The Hedgehog Signaling Molecule Gli2 Induces Parathyroid Hormone-Related Peptide Expression and Osteolysis in Metastatic Human Breast Cancer Cells

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Abstract

Parathyroid hormone-related peptide (PTHrP) is a major factor involved in tumor-induced osteolysis caused by breast cancers that have metastasized to bone. However, the molecular mechanisms that mediate PTHrP production by breast cancer cells are not entirely clear. We hypothesized that Gli2, a downstream transcriptional effector of the Hedgehog (Hh) signaling pathway, regulates PTHrP expression in metastatic breast cancer because the Hh pathway regulates physiologic PTHrP expression in the developing growth plate. Here, we show that Gli2 is expressed in several human cancer cell lines that cause osteolytic lesions in vivo and produce PTHrP (MDA-MB-231, RWGT2, and PC-3) but is not expressed in nonosteolytic cancer cell lines that do not secrete PTHrP (MCF-7, ZR-75, and T47D). Transient expression of Gli2 in MDA-MB-231 and MCF-7 breast cancer cells increased PTHrP promoter-luciferase activity dose dependently. Stable expression of Gli2 in MDA-MB-231 cells resulted in an increase in PTHrP protein in the conditioned medium. Alternatively, MDA-MB-231 cells stably transfected with Gli2-EnR, a repressor of Gli2 activity, exhibited a 72% to 93% decrease in PTHrP mRNA by quantitative real-time PCR when compared with control cells. To examine the effects of Gli2 on breast cancer–mediated osteolysis in vivo, athymic nude mice were inoculated with MDA-MB-231 cells stably expressing Gli2 or the empty vector. Following tumor cell inoculation via the left cardiac ventricle, Gli2-expressing tumors caused significantly more osteolysis. Together, these data suggest that PTHrP expression and osteolysis in vivo in human breast cancer cells is driven at least in part by Gli2. (Cancer Res 2006; 66(15): 7548-53)

Introduction

Parathyroid hormone-related peptide (PTHrP) produced by human breast cancer cells is enhanced at the site of bone metastases (1–3). In addition, blocking tumor production of PTHrP using small molecules reduces bone metastases and tumor burden in bone in a mouse model of breast cancer metastasis to bone (3, 4). However, despite the clear role of tumor-produced PTHrP in osteolytic bone metastases, the mechanisms by which PTHrP expression is enhanced in metastatic breast cancer cells are still not fully understood.

In the developing growth plate, Indian Hedgehog (Ihh) has been shown to regulate PTHrP (5). This has been shown in part by studies involving in vitro assays as well as transgenic and knockout animals (5–7). For example, Ihh-deficient mice do not express PTHrP, signifying a link between these two important molecules (6). Furthermore, Ihh- and PTHrP-deficient mice have similar and overlapping phenotypes. Both knockout mouse lines are smaller in size and exhibit abnormalities in chondrocyte differentiation compared with wild-type littermates (5, 7). The overlap in phenotypes is consistent with regulation of PTHrP by Ihh. Accordingly, we reasoned that the Hedgehog (Hh) signaling pathway may also play an important role in the regulation of PTHrP expression by tumor cells that have metastasized to bone.

The Hh family of proteins signals through the membrane receptor Smoothened (Smo) by binding to the membrane receptor Patched, which releases Smo from a repressed state and allows it to signal (8). Hh signaling in mammalian cells is mediated by the Gli family of zinc finger transcription factors composed of Gli1, Gli2, and Gli3 (8). These factors have separate and discrete functions in mammalian cells (8, 9). In addition, Gli2-deficient mice display a decrease in PTHrP expression in the growth plate (10). Because Gli2 seems to be an important factor for expression of PTHrP in the developing growth plate, we hypothesized that this pathway is responsible for regulating PTHrP transcription in breast cancer cells and that overexpression of Gli2 in breast cancer cell lines will lead to an increase in their osteolytic potential by this mechanisms. Here, we present data indicating that Gli2 overexpression in tumor cell lines occurs in those lines that also secrete high levels of PTHrP and induce osteolysis in vivo and that overexpressing Gli2 in MDA-MB-231 cells leads to increased PTHrP production and increased tumor-induced osteolysis in a well-established murine model of human breast cancer metastasis to bone.

