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In vivo inhibition of endogenous brain tumors through systemic interference of Hedgehog signaling in mice

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Abstract

The full spectrum of developmental potential includes normal as well as abnormal and disease states. We therefore subscribe to the idea that tumors derive from the operation of paradevelopmental programs that yield consistent and recognizable morphologies. Work in frogs and mice shows that Hedgehog (Hh)-Gli signaling controls stem cell lineages and that its deregulation leads to tumor formation. Moreover, human tumor cells require sustained Hh-Gli signaling for proliferation as cyclopamine, an alkaloid of the lily *Veratrum californicum* that blocks the Hh pathway, inhibits the growth of different tumor cells in vitro as well as in subcutaneous xenografts. However, the evidence that systemic treatment is an effective anti-cancer therapy is missing. Here we have used $Ptc1^{+/-}$; $p53^{-/-}$ mice which develop medulloblastoma to test the ability of cyclopamine to inhibit endogenous tumor growth in vivo after tumor initiation through intraperitoneal delivery, which avoids the brain damage associated with direct injection. We find that systemic cyclopamine administration improves the health of $Ptc1^{+/-}$;$p53^{-/-}$ animals. Analyses of the cerebella of cyclopamine-treated animals show a severe reduction in tumor size and a large decrease in the number of *Ptc1*-expressing cells, as a readout of cells with an active Hu-Gli pathway, as well as an impairment of their proliferative capacity, always in comparison with vehicle treated mice. Our data demonstrate that systemic treatment with cyclopamine inhibits tumor growth in the brain supporting its therapeutical value for human HH-dependent tumors. They also demonstrate that even the complete loss of the well-known tumor suppressor p53 does not render the tumor independent of Hh pathway function.

Keywords: Cancer; Tumor; Medulloblastoma; Brain; Mouse; Cyclopamine; Hedgehog; Gli

1. Introduction

Secreted glycoproteins of the Hedgehog (Hh) family participate in the development and homeostasis of many organs. Hh signal transduction in the target cells occurs through at least two membrane proteins, Smoothened (Smo) and Patched1 (Ptc1) (reviewed in Ingham and McMahon, 2001). In the absence of Hh, Ptc1 represses Smo. Hh inhibits Ptc1, allowing Smo to signal intracellularly and activate the Gli zinc-finger transcription factors (reviewed in Ruiz i Altaba et al., 2002a). One of the genes that is positively controlled by the Hh pathway is *Ptc1* itself, thus generating a negative feedback loop and serving as a convenient indicator of pathway activation (Goodrich et al., 1996). Inappropriate activation of the Hh pathway has been associated with familial cancers in Gorlin's syndrome (Johnson et al., 1996; Hahn et al., 1996) as well as sporadic human cancers in tissues in which Hh signaling normally plays a role during patterning, growth or homeostasis, like skin (Dahmane et al., 1997; Nilsson et al., 2000; Xie et al., 1998), lung (Watkins et al., 2003), pancreas (Thayer et al., 2003), stomach (Berman et al., 2003), prostate (Sanchez et al., 2004; Karhadkar et al., 2004) and brain (Dahmane et al., 2001; Berman et al., 2002) (reviewed in Ruiz i Altaba et al., 2002b, 2004; Pasca di Magliano and Hebrok, 2003). This has led to the search and isolation of molecules with therapeutic potential capable of modulating the Hh

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pathway (Taipale et al., 2000; Chen et al., 2002; Frank-Kamenetsky et al., 2002; Williams et al., 2003). Such molecules include cyclopamine (Keeler and Binns, 1968; Keeler, 1978), an alkaloid that inhibits Hh signaling (Incardona et al., 1998; Cooper et al., 1998) by impairing the activity of Smo (Taipale et al., 2000) as well as siRNAs against GLI1 (Sanchez et al., 2004). Cyclopamine appears to be as effective as other compounds targeting Smo (Taipale et al., 2000; Chen et al., 2002; Frank-Kamenetsky et al., 2002; Williams et al., 2003) and inhibits tumor cell proliferation in culture and in mouse skin xenografts following local subcutaneous injections (see below). However, it is not known if systemic treatment could be effective or beneficial for treating endogenous cancers as these could have acquired new mutations or changes that might alter their parade-velopmental programs (see Ruiz i Altaba et al., 2004). Here we have chosen to use cyclopamine as the in vivo use of the more promising anti-GLI RNAi technology (Sanchez et al., 2004) is not yet satisfactory. The finding that ewes that ate the plant *Veratrum californicum,* and thus ingested cyclopamine and related compounds, gave birth to cyclopic offspring but appeared normal is encouraging (Keeler and Binns, 1968; Bryden et al., 1971). Similar results have been obtained with purified cyclopamine in other species (Keeler, 1970; Sim et al., 1983) after oral administration (Keeler and Baker, 1989). Consistent with the specific action of cyclopamine as a Hh pathway blocker, these cyclopic phenotypes mimic that of loss of Shh function in mice (Chiang et al., 1996) and humans (Belloni et al., 1996; Roessler et al., 1996).

