# TGF-β Promotion of Gli2-Induced Expression of Parathyroid Hormone-Related Protein, an Important Osteolytic Factor in Bone Metastasis, Is Independent of Canonical Hedgehog Signaling

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## Abstract

Breast cancer frequently metastasizes to bone, in which tumor cells receive signals from the bone marrow microenvironment. One relevant factor is TGF-β, which upregulates expression of the Hedgehog (Hh) signaling molecule, Gli2, which in turn increases secretion of important osteolytic factors such as parathyroid hormone–related protein (PTHrP). PTHrP inhibition can prevent tumor-induced bone destruction, whereas Gli2 overexpression in tumor cells can promote osteolysis. In this study, we tested the hypothesis that Hh inhibition in bone metastatic breast cancer would decrease PTHrP expression and therefore osteolytic bone destruction. However, when mice engrafted with human MDA-MB-231 breast cancer cells were treated with the Hh receptor antagonist cyclopamine, we observed no effect on tumor burden or bone destruction. *In vitro* analyses revealed that osteolytic tumor cells lack expression of the Hh receptor, Smoothened, suggesting an Hh-independent mechanism of Gli2 regulation. Blocking Gli signaling in metastatic breast cancer cells with a Gli2-repressor gene (Gli2-rep) reduced endogenous and TGF-β–stimulated PTHrP mRNA expression, but did not alter tumor cell proliferation. Furthermore, mice inoculated with Gli2-Rep–expressing cells exhibited a decrease in osteolysis, suggesting that Gli2 inhibition may block TGF-β propagation of a vicious osteolytic cycle in this MDA-MB-231 model of bone metastasis. Accordingly, in the absence of TGF-β signaling, Gli2 expression was downregulated in cells, whereas enforced overexpression of Gli2 restored PTHrP activity. Taken together, our findings suggest that Gli2 is required for TGF-β to stimulate PTHrP expression and that blocking Hh-independent Gli2 activity will inhibit tumor-induced bone destruction. *Cancer Res; 71(3); 822–31. ©2010 AACR.*

## Introduction

Despite advances in the prevention and treatment of breast cancer, it remains the second leading cause of cancer deaths in women (1) which is, in part, due to its propensity to metastasize to distant organs such as lung and bone. Breast cancer patients who develop bone metastases suffer increased morbidity and mortality, with increased fracture risk and the possibility of hypercalcemia among other complications (2, 3). Although survival rates among breast cancer patients with controlled local disease remain high, patients with advanced disease suffer from a 71% decrease in survival (4). Therefore, it is critical that new approaches be generated for the prevention and treatment of breast cancer metastasis to bone.

Breast cancer metastasis to bone begins with initiation of a "vicious cycle" of bone destruction, commencing upon tumor cell establishment in the bone marrow and resulting in increased bone resorption, or osteolysis. Tumor cells receive signals from the bone marrow environment (e.g., transforming growth factor-β; TGF-β), which upregulates expression of the Hedgehog (Hh) signaling transcription factor, Gli2, and leads to increased expression and secretion of the osteolytic factor parathyroid hormone-related protein (PTHrP; ref. 4). PTHrP propagates the vicious cycle via PTH receptor binding on osteoblasts, leading to increased RANKL expression, which induces osteoclastogenesis. Because the bone is resorbed, active TGF-β is released from the bone matrix stimulating further tumor growth and PTHrP expression (5). Inhibition at any point in this process should reduce bone destruction. For example, neutralizing antibodies against tumor production of PTHrP inhibits osteolytic bone destruction and tumor burden *in vivo* (6, 7). Although

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a humanized anti-PTHrP antibody was developed in 2003, no official report has been made about the success of this antibody in patients (8, 9). Our laboratory has previously demonstrated that the Hh signaling transcription factor Gli2 positively regulates PTHrP expression and secretion in osteolytic breast tumor cells (10).

Canonical Hh signaling occurs through Hh ligand binding to the membrane receptor Patched (Ptch), which releases inhibition of a second membrane receptor, Smoothened (Smo). This release initiates a downstream signaling cascade resulting in translocation of the Gli family proteins to the nucleus, where they can initiate transcription (11). Gli protein activation has been demonstrated in numerous tumor types and results from a variety of mutations that occur throughout the Hh signaling pathway (12). In these tumor types, Hh receptor antagonists, like cyclopamine, have been used successfully to prevent Gli overexpression (13).

Although all Gli family members bind to the same binding sequence, they have separate and discrete functions in mammalian cells (14). We have shown that Gli2, but not the other Gli family members, enhances PTHrP expression. Furthermore, expression of Gli2 appears limited to tumor cells that have high metastatic potential, especially to bone resulting in osteolytic lesions (10).

Taken together these data suggest that inhibition of Gli2 is a potential target for the development of therapeutics aimed at preventing and treating bone metastases. Therefore, we hypothesized that inhibition of Gli2 in bone metastatic lines would decrease PTHrP expression and therefore osteolytic lesions.

## Materials and Methods

### Cells

The human breast cancer cell line MDA-MB-231 was obtained from ATCC (American Type Culture Collection) and a bone metastatic variant generated in our lab was used for all *in vitro* and *in vivo* experiments, as previously published (6, 10). MDA-MB-231 cells, the human squamous non–small cell lung carcinoma cell line RWGT2 (15), and human metastatic prostate cancer cell line PC3 (ATCC), were maintained in DMEM (Cellgro) plus 10%FBS (Hyclone Laboratories) and 1% penicillin/streptomycin (P/S; Mediatech). The murine chondrocyte cell line TMC23 and human nonosteolytic breast cancer cell line MCF-7 were cultured in α-MEM (Invitrogen) plus 10% FBS and 1% P/S. All cell lines are routinely tested for changes in cell growth and gene expression. MDA-MB-231 cells were transfected with either Gli2-His, GFP, pcDNA (empty vector) or Gli2-Rep, by Lipofectamine transfection reagent and Plus reagent (Invitrogen), per manufacturer's instructions, and stable cell lines were selected for antibiotic resistance into single-cell clones by limiting dilutions or pooled colonies under 400 μg/mL G418 selection medium and maintained in culture medium supplemented with 200 μg/mL G418.

