Tumorigenesis and Neoplastic Progression

Hedgehog Signaling Promotes Medulloblastoma Survival via *BclII*

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Activation of the Hedgehog (Hh) pathway has been identified in several cancers, including medulloblastoma, but the mechanisms by which this pathway affects tumor survival and growth are incompletely understood. We investigated whether Hedgehog might promote survival of medulloblastoma cells via up-regulation of *BclII*. We found that mRNA levels of the Hedgehog pathway effector *Gli1* were significantly associated with *BclII* expression in medulloblastoma and that *Gli1* and *BclII* are both present in regions of decreased apoptosis in nodular medulloblastoma. Transient overexpression of *Gli1* and *Gli2* in medulloblastoma cultures induced a *BclII* transcriptional reporter and increased *BclII* protein levels, whereas stable overexpression of *Gli1* was associated with increased *BclII* mRNA. The Hedgehog antagonist cyclopamine blocked expression of the Hh pathway targets *PTCH1* and *Gli1*, lowered *BclII* levels, and increased apoptosis in DAOY and UW228 medulloblastoma cells. Apoptotic induction caused by cyclopamine could be rescued in part by enforced expression of *Gli1* or *BclII*. Hh pathway blockade also sensitized medulloblastoma to the effects of the proapoptotic agent lovastatin. These data demonstrate that *BclII* is an important mediator of Hh activity in medulloblastoma and suggest new strategies for combined chemotherapeutic regimens. (Am J Pathol 2007; 170:347–355; DOI: 10.2353/ajpath.2007.060066)

Hedgehog (Hh) pathway blockade has emerged as a promising therapy for multiple types of cancer, including brain tumors and carcinomas of the prostate, digestive tract, and lung.^{1–5} Gli transcription factors are critical effectors of the canonical Hh signaling pathway and regulate expression of multiple targets, including N-myc, cyclin D, *PTCH1*, and *Gli1*.^{6,7} The Hh pathway is frequently activated in the nodular medulloblastoma sub-

type by genetic changes inactivating the inhibitory *PTCH1* receptor, although nonnodular medulloblastoma also can harbor *PTCH1* mutations.^{8–10} Expression analyses have detected elevated levels of the Hh pathway targets *PTCH1*, *Gli1*, and N-myc in nodular medulloblastoma.^{11,12} However, Hh pathway targets are expressed in medulloblastoma of the classic subtype as well.^{11,13,14}

Several groups have shown that Hh pathway blockade inhibits the growth of murine and human medulloblastoma cells in culture and in xenografts.^{1,15–17} It has also recently been demonstrated that cyclopamine or synthetic Hh antagonists can cause regression of primary medulloblastoma arising in *PTCH* heterozygous transgenic mice.^{16,17} Although most studies have concentrated on the effects of Hh inhibition on cell cycle regulation and cellular differentiation, increased apoptosis after Hh blockade has been noted as well.^{1,16,17} In this report, we focus on the mechanism by which Hh signaling regulates apoptosis, implicating *BclII* in the process. Increased expression of *BclII* has been associated with Hh signaling in keratinocytes and renal epithelial cells.^{18,19} More recently, two groups have shown that *Gli1* and *Gli2* directly bind to sequences in the *BclII* promoter in epithelial cells and induce transcription, indicating that this regulation is direct.^{20,21} Several studies have suggested that *BclII* is an important regulator of apoptosis in the cerebellar granule cell precursors from which medulloblastomas are thought to arise.^{22,23} Given these data and the direct linkage between Hh activity and *BclII* expression in nonneoplastic tissues, we investigated whether Hh might regulate cell survival in medulloblastoma via this anti-apoptotic protein.

