Medulloblastoma: mouse models and novel targeted therapies based on the Sonic hedgehog pathway

LEANDRO R. PIEDIMONTE, M.D., IAN K. WAILES, AND HOWARD L. WEINER, M.D.

Division of Pediatric Neurosurgery, Department of Neurosurgery, New York University School of Medicine, New York, New York

Understanding molecular pathways, signaling cascades, and genetic alterations activated during tumorigenesis is essential for the development of targeted cancer treatments. In children, tumors of the central nervous system are thought to arise from progenitor cells that show considerable temporal and spatial heterogeneity in a developmental environment that is different from that of the adult. Investigating the molecular basis of pediatric tumors is critical because it is likely to generate novel treatments. Animal models have brought many important advances in this field. In this review the authors discuss the mouse models based on the Sonic hedgehog pathway, which have provided a better knowledge of the genetic and molecular alterations of medulloblastoma.

KEY WORDS • brain tumor • medulloblastoma • Sonic hedgehog • animal model

OVERVIEW

Medulloblastoma is a primitive neuroectodermal tumor of the cerebellum that most often arises sporadically, although it may be part of an inherited disorder such as Gorlin, Turcot, or Li-Fraumeni syndromes. Currently, surgical removal in combination with radiotherapy and chemotherapy is the standard treatment. Nevertheless, these therapies, especially radiation, may cause unacceptable side effects such as intellectual deterioration, coordination problems, neuroendocrine difficulties, and neuropsychological deficits, making the development of new targeted treatments essential.

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It is believed that medulloblastoma originates from neuronal progenitor cells, which during the embryonic period form the external germinal layer of the developing cerebellum. These cells are precursors to granule cells, the most abundant neurons in the brain, and undergo a dramatic proliferative expansion in the external granule layer during neonatal and early postnatal development in both humans and mice. At this point, these cells exit the cell cycle, differentiate, reach maturity, and migrate inward through the Purkinje cell layer to the deeper layer of the cerebellum to assume their mature position in the internal granule layer (Fig. 1). This process of proliferation, differentiation, and migration is completed by the 3rd postnatal week in mice and generally by the 9th postnatal week in humans.

It is not clear whether a medulloblastoma arises from a cell that has acquired a transforming mutation during a period of normal proliferative expansion of the granule cell layer during development, which becomes clinically apparent sometime later, or if a postmitotic granule cell incurs damage to its DNA before reentering the cell cycle and proceeding along the course of neoplastic transformation to form a tumor. Alternatively, it may be possible that tumors arise from stem cells that have remained mitotically active and that have undergone neoplastic transformation to develop a critical mass of tumor cells that becomes symptomatic. Thus, one of the challenges in our efforts to understand the molecular basis of medulloblastoma is that the population of cells from which this tumor is thought to arise is absent at the time the lesion becomes clinically apparent, making it difficult to compare gene expression in tumors with that in adjacent, biologically active, nontumorous tissue. Progress in developing novel therapies for treatment of medulloblastoma has been hampered by the difficulty in obtaining appropriate nontumorous tissues for gene expression analysis, and also by the lack of animal models that reproduce the pathophysiological features of this tumor.

The Shh signaling pathway regulates a variety of events in the embryonic development of invertebrate and vertebrate organisms. Laboratory studies have shown that the Shh regulatory pathway plays an essential role in developmental patterning of various organs and tissues, including the neural tube. Sonic hedgehog is a secreted protein that was originally described as a regulator of cell fate determination and body segment polarity. It is known that the expression of this pathway is essential for normal cerebellar development; during embryogenesis Shh is released by Purkinje cells and binds to Ptc receptors of the external granule layer granule cells, promoting their growth and proliferation; however, its misexpression can lead to medulloblastoma formation (Fig. 1). Binding of Shh to the 12-transmembrane domain protein Ptc triggers activation of the pathway. In the absence of Shh, Ptc suppresses the activity of the seven-transmembrane domain Smo. Binding of Shh to Ptc, or inactivational mutations of Ptc, which occur in some tumors, relieve Smo from inhibition, leading to the activation of the Gli transcription

**Abbreviation used in this paper:** Shh = Sonic hedgehog.
factors and of Ptch itself (Fig. 2). 

Thus, a complex signaling pathway that now appears to be catalytically regulated between Ptch and Smo, and transcription of Gli and other target genes, is initiated.