### Animals

All animal protocols were approved by Vanderbilt University Institutional Animal Care and Use Committee and were conducted according to NIH guidelines. Female, 4-week-old athymic nude mice ($n = 8$, vehicle; $n = 13$, cyclopamine; and $n = 10$, empty vector; $n = 7$, Gli2-Rep) were anesthetized by continuous isoflurane and inoculated with 100,000 MDA-MB-231, MDA-231-cntrl (control), or MDA-231-Gli2-Rep cells resuspended in PBS via intracardiac injection into the left cardiac ventricle using a 27-gauge needle, as previously described (10, 16, 17). Mice were imaged weekly and sacrificed 4 weeks post-tumor cell inoculation. For mammary fat pad injections, female 4-week-old athymic nude mice ($n = 8$ per group) were anesthetized by continuous isoflurane and an incision was made on the ventral lower abdomen. The left inguinal mammary gland was inoculated with 1,000,000 MDA-231-Gli2-Rep or MDA-231-cntrl cells resuspended in PBS. Tumor size was assessed by caliper measurements twice per week. Mice were sacrificed 3 weeks after tumor cell inoculation, and tumors excised, measured, and weighed.

### Radiographic imaging

Mice were radiographically imaged weekly beginning 1-week post-tumor cell inoculation using a Faxitron LX-60. Specifically, mice were anesthetized deeply with ketamine/xylazine and laid in a prone position on the imaging platform. Images were acquired at 35 kVp for 8 seconds. Lesion area and number were measured using quantitative image analysis software (Metamorph, Molecular Devices, Inc.) by region of interest analysis. All data are represented as mean lesion area and number per mouse.

### TGF-β/cyclopamine treatments

Cyclopamine (LC Labs) was reconstituted in (2-hydroxypropyl)-β-cyclodextrin solution 45% (w/v) in HOH (Sigma-Aldrich). Beginning 2 weeks post-tumor cell inoculation, mice were treated daily with either 10 mg/kg of cyclopamine or control tomatadine analogue by i.p. injection, as previously published (13). For *in vitro* studies, 1 to 10 μmol/L (with data from 3 μmol/L shown) of cyclopamine or tomatadine was added to cell culture medium and cells were harvested 24 hours later. Recombinant TGF-β (R&D Systems, Inc.) was reconstituted in 4 mmol/L of HCl and 1 mg/mL bovine serum albumin (BSA), and added at 5 ng/mL to serum free cell culture medium (or as indicated in text).

### Histology/histomorphometry

Hind-limb specimens (tibiae and femora) were removed during autopsy and fixed in 10% neutral-buffered formalin (Fisher Scientific) for 48 hours at room temperature. Bone specimens were decalcified in 10% EDTA for 2 weeks at 4°C and embedded in paraffin. Bone sections (5-μm thickness) were stained with hematoxylin & eosin (H&E), orange G, and phloxine. Tumor burden in the femora and tibiae was examined under a microscope and quantified using Metamorph software (Molecular Devices, Inc.) and region of interest analysis.

### Reverse-transcriptase PCR

RNA was extracted from cells using RNeasy Mini Kit (QIAgen), per manufacturer's instructions. cDNA was

synthesized using SuperScript III First-Strand Synthesis System for reverse-transcriptase PCR (RT-PCR; Invitrogen) and random hexamers from 1 to 5 μg of total RNA per manufacturer's instructions. cDNA (1.0 μL) was used for RT-PCR using Platinum PCR SuperMix (Invitrogen). RT-PCR was conducted for the human homologues of the Hh signaling receptors Ptch and Smo. Primers for amplifying hPtch are as follows: F, 5′-CGCCTATGCCTGTCTAACCATGC-3′; R, 5′-TAAATCCATGCTGAGAATTGCA-3′. PCR was performed on the Bio-Rad iCycler with the following cycling conditions: 94°C for 2 minutes, (94°C for 30 seconds, 66°C for 1 minute, 72°C for 30 seconds) ×35 cycles, 72° for 2 minutes. Primers for hSmo amplification are as follows: F, 5′-TTACC-TTCAGCTGCCACTTCTACG-3′; R, 5′-GCCTTGGCAATCAT-CTTGCTCTTC-3′. PCR was performed with the following cycling conditions: 94°C for 4 minutes, (94°C for 30 seconds, 56°C for 1 minute, 72°C for 45 seconds) ×35 cycles, 72° for 2 minutes.

### Quantitative real-time RT-PCR

PTHrP, Gli2, and 18s mRNA expression were measured by Quantitative Real-Time RT-PCR (Q-PCR). After 48-hour incubation, cells were harvested for mRNA, and a cDNA strand was generated as described above. cDNA was serially diluted to create a standard curve, and combined with TaqMan Universal PCR Master Mix (Applied Biosystems), and primer: TaqMan PTHLH (Hs00174969_m1), TaqMan Gli2 (Hs00257977_m1), or TaqMan Euk 18S rRNA (4352930-0910024; Applied Biosystems). Samples were loaded onto an optically clear 96-well plate (Applied Biosystems) and the Q-PCR reaction was performed under the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, (95°C for 15 seconds, 60°C for 1 minute) x40 cycles on the 7300 Real-Time PCR System (Applied Biosystems). Q-PCR reactions were quantified using the 7300 Real-Time PCR Systems software (Applied Biosystems).

### Cell proliferation assay

*In vitro* cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega). Briefly, 2,000 cells per well were plated in 96-well plates in quadruplicate, and growth was measured at days indicated spectrophotometrically at 450 nm on a Synergy2 plate reader, per manufacturer's instructions.

### Western blot

Cells were harvested for protein into a 1× radioimmunoprecipitation assay (RIPA) lysis buffer (ThermoScientific) supplemented with a cocktail of protease inhibitors (Roche). Equal protein concentrations were prepared for loading with Laemmli sample buffer and electrophoresis was performed on SDS-PAGE Mini-Protein II ready gels (Bio-Rad). Separated proteins were then transferred to polyvinylidene fluoride (PVDF) in transfer buffer [25 mmol/L of Tris, 192 mmol/L of glycine, 20% (v/v) methanol (pH 8.3)] at 100 V at 4°C for 1 hour. Membranes were blocked with 1× Tris-buffered saline (TBS) buffer containing 1% Tween 20 (1× TBS with Tween, TBST) for 1 hour at room temperature and incubated with a 1:200 dilution of Omni-probe α-His antibody (Santa Cruz Biotechnology) in blocking buffer. The membrane was washed with 1× TBST and signal was detected using an enhanced chemiluminescence system (Amersham). Membrane was stripped using Restore Western Blot Stripping Buffer (Thermo Scientific), washed with 1× TBST and reprobed with a 1:5,000 dilution of β-actin antibody (Sigma) as a loading control.

