Angiogenesis, Metastasis, and the Cellular Microenvironment

TAK1–TAB2 Signaling Contributes to Bone Destruction by Breast Carcinoma Cells

Alfiya Safina¹, Paula Sotomayor¹, Michelle Limoge¹, Carl Morrison², and Andrei V. Bakin¹

Abstract

Advanced-stage breast cancers frequently metastasize to the bones and cause bone destruction, but the underlying mechanism is not fully understood. This study presents evidence that TGF-β–activated protein kinase 1 (TAK1) signaling in tumor cells promotes bone destruction by metastatic breast carcinoma cells, controlling expression of prometastatic factors including matrix metalloproteinase (MMP) 9 and COX2. Suppression of TAK1 signaling by dominant-negative TAK1 (dn-TAK1) in breast carcinoma MDA-MB-231 cells impairs bone colonization by carcinoma cells and bone osteolysis in the intracardiac injection model. Mechanistic studies showed that inhibition of TAK1 by dn-TAK1 or siRNA blocked expression of factors implicated in bone metastasis, such as MMP-9, COX2/PTGS2, parathyroid hormone–related protein (PTHrP) and interleukin 8 (IL-8), but did not affect activation of p38MAPK by TGF-β. TAK1 signaling is mediated by TAK1-binding partners TAB1, TAB2, and TAB3. Carcinoma cells express elevated mRNA levels of TAB2 and TAB3, whereas the TAB1 expression is noticeably low. Accordingly, depletion of TAB2 by siRNA reduced expression of MMP-9 and COX2. Together, these studies show that the TAK1–TAB2–TAB3 signaling axis is critical for carcinoma-induced bone lesions, mediating expression of proinvasive and osteolytic factors. These findings identify the TAK1–TAB2 axis as a potential therapeutic target in bone metastasis. Mol Cancer Res; 9(8); 1042–53. ©2011 AACR.

Introduction

Patients with advanced-stage breast cancers frequently develop skeletal metastases that cause pain, bone fractures, and other complications (1). Advanced breast cancers express elevated levels of TGF-β cytokines that promote osteoclast-mediated bone destruction (2). Although, the TGF-β pathway is an attractive target for therapeutic intervention in the skeletal metastases, TGF-β is a potent inhibitor of cell growth and functions as a tumor suppressor in the early stages of cancer. In advanced cancers, TGF-β promotes tumor spreading by enhancing invasion and angiogenesis (3). Identifying the factors mediating tumorigenic activities of TGF-β is an important step in the development of effective therapeutics preventing tumor invasion and metastasis.

Cancer progression and bone metastasis have been linked to changes in tumor microenvironment and inflammation. TGF-β cytokines regulate the composition of extracellular matrix, matrix proteolysis, and inflammatory responses (4, 5). According to a current model, carcinoma cells respond to TGF-β with upregulation of parathyroid hormone–related protein (PTHrP) that plays a key role in cancer-associated osteolysis (6). Within the bone microenvironment, PTHrP acts upon osteoblasts to stimulate production of receptor activator of NF-kB ligand (RANKL) that activates osteoclasts (7). Thus, TGF-β, both stored in the bone microenvironment and produced by carcinoma cells, promotes a “vicious cycle” within the bone, causing osteoclast-mediated bone destruction. In addition to the PTHrP-RANKL link, TGF-β may stimulate osteoclasts by regulating interleukin 11 (IL-11) connective tissue growth factor (CTGF), COX2, and matrix metalloproteinases (MMP; ref. 7).

TGF-β cytokines signal through serine/threonine kinase transmembrane type I and type II receptors (8). TGF-β binding to the receptor complex triggers activation of the type I receptor, termed activin-like receptor kinase 5 (ALK5), which activates Smad transcription factors, mitogen-activated protein kinases (MAPK), and phosphoinositide 3-kinase (PI3K)/Akt signaling (9). ALK5 phosphorylates receptor-associated Smad2 and Smad3 (R-Smads) and induces translocation of R-Smads and Smad4 to the nucleus (8). Activation of MAPKs by TGF-β may involve TGF-β–activated kinase 1 (TAK1) or small GTPases (9), whereas TGF-β–mediated activation of PI3K/Akt signaling (10, 11) requires small Rho-like GTPases (10). MAPK and PI3K/Akt signaling cascades are activated independently of Smads (11, 12). All 3 cascades have been implicated in the regulation of tumor microenvironment and invasion and potentially can contribute to the metastatic function of TGF-β (3).

