastoma biology is needed to improve patient outcomes.

This review discusses the developing concept of cancer stem cells (CSCs) as a driving mechanism for medulloblastoma treatment resistance and relapse. CSCs are a small population of cells within a tumor cell population that can recapitulate a new heterogeneous tumor if not eliminated by therapy (Figure 2). Selective targeting of CSCs alongside traditional medulloblastoma therapy can decrease the likelihood of tumor recurrence and improve the outcomes for patients. This review will discuss the current treatment modalities for medulloblastoma, review the present understanding of molecular subgroups, and describe the current state of the science of CSCs in medulloblastoma and their potential therapeutic vulnerabilities.

2. Medulloblastoma subgroups

Medulloblastoma was first recognized to be a heterogenous disease entity with three distinct histological patterns: classic, nodular/desmoplastic (including the medulloblastoma with extensive nodularity or MBEN type), and large cell/anaplastic (LCA) subtypes. Outcomes vary by subtype, with LCA tumors often being more aggressive and patients with nodular/desmoplastic tumors having better outcomes [3]. Factors which escalate a patient from average to high risk have included extent of metastatic disease [4], presence of residual tumor [4], and anaplastic histology [5].

Recently, incorporating molecular tumor subgrouping has allowed for more sophisticated risk stratification. Using genomic, transcriptomic, and epigenomic analyses [6,7]. four major molecular subgroups of medulloblastoma have been identified. The WNT subgroup is driven by activation of the Wingless signaling pathway, while the SHH subgroup is driven by activation of the Sonic hedgehog signaling pathway. The driver

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Article highlights

- Medulloblastoma is the most common central nervous system malignancy in pediatrics.
- Current treatment of primary medulloblastoma consists of maximum safe surgical resection, craniospinal irradiation, and myelosuppressive chemotherapy. Relapsed disease has a very poor prognosis with no standard therapy.
- Medulloblastoma tumors can be divided into four major molecular subgroups: WNT, SHH, Group 3, and Group 4. These subgroups vary by molecular characteristics, age at presentation, and prognosis. New clinical trials are ongoing that incorporate medulloblastoma molecular subgrouping in risk stratification.
- Cancer stem cells (CSCs) drive relapsed and refractory medulloblastoma but are resistant to traditional therapy. Killing CSCs should improve overall outcomes and new treatment paradigms are needed.
- There are common signaling pathways used by neural stem cells and medulloblastoma CSCs, including Notch, Wnt, and Hedgehog signaling.
- Currently identified markers of medulloblastoma CSCs include CD133, CD15, and CD114. These markers identify populations of cells with higher tumorigenicity and a less differentiated gene expression profile.
- Many treatments which in part target CSCs are in varying stages of exploration and development. These include approaches targeting key pathways such as Notch, Hh, Wnt, and PI3K, along with drug screening approaches using enriched CSC samples, immunotherapy, and oncolytic viruses.

mechanisms of groups 3 and 4 tumors are less clearly defined [8], though recent work has identified a number of driver mutations [9,10,11]. Notably, despite the variability in outcomes between patients with group 3 and 4 tumors, they are typically grouped together for clinical trials. Presence of *MYC* amplification or metastatic disease status will upgrade the risk of a patient regardless of subgroup, and these features are more common in Group 3 tumors (Table 1).

Prognosis widely varies between groups, with patients with WNT medulloblastoma tumors having approximately 95% 5-year overall survival rates, while patients with group 3 medulloblastoma tumors have only 50% 5-year overall survival (Table 1)[8]. Tumor subgrouping is not only prognostically useful but allows for risk stratification in new clinical trials. For example, the currently enrolling SJMB12 trial (NCT01878617) intensifies therapy for patients with intermediate and high-risk Group 3/4 tumors.

In 2017, several groups published large-scale analyses of medulloblastoma samples and were able to further refine medulloblastoma molecular subgrouping utilizing analyses of tumor genome methylation and transcriptomic profiles. Schwalbe et al [12]. analyzed 428 primary human medulloblastoma tumors using DNA methylation microarray analysis and found seven distinct subtypes of medulloblastoma within the four established groups. WNT medulloblastoma tumors remained a single group, while SHH medulloblastoma tumors were divided into infant and child subtypes and Group 3 and Group 4 tumors were subtyped into low and high-risk subtypes. Genome-wide DNA methylation and gene expression analysis of 763 human medulloblastoma samples by Cavalli et al. in 2017 [2] distinguished a total of 12 molecular subtypes in medulloblastoma: two subtypes in WNT, four in SHH, three in Group 3, and three in Group 4 (Table 2). Parallel work by Northcott et al. [9] divided Group 3/4 tumors into 8 subtypes by methylation profiling (subtypes I–VIII), with significant concordance with the six Cavalli Group 3/4 subtypes (Table 3).

In 2019, an analysis of DNA-methylation profiling and transcriptomic data from 1501 Group 3/4 medulloblastoma tumors combining the tumors from the Schwalbe, Cavalli, and Northcott cohorts resulted in a consensus identification of eight subtypes highly congruent with the original eight Northcott subtypes I–VIII (Table 3)[13]. In all analyses, subtypes I, V, and VII were a mix of canonical Group 3 and Group 4 tumors, while the other subtypes remained more discretely either Group 3 or 4 [14], suggesting that Group 3/4 medulloblastoma tumors lie along a molecular spectrum.

Each subgroup and subtype has characteristic genomic alterations. Tumors in the WNT subgroup are the most molecularly homogenous, with frequent *CTNNB1* mutations (85% of patients) [14]. Conversely, Group 3/4 tumors have

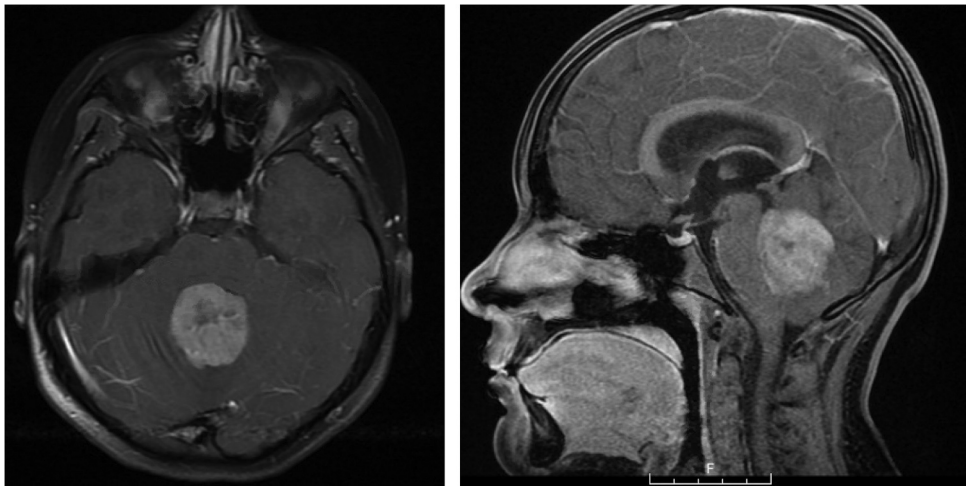

Figure 1. T1 weighted MRI imaging of a 9-year-old female patient with WNT-subgroup medulloblastoma.