The Gli family of zinc-finger transcription factors is composed of three members; in mammals Gli1, Gli2, and Gli3. 

It has been proposed that Gli1 is a key transcription factor activating the Shh pathway in development and disease; this is supported by several lines of experimental evidence and also by the prominent expression of Gli1 in several human tumors with presumed activation of the Shh pathway. 

Once activated, Gli1 may then become independent of Shh through autoregulation. An understanding of normal cerebellar development is critical in identifying the molecular alterations that trigger medulloblastoma formation, and the generation of genetically modified mice provides new tools for the dissection of these proliferative pathways.

Inappropriate activation of the Shh pathway has been associated with familial cancer. A subset of hereditary and sporadic human medulloblastomas shows mutations of one copy of PTCH, suggesting that uncontrolled activation of this mitogenic pathway, which has a crucial role in cerebellar ontogenesis, leads to medulloblastoma formation.

Inherited mutations of the PTCH gene have been identified as responsible for basal cell nevus syndrome, also known as Gorlin syndrome, which is characterized by a variety of clinical problems, and a predisposition to benign and malignant tumors in the skin and nervous system, including medulloblastoma. When it was determined that germ line mutations in ptch were associated with Gorlin syndrome, interest was focused on PTCH as a possible tumor suppressor gene that plays a role in the pathogenesis of subsets of sporadic basal cell carcinoma and medulloblastoma. Numerous missense or truncated mutations in ptch have been reported in human tumors, but correlations between these mutations and disease phenotype and severity have not been established.

Mutations in other downstream components of the pathway, Smo and SUFU, have also been described as alterations that contribute to the formation of sporadic basal cell carcinoma and medulloblastoma. Recently, it was shown that a subset of children with medulloblastoma carry germ line and somatic mutations in SUFU, another member of the Shh pathway, whose protein product has been shown to function in directing the subcellular localization of Gli1. 

Mutations of SUFU result in a truncated protein that is unable to transport Gli1 out of the nucleus, resulting in the persistent activation of transcription and signaling. Aberrant regulation of the Shh pathway and increased Gli1 expression are believed to occur in subsets of medulloblastoma, and it has been suggested that these changes also correlate with a histologically distinct group of desmoplastic tumors with a less favorable clinical outcome.

Furthermore, medulloblastomas most likely arise as a consequence of deregulated signaling that may be achieved by a number of heterogeneous pathways. Mutations in Shh pathway components have been noted in only 25% of these tumors; thus, activation of Shh signaling may...
Fig. 2. Schematic drawings showing the Shh signaling pathway. a: Patched encodes a 12 pass–transmembrane protein that in the absence of the ligand Shh serves as a negative regulator of Smo activity; Gli1 is retained in the cytoplasm, bound to the complex SUFU-FU-COS2 and is not believed to activate transcription when bound to this complex. b: In the presence of Shh, Ptch repression of Smo is relieved, and Gli1 is released from the SUFU-FU-COS2 complex, translocates to the nucleus, and activates transcription of downstream genes (including Gli1 and Ptch). c: Mutations (stars) in Ptch, Smo, and SUFU have been detected in human tumors and are believed to result in the transcriptional activation and regulation of this signaling pathway.
the age of onset and the incidence of medulloblastoma in ptch heterozygotes; in fact, 95% of the ptch1+/−; p53−/− mice developed a medulloblastoma by 12 weeks of age.40 Although p53 mutations are rare in human medulloblastoma, the inactivation of p53 may lead to genetic instability and subsequent genetic alterations that lead to transformation, cooperating with the hemizygous ptch alteration in the formation of these tumors.12,43

An alternative approach consistent with the human disease is to activate Smo, a downstream activator of the pathway that has been described as a contributor of medulloblastoma formation in a subset of patients in whom the tumor develops. Mice expressing high levels of the activated form of Smo had a high rate of medulloblastoma formation and early cerebellar hyperproliferation.24 Selective activation of the Shh pathway in cerebellar granule neuron precursors was achieved by expressing constitutively active forms of the Smo gene, SmoA1 or SmoA2, by using the NeuroD2 1-kb promoter that is principally expressed in cerebellar granule neuron precursors. The SmoA1 transgenic mice were established in three lines with varying levels of expression.

