Hedgehog Signaling Inhibition Blocks Growth of Resistant Tumors through Effects on Tumor Microenvironment

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Abstract

Hedgehog (Hh) signaling is implicated in bone development and cellular transformation. Here we show that inhibition of Hh pathway activity inhibits tumor growth through effects on the microenvironment. Pharmacologic inhibition of the Hh effector Smoothened (Smo) increased trabecular bone in vivo and inhibited osteoclastogenesis in vitro. In addition, enhanced Hh signaling due to heterozygosity of the Hh inhibitory receptor Patched (Ptc1+/−) increased bone resorption, suggesting direct regulation of osteoclast (OC) activity by the Hh pathway. Ptc1+/− mice had increased bone metastatic and subcutaneous tumor growth, suggesting that increased Hh activation in host cells promoted tumor growth. Subcutaneous growth of Hh-resistant tumor cells was inhibited by LDE225, a novel orally bioavailable SMO antagonist, consistent with effects on tumor microenvironment. Knockdown of the Hh ligand Sonic Hh (Shh) in these cells decreased subcutaneous tumor growth and decreased stromal cell production of interleukin-6, indicating that tumor-derived Hh ligands stimulated tumor growth in a paracrine fashion. Together our findings show that inhibition of the Hh pathway can reduce tumor burden, regardless of tumor Hh responsiveness, through effects on tumor cells, OCs, and stromal cells within the tumor microenvironment. Hh may be a promising therapeutic target for solid cancers and bone metastases. Cancer Res 72(4): 897–907. ©2011 AACR.

Introduction

The Hedgehog (Hh) signaling pathway plays critical roles in epithelial–mesenchymal transition and cell differentiation during embryonic development (1, 2), adult tissue homeostasis, and tumorigenesis (3, 4). In the absence of ligand, the Hh receptor Patched (Ptc1) inhibits the activator Smoothened (Smo). Upon ligand binding to Ptc1, Smo is released, resulting in pathway activation and transcription of target genes, including Gli1, Gli2, and Ptc1 (5, 6). Cyclopamine, a naturally occurring Hh inhibitor and Smo antagonist, first highlighted the importance of Hh signaling. Several next-generation analogs with increased specificity and tolerability (ref. 7; including GDC-0449, LDE225, and IPI-926) exert antitumor effects in a subset of cancer cells and are currently in clinical trials for a wide variety of tumors (8–10).

The Hh signaling pathway plays a critical role in tumorigenesis and progression in many tumor types. Mutations leading to ligand-independent Hh pathway activation have been linked to basal cell carcinoma and medulloblastoma (11, 12), whereas overexpression of the Hh ligand Sonic Hh (Shh) or mutations in Hh signaling genes (Smo, Ptc1, Gli1, and Gli2) have been implicated in the emergence and progression of numerous epithelial cancers, including breast, skin, esophagus, stomach, pancreas, liver, lung, and prostate (11, 13, 14). Hh inhibition via cyclopamine suppressed proliferation of breast carcinoma cell lines and decreased Gli1 (15, 16). These effects are not limited to primary tumors, as inhibition of the Hh pathway decreased lung and liver metastases in a mouse pancreatic cancer model together with gemcitabine (17, 18).

In addition to direct effects on tumor cell growth, Hh signaling within the host stromal microenvironment also controls tumor progression. Mice with a targeted disruption of the Hh inhibitory receptor Ptc1 develop ductal hyperplasia (15). Interestingly, it was disruption of Ptc1 in mammary stroma, rather than in mammary epithelium, that led to the ductal changes, suggesting an indirect effect of Hh signaling on tumor-initiating cells. Furthermore, paracrine Hh activation in host-derived stromal cells leads to increased tumor growth (19–21) and is necessary to support the growth of stromal-dependent B

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cell lymphoma and multiple myeloma (22). Increased intratumoral expression of Hh target gene Gli2 increased production of osteoclast (OC)-activating factor PTHrP in breast cancer cells, linking Hh signaling with tumor-induced osteolysis (23). However, in certain breast cancer cell lines that are relatively resistant to Hh signaling modulation, Gli2 expression can be induced through TGFβ signaling independent of Hh resulting in enhanced osteolysis (24). Together, these studies provide a strong rationale for evaluating Hh signaling as a therapeutic target for cancer and metastasis.