Materials and Methods

Cell culture. The human cancer cell lines MDA-MB-231, MCF-7, and PC-3 (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). The squamous cell carcinoma cell line RWGT2 (11) was maintained in DMEM plus 10% FBS.

DNA constructs. Gli1 and Gli2 constructs were kindly provided by Dr. Sasaki (Institute for Molecular and Cellular Biology, Osaka, Japan; ref. 9). Gli3 constructs were used as described previously (12). Gli2-EnR construct was kindly provided by Ilona Skerjanc (University of Western Ontario, London, Ontario, Canada) as described previously (13).

Transfections. Cells were plated at a density of 0.8 × 10^5 per well in a 24-well plate on the day before transfection. Cells were transiently transfected with 0.25 μg of the luciferase construct, 0.2 μg β-galactosidase (β-gal) as a transfection efficiency control, and the indicated amounts of expression constructs. All transfections were done using LipofectAMINE Plus (Invitrogen) reagent per manufacturer’s instructions.
Luciferase assay. Forty-eight hours after transfection, cells were harvested using 200 μL of 1× Glo lysis buffer (Promega, Madison, WI). After lysis was complete, 50 μL cell lysate was used for luciferase assay using 50 μL Bright-Glo reagent (Promega). In addition, to account for transfection efficiency, a 50-μL lysate was used to measure β-gal activity using the Beta-Glo assay (Promega) per manufacturer’s directions.

Stable cell lines. For stable transfection assays, cells were plated at 5 × 10^4 per 60-mm tissue culture dish 18 to 24 hours before transfection. Transfections were done as above using Gli2-pcDNA vector or pcDNA alone, both of which contain the neomycin resistance gene. After 48 hours, cells were plated at low density in medium containing 800 μg/mL geneticin (G418). Once colonies were formed, individual colonies were isolated and expanded. Cells were continuously maintained in medium containing 800 μg/mL G418.

Western blotting. Stable cell lines were harvested into a radio-immunoprecipitation assay lysis buffer containing a cocktail of protease inhibitors (Roche, Basel, Switzerland). Equal protein concentrations were prepared for loading with laemmli sample buffer and run on SDS-PAGE Mini-Protein II ready gels (Bio-Rad, Hercules, CA). Separated proteins were prepared for loading with laemmli sample buffer and run on SDS-PAGE. Western blotting. Stably transfected cells were Western Blotted using diluted His-Gli2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 5% milk in TBS-T at 4°C overnight. The membrane was then washed several times with TBS-T and then incubated with a horseradish peroxidase-conjugated anti-mouse antibody (Amersham, Buckinghamshire, United Kingdom) at room temperature for 1 hour. Finally, the membranes were washed multiple times with TBS-T buffer, and signals were detected using an enhanced chemiluminescence system (Amersham). Membrane was stripped and reprobed using an antibody for HSC-70 (Santa Cruz Biotechnology) as a loading control.

Semiquantitative reverse transcription-PCR. Total RNA was extracted using TRIzol (Promega, Madison, WI) according to the manufacturer’s instructions. cDNA was synthesized using SuperScript II reverse transcriptase (Promega) and oligo(dT) from 2 μg of total RNA at 42°C for 50 minutes and 70°C for 15 minutes. cDNA (2 μL) was used for PCR amplification using 100 μmol/L deoxynucleotide triphosphate, 1.5 mmol/L MgCl2, 1 unit Taq polymerase, and 10 μmol/L of Gli2 forward and reverse primers using conditions as described previously (14). β-Actin-specific primers were used as internal controls (15). PCR products were separated by electrophoresis on a 1% (w/v) agarose gel containing 0.5 μg/mL ethidium bromide.