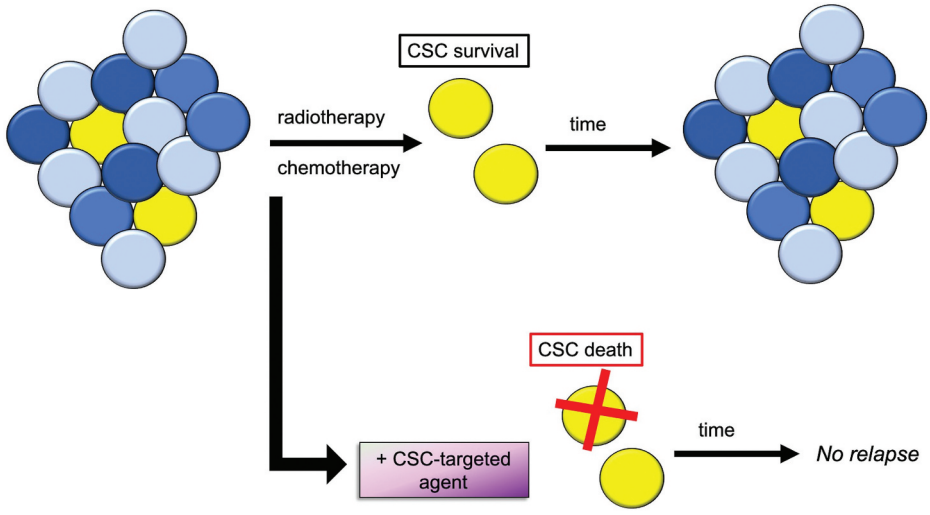

Figure 2. Rationale of targeting medulloblastoma cancer stem cells. chemotherapy and radiotherapy will kill the majority of tumor cells, but cancer stem cells may remain. these cells, left unchecked, may lead to new medulloblastoma tumors, which are typically much harder to treat than the primary tumor. Alternatively, if a treatment targeting the cancer stem cells is added to the treatment strategy, no cells remain to develop into medulloblastoma relapse.

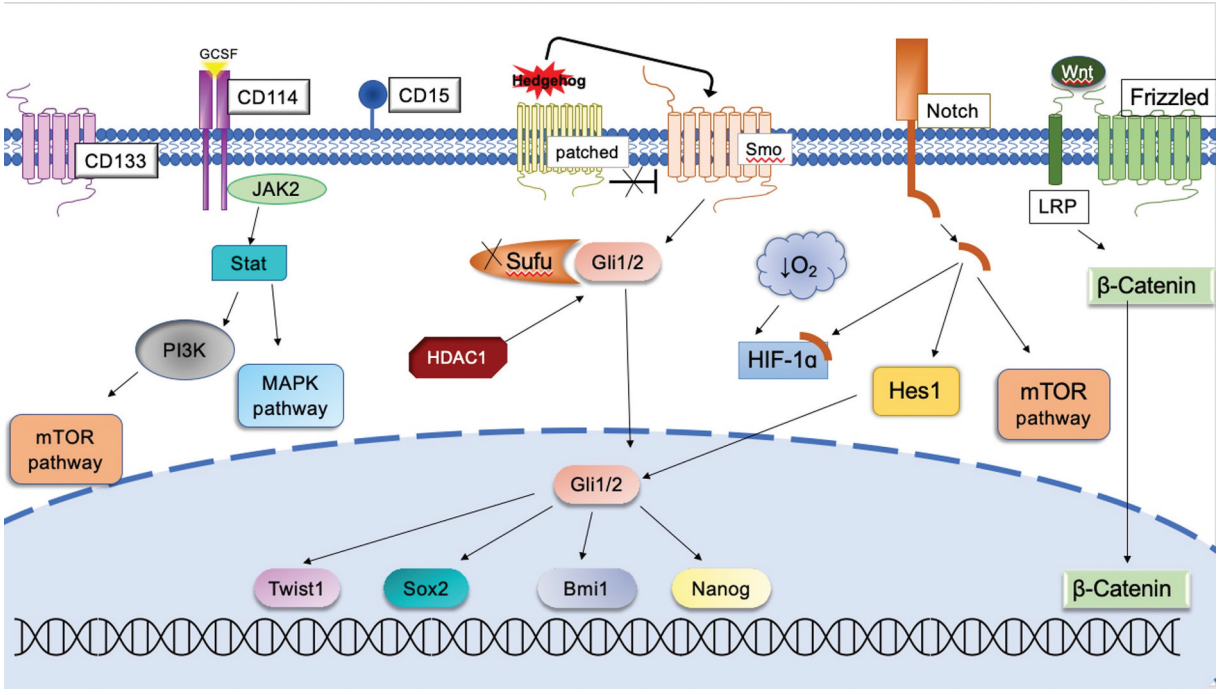

Figure 3. Cancer stem cell markers and pathways. the notch, wnt, and hedgehog pathways are involved in the maintenance of cancer stem cells, and represent key targets for cancer stem cell directed treatment strategies. The known medulloblastoma cancer stem cell markers CD133, CD15, and CD114 are shown.

overlapping but distinct driver events within the eight subtypes. For example, *MYC* amplification is found in subtypes II, III, and V, while subtype IV has not been found to have any consistent driver events [14]. Many of the key genetic changes are summarized in Tables 1,2,3, and a recent review of the molecular characterization of medulloblastoma has a detailed discussion of the current state of understanding [14].

Molecular subtyping beyond the four major subgroups has not been prospectively introduced into any open clinical trials for patients with medulloblastoma to date, but is being applied retrospectively to data from completed trials to refine risk stratification. Methylation analysis and next-generation sequencing were performed on medulloblastoma tumors from 305 patients enrolled on the SJMB03 clinical trial [15]. Group 3/4 tumors were defined by the methylation subtypes I–VIII and were stratified into low, intermediate, and high-risk groups based upon the outcomes of that study. Group 3/4 tumors with metastatic (M+) disease, *MYC* amplification, or in the subtype III group had inferior outcomes and were designated high risk. Subtype VII tumors (which are primarily group 4) were considered low risk, and all other subtypes were designated intermediate risk [15].

Table 1. Clinical characteristics of the major medulloblastoma subgroups (per Northcott 2012) [8].

	WNT	SHH	Group 3	Group 4
Frequency	10%	30%	25%	35%
Histopathology	Classic, LCA (rarely)	Desmoplastic/ nodular, classic, LCA	Classic, LCA	Classic, LCA
5-year OS	~95%	~75%	~50%	~75%
Incidence of metastasis	5–10%	10–15%	40–45%	35–50%
Pattern of relapse	Local or distal	Local	Distal	Distal
Age group*	Children, adult	Infant, adult	Infant children	Children, adult
Male: female ratio	1:1	1.5:1	2:1	3:1

LCA- Large cell anaplastic. OS- Overall survival. *Infant is defined as less than 3 years old.

3. Treatment

The standard treatment of newly diagnosed medulloblastoma consists of maximal safe resection, craniospinal radiation, and systemic cytotoxic chemotherapy. In young children, craniospinal radiation leads to unacceptable neurocognitive deficits, and treatment strategies rely on delaying radiation with intensified chemotherapy [16]. The side effects of therapy are significant, including endocrinopathy, hearing loss, neurocognitive deficits, and secondary malignancies [17], [18].

3.1. Surgical resection

The goal of surgical resection of medulloblastoma tumors is to achieve maximal safe resection. A retrospective look at 787 patients with medulloblastoma defined the difference in outcomes based on extent of resection. Compared to subtotal resection (greater than 1.5cm^2 disease remaining), gross total resection provides an increase in progression-free survival (hazard ratio [HR] 1.45, 95% CI 1.07–1.96), but no increase in overall survival (HR 1.23, 0.87–1.72) [19]. There was no improvement in progression-free survival or overall survival when comparing gross total and near total resection (less than 1.5 cm^2 remaining tissue). When analyzed by molecular subtype, group 4 tumors were the only subtype with a benefit to progression-free survival with gross total resection compared to sub-total resection, with no improvement seen with overall survival. These results challenged the paradigm of the need for gross total resection, and underlie the current recommendation not to pursue aggressive resection if there is a likelihood of resultant neurologic deficits.

3.2. Radiation therapy

Following surgical resection, patients with medulloblastoma undergo craniospinal irradiation, with a boost of additional radiation to the post-operative bed and any remaining sites of disease. Patients with average risk disease receive 23.4 Gy craniospinal irradiation (CSI) and a total dose of 54 Gy to the local tumor bed. In high-risk disease, the craniospinal radiation dose is generally 36 Gy but the tumor bed dose remains 54 Gy [20]. Ongoing clinical trials aim to find the minimum dose of craniospinal radiation in low-risk patients without sacrificing outcomes. The currently enrolling SJMB12 trial (NCT01878617) reduces the dose for the low-risk, non-metastatic WNT medulloblastoma tumors to 15 Gy CSI with 51 Gy tumor bed boost, and the study ACNS1422 (NCT02724579) is also investigating the ability to safely reduce dosing in non-metastatic low-risk WNT-MB (18 Gy CSI with 54 Gy tumor bed boost). A study of eliminating radiation from WNT medulloblastoma treatment (NCT02212574) was suspended due to an unacceptable number of treatment failures, indicating that radiation is unlikely to be eliminated entirely, even from low-risk protocols [21].