### Microcomputed tomography

Tibiae were analyzed using the Scanco μT 40. Specifically, 100 slices from the proximal tibia were scanned at 12-μm resolution. Images were analyzed using the Scanco Medical Imaging software to determine the bone volume/total volume (BV/TV), trabecular number and thickness, and connectivity density.

### Statistical analyses

All statistical analyses were performed using InStat version 3.03 software (GraphPad Software, Inc.). Values are presented as mean ± SEM, and $P$ values determined using unpaired $t$ test, where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ unless otherwise stated.

## Results

### Cyclopamine treatment does not prevent tumor-induced bone destruction *in vivo*

Cyclopamine treatment has previously been reported to block Hh signaling and inhibit tumor growth in some *in vivo* models (18) through its action as a Smo antagonist (19). Therefore, we first proposed to determine the effects of cyclopamine treatment on osteolytic bone metastases. Mice inoculated with MDA-MB-231 human breast cancer cells were treated with 10 mg/kg of cyclopamine or control tomatadine analogue daily, beginning 2 weeks postinoculation and after tumor cells had seeded to bone, similar to previously published treatment regimens which successfully inhibited tumor growth (20). Surprisingly, cyclopamine inhibited neither tumor growth nor cancer-induced bone disease in the bone metastasis model. Indeed, examination by radiography (Fig. 1A) indicated that there was a slight increase in osteolytic lesion number in mice treated with cyclopamine when compared with tomatadine-treated mice. After sacrifice, bones were collected from tumor-bearing mice from both treatment and control groups for histology and histomorphometry. Histologically, cyclopamine treatment had little or no effect on tumor burden in bone (Fig. 1B) or trabecular bone volume at multiple sites commonly examined by histology (Fig. 1C). It was concluded that the Hh receptor antagonist cyclopamine is ineffective treatment for osteolytic bone metastases in this experimental model of breast cancer–induced bone destruction.

### Canonical Hh signaling does not regulate Gli2 in MDA-MB-231 cells

To determine why MDA-MB-231 breast cancer cells are not inhibited by cyclopamine, we next examined the expression of

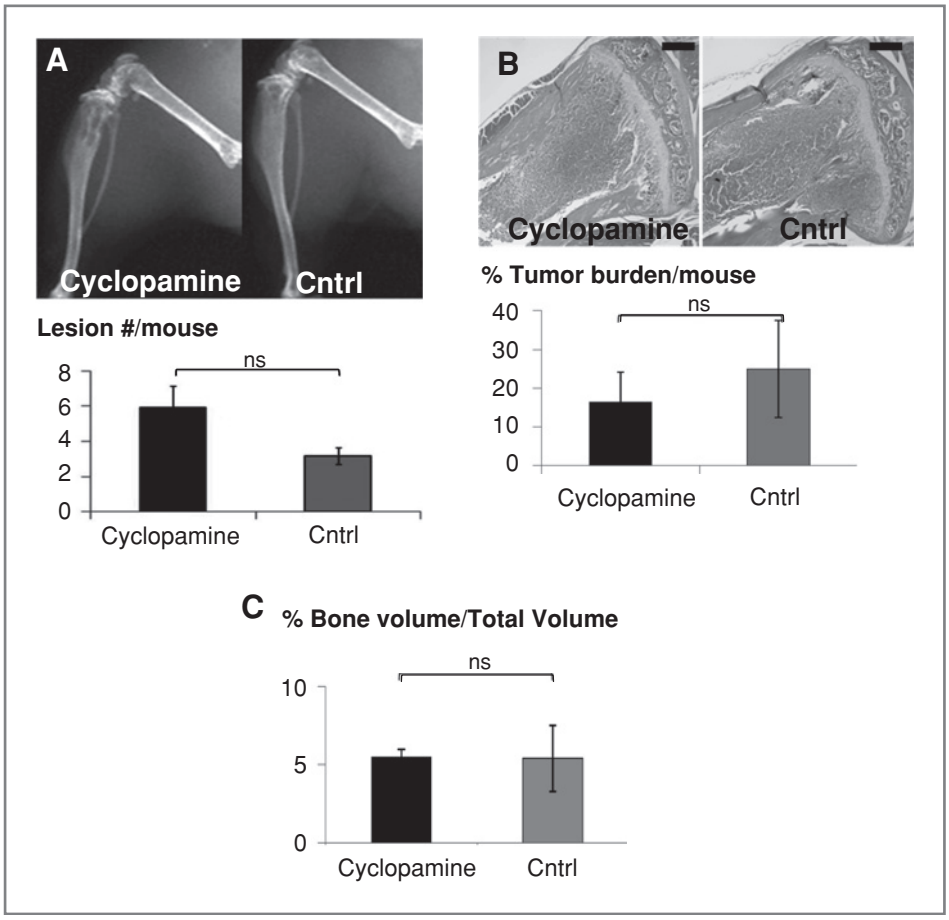


Figure 1. Cyclopamine does not reduce osteolysis or tumor burden. Mice were treated daily with 10 mg/kg of cyclopamine ($n = 13$) or control tomatadine analogue ($n = 8$) and imaged radiographically weekly. A, Faxitron analyses indicate no significant difference (ns) in lesion number in cyclopamine-treated versus nontreated mice. There was no significant (ns) change in (B) tumor burden detected by % tumor area, or (C) bone volume measured as trabecular BV/TV, on histomorphometric analyses of H&E-stained sections. The black bar on the histologic sections represents a length of 500 μm. Values = mean ± SE, and *P* values were determined using unpaired *t* test.