The Hh pathway is critical to osteoblast (OB) differentiation and chondrocyte proliferation during embryonic endochondral bone development (25, 26). Targeted disruption of several Hh pathway genes result in profound effects on bone development (27–29). Postnatal interruption of the Hh pathway leads to trabecular bone abnormalities and disrupted long bone formation (30, 31). The effects of Hh signaling interruption on adult bone have not been fully elucidated. Mice with conditional deletion of Ptch1 in mature OB using osteocalcin-Cre show increased production of RANK ligand (RANKL) by OB, which indirectly increased OC activity and bone loss (32). However, mice with heterozygous germline deletion of Ptch1 (Ptch1+/−) show increased bone mass, with enhanced bone formation being dominant to increased in vivo OC number and resorption (33). The discrepancy between the 2 models remains to be fully resolved. Interestingly, direct effects of Hh pathway signaling on primary OC have not been reported.

We hypothesized that disruption of Hh signaling would block tumor growth both directly, by targeting intratumoral survival signaling, and indirectly, by altering the host microenvironment. Indeed, OC- and OB-derived proteins, such as TGFβ, can enhance growth of bone-invading tumor cells (34), and we propose that the effects of Hh signaling in both tumor and host cells may enhance metastatic growth. In this study, we show that disruption of the Hh pathway in adult nontumor-bearing mice increased trabecular bone in part through reduced OC function and indirectly, by altering the host microenvironment. Employing pharmacologic inhibitors, we show that disruption of the Hh pathway decreased subcutaneous and bone tumor burden in vivo. Mice heterozygous for Ptch1, resulting in systemically enhanced Hh signaling, also had increased tumor burden. Moreover, Hh inhibitors decreased subcutaneous tumor burden in a cell line that is resistant to direct cytotoxic effects due to reduced SMO expression (24), showing indirect antitumor effects of targeting cells of the host microenvironment. Interestingly, MDA-MB-231 cells produce Hh ligands and knockdown of SHH decreased tumor growth through paracrine effects on stromal cell production of growth factors including interleukin (IL)-6. Thus, Hh inhibitors represent promising therapeutics due to their ability to target both tumor cells and the protumor microenvironment.

Materials and Methods

Animals
Female BALB/c mice and athymic nude mice (NCr-nude) were obtained from Taconic. Ptch1−/− (12) and Smo0/0 (25) mice on C57Bl/6 and mixed backgrounds, respectively, were previously described. Animals were housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University, St. Louis, MO. The animal ethics committee approved all experiments.

Cells
4T1 BALB/c murine breast cancer (35) and B16-F10 C57Bl/6 murine melanoma cell lines (36) were purchased from the American Type Culture Collection and modified to express firefly luciferase as previously described. A bone metastatic variant of MDA-MB-231, described in Guise and colleagues (37), was a kind gift of T. Guise (Indiana University, Bloomington, IN). Low passage stocks were utilized and regularly tested for Mycoplasma and maintenance of growth characteristics.

Drug compounds and dosing
The following drugs were used as indicated: cyclopamine (LC Labs), tomatidine (Sigma), GDC-0449 (provided by Dr. Jim Janetka, Washington University, St. Louis, MO), and LDE225 (provided by Novartis Pharmaceuticals). Cyclopamine (25 mg/kg) was administer per oral gavage twice daily for 11 to 14 days, as previously described (17); LDE225 (20 mg/kg) once daily for 21 days orally.

Microcomputed tomography
Postmortem, tibiae and femurs were scanned (µCT-40; Scanco Medical) and evaluated as described previously (36).

Bone histology and in vitro OC quantification
Decalcified and paraffin-embedded sections were stained with hematoxylin and eosin (H&E) stain or tartrate-resistant acid phosphatase (TRAP). Images were taken with an Eclipse TE300 inverted microscope (Nikon) using the 4× (H&E, TRAP, in vitro OC) or 40× (actin rings, pits) objectives. Histomorphometry was done using BioQuant Osteo.

Serum CTX and osteocalcin
Serum from overnight-fasted mice was measured by ELISA for CTX (RatLaps; Immunodiagnostic Systems) and osteocalcin (Biomedical Technologies Inc.) according to the manufacturer’s instructions.

