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Materials and Methods

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Medulloblastoma Growth Inhibition by Hedgehog Pathway Blockade

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Constitutive Hedgehog (Hh) pathway activity is associated with initiation of neoplasia, but its role in the continued growth of established tumors is unclear. Here, we investigate the therapeutic efficacy of the Hh pathway antagonist cyclopamine in preclinical models of medulloblastoma, the most common malignant brain tumor in children. Cyclopamine treatment of murine medulloblastoma cells blocked proliferation in vitro and induced changes in gene expression consistent with initiation of neuronal differentiation and loss of neuronal stem cell–like character. This compound also caused regression of murine tumor allografts in vivo and induced rapid death of cells from freshly resected human medulloblastomas, but not from other brain tumors, thus establishing a specific role for Hh pathway activity in medulloblastoma growth.

Signaling by the Hh family of secreted proteins was implicated initially in determination of embryonic cell fate, and more recently in maintenance of somatic stem cells and in specification of organ size. The latter role is illustrated in the developing cerebellum, where Hh signaling delays neuronal differentiation and induces proliferation of cerebellar granular neuronal precursors (CGNPs) (*1–4*). Medulloblastomas, which are aggressive childhood tumors of cerebellar origin, are associated with inappropriate Hh pathway activity (*5–8*). The activation of this pathway normally requires antagonism of the 12-transmembrane protein Patched (Ptch) by Hh ligand, thus releasing the seven-transmembrane protein Smoothened (Smo) for activation of target genes by the Cubitus interruptus/Gli (Ci/Gli) family of transcription factors (*9–11*).

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Ligand-independent pathway activity in medulloblastoma is caused either by mutations that render Smo insensitive to regulation by Ptch or by mutational inactivation of *Ptch*. The transcription of *Ptch* is induced by Hh pathway activity, thus generating a negative feedback loop and serving as a convenient indicator of pathway activation.

We studied Hh pathway activity and function in cerebellar tumors from mice carrying a single mutant allele of the *Ptch* gene ($Ptch^{+/-}$) (*5*). Wild-type *Ptch* mRNA expression was never detected in pure tumor tissue, indicating a lack of functional *Ptch* gene product that should result in Hh pathway activation (see supporting online material, fig. S1). The frequency of medulloblastomas in $Ptch^{+/-}$ mice was increased by a *p53* mutant background (*12*); loss of *p53* function also enabled propagation of these tumors in athymic mice as subcutaneous allografts that displayed diagnostic features of human medulloblastoma (Fig. 1, A and B; fig. S2) (*13*). From four such allografts independently originating in $Ptch^{+/-}$ mice, cultured cell lines were derived that lacked *p53* function (Fig. 1C), retained a $Ptch^{+/-}$ genotype (Fig. 1C), and displayed elevated levels of Hh pathway activity (see below). Treatment with the DNA demethylating agent 5-azacytidine (5-azaC) restored *Ptch* mRNA expression in each cell line (Fig. 1D) and dramatically reduced activity from the *Ptch-LacZ* reporter (Fig. 1E). As the *LacZ* gene disrupts the targeted *Ptch* allele, thereby reporting *Ptch* transcription, the observed decrease in β-galactosidase levels indicates a reduction in pathway activity (*5*, *14*). We confirmed the specificity of this effect by stably transfecting cells to express high levels of Gli1, which activates the Hh pathway downstream of Ptch. In such cells, 5-azaC did not block Hh pathway activity (Fig. 1E), despite restoration of *Ptch* mRNA expression (*15*). These results indicate that silencing by DNA methylation of the functional *Ptch* allele is the precipitating event in pathway activation and tumor initiation in these animals.

To test the role of Hh pathway activity in tumor growth, we used cyclopamine, a plant-derived pathway antagonist that acts at the level of Smo (*14*). Medulloblastoma-derived cell lines were cultured with cyclopamine or with tomatidine, another steroidal alkaloid with little effect on the Hh pathway (*16*). By 72 hours, cyclopamine treatment resulted in a 60 to 80% reduction in growth relative to tomatidine in cultures of all tumor-derived cell lines, whereas growth of fibroblast control cells was unaffected (Fig. 2A). Cyclopamine also completely and specifically abolished β-galactosidase activity in all murine medulloblastoma lines (Fig. 2B), demonstrating that the effect of cyclopamine on cell growth parallels the reduction in Hh pathway activity.

