Chapter 8
Hedgehog Signaling in Pediatric Brain Tumors

Joon Won Yoon, Jason Fangusaro, Philip Iannaccone, and David Walterhouse

Keywords Sonic hedgehog • Central nervous system • Cerebellum • Pediatric brain tumors • Medulloblastoma • Hedgehog inhibitors

Introduction

Sonic hedgehog (SHH) signaling plays roles in patterning and normal development of the mammalian central nervous system (CNS). Precise regulation of the pathway appears to be crucial in the CNS since dysregulation of SHH signaling has been associated with CNS birth defects and brain tumors. In this chapter, we focus on (1) the role of SHH signaling in mammalian CNS development, (2) the role of SHH signaling in pediatric brain tumors, and (3) potential clinical applications of Hedgehog (HH) pathway inhibitors in the treatment of pediatric brain tumors. We use the following conventions in this chapter: upper case = human protein (e.g. SHH, GLI1), lower case = mouse protein (e.g. shh, gli1), upper case italics = human gene (e.g. *SHH*, *GLI1*), and lower case italics = mouse gene (e.g. *shh*, *gli1*). When we are discussing a pathway in a general way without specific reference to gene, protein, or species, we use upper case without italics.

Role of SHH Signaling in CNS Development

During development, the CNS arises from the neural plate, which is composed of a single layer of cells derived from midline ectoderm. Neuroepithelial cells in the neural plate undergo rapid proliferation and morphologic changes to form the neural tube.

J.W. Yoon (✉)
Department of Pediatrics, Developmental Biology Program,
Children's Memorial Research Center, Northwestern University,
Feinberg School of Medicine and Children's Memorial Hospital,
2300 Children's Plaza, Box 204, Chicago, IL 60614, USA
e-mail: j-yoon2@northwestern.edu

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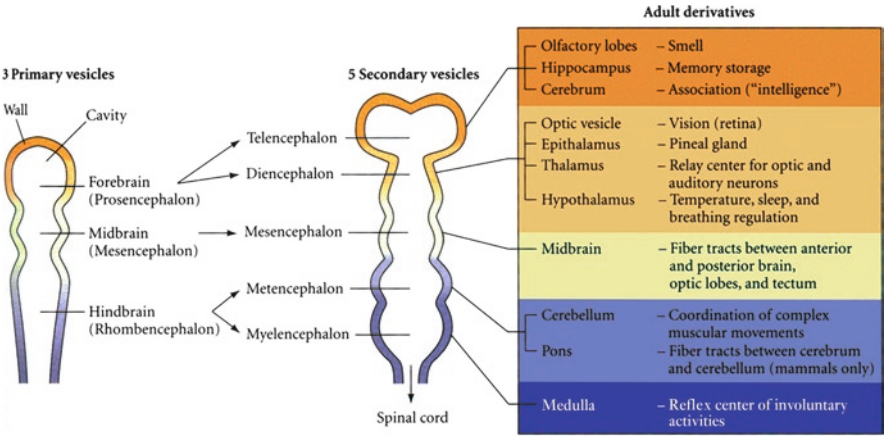

Fig. 8.1 Early human brain development. The three primary brain vesicles, five secondary brain vesicles, and their adult brain derivatives are shown. Reproduced and adapted from Developmental Biology, eighth edition with permission from Sinauer Associates, Inc.

Further proliferation of cells in the anterior region of the neural tube causes expansion and creates three primordial brain vesicles called the prosencephalic, mesencephalic, and rhombencephalic vesicles. These primary vesicles subsequently develop into secondary vesicles: the telencephalon and diencephalon (forebrain), mesencephalon (midbrain), and metencephalon and myelencephalon (hindbrain) (Fig. 8.1).

At a molecular genetic level, the events described above require complex interactions between key signaling pathways, including the SHH, Wingless (WNT), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β) pathways and their target genes. Activation of these signaling pathways has been associated with fundamental events during CNS patterning. These events include (1) establishment of polarity within the developing nervous system, (2) rapid expansion of cells in the region of the developing brain, (3) establishment of inter-brain boundaries, and (4) establishment of regional specificity. Remarkably, SHH signal transduction appears to be critically involved in each of these developmental events in a spatial- and temporal-dependent manner.

Dorso-Ventral Polarity and SHH Signaling

During early mammalian development, CNS patterning requires the establishment of axes in the neural tube. shh signaling contributes to establishing the dorso-ventral axis as the neural tube fuses at embryonic (E) day 8.5 in the mouse. shh is secreted by the notochord which lies immediately ventral to the neural tube and is also expressed by the ventral floor plate in the developing neural tube [1, 2]. Expression of *shh* in the ventral region of the developing neural tube establishes a gradient of shh within the neural tube, highest ventrally and lowest dorsally (Fig. 8.2).

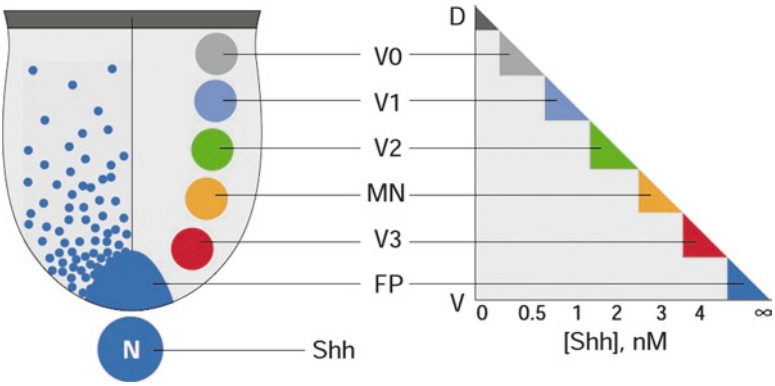

Fig. 8.2 A shh gradient regulates transcription factors that establish ventral specification in the developing CNS. The shh gradient (shown on the *right*) induces ventral floor plate and specifies five ventral cell types (shown on the *left*). *D* dorsal, *FP* floor plate, *MN* motor neuron, *N* notochord, *V* ventral, *V0–V3* ventral inter neurons 0–3. Reproduced and adapted from EMBO reports 4(8):761–765 (2003) with permission from the Nature Publishing Group

This gradient differentially regulates the expression of transcription factors that specify polarity and ultimately cell fates in the developing CNS, including *pax*6, the homeobox gene *nkx*2.2, and the floor plate marker *hnf3*-β [3, 4]. The importance of shh to early CNS development has been demonstrated in $shh^{-/-}$ mice, in which the notochord degenerates and the ventral floor plate and motor neurons fail to form. The ability of shh to induce differentiation of ventral cell types in the nervous system has been demonstrated by aberrantly expressing *shh* in the dorsal CNS. Aberrant expression of *shh* in the dorsal CNS activates dorsal expression of *hnf3-β* and causes aberrant dorso-ventral patterning [5–7].

