Hedgehog Pathway Inhibition in Chondrosarcoma Using the Smoothened Inhibitor IPI-926 Directly Inhibits Sarcoma Cell Growth


Abstract

Hedgehog (Hh) pathway inhibition in cancer has been evaluated in both the ligand-independent and ligand-dependent settings, where Hh signaling occurs either directly within the cancer cells or within the nonmalignant cells of the tumor microenvironment. Chondrosarcoma is a malignant tumor of cartilage in which there is ligand-dependent activation of Hh signaling. IPI-926 is a potent, orally delivered small molecule that inhibits Hh pathway signaling by binding to Smoothened (SMO). Here, the impact of Hh pathway inhibition on primary chondrosarcoma xenografts was assessed. Mice bearing primary human chondrosarcoma xenografts were treated with IPI-926. The expression levels of known Hh pathway genes, in both the tumor and stroma, and endpoint tumor volumes were measured. Gene expression profiling of tumors from IPI-926–treated mice was conducted to identify potential novel Hh target genes. Hh target genes were studied to determine their contribution to the chondrosarcoma neoplastic phenotype. IPI-926 administration results in downmodulation of the Hh pathway in primary chondrosarcoma xenografts, as demonstrated by evaluation of the Hh target genes GLI1 and PTCH1, as well as inhibition of tumor growth. Chondrosarcomas exhibited autocrine and paracrine Hh signaling, and both were affected by IPI-926. Decreased tumor growth is accompanied by histopathologic changes, including calcification and loss of tumor cells. Gene profiling studies identified genes differentially expressed in chondrosarcomas following IPI-926 treatment, one of which, ADAMTSL1, regulates chondrosarcoma cell proliferation. These studies provide further insight into the role of the Hh pathway in chondrosarcoma and provide a scientific rationale for targeting the Hh pathway in chondrosarcoma.

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Introduction

The Hedgehog (Hh) signal transduction pathway plays a critical role in cell differentiation and patterning during development, but is inactive in many adult cells (1). There are three Hh ligands—Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH)—that activate the pathway. In the absence of Hh ligand, the transmembrane receptor Patched (PTCH) is localized in the primary cilia and inhibits the activity of the G-protein–coupled receptor-like protein Smoothened (SMO), which is sequestered within cytosolic vesicles (2). Inhibition of the key signaling protein SMO ensures that GLI and other transcription factors are held in an inactive form via a complex that contains Suppressor of Fused (SUFU). When Hh ligands bind PTCH, repression of SMO is relieved, resulting in signaling that activates the GLI transcription factors, leading to increased expression of many genes involved in growth and development. A role for Hh pathway activation has been demonstrated in a broad range of cancers. Ligand-independent signaling occurs in some tumors due to genetic mutations in key pathway members such as PTCH (3–5); however, in many tumors, ligand-dependent Hh signaling occurs either directly within the tumor cells, or involves the cells within the tumor microenvironment.

Emerging evidence points to a critical role for Hh signaling in the pathogenesis of chondrosarcoma, a malignant tumor of cartilaginous origin (6). A potential role for Hh in chondrosarcoma was first implied by studies that evaluated Hh signaling in the biology of chondrocytes within the growth plate. IHH regulates the differentiation...
of chondrocytes during endochondral bone development. During normal bone development, expression of IHH by prehypertrophic chondrocytes leads to proliferation of growth plate chondrocytes. IHH subsequently upregulates PThrP, which inhibits chondrocyte differentiation and downregulates IHH, thus completing a tightly controlled negative feedback loop. Thus, IHH ligand functions as a central regulator of endochondral ossification and regulates the proliferation and differentiation of chondrocytes via a feedback loop essential for maintenance of the growth plate (7–9). The importance of Hh signaling in developing bone was further substantiated in recent studies where young mice treated with a Smo inhibitor developed severe bone defects, including premature differentiation of chondrocytes, thinning of cortical bone, and fusion of the growth plate (10).

In chondrosarcoma, signaling through the IHH/PThrP axis is dysregulated. The ability of PThrP to inhibit IHH expression is lost, leading to constitutive Hh signaling (11). High expression levels of the Hh-regulated genes PTCH1 and GLI1 provide evidence for constitutive Hh pathway signaling in chondrosarcoma (12). Chondrosarcoma tumor cell proliferation is increased by Hh ligand and decreased by inhibitors of the Hh pathway in cell culture. Studies using tumor explants found that exposure to Hh inhibitors downregulated expression of GLI1 and PTCH1, supporting the existence of ligand-dependent autocrine Hh signaling in chondrosarcoma.

