Chapter 4

Constitutive Activation of Activin Receptor-like Kinase 2 in Human Breast Cancer Cells inhibits Metastatic Progression and Osteolytic Bone Lesions

Maj Petersen*,†, Jeroen T. Buijs†, Evangelia Pardali*, Geertje van der Horst†, Henry Cheung†, Peter ten Dijke* and Gabri van der Pluijm†§

*Department of Molecular Cell Biology and Centre for Biomedical Genetics, †Urology, and §Endocrinology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands.

Submitted.

†Correspondence to G.van_der_pluijm@lumc.nl
Abstract

Transforming growth factor-β (TGF-β) is a crucial player in metastasis of breast cancer cells to bone. We previously reported that bone morphogenetic protein-7 (BMP-7) can counteract the effects of TGF-β in vitro and inhibit the progression and formation of osteotropic MDA-BO2 human breast cancer bone metastasis in vivo. Whether BMP-7 acts directly on the breast cancer cells and/or on the cancer-associated bone stroma has remained elusive. Here, we demonstrated that activin-like kinase receptor 2 (ALK2) is the functional BMP-7 type I receptor in MDA-BO2 cells. Subsequently, to restrict BMP-7-induced actions to MDA-BO2 cells we engineered these cells to ectopically express a constitutively active variant of ALK2, denoted caALK2. We demonstrate that caALK2 continuously activates the BMP signaling pathway as measured by Smad phosphorylation, BMP transcriptional reporter activity and target gene responses, and antagonized basal and TGF-β-induced transcriptional reporter activation. In vivo tumor-induced osteolysis and metastatic progression was mitigated in mice inoculated with MDA-BO2 cells over-expressing caALK2 compared to a control cell line. These observations suggest that cell autonomous activation of caALK2 or BMP7 over-expression in breast tumor cells inhibit osteolysis and metastatic progression possibly by interfering with the TGF-β-mediated self-amplifying cycle of bone destruction.

Abbreviations ActR-II, Activin receptor type II; ALK, Activin receptor-like kinase; caALK, constitutively active ALK; BMP, bone morphogenetic protein; BMPR-II, BMP receptor type II; BRE, BMP-responsive element; caALK2, constitutively active ALK2; EMT, epithelial mesenchymal transition; GFP, green fluorescent protein; HLH, helix-loop-helix; ID, inhibitor of differentiation/DNA binding; μ-CT, micro-computed tomography; MET, mesenchymal epithelial transition; P-Smad, phosphorylated Smad; I-Smad, inhibitory Smad; Smad, small phenotype and mothers against DPP related protein; TGF-β, transforming growth factor β.
Introduction

Women diagnosed with advanced breast cancer are at high risk of developing metastatic bone disease [1]. Tumor-induced bone remodeling result in severe patient morbidity [2, 1]. At present, no curative therapies are available for the treatment of bone metastasis and a better understanding of the underlying mechanisms of metastatic disease and the interplay between the cancer cells and the bone microenvironment is critically important in the search for new anti-metastatic therapies.

Bone morphogenetic protein (BMP)-7 is a dimeric cytokine and a member of the transforming growth factor (TGF)-β family that have pleiotropic functions in development, tissue homeostasis and in cancer progression [3]. BMPs signal via assembly of heterotetrameric BMP-receptor complexes comprised of type I and type II serine/threonine kinase receptors [4, 5, 6]. The type I receptors for BMPs include activin-like kinase (ALK)1, ALK2, ALK3, and ALK6 whereas the type II receptors are BMPRII, ActRII, and ActRIIB. ALK2 has been described as predominant type I receptor for BMP-7 [5, 6, 7, 8, 9]. Type I receptors are phosphorylated and activated by type II receptors and determine the signaling specificity within the heterotetrameric complex [10]. Upon ALK2 activation receptor-regulated (R-)Smad1, Smad5, and Smad8 can be phosphorylated and form heteromeric complexes with the common mediator Smad4. These transcriptional complexes modulate target gene expression in concert with various co-factors [5, 11, 12]. Inhibitory Smads (I-Smads), Smad6 and Smad7 antagonize activation of R-Smads [13, 14, 15].