Although shh induces differentiation of ventral neural precursor cells [6, 8, 9], further differentiation into motor neurons, interneurons, glial cells (oligodendrocytes), and other cell types in the CNS appears to require complex and incompletely understood interactions between shh signaling and the wnt, bmp, fgf, and tgf-β signaling pathways [7]. For example, shh regulates expression of *fgf8* receptors [10–13], and fgf8 together with shh induce dopaminergic neurons in the ventral region [10].

Rapid Expansion of Cells in the Region of the Developing Brain

Early mammalian brain development is characterized morphologically by rapid growth and expansion of the neural tube, which results in the formation of the brain vesicles (Fig. 8.1). The three primary vesicles have formed by week 4 of human development. This morphologic change results from both increased proliferation and reduced apoptosis of neuroepithelial cells. Several experimental approaches have been used to show roles for shh in regulating both proliferation and survival of cells that contribute to brain development. First, studies placing a transplanted

notochord, the source of shh, near the neural tube demonstrate increased proliferation, differentiation, and survival of nearby neural tube cells [14–16]. On the contrary, surgical removal of the notochord disrupts midbrain expansion by promoting cell death and inhibiting cell proliferation [17]. Second, *shh*$^{-/-}$ mouse embryos show multiple defects, including an overall reduction in the size of the brain, especially the forebrain [5]. Finally, ectopic expression of shh by electroporation into the developing midbrain region stimulates cell proliferation to regulate growth and morphology of the ventral region of the midbrain [17, 18].

Brain Boundaries

Studies in vertebrate embryos suggest that shh signaling specifically regulates genes at the midbrain–hindbrain boundary. Evidence suggests that shh functions to maintain this distinct boundary once it has formed rather than establishing the boundary [19]. Indeed, ectopic expression of *shh* by microinjection into one blastomere at the 2–4 cell stage expands the domain of expression of the shh target gene *sal* at the midbrain–hindbrain boundary [20, 21]. Disruption of shh signaling at the boundary by a mutant form of patched (ptc1) that cannot bind shh causes the midbrain–hindbrain boundary to become broader and indistinct, with midbrain and hindbrain cells inter-mixing across the expanded border [22]. Dorso-ventral cell fates are also affected in this model. As seen in other regions and periods during CNS development, blocking endogenous shh activity in the midbrain transforms cell fates from ventral to dorsal and correlates with the movement of dorsal cells into the ventral midbrain [19].

Regional Specification of the Developing Brain

shh is expressed along the entire anterior–posterior axis of the developing neural tube. It is believed that the establishment of regional specificity along the anterior–posterior axis of the developing CNS is achieved by differential expression of *shh* together with other key signaling pathways, such as the *wnt*, *bmp*, *fgf*, and *tgf-β* pathways in a regional specific manner. Roles for shh in regional specification is reviewed in the following sections.

Forebrain

shh signaling appears to regulate the size, ventral cell fate specification, and ventral patterning of the developing telencephalon. Targeted loss of shh in *shh*$^{-/-}$ mice results in multiple morphologic defects in the forebrain, including a reduction in size, fused telencephalic vesicles, and fused optic vesicles [5]. On the contrary, ectopic expression of *shh* by retroviral injection in early mouse embryos (E9.0) enhances proliferation and causes a substantial expansion in the size of the telencephalon [23].

If neural explants from the telencephalic region are incubated with *shh*-expressing cells, the neural plate in the prospective forebrain region differentiates into motor neurons which are normally observed in the ventral CNS [8]. shh treatment at E10.5 also represses expression of dorsal telencephalic markers such as *emx1* and *tbr-1* [24].

Ventralization of the telencephalon is also tightly regulated at the level of the gli family transcription factors. For example, shh inhibits expression of the repressor form of *gli3* (*gli3-R*) in the ventral telencephalon, presumably to promote active shh signaling and ventralization as well as to inhibit dorsalization [25]. Indeed, "extra-toes" mice carry a mutation in *gli3*, and E12.5 mutant embryos lack expression of dorsal marker *bmp* genes in the telencephalon, even though shh expression is unaltered [25]. Also, ventral marker genes, such as *dlx*2 and *gsh*2 are expressed in the dorsal telencephalon in "extra-toes" mice [23].

Midbrain and Hindbrain

shh signaling appears to regulate cell proliferation, apoptosis, and cell fate specification in the developing midbrain and hindbrain. Loss of *shh* expression causes decreased cell proliferation and increased apoptosis in the midbrain region. Similar results are obtained when the shh signal is reduced by surgical separation of the notochord from the floor plate, injection of cyclopamine into the lumen of the midbrain, or in the setting of $shh^{-/-}$ mice [17, 26]. These cellular changes collectively alter early expansion of the brain, causing a reduction in the size of the midbrain and ultimate collapse of the brain vesicles. Of interest, while the growth of the developing midbrain in E8.5 $shh^{-/-}$ mice is significantly reduced, the sizes of the diencephalon and hindbrain are unaffected [26].

shh signaling also specifies dopaminergic neuron cell fate in the developing midbrain [27]. Recent evidence using cultured midbrain suggests that higher level shh signaling inhibits cell proliferation and dopaminergic neuron specification, pointing out that shh signaling functions in a concentration-dependent manner to establish cellular and morphologic phenotypes [28]. In addition, in the hindbrain, shh signaling defines the ventral region and promotes hindbrain growth.

shh signaling in the midbrain and hindbrain regions is mediated through gli family transcription factors [29, 30]. Ectopic expression of *gli1* in the midbrain and hindbrain regions activates the ventral markers *ptc1* and *hnf3-β* [31]. Conditional *gli2* knockout mouse embryos at E9.0 and E11.5 demonstrate that the activator form of gli2 (gli2-A) promotes growth of the ventral midbrain and hindbrain regions, whereas the repressor form of gli3 (gli3-R) is continuously required for the overall growth of the dorsal midbrain and hindbrain, presumably by inhibiting shh signaling in these regions [27–29]. Detailed analysis in the developing hindbrain reveals that *gli*2 **–/–** mouse embryos show a more severe ventral defect in the hindbrain than in the spinal cord, since gli3 can compensate for the loss of gli2 in the spinal cord but not in the hindbrain [32].

Interactions between the shh signaling pathway and the bmp pathway appear to specify the ventral region of the hindbrain. Aberrant expression of *bmp-7* in the

floor plate region inhibits *shh* expression and interrupts dorso-ventral patterning of the hindbrain, suggesting that bmp-7 regulates shh signaling in this domain [33].