There are few therapeutic options for patients with chondrosarcoma. These tumors are largely resistant to chemotherapy and radiotherapy, therefore radical surgery constitutes the mainstay of therapy in patients. Although chemotherapy may be beneficial for patients with the rare tumor variant known as mesenchymal chondrosarcoma, there is little evidence of efficacy against dedifferentiated chondrosarcoma or the most common subtype of conventional chondrosarcoma (13–15). In patients with unresectable disease, there is no treatment option, and the outcome is ultimately fatal. The relatively low incidence of chondrosarcoma has made the study of new therapeutic agents difficult. Given the role of the Hh pathway in the biology of normal chondrocytes, and the emerging evidence for the importance of the Hh pathway in chondrosarcoma, a clinically relevant animal model of chondrosarcoma was developed to test the effect of IPI-926, a potent, orally delivered small molecule that targets the Hh pathway by specifically inhibiting Smo (5, 16). Studies were conducted in primary chondrosarcoma xenografts to assess the activity of IPI-926 on tumor establishment and growth, and to further understand the mechanism of Hh inhibition.

Materials and Methods

Two-week oral gavage study in CD-1 mice for bone plate analysis

The in vivo portion of this study was performed by the Charles River Laboratories Preclinical Services Montreal, Inc. at the Experimental Biology Center of the Institute of Pathology and Cell Biology, Charles River Laboratories Preclinical Services Montreal, Canada. After surgical removal, tumor samples were divided into explants of 5 × 5 × 5 mm each, and implanted into the subcutaneous tissues on the back of either non-obese diabetic/severe combined immunodeficient (NOD/SCID) or interleukin-2 receptor gamma chain (gamma)-null NOD/SCID (NSG) mice. For each condition tested in vivo, 10 mice with tumor explants from each human chondrosarcoma were utilized. In all chondrosarcoma in vivo studies, IPI-926 was administered orally by gavage at 40 mg/kg, five times a week on a Monday–Friday schedule. The vehicle control mice were treated with 5% cyclodextrin in PBS. Treatment was initiated on the day of tumor implant, one month after implantation, or after one passage through the NOD/SCID mice and transplantation into NSG mice. Chemotherapies were injected intravenously. Doxorubicin was administered at 5 mg/kg three times a week on a Monday, Wednesday, and Friday schedule. Cisplatin was administered at 8 mg/kg once a week. After sacrifice and removal of the xenograft, tumor volume was calculated using three-dimensional measurements as previously described (12). For the Mia PaCa-2 pancreatic carcinoma cell line in vivo study, tumor cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI-1640 media supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were prepared in sterile PBS and implanted subcutaneously at a concentration of 10⁶ cells per Ncr Nude mouse. IPI-926 was administered orally at 40 mg/kg/d for a total of 6 weeks. Twenty-four hours after the last dose, tumors were placed in 10% neutral-buffered formalin for histology or snap frozen in liquid nitrogen for RNA isolation. Sections were prepared by standard methods and stained with hematoxylin and eosin for histologic evaluation.

Primary chondrosarcoma xenograft mouse model and drug treatment

With institutional review board approval, 6 human chondrosarcoma tumor samples were obtained fresh from the operating room at Mount Sinai Hospital (Toronto, Canada). After surgical removal, tumor samples were divided into explants of 5 × 5 × 5 mm each, and implanted into the subcutaneous tissues on the back of either non-obese diabetic/severe combined immunodeficient (NOD/SCID) or interleukin-2 receptor gamma chain (gamma)-null NOD/SCID (NSG) mice. For each condition tested in vivo, 10 mice with tumor explants from each human chondrosarcoma were utilized. In all chondrosarcoma in vivo studies, IPI-926 was administered orally by gavage at 40 mg/kg, five times a week on a Monday–Friday schedule. The vehicle control mice were treated with 5% cyclodextrin in PBS. Treatment was initiated on the day of tumor implant, one month after implantation, or after one passage through the NOD/SCID mice and transplantation into NSG mice. Chemotherapies were injected intravenously. Doxorubicin was administered at 5 mg/kg three times a week on a Monday, Wednesday, and Friday schedule. Cisplatin was administered at 8 mg/kg once a week. After sacrifice and removal of the xenograft, tumor volume was calculated using three-dimensional measurements as previously described (12). For the Mia PaCa-2 pancreatic carcinoma cell line in vivo study, tumor cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI-1640 media supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were prepared in sterile PBS and implanted subcutaneously at a concentration of 10⁶ cells per Ncr Nude mouse. IPI-926 was administered orally at 40 mg/kg/d for a total of 6 weeks. Twenty-four hours after the last dose, tumors were placed in 10% neutral-buffered formalin for histology or snap frozen in liquid nitrogen for RNA isolation. Sections were prepared by standard methods and stained with hematoxylin and eosin for histologic evaluation.