Table 2. Characteristics of subgroups (per Cavalli 2017) [2].

	Cell of origin	Copy number changes	Driver events	Age at diagnosis	5-yr OS	Mets at presentation
WNTα	lower rhombic lip progenitor (BLBP$^+$, OLIG3$^+$)	6-	*CTNNB1, DDX3X, SMARCA4* mut	Child/teen	87%	8.6%
WNTβ				Teen/adult	100%	21.4%
SHHα	Granule neuron precursors (ATOH1$^+$)	9p+, 9q-, 10q-, 17p-	*MYCN* amp, *GLI2* amp, *YAP1* amp, *PTCH1* mut (less), *TP53* mut	Child/teen	69.8%	20%
SHHβ		2+	*PTCH1* or *KMT2D* mut, *SUFU* mut/del, *PTEN* del	Infant	67.3%	33%
SHHγ		9q-	*PTCH1, SMO*, or *BCOR* mut, *PTEN* del	Infant	88%	8.9%
SHHδ		10q22-, 11q23.3-, 9q-, 14q-	*PTCH1* mut, *TERT* promoter mut	Adult	88.5%	9.4%
G3α	Progenitor neural cells (Nestin$^+$)	7+, 8-, 10-, 11-, i17q		Infant/child	66.2%	43.4%
G3β			*OTX2* gain, *DDX3* loss, High *GFI1/1B* expression	Child/teen	55.8%	20%
G3γ		8+, i17q	*MYC* amp	Infant/child	41.9%	39.4%
G4α	Unipolar brush cells (EOMES$^+$ and LMX1A$^+$) and glutamatergic cerebellar nuclei (MESI2$^+$, TBR1$^+$)	7q+, 8p-, i17q	*MYCN* amp, *CDK6* amp	Child/teen	66.8%	40%
G4β		i17q	*SNCAIP* dup	Child/teen	75.4%	40.7%
G4γ		7q+, 8p-, i17q	*CDK6* amp	Child/teen	82.5%	38.7%

Amp- amplification. Del- deletion. Mut- mutation. Dup- duplication. Mets- Metastases. OS- overall survival.

Table 3. Characteristics of group 3/4 subtypes (per Northcott 2017, Sharma 2019) [9,13].

Subtype	Risk Group (5 year OS)	Involved subgroups	Cytogenetics	Driver events [10]	Key clinical features
I	Standard 77%	3 and 4	1q+	*GFI1* and *GF1B* activation *OTX2* amp	Primarily in infants
II	Very high 50%	3	1q+, 5+, 6+, 8+, i17q, 10q-, 16q-	*MYC* amp *GFI1/GFI1B* activation *KBTBD4, SMARCA4, CTDNEP1* or *KMT2D* mut	
III	Very high 43%	3	7+, i17q, 8-, 10q-, 11-, 16q-	*MYC* amp (less)	
IV	Standard 80%	3	7+, 14q+, i17q, 3-, 8-, 10-, 11-, 16q-	No common drivers	Approximately 50% in infants 'Low risk' Group 3 tumors
V	Very high 59%	3 and 4	7+, 12+, i17q, 18+, 8-, 11-, 16q-	*MYC* or *MYCN* amp	Metastatic at diagnosis in >50%
VI	Standard 81%	4	7+, 12+, i17q, 18+, 3-, 8-, 11-	*PRDM6* activation, *MYNC* amp (less)	
VII	Standard 85%	3 and 4	7+, i17q, 18+, 3-, 8-	*KBTBD4* mut	
VIII	High, risk of late relapse/ death >5 years	4	4+, 7+, i17q, 8-	*PRDM6* activation, *ZMYM3* or *KMT2C* mut	Late relapse Largest subtype (25% of all Group 3/4 tumors)

**MYC* amplification and M+ disease were also considered high risk regardless of subtype.
Amp- amplification. Mut- mutation. OS- Overall survival.

Long-term toxicities from craniospinal radiation include hearing loss, hormone deficiencies [22], secondary malignancies [23], and decreases in intellectual development [24], which are more common in patients who receive radiotherapy as infants. Children under 36 months of age with posterior fossa tumors treated with radiation therapy experience significant decreases in verbal, language, and executive function skills compared to children treated without radiation therapy [25]. Therefore, a radiation-sparing approach in patients who are high risk for unacceptable neurocognitive toxicity (such as those under 3 years old) using higher doses of chemotherapy without radiotherapy has been used. Use of proton-beam radiotherapy has been shown to reduce toxicities in tissue outside the neuraxis, such as primary hypothyroidism [26], and outcomes are comparable when using proton-beam radiotherapy instead of conventional radiotherapy [22]. However, use of proton-beam radiation is limited by availability [27]. Ongoing efforts to safely reduce radiation for lower risk patients are likely to be aided by the additional prognostic stratification allowed by molecular tumor subgrouping [28].

3.3. Chemotherapy

The last phase of medulloblastoma therapy is systemic chemotherapy. A number of chemotherapy agents have been used, including cisplatin, lomustine, vincristine, and cyclophosphamide [29,30,31]. In addition, some protocols utilize weekly carboplatin [32] or vincristine [33] during radiation treatment as radiosensitizing agents. The SJMB12 trial (NCT01878617) further adds gemcitabine and pemetrexed to intermediate and high-risk Group 3/4 tumors [34]. The only targeted agent used in upfront therapy for medulloblastoma is vismodegib, a Smoothened inhibitor [35], used in upfront therapy in SJMB12 for children with SHH-mutant tumors.

In children under 3 years of age, clinical trials have used increased chemotherapy dose intensity to obviate or delay use of radiation, including the addition of intrathecal or intraventricular methotrexate [36] and use of myeloablative chemotherapy with autologous stem cell rescue [37,38]. While the relative efficacy of radiation-sparing treatment strategies in most patients is still unclear, patients with nodular desmoplastic histology medulloblastoma, especially those subsequently sub-grouped into SHH-II [16], had improved outcomes with chemotherapy treatment without radiotherapy in the HIT 2000 [36] and ACNS1221 [16] clinical trials. Prospectively, molecular subgrouping of younger children may help guide the clinical decision to delay or avoid radiation.

3.4. Relapsed medulloblastoma treatment

Patients with relapsed medulloblastoma have dismal outcomes, with a 3-year survival rate after relapse of less than 25% [39]. The treatment of relapsed and refractory medulloblastoma is varied, and options include re-irradiation [40], additional cytotoxic chemotherapy [41], and targeted agents [42]. There are greater than 20 currently open clinical trials that are enrolling children with relapsed medulloblastoma. Many of the open studies are reviewed later in this discussion.

4. Definitions of cancer stem cells

Understanding the drivers of medulloblastoma relapse will be key to improving overall survival rates in medulloblastoma patients. Medulloblastoma CSCs likely play a key role in tumor recurrence and contribute significantly to tumor

relapses and poor outcomes seen in medulloblastoma patients, and therefore targeting of CSCs represents a potentially effective therapeutic option.