Hh signaling receptors. MDA-MB-231 cells, which express both Gli2 and PTHrP and are known to cause osteolysis, did not express Smo mRNA, and MCF-7 breast cancer cells (nonosteolytic) and RWGT2, a squamous non–small cell lung carcinoma cell line, expressed low levels of Smo mRNA (Fig. 2A). In contrast, PC-3 prostate cancer cells were found to express Smo mRNA, consistent with findings by another group that showed these cells are inhibited by cyclopamine (Fig. 2A; ref. 13).

Treatment of MDA-MB-231 cells with cyclopamine *in vitro* did not alter tumor cell growth over a 7-day treatment period (Fig. 2B), indicating that Hh signaling does not play a significant role in the growth of these cancer cells. RWGT2 cell growth was also not inhibited by cyclopamine, despite low levels of Smo expression (data not shown). We next examined cyclopamine-treated MDA-MB-231 and RWGT2 cells for PTHrP and Gli2 expression by Q-PCR. As a positive control, we also tested the cyclopamine-treated chondrocyte cell line, TMC-23. Blocking Hh signaling had no effect on PTHrP mRNA expression in either of the tumor cell lines, and cyclopamine treatment did not inhibit and in fact increased Gli2 mRNA in both the MDA-MB-231 and RWGT2 cells (Fig. 2D and E). Because Hh signaling is known to regulate PTHrP expression in proliferating chondrocytes (21), we reasoned that TMC-23 cells would be inhibited by cyclopamine, and as expected, PTHrP expression was indeed inhibited by cyclopamine treatment in TMC-23 cells (Fig. 2C). Together, these data confirm that the compound was active and suggests that osteolytic tumor cells rely on an alternative mechanism for PTHrP expression. Thus, Hh signaling inhibition by cyclopamine appears ineffective toward breast tumor cell growth or expression of osteolytic factors by tumor cells, and we conclude that elevated Gli2 expression levels in MDA-MB-231 cells is not due to enhanced Hh signaling through the canonical pathway.

### Gli2 inhibition in human bone metastatic breast cancer cells

Because cyclopamine treatment was ineffective at blocking downstream Gli2 expression in osteolytic tumor cells, targeted Gli2 inhibition was explored. To specifically inhibit Gli2 expression, tumor cells were transfected with a Gli2-repressor construct (Gli2-Rep) in which the activation domain of the Gli2 promoter has been replaced with the engrailed repressor domain as previously described (22). We have found that this construct efficiently knocks down Gli2 activity *in vitro* (10). After generating stable cell lines, positive cells were selected on the basis of neomycin resistance, with both single-cell clones and pools of transfectants generated. Because of concerns that single cell clones may result in a selection of a population with a constitutive

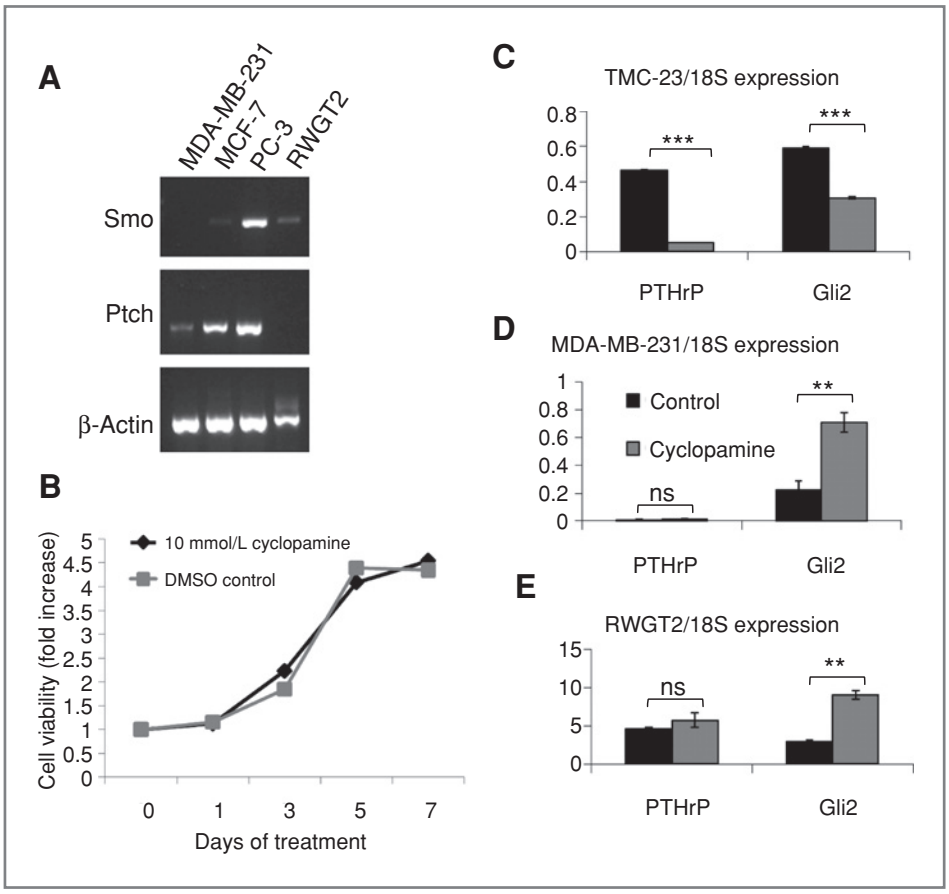


**Figure 2.** Gli2 is regulated by a Hedgehog independent mechanism. A, PCR analyses for Ptch and Smo receptor expression in osteolytic and nonosteolytic cell lines show MDA-MB-231 cells do not express Smo. B, MDA-MB-231 cells treated with 3 μmol/L of cyclopamine exhibit no significant (ns) difference in tumor cell growth by MTS assay. Tumor cells treated with 3 μmol/L of cyclopamine (gray bars) were compared with control tomatadine treatment (black bars) and examined after 24 hours for Gli2 and PTHrP mRNA expression by Q-PCR. C, Chondrocyte cell line TMC-23; D, MDA-MB-231; and E, RWGT2 cells. Values = mean ± SE, and *P* values were determined using unpaired *t* test. **, $P < 0.01$; ***, $P < 0.001$.

decrease in PTHrP or a decrease or increase in aggressiveness, we focused on the pooled populations for these studies. However, similar results were obtained for all populations tested. Positive cells were screened for the expression of Gli2-Rep with an anti-His-tag antibody by Western blot (Fig. 3A). Interestingly, expression of Gli2-Rep had no effect on tumor cell growth *in vivo* when cells were injected orthotopically into the mammary fat pad of athymic nude mice (Fig. 3B). Consistent with our previous observation that Gli2 stimulates PTHrP expression, the expression of Gli2-Rep caused a reduction in endogenous PTHrP mRNA expression in MDA-MB-231 cells (Fig. 3C).