Cerebellum

The cerebellum originates from the metencephalon. It is the largest part of the hindbrain and is connected to the other parts of the brain through projection fibers. Through these fibers, the cerebellum receives input from sensory systems and integrates the signals to coordinate and accurately time movement. The cerebellum is composed of many different types of neurons, including Purkinje neurons, granule neurons, Bergmann glia, astrocytes, interneurons, and neurons of the deep nuclei. Cerebellar development has been reviewed previously in detail [34–37].

shh signaling plays an integral role in the developmental biology of the Purkinje neurons, Bergmann glia, and granule neurons in the cerebellum (Fig. 8.3).

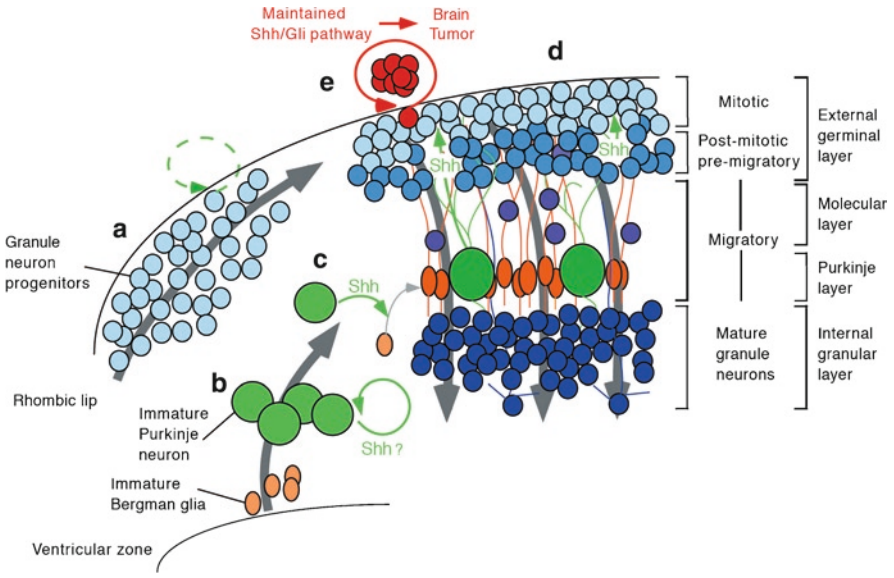

Fig. 8.3 The role of shh in cerebellar development. (**a**) Granule neuronal precursors (CGPs in text; *light blue*) migrate tangentially from the rhombic lip toward the EGL. During migration, the shh pathway may be transiently active in an autocrine manner (*dashed green arrow*). (**b**) Purkinje neurons (*green*) and Bergmann glia (*pale orange*) migrate from the ventricular zone toward the Purkinje layer. Purkinje neurons may initially use the shh pathway in an autocrine manner (*green arrow*). (**c**) shh from Purkinje neurons (*green arrows*) induces Bergmann glia maturation (*bold orange*). (**d**) In the later stage EGL, granule neuronal precursors (CGPs; *light blue*) proliferate in the outer zone and mature glia send extensions (*orange lines*) toward the inner EGL. Post-mitotic granule cells (*bold blue*) then migrate (*purple cells*) on glial fibers to form the internal granular layer (*dark blue cells*). (**e**) Constitutive shh signaling in EGL cells or failure to induce their differentiation may contribute to the development of medulloblastoma (*red arrows and cells*). Reproduced and adapted from Development 126, 3089–3100 (1999) with permission from the Company of Biologists, Ltd

During early embryonic development, Purkinje cells are derived from progenitor cells located in the ventricular zone of the neural tube, and granule neurons arise from the thickened alar plate of the embryonic rhombencephalon called the rhombic lip. Both cell types migrate into the region of the developing cerebellum, where cerebellar granule precursors (CGPs), also called granule cell precursors, granule neuron progenitors, or granule cell neuron precursors, form the external granular (or germinal) layer (EGL). Once the cells arrive in the EGL, dramatic growth of the neonatal mouse cerebellum ensues, increasing over 1,000-fold in volume. This period of growth is driven by the rapid proliferation of CGPs [38]. As a result of this rapid proliferation, granule neurons become the most abundant neurons in the cerebellum. In fact, more than 50% of the neurons in the entire mouse brain are comprised of granule neurons [39]. Amplified CGPs located in the EGL eventually exit mitosis, differentiate, and migrate internally to form the internal granular layer [39]. Bergmann glia interact with post-mitotic CGPs during their migration.

shh signaling regulates the proliferation and differentiation of CGPs and induces maturation of Bergmann glial cells. In situ hybridization studies for *shh*, *ptc1*, and *gli1* demonstrate that the shh ligand is produced by the Purkinje cells, and the *ptc1* receptor and *gli1* transcription factor are expressed in CGPs in the EGL. This expression pattern suggests paracrine signaling from Purkinje cells to CGPs in the developing cerebellum. Indeed, blocking shh activity with neutralizing anti-shh antibody disrupts CGP proliferation in the EGL [39–41]. Also, CGPs treated with shh in vitro remain undifferentiated while untreated CGPs undergo spontaneous differentiation, suggesting a role for shh in preventing their differentiation [39].

shh drives CGP proliferation by activating G_1-cyclins and N-myc [42, 43]. *atoh1* (also called *math1*) is a basic helix-loop-helix transcription factor, which is highly expressed in CGPs [44] that directly activates expression of the shh mediator *gli2*, thereby significantly promoting shh signaling [45]. Conditional knockout of *atoh1* in the post-natal mouse cerebellum reduces the size of the EGL, since CGPs cannot respond to the shh signal. In addition, *gli2* expression is significantly inhibited even in the presence of constitutively activated shh signaling in *atoh1* null conditional mutants, supporting the concept that atoh1 is a critical regulator of *gli2* and therefore shh signaling in the cerebellum [45].

Interactions of shh with several other proteins and signaling pathways are required for CGPs to exit from the proliferative cycles and begin differentiation. The extracellular matrix protein vitronectin is continuously expressed in the developing cerebellum very close to the *shh*-expressing cell population. Physical interaction of vitronectin with shh inhibits shh-mediated proliferation of CGPs and promotes their differentiation [46]. bmp-2 antagonizes shh-mediated CGP proliferation through smad5 [47] and through tieg-1, which inhibits N-myc [48]. The BTB/POZ domain-containing protein REN also antagonizes shh by negatively regulating gli1 and gli2 activity in CGPs, thereby promoting growth arrest, enhancing differentiation, and activating apoptosis of CGPs [49]. Finally, the fgf signaling pathway suppresses shh-induced proliferation of CGPs by down-regulating expression of *gli1*, *N-myc*, and *cyclin D1* [39, 50] and promotes differentiation of CGPs in the presence shh, suggesting an inhibitory role on shh during CGP differentiation [50].