Authors’ Affiliations: Departments of ¹Cancer Genetics and ²Pathology, Roswell Park Cancer Institute, Buffalo, New York

Corresponding Author: Andrei V. Bakin, Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY 14263. Phone: 716-845-1033; Fax: 716-845-1698; E-mail: andrei.bakin@roswellpark.org

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TAK1, a mitogen-activated protein kinase kinase kinase (MAP3K7), was identified as a kinase that mediates TGF-β signaling to MAPK p38 and c-JUN N-terminal kinase (JNK; ref. 13). TAK1 is also involved in bone morphogenic protein (BMP) signaling (14) as well as in inflammatory and innate immune responses (15). TAK1 is activated by association with TAK1-binding proteins TAB1, TAB2, TAB3, and BIRC4/XIAP (16). In addition to activating MAPKs (extracellular signal–regulated kinase (ERK), JNK, and p38), TAK1 is an important activator of NF-κB transcription factor (17). Our recent studies have identified TAK1 as a critical regulator of MMP-9 expression (18). MMP-9/gelatinase-B contributes to the invasive and metastatic potential of cancer cells (19–21), as well as to tumor angiogenesis, by promoting recruitment of endothelial cells (22) and pericytes (23). Indeed, breast tumor angiogenesis is significantly reduced by small hairpin RNA (shRNA) to MMP-9 (21). TGF-β signaling, MMP-9, and NF-κB are implicated in breast- and prostate-cancer–induced bone lesions (7, 24–26). NF-κB transcription factor can also contribute to osteolytic bone lesions in breast carcinoma cells by regulating COX2/PTGS2, CSF2/GM-CSF, and IL6 (26, 27). TGF-β/NFκB signaling may negatively and positively interact with NF-κB signaling in various cell systems, but the role of TAK1 in this interaction is not fully understood (28–30).

The present study explored the role of TAK1 signaling in breast carcinoma–induced bone destruction using the intracardiac injection model in severe combined immunodeficient mice (SCID) mice. Disruption of TAK1 signaling by forced expression of dominant-negative (dn) K63W mutant of TAK1 blocked the formation of osteolytic bone lesions by human breast cancer cell line MDA-MB-231. Mechanistic studies revealed the critical role of TAB2-TAB3 in the regulation of MMP-9 and COX2. These findings indicate that the TAK1/TAB2-TAB3 axis is important for TGF-β–mediated osteolytic bone lesions of breast carcinoma cells.

Materials and Methods

Antibodies, plasmids, and other reagents

TGF-β1 was obtained from R&D Systems. Antibodies were obtained: TAK1 from Sigma (T7824); phospho-HSP27 (2401) and phospho-p38 MAPK (9211) from Cell Signaling Technology; GAPDH (sc25778) from Santa Cruz Biotechnology, Inc. siRNA to human TAK1 and p38MAPK were from Dharmacon, Inc. Scramble control RNA duplexes labeled with rhodamine were from Qiagen. Kinase-inactive TAK1-K63W was a gift of Dr. Hiroaki Sakurai (31). Retroviral vector pBMN-EGFP and constructs encoding TAK1-K63W, Flag-tagged p38alpha-AF, and HA-tagged ALK5-T204D are described in the work of Safina and colleagues (18) and Bakin and colleagues (32). Plasmids encoding shRNA to human TAK1 in the pSHAG-MAGIC2 vector were obtained from the shRNA Core Resource at Roswell Park Cancer Institute (RPCI; Director Dr. Irwin Gelman).

Cell culture

Human breast cell lines MDA-MB-231, MCF10A, MCF7, T47D, MDA-MB-468, MDA-MB-435, and prostate cancer cell line PC3 were obtained from the American Tissue Culture Collection (ATCC) and cultured as recommended by ATCC. MDA-MB-231-ALK5-T204D cells expressing constitutively active ALK5/TGFβR1 are described elsewhere (21). MDA-MB-231 cells expressing shRNA to TAK1 were cultured in the presence of 0.5 μg/mL puromycin, whereas assays were conducted in the absence of antibiotics.