BMP-7 plays key regulatory roles during early embryogenesis [16, 17, 18, 19]. In a pathological setting, loss of BMP-7 mRNA expression in primary tumor is associated with increased bone metastasis and invasiveness of breast cancers cell lines [20]. Moreover, BMP-7 was shown to counteract TGF-β-induced tumorigenic responses in breast cancer cells in particular through inhibition of TGF-β-mediated epithelial mesenchymal transition (EMT) and induction of mesenchymal epithelial transition (MET) [17, 20, 21]. The dichotomous roles of TGF-β and BMP-7 on EMT and MET can be explained, at least in part, by the differential regulation of inhibitors of differentiation/DNA binding (ID) proteins [22].

Breast cancer has high predilection for the skeleton and once micro-metastatic lesions are established they induce osteolytic bone destruction through a vicious cycle of paracrine signaling between the tumor cells and the bone microenvironment [1, 2, 23]. In brief, cancer cells stimulate osteoclastic progenitor maturation and bone resorption resulting in release of matrix-bound growth factors such as TGF-β, insulin-like growth factor and others. These cytokines can then act back on the tumor cells and stimulate further growth, osteoclastogenesis and bone destruction [1, 2, 21, 23]. The MDA-BO2 breast cancer cell line is an osteotropic sub-clone of the MDA-MB-231 cell line which gives rise to highly aggressive and osteolytic bone metastasis in immunodeficient mice [24]. Over-expression of BMP-7 in MDA-BO2 cells or systemic administration of human recombinant BMP-7 to MDA-BO2 tumor-bearing animals inhibited the metastatic potential and the progression of these breast cancer cells in vivo [20]. Whether the BMP-7
exerts its effects directly on the cancer cells directly and/or on the cancer-associated stroma is not known. In this study, we engineered MDA-BO2 cells to over-express a constitutively active form of, the BMP-7 receptor, ALK2 to restrict BMP-7-induced responses solely to breast cancer cells. This cell line was characterized in vitro and compared to BMP-7 over-expressing cells in a bone tumor model in vivo. The outcome of our studies demonstrates that inhibition of de novo formation of bone metastases and tumor cell-induced osteolysis is mediated primarily through direct effects of BMP-7 on the cancer cells.

Results

ALK2 is the functional BMP-7 type I receptor in MDA-BO2 cells

We first examined the expression profile of BMP type I and type II receptors in MDA-BO2 cells by quantitative real-time PCR (Fig. 1A). ALK2 was expressed in these cells however the expression levels of ALK3 was four fold higher. Some ALK6 was expressed, whereas ALK1, a type I receptor for BMPs in endothelial cells, was not detected as expected. The BMPRII was expressed at high levels whereas ActRII and ActRIIB were detected at lower ratios (Fig. 1A).

The physiological binding property of BMP-7 to cell surface BMP type I receptors of MDA-BO2 cells was analyzed by affinity labeling with radiolabeled BMP-7 to a monolayer of cells. Ligand-receptor complexes were cross-linked and lysates immunoprecipitated with anti-sera for all BMP type I receptors. BMP-7 specifically bound to ALK2 (Fig. 1B). No detectable binding to ALK3 or ALK6 was observed for BMP-7 (Fig. 1B).

Ligand receptor binding triggers trans-phosphorylation of BMP-specific R-Smads; i.e. Smad1, Smad5, and Smad8 and subsequently heteromeric complex formation with Smad4 [13]. To ascertain that BMP-7 signals via ALK2, we transiently co-transfected cells with shRNA plasmids targeting ALK2 and the BMP responsive reporter construct (BRE-luc) which contains repeated promoter sequences of the BMP target gene ID1 [25]. When endogenous levels of ALK2 were depleted in MDA-BO2 cells both the basal and the BMP-7-induced reporter activity was significantly inhibited (Fig. 1C). This was observed with two independent shRNAs for ALK2 and strongly suggests that BMP-7 indeed signals via ALK2 in MDA-BO2 cells.