It remains uncertain whether shh plays a direct role in the migration of CGPs. shh increases migration of granule cell explants [40], whereas blocking shh activity with neutralizing anti-shh antibody inhibits the migration of the cells. However, two complementary models, which prevent shh signaling by Purkinje cells show significantly compromised expansion of the CGPs and post-mitotic granule cells, but migration is not affected [38].

Aberrant Activation of SHH Signaling in Pediatric Brain Tumors

Collectively, CNS tumors represent the most common solid tumors among children and are a leading cause of pediatric cancer-related morbidity and mortality [51–53]. Pediatric brain tumors are a heterogeneous group of malignancies that differ in scope, behavior, and biology compared to adult CNS tumors. The majority of adult brain tumors are high-grade gliomas, meningiomas, and metastases from extra-CNS solid tumors [54–56]. Metastases to the brain and meningiomas are rarely seen in pediatrics, and high-grade gliomas only represent 10–15% of all childhood brain tumors [53]. On the contrary, astrocytomas and medulloblastomas are the most common CNS tumors in children, accounting for approximately 60 and 20% of pediatric brain tumors, respectively [54–56].

Despite multimodal therapies for childhood brain tumors, including surgical resection, cytotoxic chemotherapy, and radiation therapy, there remains a significant group of patients who succumb to their disease. In addition, many children who survive sustain significant late effects related to their original tumor and therapies, including neurocognitive deficits, endocrine dysfunction, ototoxicity, and the development of secondary malignancies [57–60]. A significant amount of research is underway evaluating the molecular, biologic, and cytogenetic characteristics of pediatric CNS tumors. The hope is that future targeted therapies tailored to the specific aberrant molecular pathways within a tumor will not only improve survival, but also may help to minimize some of the late effects. The childhood brain tumor that has advanced the furthest along this research trajectory is medulloblastoma, in large part based on research directed at the SHH signal transduction pathway.

Genetic Alteration of Components of the SHH Pathway in Pediatric Brain Tumors

Based on the fundamental roles that the SHH pathway plays in cell proliferation and cell fate specification during CNS development, it is not surprising that constitutive activation of the pathway is associated with brain tumors. Constitutive pathway activation has been described in association with continuous somatic expression of the SHH ligand in a variety of cancers outside the CNS. In pediatric brain tumors,

Table 8.1 Dysregulation of SHH signaling in childhood brain tumors

Tumor	Gene	Type of abnormality	References
Medulloblastoma	*PTCH1*	Loss of function mutation	[68, 96, 112, 142]
	SMO	Activating mutation	[141]
	SuFu	Loss of function mutation	[101, 143]
	GLI2	Amplification	[102]
Ependymoma	*IHH*	Overexpression	[121]
	GLI2	Overexpression	[120, 121]
	GLI1	Overexpression	[120]
Pilocytic astrocytoma	*PTCH1*	Overexpression	[124]
	GLI1	Overexpression	[124]
Craniopharyngioma	*PTCH1*	Loss of function mutation	[119]

constitutive pathway activation is more typically independent of HH ligands and is the result of mutations in downstream components of the HH pathway. These genetic alterations may be inherited constitutional mutations associated with cancer predisposition syndromes, such as basal cell nevus syndrome, or may be somatic. The most common genetic alterations in HH pathway components in childhood brain tumors are summarized in Table 8.1.

Medulloblastoma: Clinical Aspects

Medulloblastoma is the most common malignant brain tumor in childhood [54, 61]. It is a highly malignant embryonal tumor that is believed to arise from CGPs in the cerebellum. It is considered a central primitive neuroectodermal tumor (PNET) based on the histologic appearance of the cells mimicking embryonic neuroectoderm. The name "medulloblastoma" implies that the primary tumor is located within the cerebellum. Central PNET can occur in other locations within the CNS, including the supratentorium, brainstem, and spine; however, in these locations the tumor is not referred to as medulloblastoma and only represents approximately 2–3% of all pediatric brain tumors [62, 63]. These non-cerebellar central PNETs are thought to be biologically distinct from medulloblastoma based upon genetic and biologic studies as well as worse clinical outcomes compared to medulloblastoma [62, 64]. The cell of origin for these CNS non-cerebellar PNETs is not yet known.

Medulloblastoma is more commonly seen in males than females. The peak age at diagnosis is typically between 5 and 7 years old, however, it can be seen from birth to young adulthood [54, 65]. In fact, age at the time of diagnosis is one of the few important clinical prognostic factors known in medulloblastoma. The etiology of the majority of medulloblastomas is unknown, however, there are a few rare genetic disorders that predispose some patients to medulloblastoma, including basal cell nevus syndrome, Li–Fraumeni syndrome, ataxia telangiectasia, and Turcot's syndrome [66–69].

Children with medulloblastoma typically present with a relative short history of symptoms related to obstructive hydrocephalus, including early morning headaches,

emesis, and papilledema. They can also present with signs of cerebellar dysfunction, such as truncal ataxia and unsteady gait [54]. Finally, some patients may present with symptoms related to metastatic foci of disease in other parts of the brain and spine, such as seizures and signs of spinal cord compression.

Medulloblastoma has an inherent tendency to spread throughout the CNS. Therefore, staging at diagnosis is essential and includes a complete brain and spine MRI as well as evaluation of lumbar cerebro-spinal fluid (CSF) cytology to evaluate for metastasis. Typically, the spine MRI and lumbar CSF collection is performed 10–14 days postoperatively in an effort to avoid false positives secondary to surgical debris. The modified Chang staging system is used to stage these patients at diagnosis, where M0 patients have no signs of metastasis, M1 patients only have tumor cells on lumbar cytology, M2 patients have macroscopic spread of tumor to distant parts of the brain, M3 patients have macroscopic metastases to the spine, and M4 patients have spread outside the CNS, which is exceedingly rare in the modern era [70].

Despite a significant understanding of medulloblastoma biology, biologic characteristics have not yet been incorporated into up-front treatment strategies and prognostication. Currently, the major prognostic factors utilized to stratify patients with medulloblastoma include age at diagnosis, extent of tumor resection, absence or presence of CNS dissemination/metastases, and tumor histology. Disease characteristics that render a patient at high risk include residual disease greater than 1.5 cm^2 after primary surgery, metastasis to distant parts of the brain or spine, and anaplastic histology [61]. Patients with one or more of these characteristics are typically treated with an intensified regimen that includes both craniospinal irradiation and chemotherapy. In addition, patients less than 3 years old at the time of diagnosis are also considered at high risk due to their worse clinical outcomes and are treated with unique therapy approaches. These strategies often delay or avoid irradiation, since this group of patients is highly susceptible to the deleterious effects of radiation therapy.