Quantitative real-time PCR. Specific clones were selected by their expression of the pcDNA vector as determined by standard PCR. Quantitative real-time PCR was done using cDNA prepared as described above. PCR amplification was done using the RealMasterMix (Eppendorf, Hamburg, Germany) and 0.5 μL of prepared cDNA per manufacturer’s instructions. Real-time PCR was done in triplicate using the RealPlex Machine (Eppendorf) with the following cycling conditions: 95°C for 15 seconds, 58°C for 30 seconds, and 68°C for 30 seconds. Quantification was done using the ΔΔCt, relative quantification method using cyclophilin A as an internal control and comparing all values to the empty vector containing cells. Primer sequences are as follows: PTHrP, TTAAAGCAGTACCCCCCTACCA (sense) and ATGG-jC2 for 15 seconds, 70°C for 30 seconds. PTHrP protein was measured in conditioned medium and plasma using a two-site immunoradiometric assay (IRMA) kit available from Nichols Institutes (San Clemente, CA). All samples, conditioned medium, and plasma were collected in protease inhibitors and placed immediately on ice and stored at −70°C until assay. Assays were done per manufacturer’s instructions.

Cell proliferation assay. To measure in vitro cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonylphenyl)-2H-tetrazolium (MTS) assay, cells were plated in 96-well plates and growth was measured at days indicated using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) per manufacturer’s instructions.

In vivo tumor growth assay. Cells (1 × 10^6) were injected s.c. into athymic nude mice. Tumor growth was measured approximately every 5 days by caliper measurement.

Figure 1. Gli2, but not Gli1 or Gli3, increases PTHrP promoter activity. Cells were transfected with the 1.1-kb PTHrP promoter/luciferase construct and the described Gli expression vectors. A, of the Gli proteins used in the assay, only Gli2 resulted in a significant increase in PTHrP promoter activity. MDA-MB-231 (B) and MCF-7 (C) cells cotransfected with increasing concentration of Gli2 showed a dose response to Gli2. Gli2 expression stimulated PTHrP promoter activity up to 18-fold higher in the MCF-7 cells, which do not secrete PTHrP and do not express Gli2 mRNA. By contrast, the increases in promoter activity in the MDA-MB-231 cells, which already express Gli2 and secrete high levels of PTHrP constitutively, were not as marked. Results were corrected for transfection efficiency by measuring β-gal activity from a cotransfected rsv-β-gal control. Data presented as fold increase above empty vector control. Columns mean ± SEM. * P < 0.05, ** P < 0.01; *** P < 0.001. D, expression of His-Gli2 was determined by Western blot analysis of the transiently transfected MDA-MB-231 cells using a His antibody.
Bone histomorphometry. Forelimb and hindlimb long bones and spines were collected from each mouse at sacrifice. Bones were fixed in 10% buffered formalin, decalcified in 10% EDTA, and paraffin embedded. Sections for histomorphometric analysis were stained with H&E, orange G, and phloxine. Histomorphometric analyses for tumor burden and trabecular bone volume were done using the MetaVue program (Universal Image Corp., Downingtown, PA) as described previously (16).

Results

Regulation of PTHrP promoter activity by Gli2. Based on the evidence that Hh regulates PTHrP expression during development (5), we hypothesized that the Gli family of transcription factors may also regulate PTHrP expression in the metastatic breast cancer cell line MDA-MB-231. To determine if the Gli family of transcription factors regulates PTHrP expression in breast cancer cells, we did transient transfections of the human breast cancer cells MDA-MB-231 and MCF-7 with Gli expression constructs and a PTHrP promoter-luciferase construct. We found that only Gli2 significantly increased PTHrP promoter activity of a 1.1-kb promoter-luciferase construct (4). Gli1 and Gli3 did not stimulate PTHrP promoter activity (Fig. 1A). No additive effects were observed when increasing concentrations of Gli1 or Gli3 were added alone or in conjunction with Gli2 (data not shown).

In MDA-MB-231 cells, Gli2 increases PTHrP in a concentration-dependent manner, with the highest concentrations of transfected Gli2 increasing PTHrP promoter activity 2- to 3-fold (Fig. 1B). However, in the MCF-7 cells, the increase in PTHrP promoter activity was much more prominent, with an ~18-fold increase in activity at maximum concentrations of transfected Gli2 (Fig. 1C).