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Materials and Methods

Clinical Specimens

Immunohistochemistry was performed on freshly cut, deparaffinized tumor sections after antigen retrieval using Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Primary antibody was detected using the Vectastain Elite kit (Vector Laboratories). The following antibodies were used: *BclII*, clone Ab-1 (1:500; Calbiochem, La Jolla, CA); *Gli1*, clone C-18 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA); *Gli2*, clone N-20 (1:100, Santa Cruz Biotechnology); and cleaved caspase 3, clone Asp175 (1:100; Cell Signaling Technologies, Beverly, MA). Snap-frozen primary medulloblastoma tissues were obtained from the Department of Pathology, The Johns Hopkins University School of Medicine, with institutional review board approval. RNA was extracted from medulloblastoma samples using Trizol reagent (Invitrogen, Carlsbad, CA) and further purified using RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Cell Culture

The DAOY cell line was obtained from American Type Culture Collection (Rockville, MD) and maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS). UW228 cells were the kind gift of Dr. J.R. Silber (University of Washington, Seattle, WA) and were maintained in Dulbecco's modified Eagle's medium/F12 supplemented with 10% FBS. To isolate granule cell precursors, cerebella of 7-day-old C57/B6 pups were microdissected and minced in ice-cold Hanks' buffer and washed once with 12 ml of ice-cold phosphate-buffered saline (PBS). To digest connective tissue, cerebella pieces were incubated in 15 ml of Hanks containing 0.25% trypsin (Mediatech, Herndon, VA) and 1.5 mg of DNase I (Roche, Nutley, NJ) for 7 minutes at 37°C with gentle agitation. Fifteen ml of Hanks with 10% FBS was then added to the suspension to inactivate the trypsin. After two additional washes in ice-cold Hanks, a single-cell suspension was generated by trituration with a fire-polished Pasteur pipette in 5 ml of ice-cold Hanks containing 1.5 mg of DNase I. The cell suspension was filtered through a 70- μ m nylon mesh (BD Falcon, Bedford, MA), and the cells concentrated by centrifugation at 700 rpm for 5 minutes in a table-top centrifuge. Granule cell precursors were resuspended in neurobasal medium supplemented with B27 growth supplement (Invitrogen) to a final concentration of 1×10^6 /ml. Granule cell precursors (5×10^5) were treated in poly-L-lysine-coated six-well plates (Corning, Acton, MA). SHH was produced as previously described.²⁴

Cells were transfected using Fugene6 reagent (Promega, Madison, WI) with plasmids encoding either *BclII* under control of the CMV promoter²⁵ or *Gli*-expressing pcDNA4 plasmids.²¹ Stable subclones were selected by 28 days of treatment with 1 mg/ml G418. Lovastatin (Sigma, St. Louis, MO) was solubilized in ethanol. The

BclII reporter assay was performed 48 hours after transfection using a dual-luciferase reporter assay system (Promega) and normalized to Renilla luciferase activity. Cells were plated in 24-well plates at 3.5×10^4 cells per well. On the next day, they were transfected with 475 ng of *BclII*-luciferase reporter [95% (w/w)] or an empty vector as negative control and with 25 ng of Renilla luciferase [5% (w/w)] as a transfection control. For mRNA quantification or apoptotic assays, cells were plated in six-well plates (RNA) or 24-well plates (apoptosis) and incubated overnight, and then washed once with PBS and switched to low serum (0.5% FBS) media. The following day (normally 16 hours after serum reduction), this was replaced with medium supplemented with 0.5% FBS and either ethanol (designated either vehicle or 0 μ mol/L in the figures) or cyclopamine (5 or 10 μ mol/L) in ethanol. RNA was extracted at various time points after cyclopamine treatment using Qiagen RNeasy kits. Quantification of mid-apoptotic and dead cells was performed 2 and 4 days after cyclopamine treatment with the Guava-PCA flow cytometry system and ViaCount reagent according to the manufacturer's instructions (Guava Technologies, Hayward, CA). In brief, cells were trypsinized and spun at 1200 rpm for 5 minutes, and then 200 μ l of ViaCount reagent was added to the cell pellets. Analysis was performed 10 to 20 minutes after the addition of the reagent. Maximal apoptotic induction was observed after 2 days in DAOY cells, and after 4 days in UW228 cells, and these time points are shown in the results.