4.1. Normal cerebellar development and medulloblastoma cells of origin

Normal cerebellar development begins during embryonic development and is completed several months after birth [43,44]. As medulloblastoma is an embryonal neoplasm derived from developing cells in the cerebellum, normal cerebellar development informs both the initiation of tumors and the pathways required for cancer stem cell maintenance [3]. Tumors from the different subgroups of medulloblastoma arise from cells from different parts of the developing cerebellum [10]. The upper rhombic lip is the source of the granule neuron precursors (GNPs) of the internal granule layer and external granule layer, the cells of origin for tumors in the SHH subgroup [3,45,46]. The lower rhombic lip is thought to be the site of origin of WNT subgroup tumors. Wnt signaling is active in neural stem cell proliferation, defining the midbrain-hindbrain boundary [43], and WNT medulloblastoma tumors are typically found in the fourth ventricle adjacent to the brainstem [47]. In 2019, Vladiou et al [11]. and Hovestadt et al [10]. applied single-cell RNA sequencing (scRNA-seq) to further investigate the cells of origin for each subtype as well as intratumoral cellular heterogeneity. They also clarified the cell of origin for Group 3/4 tumors; they appear to develop from the lineage of cells derived from a Nestin^{+} progenitor neural cell which differentiates into unipolar brush cells (UBC) and glutamatergic cerebellar nuclei (GCN) [10,11]. The scRNA-seq profile of Group 3/4 tumors lie along a continuum. Group 3 tumors contain higher numbers of cells that more closely resemble an undifferentiated progenitor cell, most markedly in *MYC*-amplified tumors, while Group 4 tumors are enriched in cells more similar to the more differentiated UBC and GCN cells [10]. These patterns correlate with the composition of methylation based Group 3/4 subtypes I–VIII [13]. Tumors that are in the middle of this continuum contained cells of both the differentiated and undifferentiated programs [10].

4.2. Definition of cancer stem cells

Cancer stem cells (CSCs) are cells which contain the dual properties of self-renewal [48] as well as the ability to differentiate into the original tumor cell lineages [43]. CSCs were first described in 1994 in Acute Myeloid Leukemia and were defined by elevated CD34 and reduced CD32 expression (CD34^{high} CD32^{low}) [49], opening a new chapter of cancer biology. Significant research efforts have since gone into both identifying and characterizing CSCs in multiple tumor types.

CSCs are typically small subpopulations of chemoresistant or radioresistant tumor cells with properties that make them of particular interest in understanding relapsed and refractory cancers. Incomplete elimination of CSCs by cancer therapy leaves a seed from which a new tumor can develop, with the same heterogeneity of the original tumor. New tumors may also have accumulated additional mutations and resistance to the previously used therapeutic agents, exacerbating the difficulty in eradicating recurrent disease.

4.3. Relationship between brain tumor cancer stem cells and neural stem cells

Neural stem cells (NSCs), which are both multipotent and self-renewing and which can be found in the adult brain, are the source of malignant transformation leading to brain tumors [43]. NSCs have numerous similarities with brain tumor cells as well. NSCs express the intermediate filament protein nestin [50], which is expressed on the progenitor cell of origin for Group 3 medulloblastoma [11] and also is expressed in multiple brain tumor types, including astrocytic tumors such as glioblastoma [51], oligodendroglial tumors, and ependymal tumors; nestin expression is frequently used to characterize CSC identity in brain cancers. The Notch, Wnt/β-catenin, and Hedgehog signaling pathways are additional conserved pathways that play important roles in NSC regulation throughout typical brain development and that also appear to have a role in medulloblastoma CSC maintenance and represent possible therapeutic targets (Figure 3). There are likely to be other pathways in normal neural development whose relationship with CSCs should be studied, such as neurotrophin signaling [52].

Notch activation at the cell surface leads to increased downstream expression of multiple genes, including *HES1, FOXG1, PI3KCA, AKT1, NFKB1, PPARG*, and *CCND1*. The expression of these genes modulates differentiation and cell-cycle progression in a context-dependent manner [53,54]. and in NSCs Notch signaling functions to maintain a stem-like state with delayed differentiation [55]. Notch2 is predominantly expressed in GNPs during cerebellar development and is overexpressed in some medulloblastoma patients [43].

In addition to its role in cell fate determination during neural stem cell development [56], Wingless (Wnt)/β-catenin signaling dysregulation drives the development of the WNT subgroup of medulloblastoma tumors and has been implicated in the maintenance of CSCs in several solid tumors [57]. Patients with aberrant Wnt signaling due to germline mutations in the *APC* gene have a 13-fold increased risk of developing WNT medulloblastoma tumors, in addition to increased risks of other tumors such as colorectal carcinoma [58]. The WNT signaling pathway also interacts with other key pathways implicated in both development and oncogenesis, including the RAS, PI3K, and hedgehog signaling pathways [59].

Hedgehog (Hh) signaling is involved in tissue patterning and can modulate epithelial-to-mesenchymal transition (EMT) in normal and malignant tissue [60,61]. Overactivity of this pathway can lead to tumorigenesis, and Hh activity is also increased in CSCs [62]. When an Hh ligand binds the Hh receptor, the inhibitory effect of PTCH on SMO is released. Free SMO then leads to GLI transcription factor nuclear translocation and a series of gene expression changes, resulting in altered proliferation, angiogenesis, and cell survival. SHH medulloblastoma tumors are driven by aberrant overactivity in the Hh pathway, and germline mutations of *PTCH1* causes Gorlin syndrome, a cancer predisposition syndrome with

increased incidence of both medulloblastoma and basal cell carcinoma.

Compelling questions remain unanswered regarding the relationship between cell-of-origin and CSCs. In one example, Zhang et al. utilized scRNA-seq to investigate the developmental hierarchy of cells within a *Ptch*-mutant SHH medulloblastoma mouse model, finding an OLIG2$^+$ lineage cell as a candidate tumor initiating cell driving tumorigenesis and relapse [63]. OLIG2+ cells were highest in the neural stem cell-like population, correlating with Nestin and Sox2 expression, and decreased in prevalence with differentiation along the GNP lineage. OLIG2$^+$ cells demonstrated higher sphere-forming capacity and tumorigenicity and were critical for tumor initiation in the mouse model. Also consistent with a cancer stem cell, OLIG2$^+$ cells represented only a small population in mature tumors, and were enriched after chemotherapy and in relapsed tumors. Deletion of *OLIG2* led to tumor growth inhibition. High *OLIG2* expression was also associated with inferior outcomes in SHH medulloblastomas, though not in the other medulloblastoma subgroups.

Sox2$^+$ cells within medulloblastoma further mark the link between aberrant cerebellar development and SHH medulloblastoma tumorigenesis. In normal cerebellar development, the EGL is derived from Sox2$^+$ precursors, which are transient in normal development. The persistence of the Sox2$^+$ cell population due to constitutive SHH activation is therefore suggested to initiate SHH-driven medulloblastoma tumors [64].

Work in the irradiated *Ptch1*$^{+/-}$ *mouse* SHH medulloblastoma model suggests a small population of Sox2$^+$ cells remain in mature medulloblastoma tumors and drive tumor propagation and relapse [65]. These cells were quiescent, and demonstrated higher tumor propagation at low seeding density than Sox2$^-$ cells with subsequent differentiation into the heterogeneous tumor population. They were resistant to antimitotic therapy and treatment with SHH-inhibitor vismodegib. Gene set enrichment analysis (GSEA) of Sox2$^+$ cells revealed similar transcriptional patterns to NSCs and other stem cell populations. Finally, high expression of Sox2 in patients with medulloblastoma was associated with inferior outcomes [65]. Sox2$^+$ cell growth and allograft tumor growth was inhibited with the antitumor antibiotic mithramycin, making this a drug of interest for further investigation.

Another study in *Ptch1*$^{\pm}$ mice determined that Sox2$^+$ cells are resistant to p53-dependent p21-mediated cell-cycle arrest, and radiation-enhanced p53-mediated cell cycle arrest is unable to fully eliminate Sox2$^+$ cells in p53 mutant tumors [66]. In p53^{WT} tumors, however, radiation was able to eliminate all Sox2$^+$ cells. After radiation of *Ptch1+/–p53*^{R172P} tumors, remaining Sox2$^+$ cells were observed to become highly proliferative and drive tumor regeneration, but decreased back to a small number of quiescent cells as these tumors matured. The Sox2$^+$ cells had gene expression profiles similar to Nestin-expressing precursor cells within the GCP lineage, and had high expression of *OLIG2*, which may provide a mechanism of p53-pathway resistance. They showed that high Sox2 expression was only seen in SHH-MBs, but within each of the 4 SHH subgroups, high Sox2 expression was association with poorer outcomes, regardless of p53 mutation status.