Mimicking a state of continuous BMP-7 signaling can be achieved through expression of a constitutively active form of the ALK2 receptor (caALK2). This genetically modified receptor contains an activating mutation, Q207D, in the GS domain [26]. MDA-BO2 cells with isogenic stable expression of caALK2 were generated by Gateway Flp-In recombinase. Cells stably expressing GFP were used as controls along with MDA-BO2 cells over-expressing BMP-7 [20]. The stable caALK2 cells expressed 8 fold more ALK2 compared to physiological levels in control GFP (Fig. 1D) and parental MDA-BO2 cells (data not shown).
Figure 4.1: Ectopic of constitutive activate BMP-7 type I receptor, ALK2, in MDA-BO2 cells. (A) Quantitative real-time PCR analysis of the BMP specific type I receptors ALK1, ALK2, ALK3, and ALK6 and the type II receptors ActRII, ActRIIB, and BMPRII in MDA-BO2 cells. Relative gene expression levels were corrected to β-ACTIN using the ΔΔCt method. Values are presented as relative mean ± S.D. (B) MDA-MB-231 cells were affinity-labeled with [125I]-BMP-7, cross-linked and immunoprecipitated with anti-sera to ALK2, ALK3 and ALK6. Proteins were resolved by SDS-PAGE and radioactivity detected on a Phosphoimager. (C) MDA-BO2 cells were transiently co-transfected with the BRE-luc reporter and shRNA constructs targeting ALK2. A shRNA plasmid targeting GFP was used as control. Cells were stimulated with (black bars) or without (white bars) BMP-7 for 16 hours and the relative luciferase activity determined. The BMP-7-induced BRE-luc activity was significantly inhibited when shALK2.1 (P ≤ 0.01) or shALK2.2 (P ≤ 0.001) were co-transfected compared to shGFP control transfected cells. Also, basal reporter activity was inhibited by shALK2.1 compared to shGFP control transfected cells (P ≤ 0.05). (D) Quantitative real-time PCR analysis of the relative ALK2 gene expression in GFP control or caALK2 overexpressing MDA-BO2 cells. Data is presented as mean ± S.D corrected for β-ACTIN expression levels. (E) Immunoblot analysis of P-Smad1/5 in stable MDA-BO2 cell lines. GFP control cells stimulated with BMP-7 for 45 minutes were used as a positive control. A non-specific protein band was used to control for equal loading. (F) Cells with ectopic expression of control GFP, BMP-7 or caALK2 were transiently transfected with the BRE-luc reporter and stimulated with (black bars) or without (white bars) BMP-7 for 16 hours. Basal levels of BRE-reporter activity in BMP-7 and the caALK2 cells were induced compared to control GFP cells (P ≤ 0.05).
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We next analyzed whether ectopic expression of caALK2 resulted in continuous activation of the BMP signaling cascade. Constitutive active ALK2 cells showed high endogenous levels of P-Smad1/5 compared to the control GFP cell line as observed by western blot analysis (Fig. 1E lane 4). The amount of phosphorylated Smad1/5 (P-Smad1/5) was equivalent to the P-Smad1/5 intensity in GFP control cells stimulated with 500 ng/ml BMP-7 (Fig. 1E lane 2) and slightly higher than P-Smad1/5 levels in BMP-7 over-expressing cells (Fig. 1E lane 3). When stable cell lines were transfected with the BRE-luc reporter, significant higher basal luciferase activity was observed in caALK2 and BMP-7 cells compared to control GFP cells (Fig. 1F). Exogenous addition of BMP-7 did not further increase the luciferase activity in either caALK2 or BMP-7 over-expressing cells suggesting that a plateau in BMP signaling is reached by constitutive receptor activation and BMP-7 expression (Fig. 1F). The amount of BMP-7 produced by BMP-7 over-expressing cells was estimated by harvesting conditioned medium from the cells and using this medium in a BRE-luc reported assay. This was estimated to approximately 200 ng/ml (Fig. S1). Thus, stable isogenic expression of caALK2 continuously activates the BMP signaling cascade to similar extent as ectopic expression of BMP-7 in MDA-BO2 cells.

Ectopic expression of caALK2 inhibits TGF-β signaling

Exogenous addition of BMP-7 can inhibit TGF-β mediated signaling and counteract the induction of downstream responses such as EMT [20, 17]. We hypothesized that ectopic expression of caALK2 would result in a similar inhibition of the TGF-β pathway. To explore this, we transiently transfected the three stable MDA-BO2 lines with CAGA-luc, a Smad3/4-dependent TGF-β responsive reporter [27].

