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Tumorigenesis and Neoplastic Progression

Constitutive Hedgehog Signaling in Chondrosarcoma Up-Regulates Tumor Cell Proliferation

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Chondrosarcoma is a malignant cartilage tumor that may arise from benign precursor lesions, such as enchondromas. Some cases of multiple enchondromas are caused by a mutation that results in constitutive activation of Hedgehog-mediated signaling. We found that chondrosarcomas expressed high levels of the Hedgehog target genes *PTCH1* and *GLI1*. Treatment with parathyroid hormone-related protein down-regulated Indian Hedgehog (*IHH*) expression in normal growth plates but not in chondrosarcoma or enchondroma organ cultures. Treatment of the chondrosarcoma organ cultures with Hedgehog protein increased cell proliferation rate, whereas addition of chemical inhibitors of Hedgehog signaling decreased the proliferation rate. Chondrosarcoma xenografts from 12 different human tumors were established in NOD-SCID mice. Treatment with triparanol, an inhibitor of Hedgehog signaling, resulted in a 60% decrease in tumor volume, a 30% decrease in cellularity, and a 20% reduction in proliferation rate. These results show that Hedgehog signaling is active in chondrosarcoma and benign cartilage tumors and regulates tumor cell proliferation. Our data raise the intriguing possibility that Hedgehog blockade could serve as an effective treatment for chondrosarcoma, a tumor for which there are currently no universally effective nonsurgical management options. ***(Am J Pathol 2006, 168:321–330; DOI: 10.2353/ajpath.2006.050001)***

Cartilaginous tumors are the most common neoplasms effecting bone.[1,2] They range from benign lesions, such as enchondromas and osteochondromas, to malignant chondrosarcomas. The benign lesions cause disability because of pain, limb deformity, and pathological fracture. Enchondromas and osteochondromas have a potential for malignant change, greater than 50% in some cases of multiple enchondromatosis (ie, Maffucci syndrome).[3] Chondrosarcomas can be difficult to treat as neither chemotherapy nor radiotherapy is typically effective. Radical surgery is the mainstay of treatment.[4]

The growth plate, located between the cartilaginous epiphysis and the newly generated bone in the metaphysis, is responsible for longitudinal bone growth. Within the growth plate, chondrocytes proliferate and then proceed through an orderly differentiation process eventually resulting in programmed cell death. Indian Hedgehog (IHH) and parathyroid hormone-related protein (PTHrP) are part of a negative feedback loop that tightly regulates the fate of growth plate chondrocytes.

Prehypertrophic and hypertrophic chondrocytes express IHH, which stimulates growth plate chondrocytes to proliferate. Hedgehog (HH) activates GLI-mediated transcription through the transmembrane proteins patched (PTCH) and smoothened (SMO). GLI transcription factors contain activator and repressor domains, and the cleavage and removal of the activation domain results in transcriptional repression. GLI-mediated transcriptional activation results in the up-regulation of target

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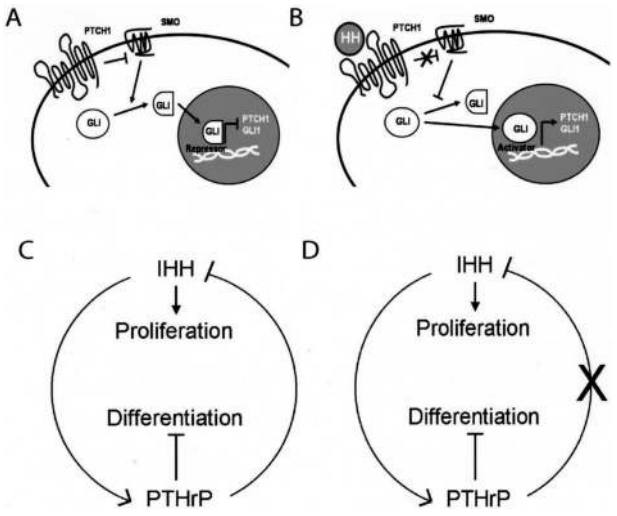

Figure 1. The Hedgehog signaling cascade and negative feedback loop with PTHrP. **A:** In the absence of Hedgehog ligand, the receptor Patched-one (PTCH1) inhibits the transmembrane protein smoothened (SMO). This inhibition maintains the Hedgehog-mediated GLI transcription factors (GLI1, GLI2, and GLI3) as repressor forms associated with cleavage of the transcriptional activating domain. SMO inhibition is associated with repressor forms of the GLI transcription factors entering the nucleus. The repressor forms of the GLI transcription factors act to inhibit expression of Hedgehog target genes, such as PTCH1 and GLI1. **B:** In the presence of Hedgehog ligand, PTCH1 no longer inhibits SMO, the GLI transcription factors are present as full-length forms that enter the nucleus activating transcription. This results in expression of the target genes *PTCH1* and *GLI1*. The up-regulation by Hedgehog ligand of components of its own signaling cascade is thought to act as a feedback loop to regulate the level of activation. **C:** Normal IHH-PTHrP signaling in the growth plate. Expression of IHH by prehypertrophic chondrocytes leads to proliferation of growth plate chondrocytes, and up-regulation of PTHrP that inhibits chondrocyte differentiation and down-regulates IHH, thus completing the negative feedback loop. **D:** Aberrant IHH-PTHrP signaling in cartilage neoplasia. The ability of PTHrP to inhibit IHH expression is lost leading to constitutive Hedgehog signaling.

genes including the transmembrane protein PTCH1 and the transcription factor GLI1. During bone development, IHH also stimulates the expression of PTHrP, which inhibits chondrocyte differentiation by preventing proliferating chondrocytes from entering the prehypertrophic stage. PTHrP additionally inhibits IHH expression to complete the negative feedback loop (Figure 1). Thus, IHH and PTHrP signaling and feedback pathways regulate the rate of chondrocyte proliferation, differentiation, as well as normal longitudinal bone development.[5–13]