For *BclII* short interfering RNA (siRNA) experiments, 4×10^4 DAOY cells were plated in triplicate and then incubated overnight, then transfected according to the manufacturer's directions using RNAiFect (Qiagen) with 1 μ g/well of a pool of scrambled, nonspecific siRNA (Dharmacon, Lafayette, CO) as negative control or *BclII* siRNA (Dharmacon) with the following oligo sequences: *BclII* forward 5'-GG-GAGAUAGUGAUGAAGUAUU-3' and *BclII* reverse 5'-P-UACUUCACUACUACUCCCCU-3'. Plates were incubated for 8 hours, and then washed once with PBS and switched to low serum (0.5% FBS) media. The following day, this was replaced with medium supplemented with 0.5% FBS and either ethanol or cyclopamine. Apoptosis analysis was performed 48 hours after treatment.

RNA and Protein Analyses

RNA levels were analyzed by real-time polymerase chain reaction (PCR) analysis performed in triplicate with SYBR Green reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions on an I-Cycler IQ real-time detection system (Bio-Rad, Hercules, CA). To minimize contaminating genomic DNA, a 15-minute on-column DNase step (Qiagen RNase-free DNase kit) was included during RNA extraction. The standard curve method was used to determine expression levels, and all values were normalized to actin. Oligo sequences were as follows: human *Gli1* forward: 5'-AC-CCGGGTCTCAAAGT-3'; human *Gli1* reverse: 5'-GG-CTGACAGTATAGGCAGAGC-3'; human *Gli2* forward: 5'-CCACATGGAGCACTACCT-3'; human *Gli2* reverse: 5'-

Figure 1. Expression of *BclII* and the Hh targets Gli1 and Gli2 is increased in the internodular regions of nodular/desmoplastic medulloblastoma. **a:** A cartoon modeling molecular and cellular factors in nodular medulloblastoma. Cells within medulloblastoma nodules have decreased levels of Gli and *BclII*, and either differentiate into neurocytes or undergo programmed cell death. **b:** *BclII* expression is low in nodules (asterisk) and high in internodular regions. **c:** Medulloblastoma cells invading the cerebellar molecular layer (arrows) are strongly immunopositive for Gli1, whereas nonneoplastic internal granule layer neurons (asterisk) are negative. **d** and **e:** Gli1 staining was frequently strong in internodular regions (arrowhead) but weak within nodules (asterisk). **f** and **g:** Expression of Gli2 was also higher between (arrows) compared with within (asterisks) nodules. Apoptotic cells with condensed nuclei lacked Gli2 protein (small arrow, inset). **h:** Cleaved caspase 3 was detected in lymphocyte-like apoptotic cells (small arrows). Original magnifications: $\times 200$ (**b, d, f, g**); $\times 100$ (**c**); $\times 400$ (**e, h**).

CCTGGCTGCAGAGATCATGG-3'; human Ptch1B forward: 5'-GACGCCGCTTCGCTCTG-3'; human Ptch1B reverse: 5'-GCCACAACCAAGAACTTGCC-3'; human *Bcl_{XL}* forward: 5'-GTCGGATCGCAGCTTGGA-3'; human *Bcl_{XL}* reverse: 5'-GCTGCATTGTTCCCATAGAGTTC-3'; human *BclII* forward: 5'-TACCTGAACCGGCACCTG-3'; human *BclII* reverse: 5'-GCCGTACAGTTCCACAAAGG-3'; mouse *BclII* forward: 5'-GGTGGTGGAGGAACTCTTCA-3'; mouse *BclII* reverse: 5'-CAGATGCCGGTTCAGGTACT-3'; human actin- β forward: 5'-CCCAGCACAAATGAAGATCAA-3'; human actin- β reverse: 5'-GATCCACACGGAGTACTTG-3'. Hprt forward: 5'-CTTTGCTGACCTGCTGGATT-3'; and Hprt reverse: 5'-GTTGAGAGATCACTCCACC-3'. Immunoblot analysis of *BclII* (clone Ab-1, 1:500) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone 6C5, 1:500,000; Research Diagnostics, Concord, MA) was performed on lysates (50 μ g) prepared using standard techniques either 2 days (DAOY) or 4 days (UW228) after cyclopamine administration and quantified using Quantity-One densitometry software (Bio-Rad).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Data graphed with error bars represent mean and SE of mean from three replicates. All experiments were repeated at least two times, with similar results. Unless otherwise specified, a two-sided Student's *t*-test was used to determine the significance of any differences.