At a median age of 25.7 weeks, almost 50% of the high-expressing line developed symptomatic medulloblastoma. Lines with lower levels of transgene expression did not develop tumors, confirming that high levels of activated Smo are necessary for Shh pathway activation. Four lines of SmoA2 transgenic mice with relatively low levels of Smo expression were created, and these animals rarely developed tumors. To assess the events that occur before tumor formation, cohorts of SmoA1 mice were killed at 8 weeks of age and their cerebellar pathological condition was evaluated. This revealed excessive granule cell proliferation in 80% of high-expressing mice. This is a model with a high incidence of tumors that do not have loss of p53 or other genetic mutations that are uncommon in human medulloblastoma, and that exhibits early disease, providing an opportunity to study disease progression from its early stages and to test novel approaches to treatment.24

In another model, our group has used Turnbull ultrasound biomicroscopy-guided in utero injections of an Shh-expressing retrovirus into the cerebellum of 13.5-day-old mouse embryos to show that direct activation of the Shh pathway can lead to tumor formation.58 The embryonic stage chosen for injection of the retrovirus, embryonic Day 13.5, was the earliest stage at which the virus could be accurately injected specifically into the cerebellar anlage by using ultrasound biomicroscopy guidance.41 Moreover, previous injections of Shh-expressing cells and retroviruses at earlier embryonic stages resulted in transformation of the dorsal neural tube into ventral structures, which could preclude cerebellar formation.16,36,59 Significantly, medulloblastomas were observed in regions of heavy Shh-expressing retrovirus infection in 76% of postnatal Days 14 and 21 mice. These data suggest that ectopic expression of Shh is sufficient to induce early postnatal tumor formation rapidly and at a high frequency.

To determine whether these early tumors would progress further with age, the cerebella of postnatal Week 13 mice that had been injected with retrovirus at embryonic Day 13.5 were analyzed. As in the earlier postnatal stages, tumors were observed in regions of heavy Shh-expressing retrovirus infection. These tumors exerted mass effect on the surrounding cerebellum and, as in the tumors evaluated at the earlier postnatal stages, had the typical histological features of human medulloblastoma.58

To test the requirement for Gli1 in tumor formation, Shh-expressing retroviruses were injected into the cerebella of embryonic Day 13.5 Gli1 heterozygous and homozygous null mutant embryos. Significantly, it was found that at postnatal Day 21, Gli1−/− mice infected with Shh retroviruses developed tumors that were indistinguishable from those seen in postnatal Day 21 wild-type and Gli1−/+ mice expressing Shh.58 These results suggest that when the Shh pathway is activated upstream, Gli1 is not required for tumorigenesis and would not be an effective drug target.

In contrast, however, in their recent study Kimura et al.,31 reported that spontaneous tumor formation in ptch1−/− mutant mice was dramatically reduced although not completely eliminated in the absence of Gli1; thus Gli1 would be a crucial contributor to medulloblastoma formation in ptch1−/− mutant mice. A possible explanation for this result is that in the Ptch1−/− model, the tumor forms in a context of normal physiological protein levels. In the Shh retrovirus approach, the absence of Gli1 and its regulatory effect on the pathway could possibly be overcome by the excess amount of Shh. This latest study, in which loss of Gli1 in ptch1−/− mice resulted in a clearly reduced incidence of medulloblastoma, supports the suggestion that Gli1 plays a crucial role in the formation of this tumor. Interestingly, in the few medulloblastomas arising in ptch1−/−; Gli1−/− mice, and in those that form in ptch1−/−; Gli1−/+ mice, the level of Gli2 expression was elevated, leading to the conclusion that there is a compensatory mechanism in which reduced Gli1 expression leads to an increase in Gli2 expression. A remarkable reduction in medulloblastoma incidence was demonstrated in ptch1−/− mice lacking Gli1 function.