The IHH-PTHrP signaling pathway is implicated in the pathogenesis of many types of benign cartilage tumors. Enchondromas, osteochondromas and chondroblastomas all express PTHrP and its receptor PTHR1.[14–17] PTHrP expression levels correlate with the proliferative rate of cartilage tumors.[16,17] We previously identified a *PTHR1* mutation (R150C *PTHR1*) that leads to excessive Hedgehog signaling in multiple enchondromatosis.[18] In addition, mice overexpressing either R150C *PTHR1* or the Hedgehog activated transcription factor Gli-2 in the growth plate developed a phenotype similar to enchondromatosis, providing an *in vivo* model supporting the role of constitutive Hedgehog signaling in the pathogenesis of enchondromatosis.[18] Similar to enchondromatosis, osteochondromas also exhibit defective Hedgehog signaling. Mutations in the EXT genes, which regulate Hedgehog diffusion, are frequently associated with osteochondromas.[19]

Holoprosencephaly, a disorder in which there is malformation of the central portions of the brain and head, can be caused by mutations in Sonic Hedgehog (*SHH*). A variety of teratogens also cause this disorder.[20,21] Cell culture studies have verified that many of the compounds that cause holoprosencephaly also block HH signaling. These compounds include *Veratrum* alkaloids (eg, cyclopamine) and inhibitors of 7-dehydrocholesterol reductase (eg, triparanol), an enzyme in the distal cholesterol synthesis pathway.

In this study we investigated IHH-PTHrP signaling in cartilage tumors. Although previous studies examined PTHrP signals in cartilage neoplasia, the role of HH signals has received little attention. Here we show that HH signals are active in chondrosarcoma and benign cartilage tumors and that feedback inhibition of PTHrP on IHH expression is absent. Blockade of HH signaling in chondrosarcoma explants and xenografts attenuated the neoplastic phenotype by decreasing tumor cell proliferation, tumor cellularity, and tumor volume. Targeting HH signaling may be an effective chemotherapeutic strategy for patients with chondrosarcoma.

Materials and Methods

Tissue Specimens

Tissues were obtained from the Sarcoma Tumor Banks at Mount Sinai Hospital and the Hospital for Sick Children, as well as the Mount Sinai Hospital Bone Bank. Consent was obtained for each specimen according to each institution's policies. Frozen specimens were processed immediately after surgical excision, cryopreserved, and stored at −70°C. Samples used for explant organ cultures and xenografts were obtained directly from the operating room. Sufficient case material for all studies was not available from each tumor.

Mutational Analysis

Single-strand conformation polymorphism analysis was used to screen for mutations in the *PTHR1* gene. Genomic DNA was extracted from specimens using Qiagen reagents (QIAamp DNA Mini kit; Mississauga, ON, Canada) and used as template for polymerase chain reaction (PCR) amplification of fragments containing an exon and its adjacent intron boundaries. The ^{33}P-ATP incorporated PCR product was denatured and electrophoresed on a native polyacrylamide gel containing 10% glycerol. Band shifts were further analyzed by sequencing (Thermo Sequenase sequencing kit; Amersham Life Science, Cleveland, OH). DNA samples from cases of enchondromatosis known to harbor the R150C variant were used as a positive control.[18]

Expression of IHH/PTHrP *Pathway Members*

Total RNA was extracted from ~50 mg of each specimen (Trizol Reagent; Life Technologies, Inc., Grand Island, NY) and examined for expression of select genes using semiquantitative reverse-transcriptase (RT)-PCR. Two hundred ng of RNA template were reverse-transcribed into cDNA (Perkin-Elmer/Roche, Branchburg, NJ). One μl of cDNA from the above RT reaction was used for each PCR reaction. Each primer pair was designed to flank at least one intron, to prevent and distinguish amplification of contaminating genomic DNA. Genes of interest were matched to internal control genes that had similar PCR kinetics, and both were analyzed over a range of cycles to ensure the result was obtained from the logarithmic phase of PCR amplification to allow direct comparison of gene expression levels.[22,23] PCR products were electrophoresed on agarose gels, stained with ethidium bromide, and photographed for densitometry (ImageQuant Software v3.0; Molecular Dynamics, Sunnyvale, CA). Relative levels of band intensity of the gene of interest were compared to the internal control gene within the linear range of amplification and normalized to a positive control sample on each gel. Appropriate negative controls were included in each RT-PCR experiment. Each sample was analyzed in triplicate over at least three different numbers of cycles.

Quantitative Assessment of IHH, PTCH, *and* GLI1 *Expression by Real-Time RT-PCR*

Gene-specific TaqMan Assay-on-Demand (PE/Applied Biosystems, Foster City, CA) was used, consisting of a fluorogenic probe and a pair of oligonucleotides. We performed standard quantitative RT-PCR reactions for IHH, PTCH1, and GLI1 on the ABI Prism 7900HT (Applied Biosystems, Foster City, CA) sequence detection system. Asparagine synthetase (*AS*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as internal control genes. The reactions were performed in duplicate in a 20-μl reaction volume using TaqMan Universal PCR Master Mix (ABI) on a 96-well plate format. We used the absolute standard curve method to determine the copy number of the transcript of interest by relating the PCR signal to a standard curve that was first constructed from RNA of known concentration. This curve was then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. cDNA from colon cell lines SW1417 and RW948 were used as reference standards for IHH and PTCH1, respectively. cDNA from a pool of 11 tumor cell lines was used as a reference standard for GLI1.

Explant Cultures

Primary tissue specimens were cut into 2-mm-thick sections and maintained in Dulbecco's modified Eagle's medium with high glucose, as previously described for 4 days.[24] Cultures were treated with either 0.1% fetal bovine serum (agonist control), 5 μg/ml Ihh-N (R&D Systems, Minneapolis, MN), 10^{-7} mol/L PTHrP (Bachem, King of Prussia, PA), 10^{-4} mol/L cyclopamine (a gift from William Gaffield, U.S. Department of Agriculture, Albany, CA), 10^{-4} mol/L tomatidine (Sigma, St. Louis, MO), or 10 μg/ml of neutralizing 5E1 anti-SHH monoclonal antibody (Hybridoma Bank, University of Iowa, Iowa City, IA). Proliferation was measured using thymidine incorporation and normalized to the total number of cells. Explant specimens were incubated with 10 μCi/ml tritiated (^{3}H-T) thymidine for the final 20 hours of culture, then digested with 0.5% pronase (Boehringer Mannheim, Laval, Quebec, Canada) for 1 hour, followed by 0.125% collagenase A (Boehringer Mannheim) for 8 hours. Cells were counted and resuspended in 0.5 ml of 1% sodium dodecyl sulfate/0.005 mol/L ethylenediamine tetraacetic acid. Nucleic acids were precipitated with 1 ml of ice-cold 25% trichloroacetic acid and pipetted onto 2.4-cm glass microfiber filters (Whatman GF/C; Whatman, Maidstone, UK) over a suction apparatus. The filters were washed twice with 2 ml of 5% trichloroacetic acid, air-dried, and placed in scintillation vials with 5 ml of scintillation cocktail, and counts per minute averaged over 5 minutes. Semiquantitative RT-PCR was used to measure expression of type X collagen (COLX), a marker of terminally differentiated growth plate chondrocytes, relative to β_2-microglobulin (β2M), an internal control gene.