Retroviral infection

MDA-MB-231 cells were infected in the presence of 6 μg/mL of polybrene with amphotropic retroviruses produced in Phoenix cells, as previously described (32). Enhanced green fluorescent protein (EGFP)-positive cells were selected by fluorescence-activated cell sorter. MDA-MB-231 cells were infected using retroviral vector pSHAG-MAGIC2 encoding shRNA to TAK1 and then were selected in the presence of 2 μg/mL puromycin. Cells were routinely maintained in the presence of 0.5 μg/mL puromycin.

siRNA

Cells were transfected with RNA duplexes using Oligofectamine reagents (Invitrogen) following the manufacturer’s protocol. The media was replenished the next day. The cells were grown for an additional 48 hours in the absence or presence of 2 ng/mL TGF-β1 followed by gelatin zymography and immunoblotting.

Immunoblot analysis

Cells were incubated in medium containing 5% serum for 24 hours before treatment with 2 ng/mL TGF-β1. Kinase inhibitors were added 1 hour before treatment with TGF-β1. Cells were lysed in buffer: 20 mmol/L Tris, pH 7.4, 137 mmol/L NaCl, 1% NP-40, 10% glycerol, 20 mmol/L NaF, 1 mmol/L Na orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride, 2 μg/mL aprotinin, and 2 μg/mL leupeptin. Immunoblot analyses of protein extracts were conducted as described (32).

Reverse transcriptase PCR analysis

Reverse transcriptase PCR (RT-PCR) was carried out as described (21). RNA extraction was carried out using Versagene RNA kit (Centra). Amplification of transcripts was conducted using 50 ng/μL of total RNA and one-step RT-PCR system from Invitrogen according to the manufacturer’s protocol. Primer sequences are presented in Table 1. The optimal number of the PCR cycles was determined for each primer set to ensure a linear range of amplification, typically 21 to 26 cycles.

Gelatin zymography

The assay was conducted as described (21). Conditioned media samples were prepared from cells incubated in serum-free Improved Modified Eagle’s Medium for 48 hours. SDS-PAGE gels were copolymerized with gelatin at a final
concentration of 1 mg/mL. After electrophoresis, the gels were renaturated in 2.5% Triton X-100 and incubated at 37°C for 24 hours in 5 mmol/L CaCl₂ and 50 mmol/L Tris-HCl buffer, pH 7.5, containing 0.05% NaN₃. The gels were stained with 0.5% Coomassie Blue R-250 and destained in 10% methanol and 5% acetic acid in water. Gelatinolytic activities were detected as transparent bands on the blue background. The band densities were evaluated using NIH Image software.

Intracardiac injection model of bone metastasis with MDA-MB-231 cells in SCID mice

Female SCID/CB17 mice, 5 weeks of age, were obtained from a colony of SCID/CB17 mice that was bred and maintained at the Department of Laboratory Animal Resources (DLAR) facility at the RPCI. All animals were kept 3 to 5 mice per cage in microisolator units and provided with water and food ad libitum according to a protocol approved by the Institute Animal Care and Use Committee at RPCI. The facility has been certified by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulation and standards of the U.S. Department of Agriculture and the U.S. Department of Health and Human Services.

Exponentially growing breast cancer cells (200,000) in 0.1 mL of sterile PBS were injected using 27-gauge needle into the left heart ventricle of mice under anesthesia with isoflurane according to the modification of the method described by the work of Arguello and colleagues (33). Formation of bone lesions was monitored biweekly using full-body X-ray imaging with a Faxitron MX-20 X-ray machine.

Histologic analysis
Long bones (femur and tibia) were removed from the mice and fixed in 10% formalin for 24 hours and decalcified in 10% EDTA (pH 7.4) for 14 days and then embedded into paraffin blocks. Serial sections were prepared and stained with hematoxylin and eosin (H&E). Bone lesions observed by radiography were confirmed by histologic examination. Rabbit polyclonal serum to GFP (NB600-308) from Novus Biological was used for detection of tumor cells in histologic bone sections.

TRAP staining for osteoclasts
Sections of the paraffin-embedded blocks of long bones (see above) were prepared. Tartrate-resistant acid phosphatase (TRAP) staining of the deparaffinized sections was carried out according to the manufacturer’s protocol (387A-1KT; Sigma-Aldrich). Images were acquired using Olympus DP25 camera and DP2-BSW software (Olympus). TRAP-positive multinuclear cells were detected at the tumor–bone interface in diaphysis and metaphysis of tumor-carrying animals. The amount of TRAP-positive multinuclear cells was evaluated in 4 random fields within the same section that included normal bone marrow and tumor areas at 400× magnification.