Patients who are greater than 3 years of age with high risk disease are currently treated with a combination of full dose craniospinal irradiation (36 Gy to the neuraxis and boost to the posterior fossa up to 54 Gy) and chemotherapy. These patients have a 5-year progression-free survival ranging from 40 to 70% [54, 61, 71, 72]. Those patients who are less than 3 years old at diagnosis are often treated with a combination of high-dose chemotherapy followed by autologous hematopoietic cell rescue with or without adjuvant radiation therapy. These patients have 5-year progression-free survival ranging from 30 to 60% in published prospective series, and many of these patients have avoided radiation therapy completely [73–75]. Superior survival has been published using the German HIT protocol for this group of young patients. This protocol uses a chemotherapy alone regimen that includes intrathecal methotrexate. However, over half of the patients reported in this series had desmoplastic histology which is believed to confer a better prognosis in younger patients [75]. Also, there is concern that the intrathecal methotrexate contributed to neurocognitive sequelae seen in these patients. For these reasons, this approach has not been universally adopted.

The current approaches to patients with standard risk of medulloblastoma, that is, age greater than 3 years at diagnosis, less than 1.5 cm² residual disease postoperatively, no signs of metastasis (as seen on MRI of the brain and spine and lumbar fluid cytology), and a classic histology, include a dose reduction of craniospinal irradiation (23.4 Gy to the neuraxis and a boost to the posterior fossa up to 54 Gy) and adjuvant chemotherapy. This strategy maintains good outcomes, and preliminary data suggest it may reduce neurocognitive sequelae [76]. These patients have a 5-year progression-free survival of approximately 85% [71].

Desmoplastic medulloblastoma is a less common histologic variant of medulloblastoma, most commonly seen in patients with basal cell nevus syndrome. The association between desmoplastic histology and basal cell nevus syndrome suggests that abnormalities in SHH signaling contribute to the development of this form of medulloblastoma. This histologic variant accounts for approximately 10–20% of sporadic medulloblastomas as well [77]. Histologically, desmoplastic medulloblastomas are characterized by pale nodular areas surrounded by densely packed cells and a significant reticulin network between these areas. The nodular areas are made up of more mature tumor cells with fewer mitoses and more abundant cytoplasm. The densely packed cells, surrounding the nodules appear more typical of classic medulloblastoma [54, 75, 78]. Interestingly, this subtype shows superior survival compared to other subtypes in patients with or without basal cell nevus syndrome [75, 77, 79].

Despite all of the aforementioned strategies, approximately 30% of patients with medulloblastoma will relapse, and unfortunately most of these patients will succumb to their disease [80, 81]. If they have not yet received radiation therapy, as is the case in some very young children, a small percentage may be salvaged using radiation therapy. Unfortunately, based on the international experience, most patients who have already received craniospinal irradiation do not appear to be curable once they recur; however, there are some data to suggest that the use of high-dose chemotherapy with autologous hematopoietic cell rescue may be of value in a select group of patients [81, 82]. Ongoing phase I and phase II trials are attempting to utilize molecularly targeted agents in an effort to improve survival at the time of recurrence.

Medulloblastoma: Biologic Aspects

Studies continue to more completely understand the molecular biology and cytogenetics of medulloblastoma. The most common cytogenetic abnormality in medulloblastoma is isochromosome 17q, which is present in up to 40% of cases [83, 84]. Numerous reports have also identified gains of chromosomes 4, 6, 7, 8, and 18 as well as losses of chromosomes 1, 2, 6, 8, 9, 10, 11, and 16 [84–86]. Approximately 5% of medulloblastomas also harbor a high level of amplification of the *N-MYC* oncogene [84].

Many groups have suggested using these biologic and molecular aberrations as a means of risk stratification. Pfister et al. developed a five-tier system based on screening 80 medulloblastoma samples by array-based comparative genomic hybridization and an independent validation of 260 samples via fluorescence in situ hybridization. This hierarchical medulloblastoma molecular staging system from worst to best outcome includes (1) *c-MYC/N-MYC* amplification, (2) 6q gain, (3) 17q gain, (4) 6q and 17q balanced translocation, and (5) 6q deletion [87]. These data show quite convincingly that molecular and cytogenetic abnormalities are powerful tools for prognostication, and one day may be more useful than the traditional risk categorization based solely on clinical characteristics.

Another group evaluated gene-expression profiles of 46 human medulloblastoma samples. Unsupervised analysis divided the samples into five distinct groups (A–E) enriched for specific genetic alterations that were later confirmed by gene sequence analyses and fluorescence in situ hybridization [88]. Some of the specific abnormalities include WNT pathway mutations and chromosome 6 deletions in subgroup B and SHH pathway mutations in subgroup D [88]. This type of analysis and separation of tumors by genetic alterations may help stratify patients for the most appropriate targeted therapies.

To date, three main molecular signaling pathways have been implicated in medulloblastoma development, including the WNT pathway, the Notch pathway, and the SHH pathway. Better understanding of the WNT pathway and medulloblastoma development has come from a rare disorder called Turcot's syndrome. Turcot's syndrome, also known as glioma-colonic polyposis syndrome, is a genetic disorder characterized by colonic polyposis and an increased risk of developing colon cancer and malignant neuroepithelial CNS tumors. Most commonly, these patients develop glioblastoma multiforme and medulloblastoma [69, 89, 90]. One of the main mutations identified in this syndrome is a defect in the adenomatous polyposis coli (APC) gene, a tumor suppressor, which is a component of the WNT pathway and helps to coordinate the proliferation and ultimate fate of neural progenitor cells. Differing phenotypes may result from mutations at unique regions within the APC gene. Activation of the WNT pathway inhibits phosphorylation of beta-catenin, allowing its translocation into the nucleus [91]. This in turn increases the expression of a variety of genes that ultimately lead to cell proliferation, inhibition of apoptosis, and differentiation within the CNS. In addition, mutations of beta-catenin and other WNT pathway genes have been described in approximately 10–20% of sporadic medulloblastomas [84, 91]. The accumulation of beta-catenin within the nucleus, suggesting WNT pathway activation, appears to predict a favorable outcome in medulloblastoma [84, 92].

The Notch signaling pathway is vital to a variety of developmental processes, including hematopoiesis, somitogenesis, vasculogenesis, and neurogenesis [93]. This pathway has been implicated in the development of a variety of malignancies, including medulloblastoma. Notch signaling is activated by four transmembrane receptors, including Notch 1–4. Notch 1 is thought to be essential to the normal development of the cerebellum, whereas Notch 2 is implicated in medulloblastoma development [54, 93]. Once the receptors bind their extracellular ligands, proteolytic cleavage

leads to the release of the intracellular domain of the receptors into the intracellular compartment and eventual translocation into the nucleus. A variety of downstream targets are then activated, such as *cyclin D1* and apoptosis associated genes [54, 94]. If unregulated, this activation is thought to drive a variety of processes, including neural "stem" cell maintenance, gliogenesis, and oncogenesis [93].