Xenograft Model

Twelve human chondrosarcoma tumor samples were obtained fresh from the operating room after surgical removal, divided into 120 explants each 5 mm $\times$ 5 mm $\times$ 5 mm, and xenografted into the subcutaneous tissues on the back of NOD-SCID mice. By gavage, one group of mice was treated with triparanol, a Hedgehog-blocking agent, three times a week at a dose of 400 mg/kg. The control mice were treated with the carrier, olive oil. Both groups of mice were sacrificed after 15 weeks of treatment. The amplex red cholesterol assay (Molecular Probes, Burlington, Ontario, Canada) was used to measure serum cholesterol to determine whether the mice were able to absorb triparanol.[25,26] To determine whether treatment with triparanol blocked HH signaling *in vivo*, the level of expression of two pathway target genes, *Gli-1* and *Ptch*, was measured in the ends of the long bones by RT-PCR. After sacrifice and removal of the xenograft, tumor volume was calculated using three-dimensional measurements as previously described.[27] Tumors were placed in 4% paraformaldehyde, and sections were stained with hematoxylin and eosin. For 12 tumor explants, the number of nuclei in 10 high-power fields of views was counted.

Immunohistochemical Studies

Immunohistochemistry was performed using antibodies against the N-terminus of PTCH (G-19; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were blocked for endogenous peroxidase, followed by pepsin treatment and incubation with primary antibody at 4°C overnight.

Table 1. Expression of IHH/PTHrP Members in Cartilage Neoplasia and Normal Tissues

	IHH	PTCH1	GLI1	GLI2	GLI3	PTHrP	PTHR1
Growth plate	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Articular cartilage	0/4	4/4	2/4	2/4	4/4	2/4	4/4
Cortical bone	2/11	11/11	11/11	11/11	11/11	11/11	11/11
Osteochondroma	0/4	4/4	4/4	4/4	4/4	4/4	4/4
Chondroblastoma	2/7	7/7	7/7	7/7	7/7	2/7	7/7
Enchondroma	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Chondrosarcoma	23/23	23/23	23/23	23/23	23/23	23/23	23/23

The fraction of each specimen type for which transcripts were detectable by RT-PCR is listed. Enchondromas and chondrosarcomas all expressed each of the factors (except for Sonic Hedgehog, which was not expressed in any of the tumors). IHH, *Indian hedgehog*; PTCH1, patched-one; PTHrP, parathyroid hormone-related protein; PTHR1, type 1 PTH/PTHrP receptor.

Primary antibody was detected using a 3,3′-diaminobenzidine color substrate immunostaining kit (Vector Laboratories, Burlingame, CA). IgG antibody was used instead of the anti-PTCH antibody as a negative control.

Mice were treated intravenously with BrdU 2 hours before sacrifice for assessment of xenograft proliferation. Immunohistochemical detection of BrdU using anti-BrdU antibodies was performed (Roche Applied Science, Laval, Quebec, Canada). Sections were counterstained with Light Green. For each section 10 high-power fields of view were analyzed. The number of BrdU-positive cells was divided by the total number of cells to determine the percent proliferation.

DNA damaged cells were detected using the terminal dUTP nick-end labeling (TUNEL) assay for *in situ* end-labeling. Recombinant terminal deoxynucleotidyl transferase (Tdt) (Life Technologies, Inc., Gaithersburg, MD) was used to detect 3′ ends of cleaved DNA. Biotin 16-dUTP (Roche Diagnostics, Laval, Quebec, Canada), avidin-horseradish peroxidase, and 3,3′-diaminobenzidine detection method was performed. The slides were counterstained with hematoxylin. For each section 10 fields of view were analyzed. The number of TUNEL-positive cells was divided by the total number of cells to determine the percentage of apoptosis. Because each tumor provided multiple explants, statistical analysis was based on the number of patient samples (ie, $n = 12$) and used to compare tumor volume, cellularity, proliferation, and apoptosis rate between triparanol-treated and control explants. Data in plots are presented as mean values with 95% confidence intervals. The P values were calculated using the two-tailed t-test.

Results

IHH *and* PTHrP *Pathway Members Are Expressed in Cartilage Tumors*

We examined the expression of key members of the IHH/PTHrP signaling pathway using RT-PCR analysis for samples in which sufficient high-quality RNA was available. IHH and PTHrP were both expressed in all 4 solitary enchondromas and all 23 chondrosarcoma specimens, although the levels were variable (Table 1). IHH was not expressed in any of the four osteochondromas and in only two of seven cases of chondroblastoma. PTHR1, the IHH receptor PTCH, and the Hedgehog responsive transcription factors[25] GLI1, GLI2, and GLI3 were consistently expressed in all tumors. SHH transcripts were not detected in any cartilage tumors.

To determine whether the HH signal is active in the various cartilage lesions, we used real-time PCR for quantitative assessment of expression of IHH as well as target genes known to be up-regulated by HH signaling (Figure 2). The level of expression of IHH was significantly lower in normal articular cartilage than in any of the other samples ($P < 0.01$). Enchondromas and low- and high-grade chondrosarcomas expressed IHH at levels similar to that of the growth plate. There was a significantly higher level of expression in intermediate grade chondrosarcomas ($P < 0.05$). However, there were only three cases each of low- and high-grade chondrosarcomas compared to 23 intermediate-grade tumors that makes it difficult to interpret the significance of the differences in the levels of expression for these grades of tumors. The levels of expression of PTCH1 and GLI1, Hedgehog target genes, were significantly increased in intermediate grade chondrosarcomas and in enchondromas, compared to the other cartilaginous lesions ($P < 0.05$). The small numbers of low- and high-grade tumors and the large 95% confidence interval ranges also makes the meaning of the differences in PTCH1 and GLI1 expression in these tumor grades uncertain. However, the elevated levels of IHH and the Hedgehog target genes suggest that Hedgehog-mediated signaling is activated in chondrosarcomas and enchondromas.

