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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 TGF β mediated tumor growth and osteolysis. We find that Runx2 directly upregulates Indian Hedgehog (IHH) and colocalizes with Gli2, a Hedgehog signaling molecule. These events further activate parathyroid hormone related peptide (PTHrP). Furthermore, Runx2 directly regulates the TGF β induced PTHrP levels. A subnuclear targeting deficient mutant Runx2 which disrupts TGF β 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 TGF β . In vivo blockade of the Runx2-IHH pathway in MDA-MB-231 cells by Runx2 shRNA inhibition prevented the osteolytic disease. Thus, our studies define a novel role of Runx2 in upregulating the vicious cycle of metastatic bone disease, in addition to Runx2 regulation of genes related to progression of tumor metastasis.

Keywords

Runx2; Bone Metastasis; Breast Cancer; PTHrP; TGF β ; Vicious cycle; shRNA-Runx2; MDA-MB-231

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, RANKL, TGF β and PTHrP, that 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 non metastatic 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).

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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, tumorigenesis and essential mediators of tumor invasion and metastasis (8,9). These well known concepts for the functional activities of the Runx2 regulated genes, implicates 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 (parathyroid hormone related peptide) by breast cancer cells that has a profound effect on tumor cell activities, survival and when present in the bone microenvironment results in osteoclastic bone resorption (1). The resorbed bone releases TGF β 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 pathological consequences in a variety of human tumors (12,13).

Here we show that Runx2 binds to the IHH promoter, 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

Non-metastatic MCF 7 and the metastatic MDA-MB-231 human breast cancer cell lines were cultured in α -MEM containing 10% fetal bovine serum (FBS) (Invitrogen Inc. CA). The procedure for adenovirus expressing wild type and mutant Runx2 (Δ 361: C-terminal deleted mutant and DBD: DNA binding mutant) are reported previously (14). Cells were treated with 5nM TGF β for 24 h.

Western blot analysis

Runx2 protein in breast cancer cells were 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 PVDF membrane and incubated with mouse monoclonal Runx2 antibody followed by incubation with HRP-conjugated secondary antibodies (Santa Cruz, CA). Immunoreactive proteins were detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL).

Chromatin Immunoprecipitations

Chromatin immunoprecipitations (ChIP) were performed as previously described (7). Briefly, formaldehyde crosslinking 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) or IgG as a control. Purified DNA was subjected to real time PCR amplification with Syber 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' AATAGCATTTGAGTGAGAATTTTAAAG-3' and reverse primer: 5' -GGATCCCTTCTGCCAAAACAA-3' for distal promoter fragment.

siRNA and shRNA 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) were obtained from QIAGEN Inc. (Stanford, CA). Cells were transfected with either Runx2 or control siRNA duplexes specific for green fluorescent protein (GFP) at a concentration of 50 nM with Oligofectamine as per manufacturer's instructions. To generate MDA-MB-231 cells expressing shRNA-Runx2, we cloned 5'-AAGGTTCAACGATCTGATTTG-3' sequence in pLVTHM vector under H1 promoter and generated lentiviral particles utilizing Invitrogen Block-it kit. MDA-MB 231 cells were infected with lentivirus and FACS sorted for GFP signal to obtain shRNA expressing cell population. Runx2 knockdown efficiency was confirmed by western blot analysis.

Real time RT-PCR analysis

Expression levels of IHH, Runx2, PTHrP, and GAPDH from MCF-7, 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 synthesized at 42°C with SuperScript II RNA polymerase (Invitrogen). The complementary DNA was then used for real time PCR reaction (Applied Biosystems, Inc., Foster City, CA). For PCR amplification, the following primers were used: IHH (forward 5'TAGGCCATAGCAGACG, reverse 5' TTAAGGCCCCATATCAGG-3'), PTHrP (1-139) forward: ACTAACGACCCGCCCTCG; reverse: GAACAAGTTTCAAGTGCGTGTGTC; PTHrP (1-141) forward: AGGAGGCGGTTAGCCCTGT reverse: TCCCATAGCAATGTCTAATTAATCTGG, PTHrP (total) forward: ACCTCGGAGGTGTCCTAAC, reverse: TCAGACCCAAATCGGACGG. Primer sequences for Runx2 and GAPDH are described previously (7). Primers and probes for the TRAP, TNF α , integrin β 3 and α v genes were all Assays-on-Demand products from Applied Biosystems and reactions were set up according to manufacturer's directions.