Medulloblastoma: Dysregulation of SHH Signaling

Evidence linking the SHH signaling pathway and medulloblastoma originated from recognizing that patients with basal cell nevus syndrome are at increased risk for medulloblastoma. Basal cell nevus syndrome is a rare autosomal dominant genetic disorder affecting 1 in 60,000 individuals [77]. It is characterized by skeletal anomalies (frontoparietal bossing, rib and vertebrae abnormalities, and dural calcifications), large body habitus, soft tissue fibromas, radiation sensitivity (increased risk of developing radiation-induced tumors such as meningioma, ependymoma, fibrosarcoma, and basal cell carcinoma), and a high incidence of basal cell carcinoma, rhabdomyosarcoma, and medulloblastoma [67, 77, 95]. Approximately 3–5% of patients with basal cell nevus syndrome will develop medulloblastoma, typically the desmoplastic variant.

Basal cell nevus syndrome is caused by inherited inactivating germ-line mutations in one patched (*PTCH1*) allele [91, 95]. During normal development of CGPs in the cerebellum, PTCH1 maintains the SHH pathway in an inactive state except when it binds SHH. On the contrary, mutant forms of PTCH1 maintain the pathway in a constitutively activated state, even in the absence of ligand. Constitutive SHH pathway activation appears to account for the birth defects and cancer predisposition in patients with basal cell nevus syndrome. Cancers presumably develop in the setting of basal cell nevus syndrome if a somatic inactivating mutation occurs in the remaining wild type *PTCH1* allele in limited cell types, including CGPs.

Loss-of-function somatic mutations in *PTCH1* have been subsequently described in approximately 10–15% of sporadic medulloblastomas [68, 96]. It is now believed that SHH pathway activation occurs in 20–30% of all medulloblastomas, largely accounted for by inactivating *PTCH1* mutations. A variety of loss of function mutations in *PTCH1* have been described in the setting of medulloblastoma, including frame shift mutations, small deletions, duplication insertions, and splice site mutations. Interestingly, the sporadic medulloblastomas that exhibit abnormal signaling via the SHH are not all desmoplastic. Constitutive activation of SHH signaling in cerebellar CGPs is believed to contribute directly to the genesis of human medulloblastoma, based on the fact that *ptc1*$^{+/-}$ mice develop medulloblastoma [97].

Mutations in components of the SHH signaling pathway that are downstream of *PTCH1* have been more rarely described. Although activating mutations in *SMO* have been widely described in basal cell carcinomas, they appear to be rare in medulloblastoma and only have been reported in recent years [98, 99]. Suppressor of Fused (SuFu) normally binds to GLI1 and inhibits GLI1-mediated transcriptional

activation by exporting GLI1 from the nucleus to the cytoplasm [100]. Several somatic mutations in *SuFu* have been described in medulloblastomas, including frame shift and exon skipping mutations [101]. The mutant forms of the SuFu protein lack the carboxy terminal domain and are unable to bind GLI1. Therefore, the SHH pathway remains in an active state since mutant SuFu cannot sequester GLI1 in the cytoplasm. A single case of medulloblastoma with *GLI2* gene amplification has been reported in a patient with the Li–Fraumeni familial cancer syndrome [102]. The significance and the biological role of *GLI2* amplification have not been studied in medulloblastoma.

Other Pathways Affecting SHH Signaling in Medulloblastoma

Although mutations in the SHH signaling genes *PTCH1*, *SMO*, and *SuFu* are believed to directly contribute to the genesis of medulloblastoma, such mutations are observed in only a subset of the tumors, suggesting that there are other mechanisms and gene pathways that can cause or play an important role in the biology of medulloblastoma either independent of SHH signaling or by dysregulating SHH signaling. Indeed, noncanonical activation of GLI family transcription factors, which mediate HH signaling, has been described in the setting of cancer. For example, TGF-β activates the expression of *GLI1* and *GLI2* through SMAD3, independent of SHH signaling in human pancreatic cancer cells [103]. *GLI1* also appears to be activated independent of the canonical HH pathway by the EWS–FLI11 oncoprotein in Ewing sarcoma [104]. A number of studies using mouse models and human specimens demonstrate interactions between SHH signaling and other pathways and genes, both in CGPs and in medulloblastoma. Recent progress in this field is summarized in Table 8.2.

Table 8.2 Interactions between SHH signaling and other pathways in medulloblastoma

Gene	Effect on pathway	References
Genes that enhance SHH signaling in medulloblastoma		
atoh1	Increases *gli2* expression	[45]
c-myc	Enhances shh tumorigenicity	[107]
yap1	Increases gli2 expression	[108]
Genes that inhibit SHH signaling in medulloblastoma		
bmp-2,4	Degrades atoh1	[105]
bFGF	Decreases *gli1*, *N-myc*, and *cyclin D1* expression	[50]
pacap	Inhibits gli1 activity	[109]
p53	Inhibits gli1 activity	[114]
REN	Decreases *gli1* expression, nuclear localization of gli1, and gli2 activity	[49]
Targets of SHH signaling in medulloblastoma		
igf2	Increases expression	[116]
insm1	Increases expression	[118]
irs1	Stabilizes protein	[117]
nhih	Increases expression	[118]

Some proteins and pathways appear to enhance HH pathway activity in CGPs and medulloblastoma. *ATOH1* is highly expressed in CGPs and in a subset of human medulloblastomas. Conditional deletion of *atoh1* in mice downregulates *gli2* expression. In fact, atoh1 directly activates *gli2* by binding a *gli2* intron, therefore, significantly promoting the activity of the shh signaling pathway [45]. The role of atoh1 in the genesis of medulloblastoma has been tested using a mouse medulloblastoma model, carrying an activating mutation in *smo*. In this background, loss of expression of *atoh1*, using an *atoh1* null conditional mutant, significantly inhibits tumor formation, suggesting that atoh1 and activation of shh signaling are required for medulloblastoma formation [45]. bmp-2 and bmp-4 down-regulate expression of *gli1* in medulloblastoma by degrading the atoh1 protein [105].

MYC family genes are amplified and overexpressed in 5–10% of medulloblastomas [106]. Overexpression of *c-myc* alone does not appear to cause medulloblastomas in mice. However, *c-myc* greatly enhances the tumorigenicity of shh signaling in CGPs, suggesting cooperation of c-myc with hh signaling in shh-mediated medulloblastoma formation [107]. The molecular mechanism of this cooperation is unknown.