Constitutively Active Hedgehog Signaling despite PTHrP *Stimulation in Cartilage Tumor Explants*

We tested whether the PTHrP/IHH signaling pathway is functional in cartilage tumors by establishing short term primary organ cultures of enchondromas from 2 patients, chondrosarcomas from 10 patients, and normal growth plates from 4 patients. Treatment of enchondromas, chondrosarcomas as well as growth plates for 4 days with 10^{-7} mol/L PTHrP (Figure 3A) resulted in delayed hypertrophic differentiation based on decreased expression of Type X collagen (COLX) mRNA, an exclusive marker of hypertrophic chondrocytes ($P < 0.01$ for all three explant types).[5] PTHrP lead to a decrease in proliferation in growth plate explants based on BrdU incor-

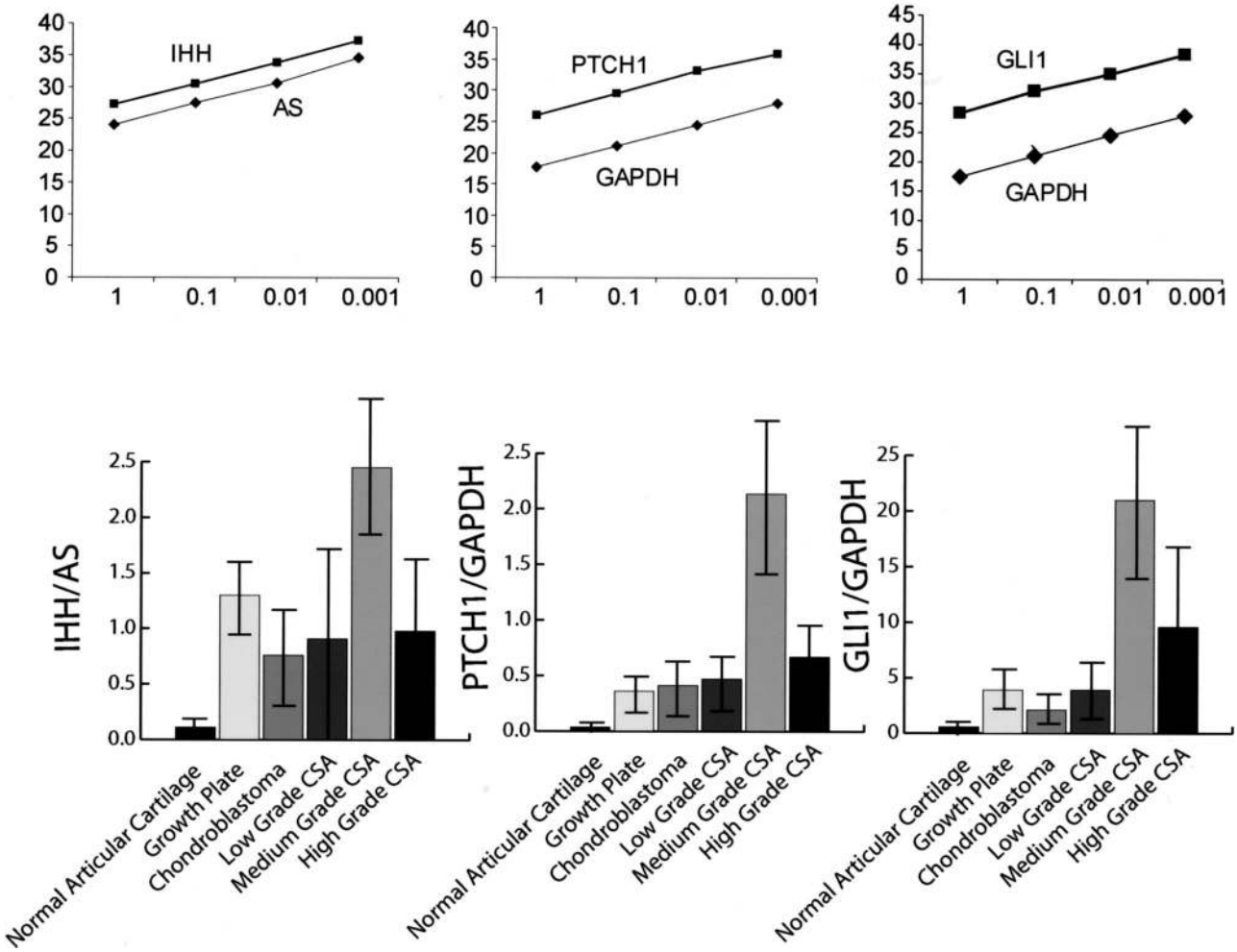

Figure 2. Evidence of active HH signaling in cartilaginous neoplasia. The level of expression of IHH, PTCH1, and GLI1 in normal articular cartilage, growth plate samples, chondroblastomas, enchondromas, and chondrosarcomas of low, medium (intermediate), and high grade was determined using real-time quantitative PCR. **Top**: Cycle threshold (Ct) versus dilution curves for the gene of interest and the control gene. **Bottom**: The ratio of the gene of interest compared to a control gene.

poration ($P < 0.05$), as would be expected based on its normal physiological role (Figure 3B), but had no effect on normal articular cartilage (data not shown). Surprisingly, PTHrP treatment did not significantly effect neoplastic chondrocyte proliferation for any of the 2 enchondromas or 10 chondrosarcomas, as measured by tritiated thymidine (^{3}H-T) uptake and BrdU incorporation ($P = 0.5$; Figure 3B). In comparison, treatment of cultures with recombinant Ihh-N increased ^{3}H-T uptake ($P = 0.02$) as well as BrdU incorporation ($P = 0.02$; Figure 3C) in all three tissue types suggesting a positive proliferative effect. Treatment also decreased the level of COLX expression (relative expression of COLX compared to β2M, as measured using semiquantitative RT-PCR, decreased from a mean of 1.3 to 0.4; $P = 0.03$). Hedgehog stimulation of cartilage tumor explants increased PTCH1 and GLI1 expression by 35% ($P < 0.05$) but had no significant effect on IHH ($P = 0.15$).