Cell line and other primary cancer xenograft mouse models and drug treatment

The following cell lines were obtained from ATCC: Panc1, BxPC3, Mia PaCa-2, L3.6PL, Colo205, SW580, and HT29, and cultured as recommended by the supplier. The purchased cell lines were not authenticated by the authors. The cell lines were not cultured beyond 20 passages for any experimental work. The cells were collected in sterile PBS and implanted subcutaneously into Ncr-
Nude mice at 2×10⁶ or 1×10⁷ cells total per mouse. The primary SCLC (LX22) xenograft cells were a gift from Neil Watkins (Monash Institute of Medical Research, Monash Medical Center, Clayton, Victoria, Australia) received in 2006 (17). The LX22 primary tumor was not further authenticated by the authors. The primary colon and ER− breast cancer models were established by purchasing fresh surgical specimen material from Asterand, dividing the explants each into 5×5×5 mm fragments and implanting them subcutaneously into NOD/SCID mice. All primary xenograft models were propagated by mouse-to-mouse passaging until enough mice were implanted for a treatment study (n = 7−10 per group). IPI-926 was dosed at 40 mg/kg/d for a total of 21 to 41 days. The control mice were treated with 5% cyclodextrin in PBS. The primary chondrosarcoma xenograft studies were established and treated as previously described. Tumors were collected and snap frozen for mRNA assessment, following IPI-926 treatment.

**RNA isolation and quantitative real-time PCR**

RNA was isolated from tumor tissue using the RNAqua-4PCR kit (Ambion). Fifty ng of RNA was added to each 25 µL reaction in One-step master mix and run on the 7300RT cycler (Applied Biosystems). RNA was isolated from at least 3 independent experiments, and was analyzed in separate PCR reactions, with a minimum of triplicates for each treatment condition and primer set. All primer and probe sets were purchased from Applied Biosystems: Human GLI1 (Hs00171790_m1), human IHH (Hs001081801_m1), human SHH (Hs00179843_m1) human PTCH1 (Hs00970980_m1), human SMO (Hs00170665_m1), human ADAMTSL1 (Hs00364599_ml), human ADAMTSL3 (Hs00324954_ml), human GAPDH (4310884E), murine Glil (Mm00494645_m1), and murine Gapdhi (4352339E). All genes tested were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and reported as relative gene expression compared with vehicle control.

**Immunofluorescence staining**

Formalin-fixed, paraffin-embedded human chondrosarcoma tissue microarrays (TMA) were purchased from Folio Biosciences for cilia staining. Before staining, all slides were placed into a 60°C oven and baked for 1 hour. Sections were deparaffinized in xylene (3 x 5 minutes) and rehydrated through a graded series of ethanol for 3 minutes each. Heat-induced epitope retrieval was carried out in the DIVA Decloaker solution using the Decloaking Chamber from Biocare Medical. Sections were blocked for 1 hour. Anti-acetylated tubulin (Sigma) at 1:2,000 and anti-CROCC (Sigma) at 1:75 were incubated overnight at 4°C. Sections were washed with Tris-buffered saline Tween-20 (TBST, 3 x 5 minutes), and anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 were added at 1:1,000. Sections were rinsed in TBST (3 x 5 minutes) and mounted using ProLong Gold anti-fade reagent with DAPI (Life Technologies). Images were captured at 40× or 63× using the TissueGnostics imaging system. The presence of cilia in the xenograft sections and TMA spots was assessed in 5 separate 40× fields of view (FOV). The tumors were designated to be positive if cilia were detected in any of the 5 FOV and negative if no cilia were identified.

**Gene expression microarray analysis**

Gene expression profiling was carried out on primary chondrosarcoma tumors from mice treated once a day orally with control or IPI-926 at 40 mg/kg on a Monday–Friday schedule (n = 4/group) for 4 weeks. The tumor RNA was used to generate cDNA which was examined on Agilent Whole Human Genome (4 x 44 K) chips. Differentially expressed genes were determined through the use of a two-sided moderated t test. Multiple test correction was applied using the Benjamini–Hochberg method to generate adjusted P values. A cut-off value of 0.05 was used to select significantly differentially expressed genes. All calculations were carried out using R version 2.14.1 (http://www.r-project.org) limma package (version 3.10.0). The data have been deposited in NCBI’s Gene Expression Omnibus (18) and are accessible through GEO Series Accession Number GSE44581.

**Chromatin immunoprecipitation analysis**

For chromatin immunoprecipitation (ChIP) analysis, cells were fixed in formaldehyde to cross-link and preserve protein/DNA interactions. The protein/DNA complexes were sheared into small fragments using sonication to produce DNA fragments of approximately 1 kb. A specific antibody against GLI1 or GLI2 and negative control IgG were used to directly immunoprecipitate the DNA-binding protein. Following immunoprecipitation, cross-linking was reversed by adding NaCl, the proteins were removed by treatment with Proteinase K, and the DNA was purified. The DNA was then analyzed by sequencing using the Solexa/Illumina Genome Analyzer II, and the resultant sequences were compared with data from the human genome.

