Decoding the roles of astrocytes and hedgehog signaling in medulloblastoma

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Highlights:

SHH is a key component in the medulloblastoma microenvironment.

SHH-signaling up regulation is essential for the development of SHH-activated medulloblastoma.

In their local SHH-medulloblastoma microenvironment, the astrocytes have strong contribution to the tumorigenic process.

The transformation of stem cells and stromal cells to SHH-expressing astrocytes are a key event for the development and progression of the medulloblastoma.
Abstract
The molecular evolution of medulloblastoma is more complex than was previously imagined as emerging evidence suggests that multiple interactions between the tumor cells and components of the tumor microenvironment (TME) are important for tumor promotion and progression. The identification of several molecular networks within the TME, which interact with tumoral cells, has provided new clues to understand the tumorigenic roles of many TME components as well as potential therapeutic targets. In this review, we discuss the most recent studies regarding the roles of astrocytes in supporting the sonic hedgehog (SHH)–activated medulloblastoma molecular subgroup, and provide an overview of medulloblastoma progression through SHH expression and signal transduction mechanisms into the complex tumor microenvironment. In addition, we highlight the associations between tumor and stromal cells as possible prognostic markers and new therapeutic strategies.

Keywords: Medulloblastoma, tumor progression, tumor microenvironment, tumor-associated astrocytes, Hedgehog signaling, tumor-astrocytes cross talk.
Introduction

The molecular signatures of the medulloblastoma tumorigenesis

Medulloblastoma is the most prevalent malignant brain tumor in children[1, 2], and comprise only 1-2% of adult brain[3–5]. Recognized as a biologically heterogeneous disease, the World Health Organization (WHO) considered four molecular subgroups: wingless-activated (WNT), sonic hedgehog-activated (SHH); Group 3; and Group 4[4, 6–8]. Recently, the picture became more complex and 12 different medulloblastoma subtypes were described, including 2 WNT, 4 SHH, 3 group 3, and 3 group 4, each subgroup characterized by specific mutations, copy number variation, transcriptomic/methylomic profiles, and clinical outcomes[4, 5, 9–12]. For the SHH-medulloblastoma subgroup, germline or somatic mutations and copy-number variation are the common drivers that affect critical genes of SHH signaling, including PTCH1 (patched 1 homologue), SUFU (suppressor of fused homologue), SMO (smoothened), TP53 among others[13, 14]. The most common genetic events, which occur in both pediatric and adult tumors, are the loss-of-functions, mutations or deletions in PTCH1 and SUFU that are negative regulators of SHH signaling[13–15]. Activating mutations and amplifications of SMO, GLI1 or GLI2 (glioma associated oncogene homologues 1 and 2), also lead to constitutive activation of Hedgehog pathway[16, 17]. Germline and somatic TP53 mutations predominantly coincident with GLI2 amplifications are found exclusively in children between the ages of 8 and 17 years[18–21]. Somatic TERT (telomerase) promoter hotspot mutations were also associated with the SHH subgroup[22, 23]. In addition, alterations in many of these genes are also identified in transgenic mouse models of SHH-medulloblastomas[24, 25].

The tumor microenvironment and its roles in medulloblastoma-SHH subgroup

The medulloblastoma can also be viewed through the lens of the tumor microenvironment (TME) and its multiple roles in cancer can offer an interesting way to identify the critical steps regulating medulloblastoma biology, disease progression and overall survival[26–36]. In addition to tumor cells, the tumor microenvironment is characterized by diverse cell populations including stem-like cells and tumor-associated components such as blood vessels[28], immune cells[34, 35], neurons, endothelial cells, microglia[37], macrophages[38, 39], astrocytes[27, 29–31, 36] and stromal cells. The communication between these unique collections of cell types is implicated in therapy resistance[40–42], immune infiltration, and inflammation[38]. Since tumor-associated cells could be the focus of
therapeutic vulnerabilities, a comprehensive understanding of the interactions between the tumor cells and the tumor-associated components can afford new opportunities for targeted discoveries. In the SHH-activated medulloblastoma, recent studies have highlighted that the cellular diversity within tumors has critical roles in supporting the growth of tumor cells, and robustness of cancer[27, 31, 35, 37–39, 43, 44]. In the medulloblastoma-prone mice with a SMO mutation, the TME has tumor cells types in a spectrum of differentiation states, and tumor-derived cells expressing makers for astrocytic and oligodendrocytic precursors[45]. These results suggest that even in a tumor with single pathway-activation mutation, diverse mechanisms may emerge to drive tumor growth, demonstrating the need to target multiple pathways simultaneously for therapeutic effectiveness.