Blocking Hedgehog signaling in tumor explant and growth plate cultures with cyclopamine, a known HH pathway antagonist, decreased tumor cell proliferation based on ^{3}H-T uptake ($P < 0.01$) and BrdU incorporation ($P < 0.02$; Figure 3D). The structurally related but inactive compound tomatidine[21] served as a negative control and did not significantly affect tumor cell proliferation according to ^{3}H-T results (1120 versus 1080 counts per minute; $P = 0.9$ for chondrosarcoma samples). Similar to the effect of cyclopamine, treatment with triparanol diminished tumor cell proliferation (1190 versus 530 counts per minute; $P < 0.05$ for chondrosarcoma samples) as did the HH-neutralizing antibody 5E1,[28] according to partially blocked ^{3}H-T uptake (1160 versus 780 counts per minute; $P < 0.05$ for chondrosarcoma samples). Western blot confirmed binding of the anti-HH antibody 5E1 to human Ihh-N. Hedgehog blockade of cartilage tumor explants decreased PTCH1 and GLI1 expression by a mean of 25% ($P < 0.05$) but had no significant effect on IHH ($P = 0.1$). These findings are consistent with the known consequences of IHH and PTHrP signaling on normal growth plate chondrocyte proliferation and differentiation, respectively.[26,28–32]

In the growth plate, chondrocyte proliferation and differentiation are co-regulated because IHH induces PTHrP expression, and PTHrP in turn negatively regulates

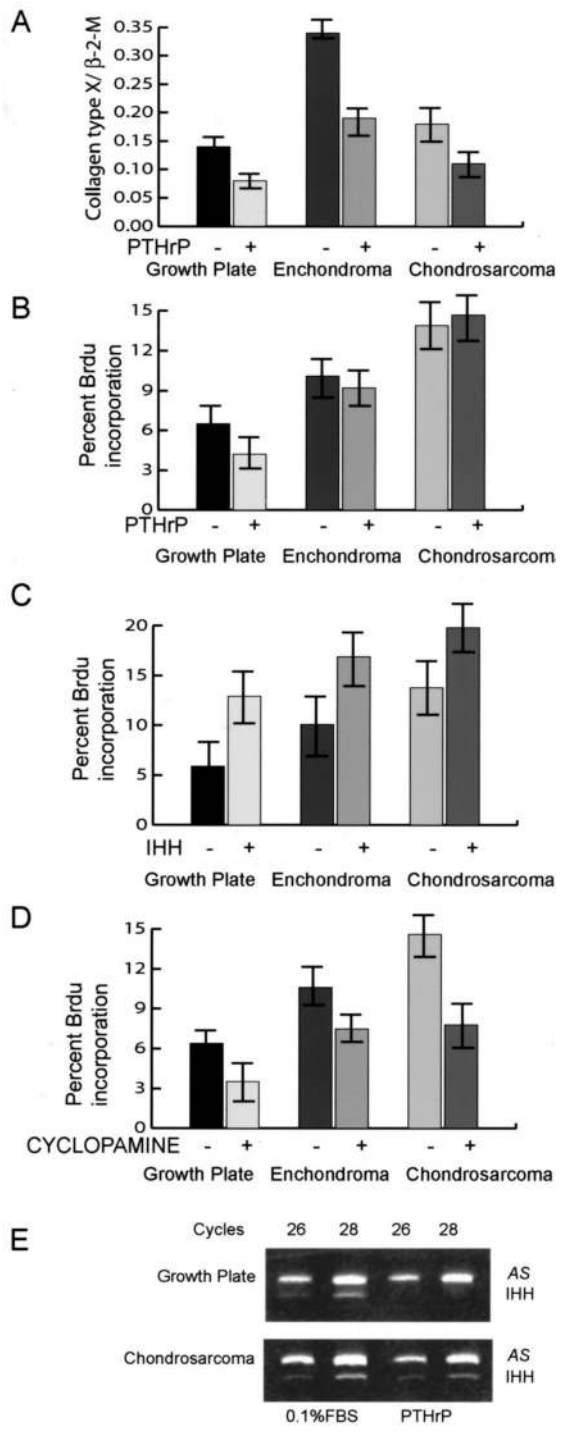

Figure 3. The effects of IHH stimulation and blockade and PTHrP treatment on growth plate, chondrosarcoma, and enchondroma explant cultures. **A:** There was down-regulation of COLX expression by semiquantitative RT-PCR after treatment with 10^{-7} mol/L PTHrP compared to treatment with carrier alone in enchondromas, chondrosarcomas, and growth plates ($P < 0.01$ for all three). Data are given as means and 95% confidence intervals. **B:** Treatment with PTHrP caused a decrease in the percentage of cells taking up BrdU in growth plate explants ($P < 0.05$), but not in enchondroma or chondrosarcoma explants. **C:** Treatment with Ihh-N increased the proliferation rate in all three tissue types ($P < 0.02$). **D:** Treatment with the Hedgehog blocking agent cyclopamine resulted in a decrease in the proliferation rate in all three tissue types ($P < 0.05$). Data on proliferation is given as the mean percentage of cells staining for BrdU over 10 high-power fields, and error bars are 95% confidence intervals. **E:** Semiquantitative RT-PCR at 26 and 28 cycles shows that IHH expression was down-regulated by PTHrP treatment in the normal growth plate but showed little change in a chondrosarcoma explant.

IHH expression (Figure 1A). As expected, down-regulation of IHH was observed in all four normal growth plate explants treated in culture with PTHrP. However, there was less down-regulation of IHH after treatment of the 2 enchondroma and 10 chondrosarcoma explant cultures with PTHrP (Figure 3E). Real-time PCR results confirmed the same findings, with PTHrP treatment of growth plate explants leading to a 33% decrease in IHH expression but no significant change in IHH levels in chondrosarcoma. These results illustrate that the normal feedback mechanism between IHH and PTHrP is absent in enchondromas and chondrosarcomas (Figure 1B), consistent with previous observations in enchondromas harboring the R150C-variant *PTHR1*.[18]

Lack of PTHR1 Mutations in Sporadic Enchondromas and Chondrosarcomas

Mutational analysis did not reveal any mutations in *PTHR1* in 50 chondrosarcomas and 6 sporadic enchondromas. We specifically did not identify the R150C-variant *PTHR1*, found in some cases of multiple enchondromatosis,[18] in any sporadic chondrosarcomas.