Basal and TGF-β-induced luciferase activities were significantly reduced in cells expressing caALK2 (Fig. 2A and B) and similar results were observed in BMP-7 over-expressing cells (Fig. 2A). Together, these results suggest that the interplay between BMP-7 and TGF-β signaling pathways is mutually competitive and may depend on formation of mixed Smad complexes [7] or bioavailability of intracellular signaling molecules such as Smad4 or transcriptional co-activators [28].

Ectopic expression of caALK2 mimics BMP-7-induced gene responses

Ectopic expression of BMP-7 or caALK2 induces similar profiles of Smad1/5 activation and BRE-luc reporter activation. The expression levels of the BMP-inducible target genes ID2, SMAD6, and SMAD7 were greatly induced after 24 hours BMP-7 stimulation in control GFP cells (Fig. 3). Elevated mRNA levels of ID2 were also observed in cells over-expressing BMP-7 and caALK2. The expression was comparable to control cells stimulated with BMP-7 (Fig. 3A). Similar enhanced basal levels of SMAD6 and SMAD7 mRNA were observed in these lines (Fig. 3B and C). SMAD6 was expressed at two fold higher levels in caALK2 cells compared to BMP-7 cells (Fig. 3B). Exogenous
Figure 4.2: Ectopic expression of caALK2 inhibits TGF-β signaling. (A) The stable MDA-BO2 cell lines were transiently transfected with the TGF-β-inducible CAGA-luc reporter and the relative luciferase activity measured. Autocrine TGF-β activity was inhibited in cells with ectopic expression of caALK2 (P ≤ 0.01) or BMP-7 (P ≤ 0.001) compared to GFP control cells. (B) GFP control and caALK2 cells were transiently transfected with the CAGA-luc reporter and cells were stimulated with (black bars) or without (white bars) TGF-β for 16 hours. The TGF-β-induced reporter activity was significantly inhibited in caALK2 cells (P ≤ 0.001) versus control GFP cells.

addition of BMP-7 to the BMP-7 MDA-BO2 cells further enhanced these gene responses by 2 fold whereas the response in caALK2 cells was saturated (Fig. 3B and C). Thus, activation of BMP-7 signaling pathways in MDA-BO2 via either over-expression of ligand or continuous activation of the receptor results in comparable target gene responses.

Figure 4.3: Target gene responses in cells over-expressing GFP, caALK2 or BMP-7. Quantitative real-time PCR analysis of (A) ID2, (B) SMAD6, and (C) SMAD7 in cells stimulated with (black bars) or without (white bars) BMP-7 for 24 hours. Data is presented as the relative gene expression compared to GAPDH as a mean of three measurements ± S.D.

We next examined if expression of either BMP-7 or caALK2 affects the viability of MDA-BO2 cells in vitro. Viability profiles for the control GFP, BMP-7, and caALK2
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Constitutive activation of ALK2 inhibits osteolysis and metastatic progression in vivo

Bone metastases from breast cancers are typically osteolytic as a result of the establishment of a vicious cycle of tumor-induced osteoclastic bone resorption and tumor progression. The net results being osteolysis and enhanced release of growth factors stored in the bone matrix, which can then act back on the cancer cells [1, 23]. This ultimately results in dramatic alterations in the micro-architecture of the bone and extensive loss of mineral content. We previously found that BMP-7 suppress the incidence and progression of metastatic bone disease [20, 29]. In order to determine if the inhibitory actions of BMP-7 is a result of direct effects of BMP7 on the breast cancer cells and/or mediated indirectly via the surrounding bone-marrow stroma, we compared the growth in bone of cells over-expressing BMP-7 versus cells over-expressing caALK2 in MDA-BO2 cells in vivo. Alternatively, MDA-BO2 cells were inoculated into the systemic circulation of immunodeficient mice via the left heart ventricle giving rise to osteolytic bone metastases [20, 29, 30]. Metastatic progression was followed in time by bioluminescent imaging (BLI) and osteolytic lesions were detected by radiography and/or micro-computed tomography (μ-CT) scanning as areas of low mineral density at the end of the experiment [30, 31].