Here we have used the $Ptc1^{+/-}$; $p53^{-/-}$ mouse model of medulloblastoma (Goodrich et al., 1997; Wetmore et al., 2001) to test if systemic treatment by intraperitoneal injection of cyclodextrin-complexed cyclopamine (Van den Brink et al., 2001) is able to inhibit tumor growth once it has been initiated, to parallel the situation in human patients. We have also used a p53 mutant background to include the loss of a tumor suppressor that is unrelated to the Hh-Gli pathway, thus allowing us to test if additional changes make the behavior of the cells forming the tumor derived from loss of Ptc1 function independent of pathway activity. We demonstrate a clear reduction in tumor size as well as an inhibition of cell proliferation in the tumors in situ, demonstrating that systemic treatments with a Hh-Gli pathway blocker is beneficial as an anti-cancer strategy, showing that ptc mutant tumors having the additional loss of p53 remain dependent of Hh pathway activity, and providing a solid base for therapeutic trials.

2. Results

2.1. Cyclopamine inhibits Ptc1 expression and the proliferation of mouse medulloblastoma cells in vitro

Mice carrying a mutant allele of the *Ptc1* gene ($Ptc1^{+/-}$) often develop cerebellar tumors that closely resemble human medulloblastomas (Goodrich et al., 1997; Hahn et al., 1998), the frequency of which increases to ~100% in the absence of p53 ($Ptc1^{+/-}$; $p53^{-/-}$) (Wetmore et al., 2001). Consistently, we have observed that by 2 months of age, 95% of such mice, but not wild type or single *p53* mutant mice, display behavioral anomalies including circling, failure to regain an upright position when over-turned and posterior paralysis. These animals have large cerebellar tumors that engulf the majority of the cerebellum, present an amorphous appearance with a smooth surface and often produce superficial local edemas, not present in control siblings (Fig. 1a,b), and die shortly afterwards due to medulloblastoma formation.

In order to test the potential of cyclopamine as an effective anti-tumor agent, we first confirmed its reported ability to inhibit tumor cell growth in vitro (Dahmane et al., 2001; Berman et al., 2002). Medulloblastomas from $Ptc1^{+/-}$; $p53^{-/-}$ mice were dissected at ~7–8 weeks of age, dissociated and plated in vitro for primary culture. Dissociated cultures of normal cerebella from 2 month-old $p53^{-/-}$ animals were prepared as control but these did not yield any cell growth (not shown) indicating that growth in the medulloblastoma cultures reflects the presence of tumor cells. Analyses of BrdU incorporation in the presence of 10 μM cyclopamine demonstrated a reduction (between 50 and 80%; >1000 cells counted per sample; $n=4$ animals) in the proliferative capacity of tumor cells in the presence of cyclopamine as compared with carrier (ethanol)-only treated samples (Fig. 1c; each experiment was done in triplicate for each animal). In these mice, the *LacZ* gene disrupts the targeted *Ptc1* allele, reporting *Ptc1* transcription and thereby serving as a readout of pathway activity (Goodrich et al., 1997). All tumor cells were $LacZ^{+}$ (e.g. Fig. 1e). The reduction in BrdU incorporation after cyclopamine treatment correlated with an obvious decrease (>60%) in the number and intensity of staining of β-galactosidase positive cells (Fig. 1e,f), indicating that cyclopamine is effective in inhibiting the Hh pathway. In two tumors tested, cyclopamine also induced apoptosis as measured by increased levels of activated Caspase 3 staining (Fig. 1d; 1000 cells counted per sample).