Macrophage and OC generation
To generate macrophages, whole bone marrow cells were cultured in αMEM with 10% FBS and 100 ng/mL M-CSF for 3 days. To generate OCs, macrophages were cultured in αMEM, 10% FBS, 50 ng/mL M-CSF, and 50 ng/mL RANKL for 6 days (36, 38). Media was refreshed every 2 days. Cells were fixed and stained for TRAP using the leukocyte acid phosphatase kit (Sigma).

Lentiviral production and infection
293T cells were transfected with plasmid of interest, pHR8.2deltaR and pCMV-VSV-G using Xtreme Gene 9
Transduced cells were selected with 2

CAGAGGTGTAA; shSHH-3

the comparative threshold cycle (Ct)

quantiﬁed target and the endogenous gene (GAPDH for OC, cyclophilin was done using SsoFast EVA Green Supermix (Bio-Rad).

no-RT control was included in each assay. Quantitative PCR with DNaseI, and reverse transcribed using iScript (Bio-Rad). A

Quantitative reverse transcription PCR

RNA was extracted using RNeasy Mini kit (Qiagen), treated with DNasel, and reverse transcribed using iScript (Bio-Rad). A

no-RT control was included in each assay. Quantitative PCR was done using SsoFast EVA Green Supermix (Bio-Rad). Experiments were conducted in duplicate for both the target and the endogenous gene (GAPDH for OC, cyclophilin for BMSC and MDA-MB-231) used for normalization. Relative quantiﬁcation of the target gene expression was calculated by

the comparative threshold cycle (Ct) method: 2−ΔΔCt in which

ΔCt = Ct(target gene) − Ct(endogenous gene) and ΔΔCt = ΔCt(vehic le) − ΔCt(treated) (see Supplementary Methods for primer sequences).

Actin ring and bone resorption assay

A total of 3,000 day 3 pre-OCs differentiated as above were plated on bovine bone slices in 96-well plates. At day 6, actin rings and resorption lacunae were visualized as previously described (36).

BrdUrd proliferation assay

A total of 2.5 × 10^5 cells/mL were plated with indicated drug concentrations. Cells were labeled with BrdUrd for 24 hours and processed according to the manufacturer's instructions (Cell Proliferation ELISA; Roche).

Immunoblotting

Fifty micrograms of protein was separated on 8% SDS-polyacrylamide gels and transferred onto a polyvinylidene ﬂuoride membrane and incubated with p-AKT-Substrate, p-ERK, or total-ERK rabbit antibodies (Cell Signaling Technology), followed by horseradish peroxidase–conjugated anti-rabbit secondary antibody (Amersham Bioscience). Speciﬁc bands were developed by enhanced chemiluminescence. Loading control was β-actin (clone AC15; Sigma).

Tumor models

Intracardiac (1 × 10^5 cells) and intratibial (1 × 10^4 cells) tumor cell injections were carried out as previously described (36). For subcutaneous injections 1 × 10^6 (4T1 and B16) or 2 × 10^6 (MDA-MB-231) tumor cells were injected in a 1:1 ratio with Matrigel (BD Biosciences) as previously described (36, 38).

In vivo bioluminescence imaging

Imaging was done on a IVIS 100 device (Caliper Life Sciences) as previously described (36), except that for subcutaneous tumor image analysis, a software-deﬁned contour region of interest was used to measure total photon ﬂux.

MTT viability assay

A total of 5,000 cells per well were plated in 96-well plates with indicated concentrations of drug. After 48 hours, 10 μL MTT (Sigma) was added for 4 hours. HCl/isopropanol was added to measure absorbance at 570 and 530 nm.

Bone marrow chimeras

Recipient mice were lethally irradiated (1,000 rads). Twenty-four hours later, 1 × 10^6 donor whole bone marrow cells were transferred intravenously into recipient mice. After 4 weeks, hematopoietic reconstitution with the donor genotype was conﬁrmed by PCR of peripheral blood and mice were challenged with the B16 tumor.

Bone marrow stromal cell culture

To generate bone marrow stromal cells (BMSC), whole bone marrow of WT C57BL/6 mice was cultured in αMEM with 20% FBS for 7 days and adherent cells replated at 5 × 10^5 cells/mL. At conﬂuence, recombinant murine SHH (Ebioscienc e) or a 50:50 dilution of tumor cell conditioned media (CM, from 3 × 10^6 cells, 24 hours in serum-free media) was added and cultured for an additional 72 hours in αMEM with 2.5% FBS.