Kimura and colleagues31 also found that medulloblastomas obtained in ptch1−/− mice exhibit high levels of Gli1 but not Gli2 expression compared with the developing cerebellum. Interestingly, the tumor incidence in Ptch1−/− mice was similar to that observed in ptch1−/−; Gli1−/+ mice, demonstrating no evidence of haploinsufficiency effect. Nevertheless, medulloblastoma from ptch1−/−; Gli1−/+ mice expressed elevated levels of Gli2. This suggests that increased expression of Gli2 may compensate for the reduced level of Gli1 to regulate target genes responsible for medulloblastoma formation in ptch1−/− mice. Furthermore, in ptch1−/−; Gli1−/+ tumors, the expression of the mutated Gli1 transcript was increased, suggesting that Gli2 function may be increased because Gli1 is a target gene of Gli2.

These data lead to the conclusion that there might be a threshold of Gli transcriptional activity required to initiate oncogenic activity that can be met by contributions from both Gli1 and Gli2. These findings show that Gli1 is important in medulloblastoma formation and that Gli2 shares this oncogenic function. The relevance of Gli1 as an important contributor in medulloblastoma formation was also underscored by the recent observation that suppression of Smo by using a small-molecule inhibitor reduces Gli1 expression in tumor cells in vivo and eradicates medulloblastoma.48

Treatments Based on These Models

The eradication of medulloblastoma in mice by blocking
the Shh pathway provides evidence that this tumor might be treated without the use of potentially risky chemothera-
py and radiation regimens.

Cyclopamine, an alkaloid agent isolated from the corn lily Veratum californicum, has been shown to suppress the response of target tissues to Shh by acting at the level of Smo.\textsuperscript{6} Cyclopamine treatment of medulloblastoma cells derived from mice blocked proliferation in vitro and induced changes in gene expression that were consistent with initiation of neuronal differentiation and loss of the neuronal stem cell–like character. It also induced rapid death of cells obtained in freshly resected human medulloblastoma.\textsuperscript{3} Furthermore, in vivo experiments showed that by the 7th day of treatment, subcutaneous injections of the highest cy-
clopamine dose (1.25 mg/day; \textasciitilde 50 mg/kg) abolished Shh pathway activity and growth of medulloblastoma allografts derived from ptch\textsuperscript{+/−}; p53\textsuperscript{−/−} animals and propagated in nude mice. These results demonstrate that cyclopamine can induce tumor regression by specific effects on the Shh path-
way. No adverse effects were noted in cyclopamine-treated animals.\textsuperscript{3} These findings demonstrate that targeting signaling components downstream of tumor-initiating mutations can be an effective therapeutic strategy, and support the de-
velopment of Shh antagonists for the treatment of medulloblastoma.

High-throughput cell-based screening assays identified several small-molecule inhibitors of the Shh pathway.\textsuperscript{52} One of these compounds, HhAntag (a benzimidazole de-
rivative), exhibited higher affinity for Smo than cyclop-
amine, blocking Shh function at greater than 10-fold lower concentrations. The HhAntag compound penetrates the blood–brain barrier after oral delivery, making it an ideal candidate for treatments of brain tumors caused by in-
creased activity of the Shh pathway. Recent experiments in which Smo was blocked using HhAntag in mice with med-
ulloblastoma resulted in suppression of several genes that are highly expressed in medulloblastoma, inhibition of cell proliferation, increase in cell death, and, at the highest dose, complete eradication of tumors.\textsuperscript{48} Long-term treat-
ment with HhAntag prolonged medulloblastoma-free sur-
vival.