2.2. Intraperitoneal injections of cyclopamine reduce medulloblastoma tumor size in vivo

In order to test the anti-tumor capacity of cyclopamine in vivo, a 1 month-old $Ptc1^{+/-}$;$p53^{-/-}$ mouse cohort was treated systemically with the drug or with the vehicle alone every other day. We have previously shown such in vivo treatment decreases Shh pathway activity in the brain as measured by a decrease in endogenous *Gli1* mRNA levels (Palma and Ruiz i Altaba, 2004; V. Palma et al., submitted). Injections were begun at 1 month of age, a time when the tumors are already detectable and the animals do not yet display any behavioral anomalies or signs of disease. In this way, we could test for possible beneficial effects on an

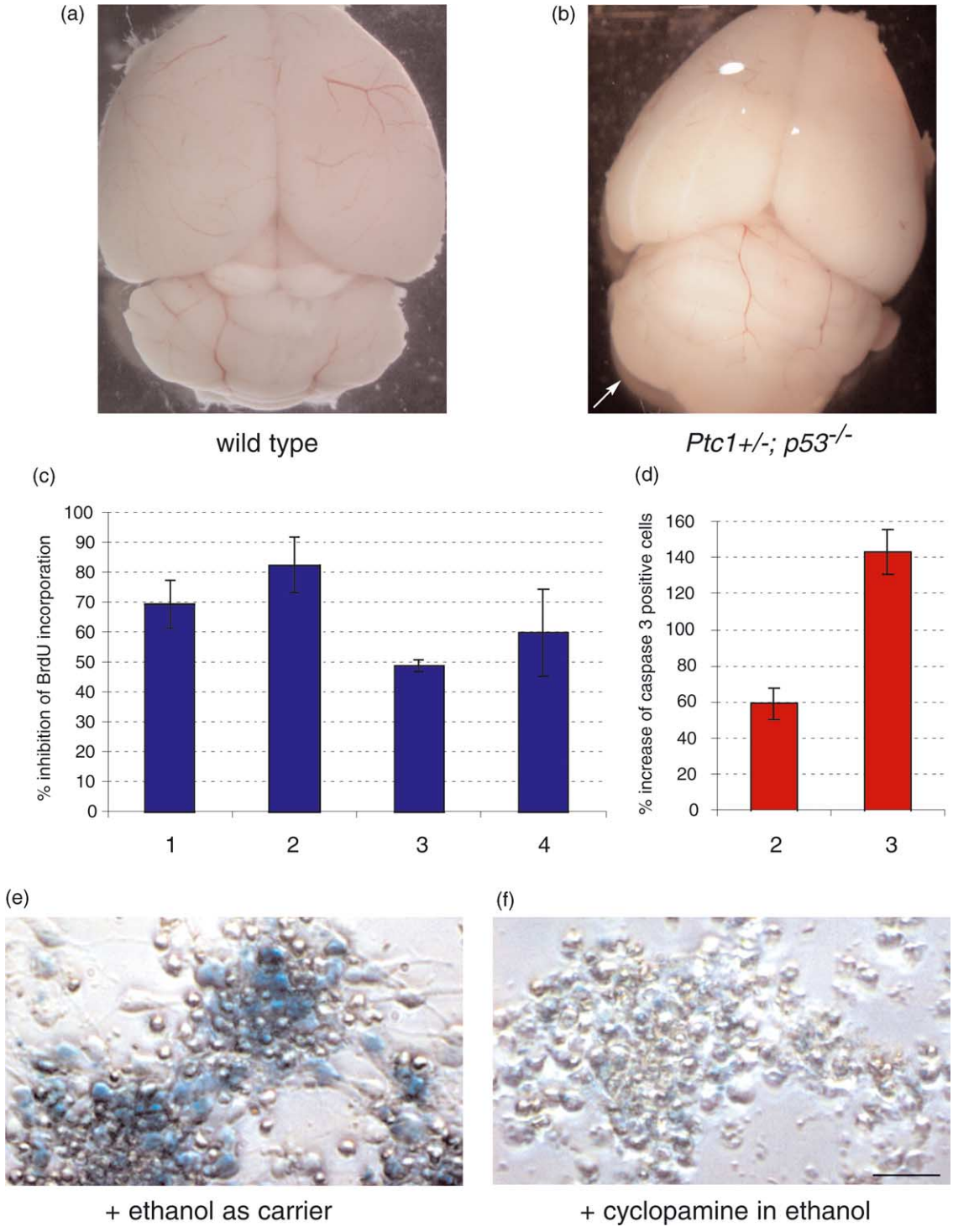

Fig. 1. Cyclopamine inhibits proliferation and survival of mouse medulloblastoma cells in vitro. (a,b) Dorsal view of a wild type (a) or a $Ptc1^{+/-}$; $p53^{-/-}$ (b) mouse brains at 2 months of age. Note the large cerebellar tumor in the mutant (arrow, b). (c) Histograms of the inhibition of the proliferation of tumor cells in vitro by cyclopamine as assessed by BrdU incorporation, presented as percentage over carrier (ethanol)-treated samples. The cells were obtained from four different mouse medulloblastomas of $Ptc1^{+/-}$; $p53^{-/-}$ mice. (d) Histogram of the increase in Caspase 3 immunolabeling, as a measure of apoptosis, in medulloblastoma cells obtained from two different mice, after treatment with cyclopamine for 48 h. The percentages denote increases over carrier-treated samples. (e,f) Example of x-gal staining revealing *Ptc1/LacZ* expression in primary mouse medulloblastoma cells treated with carrier only (e) or cyclopamine (f) for 48 h. Note that most tumor cells express the *Ptc1–LacZ* gene and that cyclopamine decreases its expression, indicating the effective inhibition of Hh pathway activity. Scale bar = 15 μm for (e,f). All the changes are significative ($P < 0.05$, Student's *t*-test). All error bars represent SEM.