Statistical analysis

Experiments were analyzed using 2-tailed Student t test or ANOVA using Prism5 (GraphPad). Errors bars represent SEM. Results were considered to reach signiﬁcance at P ≤ 0.05 and are indicated with an asterisk (*).

Results

Cyclopamine increased bone mass and suppressed OC function in nontumor–bearing mice

To understand the effect of Hh signaling on adult nontumor–bearing bone, we administered the Hh inhibitor cyclopamine to adult mice by twice daily oral gavage for 14 days and observed increased trabecular bone volume and thickness (Fig. 1A–D). Despite a nonsigniﬁcant increase in OC number (Fig. 1E), serum CTX, a marker of osteoclastic bone resorption, was signiﬁcantly decreased with cyclopamine (Fig. 1F). Serum osteocalcin, a marker of OB activity, showed a nonsigniﬁcant reduction after Hh inhibition (Fig. 1G). Thus, pharmacologic inhibition of Hh signaling led to increased trabecular bone mass, with evidence of decreased OC function in vivo.

Disruption of Hedgehog signaling inhibited ex vivo osteoclastogenesis in a cell-autonomous manner

To test whether Hh signaling had direct effects on OC formation, we disrupted Smo(ΔH) by transducing SmoΔH

bone marrow macrophages (BMM) with a lentivirus expressing Cre-recombinase (Supplementary Fig. S1A) and subjected the...
cells to osteoclastogenesis. We observed a decrease in OC size and number after Smo excision compared with green fluorescent protein (GFP)-transduced control cells (Fig. 2A and B). Furthermore, pharmacologic inhibition of SMO with cycloamine (Supplementary Fig. S1B) or LDE225 (Fig. 2C) resulted in a dose-dependent decrease in TRAP⁺ multinucleated OC formation. Likewise, LDE225 reduced the mRNA abundance of the Hh target Gli1 and of the OC differentiation markers NFATc1 (Nfatc1), β3 integrin (Itgb3), and cathepsin K (Ctsk; Fig. 2D). Furthermore, treatment of pre-OCs with recombinant murine SHH increased mRNA transcripts of Gli1, Nfatc1, and Itgb3, suggesting that Hh stimulation enhanced signaling pathways involved in OC differentiation (Fig. 2E). These results suggested that Hh signaling through SMO is critical to normal OC formation in a cell-autonomous fashion.

Enhanced Hh signaling due to Ptch1 heterozygosity increased OC function in a cell-autonomous manner

Adult Ptch1⁺/− with enhanced Hh signaling had elevated serum CTX, indicating increased bone resorption (Fig. 3A). Interestingly, subjecting equal numbers of WT and Ptch1⁺/− BMMs to osteoclastogenesis, Ptch1⁺/− OC formed increased actin rings and resorption lacunae when plated on bone (Fig. 3B and C), but showed no difference in osteoclastogenesis on plastic (Supplementary Fig. S2A). We observed increased proliferation of Ptch1⁺/− BMMs (Fig. 3D) and increased levels of phospho-AKT substrates (p-AKTs) in day 3 Ptch1⁺/− pre-OC (Fig. 3E). However, we did not observe a difference in the expression of phosphorylated ERK in BMMs (Supplementary Fig. S2B), nor in rate of apoptosis of Ptch1⁺/− OCs (Supplementary Fig. S2C). From these data, we concluded that enhanced Hh signaling promoted macrophage proliferation and OC function.

Hedgehog pathway inhibition with cycloamine decreased bone metastases in a murine breast cancer model

To test whether Hh inhibition would reduce bone tumor burden, we challenged immunocompetent BALB/c mice with osteolytic murine 4T1 mammary breast carcinoma cells (35). Cycloamine significantly decreased tumor burden in bone after either intracardiac (Fig. 4A–D) or intratibial
These data showed that Hh antagonism decreased bone metastatic tumor growth in immunocompetent mice.