Results

Gli and *BclII* Are Preferentially Expressed in the Internodular Regions of Desmoplastic Medulloblastoma

In nodular/desmoplastic medulloblastoma, the internodular regions are similar to the external germinal layer of the developing cerebellum in that they are comprised of primitive-appearing, highly proliferative cells. Nodules, in contrast, are characterized by cell cycle exit and increases in both apoptosis and neuronal differentiation, and resemble maturing granule cell precursors (Figure 1a).^{26,27} *BclII* levels are increased in the internodular regions (Figure 1b),²⁸ and we investigated whether the Hh pathway was active here as well. We used expression of Gli1 and Gli2 as markers of Hh activity, and detected these pathway targets immunohistochemically in medulloblastoma cells, but not in adjacent nonneoplastic cerebellar tissue (Figure 1c and data not shown). In 5 of 10 nodular lesions, nuclear Gli1 staining was more intense in the internodular regions than within nodules (Figure 1, d and e). A similar expression pattern was observed for Gli2 in five of eight lesions, and in one very nodular tumor Gli2 expression was essentially restricted to internodular cells (Figure 1f). In other tumors, a fraction of cells within nodules were weakly positive for Gli2, although the small cells with condensed nuclei were always immunonegative for Gli1 and Gli2 (Figure 1g). We believe these small lymphocyte-like cells within nodules are undergoing programmed cell death because they are immunopositive for cleaved caspase 3 (Figure 1h) and have been previously shown to be terminal dUTP nick-end labeling-pos-

Figure 2. Analysis of Gli and *BclII* mRNA expression. **a:** Gli1 expression was measured by quantitative RT-PCR in RNA extracted from five nodular, six classic, and seven anaplastic snap-frozen medulloblastomas, as well as the DAOY cell line. **b:** *BclII* mRNA levels in these 18 samples are compared with those of Gli1, with each tumor represented by a filled triangle. The subtype of tumors with above-median Gli1 or *BclII* expression is further designated using an open square, diamond, or circle.

itive.²⁹ The fact that both Gli and *BclII* proteins are expressed in internodular regions of decreased apoptosis suggests that Hh activity may regulate cell death in tumors via *BclII*.

Gli1 Expression Is Significantly Associated with BclII in Medulloblastoma

We used quantitative reverse transcriptase (RT)-PCR to more precisely measure expression of the Hh pathway target Gli1 in and a second, independent set of medulloblastoma samples. It has previously been suggested that Gli1 expression is elevated in desmoplastic medulloblastoma as compared with classic ones.¹² Consistent with this, Gli1 expression was high in four of the five desmoplastic tumors we tested (Figure 2a). In our series, however, two of six classic medulloblastomas and three of seven anaplastic medulloblastomas also had relatively high Gli1 mRNA levels, and the differences between nodular/desmoplastic lesions and these groups were not statistically significant. Thus, activation of the Hh pathway does not seem to be restricted to the nodular/desmoplastic medulloblastoma subtype.

