Runx2 Transcriptional Activation of Indian Hedgehog and a Downstream Bone Metastatic Pathway in Breast Cancer Cells

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Abstract

Runx2, required for bone formation, is ectopically expressed in breast cancer cells. To address the mechanism by which Runx2 contributes to the osteolytic disease induced by MDA-MB-231 cells, we investigated the effect of Runx2 on key components of the "vicious cycle" of transforming growth factor β (TGFβ)-mediated tumor growth and osteolysis. We find that Runx2 directly up-regulates Indian Hedgehog (IHH) and colocalizes with Gli2, a Hedgehog signaling molecule. These events further activate parathyroid hormone-related protein (PTHrP). Furthermore, Runx2 directly regulates the TGFβ-induced PTHrP levels. A subnuclear targeting deficient mutant Runx2, which disrupts TGFβ-induced Runx2-Smad interactions, failed to induce IHH and downstream events. In addition, Runx2 knockdown in MDA-MB-231 inhibited IHH and PTHrP expression in the presence of TGFβ. *In vivo* blockade of the Runx2-IHH pathway in MDA-MB-231 cells by Runx2 short hairpin RNA inhibition prevented the osteolytic disease. Thus, our studies define a novel role of Runx2 in up-regulating the vicious cycle of metastatic bone disease, in addition to Runx2 regulation of genes related to progression of tumor metastasis. [Cancer Res 2008;68(19):7795–802]

Introduction

Bone provides a favorable site for cancer metastasis and the aggressive tumor behavior of breast, prostate, and lung metastatic cancer cells. Tumor cells anchor to the bone matrix proteins and respond to the local production of growth factors, including cytokines, matrix metalloproteinases (MMP), receptor activator of nuclear factor-κB ligand (RANKL), transforming growth factor (TGFβ), and parathyroid hormone-related protein (PTHrP), which promote activation of transcription factors and bone-resorbing osteoclasts (1, 2). The resulting tumor growth and severe osteolytic disease leads to the end stage of cancer (3).

Runx2 is highly expressed in bone metastatic cancer cells but not in nonmetastatic cancer cells (4). Runx2, a key factor for bone formation, activates several genes (*p21, RANKL, MMP2, MMP9, MMP13, VEGF, OP*, and *BSP*) required for bone development and turnover (5). Importantly, these genes are regulated by Runx2 in metastatic breast cancer cells (6, 7). This cohort of Runx2-regulated genes has been identified as both markers of progression of tumor growth and tumorigenesis and essential mediators of tumor invasion and metastasis (8, 9). These well-known concepts for the functional activities of the Runx2-regulated genes implicate this master transcription factor in multiple events associated with metastatic tumor growth and osteolytic disease in bone. What remains unclear, however, is Runx2 linkage to the osteolytic disease/pathway operative in bone microenvironment by cancer cells. Indeed, a Runx2 mutant protein, which abolished functional activity in subnuclear domains, inhibited the bone osteolysis associated with breast cancer cells (2, 4, 10). However, the mechanism by which Runx2 is permissive for osteolysis remains a compelling question.

To understand the mechanism by which Runx2 participates in metastatic bone disease, we investigated Runx2 regulation of key components of the "vicious cycle" of tumor growth and bone resorption. This cycle involves overproduction of PTHrP by breast cancer cells that has a profound effect on tumor cell activities and survival and, when present in the bone microenvironment, results in osteoclastic bone resorption (1). The resorbed bone releases TGFβ-stimulating tumor cell proliferation and consequently increased PTHrP secretion, thus continuing the vicious cycle (11). Furthermore, PTHrP is regulated by Gli, a Hedgehog signaling factor, and this pathway leads to pathologic consequences in a variety of human tumors (12, 13).

Here, we show that Runx2 binds to the Indian Hedgehog (IHH) promoter and activates its expression in cancer cells and that this regulation further increases PTHrP levels, resulting in operation of vicious cycle in cancer cells. Our studies show that Runx2 directly contributes to the osteolytic process by regulating the IHH-PTHrP pathway in breast cancer cells that leads to osteoclastogenesis *in vivo*. We propose that metastatic cancer cells, by having higher levels of Runx2, are able to activate components of the vicious cycle and target genes that increase bone loss and promote tumor progression in bone, resulting in the metastatic bone disease.

Materials and Methods

Cell culture, transient transfections, and treatments. Nonmetastatic MCF7 and the metastatic MDA-MB-231 human breast cancer cell lines were cultured in α-MEM containing 10% fetal bovine serum (Invitrogen, Inc.). The procedure for adenovirus expressing wild-type (WT) and mutant Runx2 [COOH-terminal deleted mutant (Δ361) and DNA-binding mutant (DBD)] are reported previously (14). Cells were treated with 5 nmol/L TGFβ for 24 h.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Western blot analysis. Runx2 protein in breast cancer cells was detected as described previously (7). For Western blot analysis, whole-cell lysates were mixed with direct lysis buffer and separated in a 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and incubated with mouse monoclonal Runx2 antibody followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology). Immunoreactive proteins were detected using an enhanced chemiluminescence kit (Pierce).

Chromatin immunoprecipitations. Chromatin immunoprecipitations (ChIP) were performed as previously described (7). Briefly, formaldehyde cross-linking was performed followed by sonication to obtain DNA fragment with average size of 0.3 kb. Protein-DNA complexes were immunoprecipitated using Runx2 antibody (M-70; Santa Cruz Biotechnology) or IgG as a control. Purified DNA was subjected to real-time PCR amplification with SYBR Green dye on ABI real-time thermocycler. IHH promoter fragments (1.5 kb and −502 bp) containing Runx element were amplified using forward primer 5′-CTCAGAAGCCCAAGGAAGAGT-3′ and reverse primer 5′-CACTACCCACTCCTTTATGCCC-3′ for proximal promoter fragment and forward primer 5′-AATAGCATTTGAGTGAGAATTTTTAAG-3′ and reverse primer 5′-GGATCCCTTCTGCCAAAACAA-3′ for distal promoter fragment.