For the intra-osseous tumor growth model, series of longitudinal and transverse μ-CT and radiographs were compared to assess the extent of tumor-mediated osteolysis in the three experimental groups (Fig. 4). Animals injected with caALK2 or BMP-7 over-expressing tumor cells displayed significantly less bone destruction compared to mice inoculated with control MDA-BO2 GFP cells. (P=0.04 and P=0.017 for caALK2 and BMP-7 ectopic expressing cells, respectively; Fig. 4A and B). The bone density was measured by transverse image analysis of the proximal tibiae at one fixed position and given as the % area covered by mineralized bone. The μ-CT volume measurements were significantly reduced in MDA-BO2 GFP xenografted animals compared to non-tumor bearing tibiae (P≤0.001), as expected (Fig. 4C). The radiographic observations were further supported by morphometrical analysis by Masson-Goldner histological staining of bone sections (Fig. 4D). This revealed extensive bone loss and complete replacement of the bone marrow with breast cancer cells in bone sections from mice inoculated with MDA-BO2 GFP cells. Osteolytic bone destruction was again significantly less in mice inoculated with both BMP-7 and caALK2 expressing cells (Fig. 4C and D).

We next used the experimental in vivo model of bone metastasis, which recapitulates later stages of metastatic progression namely, survival in the blood stream, extravasation, bone marrow colonization and establishment of bone metastases [32, 31, 20]. Animals were injected with stably expressing GFP or caALK2 MDA-BO2 cells and metastatic...
Figure 4.4: Active BMP signaling in MDA-BO2 cells inhibits osteolytic bone resorption.

(A) Representative μ-CT images of the longitudinal plane and the corresponding transversal sections acquired below the growth plate of non-injected tibiae or mice injected with GFP control, caALK2 and BMP-7 MDA-BO2 cells. Micro-CT scans were acquired at the end of the experiment 45 days after tumor cell inoculation. Mice injected with GFP control cells display extensive tumor-induced osteolysis and complete loss of normal bone architecture (arrows indicate osteolytic lesions). Animals injected with caALK2 or BMP-7 over-expressing cells display significantly less osteoclastic bone destruction. This is quantified in the figure bone surface area. Abbreviations: F, fibula; T, tibia. (B) Quantification of the tumor burden and (C) percentage bone volume area on μ-CT images (n=6) measured by binary image analysis. The tumor burden and osteolytic bone destruction were significantly higher in GFP control MDA-BO2 versus BMP-7 and caALK2 MDA-BO2 inoculated mice **P≤0.001 and *P≤0.05 in caALK2 and BMP-7 MDA-BO2 versus GFP MDA-BO2 inoculated mice. Data is presented as mean ± S.D. (D) Histomorphometric analysis of proximal tibiae. Masson’s-Goldner trichrome staining was used to visualize mineralized bone. Massive loss of cortical and trabecular bone is observed in control GFP injected animals. Images were captured at 4 x magnification. Abbreviations: B, bone; T, tumor.
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Figure 4.5: Ectopic expression of caALK2 in MDA-BO2 cells inhibits breast cancer metastasis. (A) In vivo BLI at day 35 of three representative mice injected with MDA-BO2 luc cells stably expressing GFP control or caALK2. Dorsal images are shown. (B) Average total flux of BLI from long bones of each experimental group followed in time. MDA-BO2 GFP (black square, black line) and caALK2 (grey circle, grey dotted line). Asterisks indicate statistically significant difference in total flux at day 35 between GFP MDA-BO2 and caALK2 MDA-BO2 long bone metastases ***P≤0.001. (C) Average number of metastases per animal in each experimental group. Significant less metastases are observed in the animals inoculated with caALK2 versus GFP control cells at day 35 *P≤0.05. (D) Representative radiographies of GFP (left) and caALK2 (right) inoculated mice at day 35. (E) Quantification of the average area of osteolytic lesion measured on radiographies on hind legs of mice with bone metastases. Results are expressed at mean relative area ± S.E.M. *P≤0.05 compared to GFP MDA-BO2 inoculated mice. (F) Histology of tibiae with metastases. Near complete loss bone architecture is observed in control GFP MDA-BO2 injected animals. Images were acquired at 4 x magnification. Abbreviations: B, bone; GP, growth plate; T, tumor.
progression was assessed by BLI (Fig. 5A and B). Tumor burden in long bones (Fig. 5B) was significantly decreased in mice inoculated with caALK2 MDA-BO2 cells compared to GFP control MDA-BO2 tumor-bearing mice (P ≤ 0.001). Also, the average number of metastasis at the end of the experiment was significantly reduced in caALK2 MDA-BO2 inoculated mice compared to GFP MDA-BO2 metastases-bearing animals (P ≤ 0.05) (Fig. 5C). In line with these findings, mice inoculated with the caALK2 over-expressing cells displayed less osteolytic lesions (Fig. 5D and E) compared to mice with GFP control MDA-BO2 bone metastases (P ≤ 0.05) as evaluated by radiography. Histomorphometric analysis further confirmed these findings (Fig. 5F). Mice inoculated with GFP control cancer cells displayed a near complete loss of normal bone architecture and tumor cells had largely replaced the bone marrow.