already initiated tumor in an otherwise intact brain, paralleling the human condition. After 1 month of treatment, at 2 months of age, we observed the typical medulloblastoma-associated abnormalities in carrier treated animals, including a decrease in overall activity, rough hair, decrease feeding, circling and the inability to gain an upright position when overturned, as well as signs of abnormal occipital prominences in the head. In contrast, cyclopamine-treated animals presented a near normal behavior at this time. Minor anomalies in gait and posture were seen in one animal resembling those of untreated cohorts animals with early medulloblastoma at ~1.3 months of age (not shown). This mouse had the largest tumor of the treated cohort (with an increase in ~23% of cerebellar volume over control; Fig. 3). This indicates that cyclopamine treatment is beneficial and that it is not toxic at the doses used even though it is predicted to affect other Hh-related events including, for example, the renewal of the gastric mucosa (Van den Brink et al., 2001). Animals were then sacrificed and their brains collected. The tumors of carrier-treated mice were apparent on gross examination (Fig. 2a), were prominent in sagittally bisected brains (Fig. 2d) and were similar to uninjected *Ptc1*$^{+/-}$; *p53*$^{-/-}$ siblings of the same age (not shown). The brains of *Ptc1*$^{+/-}$ control mice were normal or had minor signs of tumor (Fig. 2c). In contrast, cyclopamine-injected *Ptc1*$^{+/-}$; *p53*$^{-/-}$ siblings had much smaller tumors and the foliation of the cerebellum was visible (Fig. 2b,e). The rest of the brain of cyclopamine- and carrier-injected animals, as well as the body appeared normal after a general pathological examination of all tissues and viscera (not shown). Similarly, wild type animals treated systemically with cyclopamine also did not show any gross pathological defects (Palma and Ruiz i Altaba, 2004). The present expense of cyclopamine and the length of time required to obtain *Ptc1*$^{+/-}$; *p53*$^{-/-}$ cohorts made experiments with higher doses and longer treatments impractical. Histological analyses showed that the expression of β-galactosidase from the *LacZ* targeted *Ptc1* allele (Goodrich et al., 1997) was active in all tumors (Fig. 2f–h). In carrier treated animals the tumors invaded almost all the cerebellum (Fig. 2g), whereas in cyclopamine-treated mice the number of *lacZ* positive cells was severely reduced (Fig. 2h) indicating that the systemic injections of cyclopamine are able to inhibit the Hh pathway in the mice cerebella and that this inhibition is sufficient to curtail medulloblastoma growth.