Small interfering RNA and short hairpin RNA treatment. Breast cancer cells were transfected with small interfering RNA (siRNA) duplexes at 30% to 50% confluency using Oligofectamine (Invitrogen Life Technologies). siRNA specific for human Runx2 oligo #1 r(CUCUGCACCAAGUCCUUUU)d(TT) and oligo #2 r(GGUUCAACGAUCUGAGAUU)d(TT) was obtained from Qiagen, Inc. Cells were transfected with either Runx2 or control siRNA duplexes specific for green fluorescent protein (GFP) at a concentration of 50 nmol/L with Oligofectamine as per the manufacturer's instructions. To generate MDA-MB-231 cells expressing short hairpin RNA (shRNA)-Runx2, we cloned 5′-AAGGTTCAACGATCTGATTTG-3′ sequence in pLVTHM vector under H1 promoter and generated lentiviral particles using Invitrogen BLOCK-iT kit. MDA-MB-231 cells were infected with lentivirus and by fluorescence-activated cell sorting for GFP signal to obtain shRNA-expressing cell population. Runx2 knockdown efficiency was confirmed by Western blot analysis.

Real-time reverse transcription-PCR analysis. Expression levels of IHH, Runx2, PTHrP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from MCF7, MDA-MB-231, and PC3 cells were analyzed after Runx2 siRNA treatment or adenovirus transduction. Total RNA was isolated using Trizol reagent according to the manufacturer's specification. Purified RNA was oligo(dT) primed and cDNA was synthesized at 42°C with SuperScript II RNA polymerase (Invitrogen). The cDNA was then used for real-time PCR (Applied Biosystems, Inc.). For PCR amplification, the following primers were used: IHH, 5′-TAGGCCATAGCAGACG (forward) and 5′-TTAAGGCCCATATCAGG-3′ (reverse); PTHrP (1-139), ACTAACGACCCGCCCCTCG (forward) and GAACAAGTTTCAAGTGCGTGTGTC (reverse); PTHrP (1-141), AGGAGGCGGTTAGCCCTGT (forward) and TCCCATAGCAATGTCTAATTAATCTGG (reverse); and PTHrP (total), ACCTCGGAGGTGTCCCCTAAC (forward) and TCAGACCCAAATCGGACGG (reverse). Primer sequences for Runx2 and GAPDH are described previously (7). Primers and probes for the tartrate-resistant acid phosphatase (TRAP), tumor necrosis factor α (TNFα), integrin β3, and αv genes were all Assays-on-Demand products from Applied Biosystems and reactions were set up according to the manufacturer's directions.

Histologic analysis and immunohistochemistry. H&E staining was performed on paraffin sections from tibial tissues harvested after 15 d of cancer cell injection and fixed in 4%paraformaldehyde and decalcified in 14% EDTA solution as previously described (15). Ki-67 (1:100, rabbit polyclonal; Santa Cruz Biotechnology) immunohistochemistry was performed by using antigen retrieval with antigen unmasking solution (DakoCytomation) diluted 1:100. Sections were incubated overnight with Ki-67 antibody followed by incubation with HRP-conjugated secondary antibodies and developed with 3,3′-diaminobenzidine. TRAP enzyme detection for showing osteoclast activities was performed using reagent kits from Sigma-Aldrich Biotechnology. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was performed using the *In situ* Cell Death Detection kit (Roche). Proliferation of the stable MDA-MB-231 cells expressing shRNA-Runx2 or scramble was quantitated by flow cytometry of bromodeoxyuridine (BrdUrd)-labeled cells with FITC-BrdUrd antibody (BD Biosciences; ref. 16).

***In vivo* experiments.** Groups of six severe combined immunodeficient (SCID) mice were injected with MDA-MB-231 cells (expressing shRNA-Runx2 or scramble sequence; 1×10^5 cells in 100 μL PBS) in medullar cavity of tibia. X-ray analysis was performed on Faxitron soft X-ray machine to monitor osteolysis.

Results

Runx2 is ectopically expressed in breast cancer cells and regulates IHH-PTHrP signaling. Deregulation of Runx factors is associated with several types of cancers, with Runx2 being highly expressed in bone metastatic breast cancer cells (17, 18). To further understand the molecular mechanism by which Runx2 contributes to the metastatic bone disease, we examined the linkage between Runx2 and factor(s) responsible for osteolysis, PTHrP. Secretion of PTHrP from cancer cells activates and recruits osteoclasts to bone surface and causes bone destruction (1, 3, 19). Recently, Hedgehog signaling has been implicated in regulation of PTHrP in breast cancer cells (12). Consistent with the previous results, Fig. 1*A* shows the robust endogenous Runx2 levels in the highly metastatic MDA-MB-231 cells and near absence in the nonmetastatic MCF7 cell line. The MDA-MB-231 cells also show high levels of IHH and PTHrP as detected by real-time PCR analysis (Fig. 1*B* and *C*). These