**Explant culture proliferation assay**

Human chondrosarcoma tissue organ cultures were established from primary tumor explants (12). Five explant tumor tissue cultures, each derived from a different patient, were treated for 24 hours with 1 µmol/L Hh antagonist IPI-926, 0.5 µg/mL recombinant Hh ligand SHH-N, or 100 µL of ADAMTSL1 conditioned medium, as reported elsewhere (19–21). A dose–response experiment was conducted with 0, 0.5, 1, 2, 4, and 8 µg/mL purified ADAMTSL1 protein. A proliferation assay was performed 24 hours following treatment (totaling 48 hours treatment) with ADAMTSL1 according to the manufacturer’s protocol [Cell Proliferation ELISA, BrdU (colorimetric), Roche].

**Statistical analysis**

A Student t test or ANOVA with Student t test was run using the JMP software to determine the statistical
The significance of each experiment. The level of significance was set at 0.05 for all experiments. The in vivo tumor data are expressed as mean ± SEM.

Results

Primary chondrosarcoma tumor cells contain essential Hh pathway signaling components

The Hh pathway is active in normal chondrocyte development and promotes active bone plate growth. Studies in juvenile mice demonstrated that Hh pathway blockade leads to premature growth plate closure in the long bones (10). Indeed, Hh signaling in normal mouse chondrocytes is inhibited by IPI-926 treatment resulting in a decrease in proliferating chondrocytes and premature growth plate closure (Supplementary Fig. S1).

To investigate whether there is evidence of active Hh pathway signaling in primary chondrosarcoma xenografts, human Hh pathway gene expression was assessed using quantitative real-time PCR (qRT-PCR). Bulk tumor specimens from surgically resected human conventional chondrosarcomas were implanted subcutaneously into NOD/SCID immunocompromised mice. Following tumor engraftment and growth for at least 6 weeks, tumors were excised and RNA was extracted. Similar to normal chondrocytes within the growth plate, the chondrosarcoma tumor cells expressed high levels of human IHH mRNA compared with SHH mRNA (Fig. 1A). In addition, high levels of human PTCH1, SMO, and GLI1 mRNA were also detected (Fig. 1B).

Chondrosarcoma xenografts were also evaluated for the presence of primary cilia, as these provide an important cellular scaffold for Hh-induced pathway activation (2). Cilia were visualized by staining the microtubules with an antibody against acetylated tubulin and the basal bodies at the base of the cilium with an antibody against rootletin. Immunofluorescence staining demonstrated that primary cilia were present in chondrosarcoma tumor cells in three of the xenografts available for testing (Fig. 1C). To investigate the prevalence of primary cilia in a larger set of primary chondrosarcoma tumor samples, commercially obtained conventional chondrosarcoma TMAs were stained. Microscopic analysis of chondrosarcomas revealed that a majority (21/28; 75%) exhibited cilia expression (Fig. 1D).

IPI-926 treatment inhibits Hh pathway signaling in chondrosarcoma xenografts

To determine whether IPI-926 could inhibit Hh signaling in chondrosarcoma tumor cells, human GLI1, PTCH1, and SMO mRNA levels were assessed in xenograft tumor tissue following treatment with IPI-926 at 40 mg/kg for a minimum period of 3 weeks. Twenty-four hours following the last dose of vehicle or IPI-926 treatment, tumors were harvested and mRNA levels of human Hh genes were assessed. Treatment with IPI-926 resulted in a 5- to 12.5-fold downregulation of human GLI1 (P < 0.01) and a 2- to 3-fold downregulation of PTCH1 (P = 0.05) expression, compared with vehicle controls (Fig. 2A and B). A lack of corresponding decrease in expression of SMO was anticipated, but SMO expression actually increased following treatment with IPI-926 (Fig. 2B). This increase in SMO expression occurred in response to the effect of IPI-926, which inhibits Hh signaling by targeting SMO at the protein level. These data suggest that inhibition of the Hh pathway occurs in the cancer cells themselves.