Gli2 expression correlates with PTHrP secretion and osteolysis. Because Gli2 specifically regulated PTHrP promoter activity, we examined several cancer cell lines, including the squamous cell carcinoma RWGT2, prostate cancer cell line PC-3, and the breast cancer cell lines MDA-MB-231, MCF-7, ZR-75, and T47D, for expression of Gli2 mRNA and PTHrP protein production. Expression of Gli2 was examined by reverse transcription-PCR (RT-PCR) in several different tumor cell lines (Fig. 2, inset). In addition, secreted PTHrP protein levels were measured in the medium of each of these cell lines. We found that Gli2 was highly expressed in cancer cell lines that had previously been shown to metastasize to bone in mouse models of bone metastases, cause osteolysis, and secrete high levels of PTHrP (Fig. 1). However, cell lines, such as T47D and MCF-7, which do not cause osteolytic bone metastases (16), do not express both detectable levels of secreted PTHrP and Gli2 mRNA. These data indicate that a positive correlation exists between Gli2 expression, PTHrP, and osteolysis and suggest that Gli2 may play a role in regulating PTHrP expression in breast cancer bone metastases.

Antagonists of Gli2 block PTHrP promoter activity and expression. To verify that this up-regulation of PTHrP by Gli2 is specific, we used various inhibitors of Gli2-induced gene activation. Firstly, we used a mutant version of Gli2 (Gli2-∆C4), in which the activation domain has been deleted (9). This construct binds to Gli target genes but does not activate transcription. Using this construct in transient transfections of MDA-MB-231 and MCF-7 cells, we were able to block Gli2-induced PTHrP promoter activity (Fig. 3B).

In addition to the Gli2-∆C4 construct, we used a Gli2 chimeric construct fused with the repressor domain of the engrailed protein (Gli2-EnR; ref. 13). This construct dramatically blocked Gli2 activation of PTHrP promoter activity to a level well below that of basal promoter activity (Fig. 3A). To confirm that Gli2-EnR blocked endogenous PTHrP expression, MDA-MB-231 cells were stably transfected with Gli2-EnR. We found that these MDA-Gli2-EnR cells exhibited a 72% to 93% decrease in PTHrP expression by quantitative real-time PCR when compared with MDA-MB-231 cells transfected with the empty vector (Fig. 3C). These data indicate that Gli2 is an important regulator of PTHrP expression.

Gli2 overexpression increases PTHrP protein in conditioned medium. MDA-MB-231 cells were also stably transfected with the same Gli2 construct that was used in the transient transfections described above. We found that clones that expressed high levels of the Gli2 construct also expressed high levels of PTHrP protein by IRMA (Fig. 4A). The stable clone that expressed the highest levels of Gli2 (MDA-Gli2) produced as much as 10-fold higher PTHrP protein than empty vector controls (MDA-EV). This result indicates
that Gli2 can regulate PTHrP secretion levels as well as transcriptional regulation. Therefore, we rationalized that Gli2 overexpression would also increase the osteolytic bone destruction caused by MDA-MB-231 cells by increasing PTHrP secretion by the tumor cell line.

**Gli2 overexpression in MDA-MB-231 cells does not significantly alter cell growth in vivo.** In some systems, Hh signaling and its components are important for tumor cell growth. In fact, blocking Hh signaling with an Hh inhibitor in PC-3 cells abrogates tumor cell growth (17). Before doing metastasis studies, we investigated what effect, if any, Gli2 expression had on tumor cell growth. This was done both in vitro by MTS assays and in vivo by monitoring the growth of tumor cells inoculated s.c. In vitro cell growth was monitored everyday for 7 days. No significant differences in cell growth in vitro were observed between the MDA-EV cells and the MDA-Gli2 cells (Fig. 4B). In addition, there were no significant differences between MDA-EV and MDA-Gli2 cell growth in vivo following s.c. inoculation of tumor cells and monitoring with caliper measurements (Fig. 4C).