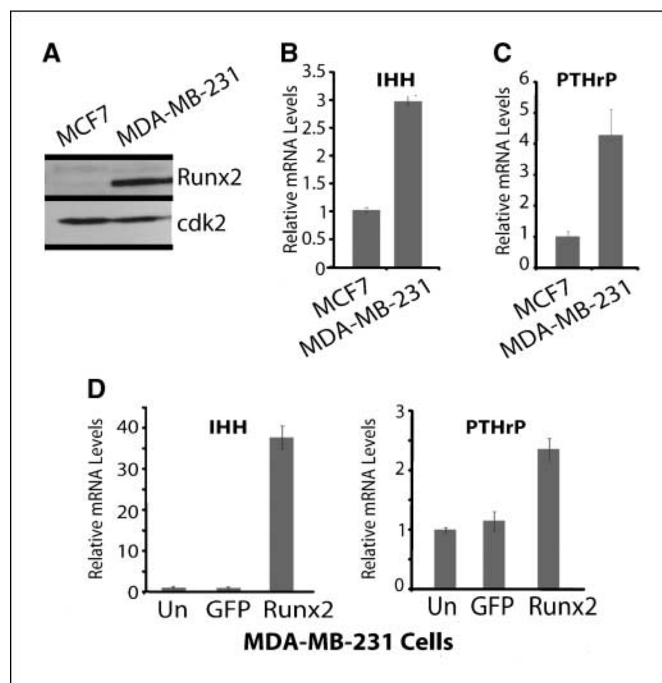

Figure 1. Runx2 is expressed in metastatic breast cancer cells and is a major determinant of transcriptional activation of IHH and PTHrP gene expression. *A,* total protein from MCF7 and MDA-MB-231 was subjected to Western blot analysis to detect Runx2 protein by mouse monoclonal antibody (1:2,000). *Bottom,* the expression level of cyclin-dependent kinase 2 (*cdk2*) is shown as internal loading control. Total RNA (5 μg) from MCF7 and MDA-MB-231 cells was used to detect mRNA levels of IHH (*B*) and PTHrP (*C*) by qRT-PCR analysis. Expression levels are normalized to GAPDH. *D,* MDA-MB-231 cells were transduced with adenovirus expressing Runx2 or GFP. Total RNA from MDA-MB-231 cells untreated (*Un*) or transduced with Runx2 or GFP virus was subjected to qRT-PCR analysis to detect IHH (*left*) and PTHrP (*right*) mRNA levels after 48 h of viral transduction. The mRNA levels were normalized to GAPDH.

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results suggest a causal relation between Runx2 protein and expression levels of IHH and PTHrP in breast cancer cells. We further examined the mechanism of IHH activation and its downstream signaling events modulated by Runx2 in breast cancer cells.

Downstream events of IHH signaling in breast cancer cells result in activation of PTHrP and consequently activates osteoclasts through RANKL. To understand the regulation of IHH expression in breast cancer cells, we transduced MDA-MB-231 cells with adenovirus expressing GFP or WT Runx2 protein. Cells were harvested at 6, 12, 24, and 48 h after infection and total RNA was examined for IHH expression by quantitative reverse transcription-PCR (RT-PCR) analysis. Runx2 transduction efficiency and protein levels were confirmed by GFP signal and Western blot analysis (Supplementary Fig. S1*A*). Ectopic expression of Runx2 results in a robust activation of IHH mRNA levels after 6 h of adenovirus infection, which further increased up to 35-fold by 48 h (Fig. 1*D, left*), whereas control GFP virus failed to increase basal level of IHH at any time point examined. Furthermore, we find a significant 2- to 3-fold induction in total PTHrP levels in MDA-MB-231 cells transduced with Runx2 adenovirus (Fig. 1*D, right*). The two isoforms of PTHrP (1-139 and 1-141) were examined by real-time PCR analysis and both are activated to the same extent by Runx2 in MDA-MB-231 cells (Supplementary Fig. S1*B* and *C*). We further confirmed this positive regulation of IHH and PTHrP by Runx2 in MCF7 cells (Supplementary Fig. S1*D* and *E*) and PC3 bone metastatic prostate cancer cells (Supplementary Fig. S1*F* and *G*) and find similar results as in MDA-MB-231 cells.

Runx2 COOH-terminal multifunctional transactivating domain is critical for IHH and PTHrP expression and directly regulates the *IHH* gene. To evaluate the functional domain of Runx2 responsible for IHH induction in breast cancer cells, we tested the Runx2 mutant protein that is defective in the COOH-terminal activities required for forming transcriptionally active complexes in subnuclear domains. The MDA-MB-231 cells were infected with adenovirus expressing WT and Runx2 Δ361 mutant protein. In Fig. 2*A*, activation of IHH and PTHrP is reduced by 75% compared with WT protein. The *in vivo* phenotype of this mutant is complete loss of bone formation (20). Runx2 Δ361 mutant protein lacks an activation/repression functional domain but retains normal DNA-binding activity. Therefore, to show that Runx2 activates IHH-PTHrP directly, we examined DBD mutant protein and find that it failed to induce IHH or PTHrP in MDA-MB-231 cells (Fig. 2*A*). Expression levels of these mutants are shown in Supplementary Fig. S1*A* as examined by Western blot analysis. The PTHrP expression levels were reduced to the range of basal levels by both Runx2 Δ361 and DBD mutant, suggesting that the decrease is a secondary response to IHH. The striking block in IHH activation by Runx2 DBD mutant indicates a direct protein-DNA interaction required for transcriptional activation. The IHH response to the mutant Runx2 proteins suggests that both signaling component due to Runx2 COOH-terminal function and direct DNA binding to IHH promoter are necessary.

To determine if Runx2 activation of IHH is a result of recruitment of Runx2 on the IHH promoter, we performed ChIP assay in MDA-MB-231 cells. Four putative Runx-binding elements 5′-TGTGGT-3′ are present within the 1.5 kb of 5′ flanking sequence of human *IHH* gene (Fig. 2*C*). We used Runx2 and IgG control antibody and immunoprecipitated DNA was analyzed for *in vivo* occupancy of the IHH promoter by quantitative PCR (qPCR) analysis. The IHH proximal promoter Runx site shows greater binding of Runx2 compared with the distal promoter fragments (Fig. 2*D*). These results show that Runx2 activates IHH by directly binding to its promoter elements in breast cancer cells.