To quantify the anti-tumor activity of cyclopamine in vivo we measured the cerebellar area of one sagittal section every 100 μm for each animal and added the results for the whole cerebellum per animal to obtain a 'volume' value. *Ptc1*$^{+/-}$; *p53*$^{-/-}$ mice showed an enhanced volume as compared to cerebella of sibling control animals due to the presence of medulloblastomas (Fig. 3a). No intracerebellar edemas or hemorrhages were detected upon sectioning (not shown). The size of the cerebellum of *Ptc1*$^{+/-}$;*p53*$^{-/-}$ animals was as large as 175% that of normal siblings, whereas the size of the cerebella of cyclopamine-treated animals showed only a 10–20% increase, indicating an impairment of tumor growth by cyclopamine (Fig. 3a). The presence of remaining tumor cells in cyclopamine-treated brains that express β-gal (Fig. 2f) suggest that the doses used here inhibit tumor growth but do not shut down the Hh pathway completely as the normal endogenous expression of *Ptch1–LacZ* in the cerebellum is still present (Fig. 2).

2.3. Cyclopamine-treatment inhibits tumor cell proliferation in vivo

A decrease in tumor size could, in principle, result from a decrease in cell proliferation and/or an increase in cell death. Analyses of cell proliferation through phospho-Histone H3 immunolabeling, as a measure of cells in S phase, in cerebellar sections showed a reduction in cyclopamine versus carrier-treated samples after 1 month of treatment (Fig. 3b,c). In contrast, we were unable to detect a significant difference in the level of apoptosis between the two groups of animals as determined by Caspase 3 staining (11.8 ± 2.2 positive cells per field (20 fields per brain) in HBC treated, versus 13.8 ± 4.8 in cyclopamine-treated mice ($P = 0.7$).

3. Discussion

Solid cancers may be diseases of stem cell lineages in which paradevelopmental programs dictate the organization of tumoral tissues in attempts to recapitulate normal organogenesis (Ruiz i Altaba et al., 2004). In this case, there may be cancer stem cells that are responsible for the continued persistence of cancers and their recurrence after intervention, much as in the case of leukemias (discussed in Reya et al., 2001). In the brain, the possibility that brain tumors contain cancer-stem cells is supported by assays of self-renewal in vitro (Ignatova et al., 2002; Singh et al., 2003; Hemmati et al., 2003; P.S. and A.R.A., submitted). Moreover, we and others have shown that Hh-Gli signaling is required for normal precursor and stem cell behavior in the mouse brain (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Wallace, 1999; Dahmane et al., 2001; Lai et al., 2003; Machold et al., 2003; Palma and Ruiz i Altaba, 2004). Taken together, these findings suggest that Hh-Gli signaling blockade may be effective to combat cancer in vivo. Indeed, established cell lines, xenografts and primary culture assays strongly support this idea. However, in vivo data with endogenous tumors has been lacking. Here we demonstrate for the first time that anti-cancer therapy of a whole live animal with a Hh pathway inhibitor is effective and beneficial.

The tumor we have chosen to test derives from the loss of Ptc1 function (perhaps through loss of heterozygosity or epigenetic silencing events (Berman et al., 2002), which