Histological analysis and Immunohistochemistry

Hematoxylin and eosin staining was performed on paraffin sections from tibial tissues harvested after 15 days of cancer cells injection and fixed in 4% paraformaldehyde and decalcified in 14% EDTA solution as previously described (15). Ki-67 (1:1,00 rabbit polyclonal, Santa Cruz) immunohistochemistry was performed by using antigen retrieval with antigen unmasking solution (Dako Cytomation) diluted 1:100. Sections were incubated overnight with rabbit polyclonal Ki-67 antibody (Santa Cruz, CA) followed by incubation with HRP-conjugated secondary antibodies and developed with 3,3'-diaminobenzidine. Tartrate-resistant acid phosphatase (TRAP) enzyme detection for demonstrating osteoclast activities was performed using reagent kits from Sigma-Aldrich Biotechnology (St. Louis, MO). Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using the *In situ* Cell Death detection kit (Roche, IN). Proliferation of the stable MDA-MB-231 cells expressing shRNA-Runx2 or Scramble was quantitated by flow cytometry of BrdU labeled cells with FITC-BrdU antibody (BD Biosciences, CA) (16).

In vivo experiments

Groups of six SCID mice was injected with MDA-MB-231 cells (expressing shRNA-Runx2 or scramble sequence; 1×10^5 cells in 100 μ l 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 HH signaling has been implicated in regulation of PTHrP in breast cancer cells (12). Consistent with the previous results, Fig.1a shows the robust endogenous Runx2 levels in the highly metastatic MDA-MB-231 cells and near absence in the non metastatic MCF7 cell line. The MDA-MB-231 cells also show high levels of IHH and PTHrP as detected by real time PCR analysis (Fig. 1b, c). These 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 wild type Runx2 protein. Cells were harvested at 6, 12, 24 and 48 h after infection and total RNA examined for IHH expression by RT-qPCR analysis. Runx2 transduction efficiency and protein levels were confirmed by GFP signal and western blot analysis (Supplementary Fig. S1a). 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. 1d, left panel), whereas control GFP virus failed to increase basal level of IHH at any time point examined. Furthermore, we find a significant two-three fold induction in total PTHrP levels in MDA-MB-231 cells transduced with Runx2 adenovirus (Fig. 1d, right panel). 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.S1b 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.S1f and g) and find similar results as in MDA-MB-231 cells.

Runx2 C-terminal multifunctional trans-activating 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 C-terminal activities required for forming transcriptionally active complexes in subnuclear domains. The MDA-MB-231 cells were infected with adenovirus expressing wild type (WT) and Runx2 C-terminal deletion (Δ 361) mutant protein. In Fig. 2a activation of IHH and PTHrP is reduced by 75% compared to wild type protein. The in vivo phenotype of this mutant is complete loss of bone formation (20). Runx2 Δ 361 mutant protein lacks an activation /repression functional domain but retains normal DNA binding activity. Therefore to demonstrate that Runx2 activates IHH-PTHrP directly, we examined DBD (DNA Binding Domain) mutant protein and find that it failed to induce IHH or PTHrP in MDA-MB-231 cells (Fig. 2a). Expression levels of these mutants are shown in supplementary Fig. S1a as examined by western blot analysis. The PTHrP expression

levels were reduced to the range of basal levels by both Runx2 $\Delta 361$ 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 a signaling component due to Runx2 C-terminal function and as well as direct DNA binding to IHH promoter is necessary.