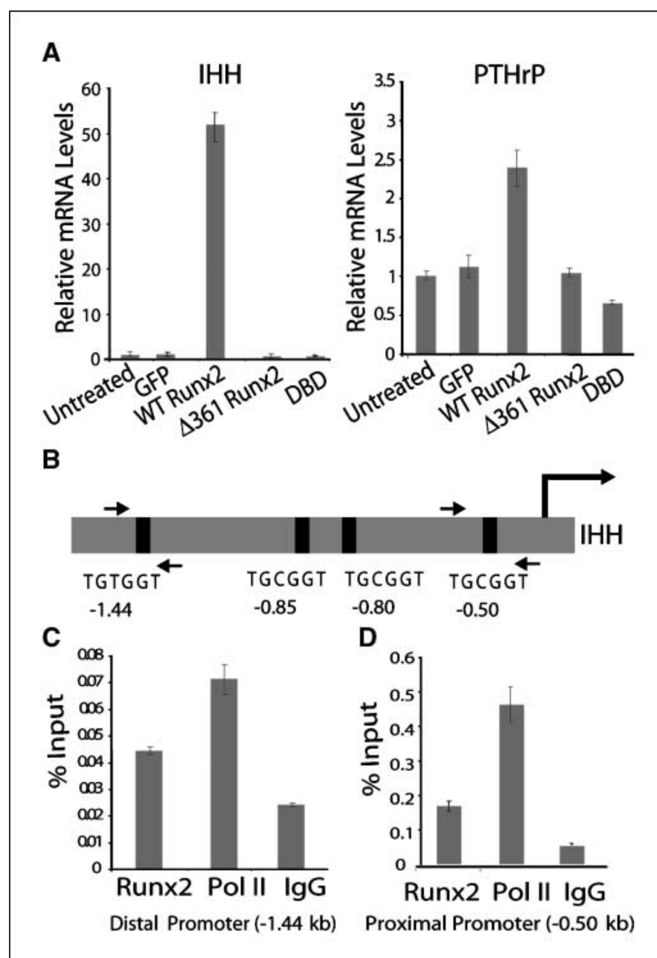

Figure 2. Runx2 COOH-terminal multifunctional transactivating domain is critical for IHH and PTHrP expression and directly regulates the human *IHH* gene. *A,* MDA-MB-231 cells were transduced with WT Runx2, Δ361, and DBD Runx2 or GFP control adenovirus. Total RNA was used to detect mRNA levels of IHH and PTHrP by qRT-PCR analysis. *B,* the schematic shows 1.5-kb 5′-upstream promoter fragment of *IHH* gene. The putative Runx-binding elements are indicated within proximal and distal domains. MDA-MB-231 cells were subjected to ChIP assay with Runx2, Pol II, or control IgG antibody. Immunoprecipitated DNA was further amplified by real-time qPCR analysis with the primer pairs for proximal promoter (*C*) and distal promoter (*D*).

Runx2-mediated PTHrP regulation requires IHH signaling. Our results suggest that Runx2 can induce IHH and PTHrP expression in breast cancer cells. A recent report shows that IHH signaling molecule, Gli2, is involved in PTHrP regulation in bone metastatic cancer cells (12). To understand the contribution of IHH signaling in Runx2-mediated PTHrP induction, we used Hedgehog signaling inhibitor cyclopamine and examined PTHrP induction with overexpression of Runx2 in breast cancer cells. Cyclopamine blocks interaction of Smoothened and Patch receptors and thus inhibits downstream Hedgehog signaling events to its targets. Blocking Hedgehog signaling results in loss of Runx2-mediated induction of PTHrP after 12 h (Fig. 3*A*) or 24 h (Fig. 3*B*) of cyclopamine treatment. These results show that both Runx2 and

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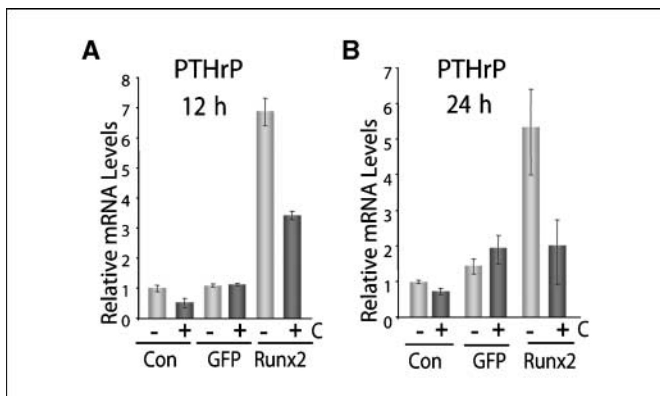

Figure 3. IHH signaling is required for Runx2-mediated regulation of PTHrP. The MDA-MB-231 cells were transduced with Runx2 or GFP adenovirus followed by Hedgehog inhibitor cyclopamine (*C*; 5 μmol/L) treatment. Total RNA from cells harvested at 12 h (*A*) or 24 h (*B*) was used to detect PTHrP levels by qRT-PCR analysis.

IHH signaling are required for PTHrP expression in breast cancer cells.

Runx2 is required for TGFβ-mediated activation of PTHrP and cyclin D1 in breast cancer cells. During metastasis, tumor cells secrete factors that stimulate osteoclast-mediated bone resorption, which releases active factors from the bone matrix, particularly TGFβ. It has been shown that TGFβ stimulates PTHrP and osteolytic metastases via Smad and mitogen-activated protein kinase (MAPK) signaling pathways (21). Several studies also showed that Runx2 is a key component in TGFβ signaling by interacting with Smad proteins (14, 22, 23). The present study indicates that levels of PTHrP are regulated by Runx2. To further investigate the role of Runx2 in TGFβ-induced PTHrP levels in breast cancer cells, we treated MDA-MB-231 cells with Runx2 siRNA oligos to deplete Runx2 protein in the presence and absence of TGFβ and examined PTHrP mRNA levels (Fig. 4). The knockdown of Runx2 protein after siRNA was confirmed by Western blot analysis, which shows >75% depletion compared with controls in both the absence and presence of TGFβ (Fig. 4*A*). Runx2 knockdown had no effect on basal IHH expression but did decrease IHH in TGFβ-treated samples (Fig. 4*B*). Consistent with previous reports (21), we find a 10-fold increase in PTHrP expression on TGFβ treatment (Fig. 4*C, left*). Runx2 knockdown results in significant down-regulation of TGFβ-induced PTHrP levels compared with nonspecific siRNA or untreated control samples. To address if the decrease in PTHrP by Runx2 is a consequence of decreased IHH, we examined PTHrP expression in the presence of cyclopamine and find a 2-fold decrease in TGFβ-induced PTHrP levels (Fig. 4*C, right*). Therefore, the decreased IHH by knockdown of Runx2 (Fig. 4*B*) accounts for decreased PTHrP expression.

We performed complementary studies where MDA-MB-231 cells were transduced with Runx2 or control GFP adenovirus and examined the PTHrP levels after TGFβ treatment. Our results indicate that overexpression of Runx2 further enhances TGFβ-mediated expression levels of PTHrP up to 2- to 3-fold over endogenous levels in breast cancer cells (Fig. 4*D*). These results indicate that Runx2 is a key regulator of IHH as well as TGFβ-induced PTHrP expression.