Finally, the transcriptional coactivator yes-associated protein (*yap1*) that is a key factor in Hippo signaling pathway is amplified in some medulloblastomas [108]. It activates transcription of target genes by interacting with a tea domain family transcription factor (tead). The yap1–tead1 complex drives *gli2* transcription by directly interacting with the CATTC consensus sequence in the *gli2* promoter and thus promotes constitutive shh pathway activation in medulloblastoma.

On the contrary, other proteins and pathways appear to inhibit SHH signaling in CGPs and in medulloblastoma. bFGF dramatically downregulates the expression of *gli1*, *N-myc*, and *cyclin D1*, and thereby suppresses shh-induced proliferation of CGPs [39, 50]. The fgf pathway also inhibits *gli11* expression and proliferation of medulloblastoma cells derived from $ptc1^{+/-}$ mice, suggesting an inhibitory role in the genesis of shh-induced medulloblastoma [50].

Pituitary adenylyl cyclase-activating peptide (pacap) activates PKA and functions as a powerful inhibitor of medulloblastoma formation. In fact, double heterozygote $ptc1^{+/-}$ $pacap^{+/-}$ mice demonstrate a 2.5-fold increase in medulloblastoma incidence [109]. pacap inhibits gli1 in medulloblastoma cells by activating PKA. The tumor suppressor *REN* is deleted in 39% of sporadic human medulloblastomas and inhibits the growth and the tumorigenicity of medulloblastomas [49, 110]. REN inhibits *gli1* expression and gli1 activity in medulloblastoma by blocking Dyrk1-mediated nuclear localization of gli1. REN also impairs gli2-dependent gene transcription.

Finally, inherited germ-line *p53* mutations are associated with the development of medulloblastoma in some patients with Li–Fraumeni syndrome [111]. Normally, only a small subset of $ptc1^{+/-}$ mice develop medulloblastoma [112]. However, $ptc1^{+/-}$ $p53^{-/-}$ mice develop medulloblastoma significantly more frequently (>95%) and at an earlier age [113]. This finding strongly suggests that loss of p53 and constitutive activation of the shh signaling pathway interact functionally. The mechanism of

this interaction in medulloblastoma is not known. However, recent studies suggest a feedback loop between p53 and gli1. p53 inhibits the transcriptional activity, nuclear localization, and level of expression of gli1, while gli1 inhibits the activity p53 [114]. Thus, loss of p53 may enhance GLI1 activity and thereby medulloblastoma formation. In addition, increased expression of *p53* has been observed following the transfection of *GLI1* into rat kidney epithelial cells (RK3E cells) and in the subset of medulloblastomas with HH pathway activation, suggesting that GLI1 may regulate *p53* expression [115].

shh signaling appears to regulate several genes in the "insulin regulatory pathway" in both CGPs and medulloblastoma. Insulin-like growth factor 2 (igf2) promotes cell proliferation in developing embryos. Normally, *igf2* is expressed in the meninges and at lower level in CGPs. However, *igf2* is highly expressed in medulloblastomas that develop in *ptc1*$^{+/-}$ mice. In addition, *igf2* expression in CGPs is directly activated by shh in vitro and is inhibited by cyclopamine treatment in medulloblastoma cell lines [116]. Loss of *igf2* expression decreases medulloblastoma formation in *ptc1*$^{+/-}$ mice, suggesting a role as a vital downstream target of the shh signaling pathway in medulloblastoma. Another insulin-related gene that is necessary for proliferation of CGPs and aberrantly activated in medulloblastoma is the insulin receptor substrate 1 (*irs1*) [117]. shh signaling stabilizes the irs1 protein by inhibiting the mTOR pathway that directs the degradation of irs1. Neural basic helix-loop-helix 1 (*nhlh1*) and insulinoma-associated 1/IA1 (*insm1*) are also highly expressed in rapidly expanding CGPs and medulloblastomas [118]. Both *nhih1* and *insm1* are activated transcriptionally by shh signaling in cultured CGPs, and activation of *nhih1* is directly mediated by gli1. Understanding the interactions between genes/proteins in the HH signaling pathway with those of other pathways that modulate HH signaling, as well as identifying vital effects of HH signaling in CGPs and medulloblastomas, will be important when developing targeted therapy and making informed decisions about which agents to test in combination.

Dysregulation of SHH Components in Other Pediatric Brain Tumors

Limited information is available about genetic alterations or aberrant activation of the SHH signaling pathway in other pediatric brain tumors. Craniopharyngiomas, which arise from the embryonic remnants of Rathke's pouch and account for 5.6–6.2% of all pediatric brain tumors [52], have been reported in members of a family with basal cell nevus syndrome [119]. Analysis of the *PTCH1* gene in this family shows an insertion mutation, causing a frame shift. The craniopharyngiomas from these patients have loss of heterozygosity in the *PTCH1* region, suggesting potential involvement of SHH signaling in this tumor [119]. It is of interest that the shh, bmp, and fgf pathways appear to be involved in establishing dorso-ventral polarity in Rathke's pouch during development.

A study conducted to identify an ependymoma-specific gene signature identified overexpression of *GLI1* and *GLI2*, suggesting a potential role for HH signaling in this type of tumor [120]. More recently, gene-expression analysis using 34 ependymoma samples demonstrated that a subset of HH pathway components were highly expressed, including *GLI2* and Indian Hedgehog (*IHH*) [121]. During development, ependymal cells are derived from *nkx6.1*-expressing ventral neuroepithelial cells, which are regulated by shh [122]. A mechanism for HH pathway dysregulation in ependymomas has not been reported.

Pilocytic astrocytoma is a very heterogeneous tumor that is the most frequently occurring brain tumor during childhood [52]. It typically arises in the cerebellum (40–70%) and generally is benign with an excellent survival rate [123]. The level of *PTCH1* mRNA is elevated in approximately 45% of pilocytic astrocytomas and its level is inversely correlated with the age of patient [124]. Higher expression of *PTCH1* and *GLI1* is associated with younger age at diagnosis and more rapid tumor growth, suggesting a role for the pathway in regulating proliferation. It will be important to expand the analyses of HH signaling in these tumors and to see if any genetic alterations are associated with the activation of the SHH pathway in any of these non-medulloblastoma tumors.

Potential Clinical Applications of HH Pathway Inhibitors in the Treatment of Patients with Pediatric Brain Tumors

Specifically targeting the SHH pathway as cancer therapy becomes possible as our understanding of the pathway improves. An observation reported in 1962 provided the first evidence that blocking the SHH pathway is feasible. It was noted that when pregnant ewes ingested the *Veratrum californicum* plant during their first trimester, they bore lambs with congenital cyclopean-type malformations [125]. Later, a steroidal alkaloid, called cyclopamine, was isolated from this same plant, which induced midline deformities in lamb fetuses [126]. It is now known that cyclopamine directly binds to the heptahelical bundle of SMO, likely changing the protein's conformation and thereby inactivating the SMO protein. In vitro, cyclopamine has been shown to inhibit SHH-dependent expression of *GLI1*, *GLI2*, and *PTCH1* and cause medulloblastoma cell cycle arrest [127]. In murine medulloblastoma tumor allograft and xenograft models, cyclopamine induces rapid tumor cell death [128, 129]. Unfortunately, due to its pharmacokinetic and side effect profile cyclopamine is not ideal for clinical use in humans [130]. Cyclopamine has poor solubility, acid sensitivity, weak potency and is a known teratogen [131]. More recently, a variety of naturally occurring and synthetic small molecule antagonists have been discovered [127, 128, 132].