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

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 utilized Hedgehog signaling inhibitor cyclopamine and examined PTHrP induction with overexpression of Runx2 in breast cancer cells. Cyclopamine blocks interaction of Smoothened (Smo) and Patch (Ptc) receptors and thus inhibits downstream HH signaling events to its targets. Blocking Hedgehog signaling results in loss of Runx2 mediated induction of PTHrP after 12h (Fig. 3a) or 24 h (Fig. 3b) of cyclopamine treatment. These results demonstrate that both Runx2 and IHH signaling are required for PTHrP expression in breast cancer cells.

Runx2 is required for TGF β mediated activation of PTHrP and CyclinD1 in breast cancer cells

During metastasis, tumor cells secrete factors that stimulate osteoclasts mediated bone resorption which releases active factors from the bone matrix, in particular transforming growth factor-beta (TGF β). It has been shown that TGF β stimulates PTHrP and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways (21). Several studies also showed that Runx2 is a key component in TGF β 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 TGF β induced PTHrP levels in breast cancer cells, we treated MDA-MB-231 cells with Runx2 siRNA oligos to deplete Runx2 protein in presence and absence of TGF β and examined PTHrP mRNA levels (Fig. 4). The knockdown of Runx2 protein after siRNA was confirmed by western blot analysis which shows more than 75% depletion compared to controls in both the absence and presence of TGF β (Fig. 4a). Runx2 knockdown had no effect on basal IHH expression, but did decrease IHH in TGF β treated samples (Fig.4b). Consistent with previous reports (21) we find a 10 fold increase in PTHrP expression upon TGF β treatment (Fig. 4c left panel). Runx2 knock down results in significant down-regulation of TGF β induced PTHrP levels compared to non specific 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 TGF β induced PTHrP levels (Fig. 4c right panel). 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 TGF β treatment. Our results indicate that over expression of Runx2 further enhances TGF β mediated expression levels of

PTHrP up to 2–3 fold over endogenous levels in breast cancer cells (Fig. 4d). These results indicate that Runx2 is a key regulator of IHH as well as TGF β induced PTHrP expression.

We further addressed how Runx2 levels affect the direct PTHrP down-stream target gene, cyclin D1 (24). Cyclin D1 is a key cell cycle regulatory protein with demonstrated oncogenic activity in a variety of malignancies including breast cancer (25). Importantly, Cyclin D1 is a fundamental target gene of TGF β and PTHrP during skeletal growth (26,27). To examine the contribution of Runx2 in Cyclin D1 regulation by PTHrP and TGF β , we performed *in situ* immunofluorescence and RT-PCR studies to detect endogenous Cyclin D1 in TGF β treated MDA-MB-231 cells expressing either shRNA for Runx2 or scramble sequence. We find that TGF β treatment in MDA-MB-231 cells increases Cyclin D1 levels analogous to regulation in normal skeletal cells (Fig. 5a, top panels: scramble). However, knockdown of endogenous Runx2 in these cells abolished the TGF β mediated Cyclin D1 induction as shown by *in situ* immunofluorescence (Fig. 5a, lower panels) and quantitative RT-PCR analysis (data not shown). Taken together, these studies demonstrate that Runx2 is a key transcription factor in regulating the complete pathway from TGF β to PTHrP to Cyclin D1 in breast cancer cells.