5. Specific markers of medulloblastoma cancer stem cells

A number of methods have been employed to identify markers of CSCs in medulloblastoma tumors, which have been limited by the small percentages of CSCs found in individual tumors. We summarize the most well studied CSC markers in medulloblastoma and the current understanding of their functional roles [43,67–69]. The strategy for identifying new putative CSC markers is not uniform, and there are likely additional markers not yet described. Methods of identifying cancer stem cells have matured with technologic advances, from the relatively nonspecific method of side population identification, to the present where single-cell RNA sequencing (sc-RNA seq) allows for a complete transcriptomic profiling of each individual cell in heterogenous populations.

An early method of characterizing a CSC population was through identification of a 'side population,' using the cell-permeable DNA binding dye Hoechst 33,342. A side population which has high levels of ABCG2 transporters pumps out Hoechst 33,342, defining a stem cell-enriched population [70]. This method has been used to identify putative CSC populations in several cancer types [71,72–74]. Since identification of side populations is less reliable and often inconsistent, side population analyses may remain a complimentary approach to any future CSC profiling but are unlikely to stand alone.

5.1. CD133

The most well-studied cell surface marker of medulloblastoma CSCs is CD133, or prominin-1 (*PROM1)*. CD133 is a 120 kDa five-transmembrane cell surface protein originally shown to be a hematopoietic stem cell marker [75,76]. and subsequently found to be a marker of normal human NSCs [77]. CD133 is also described as a CSC marker for many other solid tumors [55], and subsets of CD133+ cells have been found in all molecular subgroups of medulloblastoma [78].

The CSC properties of CD133-expressing medulloblastoma cells were first described in 2003 [79]. Medulloblastoma tumors were dissociated and grown in serum-free NSC media, and a fraction of cells maintained undifferentiated tumor spheres that demonstrated nestin and CD133 expression while lacking expression of differentiation markers such as β-III-tubulin. After cell sorting, the CD133+ fraction alone was capable of self-renewal. Under differentiating conditions, CSCs subsequently became negative for CD133 and nestin. Subsequent studies have verified that neurosphere conditions enrich for CD133+ cells [80].

CD133+ medulloblastoma cells were then tested *in vivo*, where they were uniquely able to initiate tumors in NOD-SCID mice and could be serially transplanted [81]; the CD133+ xenografts expressed neural precursor markers nestin and vimentin. CD133- cells, however, were not able to produce xenograft tumors.

Data further suggest that CD133 expression is correlated with outcomes. A study of 45 medulloblastoma samples

demonstrated a statistically significant decrease in overall survival and progression-free survival in those with high expression of *PROM1* [82]. A similar trend was seen in a series of 95 adult gliomas [83].

The work of Annabi et al. described the interaction between the tumor microenvironment and CD133+ cells. When implanting tumors from Daoy medulloblastoma cells or U87 glioblastoma cells into nude mice, subcutaneous tumors did not express CD133; however, the tumors formed from intracerebral injection did express CD133, indicating a differential response to the microenvironment. In addition, neurosphere cultures of Daoy cells demonstrated an induction of CD133 as well as metalloproteinases MT1-MMP and MMP-9, and silencing of the metalloproteinases reduced neurosphere-forming ability of Daoy cells [84], which may explain some of the invasive phenotype of CD133+ cells.

The level of oxygen in the tumor microenvironment also impacts the expression of CD133; Daoy cells show increased CD133 expression in a hypoxic environment *in vitro* and are resistant to radiation while hypoxic [85]. Further exploring the relationship of radiation and CD133, Sun et al. characterized the radioresistant fraction of ONS-76 cells [76,86]. The three clones with the highest post-radiation increase in CD133 expression had more tumor sphere formation, higher side population fractions, and a higher number of colonies which survived radiation. CD133+ cells had faster growth than CD133- cells, and the growth rate was higher from the resistant clones than the parental line [86]. Yu et al. found that Daoy neurospheres, which highly expressed CD133, Sox2, BMI1, and nestin, were radioresistant, resistant to apoptosis from TNF-related apoptosis-inducing ligand (TRAIL), and were not radiosensitized by TRAIL treatment when compared to the non-neurosphere cells [87].

Comparative analysis of putative stem cell populations with non-stem-like cancer cells from the same disease is now possible on a larger scale, which may further elucidate the signaling differences between the two populations and identify therapeutic targets. Studies to date have found detectable differences in gene and protein expression in CD133± cells and generally support CSCs being less differentiated [88], suggesting avenues for therapeutic targeting. Increased expression of anti-apoptotic genes in CD133+ cells (*CFLAR, CASP8, BCL2* and *BAX*) is likely linked to known resistance to therapy [87]. A proteomic evaluation of neurospheres from Daoy, UW-228, and ONS-76 medulloblastoma cell lines confirmed a more undifferentiated profile as well as expression of CD133 and nestin, but a clear proteomic profile separating CSCs from other tumor cells remains elusive [80].

The exact function of CD133 in medulloblastoma is not fully known, but genomic and proteomic profiles of CD133+ cells have begun to shed light on its function. Prior studies have demonstrated that CD133 expression is associated with known CSC signaling pathways, including the Notch, Hedgehog, and Wnt pathways [62]. In normal NSCs, Notch signaling promotes proliferation and supports a de-differentiated state [62], making it a logical target of investigation in medulloblastoma CSCs. Ligand binding to the Notch receptor causes intramembranous cleavage of Notch by the γ-secretase complex [89]. Fan et al. found that Notch blockade via a γ-secretase inhibition preferentially affected the CSC population, with reduction in the CD133+ population by five-fold and elimination of the Hoechst side population, indicating a possible vulnerability of CSCs to Notch blockade [89]. They also demonstrated decreased proliferation and increased differentiation of CD133+ cells after treatment. *In vivo*, Notch blockade led to decreased xenograft tumor formation. The CD133+ fraction of medulloblastoma cell lines exhibited higher Notch signaling, suggesting a higher dependence on this pathway. As γ-secretase inhibitors have been tested in several clinical trials, this vulnerability is a promising avenue to explore [90].

In normal neural precursors and in medulloblastoma cells, HIF-1α interacts with Notch to maintain the undifferentiated state, and medulloblastoma precursor cell expansion was supported by hypoxia [91]. Stimulating Notch1 activation with its ligand DLL4 under hypoxic, but not normoxic, conditions led to increased numbers of CD133+ cells, and DLL4 treatment had no effect on cell expression of other CSC markers. Blocking Notch signaling did not change the number of CD133+ cells but did decrease the number of nestin+ cells and increase the number of βIII-tubulin-expressing cells regardless of oxygen concentration. Conversely, normoxia supported differentiation, and γ-secretase inhibition blocked Notch activation and caused neuronal differentiation with decreased SOX2 expression and increased Math1 (a cerebellar external granule layer marker [92]) and βIII-tubulin expression. HIF-1α siRNA silencing also induces differentiation of medulloblastoma precursors. Lastly, HIF-1α+ cells in medulloblastoma tumors were found to have a higher prevalence of CD133+ cells, as well as enrichment of nestin+, Notch1+, and Hes1+ cells, and reduced βIII-tubulin+ cells [91]. While HIF-1 and HIF-2 may lead to the activation of the Notch, Wnt, and Hh pathways, they also activate dendritic cells and effector T cells, so concomitant use of immunotherapy may be able to address the radioresistance of CSCs [55].

Hedgehog signaling is also key to medulloblastoma development, and Wang et al. elucidated a relationship between CD133 and the Hedgehog signaling pathway [93]. CD133+ Daoy cells showed an increase in expression of Hh receptor genes *Smo* and *Ptch1*, while CD133- cells showed increased expression of *Shh* [93]. Hh antagonist KAAD-cyclopamine decreased the *Gli1* and *Ptch1* expression in CD133+ but not CD133- cells and also led to reduced CD133 protein expression. *Math1* and *MYCN* (a Hh target gene [94]) expression are also higher in CD133+ cells [93]. Bmi1 is downstream of Hh and is a key regulator of hematopoietic, neural, and brain tumor stem cell populations [93]. Incubating Daoy cultures with Shh ligand increased *Bmi1* expression through preferential binding of *Gli1* at the *Bmi1* promoter, and a positive feedback loop exists where downstream effectors of Bmi1 further activates Shh pathway genes. Bmi1 expression is also seen at higher levels in tumors from groups 3 and 4 medulloblastoma.