Statistical analysis
Continuous data were compared using the Student’s t test. Differences were considered significant when P < 0.05.

Results
TAK1 is required for breast carcinoma–induced bone lesions
Both the TGF-β and NF-κB signaling pathways have been implicated in the formation of skeletal metastases (7, 24–26), although the underlying mechanisms are likely to be different, as these pathways have opposite effects in various cell systems (28–30). Our recent study has linked TAK1 to tumor invasion and angiogenesis via the TGF-β–TAK1–NF-κB–MMP-9 axis (18), whereas the role of TAK1 in skeletal metastases has not been addressed. The present study assessed the role of TAK1 in the bone metastatic process using the human breast carcinoma

### Table 1. Primer sequences

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<th>Reverse primer</th>
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<td>GM-CSF, BC113999</td>
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<tr>
<td>IL8, NM_000584</td>
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<td>TTCCAGAGGTTTTCACTTGTC</td>
<td>196</td>
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<td>IL11, NM_000641.2</td>
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<td>CCAACCTGTCGCTGAAATA</td>
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</tr>
<tr>
<td>Mmp9, NM_004994</td>
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MDA-MB-231 cell line in the mouse intracardiac injection model (34–36). In this model, the inoculation of carcinoma cells into the left cardiac ventricle of immunodeficient mice results in osteolytic bone lesions, which closely match bone metastases observed in patients (37, 38). The breast carcinoma MDA-MB-231 cell line was established from the pleural effusions of a breast cancer patient with metastatic disease (39). Suppression of TAK1 signaling in MDA-MB-231 cells was achieved by expressing the dn-TAK1-K63W mutant allele from a bicistronic EGFP-encoding retroviral vector (Fig. 1A). The empty vector control and dn-TAK1 cells express EGFP from the internal ribosome entry site (IRES) and have been characterized elsewhere (18). First, we optimized a number of injected cells, a time frame of development of bone lesions, and a method for the decalcification of bones. Formation of osteolytic bone lesions was monitored using full-body X-ray imaging. Radiographs showed that 80% of mice injected with the EGFP control cells developed osteolytic lesions in limbs at 4 weeks after inoculation (Fig. 1A). Histologic analysis revealed that the control cells occupy bone marrow cavities in metaphysis (in the proximity of the growth plate) and diaphysis of tibia and femur bones (Fig. 1B). With the exception of one case with a single metastatic site, mice from the dn-TAK1 group did not develop osteolytic bone lesions either radiographically or histologically at 4 weeks after inoculation (Fig. 1B–D). Dn-TAK1 cells were absent from the bone marrow cavity in diaphysis (Fig. 1D). These findings indicate that the dn-TAK1 cells exhibit a deficiency in the bone invasiveness compared with the control cells, and this is consistent with a reduced invasive and metastatic potential of MDA-MB-231–dn-TAK1 cells (18). Dn-TAK1 did not affect the tumor cell growth in culture but markedly reduced tumor angiogenesis (18). Thus, the reduction in bone metastases by dn-TAK1 could not be explained by the growth defect, and it is likely associated with tumor–host interactions in the tumor angiogenesis and/or in the “vicious cycle” within the bone.

**TAK1 contributes to osteoclastogenesis induced by breast carcinoma cells**

In the intracardiac bone metastasis model, MDA-MB-231 cells induce the osteolytic bone lesions that are caused
primarily by the disproportionate activity of osteoclasts (6, 7). The osteoclasts are derived from the pluripotent hematopoietic precursors in the bone marrow, and both TGF-β and NF-κB promote osteoclast differentiation and maturation (6, 26). To explore the effect of dn-TAK1 on osteoclasts, bone sections were stained for the enzymatic activity of TRAP, a marker of osteoclasts. The activated osteoclasts were identified as TRAP-positive and multinucleated (>3 nuclei) cells (Fig. 2). Microscopic inspection revealed high amounts of osteoclasts at the bone–tumor interface of the EGFP control group compared with the bone–normal marrow interface in metaphysis and diaphysis (arrows), as well as within the tumor area (stars) versus the normal bone marrow (Fig. 2). The amount of osteoclasts in the dn-TAK1 tumor sections and tumor–bone border were significantly reduced compared with the EGFP control areas (Fig. 2). Thus, these findings show that carcinoma cells increase the amount of activated osteoclasts in the bone environment and suggest that dn-TAK1 affects either homing or tumor–bone interactions, reducing the osteoclast response.