To address the relationship between Hh activity and *BclII* expression, we compared Gli1 mRNA levels in these 18 primary medulloblastomas with *BclII* expression (Figure 2b). Interestingly, of the nine tumors with above-median Gli1 mRNA levels, eight also had above-median *BclII* levels, a

Figure 3. Gli positively up-regulates *BclII* expression. **a:** Transient transfection of constructs encoding Gli1 or Gli2 into DAOY or UW228 cell lines significantly induced transcription of a *BclII* promoter-luciferase reporter as compared with empty vector. **b:** Stimulation of Hh signaling in cerebellar granule cell precursor cultures using SHH ligand results in a significant increase in *BclII* mRNA levels measured using quantitative RT-PCR. **c:** Both Gli1 and *BclII* mRNA levels are significantly increased in two DAOY subclones (g5, g29) stably expressing Gli1 compared with a vector-transfected clone (v). **d:** *BclII* protein expression, normalized to GAPDH, is elevated in DAOY cells stably transfected with Gli1. Mean levels from two experiments are shown above representative bands. * $P < 0.05$, ** $P < 0.005$ for two-sided *t*-tests; ns, not significant.

statistically significant association ($P = 0.003$, two-tailed Fisher's exact test). The one medulloblastoma with low Gli1 levels but high *BclII* expression was a diffusely anaplastic tumor that quickly metastasized. We have previously shown that anaplastic medulloblastomas have an increased number of chromosomal abnormalities,³⁰ and other genetic alterations might account for the elevated *BclII* levels in this single case with low Hh activity. Indeed, although the correlation between Gli1 and *BclII* was not significant in the entire cohort, when the anaplastic tumor with extremely high *BclII* levels was excluded, they were significantly correlated in the remaining 17 cases (Pearson's $r = 0.69$, $P = 0.002$). Overall, our data indicate that increased Hh activity is associated with higher *BclII* expression, potentially resulting in decreased apoptosis.

Gli1 and Gli2 Positively Regulate BclII Expression in Medulloblastoma

We next sought to determine whether Gli1 and Gli2 regulate *BclII* expression in medulloblastoma cultures. The DAOY cell line was initially derived from a nodular medulloblastoma and has recently been shown using luciferase-based reporter assays to have Gli transcriptional activity.^{31,32} We also easily detected Gli1 mRNA in UW228 cells, although at significantly lower levels than in DAOY. Using a reporter containing the *BclII* promoter region, we found that transient transfection of Gli1 or Gli2 into either cell line significantly induced reporter activity (Figure 3a). Gli1 and Gli2 had similar induction maxima, and we did not observe preferential induction of the reporter by Gli2 as was previously described in keratin-

ocytes.³³ We also found that induction of Hh signaling in cultured granule cell precursors by application of sonic hedgehog (SHH) ligand induced expression of *BclII* mRNA, suggesting that anti-apoptotic effects of Hh signaling in tumors may parallel mechanisms operating during normal cerebellar development (Figure 3b).

We next generated subclones of DAOY stably transfected with Gli1 expressed under the control of a constitutive promoter. Two representative clones (Gli1-5, Gli1-29) had Gli1 mRNA levels increased approximately sevenfold and threefold greater than those in vector-transfected cells (Figure 3c). We were not able to isolate subclones with higher levels of Gli1 mRNA, suggesting that DAOY cells may not tolerate extremely elevated Gli1 expression, nor were we able to isolate clones expressing Gli2. In agreement with our transient transfection experiments, stable increases in Gli1 expression were associated with significantly higher *BclII* mRNA expression (Figure 3c). *BclII* luciferase reporter activity was increased approximately twofold in the Gli1-overexpressing cells (data not shown). The effect of Hh signaling on anti-apoptotic proteins seemed to be somewhat selective because increased Gli1 expression did not up-regulate the Bcl family member *Bcl_{XL}* (Figure 3c). *BclII* protein levels were also increased in the DAOY clone with elevated Gli1 (Figure 3d).