We further addressed how Runx2 levels affect the direct PTHrP downstream target gene, *cyclin D1* (24). Cyclin D1 is a key cell cycle regulatory protein with shown oncogenic activity in a variety of malignancies, including breast cancer (25). Importantly, *cyclin D1* is a fundamental target gene of TGFβ and PTHrP during skeletal

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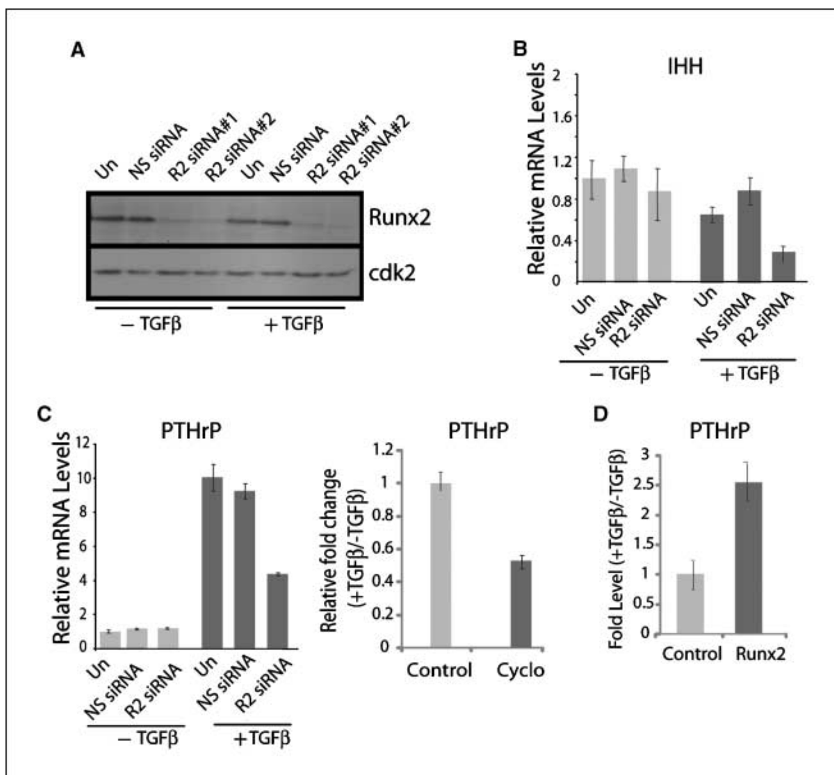

Figure 4. Runx2 contributes to regulation of the TGFβ-mediated activation of PTHrP in cancer cells. MDA-MB-231 cells were transfected with Runx2 siRNA oligo #2 or control oligos (*NS*) for 36 h followed by TGFβ (5 nmol/L) treatment for another 24 h. *A,* the knockdown levels of Runx2 were confirmed by Western blot analysis. Cells were harvested for total RNA to detect IHH (*B*) or PTHrP (*C*) levels by qRT-PCR analysis. *C, right,* PTHrP levels in MDA-MB-231 cells treated with cyclopamine (*Cyclo*; 5 μmol/L) followed by TGFβ induction (5 ng/mL) for 24 h. Overexpression of Runx2 increased TGFβ responsiveness to PTHrP. *D,* PTHrP mRNA levels in MDA-MB-231 cells transduced with control or Runx2 adenovirus followed by TGFβ treatment.

growth (26, 27). To examine the contribution of Runx2 in cyclin D1 regulation by PTHrP and TGFβ, we performed *in situ* immunofluorescence and RT-PCR studies to detect endogenous cyclin D1 in TGFβ-treated MDA-MB-231 cells expressing either shRNA for Runx2 or scramble sequence. We find that TGFβ treatment in MDA-MB-231 cells increases cyclin D1 levels analogous to regulation in normal skeletal cells (Fig. 5*A, top, Scramble*). However, knockdown of endogenous Runx2 in these cells abolished the TGFβ-mediated cyclin D1 induction as shown by *in situ* immunofluorescence (Fig. 5*A, bottom*) and quantitative RT-PCR (qRT-PCR) analysis (data not shown). Taken together, these studies show that Runx2 is a key transcription factor in regulating the complete pathway from TGFβ to PTHrP to cyclin D1 in breast cancer cells.

Runx2 colocalizes with Hedgehog signaling molecule Gli2. Hedgehog signaling is mediated by Gli proteins to its downstream targets (e.g., PTHrP; ref. 12). To understand how Runx2 modulates Hedgehog-PTHrP pathway and its mediators, we examined colocalization of Runx2 with Gli1 and Gli2 by *in situ* immunofluorescence. We find significant increase in colocalization of endogenous Runx2 molecule with Gli2 (Fig. 5*B*) in the TGFβ-treated (35%) MDA-MB-231 cells compared with treatment control (3%). There was no significant colocalization of Runx2-Gli1 detected (data not shown) in MDA-MB-231 cells. These results indicate that Runx2 cooperates with Hedgehog signaling molecule specifically through Gli2 in regulation of downstream target genes in metastatic breast cancer cells in response to TGFβ treatment.

Depletion of Runx2 results in decreased osteoclasts formation. It has been previously documented that Runx2 expression in highly bone metastatic breast MDA-MB-231 and prostate PC3 cells is associated with osteolytic disease (4, 7). To determine the relationship between this activity of Runx2 and PTHrP pathway, we developed stable MDA-MB-231 cells expressing shRNA for Runx2. Depletion of Runx2 was confirmed by Western blot analysis (Fig. 6*A, left*). To test the hypothesis that Runx2 in MDA-MB-231 cells alters the response to PTHrP that leads to induced RANKL production from osteogenic lineage cells in bone microenvironment, we cocultured shRNA-Runx2 or control cells with MC3T3 osteoblastic cells. The conditioned medium from this coculture was then used (in a 50:50 ratio with fresh medium) to differentiate *ex vivo* mouse bone marrow cell osteoclasts in the absence of exogenous RANKL. Our result shows significantly less formation (70% reduction) of multinucleated cells detected by osteoclast-specific TRAP staining (day 7) in cells incubated with shRNA-Runx2 breast cancer cell conditioned medium compared with control cells (Fig. 6*A, right*). The RT-PCR analysis of genes expressed in the day 7 bone marrow cultures revealed down-regulation of osteoclast markers (TRAP, cathepsin K, TNFα, and integrins αv and β3) in cells cultured in shRNA-Runx2 conditioned medium in comparison with controls (Supplementary Fig. S2). These results are consistent with the known activation of PTH/PTHrP in increasing osteoclastogenesis and inhibition of the PTHrP signaling in decreasing osteoclast formation (11, 28, 29) and now provide direct evidence for Runx2 in breast cancer cells in contributing toward the activation of osteoclasts in the bone microenvironment by cancer cells through regulation of IHH and PTHrP expression.