HH *Blockade Decreases Proliferation, Cellularity, and the Size of Xenograft Chondrosarcomas*

Cholesterol assays showed that the serum cholesterol of mice treated with triparanol was half that of mice treated with the carrier (Figure 4A), indicating the mice were able to absorb triparanol ($P < 0.01$). RT-PCR showed decreased expression of the HH target genes *Ptch* (Figure 4C) and *Gli-1* in the ends of long bones of triparanol-treated mice, indicating triparanol was functional in decreasing HH signaling.

Hedgehog pathway gene expression was assessed in the xenografts by real-time PCR (Figure 4B). Xenograft expression of PTCH1 and GLI1 decreased with triparanol treatment ($P = 0.02$) and IHH increased slightly, although not at statistical significance ($P = 0.06$). Anti-PTCH immunohistochemistry of chondrosarcoma xenografts from mice treated with triparanol showed less cellularity and less PTCH staining then control mice (Figure 4, D–F). Overall, there was a 20% decrease ($n = 12$, $P < 0.05$) in the proportion of tumor cells that stained positive for PTCH in triparanol-treated mice (Figure 4G). The histological grades of the chondrosarcomas used for xenografts were low ($n = 1$), intermediate ($n = 9$), and high ($n = 2$). Neither the levels of gene expression in the xenografts nor changes in expression due to triparanol treatment correlated with tumor grade.

Tumors in triparanol-treated mice decreased in volume by 30% compared to their size at the time of implantation ($P = 0.01$; Figure 4H). In comparison, tumors in control mice treated with carrier alone increased in volume by 30%. Overall, xenografted chondrosarcoma in mice treated with triparanol for 15 weeks decreased in volume by 60% ($n = 12$, $P = 0.01$) compared to tumors in control

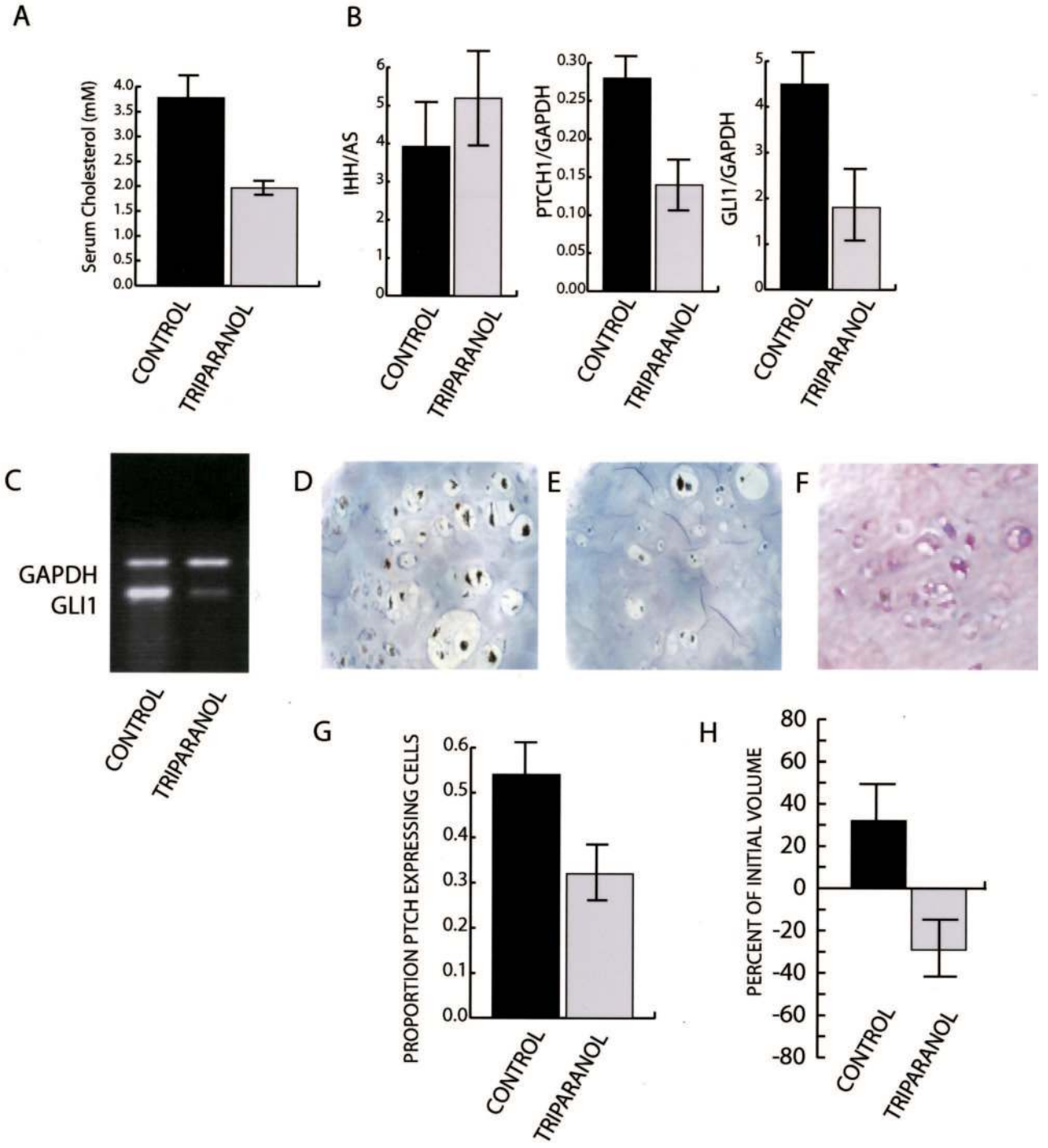

Figure 4. Triparanol treatment of chondrosarcoma xenografts results in HH blockade and decreased tumor size. **A:** Triparanol treatment decreased the serum cholesterol levels of mice. **B:** Real-time PCR from tumor samples implanted into immunodeficient mice show that triparanol decreased PTCH1 and GLI1 expression ($P = 0.02$). Interestingly, there was a slight increase in IHH expression in treated samples, but this did not reach statistical significance ($P = 0.06$). **C:** Semiquantitative RT-PCR showing down-regulated expression of the HH target gene *Gli1* in the ends of the long bones of mice treated with triparanol. **D–F:** Anti-PTCH immunohistochemistry of chondrosarcoma samples from mice treated with carrier (**D**) compared to triparanol (**E**) (**F** shows a control without primary antibody). **G:** There was a decrease in the percentage of tumor cells expressing PTCH ($P < 0.05$) when treated with triparanol. **H:** Xenografted tumors of mice treated with triparanol showed a decrease in size from initial implantation as well as compared to the controls ($P = 0.01$). Error bars indicate 95% confidence intervals. Original magnifications, ×40.

mice. Changes in xenograft tumor size due to triparanol did not correlate with tumor grade.