To verify that tumor cells maintained the expression of BMP-7 or caALK2 in vivo tumor cells derived from bone tumors or metastases were analyzed by quantitative real-time PCR (Fig. S3). BMP-7 and caALK2 over-expression was retained to similar levels as in the parental cell lines. In addition, functionality of caALK2 in bone metastases was confirmed by immunohistological staining of P-Smad1. Enhanced nuclear P-Smad1 staining was observed in caALK2 expressing sections compared to control GFP metastases (Fig. 6).

Taken together, our observations suggest that the inhibitory action of BMP-7 on tumor growth and metastatic progression is mediated via direct effects on breast cancer cells.

![Figure 4.6](image)

Figure 4.6: Immunohistological staining of P-Smad1 in breast cancer metastasis (A) P-Smad1 immunostaining of GFP and caALK2 overexpressing tumor metastasis. (B) Image quantification of P-Smad1 staining in metastatic bone sections. Significant more P-Smad1 staining is observed in caALK2 versus GFP control bone metastases (P ≤ 0.001). Error bars indicate mean ± S.D.

**Discussion**

Skeletal metastases are observed in the majority of breast cancer patients diagnosed with advanced disease [33, 34]. Evidence is mounting that the formation and progression of skeletal metastasis is critically dependent on tumor-induced osteoclastic bone...
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destruction, which requires a complex interplay of cancer cells and the bone stromal microenvironment. [1, 2, 23, 31]. TGF-β is a major inducer of this vicious cycle of skeletal metastasis and understanding the multifaceted mechanisms and the role of TGF-β and BMP signaling herein is critically important for the development of anti-metastatic therapies.

We previously showed that over-expression of BMP-7 in osteotropic breast cancer cells or systemic administration of human recombinant BMP-7 to tumor-bearing animals inhibit de novo formation and progression of osteolytic metastasis [20, 29]. However, whether BMP-7 confers these anti-metastatic effects by acting directly on the cancer cells and/or via the bone microenvironment has remained elusive.

Here, we demonstrate that BMP-7 specifically binds and signals via the type I receptor ALK2 in MDA-MB-231/BO2 cells. We engineered MDA-BO2 cells to stably express a constitutively active form of ALK2 [26]. This enabled us to study ligand-independent BMP-7 signaling confined to the cancer cells and compare to BMP-7 over-expressing MDA-BO2 cells which in addition secrete ligands into the surrounding microenvironment. Ectopic expression of caALK2 in MDA-BO2 cells exhibited sustained phosphorylation of Smad1/5/8 and elevated BMP-mediated transcriptional activity, thus mimicking a state of continuous BMP-7 signaling in the cancer cells. Furthermore, BMP-responsive target genes such as ID2, Smad6, and Smad7 were highly elevated in caALK2 MDA-BO2 cells. Autocrine and TGF-β-induced transcriptional reporter activity was mitigated by over-expression of caALK2 or BMP-7, as was previously observed when MDA-BO2 cells were co-stimulated with BMP-7 and TGF-β [20, 29].

We next compared the metastatic ability and intra-bone tumor growth of these cell lines in vivo. Forced expression of caALK2 or BMP-7 in MDA-BO2 cells resulted in significantly decreased tumor-induced bone resorption. In addition to intra-bone tumor growth, over-expression of caALK2 strongly inhibited the development of bone metastases in nude mice after intra-cardiac inoculation. Also, the number of metastasis was reduced and osteolytic lesion area decreased. These findings are in line with our previously reported observations with BMP-7 over-expressing MDA-BO2 cells or recombinant BMP-7 treatment of mice with breast cancer metastasis [20].