Runx2 knockdown blocks breast cancer cell–mediated osteolysis *in vivo*. We show that Runx2 activates TGFβ-mediated PTHrP levels in breast cancer cells and depletion of Runx2 in MDA-MB-231 cells decreases osteoclast differentiation derived from bone marrow cells. To further evaluate direct consequence of Runx2 knockdown in breast cancer cell–mediated osteolysis induced by PTHrP *in vivo*, we performed intratibial injections of MDA-shRNA-scramble or MDA-shRNA-Runx2 cells in SCID mice (n = 6) and assessed the bone loss up to 3 weeks after injections. Our results indicate that MDA-shRNA-scramble cells produce massive osteolysis within 15 days of injections in all (n = 6) mice, whereas MDA-shRNA-Runx2 cells did not show any significant evidence of osteolytic disease in five of six mice (Fig. 6*B*, osteolysis indicated by *arrows*). Only one mouse had evidence of minor trabecular bone loss as examined by radiography (Fig. 6*B*, minor osteolysis indicated by *).

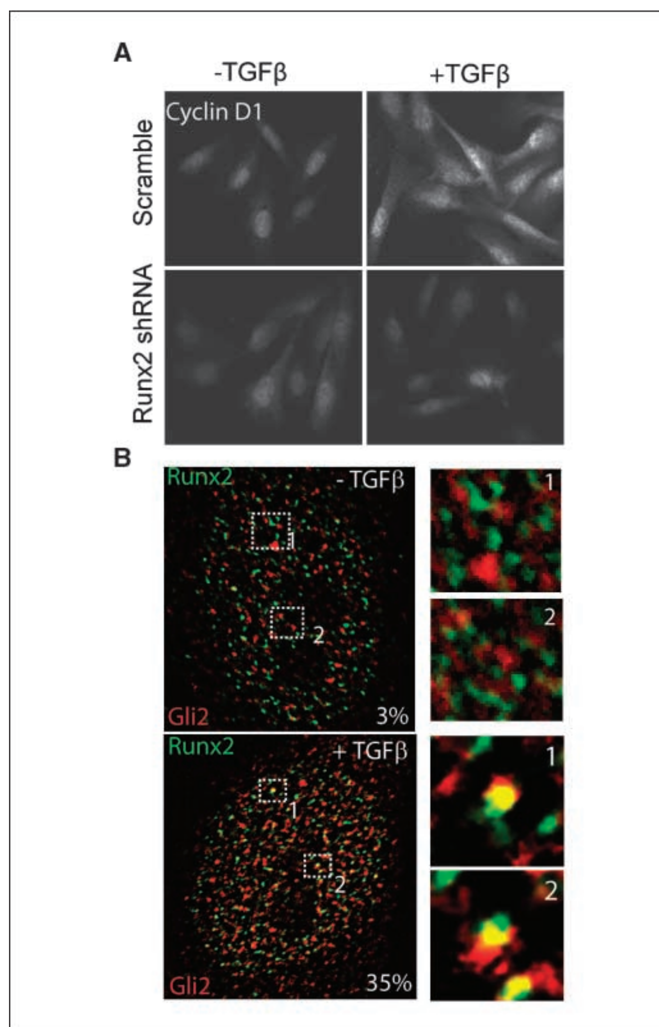

Figure 5. Runx2 regulates TGFβ and PTHrP signaling for their target gene, *cyclin D1*, and colocalizes with Gli2, a Hedgehog signaling molecule. Depletion of Runx2 in MDA-MB-231 cells blocks cyclin D1 nuclear localization in response to TGFβ. *In situ* immunofluorescence (*A*) of endogenous cyclin D1 in MDA-MB-231 cells expressing scramble or shRNA-Runx2 and treated with TGFβ (5 ng/mL) or control for 18 h. *B,* immunolocalization of endogenous Gli2 (*red*) and Runx2 (*green*) protein in MDA-MB-231 cells indicates increased colocalization (*yellow*) from 3% (*−TGFβ*) to 35% in the presence of TGFβ as shown in magnified fields (*right*).

We next addressed the cellular changes in the tumor-bone microenvironment resulting in decreased metastatic bone disease (Fig. 6*C*). We performed detailed characterization of the tibial bones by histologic, Ki-67, and TUNEL analysis. In the shRNA-scramble control group, large tumor areas were present within the marrow cavity of the tibial sections and in muscle tissue outside