Astrocytes and medulloblastoma microenvironment: the new player within the complex ecosystem

Due to the increasing evidence between wound healing and developing tumors, recent studies are now investigating the complex functions of astrocytes in supporting medulloblastoma growth, as these specialized glial cells are involved in the functional recovery of the central nervous system (CNS)[27, 30, 31, 46–62]. Astrocytes are specialized and heterogeneous cells that are essential to modulate local blood flow as well as to maintain homeostasis of extracellular fluids, ions, and transmitters[59, 60]. In the healthy CNS, these glial cells participate in synaptic function and plasticity, among others dynamic activities that are crucial for neural circuit and neurological function, and behavior[59, 60]. In this context, emerging studies have identified the SHH signaling as an essential regulator of the molecular identity and functional properties of astrocytes[61, 62]. Under normal conditions astrocytes express the components of the Shh pathway, but do not secrete the SHH protein[63, 64]. Recent studies have shown that SHH pathway is active in astrocytes of the mature forebrain in vivo through the SHH-transduction system, including the receptor PTCH as well as Gli transcription factors[64, 65]. Others studies also demonstrated that SHH is expressed mainly by neurons in several brain areas including dopaminergic neurons[66], the Purkinje cells in the cerebellum and mossy cells in the hippocampus but not in astrocytes or oligodendrocytes[67]. In addition, under physiological stress or pathological conditions, it has been reported that astrocytes may be able to produce and become powerful sources of the SHH protein[68–71].

In the cerebellum, the specialized, unipolar astrocytes called Bergmann glia (BG) have been shown to be capable of responding to Purkinje-derived SHH signals in postnatal
stages through adulthood[72]. Mice in which SMO is postnatally ablated in BG demonstrate a reduced proliferation granule cells precursors (GCP) and increased differentiation accompanied by a loss of SHH activity. In these animals WNT signaling is ectopically elevated in GCP suggesting that this pathway is involved in cross-talk with the SHH pathway in regulating GCP proliferation[72].

During CNS pathologies, the astrocyte reactivity (AR)[48–55, 59, 61], an ubiquitous, complex and multistage process, is known to be involved in different disorders including traumatic[49], inflammation[46], stem-cell repair[51], regeneration[52], peripheral metabolic disorders[53], neurodegenerative diseases[50, 54], as well as tumor progression[44, 55, 57, 73–76]. In the context of brain metastasis, reactive astrocytes have a dual role that limit disease progression during early stages, and later on, foster tumor growth[55, 75]. During tumor progression, reactive astrocytes are key components of the microenvironment, and their function, and crosstalk with other components of TME have been the target of neuro-oncology research[27, 31, 43, 73–76]. Astrocytes can act through paracrine secretion of degradative enzymes, cytokines, chemokines, and growth factors[46] and have multiple primaries and branching endfeet that interacts with tumors cells, facilitate growth, proliferation, survival and invasion. In this way, recent studies have demonstrated that, in brain tumors, astrocytes secrete cytokine and trophic factors and contribute to tumor growth, metastasis and resistance to current therapy[51, 53]. In primary gliomas and brain metastases, astrocytes establish gap junctions with tumor cells, and these functional connections are regulated by signaling molecules, such as the connexin[53]. In response to such non-cell-autonomous stimuli, astrocytes can produce a multitude of molecular signals that can in turn influence many different neural and non-neural cell types, including cells involved in innate immune responses[73]. In parasite infections, astrocytes secrete SHH that, in turn, induces the production of GRP78, an endoplasmic reticulum (ER) chaperone of the heat shock protein family[30]. Under ER stress, it is believed that activation of GRP78 may increase cell survival through the unfolded protein response and may also protect cells from ER stress-induced apoptosis by activating Bcl-2 and inhibiting Bak, Bax, Caspase, and CHOP[30]. In fact, astrocytes facilitate the formation of medulloblastoma tumoroids by secreting SHH and generating astrocyte-derived extracellular matrix[36]. Likewise, in an elegant set of studies using a Math1-Cre/Ptch1+/- mice, Liu et al.[27] revealed that astrocytes are enriched in medulloblastoma, where there is an abundant expression of SHH mRNA only in the tumor tissue but not in normal tissue. Using several strategies these authors showed that in the medulloblastoma tissue, only the tumor-associated astrocytes expressed SHH[27].
The roles of astrocytes in the medulloblastoma microenvironment have been investigated and the studies demonstrated that astrocytes secreted CD133, a key cancer stem cell marker, involved in medulloblastoma tumorigenicity, and alter gene expression, increase invasion and adhesion of medulloblastoma cells[58]. Astrocytes can also have direct influence on brain tumor stem cells that are activated by several ligands, including SHH, which enrich the stem cell population. These interactions are bi-directional and tumor stem cell can provide signals that affect the surrounding astrocytes. Interestingly, Liu et al.[76] examined the effects of tumor-associated astrocytes (TAA) in regulating the stemness properties of medulloblastomas stem-like cells in disseminate tumors. These authors showed that TAA produced CCL2 (chemokine ligand 2) to shape the inflammation microenvironment, through Notch signaling activation[76].