**TAK1 contributes to the activity of the NF-κB/MMP-9 axis in breast carcinoma cells**

Previous studies have suggested that tumor TGF-β signaling regulates expression of factors promoting the formation of osteoclasts (6, 40, 41). In MDA-MB-231 cells, TGF-β facilitates the bone osteolysis program by inducing IL11 and CTGF via Smads (40, 41), as well as upregulating PTHrP via p38MAPK (6). Previous studies have shown that dn-TAK1 does not inhibit Smad signaling (18) but rather reduces activity of NF-κB that regulates expression of MMP-9 and other osteolytic factors (18, 26, 27). To assess the effect of dn-TAK1 on p38MAPK signaling, we examined phosphorylation of p38 and its target HSP27 by immunoblotting. Dn-TAK1 did not block phosphorylation of p38 and HSP27 in response to TGF-β in MDA-MB-231...
cells (Fig. 3A). Then, the effect of dn-TAK1 on MMP-9 expression in response to TGF-β and inflammatory cytokine IL1-β was explored using gelatin zymography. Dn-TAK1 abrogated upregulation of MMP-9 by TGF-β or IL1-β alone, as well as by their combination (Fig. 3B). To confirm this finding, TAK1 was suppressed by shRNA in MDA-MB-231 cells (Fig. 3C). Gelatin zymography revealed a significant reduction of MMP-9 levels by depletion of TAK1 (Fig. 3D), whereas suppression of TAK1 did not affect phosphorylation of HSP27, a target of p38 (Fig. 3E). Together, these findings show that TAK1 contributes to TGF-β and IL1-β responses in breast carcinoma cells, and MMP-9 is a target of both pathways, whereas p38 signaling is not affected by the TAK1 suppression.

**TAK1 and MMP-9 in breast carcinoma cell lines**

Our findings show that TAK1 contributes to the invasive potential of tumor cells and tumor colonization of the bone marrow. TAK1 mediates expression of metalloproteinase MMP-9 in MDA-MB-231 cells (Fig. 3), and MMP-9 is capable of degrading the bone matrix as well as it is critical for tumor angiogenesis (19–22). Thus, the TAK1–MMP-9 axis may represent an essential pathway in tumor-induced bone osteolysis. To assess whether this axis operates in other breast carcinoma cells, we first evaluated expression of TAK1 and TABs in normal and carcinoma cell lines using RT-PCR with a range of cycles to ensure the proportionality of amplification. RT-PCR analysis showed that TAK1, TAB2, TAB3, and TRAF6 are expressed in all tested cell lines, whereas TAB1 levels were significantly lower (Fig. 5A and B). TAB2 mRNA levels were more than 50-fold higher than TAB1. These results were confirmed by the Affymetrix microarrays (data not shown). The analysis also showed that TAK1 and TAB2 are expressed at elevated levels in tumor cell lines. To assess the contribution of TAB2 to the MMP-9 expression, we examined the effect of siRNA to TAB2 on MMP-9 in breast and prostate carcinoma cell lines. RT-PCR showed that TAB2 levels were efficiently depleted by siRNA to TAB2 in breast carcinoma MDA-MB-231 cells (Fig. 4A). Then, the effect of dn-TAK1 on MMP-9 expression in response to TGF-β and inflammatory cytokine IL1-β was explored using gelatin zymography. Dn-TAK1 abrogated upregulation of MMP-9 by TGF-β or IL1-β alone, as well as by their combination (Fig. 3B). To confirm this finding, TAK1 was suppressed by shRNA in MDA-MB-231 cells (Fig. 3C). Gelatin zymography revealed a significant reduction of MMP-9 levels by depletion of TAK1 (Fig. 3D), whereas suppression of TAK1 did not affect phosphorylation of HSP27, a target of p38 (Fig. 3E). Together, these findings show that TAK1 contributes to TGF-β and IL1-β responses in breast carcinoma cells, and MMP-9 is a target of both pathways, whereas p38 signaling is not affected by the TAK1 suppression.