The xenografted chondrosarcomas of triparanol-treated mice had 40% ($n = 12$, $P < 0.05$) less cells per high-power field of view than mice treated with the carrier alone (Figure 5, A–C). In addition, the triparanol-treated xenografts also showed less clonality (ie, only one or a few cells per lacunae), more quiescent-appearing nuclei as opposed to hyperchromatic nuclei seen in the control (typical of proliferating cells), a higher proportion of lacunae without nuclei,

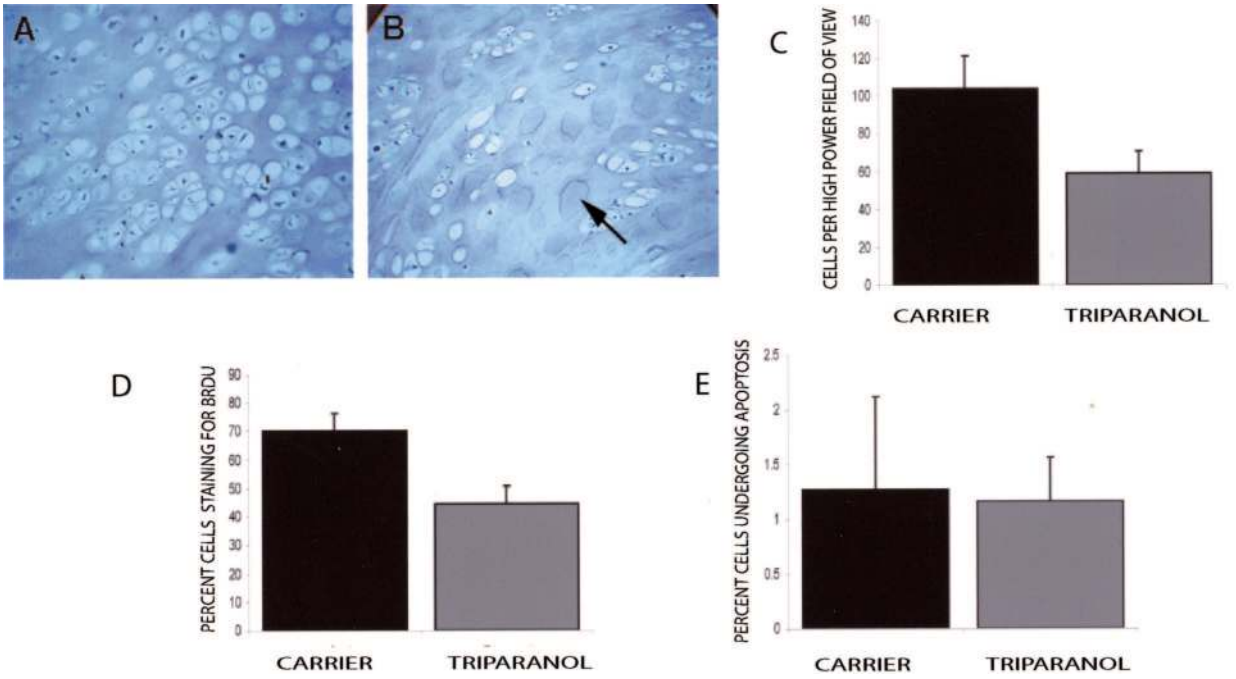

Figure 5. HH blockade decreases proliferation in xenografted chondrosarcomas. H&E staining of xenografted chondrosarcoma from mice treated with carrier (**A**) and triparanol (**B**) after 15 weeks of treatment. Triparanol treatment of mice increased the number of areas where cells dropped out and lacunae filled with matrix (**B**, **arrow**), and also decreased the number of tumor cells (**C**) ($n = 12$, $P < 0.05$). Anti-BrdU immunohistochemistry shows that triparanol decreased the proliferation rate (**D**) ($P < 0.05$) compared to the carrier. **E:** Triparanol treatment did not effect tumor cell apoptotic rate ($P = 0.86$). Error bars indicate 95% confidence intervals. Original magnifications, ×20.

and an increase in areas where cells dropped out and the lacunae filled with matrix. These findings suggest decreased proliferation in tumors from mice treated with triparanol. The results of BrdU incorporation assays support these findings and show a 25% reduction in the proliferation rate ($n = 3$, $P < 0.05$) in the tumors of triparanol-treated mice compared to controls (Figure 5D). TUNEL staining revealed no significant difference of the apoptotic rate in the tumors in mice treated with triparanol compared to those receiving carrier alone (Figure 5E).

Discussion

The normally tightly regulated IHH/PTHrP feedback loop is absent in cartilage neoplasia. We found that HH-mediated signaling was constitutively active in both chondrosarcomas and enchondromas. This finding suggests that HH signaling is not responsible for malignant transformation but likely plays a role in cartilage tumorigenesis by maintaining tumor cells in a less differentiated but proliferative state. Rozeman and colleagues[33] examined only a small number of cartilage tumors by real-time PCR but similarly found expression of IHH and Hedgehog pathway genes. Although we found that IHH/PTHrP pathway members were consistently expressed in cartilage tumors, the levels were variable. Even among the chondrosarcomas, there was no correlation between higher tumor grade and increasing gene expression level. These results may help explain some of the clinical heterogeneity associated with cartilage neoplasia.