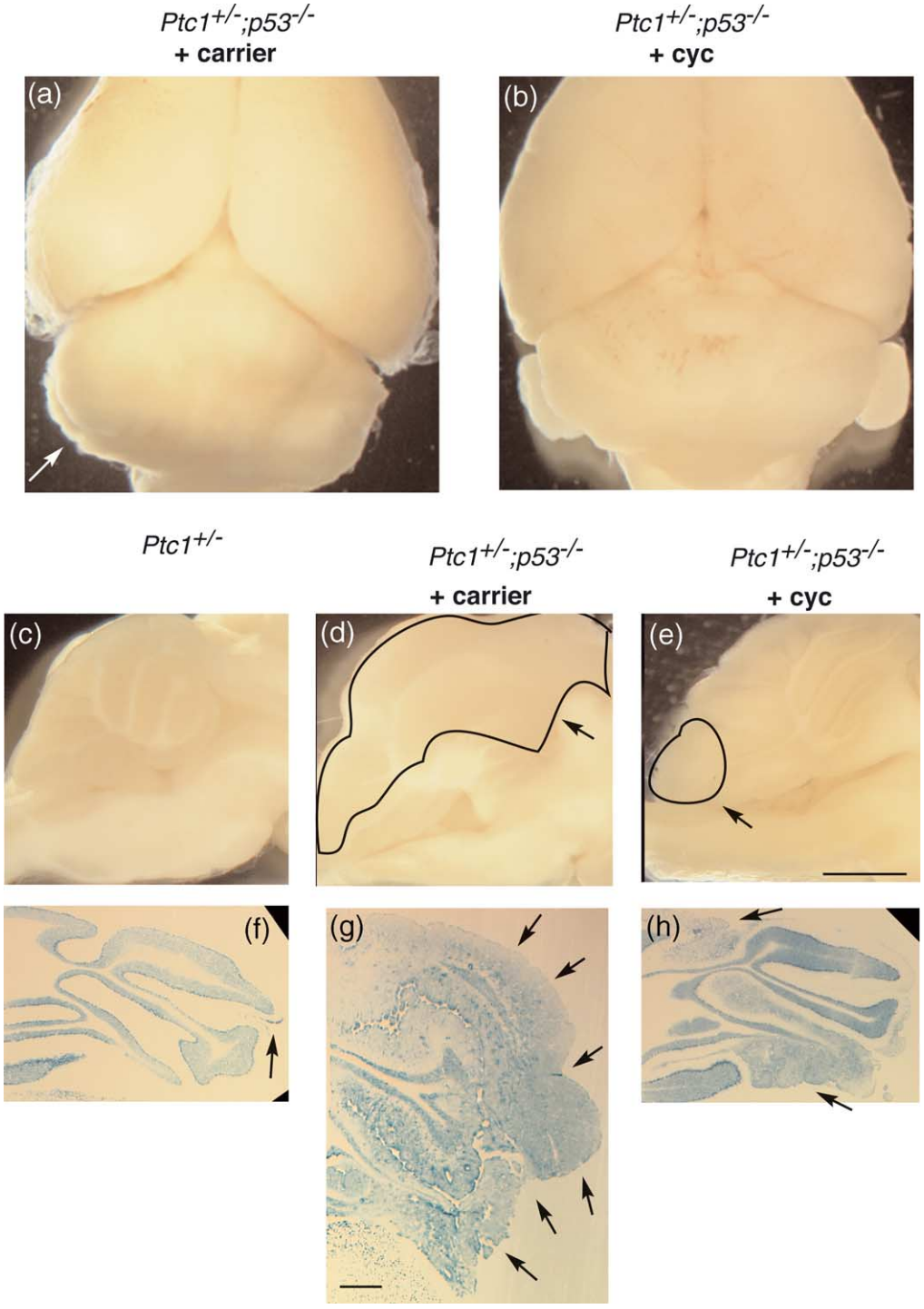

Fig. 2. Systemic cyclopamine treatment decreases tumor size in *Ptc1*$^{+/-}$; *p53*$^{-/-}$ mice. (a,b) Example of the dorsal view of *Ptc1*$^{+/-}$; *p53*$^{-/-}$ mouse brains treated for a month with cyclodextrin alone as carrier (a) or cyclodextrin-associated cyclopamine (b). The cerebellum of the cyclopamine-treated mouse appears almost normal (b) compared to the carrier treated in which a big tumor has deformed the normal foliation of the cerebellum (arrow, (a)). (c–e) Midline view of bisected cerebella of *Ptc1*$^{+/-}$ (c) or *Ptc1*$^{+/-}$; *p53*$^{-/-}$ mice treated with cyclodextrin alone (d) or cyclodextrin-associated cyclopamine (e). Foliation patterns are apparent in (c) and (e) only. Approximate tumor boundaries are denoted by a black line (arrows). (f)–(h) Cross-sections of cerebella shown in upper panels (c)–(e), respectively) after x-gal staining revealing *Ptc1/LacZ* expression. All tumors and normal granule cells express β-gal. Tumor areas are denoted by arrows. Scale bar = 150 μm for (c)–(e), 40 μm for (f)–(h).

translates into the activation of the Hh pathway at the level of the receptor complex (Goodrich et al., 1997). In addition, the complete homozygous loss of the p53 tumor suppressor gene accelerates tumor formation and the penetrance of the tumorigenic phenotype to ∼100% (Wetmore et al., 2001). Inhibition of such Ptc1/p53 mutant tumors by blockade of the Hh pathway indicates that in tumors with at least two hits, as many others are likely to accumulate in