Further work is needed to characterize the exact relationship between CD133 expression and stemness in cancer, as the link is likely complex. A prior study demonstrated CD133 expression on nearly all colonic

tumor cells in a colon cancer model and that metastatic CD133- cells formed more aggressive tumors and expressed other putative CSC markers such as CD44, raising questions regarding the exclusivity of CD133 expression to CSCs [95]. In another study of brain tumor cells from human primary gliomas and medulloblastomas, CD133+cells were both Ki67- and Ki67+, and CD133- cells had markers of progenitor cells, including nestin, TUC-4, and DCX. In addition, this study demonstrated tumor formation with pure CD133- cell populations [96], suggesting that further study of the role of CD133 in medulloblastoma CSCs is clearly needed.

5.2. CD15 (SSEA-1)

CD15 (SSEA-1) has been identified as a candidate medulloblastoma CSC marker in the *PTCH*± mouse model for SHH medulloblastoma [97]. A variable number of CD133+ cells were found within tumors, most with <5% CD133+. CD133 + cells from *PTCH* ± tumors did not form neurospheres at clonal density as they have been found to do in human tumors. *PTCH*± tumor derived, CD133+ sorted cells were unable to propagate tumors in SCID-beige mice, but unsorted and CD133- cells did. In this model, the cells which could propagate tumors instead were primarily expressing the carbohydrate antigen CD15, also known as Lewis X/stage-specific embryonic antigen 1 (SSEA-1), and the neuronal progenitor marker Math1. CD15 expression has been identified on normal neutrophils [98] as well as in several cancers [99,100,101]. In the central nervous system, CD15 expression has been found in the progenitors of both adult and embryonic nervous systems [102,103], and was found in a subset of granule neuron precursor cells of neonatal *Math1-GFP*+ mice [97].

PTCH ± tumor-derived CD15+ cells consistently propagated tumors, which recapitulated a heterogeneous, mixed CD15 +/CD15- tumor resembling the parental tumor. Conversely, CD15- cells were unable to propagate tumors. Gene expression profiling of the CD15+ population demonstrated increased expression of genes which regulate proliferation and self-renewal when compared to the CD15- population. These findings suggest a role for CD15 in CSC maintenance. CD15 was then identified on a subset of human medulloblastoma samples by immunohistochemistry and flow cytometry. Ward et al. similarly found a population of CD15+ cells in tumors from *PTCH* ± mice with higher rates of propagation and resultant heterogeneous tumors [104].

In $Ptch1^{lox/lox};GFAP^{cre}$ mouse-derived medulloblastoma tumors, a population cells with CD24+/CD15+ co-expression formed tumors much more readily than CD24+/CD15-, CD24-/CD15+, or CD24-/CD15- cells, suggesting an improved tumor initiating ability in the co-expressing cell population [105]. CD24 is a cell adhesion glycosylphosphatidylinositol anchor protein that is expressed similarly in SHH, Group 3, and Group 4 medulloblastoma tumors, but has decreased expression in WNT medulloblastoma samples. It may be that a combination of surface markers best identifies the most stem-like population of medulloblastoma cells.

5.3. CD114

In 2015, it was published that cancer cell lines from multiple tumor types, including medulloblastoma, contained subpopulations that demonstrated cell surface expression of the granulocyte colony stimulating factor receptor (GCSF-R, CD114) [106]. CD114 has previously been described as a possible marker of CSCs in neuroblastoma [107], as CD114 expression defined a discrete subpopulation within neuroblastoma cell lines with self-renewal, pluripotency, and enhanced tumorigenicity. This CD114+ cell subpopulation was also distinct from previously characterized tumor-initiating cell subpopulations defined by CD133 expression, neurosphere assays, and side population staining. Further studies using limiting dilution and competitive lineage-tracing assays demonstrated CD114+ cells were capable of both self-renewal and differentiation. The gene expression patterns of CD114+ cells closely resembled embryonic and induced pluripotent stem cells and were similar to premigratory neural crest cells, while the CD114- subpopulation demonstrated gene expression patterns consistent with migratory neural crest cells representing a later stage of differentiation. CD114+ cells also were treatment-resistant, and CD114+ neuroblastoma cells were enriched in post-chemotherapy patient samples, and further increased in post-chemotherapy metastases. Xenograft tumors treated with chemotherapy demonstrated similar increases in the prevalence of CD114+ cells.

A subsequent study demonstrated that CD114-positive neuroblastoma CSCs were responsive to G-CSF, with an increase in the percentage of cells in S-phase seen after G-CSF treatment. Mouse neuroblastoma xenograft tumors had increased size, a higher percentage of CD114+ cells, and increased incidence of metastases with G-CSF treatment. STAT3 inhibition specifically targeted CD114+ cells in neuroblastoma tumors and sensitized tumors to chemotherapy [108]. These data strongly suggest a role of CD114 as a CSC marker in neural tumors and suggest that G-CSF treatment can positively modulate this cell population [109].

CD114 cell surface expression was subsequently demonstrated to be present in a subpopulation of medulloblastoma cells across established cell lines, PDX tumors and patient samples, and CD114+ cells were more resistant to chemotherapy than CD114- cells [110]. CD114+ cells also grew more slowly and responded to G-CSF with increased growth, and the percentages of CD114+ cells were increased after chemotherapy. Furthermore, treatment of medulloblastoma cells with chemotherapy followed by G-CSF, mimicking the treatment schema used in patients, led to further increases in the percentage of CD114+ cells. Levels of *NRP1* [111], *MSI1* [112], *TWIST1* [113], *MYCN* [114] and *SOX2* [115] expression were increased in CD114+ cells, supporting an undifferentiated, CSC-like phenotype [110]. G-CSF is used clinically in nearly all children with medulloblastoma in order to manage che-

motherapy-induced myelosuppression, raising concern that G-CSF may be supporting the growth of a CSC population.

6. Targeting medulloblastoma cancer stem cells

Therapeutic strategies directed against CSCs would likely reduce the incidence of treatment failure, tumor relapse, and death from disease. There are multiple putative targets that have been explored to date. While newer targeted agents are currently being evaluated in clinical trials for patients with relapsed and refractory disease [42], there are no specific agents being evaluated that selectively target CSCs in medulloblastoma. Most available options that may also eliminate CSCs are those which work on the downstream signaling pathways of the CSC markers. However, the development of monotherapies that specifically target CSCs may be limited by crosstalk between critical intracellular signaling pathways [59], and the development of resistance. Conversely, downregulation of one pathway may lead to suppression of the interconnected pathways, which may enhance treatment efficacy. Due in part to signaling pathway crosstalk, a rational evaluation of therapeutic targets of CSCs may therefore extend beyond known canonical pathways.

6.1. Targeted agents

Notch pathway inhibition: Notch inhibition via γ-secretase inhibitors [89] is an active area of drug development. A Phase I trial of the γ-secretase inhibitor RO4929097 monotherapy in adults with solid tumors demonstrated tolerability and early efficacy with radiographic response [116], and in a phase I study of RO4929097 combined with bevacizumab in adults with glioblastoma similar tolerability was observed, and 2 of 12 patients demonstrated a radiographic response [117]. A phase I trial of RO4929097 in combination with temozolomide and radiation therapy has also been completed in adult patients with brain tumors including medulloblastoma (NCT01119599), although results are not yet published. MK-0752 is a γ-secretase inhibitor that was well-tolerated, with toxicities including secretory diarrhea and skin rashes, by children with recurrent brain tumors in a recently completed phase I study (NCT00572182) [118]. A phase II clinical trial is ongoing in children with desmoid tumors, which are driven by aberrant β-catenin signaling, investigating monotherapy with the γ-secretase inhibitor nirogacestat (PF-03084014) (NCT04195399) after efficacy was demonstrated in adults [119]. As γ-secretase is known to have more than 90 substrates in addition to the Notch receptor [60], there is potential for significant additional off-target toxicity, particularly in children with years of future growth and development. To avoid these potential toxicities, Notch inhibition with antibodies targeting the Notch ligand DLL4 have been studied in phase I trials in adults with advanced solid tumors [60], with some responses seen [120, 121]. Currently a phase I trial of anti-DLL4 antibodies, NOV1501 (ABL001), is enrolling adults with relapsed solid tumors (NCT03292783). The multi-kinase inhibitor pazopanib both has been shown to decrease serum DLL4 levels in adults with renal cell carcinoma [122] and has efficacy against medulloblastoma in *in vitro* and pre-clinical *in vivo* studies [123]. The efficacy of DLL4 inhibition or reduction in medulloblastoma CSCs, however, has not yet been investigated.