**MDA-MB-231 cells overexpressing Gli2 cause increased osteolytic lesion number and area by radiography.** To determine the role of Gli2 in breast cancer–mediated osteolysis, we evaluated MDA-Gli2 and MDA-EV cells in a mouse model of breast cancer metastasis to bone. Four-week-old female athymic nude mice were inoculated with MDA-Gli2 or MDA-EV cells via the left cardiac ventricle. Baseline radiographs, body weights, blood

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**Figure 3.** Inhibitors of Gli2 binding blocks the Gli2-mediated increase in PTHrP promoter activity. MDA cells were transiently transfected with a Gli2 expression construct and the 1.1-kb PTHrP promoter-luciferase construct in the presence of increasing amounts of Gli2-EnR (A), a Gli2-specific inhibitor construct (13). Another inhibitor, Gli2-ΔC4 (B), was also transfected in increased concentrations. This construct also functioned to block Gli2 but not at as low concentrations as the EnR. Inset, expression level of Gli2-ΔC4 in the transient transfections was determined by Western blot. C, endogenous PTHrP expression was measured by quantitative real-time PCR in MDA-MB-231 cells stably transfected with Gli2-EnR. Data are expressed as fold increase over an empty vector control construct. Columns, mean; bars, SE. *****, P < 0.001.

**Figure 4.** MDA-MB-231 human breast cancer cells stably overexpressing Gli2 have increased PTHrP levels, but Gli2 overexpression does not alter in vitro or s.c. tumor cell growth. A, PTHrP levels were measured in the conditioned medium of single cell-derived clones of MDA-MB-231 cells measured by IRMA. Gli2 expression levels by Western blot in these clones using a His antibody for detection of His-tagged Gli2 and an antibody for HSC-70 as a loading control. Gli2-overexpressing cell lines also secreted relatively more PTHrP than did the empty vector (EV) clones used in this assay. This confirms that Gli2 expression corresponds to PTHrP expression. B, MDA-EV or MDA-Gli2 cells were plated, and their growth was monitored by MTS assay for up to 7 days. No significant difference was apparent between the growth rates of these two cell lines. C, athymic nude mice were inoculated s.c. with 1 × 10^6 cells per mouse (n = 8). Tumor growth was followed by caliper measurements over 25 days. There was no difference in the growth rates of the MDA-EV or MDA-Gli2 stable cell clones. **P = not significant.**
compared with MDA-EV tumor-bearing mice (Fig. 6). Mice bearing MDA-Gli2 tumors displayed a significant decrease in trabecular bone volume (Fig. 5C). MDA-Gli2 tumor-bearing mice have increased osteolytic lesion number and area. A, representative radiographs of mice inoculated with MDA-Gli2 (top) or MDA-EV (bottom) cells. Arrows, more obvious osteolytic bone lesions caused by the Gli2-transfected cells. Quantitation of these lesions using the computerized imaging quantitation program MetaMorph Systems indicates that mice bearing MDA-Gli2 tumor cells exhibit a significant increase in osteolytic lesion number ($P = 0.0056$; B) and lesion area ($P = 0.0022$; C).

Discussion

This study implicates Gli2 in the regulation of PTHrP protein in breast cancer cells that have metastasized to bone. Our studies show that Gli2 specifically up-regulates PTHrP promoter activity as well as PTHrP protein production in tumor cells stably transfected with Gli2. Furthermore, cancer cell lines that cause osteolytic bone metastases in the mouse model used here produce Gli2 in parallel with PTHrP. In contrast, cancer cell lines that do not cause osteolysis in this model and do not produce PTHrP also do not express Gli2. This suggests a positive correlation between Gli2 expression, PTHrP production, and osteolysis. Overexpression of Gli2 in the MDA-MB-231 breast cancer cells increases tumor-induced osteolysis in a mouse model of bone metastasis as shown by radiographic analysis and decreases trabecular bone volume while increasing tumor burden in bone as shown by quantitative bone histomorphometric analysis. These results suggest that Gli2 may play an important role in the vicious cycle of bone destruction caused by breast tumor cells that have metastasized to bone.