Pharmacological Blockade of Hh Signaling Decreases *BclII* Levels and Induces Apoptosis in Medulloblastoma

We next investigated whether Hh pathway blockade in medulloblastoma cells using the steroidal alkaloid cyclopamine would reduce *BclII* expression. A significant reduction in Gli1 mRNA level was seen after 18 hours of cyclopamine therapy, whereas 48 hours of treatment further reduced expression of Gli1 and a second pathway target, PTCH1 (Figure 4a). In separate experiments, Gli1 levels were also significantly lowered in UW228 cells by cyclopamine. Basal levels of Gli2 were not reduced by Hh pathway blockade in these cells. Indeed, a significant increase in Gli2 level was seen using the lower dose of drug. PTCH levels also increased significantly when DAOY cells were treated with 10 μ mol/L cyclopamine compared with a 5 μ mol/L. The cause(s) of these increases are not clear, but could relate to feedback loops specific to Gli2 and PTCH. Gli1 mRNA levels in the DAOY-Gli1 stable transfectants remained above those in untreated DAOY cells, even in the presence of 10 μ mol/L cyclopamine (data not shown). *BclII* levels were also reduced in DAOY cultures after cyclopamine administration, consistent with our hypothesis that expression of this anti-apoptotic factor is dependent on a basal level of Gli1 activity. Normalized *BclII* reporter levels were lowered more than fourfold after 30 hours of treatment with 5 μ mol/L cyclopamine, whereas stably transfected cells expressing Gli1 under the control of a constitutive promoter were refractory to such inhibition (Figure 4b). In UW228 cells, application of 5 μ mol/L cyclopamine low-

Figure 4. Analysis of Hh targets and *BclII* expression after cyclopamine treatment. **a:** Levels of Gli1 and PTCH1, but not Gli2, are significantly reduced by 5 μ mol/L cyclopamine compared with vehicle-treated controls. **b:** *BclII* luciferase reporter assays after 5 μ mol/L cyclopamine exposure reveal progressive inhibition of *BclII* expression throughout 30 hours. *BclII* expression was not inhibited in DAOY cells stably expressing Gli1 (Gli1-5). **c:** Hedgehog pathway blockade in DAOY and UW228 cells by cyclopamine for 48 hours also inhibited BCL2 protein expression normalized to GAPDH. * $P < 0.05$, ** $P < 0.005$ for two-sided *t*-tests; ns, not significant.

ered *BclII*-reporter activity twofold throughout 3 days compared with vehicle-treated cells (data not shown). Western blot analysis confirmed that *BclII* protein levels in DAOY and UW228 cells after Hh pathway suppression were half or less of those in controls (Figure 4c).

Because decreased Hh activity and *BclII* expression are observed in medulloblastoma nodules with increased cell death (Figure 1), we examined whether the reduced *BclII* expression in culture after cyclopamine treatment also occurred in concert with apoptotic induction. Floating or refractile cells were more numerous in cyclopamine-exposed cultures, suggesting the compound was causing cell death (Figure 5a). Indeed, a statistically significant increase in the percentage of apoptotic DAOY and UW228 cells was documented

Figure 5. Hh pathway blockade induces apoptosis in medulloblastoma. **a:** Vector-transfected DAOY cell cultures exposed to 10 μ mol/L cyclopamine for 48 hours contained numerous refractile or floating cells. Cell death seemed more limited in cultures treated with vehicle, and in cyclopamine-treated DAOY cells stably expressing Gli1 (Gli1-5). **b:** Quantification of apoptotic cells after cyclopamine treatment confirmed that it significantly induces apoptosis. Enforced Gli1 expression blocked this increase in cell death in the stable DAOY Gli1-5 subclone. **c:** DAOY cells were transfected with siRNA targeting *BclII* or scrambled control (C) siRNA, and then treated with vehicle or 5 μ mol/L cyclopamine. The addition of cyclopamine to cells already depleted of *BclII* did not cause significant additional apoptosis. Decreased levels of *BclII* protein are documented in a Western blot positioned below the first two bars. **d:** Two subclones stably transfected with a *BclII*-expressing plasmid were not sensitive to apoptotic induction by cyclopamine. **e:** Combined treatment with cyclopamine and lovastatin acts synergistically to induce apoptosis in medulloblastoma. * $P < 0.05$, ** $P < 0.005$ for two-sided *t*-tests; ns, not significant.