Runx2 co-localizes with Hedgehog signaling molecule Gli2

Hedgehog signaling is mediated by Gli proteins to its downstream targets, e.g. PTHrP (12). To understand how Runx2 modulates Hedgehog-PTHrP pathway and its mediators we examined co-localization of Runx2 with Gli1 and Gli2 by *in situ* immunofluorescence. We find significant increase in co-localization of endogenous Runx2 molecule with Gli2 (Fig. 5b) in the TGF β treated (35%) MDA-MB-231 cells compared to treatment control (3%). There was no significant co-localization of Runx2-Gli1 detected (data not shown) in MDA-MB-231 cells. These results indicate that Runx2 co-operates with Hedgehog signaling molecule specifically through Gli2 in regulation of downstream target genes in metastatic breast cancer cells in response to TGF β 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 are 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. 6a left panel). 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 co-cultured shRNA-Runx2 or control cells with MC3T3 osteoblastic cells. The conditioned media from this co-culture was then used (in a 50:50 ratio with fresh media) to differentiate ex vivo mouse bone marrow cells osteoclasts in the absence of exogenous RANKL. Our result shows significantly less formation (70% reduction) of multinucleated cells detected by osteoclast specific tartrate resistant acid phosphatase (TRAP) staining (day 7) in cells incubated with shRNA-Runx2 breast cancer cell conditioned media compared to control cells (Fig. 6a right panel). The RT-PCR analysis of genes expressed in the day 7 bone marrow cultures revealed down regulation of osteoclast markers (TRAP, cathepsin K, TNF- α , integrin α v, and β 3) in cells cultured in shRNA-Runx2 conditioned media in comparison to controls (Supplementary Fig. S2). These results are consistent with the known activation of PTH/PTHrP 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 towards 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 TGF β 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 knock down in breast cancer cell mediated osteolysis induced by PTHrP in vivo, we performed intra-tibial injections of MDA-shRNA-scramble or MDA-shRNA-Runx2 cells in SCID mice (n=6) and assessed the bone loss up to 3 weeks post-injections. Our results indicate that MDA-shRNA-scrambled cells produce massive osteolysis within 15 days of injections in all (n=6) mice while MDA-shRNA-Runx2 cells did not show any significant evidence of osteolytic disease in n=5/6 mice (Fig. 6b, osteolysis indicated by arrows). Only one mouse had evidence of minor trabecular bone loss as examined by radiography (Fig. 6b, minor osteolysis indicated by *).

We next addressed the cellular changes in the tumor–bone microenvironment resulting in decreased metastatic bone disease (Fig. 6c). We performed detailed characterization of the tibial bones by histological, 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 the bone. We find increased presence of large multinucleated osteoclasts in shRNA-scramble group accounting for complete loss of trabecular and cortical bone compared to shRNA-Runx2 animals as examined by TRAP staining (Fig. 6c). Minimal change in bone tissue organization was observed in the shRNA-Runx2 group animals (Fig. 6c). 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 to scramble controls by Ki-67 staining (Fig. 6c). 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. S3a). 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 BrdU labeling (Supplementary Fig. S3b and c). Therefore, the major consequence of shRNA-Runx2 appears to be an inhibition of the TGF β –tumor cell growth response and a significant block of the osteolysis event. Thus, these results demonstrate a direct role of Runx2 in TGF β –PTHrP mediated activation of the bone destructive vicious cycle operative in breast cancer cells (Fig. 6d).

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 C-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 demonstrated by the inability of Runx2 to activate PTHrP in the presence of HH signaling inhibitor, cyclopamine. Importantly, we show that Runx2 facilitates PTHrP signaling as a mediator of TGF β induced PTHrP levels and osteolysis by bone metastatic breast cancer cells as 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 TGF β –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 TGF β -PTHrP signaling pathway.