The effect of cyclopamine treatment on murine medulloblastoma cell growth is largely mediated by inhibition of cell proliferation, because culture of the $PZp53^{MED1}$ allograft line with cyclopamine reduced DNA synthesis by 90%, as compared to tomatidine-treated cells (Fig. 2C), with only low levels of apoptosis (less than 1% of cells) (*15*). This effect depends on specific pathway inhibition, because $PZp53^{MED1}$ cells engineered to overexpress Gli1 retained high levels of β-galactosidase activity (*15*) and cell growth (fig. S3A) upon treatment with cyclopamine.

To determine the effect of Hh pathway inhibition on cellular pathways that regulate proliferation or differentiation, we examined the expression of cell cycle components and cerebellar neuronal differentiation markers in control and cyclopamine-treated $PZp53^{MED1}$ cells. Cyclins D1, D2, E1, and hyperphosphorylated Rb,

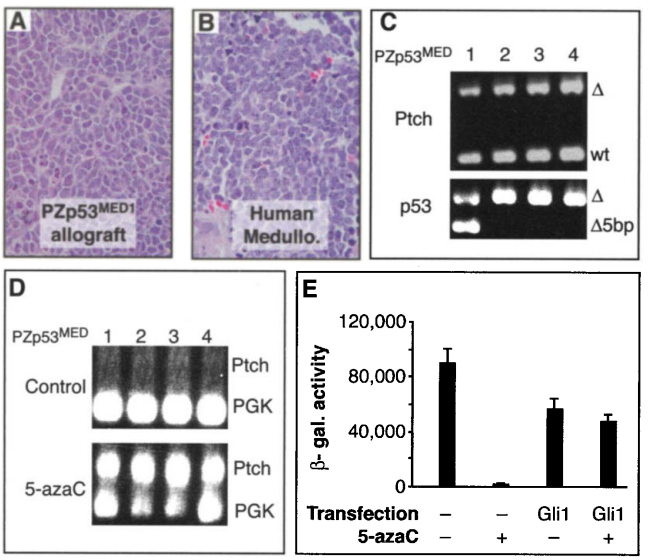

Fig. 1. Epigenetic basis of Hh pathway activation in a mouse medulloblastoma model. (**A**) Photomicrograph of PZp53^{MED1} allograft histology (hematoxylin and eosin stain) shows sheets of primitive round, basophilic (blue) cells with large, variably shaped nuclei and scant cytoplasm. Magnification is 200×. The morphology is similar to that shown in (**B**), which is human medulloblastoma (at the same magnification) with moderate anaplasia (abnormal nuclear morphology). (**C**) Genotypes of four medulloblastoma allograft lines arising in offspring of *Ptch*$^{+/-}$/*p53*$^{+/-}$ mice. Three lines were homozygous for the targeted *p53* allele (Δ) and a fourth (line 1) was heterozygous for the targeted *p53* allele and for an allele carrying a 5-bp deletion (Δ5bp). (**D**) Multiplex reverse transcription–polymerase chain reaction (RT-PCR) analysis of RNA from the same four cell lines with primers against Ptch and phosphoglycerate kinase (PGK) shows absence of detectable *Ptch* mRNA and presence of PGK mRNA (positive control) in control cultures treated with ethanol vehicle. Epigenetic inactivation of *Ptch* is indicated by expression of *Ptch* mRNA in all four cell lines after culture in the presence of the demethylating agent 5-azacytidine (5-azaC). (**E**) β-galactosidase (β-gal.) activity from the *Ptch-LacZ* reporter is abolished in untransfected PZp53^{MED1} cells treated with 5-azaC, whereas β-gal. activity in PZp53^{MED1} cells stably transfected with Gli1 is unaffected by 5-azaC. Error bars represent standard error of the mean (SEM) for triplicate cultures.