Hedgehog pathway inhibition: SMO inhibitors are being evaluated in ongoing clinical trials for children with SHH medulloblastoma tumors. Phase I and II trials of vismodegib in patients with recurrent or refractory have been completed, and prolonged progression-free survival (PFS) was seen in patients with SHH tumors but not non-SHH tumors [35]. All patients in these studies did eventually experience progressive disease with vismodegib monotherapy, suggesting the existence or development of a treatment-resistant cell population. Even within the SHH medulloblastoma tumor group there was variability in response, and patients with *TP53*-mutant SHH medulloblastoma tumors were less likely to respond [124]. Vismodegib is currently under investigation as an adjunct to traditional chemotherapy in patients with newly diagnosed SHH medulloblastoma tumors in SJMB12 (NCT01878617) and in relapsed medulloblastoma in SJDAWN (NCT03434262). Unfortunately, because of the potential for rapid premature growth plate fusion, use of vismodegib is limited to children who have already achieved skeletal maturity [125,126]. In a phase 0/II trial in patients with glioblastoma, vismodegib monotherapy did not prolong patient survival but was found to penetrate into tumor tissue, resulting in decreased proliferative capacity of CD133+ neurospheres isolated from the treated tumors [127]. The use of SMO inhibitors continues to hold promise but likely requires a combination treatment strategy to overcome resistance. An increase in PI3K signaling activity was associated with resistance to SMO inhibition in SHH medulloblastoma tumors, and addition of inhibitors of PI3K and of PI3K/mTOR delayed the development of resistance to SMO inhibition in mouse medulloblastoma xenograft tumors [128]. PI3K activity has also been associated with medulloblastoma tumor formation as well as the development of metastatic disease in preclinical models [129,130]. and PI3K inhibition has demonstrated efficacy against medulloblastoma cells and tumors [131,130]. In preclinical *in vivo* studies, this effect was enhanced with concurrent mTOR inhibition, and the percentage of stem cells was decreased with combined PI3K/mTOR inhibition [132]. Further studies have shown that Akt inhibition with perifosine can re-sensitize medulloblastoma nestin^{+} stem cells to radiation treatment [129]. The National Cancer Institute Pediatric MATCH clinical trial is currently enrolling children with recurrent solid tumors, including medulloblastoma, with *PI3K/MTOR* activating mutations for treatment with samotolisib, a dual PI3K/mTOR inhibitor (NCT03213678).

Histone deacetylases (HDACs) modulate gene expression, and class I HDACs were shown to modulate Hedgehog signaling through *Gli1* and *Gli2* deacetylation, with resultant transcriptional activation [133]. HDAC inhibition has been shown to reduce medulloblastoma cell viability and also appears to promote differentiation as evidenced by decreased CD133 and BMI1 expression, and this effect was amplified with concurrent MEK1/2 inhibition [78,134]. Antitumor effects were also seen with both pharmacologic HDAC inhibition [135,136], and genetic knockdown of HDAC

gene expression [136]. Clinical trials evaluating HDAC inhibitors in patients with medulloblastoma are underway (NCT00867178, NCT01076530), but the efficacy of these agents has not yet been established.

Wnt pathway inhibition: A number of Wnt signaling pathway inhibitors are currently in preclinical development. Sulindac and celecoxib, two well characterized non-steroidal anti-inflammatory drugs, have been shown to inhibit Wnt signaling [60]. A preclinical study of celecoxib treatment enhanced radiosensitivity of CD133+ Daoy cells *in vitro* and enhanced the anti-tumor effect of radiation *in vivo* [137]. Celecoxib is a component of several anti-angiogenic 'metronomic' treatment protocols that have been used in children with several malignancies including recurrent medulloblastoma [138], with three of eight patients with relapsed medulloblastoma demonstrating at least stable disease on a regimen of thalidomide, celecoxib, fenofibrate, and alternating etoposide/cyclophosphamide [139]. Celecoxib is part of an anti-angiogenic regimen being tested in an ongoing phase II clinical trial for children with recurrent medulloblastoma, ependymoma and ATRT (NCT01356290). Additionally, the γ-secretase inhibitor PF-03084014 inhibited both the Notch and Wnt pathways in a colorectal cancer model and was able to reduce medulloblastoma xenograft tumor growth [140]. Alternative strategies for inhibition of Wnt signaling include suppression of the CREBBP-β-catenin interaction, suppression of Wnt protein processing and secretion through Porcupine, and monoclonal antibody therapy directed at Wnt receptors and Wnt ligands, such as the ongoing study of DKN-01, an anti-DKK1 monoclonal antibody (NCT03645980). Therapeutic agents which demonstrate evidence of preclinical and clinical efficacy and safety represent promising agents for further study in the treatment of medulloblastoma, both through targeting Wnt subgroup of medulloblastoma tumors as well as specific targeting of medulloblastoma CSCs.

Cyclin dependent kinase (CDK) inhibition: Cyclin D1 and CDK6 are upregulated in CD133+ Daoy cells [88], suggesting that CDK inhibitors may additionally target CSCs. CDK6 amplifications are seen most often in group 4 medulloblastoma [9], and been associated with adverse clinical outcomes [141]. CDK inhibitors have demonstrated preclinical efficacy in medulloblastoma models [142,143], including a mouse model of high risk *MYC*-driven group 3 tumors [144]. The CDK4/6 inhibitor ribociclib is currently being evaluated in children with relapsed medulloblastoma in early phase clinical trials (SJDAWN, NCT03434262).

6.2. Novel therapeutic approaches

Oncolytic viruses: Engineered viruses have the potential to specifically target and kill tumor cells while limiting effects on normal cells and tissues, and may have more effective cytotoxicity against CSCs than chemotherapy and radiation as the mechanism of action is cell cycle independent. Modified measles virus injected intratumorally is under study in a phase I trial in children with relapsed medulloblastoma and ATRT (NCT02962167) based on positive preclinical data in medulloblastoma [145]. Measles virus has also been successfully retargeted against CD133 in a murine model of glioma, killing $CD133^+$ cells but not $CD133^-$ cells, extending animal survival and sparing hematopoietic cells [146]. Engineered oncolytic Herpes Simplex Viruses G207 and M002 have been shown to kill medulloblastoma cells, also eliminating the CD133+ and CD15+ fractions, in xenograft tumors [147], and a phase I trial of G207 in children with relapsed brain tumors including medulloblastoma is underway (NCT03911388). Zika virus, which targets neural precursor cells during infection, can be used to target brain CSCs. In medulloblastoma and ependymoma, Zika virus kills stem cells in a SOX2-dependent manner [148], which may be manipulated for clinical oncolytic viral therapy in the future.