**Smo antagonists exert direct cytotoxic effects on 4T1 breast cancer cells**

*In vitro* analyses showed that 4T1 tumor cells have intact Hh signaling pathways that are responsive to SMO antagonism. Cyclopamine decreased the viability and proliferation of 4T1 cells in a dose-dependent manner (Fig. 5A and B). mRNA expression of the downstream target *Gli1* was also significantly decreased with cyclopamine (Fig. 5C). The small molecule SMO inhibitor GDC-0449 (Supplementary Fig. S3A) also decreased 4T1 viability, whereas tomatidine, an inactive structural analog of cyclopamine, had no effect (Supplementary Fig. S3B). Compared with vehicle controls, subcutaneous growth of 4T1 cells in BALB/c mice was significantly reduced by cyclopamine (Fig. 5D). These data were consistent with a direct inhibition of tumor growth; however, the specific contribution of host-targeted SMO antagonism could not be evaluated in this model.
Enhanced Hh signaling due to Ptch1 heterozygosity indirectly enhanced tumor growth

To examine the possibility that Hh signaling may play a role in various tissues through mechanisms independent of direct antitumor actions, we evaluated tumor growth in Ptch1+/−/C0 mice. Following intracardiac injection of osteolytic B16 cells into immunocompetent C57Bl/6 mice, metastatic bone tumor burden was significantly increased in Ptch1+/−/C0 mice compared with WT littermates (Fig. 6A–D). We also observed an increase in subcutaneous B16 tumor burden in Ptch1+/−/C0 mice (Fig. 6E), suggesting that the tumor-promoting effects of enhanced Hh signaling in the host are not specific to the bone environment. To test whether this was due to cells of hematopoietic origin (including myeloid and immune cells), we established radiation chimeras of WT and Ptch1+/−/C0 recipient mice reconstituted with reciprocal WT or Ptch1+/−/C0 bone marrow. In both WT and Ptch1+/−/C0 recipients, reconstitution with Ptch1+/−/C0 hematopoietic cells increased B16 subcutaneous tumor burden.
Antitumor Effect of Hedgehog Inhibition in Host Microenvironment

Figure 5. SMO antagonists exert direct cytotoxic effects on 4T1 breast cancer cells. 4T1 cells treated for 24 hours with cyclopamine had decreased A, viability as measured by MTT assay, B, proliferation as measured by BrdUrd incorporation, and C, expression of Gli1 by qRT-PCR (10 μmol/L cyclopamine). D, tumor weight on day 11 after subcutaneous injection of 4T1 cells into WT BALB/c mice after vehicle or cyclopamine (n = 9 per group) treatment starting on day −1. qRT-PCR, quantitative reverse transcription PCR.

growth compared with reconstitution with WT bone marrow (Fig. 6F and G). Flow cytometry showed no significant differences in the extent of hematopoietic reconstitution (Supplementary Fig. S4A–C). These data suggested that host Hh signaling, in part through hematopoietic cells, influenced tumor growth indirectly through the microenvironment, independent of direct effects on tumor cells.

MDA-MD-231 breast cancer cells are resistant to direct cytotoxic effects of SMO antagonists

MDA-MB-231 human breast cancer cells are reported to have nondetectable levels of Smo transcripts and to be resistant to killing by SMO antagonists (7, 39). In agreement, we did not observe significantly decreased viability even at micromolar concentrations of LDE225 (Fig. 7A). Reports of cytotoxicity in sensitive tumor cell lines are in the nanomolar range (40), thus showing relative resistance of this tumor to in vitro cytotoxic effects. Furthermore, LDE225 treatment did not decrease the expression of Hh target genes PTCH1 or GLI2 in MDA-MB-231 cells (Supplementary Fig. S5A and B). These data suggested that MDA-MB-231 cells are resistant to direct effects of pharmacologic Hh inhibition, allowing for a system in which to examine the microenvironment-targeted effects of SMO antagonists.

Hh signaling in host microenvironment cells influence tumor growth in vivo

To evaluate the roles of Hh inhibition directly on cells present in the tumor microenvironment, subcutaneous growth of MDA-MB-231 Hh inhibition "resistant" tumor cell lines was evaluated. Although the decreased growth of Hh inhibition "sensitive" 4T1 cells could be attributed to direct cytotoxic effects on the tumor cells (Figs. 4 and 5), effects of SMO antagonists on growth of "resistant" MDA-MB-231 tumors would be specifically due to modulation of the host microenvironment. In nude mice, subcutaneous growth of MDA-MB-231 cells was significantly reduced by LDE225 (Fig. 7B), showing that Hh inhibition isolated to host cells can modulate tumor growth.