using ViaCount reagents after 48 hours of cyclopamine exposure (Figure 5b). Similar results were seen when apoptotic induction was measured using annexin assays after cyclopamine treatment (data not shown). Enforced Gli1 expression rescued DAOY cells from increased cell death. The Gli1-5 and Gli1-29 stable subclones both blocked apoptotic induction after addition of 5 μ mol/L cyclopamine (data not shown), but only the Gli1-5 subclone expressing higher levels of Gli1 and *BclII* was able to rescue this effect at the 10 μ mol/L dose (Figure 5b). These data suggest that the effect of cyclopamine on medulloblastoma apoptosis is mediated through the Hh pathway.

To further define the role *BclII* plays in apoptosis induced by Hh pathway blockade, we transiently reduced *BclII* expression in DAOY cells using siRNA. Quantitative RT-PCR analysis confirmed a more than 80% decrease in *BclII* transcript levels in DAOY and UW228 cells transfected with targeted siRNA compared with control siRNA (data not shown). This reduction in *BclII* was associated with a significant increase in the percentage of apoptotic cells (Figure 5c). Cyclo-

pamine also significantly increased apoptosis in cells transfected with control siRNA, although the level of induction was less than other experiments, perhaps because of the siRNA transfection protocol. If down-regulation of *BclII* via decreased Gli activity is a major proapoptotic mechanism of cyclopamine, one would predict that in the context of already-reduced *BclII*, Hh blockade would not cause a significant additional increase in cell death. We therefore exposed cells treated with *BclII* siRNA to 5 μ mol/L cyclopamine and saw only a small, nonsignificant increase in apoptosis in both DAOY (Figure 5c) and UW228 (data not shown), suggesting that *BclII* was a major mediator of this effect. To further address the role of *BclII* in cyclopamine-mediated apoptosis, we introduced a construct encoding *BclII* under the control of a constitutive promoter into DAOY cultures then selected two stable subclones (*BclII*-1, *BclII*-7). Constitutive expression of *BclII* fully protected tumor cells against the cyclopamine-induced increase in programmed cell death (Figure 5d). These data strongly suggest that apoptotic

induction after Hh blockade is dependent to a significant degree on *BclII* down-regulation.

Hh Blockade Sensitizes Medulloblastoma Cells to Combined Chemotherapeutic Regimens

Chemotherapeutic drugs are often given in combination, and the observation that *BclII* levels are reduced in cells treated with cyclopamine suggests that Hh inhibition may sensitize tumor cells to other proapoptotic stimuli. We tested this hypothesis by co-treatment of DAOY cultures with cyclopamine and the HMG-CoA reductase inhibitor lovastatin, which has been shown to induce apoptosis in medulloblastoma.³⁴ Apoptosis was significantly induced from 7 to 17% of cells by 5 μ mol/L lovastatin (Figure 5e). In addition, co-treatment with both 5 μ mol/L cyclopamine and 5 μ mol/L lovastatin induced apoptosis in 63% of cells compared with less than 20% programmed cell death in cultures treated with the same concentration of either agent alone (Figure 5e). This increase in sensitivity was partially rescued by constitutively expressing Gli1 because only 26% of Gli1-5 cultures were apoptotic when treated with 5 μ mol/L levels of both drugs, a statistically significant decrease from 63%. Interestingly, enforced Gli1 expression significantly diminished the effect of lovastatin treatment alone ($P = 0.02$), suggesting Hh effectors act downstream of lovastatin in protecting against cell death. It is possible that lovastatin could influence the Hh pathway directly because sterol depletion has been shown to reduce Smoothened activity and diminish responsiveness to the Hh signal.³⁵