These experiments with ptch\textsuperscript{+/−}; p53\textsuperscript{−/−} genetically engi-
neered mice themselves demonstrated that the highest dose of HhAntag (100 mg/kg) administered twice daily for 4
days completely suppressed the Shh pathway. In addition, the mouse brain showed a decrease in total tumor mass. Three-week-old mice with tumors treated twice daily for the longer time of 2 weeks at 20 mg/kg had much smaller
tumors than untreated mice. In mice treated with 100
mg/kg twice a day for 2 weeks, no tumor mass could be de-
tected, and the structure of the cerebellum was restored to
normal. When mice were treated for a prolonged period of
time with an intermediate dose of 100 mg/kg once daily, they stayed tumor free significantly longer than untreated
mice, and they showed no side effects of the drug. This
work is particularly significant because it illustrates the use of genetically engineered mouse models of cancer in the
process of drug development. These preliminary findings suggest that long-term treatment with HhAntag should be
considered for trials in patients, but because the lower dose
did not permanently eliminate the cancer, careful studies
designed to determine the best dosing regimen are needed
before the Shh inhibitors are used in clinical trials.\textsuperscript{48}

**CONCLUSIONS**

Animal models provide an environment within which to analyze the molecular interactions between tumor and host
cells. Together with in vitro studies, animal models provide a system for investigating the genetic pathways that deter-
imine the transformations of cells in an appropriate in vivo
biological context and for determining how best to counter-
act these aberrant pathways therapeutically. Our under-
standing of human disease has gained much from the inves-
tigation of fundamental signaling pathways in model
organisms. The Shh pathway is known to play a critical role
in normal cellular expansion and in the patterning of the
early embryo of vertebrates and invertebrates, and is also
implicated in human cancer. It is clear that developing ratio-
nal therapeutic approaches for medulloblastoma is essen-
tial. Although many children with medulloblastomas are
cured of their tumor by surgery, radiation, and chemothera-
py, they are frequently devastated by the treatment.

Haploinsufficiency of ptch appears to be sufficient to ini-
tiate the process of transformation, but the additional genet-
ic changes that contribute to the unrestrained proliferation of cells remains to be determined.\textsuperscript{19,60} Recent findings indi-
cate that Gli1 plays an important role in medulloblastoma formation in ptch\textsuperscript{+/−} mice, and studies have demonstrated an upregulation of Gli2 in the absence of Gli1 during
demulloblastoma formation. The ultimate mechanism responsible for controlling target genes in the Shh pathway and the identity of the genes responsible for tumorigenesis remain to be uncovered. Nevertheless, these data support the use of Gli1 expression as a molecular target for devel-
oping novel therapies to treat the subset of medulloblas-
tomas associated with increased Shh signaling pathway activity for their growth.

Recently, tumor-bearing, genetically engineered mice
were used to test a novel compound, HhAntag, that was
identified as blocking Smo with high affinity. Although
mutations in ptch are found only in a small percentage of
demulloblastomas, the elevated Shh signaling activity seen in a larger subset of these tumors is not explained by mu-
tation, and the pharmacological intervention available does
not directly attack the mutated gene product. Nevertheless,
mutations in different components of the Shh pathway have
been identified and may account for most of the medul-
loblastomas with high Gli expression. The Shh signaling
pathway likely plays a central role in a large range of tu-
mors, which makes pharmacological blockade of the path-
way an extremely attractive strategy. The data recently
found strongly suggest that such a strategy may well work
in a subset of medulloblastoma that depends on Smo activ-
ity for their growth.

The new genetically defined mouse models of human
cancer recapitulate genetic events that are causally related
to the formation of the tumor and offer the possibility to test
critical targets for therapy. The Shh signaling pathway has
now been validated as a therapeutic target in medulloblas-
toma by using this approach. Other advantages of geneti-
cally induced tumors are that they arise in situ and fre-
quently are histologically very similar or indistinguishable
from the human tumors. The substantial power of mouse
modeling has been developed over the last two decades pri-
marily to understand the biological aspects of tumors. We
hope that work with these models will lead to new insight

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into medulloblastoma and to better therapies directed by the molecular alterations.

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Address reprint requests to: Howard L. Weiner, M.D., Division of Pediatric Neurosurgery, Department of Neurosurgery, New York University School of Medicine, 317 East 34th Street, New York, New York 10016. email: howard.weiner@med.nyu.edu.