As others and we have previously shown that TGF-β stimulates Gli2 and PTHrP expression (10, 23, 24), we hypothesized that blocking Gli2 activity would inhibit the ability of TGF-β to increase PTHrP expression. We therefore treated MDA-231-Gli2-Rep cells and MDA-231-cntrl (empty vector-transfected) cells with 5 ng/mL TGF-β in serum-free medium and measured PTHrP expression by Q-PCR. Inhibition of Gli2 activity completely blocked the TGF-β–induced increase in PTHrP expression (Fig. 3D). Importantly, the basal level of PTHrP in MDA-MB-231 cells was not affected by Gli2 inhibition when cells were grown in serum-free medium (Fig. 3D), as compared with its effect on cells grown in 10% FBS containing biologically active TGF-β (Fig. 3C). Our data suggest that direct Gli2 inhibition can attenuate TGF-β induction of PTHrP, and thereby abrogate subsequent PTHrP-mediated bone destruction.

### Gli2 inhibition reduces tumor-mediated osteolytic bone destruction

To determine if blocking Gli2 activity could indeed inhibit tumor-induced osteolysis, we inoculated pooled MDA-231-Gli2-Rep and MDA-231-cntrl cells into athymic female nude mice via the left cardiac ventricle. After 4 weeks, extensive osteolytic lesions were visible radiographically in the femora/tibiae of the control mice. In contrast, the Gli2-Rep mice had either small or undetectable lesions (Fig. 4A). Quantification of lesion number and size on radiographs indicated that mice bearing MDA-231-Gli2-Rep tumor cells had significantly fewer and smaller lytic lesions than the mice bearing control cells (Fig. 4B and C). Similar results were obtained for mice inoculated with Gli2-Rep cells derived from single cell clones (Supplementary Fig. 1).

In addition to radiographic analyses, tibiae were examined by *ex vivo* microcomputed tomography (micro-CT). There was a significant decrease in trabecular bone volume in the MDA-231-cntrl tumor-bearing mice compared with the MDA-231-Gli2-Rep tumor-bearing mice (Fig. 5A), although there were no other significant changes in micro-CT parameters such as trabecular number, trabecular thickness, or connectivity density (Supplementary Fig. 2). Bone volume of mice inoculated

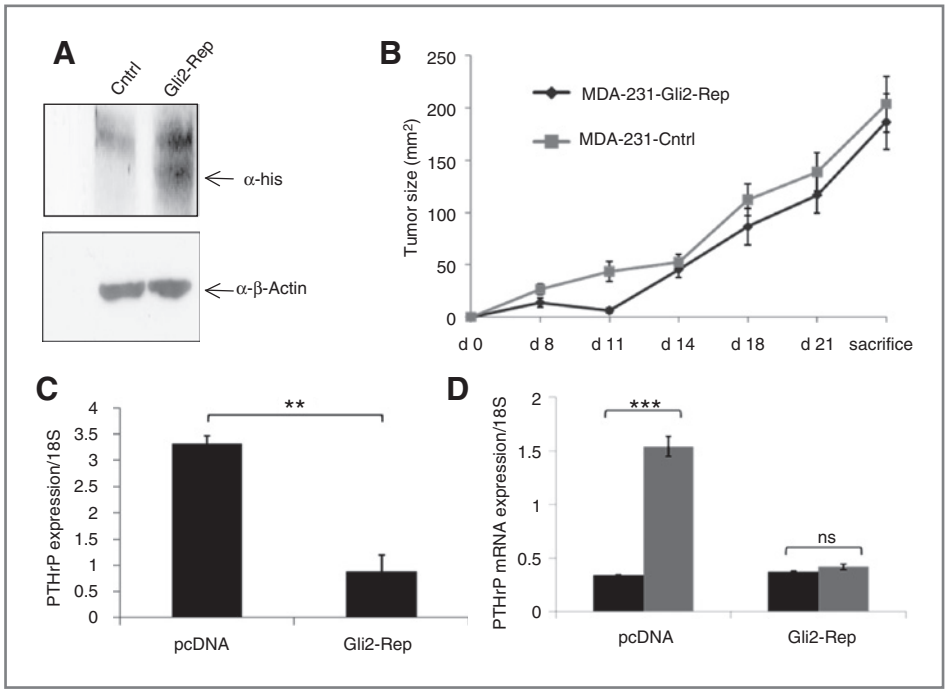


**Figure 3.** Gli2-Rep blocks downstream PTHrP, but not tumor growth directly. Pooled populations of MDA-MB-231 cells transfected with the Gli2-Rep construct and selected for antibiotic resistance were examined by (A) Western blot to verify efficient expression of Gli2-Rep. B, MDA-231-Gli2-Rep ($n = 8$) or MDA-231-cntrl ($n = 8$) cells injected into the mammary fat pad of mice and sacrificed after 3 weeks to determine tumor cell growth *in vivo*. C, MDA-231-Gli2-Rep cells grown in culture medium supplemented with 10% FBS and examined for PTHrP mRNA expression by Q-PCR have reduced PTHrP mRNA expression. D, MDA-231-cntrl or MDA-231-Gli2-Rep cells grown in serum-free medium and treated with 5 ng/mL exogenous TGF-β. Values = mean ± SE, and *P* values were determined using unpaired *t* test. **, $P < 0.01$; ***, $P < 0.001$.

with MDA-231-Gli2-Rep cells was not significantly different from non–tumor-bearing, age- and sex-matched mice (BV/TV = 0.0448–0.1518). These data indicate that Gli2 inhibition in MDA-MB-231 cells decreased the ability of the tumor cells to induce bone destruction.