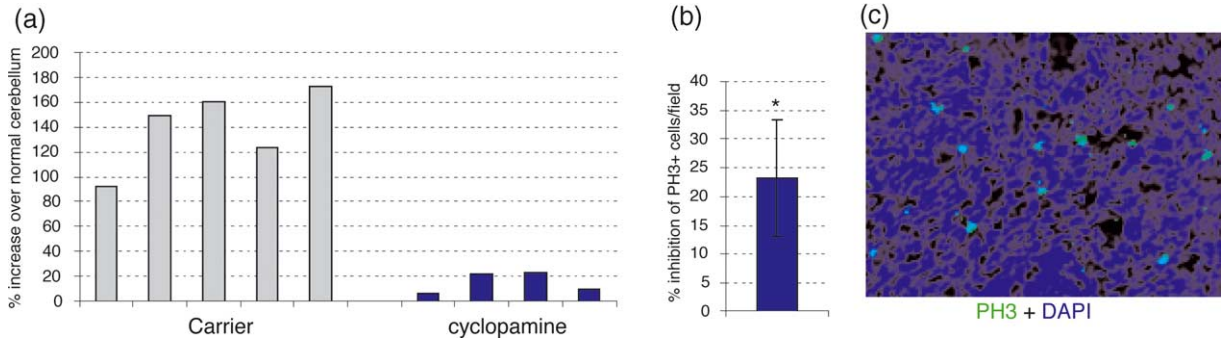

Fig. 3. Quantification of the effects of cyclopamine in vivo and effects on cell proliferation. (a) Quantification of cerebellar 'volume' in carrier and cyclopamine-injected $Ptch^{+/-};p53^{-/-}$ mice as percentage of the normal weight of the cerebellum of wild type siblings. (b) Inhibition of proliferation as measured by phospho-Histone 3 expression in tumors by cyclopamine treatment in vivo, as those shown in (Fig. 2b,e,h), over carrier-treated controls (Fig. 2a,d,g). The increase is significant ($P<0.05$). The relatively large error bar reflects differences in proliferation observed in different tumor areas. >10 Sections were counted in two representative animals of each condition. (c) High-power photomicrograph of phospho-Histone H3 (PH3) immunolabeling of a section of medulloblastoma showing a number of positive nuclei (green). All nuclei are counterstained with the DNA dye DAPI (blue). Asterisks in the histograms in this and all figures denote significative changes ($P<0.05$, Student's *t*-test).

the cells of tumor mass, cancer growth still requires sustained Hh signaling.

Sustained Hh-Gli signaling is involved in many different types of sporadic human tumors (Ruiz i Altaba et al., 2002b, 2004; Pasca di Magliano and Hebrok, 2003). These include those that can cause fulminant death such as gliomas (Dahmane et al., 2001; P.S. and A.R.A., submitted), medulloblastomas (Dahmane et al., 2001; Berman et al., 2002), pancreatic adenocarcinomas (Thayer et al., 2003), and prostate cancers, which are lethal in advanced stages and represent the most common tumor in men (Sanchez et al., 2004; Karhadkar et al., 2004), as well as the most common cancer type, sporadic basal cell carcinomas of the skin (Dahmane et al., 1997; Williams et al., 2003). Thus, the search for effective inhibitors of Hh signalling is a priority. Indeed, considerable effort is being devoted to the search and study of Hh pathway inhibitors that may be effective anti-cancer agents. Cyclopamine (Keeler and Binns, 1968; Keeler, 1978) is the first small molecule HH pathway blocker (Incardona et al., 1998; Cooper et al., 1998; Taipale et al., 2000) and it has proved efficient in inhibiting the proliferation of a variety of human cancer cells in vitro (e.g. Dahmane et al., 2001; Berman et al., 2002, 2003; Thayer et al., 2003; Watkins et al., 2003; Sanchez et al., 2004). It has also been used to inhibit the growth of mouse subcutaneous skin xenografts of medulloblastomas (Berman et al., 2002) and digestive tract and pancreatic human cancers (Thayer et al., 2003; Berman et al., 2003) after local subcutaneous injections. Our data provide a critical extension of these findings and show that systemic cyclopamine treatment inhibits the growth of endogenous Hh-dependent tumors. Thus, our results suggest that systemic or localized in vivo blockade of HH signaling will be also effective to combat many or all HH-related human cancers including those from the prostate, pancreas, lung, skin and brain.