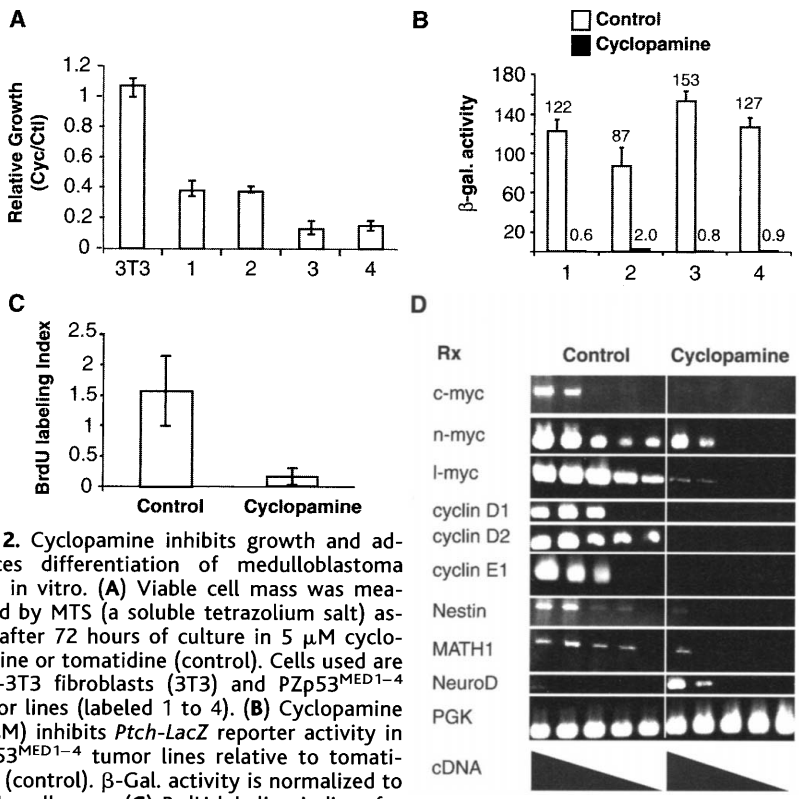

Fig. 2. Cyclopamine inhibits growth and advances differentiation of medulloblastoma cells in vitro. (**A**) Viable cell mass was measured by MTS (a soluble tetrazolium salt) assay after 72 hours of culture in 5 μM cyclopamine or tomatidine (control). Cells used are NIH-3T3 fibroblasts (3T3) and PZp53^{MED1-4} tumor lines (labeled 1 to 4). (**B**) Cyclopamine (5 μM) inhibits *Ptch-LacZ* reporter activity in PZp53^{MED1-4} tumor lines relative to tomatidine (control). β-Gal. activity is normalized to viable cell mass. (**C**) BrdU labeling indices for PZp53^{MED1} cells cultured with 5 μM cyclopamine or tomatidine (control). (**D**) Semiquantitative RT-PCR analysis showing mRNA expression in PZp53^{MED1} cells cultured in tomatidine (control) or cyclopamine for 72 hours. cDNA was synthesized from 1 μg of RNA from each culture. PCR was carried out on undiluted cDNA and on fourfold serial dilutions of cDNA, as indicated by the descending wedge.

which are induced by Shh in CGNPs and promote transit through prereplicative (G_1) cell cycle checkpoints (*4*), are also expressed in tomatidine-treated PZp53^{MED1} cells but are suppressed upon treatment with cyclopamine (Fig. 2D; fig S3B). In addition to down-regulation of a proliferative program, cyclopamine treatment appears to initiate differentiation in these cells, as indicated by reduced expression of the neurofilament nestin, a neuronal stem cell marker, and of the bHLH transcription factor Math1, a marker of proliferating CGNPs (*17*). Furthermore, cyclopamine treatment reduced expression of N-, c-, and L-myc (Fig. 2D, fig S3C) (*18*) and increased expression of NeuroD, a marker of postmitotic cerebellar granular neurons (Fig. 2D) (*19*). Morphological neuronal maturation was not observed in vitro, however, indicating that Hh pathway blockade only partially advances differentiation.