Hh pathway inhibition in reactive stroma was also evaluated by analyzing murine Gli1 mRNA using species-specific primers. Murine Gli1 expression levels were measured in the TMA (Fig. 1D).
chondrosarcoma xenografts were found to be substantially lower than the human levels, but were still significantly downregulated in the IPI-926-treated group (Fig. 2C). In addition, the level of murine Gli1 expression was lower in the chondrosarcoma xenografts than that observed in epithelial-derived tumor xenografts, such as the MIA PaCa-2 pancreatic carcinoma xenograft. Although treatment with IPI-926 resulted in downregulation of murine Gli1 expression in both chondrosarcoma and pancreatic carcinoma xenografts (2.4-fold, \( P < 0.04 \), and 42-fold, \( P < 0.0001 \), respectively), only in the chondrosarcoma cells was human GLI1 expression similarly diminished (\( P < 0.04 \)). These results are consistent with the histology of chondrosarcoma tumors, which primarily consist of tumor cells embedded in a relatively acellular matrix with few surrounding stromal cells present (Supplementary Fig. S2). The results of human and murine GLI1 expression analysis in a variety of human tumor xenografts treated with IPI-926 are summarized in Supplementary Table S1.

Collectively, these data indicate that the majority of the chondrosarcoma tumor cells tested are directly responsive to Hh pathway inhibition by IPI-926, whereas epithelial-derived cancer cells, like pancreatic tumors, are not. In addition, these data are consistent with the proposal that there is ligand-dependent activation of Hh signaling in chondrosarcoma, which occurs through both autocrine and paracrine mechanisms as signaling can be inhibited by IPI-926 treatment.

Hh inhibition in primary chondrosarcoma xenografts leads to decreased tumor volume, decreased tumor cellularity, and tumoral calcification

Experiments were designed to determine whether IPI-926 has an effect on chondrosarcoma xenograft establishment and tumor growth. Two study designs in mice were tested: in one study design, treatment was initiated on the day of implantation, and in the second study design, treatment was initiated one month after implantation to allow time for tumor establishment. IPI-926 was administered for 6 weeks. Tumors were excised, and the end-point tumor volume was recorded. There were no observed effects on tumor establishment as all chondrosarcomas were able to grow as xenografts despite treatment with IPI-926 starting on the day of implantation. In both study designs, IPI-926 administration resulted in significantly decreased tumor volumes compared with controls. Overall, there was a mean reduction in tumor volume of 44% with IPI-926 treatment (\( n = 5, P < 0.02 \); Fig. 3A). At the end of each study, tumors were also collected and processed for histologic evaluation. Hematoxylin and eosin–stained samples revealed decreased tumor cellularity, and interestingly, increased areas of calcification in the tumors from drug-treated mice (Fig. 3B). These observations suggest that IPI-926 may drive chondrosarcoma cells to differentiate, leading to calcification, a process observed in normal growth plate chondrocytes.

The response of chondrosarcoma xenografts to IPI-926 in comparison with the chemotherapeutic agents,
doxorubicin and cisplatin, was also investigated. Although it is generally accepted that chondrosarcoma is resistant to conventional sarcoma chemotherapy, it was important to determine whether there was any chemotherapeutic response of the primary chondrosarcoma tumors relative to the changes already described above by Hh inhibition with IPI-926. Mice in which chondrosarcoma xenografts were established were treated with doxorubicin, cisplatin, or IPI-926 for a duration of 3 weeks. Twenty-four hours after the last dose of each therapy, tumors were collected and weighed and then processed for mRNA expression analysis. Compared with the control group, only IPI-926 and cisplatin-treated xenografts showed significant tumor growth inhibition of 46% (P = 0.009) and 54% (P = 0.01), respectively (Fig. 3C). qRT-PCR analysis confirmed that only IPI-926 significantly inhibited human GLI1 expression (P = 0.0001) in the chondrosarcoma cells (Fig. 3D).

Identification of ADAMTSL1 as a Hh-regulated gene that alters chondrosarcoma proliferation

Hh signaling might contribute to the neoplastic phenotype by regulating the expression of downstream target genes in the chondrosarcoma tumor cells. To identify such targets, human gene profiling was carried out by expression microarray analysis of primary chondrosarcoma tissue treated with IPI-926 or control. Using the analysis described in the Materials and Methods resulted in an initial list of 28,587 differentially expressed genes that was filtered down to 9,240 genes. A cut-off value of 0.05 was used to select the significantly differentially expressed genes, and the top 10 upregulated or downregulated by IPI-926 treatment are listed in Table 1.

One of the 10 most downregulated genes under Hh blockade was ADAMTSL1. We chose to study this gene further because its Caenorhabditis elegans homolog is known to be involved in apoptosis and as such it could play an important role in neoplasia; however, it is not known to be a Hh target gene (22, 23). This gene encodes a secreted protein whose function is unclear, but shares similarities with members of the disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif (ADAMTS) family (24). In chondrosarcoma xenografts from mice treated with IPI-926, downregulation of expression of ADAMTSL1, but not its closest homolog ADAMTSL3, was verified by qRT-PCR (P = 0.002;
Chondrosarcoma xenografts were grown as tissue explants in culture (12) and subjected to either Hh blockade with IPI-926 or Hh stimulation with recombinant SHh-N. Hh blockade resulted in significant downregulation of \( GLI1 \) and \( ADAMTSL1 \) expression, while pathway stimulation led to significant upregulation of both genes (\( P < 0.01 \; \text{(Fig. 4A)} \)).