**SHH-activated medulloblastomas: the indispensable roles of astrocyte-SHH secretion in tumor progression**

Under physiological brain conditions, astrocytes in the cerebellum provide important functions that support proliferation and migration of granule cell precursors[72, 77–80]. During tumor growth and progression, it is believed that these astrocytes play a critical role in supporting medulloblastoma by secreting the mitogen SHH into the tumor microenvironment[27, 31, 36, 72]. Beside the mutational landscape of SHH signaling components promoting medulloblastoma tumorigenesis, an interesting current topic is the contribution of SHH signaling in medulloblastoma initiation and progression. SHH signaling has been hypothesized to influence the medulloblastoma in a paracrine manner by being secreted to the stroma, which in turn signals to the tumor[31]. This could be analogous to the reciprocal signaling networks that SHH establishes during embryonic development[81, 82] or in the nigro-striatal system[66]. In an autocrine manner, SHH ligands secreted by TEM cells including astrocytes activate the signaling in the surrounding stroma, which provides a favorable microenvironment for tumor growth[83].

Sonic hedgehog signaling is a highly conserved pathway that have been intensively studied in multiples roles during normal development by regulating processes involved in tissue patterning, proliferation, and differentiation[84–86]. In its canonical pathway, SHH acts on target cells and involves the binding of SHH protein to its receptor *PTCH1*, relieving the inhibition exerted by *PTCH1* on *SMO*[87, 88]. Activated *SMO* elevates the transcription of the activators and repressors of the *Gli/Ci* zinc finger transcription factors, resulting in a series of downstream events that ultimate leads the transcription of SHH target genes[84, 86].
In general, Gli1 and Gli2 act as transcriptional activators, while Gli3 represses gene transcription. In SHH-producing cells, SHH may act in an autocrine manner or is secreted in a soluble form through the extracellular milieu to act in a paracrine manner on several long-range target cells[66].