Tumor-derived Sonic hedgehog increased tumor growth through effects on the microenvironment

Although MDA-MB-231 cells are unresponsive to canonical Hh pathway stimulation, their production of Hh ligands, particularly SHH (Fig. 7C), could stimulate Hh signaling in surrounding tissues in a paracrine fashion. To investigate the effects that tumor-produced Hh ligands have on the microenvironment, SHH expression was decreased in MDA-MB-231 cells by approximately 70% using 2 lentivirally expressed shRNAs (Fig. 7D). Following knockdown, cells maintained in vitro proliferation rates similar to parental cells (Supplementary Fig. S5C). In vivo, MDA-shSHH-2 and MDA-shSHH-3 formed significantly smaller subcutaneous tumors than parental or control MDA-shLacZ tumors, showing that tumoral production of Hh ligands can increase growth of tumors that fail to respond to canonical Hh stimulation in an autocrine signaling (Fig. 7E).

Stromal cells within the microenvironment produce a variety of tumor-supporting growth factors. To evaluate the effects of tumor-derived SHH on stromal cells, we added CM from parental MDA or MDA-shLacZ cells to murine BMSCs and found that increased transcription of Gli1 to similar levels as recombinant Shh (Supplementary Fig. S5D). In contrast, CM from MDA-shSHH-2 or MDA-shSHH-3 failed to induce BMSC Gli1, suggesting that Hh signaling was blunted. BMSC production of IL-6, a protumor and proosteoclastogenic factor, was increased by recombinant SHH (Fig. 7F). CM from parental MDA or MDA-shLacZ further induced IL-6 transcription (Fig. 7F) and secretion (Supplementary Fig. S5E) in BMSCs, whereas that of MDA-shSHH-2 or MDA-shSHH-3 cells induced it to a lower extent. Although tumor cells produce an abundance of factors that affect numerous stromal cells signaling pathways, this data suggested that BMSC production of IL-6 is due in part to stimulation of the Hh pathway. These data suggested that Hh inhibition in host microenvironment cells can reduce tumor burden indirectly, even when tumor cells themselves are resistant to direct Hh inhibition.

Discussion

Although the majority of current pharmaceuticals used in the treatment of cancer directly target tumor cell growth and survival, a growing body of evidence has shown that many components of the host microenvironment are critical to tumorigenesis and represent additional therapeutic targets. Thus, therapeutic manipulation of this pathway has the potential to decrease tumor growth both through direct and indirect mechanisms. Intratumoral Hh pathway signaling has been

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shown to be vital for the growth and maintenance of many tumor types (3, 4). However, a number of tumors have been shown to be refractory to the direct effects of pharmacologic Hh inhibition with SMO antagonists due to natural or acquired mutations in Smo (24, 41) or amplification of downstream effector Gli2 (40, 42).

Previous reports have shown that tumor growth was blunted when paracrine Hh signaling was inhibited in stromal components of the microenvironment, even when the tumor itself is SMO independent (19, 22, 43). The Hh inhibitor GDC-0449 induced dramatic reductions in the growth of tumors with activating Hh mutations (44, 45). Interestingly, GDC-0449 has little direct effect on tumors without Hh mutations; however, it significantly blocked Hh signaling in tumor stroma and decreased tumor burden (46).

In this article, we found that the SMO antagonist LDE225 had potent in vivo antitumor activity in MDA-MB-231, an aggressive breast tumor cell line relatively resistant to Hh pathway modulation due to undetectable levels of Smo (7, 39). Furthermore, we show that tumoral production of the Hh ligand SHH supported growth of subcutaneous tumors in vivo. We provide evidence that this effect is due to paracrine stimulation of the Hh signaling pathway in stromal cells, resulting in the increased production of growth factors, including, but not limited to, IL-6. We also show that enhanced Hh signaling in the host environment of Pchh1+/− mice promoted growth of bone metastases and subcutaneous tumors, in part, through contributions of hematopoietically derived cells, including, but not limited to, immune cells and OC. Although the mechanisms underlying the described phenotypes still need to be further elucidated, these data underscore a role for Hh inhibition in cells of the microenvironment, even in Hh-unresponsive tumor cells, and highlights the potential for increased clinical utility of Hh inhibition in cancer treatment.

In addition to its role in tumorigenesis, Hh signaling is crucial to proper development and maintenance of many host tissues including bone (25, 26). However, studies into the role of Hh signaling in postnatal bone have yielded disparate results. Enhancing Hh signaling through germline Pchh1 heterozygosity resulted in increased bone density (33), whereas conditional homozygous deletion of Pchh1 in mature OB decreased bone density (32). Both groups observed increases in bone formation and resorption; however, the effects of Hh signaling on the OC were attributed to be indirect via increased OB expression of RANKL. Here we report a previously unrecognized cell-autonomous role for Hh signaling in the differentiation of bone-resorbing OCs.