The MULAN website (http://mulan/dcode.org) was used to examine the promoter region of human \( ADAMTSL1 \). Multiple GLI consensus binding sites were identified in its promoter region (Fig. 4B), and ChIP was used to verify GLI binding to the \( ADAMTSL1 \) promoter. ChIP with primer pairs adjacent to the GLI1 and GLI2 consensus binding site were identified (Fig. 4B) and an antibody to GLI1 or GLI2 detected both GLI1 and GLI2 bound to this site (Fig. 4C). Fragments immunoprecipitated using antibodies against GLI1, GLI2, and IgG were sequenced and the GLI1 and GLI2 fractions aligned uniquely to the intergenic region of the \( ADAMTSL1 \) gene. This verified binding of both GLI1 and GLI2 to the consensus sequence site in the \( ADAMTSL1 \) promoter. To determine whether this protein has functional significance in chondrosarcoma, primary tumors were grown in cell culture and treated with ADAMTSL1 or the related protein ADAMTSL3-conditioned medium. Treatment with ADAMTSL1, but not ADAMTSL3 (data not shown), led to a 2-fold increase in cell proliferation as measured by BrdUrd incorporation (\( P = 0.04 \); Fig. 5A). When treated with increasing concentrations of purified ADAMTSL1 protein, cell proliferation increased in a dose-dependent manner (\( P < 0.005 \); Fig. 5B). Thus, in chondrosarcoma, Hh-regulated genes include those whose protein product modulates the neoplastic phenotype. This supports the concept that Hh signaling within chondrosarcoma cells themselves alters cellular behavior in a manner that drives the neoplastic phenotype.

### Table 1. The top 20 genes modulated with IPI-926 treatment, rank ordered by fold change with treatment

**Top 10 upregulated genes**

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<thead>
<tr>
<th>Gene symbol</th>
<th>Log fold change</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ITH5(^a)</td>
<td>7.02</td>
<td>Inter-alpha-trypsin inhibitor heavy chain family, member 5</td>
</tr>
<tr>
<td>SLC2A4(^a)</td>
<td>2.63</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 4</td>
</tr>
<tr>
<td>ANGPTL7(^a)</td>
<td>2.13</td>
<td>Angiopoietin-like 7</td>
</tr>
<tr>
<td>HAS1</td>
<td>2.11</td>
<td>Hyaluronan synthase 1</td>
</tr>
<tr>
<td>FGF18</td>
<td>1.98</td>
<td>Fibroblast growth factor 18</td>
</tr>
<tr>
<td>KLRC1</td>
<td>1.92</td>
<td>Killer cell lectin-like receptor subfamily C, member 1</td>
</tr>
<tr>
<td>UBD</td>
<td>1.88</td>
<td>Ubiquitin D</td>
</tr>
<tr>
<td>C1QTNF9B</td>
<td>1.58</td>
<td>C1q and tumor necrosis factor–related protein 9B</td>
</tr>
<tr>
<td>SPPR2D</td>
<td>1.32</td>
<td>Small proline-rich protein 2D</td>
</tr>
<tr>
<td>AADY4</td>
<td>1.39</td>
<td>Adenylyl cyclase 4</td>
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</table>

**Top 10 downregulated genes**

<table>
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<tr>
<th>Gene symbol</th>
<th>Log fold change</th>
<th>Description</th>
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<tbody>
<tr>
<td>GLI1(^a)</td>
<td>-3.27</td>
<td>GLI family zinc finger 1</td>
</tr>
<tr>
<td>KCNIP1(^a)</td>
<td>-3.25</td>
<td>Kv channel interacting protein 1</td>
</tr>
<tr>
<td>PLCXD3(^a)</td>
<td>-2.91</td>
<td>Phosphatidylinositol-specific phospholipase C, X domain containing 3</td>
</tr>
<tr>
<td>SLC13A5(^a)</td>
<td>-2.86</td>
<td>Solute carrier family 13 (sodium-dependent citrate transporter), member 5</td>
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<tr>
<td>HHIP(^a)</td>
<td>-2.64</td>
<td>Hedgehog interacting protein</td>
</tr>
<tr>
<td>GDF10(^a)</td>
<td>-2.43</td>
<td>Growth differentiation factor 10</td>
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<tr>
<td>C7(^a)</td>
<td>-2.36</td>
<td>Complement component 7</td>
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<td>FAM150B(^a)</td>
<td>-2.30</td>
<td>Family with sequence similarity 150, member B</td>
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<td>HHILP2(^a)</td>
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<td>HHIP-like 2</td>
</tr>
<tr>
<td>ADAMTSL1(^a)</td>
<td>-1.81</td>
<td>ADAMTS-like 1</td>
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\(^a\)Adjusted \( P \) value < 0.05.
implantation or 6 weeks following engraftment. Histologic analysis identified changes in tumor morphology, including decreased cellularity and increased calcification, following IPI-926 treatment. Both of these features are indicative of induced cartilage cell differentiation as is normally seen in growth plate chondrocytes in response to Hh blockade. Further evidence for an on-target effect of IPI-926 was decreased proliferation of chondrocytes and premature growth plate closure in the mice following treatment, consistent with the expected normal physiologic role of Hh blockade (10).