Consistent with disrupting TGF-β–induced propagation of tumor-induced osteolysis, histomorphometric analyses of the tibiae revealed a significant decrease in tumor burden in Gli2-Rep tumor-bearing mice relative to empty vector control tumor-bearing mice (Fig. 5B), and this was accompanied by a reduction in detectable PTHrP protein expression identified by immunohistochemistry (Fig. 5C).

### TGF-β regulation of Gli2 signaling in human osteolytic breast cancer cells

Because TGF-β is known to regulate PTHrP, and other groups have shown that TGF-β regulates Gli2 expression in hepatocarcinoma cells (24), we set out to determine if TGF-β stimulates Gli2 expression in osteolytic tumor cells. MDA-MB-231 cells were tested to determine if TGF-β enhances Gli2 mRNA expression following treatment with 5 ng/mL exogenous TGF-β for 48 hours. Consistent with previous studies in other cell types, TGF-β significantly enhanced Gli2 and PTHrP mRNA expression (Fig. 6A) in MDA-MB-231 cells by Q-PCR. As expected, TGF-β also increased PTHrP protein secretion (Fig. 6B).

To determine if TGF-β signaling is necessary for Gli2 upregulation in bone metastatic breast cancer cells, we disrupted this signaling pathway through overexpression of a dominant negative TGF-β receptor type II (DNRII) construct in the MDA-MB-231 cells, as previously published (17). When TGF-β signaling was abolished in this way, both Gli2 (Fig. 6C) and PTHrP mRNA expression (Fig. 6D, right) were significantly decreased. These data suggest that Gli2 inhibition impairs the ability of TGF-β to stimulate PTHrP production, resulting in abrogation of tumor-induced bone destruction. In addition, overexpression of Gli2 protein in the MDA-231-DNRII cells resulted in a dramatic increase in PTHrP promoter activation and a partial rescue of PTHrP mRNA expression (Fig. 6D), further suggesting that Gli2 expression is downstream of TGF-β signaling. The stimulatory effect of TGF-β on PTHrP expression is thus likely to be mediated through Gli2.

## Discussion

Our results suggest for the first time that Gli2 inhibition can prevent the formation of breast cancer bone metastases. Many tumor types have mutations that lead to a constitutive activation of Gli proteins, demonstrating a clinical need for identifying targets to inhibit this pathway. Current therapeutic approaches focus primarily on the development of Smo antagonists, such as cyclopamine analogues. However, our data suggest that at least in some cancers, cyclopamine analogues are unlikely to be effective in tumor-induced osteolysis and that direct inhibition of Gli2 activity downstream of Hh receptors has better therapeutic potential for tumor-induced bone disease.

Our studies indicate that several osteolytic tumor cell lines, including human breast cancer MDA-MB-231 cells, either do not express Smo or express it at very low levels, providing an explanation for the observed failure of cyclopamine to inhibit PTHrP expression or tumor-induced bone disease. In light of these data, the expression levels of Hh receptors, Smo and Ptc, would have been useful prior to *in vivo* studies. However, it is generally assumed that elevated Gli expression is mediated

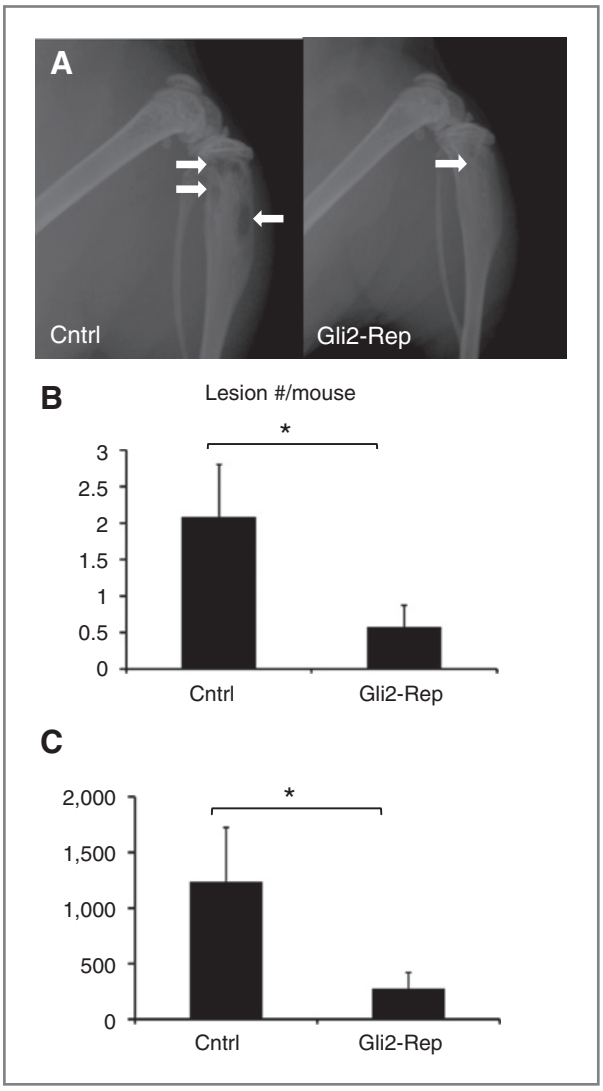


Figure 4. Gli2-Rep blocks tumor-induced bone disease. Mice inoculated with MDA-231-Gli2-Rep ($n = 7$) or MDA-231-cntrl ($n = 10$) cells via intracardiac injection were imaged radiographically weekly. A, Faxitron images depict fewer lesions in mice inoculated with MDA-231-Gli2-Rep cells. There was a significant reduction in average lesion number per mouse (B) and average lesion area per mouse (C), as measured by region of interest (ROI) analysis. Values = mean ± SE, and $P$ values were determined using unpaired $t$ test. *, $P < 0.05$.