Figure 6. Enhanced Hh signaling due to Pchh1 heterozygosity indirectly enhanced tumor growth. A–D, following intracardiac injection of B16 cells, tumor burden was increased in Pchh1+/− (n = 6) mice as compared with WT littermates (n = 5) by (A and B) BLI and (C and D) histomorphometric analysis of H&E-stained tibiae on day 12. M = marrow; T = tumor. E, tumor weight on day 11 after subcutaneous injection of B16 cells (WT, n = 4; Pchh1+/−, n = 5). F and G, B16 subcutaneous tumor burden by BLI on day 14 in WT and Pchh1+/− reciprocal bone marrow chimeras. F, subcutaneous tumors of WT recipients of Pchh1+/− bone marrow (n = 5) compared with recipients of WT bone marrow (n = 5; P = 0.3009). G, Pchh1+/− recipients reconstituted with Pchh1+/− bone marrow (n = 5) showed a trend toward increased tumor growth compared with those receiving WT bone marrow (n = 4; P = 0.0760). BLI, bioluminescence imaging.
We found that genetic and pharmacologic Hh inhibition decreased OC differentiation in vitro. Furthermore, transcription of target gene Gli1 and key genes involved in OC differentiation (Nfatc1, Itgb3, and Ctsk) were reduced with Hh inhibition. These results are in agreement with a recent report showing that RAW cell differentiation into OCs could be inhibited with cyclopamine (47). Ptch1−/−/C0 mice, in which Hh signaling is enhanced, are known to have increased bone mineral density and OB activity (33). Concurrently, we observed Ptch1−/−/C0 mice have enhanced OC activity in vitro. Together, these data suggest a direct, cell-autonomous role for Hh signaling in the OC.

We report that systemic Hh inhibition with cyclopamine increased bone density of adult WT mice, whereas a previous study found decreased bone density (33). Several differences between treatment protocols (i.e., administration and dosing), and importantly, recognized gender differences in bone biology, may explain these seemingly paradoxical results. The previous study dosed male mice with 10 mg/kg cyclopamine intraperitoneally once a day (33), whereas we used 25 mg/kg orally twice daily in females. Twice daily administration and/or increased drug dosage could result in more continuous or potent inhibition of Hh and OC function, leading to increased bone density. Similarly, continuous administration of parathyroid hormone has catabolic effects on bone, whereas intermittent dosing is anabolic (48). Hh signaling in bone homeostasis seems tightly regulated by the strength of signaling, as suggested in discrepant results between heterozygous (33) and homozygous loss of Ptch1 (32). Overall, our results and those of others (32, 33) suggest that level and regulation of Hh signaling in the bone microenvironment is important to properly regulate the extent of bone formation and resorption occurring under nonpathologic conditions.

As OC and tumor cells are both known to produce growth factors that support the activity of the other, the bone microenvironment is a hotspot of tumor metastasis, known as "the vicious cycle" (34). Thus, blunting tumor-driven manipulation of bone remodeling and turnover can indirectly decrease the expansion of tumor in bone. Here we show that mice treated with cyclopamine had decreased tumor burden in bone. However, as subcutaneous tumor
growth was also decreased, the antitumor activity of Smo antagonists was due at least partially to non-bone cell effects, including those directly on tumor cells and on host stromal cells.

In conclusion, our data show that components of the Hh signaling pathway are promising therapeutic targets for cancer as they have the ability to decrease tumor growth both by exerting direct antitumor effects and by making the host microenvironment less hospitable to tumors. Hh inhibition has now been shown to act on a variety of host microenvironmental cells, including stroma, hematopoietic cells, and vasculature (49, 50) to contribute to the overall therapeutic effect. In addition to these effects on solid tumors, targeting the Hh pathway is a particularly attractive target for the treatment of bone metastases as it may prove beneficial in interrupting the vicious cycle of OB, OC, and tumor cells and effectively decrease both tumor burden and tumor-associated osteolysis, which are linked to high rates of mortality and morbidity. As several small-molecule Hh signaling inhibitors are currently being clinically evaluated for efficacy in a variety of tumor types, their effect on Hh signaling in cells of the tumor microenvironment warrants active investigation.

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

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