The involvement of SHH signaling in medulloblastoma pathogenesis have been studied extensively, and although the link between the SHH signaling pathway and tumorigenesis is very heterogeneous, it is known that the aberrant activation of SHH signaling leads to the growth, proliferation, and invasion of tumor cells[43, 45, 83, 89–92]. Mouse models of medulloblastoma are generated by engineering mutations or mis-expressing the murine forms of genes mutated in human medulloblastoma. In these models approximately 15% of mice with heterozygous PTCH1 mutation develop tumors in their cerebella resembling the SHH group medulloblastoma in human[24, 93, 94]. Conditional deletion of PTCH1 in cerebellar granule cell precursors (Math1) caused medulloblastoma formation with 100% penetrance[95–97]. These tumors express both GFAP and neurofilament, suggesting that tumors arise from a stem cell capable of differentiating along neuronal or glial lineages. Also, mice bearing an active form of SMO or activation of neural stem cells (GFAP and Olig2 positive cells) develop medulloblastoma, suggesting that the activation of SHH pathway are responsible for medulloblastoma formation[90, 92, 94, 97, 98]. In order to investigate the early phases of SHH-associated medulloblastoma initiation and progression, Di Pietro et al.[29] used a novel GPR37L1−/−PTCH1+/− mouse strain that exhibit an increase expression of cerebellar SHH, SMO, PTCH1, down-regulation of GCP proliferation and a delay in the medulloblastoma occurrence. The GPR37L1 that is expressed in most glial cells, including cerebellar Bergmann glia, interacts with PTCH1, and this genetic ablation affected the trafficking of PTCH1 in an autocrine crosstalk way. Using a Math1-Cre/PTCH1+/− mice, Liu et al.[27] revealed that astrocytes are enriched in medulloblastoma, where there is an abundant expression of SHH mRNA only in the tumor tissue. These authors showed that in the medulloblastoma tissue, only the tumor-associated astrocytes expressed SHH, suggesting that these astrocytes also secrete this ligand. SHH also contributes to proliferation, and these authors showed that it was downregulated in medulloblastoma cells in vitro. When exogenous SHH was added to the cultures, there was an increase in medulloblastoma cell proliferation[27]. This result was further confirmed within an organotypic slice culture, where the blocking activity of SHH with 5E1 treatment reduced the level of medulloblastoma cell proliferation, without an increase in apoptosis or cell death. Using a mouse model with ablationof GFAP-TK, Liu el al.[76] also showed a
significant suppression of medulloblastoma growth in vivo by blocking tumor cell proliferation while promoting differentiation. In addition, using a Math1-Cre/PTCH1+ /Nestin mice model, in which medulloblastoma cells gradually increase the levels of Nestin, an intermediate filament protein that plays an inhibitory function of Gli3 to fuel SHH signaling, these authors showed that SHH was able to induce Nestin mRNA in these mice, via tumor-associated astrocyte (TAA)[27, 76]. Genetic ablation of TAA dramatically inhibited Nestin expression in medulloblastoma cells, resulting in reduced proliferation and a block in tumor growth. These findings revealed that SHH can signal through a pathway that is independent from PTCH1, and revealed a novel non-canonical signaling pathway in neoplastic cell that involves SHH, SMO and Nestin[27].

Although tumor cells from the above medulloblastoma models can be readily purified and cultured, these cells do not sustain SHH signaling in vitro[99]. Also, primary medulloblastoma cells tend to differentiate in vitro, which negates the possibility to passage or preserve the medulloblastoma cell lines[27]. Using medulloblastoma cells isolated from Math1-Cre/PTCH1+ /mice, it was shown that these cells autonomously cease their proliferation and initiate differentiation and all the SHH pathway target genes significantly declined in time under adherent culturing conditions[36]. However, these authors observed a supportive role of astrocytes that secrete SHH promoting the tumoroids that retain tumorigenicity. The blockade of the SHH secretion or the removal of astrocytes inhibited the formation of these tumoroids, suggesting that SHH signaling from astrocytes plays an important function in supporting tumor-growth[36].

The interplay between the TME and cellular differentiation is another exciting new area of investigation in cancer biology related to SHH-driven medulloblastoma. A recent study showed that tumor-derived astrocytes are involved in the reprogramming the microenvironment[31]. Using a Mosaic Analysis with Double Markers (MADM) to observed lineage tracing in SHH medulloblastomamice models, Yao et al[31] found that astrocytes within TME trans-differentiated from granule neuron precursors that never differentiate into astrocytes under physiological conditions[31].These authors also performed the transcriptome profile of these “astrocytes-like” cells and observed that they closely resemble to normal astrocytes[31]. In vitro cultures experiments, these tumor-derived astrocytes (TuAstrocytes) exhibit tumor-supporting roles by accelerating the growth of tumor cells in a paracrine effect. TuAstrocytes promoted the growth of tumor by secreting interleukin-4, which in turn induce the tumor-associated macrophages to secret insulin-like growth factor 1[31]. These results support the complex relationship between the TME and the tumor cells, and highlight an
intricate TME community that trans-differentiate, as well as a multilateral paracrine network supporting the growth of tumor cells[31, 74].