Together, these results suggest that de novo bone metastasis formation and subsequent osteolytic bone destruction is regulated by tumor cell autonomous signaling. The antagonistic actions of over-expressing either caALK2 or BMP-7 on TGF-β signaling seem to play an important anti-metastatic role. Inhibition of bone metastasis formation and osteolytic progression was previously reported when TGF-β signaling was blocked through ectopic expression of a dominant negative TGF-β type II receptor [23] or by systemic treatment with kinase inhibitors to ALK5 [35, 36, 37].

Though the exact downstream mediators which are responsible for the dichotomous roles of BMP-7 and TGF-β in metastasis remain unknown, it is thought to be, in part, dependent on the differential regulation of ID proteins [22]. TGF-β is known to repress ID expression, whereas BMPs induce and stabilize expression of ID genes [22, 38]. We found that both ID1 promoter activity and ID2 expression were enhanced in caALK2 cells. Conversely, ID1 and ID3 have also been identified as prime mediators of lung
metastases in MDA-MB-231 cells [39] and targeted silencing of ID1 in 4T1 breast cancer cells inhibited lung metastatic spread of 4T1 cells [40]. Thus, mechanisms of metastasis might be distinct depending on the route of administration and the specific cell subtype applied. To elucidate the underlying mechanisms of inhibition of metastatic bone disease by which active BMP signaling inhibits osteolysis we examined a set of bone-metastatic genes reported to mediate different steps of the metastatic cascade [23, 41, 42]. However, we found no difference in the expression levels of IL-11, CTGF or PTHrP in caALK2 and BMP-7 over-expressing cells compared to GFP control MDA-BO2 cells.

We further show that Smad6 and Smad7 were significantly up-regulated in caALK2 and BMP-7 over-expressing cells indicative of enhanced proteasomal degradation of type I receptors and R-Smads [43, 44, 45]. Sustained up-regulation of these genes could have dramatic effects on TGF-β and BMP signaling in the tumor cells. Over-expression of Smad7 in melanoma and breast cancer cells was recently shown to inhibit the formation of metastasis [14, 15]. We speculate that since the BMP signaling pathway is continuously activated, by either caALK2 or BMP-7, the potential inhibitory actions of Smad7 may be more pronounced on TGF-β signaling compared to the effects on BMP-7 signaling pathway. In order to elucidate this, knockdown studies of Smad7 in cells ectopically expressing caALK2 or BMP-7 would have to be performed.

In a recent report, continuous activation of ALK3, a receptor for BMP-2 and BMP-4 [46], was shown to mediate invasion and metastasis of MDA-MB-231 breast cancer cells. This pro-metastatic function of caALK3 could be blocked by over-expression of a mutated dominant negative ALK3 receptor [47]. We specifically show that BMP-7 signal via ALK2 in MDA-BO2 cells and that over-expression of caALK2 gives rise to a less metastatic phenotype in vivo. Thus, active BMP-7 signaling in tumor cells distinctively induce anti-tumorigenic properties in these human breast cancer cells. Together, these observations highlight the importance of discriminating between different BMPs when characterizing their functions.

In summary, we demonstrate that continuous activation of BMP-7 signaling via ectopic expression of caALK2 or BMP-7 ligand can inhibit tumor-induced osteolysis and progression of breast cancer bone metastases. Our results suggest that therapeutic targeting of TGF-β signaling and enhancement of BMP-7 signaling can prevent progression of skeletal metastases. We are currently analyzing potential downstream target genes of TGF-β and BMP-7 signaling that are affected by active BMP-7 signaling. Identifying such genes could potentially allow us to target the pro-metastatic function of TGF-β or augment the anti-metastatic activities of BMP-7 signaling.

Materials and Methods

Cell culture and reagents

The human breast cancer cell lines MDA-MB-231 luc and MDA-MB-231/BO2 FRT luc cells (referred to as MDA-BO2) were cultured as previously described [20, 48]. Human recombinant BMP-7 (RD Systems) was used at 500 ng/ml unless otherwise indicated. Human recombinant
TGF-β3 was used at 5 ng/ml and obtained from K. Iwata (OSI Pharmaceuticals, Inc., Melville NY, USA). Antibodies recognizing BMP type I and type II receptors are described in [8] and antibody to phosphorylated Smad 1/5 (P-Smad1/5) is described in [11]. Lentiviral shRNA constructs targeting ALK2 were TRCN 0000 000 442 and TRCN 0000 000 444 (Sigma-Aldrich). The non-targeting control lentiviral shRNA plasmid was SCH002 (Sigma-Aldrich).