Most of the small molecule antagonists to the SHH pathway also target SMO [132]. Romer and Curran have evaluated a small molecule inhibitor that binds and inhibits SMO called HhAntag-691 (Genentech). This compound is a benzimidazole derivative, which readily enters the brain of mice [128]. When $ptc1^{+/-}$ $p53^{-/-}$ mice

with medulloblastomas are treated with HhAntag-691, there is dose-dependent down-regulation of several genes overexpressed in medulloblastomas, including *gli1*, *ptc2*, and *atoh1* [128]. Importantly, treatment of these mice improves tumor-free survival with minimal noted toxicities [128, 133].

Rubin and de Sauvage conducted cell-based screens for novel compounds that block SHH-activated gene transcription [134]. A variety of agents were discovered that target the pathway. One in particular, HhAntag, was initially very promising as it had oral bioavailability and showed potent antitumor activity in both murine *ptc1*-mutated medulloblastoma and human xenograft models [133]. Unfortunately, when evaluated in humans, it was determined that this drug had low potency and unpredictable pharmacokinetics. Therefore, newer drugs, such as GDC-0449 (Genentech) have been developed to improve the pharmacokinetics and potency of HhAntag. GDC-0449 also blocks the SHH pathway by binding and inhibiting SMO. Preclinical studies evaluating absorption, distribution, metabolism, and tumor responses have been promising. The compound's characteristics include the following: low plasma clearance, a volume of distribution estimated to be approximately equal to total body water, high protein binding, and oral bioavailability ranging from 13 to 53% in different species [135]. It is currently undergoing evaluation in phase I and phase II studies in adults with a variety of cancers, including basal cell carcinoma, stomach cancer, pancreatic cancer, breast cancer, lung cancer, ovarian cancer, glioblastoma multiforme, and medulloblastoma. There is also an ongoing pediatric brain tumor consortium (PBTC) phase I trial evaluating the use of GDC-0449 in children and adults with recurrent medulloblastoma [136].

There will be many challenges and questions to address while introducing inhibitors of HH signaling into medulloblastoma therapy. Activation of HH signaling is believed vital in tumor initiation and maintenance for some medulloblastomas, often but not exclusively of the desmoplastic histologic subtype. We would expect HH inhibitors to be most effective in this subset of tumors. Therefore, a reproducible and clinically useful method to identify active HH signaling must be established in medulloblastoma. Immunohistochemistry for components of the HH signal transduction pathway, such as PTCH1 and GLI1 may be promising. On the contrary, using gene-expression profiles to identify subsets of patients is still difficult in real time across centers.

Patients with desmoplastic histology tend to be young and have a more favorable prognosis. Moving newer agents into therapy for this subset of patients may be challenging; but may ultimately be of considerable value in improving outcomes and especially limiting toxicity. Typically, phase I and phase II studies are conducted in the setting of recurrence, as is the case with the ongoing Phase I PBTC HH inhibitor study. The youngest patients have been initially excluded based on the concern of a role for HH signaling in post-natal bone growth and development. Indeed, chondrocyte and osteoblast development require IHH, and there have been significant defects noted in long bone development in IHH knockout mouse models [137]. Mutant mice have reduced chondrocyte proliferation, failure of osteoblast development in endochondral bones, and premature closure of growth plates [137]. Interestingly, in humans, IHH mutations have recently been associated with a

disorder known as acrocapitofemoraldysplasia [138]. This is a rare autosomal recessive growth disorder characterized by short stature, short limbs, brachydactyly, large head, narrow thorax, and pectus deformities [138, 139]. Osteopontin has also been identified as a target of GLI1 [140].

Efficacy of HH inhibitors may differ at the time of diagnosis versus at the time recurrence. Using a HH inhibitor at the time of diagnosis may optimize chances of observing a therapeutic benefit, since cells have not yet been exposed to agents that may select for resistant clones, however, treating at the time of recurrence may more effectively allow identification of agents that treat drug-resistant clones and more effectively build upon current therapeutic approaches. The optimal approach for testing HH inhibitors remains uncertain.

As with other agents, HH inhibitors may be most beneficial when either paired with traditional chemotherapy and radiotherapy or with other biologically active agents. Identifying other pathways that enhance or inhibit SHH signaling in medulloblastoma may help to inform decisions concerning drug combinations. For example, HH signaling in medulloblastoma activates the "insulin regulatory pathway." Inhibitors of this pathway are currently undergoing testing in a wide range of tumors. Targeting the upstream HH pathway and downstream "insulin regulatory pathway" may enhance efficacy. Identification of critical downstream targets will be essential [115].

A growing body of literature suggests a potential pitfall to the use of a HH inhibitor that targets SMO. There is now evidence that SMO's targets, GLI1, GLI2, and/or GLI3, may be activated in ways other than through the canonical HH pathway. Activation of GLI family transcription factors may then bypass the effect of the SMO inhibitor. A more complete understanding of ways to activate GLI family transcription factors in medulloblastoma and in other cancers is needed. In addition, an amino acid substitution in SMO was recently reported in human medulloblastoma, conferring resistance to GDC-0449, rather than disrupting the pathway which suggests another possible mechanism of resistance to this agent [141].

Summary

Childhood brain tumors are significantly different from their adult counterparts, since the latent period is very short, growth is fast, and the cell populations causing the tumors arise from the embryonic cells. The causes of childhood brain tumors remain incompletely understood. However, significant progress in the genetics and biology of childhood brain tumors has been made in the past 15 years. In particular, important genes and signaling pathways involved in the development of childhood brain tumors have been identified [106]. We describe recent important discoveries of the role of SHH signaling pathway in brain development and tumorigenesis. SHH signaling appears to play fundamental roles in regulating proliferation and differentiation during development of a variety of cell types in the CNS. A role for SHH signaling in the normal development of CGPs in the cerebellum appears to

reflect a role that the pathway plays in the genesis of some medulloblastomas. For this reason, drugs targeting major components of the SHH signaling pathway and interacting genes may prove to be a valuable alternative or adjunctive approach for the treatment of some children with medulloblastoma and potentially children with other brain tumors.

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