We further investigated cyclopamine effects in vivo and found by the 7th day of treatment that subcutaneous injections of the highest cyclopamine dose (1.25 mg/day; ~50 mg/kg) had abolished β-galactosidase activity (Fig. 3A) and growth (Fig. 3B) of medulloblastoma allografts propagated in nude mice. In a second experiment, allograft tumors from Gli-transfected cells (n = 7) grew despite cyclopamine treatment (Fig. 3C), whereas tumors from untransfected cells (n = 4) decreased in volume. Microscopic histologic analysis further demonstrated the complete disappearance of two of these tumors and a dramatic reduction in cell proliferation in the two that remained (fig. S4). These results demonstrate that cyclopamine can induce tumor regression by specific effects on the Hh pathway. No adverse effects were noted in cyclopamine-treated animals.

To explore the potential therapeutic utility of Hh pathway blockade, cells from freshly resected human medulloblastomas were cultured with KAAD-cyclopamine, a potent derivative of cyclopamine (*14*). Tumor cells initially displayed higher levels of *Ptch* mRNA than did normal cerebellum, indicating elevated Hh pathway activity, and this activity was reduced by treatment with either drug (fig. S5, A and B). Treatment with KAAD-cyclopamine also induced a significant decrease in cell viability within 48 hours (Fig. 4A); cell viability continued to decline after prolonged exposure (Fig. 4B), and fewer than 0.1% of cyclopamine-treated cells survived, after 1 week, as compared to typically greater than 10% of vehicle-treated controls. The effects of cyclopamine were similar to those of KAAD-cyclopamine (Fig. 4C) at pathway-inhibitory doses (*14*). Primary cultures from a glioblastoma and from an ependymoma, in contrast, failed to respond to cyclopamine (Fig. 4A). The dramatic reduction in cell viability of primary cultures from medulloblastomas, but not from other types of brain tumors, and the unaltered growth of embryonic fibroblasts (Fig. 2A) further suggests

that cyclopamine specifically affects medulloblastomas and is not generally cytotoxic.

In contrast to reports suggesting that partial loss of *Ptch* function suffices to initiate tumor formation (*20*, *21*), we found that medulloblastomas arising in $Ptch^{+/-}$ mice lack detectable *Ptch* mRNA. The absence of *Ptch* mRNA and consequent pathway activation, apparently caused by DNA methylation of the normal *Ptch* allele, is critical not only for initiation but also for growth of the tumor, because pathway suppression by reactivation of the methylated *Ptch* allele or by cyclopamine treatment blocks growth. Loss of *p53* function in murine medulloblastomas appears to favor allograft and cell line growth, perhaps by compromising the ability of cells to undergo apoptosis (*22*). Human *p53* mutations are undetectable in nearly all (92 to 99%) sporadic medulloblastomas (*23*), which may account for increased apoptosis in primary human medulloblastoma cells cultured with Hh pathway antagonists. In light of the relatively low frequency (~30%) of sporadic human medulloblastomas linked to mutational activation of the Hh pathway (*24*, *25*), it is perhaps surprising that elevated Hh pathway activity appears to be characteristic of these tumors and is required for their growth (Fig. 4; fig. S5). The basis for this requirement is not clear, but may relate to known Hh functions as a stem cell factor in other tissues (*9*, *26*) and as a mitogen in developing cerebellum (*1*–*4*). Hh pathway activity thus may play a role in maintenance of the capacity of medulloblastoma tumor stem cells (*10*) to undergo self-renewal and in the proliferation of their progeny. This dual role would be consistent with the high-level expression of the neuronal stem cell marker nestin in murine and human tumors (fig. S3C) (*27*) and with the rapid cell death of almost all human medulloblastoma cells when cultured with Hh pathway antagonists. Whatever its biological basis, the general requirement for Hh pathway activity in medulloblastoma growth represents a potential therapeutic opportunity, because cyclopamine and other pathway antagonists can be administered in effective doses with no apparent detrimental effects in rodents and other mammals (*28*).