For most tumors, it remains controversial whether Hh signaling occurs in the tumor cells themselves, stromal cells, or both. Chondrosarcoma is an example of a tumor with ligand-dependent Hh signaling as it expresses high levels of Ihh, and only rarely harbors mutations in pathway-specific genes (11, 26–28). We utilized species-specific RT-PCR primers to address the controversial issue of whether Hh functions through an autocrine or paracrine mechanism in chondrosarcoma. This tumor is unique in that it exhibits evidence of both autocrine and paracrine Hh signaling, IPI-926 led to significant reductions in

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**Figure 4.** Positive correlation between Hh activity and human ADAMTSL1 expression. A, qRT-PCR analysis of chondrosarcoma xenografts from mice treated with IPI-926 confirms the downregulation of human ADAMTSL1 expression ($P = 0.002$), but not ADAMTSL3. Treatment of chondrosarcoma explants in culture with the Hh antagonist IPI-926 significantly reduced GLI1 and ADAMTSL1 expression ($P < 0.01$), whereas the Hh agonist Shh significantly increased GLI1 and ADAMTSL1 expression ($P < 0.01$). B, diagrammatic representation of the human ADAMTSL1 gene promoter showing putative Gli consensus binding sites from MULAN analysis. The vertical black line indicates a GLI1 and GLI2 consensus binding site in the intergenic DNA. ChIP was performed on a primary chondrosarcoma treated with Shh ligand. A PCR and gel electrophoresis is shown for primers at the GLI1 and GLI2 consensus binding sites from fragments immunoprecipitated using a GLI1, GLI2 or IgG antibody. Amplification was seen with the GLI1 and GLI2 antibody, but not control IgG (input DNA is a positive control). Sequence reads of fragments from GLI1 and GLI2 immunoprecipitated fractions aligned uniquely to the intergenic region of the ADAMTSL1 gene.

**Figure 5.** ADAMTSL1 increases proliferation of chondrosarcoma cells. Conditioned media (A) or purified ADAMTSL1 protein (rADAMTSL1; B) was added to the media of chondrosarcoma explants and proliferation rate was assessed by a BrdUrd uptake assay. Treatment with ADAMTSL1 leads to a 2-fold increase in cell proliferation ($P = 0.04$). B, dose-dependent increase in proliferation was observed when increasing doses of purified ADAMTSL1 were added to three explants derived from different donors ($P < 0.005$). The average increase in proliferation of the three donors is graphed. *, significant differences in expression.
expression of PTCH1 and GLI1 in both human tumor cells and mouse stroma in chondrosarcoma xenografts, indicating that active Hh pathway signaling was present and functional in each cellular compartment. However, there was a much greater reduction in expression of the human Hh target gene GLI1 in chondrosarcoma tumor cells compared with murine Gli1 in the stroma, likely due to the much higher levels of GLI1 expression in the human tumor cells at baseline and the relative acellularity of stromal tissues seen histologically in chondrosarcoma.

We found murine Gli1 expression to be dramatically higher in the MIA PaCa-2 pancreatic cancer xenografts compared with the chondrosarcoma tissue, and this was predominantly due to the higher prevalence of mouse stromal cells. This is also consistent with the extensive desmoplasia typical of pancreatic cancer that is known to be Hh-driven and constitutes an important feature of this particular tumor microenvironment (29, 30). Interestingly, IPI-926 treatment of mice bearing MIA PaCa-2 pancreatic xenografts led to diminished Hh signaling only in stromal cells but not in the pancreatic cancer cells themselves, indicating that paracrine Hh signaling is likely involved in pancreatic cancer. Other solid tumor xenografts that we examined from colon, breast, and lung cancers demonstrated similar findings, in that Hh-responsive signaling was limited to the mouse stroma in the tumor microenvironment (Supplementary Table S1). This is consistent with recent data showing that in solid epithelial-derived malignancies, tumor cell-derived Hh ligands do not stimulate the tumor cells themselves, but instead activate the Hh pathway in the surrounding stromal cells in a paracrine manner (31–35).