**Therapeutic approaches and the intratumoral heterogeneity of SHH-medulloblastomas**

Genetic alterations in key components of the Sonic hedgehog pathway led to activation of constitutive SHH signaling[13, 14, 93]. Thus, therapies strategies for SHH-pathway-dependent cancers primarily aimed to inhibit the components of SHH pathway, including SHH ligand itself, *SMO*, and *GLI* proteins[100–102]. Historically, the standard chemotherapy regimen for medulloblastoma largely included cisplatin, vincristine, carboplatin, cyclophosphamide and lomustine. These alkylating agents are very toxic and generate many side effects[103]. The serendipitous discovery of the steroidal alkaloid cyclopamine that inhibit *SMO* and suppress SHH signaling, enable the pharmacological modulation of the SHH signaling pathway as a therapeutic[87, 104, 105]. In fact, the most successful strategy has been to target *SMO* with small molecule compounds, and two FDA-approved drugs use this strategy[106, 107]. In medulloblastoma, the two novel *SMO* inhibitors, sonidegib (LDE225) and vismodegib (GDC-0449) have been used as a therapeutic that specifically target genes[108, 109]. Unfortunately, in children, there is a greater concern for developmental toxicities since the SHH pathway is primarily active during development[110]. Also, SHH-medulloblastoma commonly harbors mutations that result in emergence of resistance to *SMO* inhibitors and can occur rapidly[111, 112]. To avoid and overcome *SMO* inhibitor resistance, combination therapies will likely be need, and there is a continuing effort to identify and therapeutically target others transcription factors[113, 114].

Regardless the canonical hedgehog pathway, recent advances in SHH-medulloblastomas research has expanded the list of potential biomarkers that involve other molecular targets[115]. Liu et al[27] demonstrated that astrocyte-derived SHH is responsible for induction of Nestin expression only in *PTCH1*-deficient GCPs and medulloblastoma cells, in a *SMO*-dependent, but Gli1-independent manner. This paradoxical effect of SHH implies a role for additional SHH receptors such as *BOC*[116], and orient research in new directions towards viable active molecular targets in the SHH pathway. Related to clinical trials, protein kinase inhibitors are being explored in the treatment of medulloblastomas[117]. The phase I clinical trial sponsored by St. Jude Children’s Research Hospital explores a selective inhibitor of checkpoint kinase 1, which mediates cell cycle checkpoint control and DNA repair, also playing a role in chemotherapy resistance (https://clinicaltrials.gov/ct2/show/NCT01601184).
Targeting other key pathways together with SHH signaling is a considerable strategy as there is considerable heterogeneity among SHH-medulloblastoma, suggesting that non-transcriptional mechanisms are also involved in SHH signaling mediated tumorigenesis in medulloblastomas[118]. Among the medulloblastoma groups, SHH-medulloblastoma displays the highest number of associated macrophages (TAMs), which are critical participants in tumor progression, and can be a potential therapeutic target[38, 39, 119]. Margol et al[38] showed that expression of inflammation-related genes including TAM-related genes, CD163 and CSF1R, is higher in SHH as compared to the other medulloblastoma subgroups, suggesting that combination therapy aimed to the microenvironment in addition to the tumor’s cells may improve and extend current therapeutics options.

Novel targets and therapeutic opportunities for medulloblastomas: a potential application of astrocytes-SHH medulloblastoma cross talk research

The current understating of the TME contributions for the growth of tumor cells shifted the focus of neuro-oncology research, moving from exclusively targeting tumoral cells to targeting the tumor microenvironment, or signals coming from it, as well as the interactions between them. From multiple studies, it has become clear that the interplay between tumor cells and cells of the tumor microenvironment orchestrate events that are critical to tumor progression, and in this way, many cellular and molecular elements of the microenvironment are emerging as attractive targets for therapeutic strategies. Although granular cell precursors (GCPs) have been presumed to be the medulloblastoma cells of origin, and the search for druggable targets in the medulloblastoma have focused for a while on these cells, protein receptors and peptide factors from other cellular sources that impact SHH-driven medulloblastoma have attracted the attention of researchers more recently.