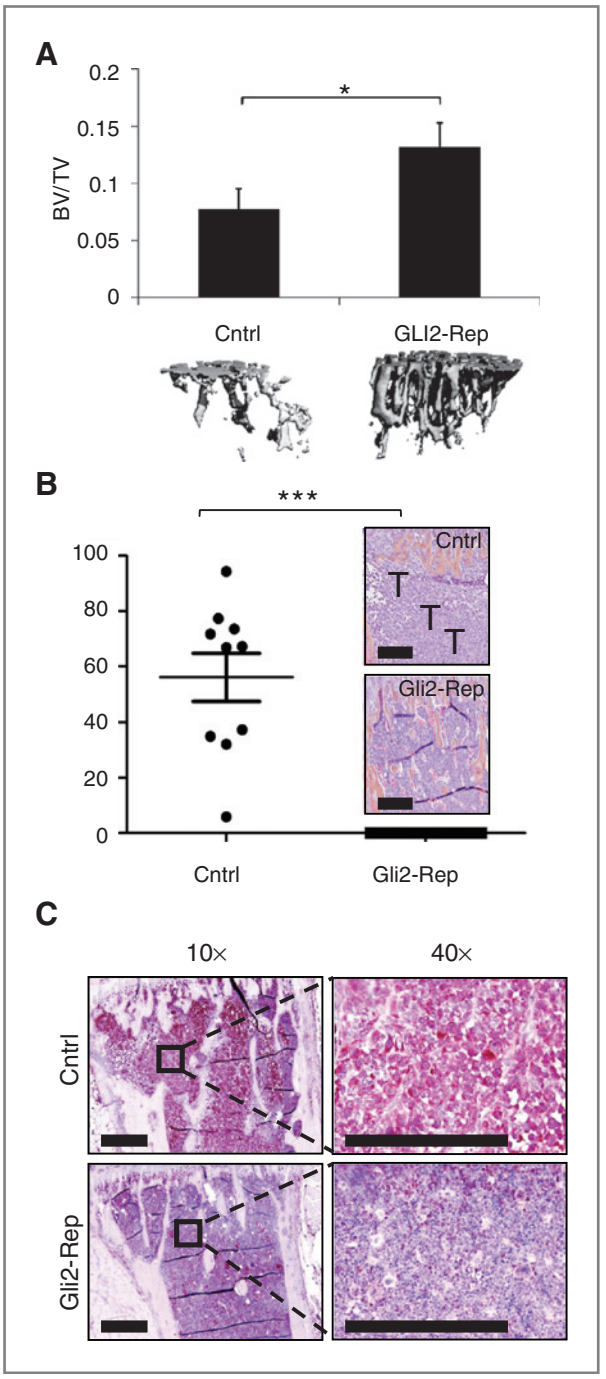


Figure 5. Gli2-Rep reduces osteolysis and tumor burden. A, *ex vivo* micro-CT (Scanco) analyses indicate a significant reduction in osteolysis in MDA-231-Gli2-Rep–inoculated mice ($n = 7$ tibiae) compared with MDA-231-Cntrl inoculated mice ($n = 10$ tibiae). Histomorphometric analyses of bone sections from these mice show (B) significant inhibition of tumor burden in MDA-231-Gli2-Rep–inoculated mice, and (C) decreased immunohistochemical staining for PTHrP in sections from MDA-231-Gli2-Rep mice. The black bar on the histologic sections represents a length of 500 μm. Values = mean ± SE, and $P$ values were determined using unpaired $t$ test. *, $P < 0.05$; ***, $P < 0.001$.

primarily via upstream Hh receptor activation. Although recently Smo-independent mechanisms have been described in prostate cancer (25), this is the first report with regards to bone metastases of Gli overexpression mediated via a Smo-independent mechanism. Surprisingly, we found that cyclopamine treatment actually increased Gli2 expression, though these results were not significant in some osteolytic tumor cells. While the mechanism of this increase remains unclear, Zhang and colleagues recently reported that cyclopamine has "off-target" effects on other signaling pathways (26). In addition, other groups have reported that cyclopamine impacts normal bone marrow cell compartments (27–31). Because