Personalized Medicine: Personalized medicine strategies that choose treatment regimens based on specific molecular and genetic tumor features and that are tailored to each individual patient are becoming increasingly feasible [149]. While this strategy is most often employed by using genomic evaluation to identify targetable oncogenic mutations, pediatric tumors generally have a much lower mutational burden than adult tumors, and oncogenic driver mutations are significantly less common in pediatric malignancies. Despite this potential limitation, the currently ongoing National Cancer Institute Pediatric MATCH trial [150] is using genomic analyses to determine the efficacy of agents directed in 17 matched mutation-drug pairs in pediatric patients with relapsed solid tumors, including medulloblastoma. This strategy has limited utility for patients with no clear targetable mutations. *Ex vivo* drug screening is an alternative strategy to identify individualized treatment strategies in which tumor cells are exposed to a battery of candidate anti-tumor agents, and the relative change in tumor cell viability is then used to identify the likely most effective agents for that specific tumor. Incorporation of high-throughput drug screening data into precision drug selection has been shown to be feasible on a series of mouse PDX tumors, setting the stage for replicating this model in patients [151]. A notable drawback to this strategy is that the chemosensitivities of CSCs may be missed by high throughput drug screening approaches, potentially leaving residual viable cells that could contribute to relapsed disease in patients. Attempts to target CSCs using targeted drug screening include the ChemoID assay that enriches for CSCs and compares chemotherapy efficacy in both CSCs and non-CSC tumor cells [152]. In prior studies of this assay in glioblastoma tumors, differential responses were seen between individual patients to each chemotherapy agent tested and also between bulk tumor cells and CSCs from the same tumor, with the CSCs showing greater chemoresistance to most drugs, and the efficacy of this approach is currently being studied in patients with glioblastoma and ovarian cancer [153]. Further work is needed to assess the utility of high throughput drug screening and CSC-enriched drug screening in patients with medulloblastoma.

Immunotherapy: Immunotherapy, including cell-based therapy, is a rapidly developing novel therapeutic strategy that may provide unique advantages in eliminating CSCs, as they can be designed to specifically target a given cell surface marker and can have an enduring effect as cells remain

in circulation. Previous clinical trials in adult patients with cancer have evaluated dendritic cells targeted against CSC antigens as cancer vaccines, including trials in patients with glioblastoma (NCT03548571), although trials have not included patients with medulloblastoma [55]. Preclinical investigations into the use of chimeric antigen receptor-T cells (CAR-T) in medulloblastoma have shown promise [154,155], and are progressing into early phase clinical trials. Currently for adult patients with medulloblastoma, there are phase I studies open using a CAR-T against IL13R-a (NCT04661384), and in children and adults with medulloblastoma phase I studies using a CAR-T against B7-H3 (NCT04185038), HER2 (NCT03500991, NCT02442297), and EGFR806 (NCT03638167) are enrolling. Using CAR-T cells specific against CSC surface markers such as CD133 could be an effective adjunct to existing medulloblastoma therapy, especially in high-risk disease.

7. Challenges of cancer stem cell research and future models

The study of CSCs has a number of technical limitations. Due to the small population of CSCs within any tumor sample, the amount of material available for analysis can be limited. Additionally, the culture media and environmental conditions used to select and maintain CSCs *in vitro* clearly impacts their molecular phenotype, along with possible phenotypic changes that occur with serial passages in culture that are CSC marker independent [67], leading to challenges in the interpretation of *in vitro* studies of CSCs. The use of established cell lines allows for large volumes of sample to be generated quickly, and are often a useful tool in first investigating a line of inquiry. However, they have inherent limitations, and therefore should be used with caution and findings should be supported with validation in another model. For example, the Daoy cell line has been used widely as a model of *TP53*-mutant SHH-MB since its creation in 1985, and in many of the studies described in this review. However, this line also has mutations in *NF1* and *CDKN2A*, which are more commonly found in high-grade glioma than medulloblastoma [156]. While this does not outright invalidate the use of Daoy cells, it underlines the need to develop rigorously profiled stem cell models for future research.

Medulloblastoma subgroup heterogeneity, including their unique developmental trajectories and cells-of-origin, implies that advances in our knowledge of the biology of tumors from individual molecular subgroups may not apply to tumors from other subgroups. The discovery of common CSC surface markers across all tumor molecular subgroups suggests that therapies targeting these CSCs will be effective in all medulloblastoma tumors. Future work in medulloblastoma CSCs will build upon prior models, but new strategies for identifying, isolating, and studying CSCs are being developed. The application of single cell sequencing has allowed for advanced comparisons of individual cells within the bulk tumor, which is key in studying small cell populations such as CSCs. In addition, new strategies have been developed to prospectively analyze the *de novo* development of the tumor, such as the use of induced pluripotent stem cell (iPS)-derived neuroepithelial-like stem cells (NES) to explore the pathogenesis of medulloblastoma tumors [157].

Understanding relapsed tumor evolution and the biology of relapsed medulloblastoma also remains a developing field, with much to be learned regarding the specific role of CSCs [158], as preventing relapse is the ultimate therapeutic goal.

8. Conclusion

CSCs in medulloblastoma demonstrate resistance to the radiation and cytotoxic chemotherapy typically used as frontline therapy for patients. Targeting CSCs is a promising strategy to reduce patient morbidity and mortality from relapsed and refractory disease, and further research is needed to better identify and target these cells. A variety of therapeutic strategies are currently under investigation, including drugs which target the Notch, Wnt, Hedgehog, and associated signaling pathways, oncolytic viruses with a tropism for CSCs, immunotherapy with CAR-T cells, and high-throughput drug screening strategies to develop personalized treatment approaches.

9. Expert opinion

The overall survival rates of children with medulloblastoma using current treatment regimens is among the highest of all pediatric central nervous system malignancies, with the majority of children surviving their disease. This success comes at a steep cost, however, with many children having life-long systemic side effects. Furthermore, children with refractory disease or who develop disease recurrence have very poor prognosis, and new treatment strategies are urgently needed. Our understanding of medulloblastoma pathogenesis has continued to increase; however, with significant insights into the drivers of medulloblastoma development and of the molecular heterogeneity which separates different subgroups. Less is known about the intratumoral heterogeneity and hierarchy and how CSCs can recapitulate tumors after the completion of standard of care therapy.

The study of CSCs has significant challenges, due in part to their scarcity within a tumor sample. Optimizing CSC-enriching growth conditions may help, but perhaps more useful are advances in technology such as single-cell sequencing, flow cytometry, and other high-sensitivity technologies that can study cell differences without requiring CSC isolation. These approaches allow for analyses of CSCs directly from patient samples, without the need to introduce variabilities from cell isolation and *ex vivo* cell culture.

The ultimate clinical goal of studying and understanding CSC biology is developing therapies that directly target them. While precision medicine approaches can be used to identify potential targets within a patient's bulk tumor, they do not address the possible chemoresistance of CSCs. Early use of CSC-specific precision medicine therefore represents a promising avenue to pursue. Targeted agents capable of exploiting the pathways known to underlie CSC maintenance, such as γ-secretase inhibitors, also hold great potential, and more research in this area is needed.

A further challenge in our understanding of CSC biology is the absence of agreement on what constitutes

a medulloblastoma CSC. While CD133 is the best understood marker for medulloblastoma CSCs, its function in medulloblastoma is not well understood, leading to difficulties in designing therapies directed against $CD133^{+}$ medulloblastoma cells. More recently described markers CD15 and CD114 are even less well understood. Sox2 and Olig2 expression have been shown to mark a CSC population, but are not cell surface markers and as such would need to be targeted differently. CD114 represents an interesting and potentially clinically relevant CSC marker, as its ligand is the granulocyte colony stimulating factor, which is given as part of medulloblastoma chemotherapy protocols, potentially promoting the survival and stimulating the growth of this CSC population. Other previously undescribed markers of CSCs may also exist. The number of molecular subgroups within MB and unique cell of origin for each subgroup raises additional questions regarding uniformity of these marker of CSCs across each subgroup. These questions may be best explored by prospectively analyzing the stages of MB development from a precancerous state.

The future holds great promise for our understanding of CSCs and in our ability to identify novel strategies to target CSCs in medulloblastoma tumors. High sensitivity assays, high throughput drug screening, and development of new models of medulloblastoma hold promise to all contribute to dramatically increasing our understanding of medulloblastoma CSCs, including our understanding of how to selectively target and eliminate them. The exact relationships between neural stem cells and normal neural development, medulloblastoma cell-of-origin, and cancer stem cell markers remain incompletely defined and a rich area of potential study. Safely eliminating CSCs without negatively impacting regular childhood neural development is an additional concern which will need to be considered with any novel therapeutic strategy and may limit which markers are viable targets. Additional strategies to more specifically target CSCs in each of the individual molecular subgroups of medulloblastoma and to use precision medicine approaches to more selectively target CSCs are also within view and may be achieved within the subsequent years.

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