The G protein–coupled receptor (GPCR) family of proteins are widely dysregulated in cancer and yet are underexploited in oncology. Recent studies have shown that GPCRs can play multiple roles in cancer progression, including proliferation, survival, angiogenesis, metastasis, therapy resistance, and immune evasion upon their activation by ligands produced by cancer cells or by the multiplicity of cells within the tumor stroma[120]. The mitogenic ciliary functions of GPR37L1 in SHH–SMO signaling are particularly attractive to target cancer via the tumor microenvironment. The GPR37L1, an orphan G-protein-coupled receptor is a selective marker of cerebellar BG astrocytes[121, 122], and specifically colocalize, and interact with the PTCH1 protein in discrete areas of these Bergmann glia cell
membranes in newborn mice[121]. The Bergmann glial cells possess the primary cilia (PC), antenna-like organelles, which are required for sensing and transducing extracellular stimuli[123]. These PC are essential for the regulation of several signaling pathways such as SHH and WNT, and they can promote tumorogenesis in medulloblastoma[123, 124]. Primary cilia were reliably detected in all cells of pre-neoplastic MB in PTCH1+/− mice[29]. Thus, the specific detection of primary cilia could be usefully applied for the study of early, pre-neoplastic MB lesions[124].

Mice GPR37L1−/− present precocious Bergmann glia and Purkinje neuron maturation, and increased levels of Purkinje secreted SHH, as well as SMO and the intracellular effectors of the SHH-SMO cascade, N-Myc, Gli2[121]. Di Pietro et al. (2019) using GPR37L1, PTCH1 double mutant mice showed that this genetic ablation of GPCR37L1 in this prone medulloblastoma mice, can reduce the occurrence and severity of post-natal tumor[29] (Figure 1). These authors speculated that this receptor could be involved in the process of BG’s modulation of SHH production by Purkinje neurons, and suggested the involvement of WNT3, a specific inhibitor of SHH-induced neuronal mitogenesis[29].
Figure 1. In the absence of Hedgehog ligand (SHH), the negative regulator *PTCH1* are present on the ciliary membrane. In this state (Inactivated), suppressor of fused (*SUFU*) forms a complex with the *GLI* transcription factors at the tip of the cilium. SHH binding to *PTCH1* (Activated) induces its translocation away from the cilium and promotes the entry of the activating receptor *SMO*. This process allows the migration of active *GLI* (*GLI*<sup>A</sup>) into the nucleus where transcription of SHH target genes is activated. Mice heterozygous for loss-of-function *PTCH1* mutations have a higher incidence of medulloblastoma[24, 93, 94]. In a GPR37L1, *PTCH1* double mutant mice, the lack of GPR37L1 reduced the post-natal tumor occurrence and decreased incidence of more aggressive tumor type[29].

The secreted neuro- and glioprotective glycoprotein prosaposin (PSAP) have been shown to interact with and activate this GPR37L1 receptor in Purkinje cells and interneurons in the cerebellum[125]. PSAP has been identified as a potent neurotrophic factor that promoted neurite outgrowth and prevented programmed cell death of cerebral granule neurons in vitro[126]. PSAP can promote glioma cell proliferation via the TLR4/NFκB signaling pathway[127]. Jian et al. 2018 using TAK-242, the TLR4-specific blocking agent, confirmed the effects of PSAP on glioma growth[127]. As GPCRs are the most “druggable” class of proteins currently known, the GPR37L1 become highly valuable targets for the development of novel therapies, and their use of specific blocking agent may be an important target for medulloblastoma treatment.

Added with the findings highlighted here, the effects of SHH secretion by tumor astrocytes on the tumor progression, and the adaptative trans differentiation of the tumor and their reliance on astrocytic signals open new perspectives for discovering multiple potential therapeutic targets, within the tumor-associated glial cells. With the current developments in genomics that has delivered large amount of genetic and epigenetic data from the subsets of human medulloblastoma, the design of the future medulloblastoma therapy drugs must rely on the identification of genes that are specifically expressed in these tumor-associated astrocytes. These genomic data will provide a more comprehensive picture of the molecular scenario of the players driving tumor progression and will help to find ways to target those genes, thus increasing the probability of suppressing tumor progression, and/or reversing the pro-malignancy effects of the SHH secretion by tumor associated astrocytes. In the specific context of SHH-MB, further research studies will require strategies to manipulate SHH signaling in reactive astrocytes in order to identify SHH-dependent transcriptional signatures. Furthermore, blocking the astrocytes from secreting SHH into the TME has the potential to
influence the tumor progression, and may bring novel intervention strategies for this child malignant disease.

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