As expected, HH treatment of tumor explants up-regulated cell proliferation as well as expression of the Hedgehog target genes *PTCH1* and *GLI1*. HH-agonist treatment also blocked tumor cell differentiation, likely due to an additional downstream effect of IHH increasing PTHrP (Figure 1). In comparison, the results of tumor explant exposure to HH blocking agents further supports the existence of autocrine signaling in cartilage neoplasia that is HH ligand-dependent. All cartilage tumor explants exhibited inhibition of cell proliferation, up-regulation of differentiation and decreased PTCH1 and GLI1 expression when exposed to cyclopamine or triparanol, which function downstream of IHH, as well as an anti-HH antibody.[28] Similar proliferative and differentiation effects of cyclopamine have been described previously in a murine medulloblastoma model.[34]

Although treatment of chondrosarcoma and enchondroma explants with PTHrP alone delayed cell differentiation, the ability of PTHrP to down-regulate IHH expression was lost in these cartilage tumors. In addition, PTHrP treatment did not alter cell proliferation in cartilage tumor explants. This indicated that the normal feedback of PTHrP on IHH signaling is absent, leading to constitutive Hedgehog signaling (Figure 1). PTHR1 mutations found in some enchondromas have differential effects on the downstream second messengers cAMP and IP_3 in response to treatment with PTHrP.[18] This may explain why treatment of cartilage tumors with PTHrP affected differentiation but not IHH expression or cell proliferation. The literature has reported disparate effects of PTHrP signaling on cell proliferation.[9–11,32] The normal interactions

between PTHrP and HH signaling that appear to be absent in neoplasia might provide important clues to some of the differences of PTHrP behavior in different biological contexts. In this regard, it is interesting that PTHrP caused a reduction in cell proliferation in growth plate chondrocytes but not in articular cartilage.[35] This raises the question of whether down-regulation of proliferation by PTHrP is an unusual event in the growth plate compared to other cartilage entities. Despite this, we did identify proliferation rates as well as levels of expression of IHH and HH target genes that were significantly higher in cartilage tumors compared to normal articular chondrocytes. We also showed that Hedgehog blockade reduced proliferation and PTCH1 and GLI1 expression in cartilage tumor explants and xenografts. This data further supports the importance of the lack of normal Hedgehog regulation in these tumors.

Clinical data suggests that enchondromas are frequent benign precursors to chondrosarcomas.[3] Our data provides evidence of a biochemical link between these tumors. Lack of normal regulation of the HH signaling pathway may be involved in the initiation of cartilaginous neoplasia, while additional secondary genetic events may be necessary for transformation into a malignancy. We are currently investigating this concept in our mouse models of cartilage neoplasia. We identified *PTHR1* mutations in only two of six patients with Ollier's disease but in no solitary enchondromas or chondrosarcomas. In a study of cartilaginous tumors from 31 patients with Ollier's disease, Rozeman and colleagues[36] failed to identify any *PTHR1* mutations. Although *PTHR1* mutations appear to be a rare event in cartilaginous neoplasia,[18] ligand-dependent constitutive Hedgehog activation is common. This situation is analogous to other neoplastic processes, such as basal cell carcinoma, in which a neoplastic syndrome is associated with specific mutations leading to ligand-independent activation of a signaling pathway; sporadic lesions also exhibit pathway activation but less commonly harbor the same mutations.[37]

Triparanol treatment of xenograft tumors led to a substantial decrease in tumor size, cellularity, proliferation rate, and level of expression of PTCH1 and GLI1. There was a more dramatic difference in cell number than tumor size. One explanation for this is that chondrosarcomas contain large amounts of matrix and resorption of the matrix lags behind cellular changes. Although we did not identify any difference in TUNEL staining in our histological evaluation of triparanol-treated xenografts, we frequently saw regions in the tumor matrix that may have contained cells that died (Figure 5). There may be a slow rate of cell death over time resulting in a difference between treatment and carrier that is too small to detect using TUNEL staining.

Several different types of compounds that block HH signaling have been identified by their teratogenic effects. One such compound used in this study, cyclopamine, may cause birth defects, such as limb malformations and holoprosencephaly, based on animal data.[20,21] However, cyclopamine has also been demonstrated to possess a potential anti-cancer role in breast cancer, medulloblastoma, digestive tract tumors, pancreatic cancer, and prostate cancer.[34,38–41] Similar to the case in chondrosarcoma, HH signaling appears to play an important role in controlling the proliferation rate of all these tumors. Therefore, agents that specifically block HH signaling may be effective in the treatment of a wide variety of tumors.

In addition to triparanol's role as a Hedgehog inhibitor, it also lowers serum cholesterol levels.[42,43] Conceivably, the effects seen in the xenografted tumors may be due in part to the change in serum cholesterol. However, both triparanol and cyclopamine treatment of tumor explants *in vitro* resulted in similar cellular changes in our xenograft tumors. Triparanol treatment resulted in a decrease in the expression of downstream HH target genes, suggesting that at least part of the effect of triparanol on the cartilaginous tumors is related to its ability to block HH signaling. Triparanol has been shown to interfere with posttranslational modification of IHH as well as the sterol sensing domain of its receptor PTCH1, thereby affecting both the active signal as well as signal transduction and leading to down-regulation of the HH signaling pathway.[44] Although triparanol proved to be useful in this study for establishing the efficacy of HH-blockade in a xenograft model of cartilage neoplasia, it is unlikely to become a treatment for patients based on its toxicity profile. It was used briefly in the 1960s as a cholesterol-lowering agent but was withdrawn from use because of complications including cataracts and alopecia. A small-molecule inhibitor of the HH-pathway has recently been used successfully in an *in vitro* model of basal cell carcinoma as well as a xenograft model of medulloblastoma.[45,46] This or similar synthetic compounds may form the basis of future clinical trials for tumors that are dependent on HH signaling.

Chondrosarcomas are generally radiation- and chemotherapy-resistant, such that radical surgical treatment is commonly used.[4] Our results suggest that blockade of HH signaling can alter tumor cell behavior, specifically attenuating proliferation in chondrosarcoma. This study suggests the prospect that targeting HH signaling in chondrosarcomas may be an effective chemotherapy treatment for these otherwise difficult to manage tumors. Hedgehog blockade would likely not be cytotoxic in the way conventional chemotherapy is and therefore might be useful as part of a long-term suppressive strategy.

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