Our results that Gli2 and PTHrP expression are positively correlated further support our hypothesis that Gli2 regulates PTHrP production in breast cancer cells. We chose to focus on Gli2 in these studies because our initial studies showed that the other Gli family members did not affect PTHrP promoter activity. In addition, others have shown that Gli2 is overexpressed in several tumor cell types, including medulloblastomas and basal cell carcinomas (14, 15, 18), and that it is required for normal mammary development (19). These findings suggest that the breast cancer cells metastatic to bone use a normal developmental process (i.e., the Hh signaling pathway), which in turn leads to increased PTHrP production and osteolysis. Furthermore, our data show that this relationship is not only exclusive to breast cancer cell lines but also apparent in other osteolytic cell lines, including the prostate cancer cell line PC-3 and the squamous cell carcinoma cell line RWGT2 (Fig. 2). Ideally, we would have correlated Gli2 protein levels with the PTHrP protein levels measured by IRMA in these studies. However, due to the lack of a suitable antibody to Gli2, we were unable to measure endogenous Gli2 protein in these cell lines. The complete lack of detectable Gli2 mRNA expression...
In the nonosteolytic tumor cell lines suggests that these lines also do not express Gli2 protein.

Our studies clearly show that Gli2 and not the other Gli family members stimulates PTHrP production. We were initially surprised that Gli1, which has been reported as a positive effector of Hh signaling, did not also regulate PTHrP promoter activity. However, recent evidence suggests that, in cancer cells, Gli2 is the major regulator of target gene expression (9, 10, 20). Furthermore, it has been shown that Gli1 is a downstream target of Gli2 (21) and that Gli2 is required for the Hh response, whereas Gli1 is dispensable in some cell types (20, 22). Taken together, these data strongly suggest that Gli2 is the major regulator of Hh signaling in metastatic breast cancer cells to bone.

Blocking the capacity of Gli2 to activate transcription by deleting the activating domain of Gli2 blocked PTHrP promoter activity. We found that, at high concentrations of the Gli2-EnR fusion construct or the Gli2-ΔC4 deletion construct, PTHrP promoter activity was blocked below basal levels. Furthermore, cells overexpressing the Gli2-EnR construct showed a dramatic decrease in PTHrP expression. These data suggest that PTHrP production in breast cancer cells is dependent on Gli2 and that blocking Gli2 effects on PTHrP expression in metastatic tumors could be a valuable therapeutic approach to inhibit osteolytic bone metastases.

Hh signaling is critical for tumor cell growth. When Hh signaling has been blocked using the Smo inhibitor cyclopamine, cell growth is decreased and apoptosis is induced. In vitro tumor cells, including the medulloblastoma cell line PZp53med (18), prostate cancer cells lines PC-3, Du145, and 22RV1 (17), and the colorectal tumor cell lines AA/C1, RC/G2, CaCO2, HT29, and SW48 (23), are growth inhibited by cyclopamine. To determine if Gli2 expression in MDA-MB-231 cells would affect cell growth, we monitored the growth inhibited by cyclopamine. To determine if Gli2expression in the nonosteolytic tumor cell lines suggests that these lines also do not express Gli2 protein.

In addition, this finding verifies that the effects seen in bone are not simply due to differences in tumor cell growth.

The fact that Gli2 can increase PTHrP expression and subsequent osteolysis by tumor cells that have metastasized to bone raises the question of what causes increased expression of Gli2. We are currently examining possible mechanisms to explain how Gli2 regulates PTHrP transcription and why Gli2 is overexpressed in the osteolytic cell lines. With respect to the latter, we expect that, in MDA-MB-231 and RWG2T2 cells, there are likely mutations at some point in the Hh signaling pathway that cause Gli2 expression levels to remain high as described previously in PC-3 cells (16). Interestingly, we have found that Gli2 increases mRNA expression levels ~20% by real-time PCR (data not shown) compared with a ~10-fold increase in protein production. This suggests that Gli2 may regulate PTHrP expression at multiple levels. To address this, experiments are currently under way to investigate mRNA stability and protein regulation. Regardless of the exact mechanisms involved, Gli2 is clearly an important factor in the regulation of PTHrP in human breast cells.

In conclusion, these data indicate that Gli2 regulates both PTHrP promoter activity and production by breast tumor cells. In addition, overexpression of Gli2 increases osteolysis and tumor burden. Furthermore, blocking transcriptional activation by Gli2 decreases PTHrP promoter activity. These data clearly implicate Gli2 and its regulation as potential therapeutic targets for drug discovery with the goal of decreasing osteolytic destruction by breast tumor cells that have metastasized to bone.

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