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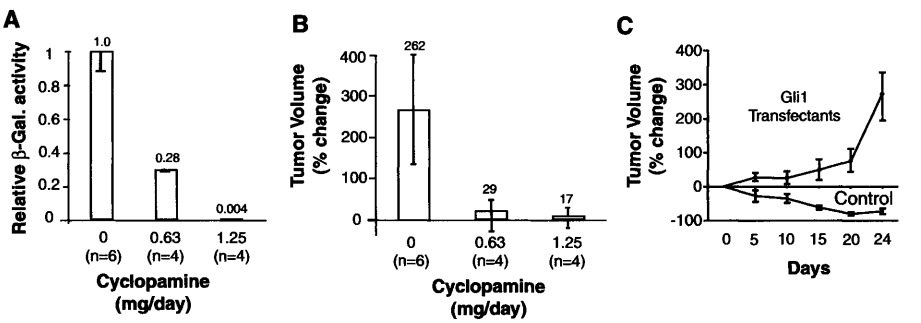

Fig. 3. Cyclopamine inhibits medulloblastoma growth through specific effects on the Hh pathway. (**A**) Dose-dependent *Ptch-LacZ* reporter inhibition in $PZp53^{MED1}$ tumor allografts in tumor tissue from mice treated for 7 days with vehicle (0), or cyclopamine (0.63 or 1.25 mg/day). Error bars represent SEM. (**B**) Change in tumor allograft volume (%) in the same experiment. In this experiment, treatment was initiated after tumors reached an average size of 181 mm^3. (**C**) Cyclopamine treatment induces regression of control-transfected tumors but not of tumors overexpressing the downstream Hh effector Gli1. Athymic mice bearing tumor allografts from control or Gli1-transfected $PZp53^{MED1}$ cells were grown to ≤125mm^3 before treatment with daily subcutaneous injections of cyclopamine (1.25 mg/day) for 24 days.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S5
Table S1

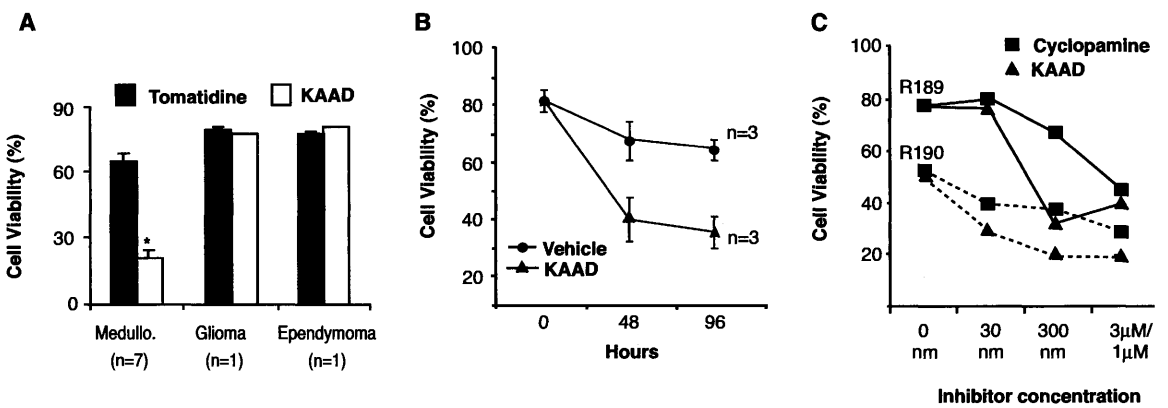

Fig. 4. Cyclopamine causes loss of viability of cultured tumor cells from primary human medulloblastomas.(**A**) Loss of cell viability in response to KAAD-cyclopamine (1 μM) 48 hours after surgical resection of tumor. Viability was assessed by absence of propidium iodide and annexin V–fluorescein isothiocyanate staining. Error bars represent standard error of the mean. (**B**) Loss of cell viability in response to KAAD-cyclopamine (1 μM) over time. (**C**) Dose response curves measuring loss of cell viability in response to increasing concentrations of cyclopamine (0 to 3 μM) and KAAD-cyclopamine (0 to 1 μM) in two medulloblastomas.