There are few examples of autocrine Hh signaling in tumor cells beyond the demonstration of chondrosarcoma in the current study. Recent evidence for constitutive Hh activation was observed in models of small and non–small cell lung cancer, melanoma, and chronic myelogenous leukemia, in which the pathway is activated in the tumor cells (36–40). However, the presence of transcripts for Hh target genes does not necessarily represent active signaling, without evidence that the expression can be modulated by pathway inhibition (35, 41). In addition, results from studies using high doses of cyclopamine may be confounded by the off-target effects of cyclopamine at concentrations in the micromolar range. In some tumors where paracrine and autocrine Hh signaling coexists, the balance between the two may regulate cancer progression (42, 43).

The antitumor activity of IPI-926 in chondrosarcoma xenografts could be due to multiple factors and pathways known to be regulated by Hh pathway blockade. To identify potential downstream targets of the Hh pathway in chondrosarcoma, differentially expressed genes were identified in tumor xenografts using microarray analysis. Not surprisingly, GLI1 and two Hh-associated proteins (HHIP and HHIPL2) were among the most downregulated genes. Another gene which was highly downregulated as a result of Hh blockade with IPI-926 was ADAMTSL1, which codes for a secreted protein of unknown function that is a member of the ADAMTS superfamily (24). ADAMTSL1 lacks the proteolytic activity typical of most ADAMTS family members but has the characteristic thrombospondin type-1 repeats (TSR). ADAMTSL1 is a mammalian ortholog of MADD-4, a recently discovered protein which functions through the netrin receptor UNC-40/DCC in C. elegans (23). As a potential mediator of netrins, ADAMTSL1 may play an important role in cell survival and neoplasia (22). Targeting ADAMTSL1 and a putative receptor that lies downstream of Hh pathway signaling may represent a promising therapeutic strategy to pursue for patients with chondrosarcoma.

A potential means of improving the poor results associated with systemic therapy for patients with chondrosarcoma would be to consider targeting Hh or ADAMTSL1 together with conventional chemotherapy, as the combination could act synergistically. Compared with the significant antitumor effects of IPI-926 in the chondrosarcoma xenograft model, cisplatin also led to a significant reduction in tumor growth independent of Hh inhibition, as there was not a concomitant decrease in Gli1 expression. Given that chondrosarcoma is considered to be chemotherapy resistant, these data suggest that further investigation may be warranted into the potential efficacy of cisplatin in chondrosarcoma and whether combination with IPI-926 or other novel therapeutics could provide additional benefits. For example, inhibition of Hh signaling in combination with chemotherapy has been shown to reverse chemoresistance in tumor models of pancreatic cancer, lung cancer, and lymphoma (38, 44, 45). In tumors such as chondrosarcoma, which rely on ligand-dependent Hh activation and exhibit high levels of GLI1 and PTCH1 expression, downregulation of GLI1 and PTCH1 could serve as biomarkers for response to Hh pathway inhibition. In addition, as most chondrosarcomas have an identifiable primary cilium, the presence of cilia may serve as a biomarker for response to Hh blockade, as recently demonstrated in a transgenic mouse model of cartilage neoplasia (46). As a potential treatment for patients, IPI-926, a potent and selective SMO antagonist, may have advantages as targeted therapy for Hh-dependent tumors compared with other pathway inhibitors. For example, triparanol has significant toxicity in humans in the form of inducing alopecia and cataracts (12), whereas cyclopamine is plagued by a lack of selectivity at exposures necessary for Hh pathway inhibition (35).

Chondrosarcoma is a malignant tumor of cartilage in which there is ligand-dependent activation of Hh signaling both within the tumor cells and the surrounding stromal compartment. The results of these studies provide the rationale for investigating Hh pathway blockade as a novel therapeutic option for patients with chondrosarcoma. Although the early results of a single-arm trial with the Hh blocking drug vismodegib found that 9 of 30 chondrosarcoma patients were progression-free at 6 months follow-up, longer follow-up of this single-arm trial and as a randomized trial with IPI-926 in patients...
with advanced chondrosarcoma were both stopped as they did not meet their clinical objectives (47, 48). Ongoing translational research on tumor samples from these two studies may identify patient subsets that have derived benefit from Hh blockade. Despite this setback, new research findings in chondrosarcoma may lead to the development of other opportunities for novel treatments. For example, isocitrate dehydrogenase (IDH) mutations were recently identified in approximately 50% of chondrosarcomas and drugs are currently under development to inhibit mutant IDH enzymatic activity (49). In addition, genomic sequencing and functional evaluation of signaling pathways have both led to the identification of specific genes that may be targetable as part of novel therapeutic strategies (27, 50).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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