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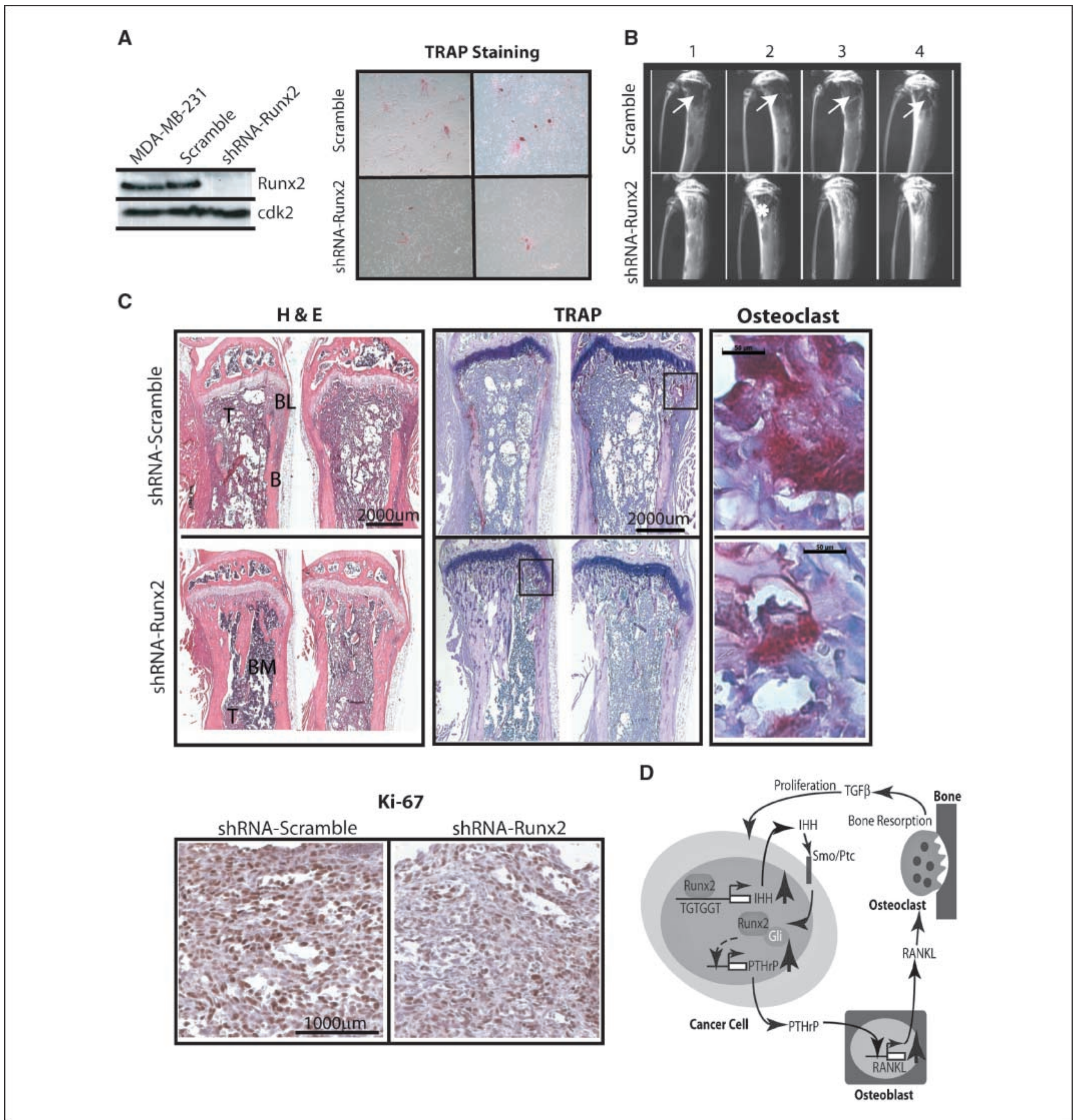

Figure 6. Runx2 decreases osteoclastogenesis *in vitro* and prevents osteolytic bone disease *in vivo*. Runx2 knockdown in cancer cells decreases osteoclasts formation from bone marrow–derived cells. *A,* Western blot analysis of whole-cell extract from MDA-MB-231 cells stably expressing shRNA-Runx2 or scramble sequence showing Runx2 protein levels. TRAP staining of bone marrow–derived cells cultured (day 7) in conditioned medium from coculture of MC3T3 cells with either MDA-shRNA-Runx2 or MDA-scramble cells. *B,* Runx2 knockdown in cancer cells reduces osteolysis *in vivo*. Radiographs of tibia injected with MDA-MB-231 cells expressing shRNA-scramble or shRNA-Runx2 at 15 d after injection. *C,* histology and immunohistochemistry of bones injected with either MDA-shRNA-scramble or MDA-shRNA-Runx2 stable cells. H&E- and TRAP-stained panels of sections from tibia (from mouse #1 and #2 as shown in *B*) indicating bone lesions (*BL*) and osteoclast activity in bone microenvironment in shRNA-scramble controls. *B,* bone tissue; *T,* tumor cells. TRAP panel shows robust osteoclast activity in shRNA-scramble compared with shRNA-Runx2 bone samples. Note that in mouse #1 of shRNA-Runx2 sample, there is more osteoclast activity than in mouse #2 consistent with radiography in *B*, but the osteoclasts are fewer and smaller than in scramble control. *Osteoclast panel,* magnified boxed areas show the presence of large multinucleated osteoclasts in shRNA-scramble group. *Ki-67 panel,* cancer cell proliferation determined by Ki-67 staining of an island of tumor cells from shRNA-scramble or shRNA-Runx2 tibias. *D,* illustration of Runx2 regulation of IHH-mediated and TGFβ-PTHrP–mediated activation of the bone-destructive vicious cycle operative in breast cancer cells. Runx2 directly activates IHH and colocalizes with Gli2 protein in cancer cells. PTHrP-induced RANKL production from osteogenic lineage cells in bone microenvironment further activates osteoclast. The resorbed bone releases TGFβ, thereby stimulating tumor cell proliferation and consequently increased PTHrP secretion and more bone resorption, thus continuing the vicious cycle. It is not clear how Runx2 activates PTHrP through interactions with coregulatory factors that increase PTHrP expression.