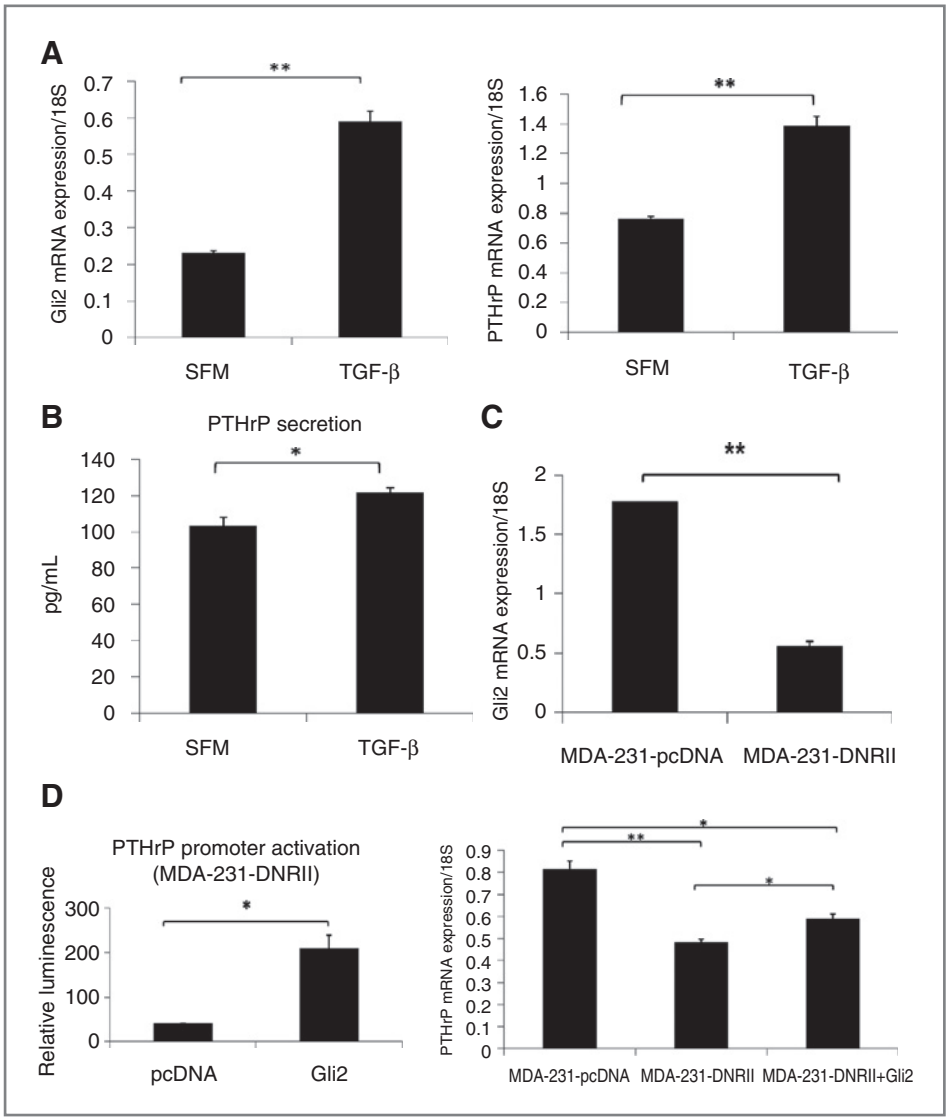


**Figure 6.** TGF-β regulates Gli2 expression in MDA-MB-231 human osteolytic breast tumor cells. A, Gli2 mRNA expression (left) increases significantly in MDA-MB-231 cells cultured in serum-free medium (SFM) supplemented with 5 ng/mL recombinant TGF-β. As previously published, PTHrP mRNA expression (right) increases in MDA-MB-231 cells following 5 ng/mL TGF-β treatment. B, stimulation with 5 ng/mL TGF-β modestly enhances PTHrP protein secretion in MDA-MB-231 cells. C, MDA-MB-231 cells stably transfected with the dominant-negative TGF-β receptor type II (DNRII) construct, which abrogates TGF-β signaling, have reduced Gli2 mRNA when compared with empty vector control pcDNA-expressing MDA-MB-231. D, MDA-231-DNRII cells exhibit low levels of PTHrP promoter activity, but when Gli2 is overexpressed by transfection with a Gli2 expression vector, PTHrP promoter activation is significantly enhanced (left) and PTHrP mRNA expression (right) is partially restored. Values = mean ± SE, and *P* values were determined using unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$.

cyclopamine had no significant effect on overall bone disease in our model, it is unlikely that the compound had any effect on other cells in the bone tumor microenvironment. However, because duration of treatment in our experiments was relatively short, we cannot rule out that longer term treatments may alter cells within the bone marrow. These data suggest that Smo antagonists are unlikely to be effective and may even have detrimental effects in some cases of tumor metastasis to bone. It will be necessary to establish how commonly breast and other bone-metastasizing cancers are deficient in Smo expression. These studies are on-going in our laboratory.

Because Smo was not expressed in MDA-MB-231 cells, our alternative approach was to inhibit Gli2 activity directly using a Gli2-Rep construct. In this construct, which was previously used to study muscle development in developing organisms, the activation domain of Gli2 has been replaced with the repressor domain of the engrailed protein, resulting in a repression of Gli target genes (22). Inhibiting Gli2 activity in this way reduced PTHrP expression and inhibited tumor-induced bone destruction, which is reflected in the reduction in average osteolytic lesion area as well as number of osteolytic lesions in experiments utilizing populations of MDA-MB-231 cells overexpressing Gli2-Rep derived from single cells (Supplementary Fig. 1). To eliminate the possibility that these changes may have been due to constitutive variation in PTHrP expression in individual MDA-MB-231 cells and not due to the inhibition of Gli2 activity, we demonstrated similar results in pooled populations of cells expressing the Gli2-Rep construct (Figs. 4 and 5).

The inhibition of bone destruction and tumor growth observed in the Gli2-Rep tumor-bearing mice identifies Gli2 as a promising target for developing therapeutic approaches in the prevention and treatment of tumor-induced bone disease. Postnatal expression of Gli2 is primarily limited to the growth plate (32) and the hair follicle (33, 34), making Gli2 an ideal clinical target with low potential for significant

off-target effects in adult patients. Although there are several reports of potential compounds that inhibit Gli activity (11, 19, 25, 35, 36) only those that inhibit downstream of Smo will be beneficial in cells deficient in Smo such as those used in this work. One promising group of small molecules is the Gli-antagonist (GANT) compounds, which inhibit Gli function irrespective of the mutation leading to the activation of Gli (25). However, perhaps due to limited availability as a result of lack of large-scale synthesis of these compounds, they have been tested in relatively few models (25, 37). Importantly, given the frequency of Smo-independent Hh activation in prostate cancer, Smo antagonists are unlikely to be effective in nearly 50% of prostate tumors. The frequency of Hh activation in clinical samples of primary breast cancer (38) and in bone metastases from any primary tumor type is currently unclear, but preclinical studies suggest an important role for Hh in tumor bone disease (10, 39). Although ultimately, we and others will need to examine the frequency of Hh mutations in tumors that metastasize to bone in clinical samples.

We observed a decrease in tumor burden at sites of bone metastases in the Gli2-Rep intracardiac model, although tumor growth was unaltered in the Gli2-Rep orthotopic mammary fat pad model. Because canonical Hh signaling did not appear to be regulating Gli2 expression, and recent reports suggested that TGF-β could regulate Gli2 signaling, we reasoned that the observed decrease in tumor growth was caused by indirect blockade of TGF-β stimulation of PTHrP. In this study, we demonstrated that Gli2 is in fact required for TGF-β to increase PTHrP expression. Importantly, this suggests that when Gli2 is not activated, TGF-β in the bone matrix will no longer have a stimulatory effect on PTHrP secretion from breast cancer cells and consequently tumor cell growth and bone resorption will be inhibited. Although our data indicate that Gli2 is required for TGF-β regulation of PTHrP, this does not exclude other signaling pathways in the regulation of Gli2 and PTHrP. In fact, Dennler and colleagues recently demonstrated that a combination of Wnt and TGF-β signaling regulates Gli2 transcription (24). Our current efforts are aimed at identifying and characterizing Hh signaling-independent regulators of Gli2 expression in tumor cells that have metastasized to bone.

Overall, this work demonstrates that Gli2 inhibition reduces tumor growth and bone destruction, suggesting that targeting Gli2 may be a valid approach to inhibit bone metastases. Studies are on-going to determine pathways that regulate Gli2 and PTHrP and to test the efficacy of small-molecule inhibitors of Hh signaling that target downstream of Hh receptors.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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