Under physiological 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 HH 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 TGF β and take advantage of gene regulatory mechanisms operative under the physiological conditions utilizing master transcription factors like Runx2 for their survival and promoting osteolytic disease through activation of the IHH-PTHrP pathway. Notably, the Runx2 C terminus contains the Smad interacting domain that responds to the TGF β /BMP 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 like 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 pathological 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 (ECM 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 TGF β induced PTHrP levels in breast cancer cells. The presence of TGF β greatly enhanced the effects of overexpression and knockdown of Runx2 on IHH and PTHrP expression. Physical interaction between TGF β 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, MAP kinase signaling and the synergy between Smad and Ets proteins has been shown for TGF β mediated regulation of PTHrP in MDA-MB-231 cells (21). Ets, like Smad proteins also interacts with Runx factors (40). Our finding of co-localization 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 co-factors like Smads, Ets and Gli2 (as we show) are key events in activation of IHH and PTHrP in cancer cells. Our results from histological analysis and Ki-67 staining of tumors demonstrate that Runx2 depletion in cancer cells decreases their growth responsiveness to TGF β 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 TGF β mediated PTHrP regulation in breast cancer cells that facilitates the vicious cycle of cancer cell survival and osteolytic bone disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 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 were subjected to western blot analysis to detect Runx2 protein by mouse monoclonal antibody (1:2000). The expression level of cdk2 is shown as internal loading control (lower panel). Total RNA (5µg) from MCF7 and MDA-MB-231 cells was utilized 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 qRT-PCR analysis to

detect IHH (left panel) and PTHrP (right panel) mRNA levels after 48h of viral transduction. The mRNA levels were normalized to GAPDH.

Fig. 2. Runx2 C-terminal multifunctional trans-activating domain is critical for IHH and PTHrP expression and directly regulates the human IHH gene

(a): MDA-MB-231 cells were transduced with wild type Runx2, C-terminal deletion mutant ($\Delta 361$) and DNA binding mutant (DBD) Runx2 or GFP control adenovirus. Total RNA was utilized to detect mRNA levels of IHH and PTHrP by qRT-PCR analysis. (b): The schematic shows 1.5kb 5'-upstream promoter fragment of IHH gene. The putative Runx binding elements are indicated within proximal and the distal domains. MDA-MB-231 cells were subjected to chromatin immunoprecipitation 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).

Fig. 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 (5nM) treatment (C). Total RNA from cells harvested at 12h (a) or 24h (b) was utilized to detect PTHrP levels by qRT-PCR analysis.

Fig. 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 (5nM) treatment for another 24 h. **(a):** The knock down levels of Runx2 was 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 panel):** PTHrP levels in MDA-MB-231 cells treated with cyclopamine (Cyclo: 5μM) followed by TGFβ induction (5nM) for 24 h. **Over expression 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.

Fig. 5. Runx2 regulates TGFβ and PTHrP signaling for their target gene, CyclinD1 and co-localizes 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 immuno-fluorescence (**a**) of endogenous cyclinD1 in MDA-MB-231 cells expressing scramble or shRNA-Runx2 and treated with TGFβ (5ng/ml) or control for 18 hours. (**b**): Immuno-localization of endogenous Gli2 (red color) and Runx2 (green color) protein in MDA-MB-231 cells indicates increased co-localization (yellow) from 3%(-TGFβ) to 35% in the presence of TGFβ as shown in magnified field (side panels).

d

Fig. 6. Runx2 decreases osteoclastogenesis in vitro and in vivo prevents osteolytic bone disease
 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 sh-RNA-Runx2 or Scramble sequence showing Runx2 protein levels. TRAP staining of bone marrow derived cells cultured (day 7) in conditioned medium from co-culture 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 days post injection. (c): Histology and IHC of bones injected with either MDA-shRNA-Scramble or MDA-shRNA-Runx2 stable cells. Hematoxylin-eosin and TRAP stained panels of sections from tibia (from mouse #1 and 2 as shown in Fig. 6b) 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 to shRNA-Runx2 bone samples. Note that in the mouse #1 of shRNA-Runx2 sample there is more osteoclast activity than mouse #2 consistent with radiography in Fig. 6b, but the osteoclast 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 shows 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 and TGFβ-PTHrP mediated activation of the bone destructive vicious cycle operative in breast cancer cells. Runx2 directly activates IHH and co-localizes 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 co-regulatory factors that increase PTHrP expression.