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the bone. We find increased presence of large multinucleated osteoclasts in shRNA-scramble group accounting for complete loss of trabecular and cortical bone compared with shRNA-Runx2 animals as examined by TRAP staining (Fig. 6*C*). Minimal change in bone tissue organization was observed in the shRNA-Runx2 group animals (Fig. 6*C*). Thick bone trabeculae are found under the growth plate. The medullary cavity was composed of mostly normal intact marrow tissue with a few small aggregates of tumor cells with minimal osteolysis observed on the endosteal surface. Interestingly, we find a 37% decrease in the population of actively proliferating tumor cells in small tumor aggregates found in the group expressing shRNA-Runx2 compared with scramble controls by Ki-67 staining (Fig. 6*C*). We further examined apoptotic index in tumor cells by TUNEL assay on tibial sections and find no significant difference of apoptotic cell number between shRNA-scramble and shRNA-Runx2 animals (Supplementary Fig. S3*A*). These observations suggested that shRNA-Runx2 cells have decreased growth responsiveness in the bone microenvironment. To further strengthen this conclusion, we examined *in vitro* cell proliferation of MDA-MB-231 cells expressing shRNA-Runx2 and find no significant difference from shRNA-scramble–expressing cells as determined by BrdUrd labeling (Supplementary Fig. S3*B* and *C*). Therefore, the major consequence of shRNA-Runx2 seems to be an inhibition of the TGFβ-tumor cell growth response and a significant block of the osteolysis event. Thus, these results show a direct role of Runx2 in TGFβ-PTHrP–mediated activation of the bone-destructive vicious cycle operative in breast cancer cells (Fig. 6*D*).

Discussion

The present study provides the direct evidence linking Runx2 to PTHrP and the vicious cycle of osteolytic disease by activation of the *IHH* gene in breast cancer cells. Runx2 mediates IHH transcription in both breast and prostate cancer cells and this event further activates PTHrP expression. The DNA-binding activity and the COOH-terminal domain of Runx2 are essential for IHH activation, indicating that the subnuclear organization of the Runx2 regulatory complex is required for this transcriptional event. This activation is physiologic in that it leads to IHH downstream signaling for PTHrP induction to occur as shown by the inability of Runx2 to activate PTHrP in the presence of Hedgehog signaling inhibitor, cyclopamine. Importantly, we show that Runx2 facilitates PTHrP signaling as a mediator of TGFβ-induced PTHrP levels and osteolysis by bone metastatic breast cancer cells, and a complete block in the signaling pathway by shRNA-Runx2 prevented bone resorption by MDA-MB-231 cells *in vivo*. In conclusion, we propose that Runx2 is an important component of TGFβ-IHH-PTHrP axis operative in breast cancer cells that contributes to the osteolytic disease. Our earlier study showed that blocking the subnuclear targeting function of Runx2 inhibits osteolytic lesions (10). Thus, the present study defines a contributory mechanism to account for those findings where blocking Runx2 inhibits the TGFβ-PTHrP signaling pathway.

Under physiologic conditions in developing chondrocytes, IHH is regulated by Runx2 (30, 31). Our results show a similar regulatory mechanism in bone metastatic breast cancer cells. In the developing growth plate, IHH regulates PTHrP levels (32) and a similar mode of regulation is operative in breast cancer cells where PTHrP expression and osteolysis is driven at least in part by Gli2 (12), a mediator of Hedgehog signaling. Thus, our present findings support the concept that cancer cells in bone assume an osteomimicry property (33). Cancer cells in the bone microenvironment respond to TGFβ and take advantage of gene regulatory mechanisms operative under the physiologic conditions using master transcription factors such as Runx2 for their survival and promoting osteolytic disease through activation of the IHH-PTHrP pathway. Notably, the Runx2 COOH terminus contains the Smad-interacting domain that responds to the TGFβ/bone morphogenetic protein signal transduction pathway (14). This domain is required for IHH expression and PTHrP signaling. Thus, our studies provide a novel insight into how metastatic cells become pseudo-osteoblast and secrete bone-resorptive cytokines, such as PTHrP (1, 3) and RANKL (34). The vicious cycle, which functions due to these factors and is partly controlled by a master regulatory transcription factor Runx2, is responsible for pathologic conditions of cancer and causes severe pain and fracture in breast cancer patients.

We and others have previously reported that bone metastatic breast cancer cells produce high amounts of Runx2 transcription factor (6, 7, 35). Runx2 may not only facilitate the bone osteolysis but also promotes metastasis and tumor growth in bone. Runx2 increases expression of several downstream target genes (*MMP9, MMP13, VEGF, MMP2, RANKL*, and *BSP*) associated with metastatic activity of cancer cells (6, 36). Therefore, Runx2 may contribute to the onset of metastasis, as well as the vicious cycle in bone microenvironment. The mechanism for breast cancer cell survival and growth, which finally leads to the destruction of bone tissue, is still not fully understood. The presence of Runx2 in metastatic cells also controls activation of genes related to both cell growth and tissue destruction. Runx2 down-regulates p21, inhibitor of cell growth, and induces MMPs (extracellular matrix–degrading enzymes) and RANKL, an activator of osteolytic resorption. We now add to this list of Runx2-destructive activities in cancer cells induction of PTHrP.

A key finding in our studies is that Runx2 directly regulates basal expression of IHH and mediates TGFβ-induced PTHrP levels in breast cancer cells. The presence of TGFβ greatly enhanced the effects of overexpression and knockdown of Runx2 on IHH and PTHrP expression. Physical interaction between TGFβ-induced Smads and Runx2 has been established in several cell types (6, 37–39). Thus, a Runx2-Smad complex may be contributing to regulation of these genes. In addition, MAPK signaling and the synergy between Smad and Ets proteins has been shown for TGFβ-mediated regulation of PTHrP in MDA-MB-231 cells (21). Ets, like Smad proteins, also interacts with Runx factors (40). Our finding of colocalization of Runx2 and Gli2 in breast cancer is consistent with the recently reported interaction between Runx2 and IHH signaling molecule Gli2 that is important for Runx2 function in osteoblast differentiation (41). These reports together with our results suggest that Runx2 interaction with its cofactors, such as Smads, Ets, and Gli2 (as we show), is a key event in activation of IHH and PTHrP in cancer cells. Our results from histologic analysis and Ki-67 staining of tumors show that Runx2 depletion in cancer cells decreases their growth responsiveness to TGFβ in the bone microenvironment. The reduced growth response of cancer cells further inhibits the activation of osteoclasts and bone erosion, thus blocking the vicious cycle.

In conclusion, we have presented direct evidence that Runx2, in addition to promoting expression of metastasis related markers, also activates IHH and is an integral part of TGFβ-

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mediated PTHrP regulation in breast cancer cells that facilitates the vicious cycle of cancer cell survival and osteolytic bone disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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