**TAK1–TAB2 signaling in carcinoma cells**

TAK1 signaling is controlled by TAK1-binding partners TAB1, TAB2, and TAB3 (16). TAB1 regulates TAK1 activation in some cell lines leading to phosphorylation of TAK1 at Thr187 (16, 42), whereas TAB2 and TAB3 link TAK1 to K63-ubiquitin-dependent signaling by TRAF6, TRAF2, and TRAF5 (43–46). To assess the contribution of TABs to the TAK1–MMP-9 axis, we first determined the expression levels of TAK1 and TABs in normal and carcinoma cell lines using RT-PCR with a range of cycles to ensure the proportionality of amplification. RT-PCR analysis showed that TAK1, TAB2, TAB3, and TRAF6 are expressed in all tested cell lines, whereas TAB1 levels were significantly lower (Fig. 5A and B). TAB2 mRNA levels were more than 50-fold higher than TAB1. These results were confirmed by the Affymetrix microarrays (data not shown). The analysis also showed that TAK1 and TAB2 are expressed at elevated levels in tumor cell lines. To assess the contribution of TAB2 to the MMP-9 expression, we examined the effect of siRNA to TAB2 on MMP-9 in breast and prostate carcinoma cell lines. RT-PCR showed that TAB2 levels were efficiently depleted by siRNA to TAB2 in breast carcinoma MDA-MB-231 cells (Fig. 4A).
and MCF7 as well as in prostate carcinoma PC3 cells (Fig. 5C). The specificity of TAB2 knockdown was confirmed in MDA-MB-231, where siRNA to TAB2 did not affect TAB1 and TAB3 levels (Fig. 6C). Production of MMP-9 was evaluated by zymography. These assays revealed that depletion of TAB2 markedly reduced MMP-9 production in all tested cell lines (Fig. 5D). Collectively, these findings show that TAB2 is required for MMP-9 production by breast and prostate carcinoma cells. It appears that TAB1 is expressed at low levels in human carcinoma cell lines.

**Targets of TAK1–TAB2 signaling in carcinoma cell lines**

To identify TAK1 effectors contributing to the bone metastatic potential of carcinoma cells, the mRNA levels of
known TGF-β and IL1-β targets were assessed by semi-quantitative RT-PCR. Dn-TAK1 reduced basal mRNA levels of PTGS2/COX2, MMP-9, IL8, and PTHrP in MDA-MB-231 cells (Fig. 6A). TGF-β-mediated induction of PTGS2, MMP-9, and IL8 was also inhibited by dn-TAK1, whereas upregulation of PTHrP was not affected. As expected, the mRNA levels of IL11 and CTGF were not affected by dn-TAK1, as expression of these genes is controlled by TGF-β/Smad signaling (41). The comparison of PTGS2/COX2 and IL8 levels in tumor cells expressing dn-TAK1 and shRNA-TAK1 revealed that suppression of TAK1 by either dn-TAK1 or shRNA markedly reduced basal and TGF-β-induced levels of PTGS2, whereas primarily basal levels of IL8 were affected (Fig. 6B). Finally, we evaluated the contribution of TAB1, TAB2, and TAB3 to PTGS2 expression using siRNA. The specificity of siRNA-mediated depletion was confirmed by RT-PCR (Fig. 6C). The analysis revealed that PTGS2 expression was reduced by depletion of TAB2 and TAB3, whereas suppression of TAB1 had no effect (Fig. 6C). These findings show that the TAK1–TAB2–TAB3 signaling axis can operate in both breast and prostate carcinoma cells. The link of TAK1 to MMP-9 and COX2 may explain a defect in bone colonization by dn-TAK1-expressing tumor cells. MMP-9–deficient mice have abnormalities in vascularization and ossification of the skeletal growth plate (48). MMP-9 is also critical for matrix-degrading activity of tumor cells (21), and the release of growth factors in bone environment (22, 24). High levels of COX2/PTGS2 in primary tumor correlate with bone marrow micrometastases in breast cancer patients (49). Forced expression of COX2 enhances bone degradation in the bone metastasis model of breast carcinomas (50). The reduction of PTHrP and IL8 levels by dn-TAK1 may contribute to a decrease in the activated osteoclasts in the dn-TAK1 group. PTHrP is a major component of the "vicious cycle" within the bone, causing osteoclast-mediated bone destruction (Fig. 7B). TAK1 is required primarily for the basal expression of PTHrP, but, apparently, it has limited input in the TGF-β-mediated upregulation of PTHrP that requires p38MAPK (6). This is consistent with unaltered activation of p38 signaling in dn-TAK1 cells (Fig. 3). The link of TAK1 to expression of IL8 could be of a particular importance. Chemokine IL8 activates macrophages and osteoclasts enhancing bone degradation and angiogenesis (47, 51, 52). Both PTHrP and IL8 may act directly on osteoclasts or...
upregulate expression of RANKL, receptor activator of NF-κB ligand, by osteoblast/stromal cells (47, 52). Breast carcinoma cells do not secrete RANKL and did not support osteoclastogenesis in in vitro experiments (our unpublished results). Hence, PTHrP and IL8 are likely to mediate tumor-induced osteoclastogenesis in the bone marrow environment. Together, these findings argue that the TAK1–TAB2-TAB3 axis represents an essential pathway for the bone metastatic potential of tumor cells, controlling expression of bone invasive factors MMP-9 and COX2 as well as osteolytic factors PTHrP and IL8 (Fig. 7).