Discussion

Medulloblastomas are the most common malignant brain tumors in children and are associated with significant morbidity and mortality.³⁶ Current treatment regimens use radiation and chemotherapeutic agents that affect dividing cells in a relatively nonspecific manner and are associated with serious long-term side effects.^{37,38} This has prompted great interest in mechanism-based therapies that might target tumor cells more selectively. The Hh pathway plays a critical role in both normal cerebellar development,³⁹ and in medulloblastoma formation.^{8,9,15,40} Several studies have demonstrated that Hh pathway inhibitors can slow or arrest the growth of both human and murine medulloblastomas.^{1,15–17} However, for these promising new agents to be used most effectively, we must first determine what groups of medulloblastoma patients might benefit from their use. A better understanding of the mechanism(s) by which Hh blockade regulates the survival of tumor cells will also be critical if we are to rationally develop such therapies. In this study, we examine Hh pathway activity in a range of medulloblastoma subtypes, and the impact of Hh activity on *BclII* expression and apoptosis.

It has previously been shown that Hh activity, as evidenced by elevated expression of the Hh pathway targets PTCH1 and Gli1, is predominantly found in nodular/desmoplastic medulloblastomas.¹² This suggests that Hh

blockade might only affect tumors of this subtype, which accounts for ~29% of cases.⁴¹ Our results contrast somewhat with this earlier study. We examined 18 primary medulloblastomas of the nodular/desmoplastic, classic, and anaplastic subtypes and detected mRNA encoding Gli1 in all cases. We observed high-level Gli1 expression in a larger percentage of the nodular/desmoplastic cases than classic or anaplastic ones, but several individual tumors in each of these other subtypes had elevated Gli1 levels as well. Differences between our study and the prior report could be attributable to sampling issues or to differences in techniques, as the earlier data came from oligonucleotide expression arrays, although we measured Gli1 levels using quantitative RT-PCR. The relatively widespread activation of the Hh pathway in medulloblastoma suggested by our Gli1 expression data are consistent with the broad response to cyclopamine previously documented in newly resected human medulloblastoma.¹ Taken together, these data suggest that therapies targeting Hh may be effective in more than one subtype of medulloblastoma.

Apoptosis induced by Hh pathway blockade seems to be caused at least in part by changes in the expression of the anti-apoptotic protein *BclII*. We found that increased expression of the Hh targets Gli1 and Gli2 positively regulated *BclII* transcription, whereas pharmacological suppression of Hh activity resulted in decreased *BclII* expression and increased apoptosis. Our data provide a mechanism explaining the induction of apoptosis previously reported in murine medulloblastoma after Hh blockade,¹⁶ and extend the results to include human cell lines. Unlike previous observations in keratinocytes, we find that Gli1 is as potent as Gli2 in inducing *BclII* expression, suggesting that the effects of the various Gli factors may be somewhat tissue-dependent. The regulation of *BclII* by Gli1 also contrasts with the Patched-induced cell death reported in developing nonneoplastic neuroepithelial cells, in which Patched acts as a dependence receptor.^{42,43} In those experiments, cell death induced by the absence of SHH ligand did not seem to function via the canonical SMO/GLI transduction cascade. Our work does not exclude the possibility that Patched acts as a dependence receptor in tumors, but it does indicate that Gli1 also plays a role in regulating cell survival via *BclII*.

Our studies of primary tumor samples also support the concept that Gli factors regulate *BclII* expression and apoptosis in medulloblastoma. Gli and *BclII* proteins are often most highly expressed in the internodular regions of desmoplastic medulloblastoma. Interestingly, apoptosis is suppressed in these zones between nodules, perhaps attributable to the increase in *BclII* expression driven by Gli. In addition, we found that expression of Gli1 mRNA was significantly associated with that of *BclII* in a wide range of primary tumors, and 89% of cases with above-median Gli1 levels also had above-median *BclII*. Although the magnitude of Gli1 and *BclII* expression was not significantly correlated in the entire group, the fact that tumors with high Gli1 generally also had high *BclII* suggests that a certain threshold of Gli1 activity might drive *BclII* expression. A final result of potential clinical significance was the synergistic induction of cell death

we observed after Hh blockade and lovastatin exposure. This suggests that cyclopamine or other Hh inhibitors might sensitize medulloblastoma to the pro-apoptotic effects of radiation or other chemotherapeutic agents.

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