The absence of significant increase in apoptosis in the cyclopamine-treated tumors in vivo that we have examined contrasts with the increase of activated Caspase 3 staining seen in vitro. This difference could be due to the action of different effective doses in the two scenarios. Indeed, apoptosis driven by Hh pathway blockade is often more evident in vitro after a prolonged period of treatment or at high doses (data not shown). It remains possible that the levels of inhibitors that reach the brain in our present protocol are not sufficient to induce apoptosis in tumor cells. This may be consistent with the detection of apoptosis by TUNEL assays in pancreatic tumor xenografts following subcutaneous cyclopamine delivery (Thayer et al., 2003) where the effective concentrations of the drug are likely to be much higher. Additional pharmacokinetic studies will be required to determine the amounts of cyclopamine, or of potential active byproducts, that reach the target cells in vivo, as well as the maximal tolerable doses. Notwithstandingly, we show that systemic cyclopamine treatment affects the brain, with cyclopamine either crossing the blood/brain barrier or being effective in tumors that may have a broken barrier.

More generally, our data indicate that understanding normal embryonic development and organ function, including research into stem cells, is critical to understand diseases that may derive from mispatterning events such as cancer. Indeed, developmental biology suggest that cancers are cell group, tissue or organ diseases, in contrast to the view derived from cell biology which focused on cell intrinsic events such as the cell cycle. The present results thus urge the further exploration and definition of paradevelopmental programs that dictate tumor morphogenesis, as a parallel effort with the study of normal ontogeny, in order to have a full view of developmental potential. The data also beg for the initiation of clinical trials with Hh-Gli inhibitors for lethal cancers.

4. Experimental procedures

4.1. Animals

Ptc1$^{+/-}$ mice were generated and maintained on a mixed C57B1/6/J background and crossed with mice carrying a targeted disruption in *p53* (Jackson Laboratories, Bar Harbor, ME) in the same background. Given the embryonic lethality of homozygote *Ptc1*$^{-/-}$ mice and the small litter size, the generation of a 9 sibling *Pct1*$^{+/-}$; *p53*$^{-/-}$ cohort took ~15 months. At 2 months of age, control- and cyclopamine-treated animals were deeply anesthetized and brains fixed with 4% paraformaldehyde, removed and the presence of tumor confirmed by gross examination. Brains were then embedded in freezing medium (OCT), sectioned at 12 μm on a cryostat and processed for inmunohistochemistry or Xgal staining. For cell culture, the animals were euthanized and the cerebellum dissected and its cells dissociated (Dahmane and Ruiz i Altaba, 1999; Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004).

4.2. Cell culture

Cells dissociated from dissected mouse medulloblastomas (taking only the core of the tumor mass) were plated at 40,000 cells/well onto polyornythine/laminin coated Lab-Tek chambers slides in Neurobasal Medium (see Palma and Ruiz i Altaba, 2004). Two days after dissociation, cells at 70% confluence were treated with 10 μM cyclopamine or carrier for 48 h. BrdU was added at 3 μM 16 h before fixation.

4.3. Cyclopamine treatment

Cyclopamine (Toronto Research Chemicals; ~760 $/10 mg) and dissolved in ethanol for a stock at 10 mM for cell culture experiments or in a solution of 45% of 2-hydropropyl-β-cyclodextrin (HBC, Sigma) in PBS at 1 mg/ml by incubating 1 h at 65° for mouse injections (Van den Brink et al., 2001). Aliquots were stored at −20° until use. Animals were first injected at 1 month of age, when they are still asymptomatic, and on every other day afterwards, with 10 mg/kg of HBC–cyclopamine or the carrier alone, intraperitoneally (IP).

4.4. Inmunohistochemistry

The following antibodies were used: monoclonal anti-BrdU (Becton-Dickinson), polyclonal anti-phospho-Histone3 (UPSTATE biotechnology), polyclonal anti-caspase3 activated (Cell Signaling), and fluorescein-conjugated secondary antibodies (Boehringer Mannheim). All probability values for the quantification of results were obtained using the Student's *t*-test.

4.5. X-gal staining

Cerebellar sections were fixed in 2% paraformaldehide, 0.2% glutaraldehide, washed twice in phospate buffer with deoxycolate and NP40, and incubated in the same buffer containing 1 mg/ml of X-Gal. Reactions were stopped after 3 h incubation at 37°.

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