Human cancer cells that produce osteolytic lesions frequently express elevated levels of IL1-β and TNF-α, which are involved in the stimulation of osteoclast differentiation and activation (52). The present study found that proinflammatory cytokines IL1-β and TNF-α cooperate with TGF-β in production of MMP-9 by breast carcinoma cells and TAK1 is required for this response. Elevated levels of MMP-9 are found in breast and prostate cancer patients with bone metastases (53) and in pediatric osteosarcomas (54). Inhibition of MMP-9 or MMP-2 reduces bone-degrading capacity of tumor cells in vitro and in vivo (55, 56). TGF-β is a potent anti-inflammatory cytokine (4, 5), and in normal epithelial cells, TGF-β negatively regulates NF-κB transcriptional activity (57, 58), whereas proinflammatory cytokines repress Smad-dependent transcription (59, 60). The anti-inflammatory effect of TGF-β is likely mediated by Smads, as Smad3-null mice develop massive inflammation (5) and Smad3/Smad4 mediate suppression of NF-κB in renal inflammation (61, 62). In tumor cells, however, TGF-β can activate the NF-κB transcriptional response (28, 63). Breast carcinoma MDA-MB-231 cells express high levels of NF-κB activity (64) and constitutively active Ras-ERK signaling (65), which can inhibit...
Smad activity (66, 67). Thus, the attenuation of Smad signaling might contribute to a positive interaction of TGF-β and proinflammatory cytokines in tumor cells.

Our studies provide insights into the mechanism of TAK1-mediated upregulation of COX2/PTGS2 and MMP-9. TAK1 can interact with the TAB1–XIAP complex (68, 69), regulating p38MAPK and NF-κB, and/or with the TAB2–TAB3–TRAF complexes, leading to the K63–ubiquitin–dependent activation of NF-κB and JNK (46). Disruption of TAK1 signaling by dn-TAK1 or by siRNA did not affect p38MAPK signaling in MDA-MB-231 cells. In agreement with this finding, breast and carcinoma cells express low levels of TAB1. These results argue that the interaction of TAK1 with the TAB1–XIAP complex is either transient or involves only a fraction of TAK1. In contrast, expression levels of the TAB2–TAB3–TRAF factors and TAK1 were comparable and suppression of TAB2 or TAB3 reduced expression of COX2/PTGS2 and MMP-9 in carcinoma cells. In support of these findings, recent studies have found that TAB1-null cells exhibit normal activation of TAK1–NF-κB signaling by IL1-β and TNF-α (70, 71).

High levels of TGF-β and proinflammatory cytokines in tumor microenvironment may facilitate the selection of tumor cells that are capable of utilizing the protumorigenic functions of both pathways. In the natural history of the tumor, the TAK1–TAB2–TAB3 signaling axis may emerge as a moderator of the 2 pathways favoring invasion and growth of tumor cells in the bone environment (Fig. 7). This signaling axis may function in tumor cells independently of TGF-β/Smad and TGF-β/Src signaling events that regulate expression of IL11 and CTGF (41, 72, 73), as these factors are not affected by dn-TAK1. Thus, the TAK1–TAB2–TAB3 axis may represent a novel target for anticancer therapy as well as a biomarker of bone metastasis capable cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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