Cloning and generation of caALK2 over-expressing cell line

The caALK2 plasmid was kindly provided by Dr. K. Miyazono [26]. Constitutive active ALK2 was enzymatically cleaved from pENTR backbone and inserted into the pEF5/FRT/V5 vector by Gateway technology (Invitrogen). MDA-BO2/luc Flp-In cells were transiently cotransfected with the pOG44 Flp recombinase expression plasmid and the pEF5/FRT/V5/caALK2 construct and selected with antibiotics to generate stable isogenic caALK2 expressing cells [20].

[^125I]-BMP-7 binding assay

Iodination of BMP-7 was performed according to the chloramine T method and cells were affinity-labeled with the radioactive ligand (Amersham) as previously described [49, 5, 8]. In brief, a monolayer of cells were incubated with [^125I]-BMP-7 for 3 hours on ice and cross-linked with 54 mM disuccinimidyl suberate (DSS) and 3 mM bis (sulfo)succinimidyl suberate (BS3, Pierce). Cell lysates were incubated with specific anti-sera for type I and type II receptors for 3 hours and immune complexes were precipitated with proteinA Sepharose (Amersham). Samples were boiled in SDS sample buffer and separated by SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham).

Quantitative real-time PCR

Total RNA was isolated using TRizol Reagent (Invitrogen) and first strand cDNA synthesis and real-time PCR performed as previously described [50]. Samples were run in triplicates for each primer set and the relative gene expression levels were assessed as the threshold cycle (Ct) values of the target and reference gene normalized for GAPDH or ACTIN. Values are presented as mean ± S.D. The following primers were used; ID2, forward: 5’-TCA GCC TGC ATC ACC AGA GA-3’ and reverse: 5’-CTG CAA GGA CAG GAT GCT GAT -3’. SMAD6 forward: 5’-TCT CCT CGC GAC GAG TAC AAG-3’ and reverse: 5’-GGA GCA GTG ATG AGG GAG TTG-3’. SMAD7 forward: 5’-AGA GGC TGT GTT GCT GTG AAT C-3’ reverse: 5’- GCA GAG TCG GCT AAG GTG ATG-3’.

Cell transfection and cell viability assay

Cells were transiently transfected with BRE renilla luciferase [25] or the CAGA renilla-luc [27] reporter constructs using FugeneHD (Roche) according to the manufacturer’s protocol. The following day, cells were stimulated 15 hours (or after three days for shRNA experiments) with the respective ligand. Cells were washed, lysed and the relative renilla/firefly luciferase activity measured [50, 20]. Transfection was done in triplicate and representative experiments are shown. For shRNA co-transfection assays, a ratio of 1:6 was used of BRE-luc versus shRNA. Cell viability was measured performed as previously described [32, 50].
Western blot analysis

Cells were stimulated with BMP-7 for 45 minutes, washed with PBS and lysed in SDS-sample buffer. Proteins were separated on SDS-PAGE and subjected to western blotting as previously described [50].

In vivo intra-bone tumor growth and bone metastasis models

Six week old female BALBc nude mice (Charles River) were anesthetized with isofluorane and inoculated with 100,000 freshly harvested MDA-BO2-luc cells suspended in 10 μl sterile PBS (n=5 per group). The surgical procedure was as follows; an incision was made just below the knee and two holes were drilled in the longitudinal plane of the proximal diaphysis using a 27G needle. Tumor cells were then directly inoculated into the bone marrow cavity and the wound closed with sutures [20]. For the bone metastasis model 1x10^5 freshly harvested cells in 100 μl PBS were inoculated into the left heart ventricle (n=10 per group) [20]. Injections were done with 27G syringes. Tumor growth progression was followed weekly by bioluminescent imaging on the IVIS-100 (Caliper Life Sciences). All animal experiments were approved and carried out according to the guidelines provided by the local animal welfare committee.

Radiography and μ-CT imaging

Radiographies were acquired as described in [20] and μ-CT scanning was performed with Skyscan 1076 micro-CT (Skyscan) on fixed tibial samples. Acquisition parameters were as follows; 750 ms exposure, 0.9 degree rotation at a source voltage of 40kV. 3D scans were reconstructed using the NRecon and Dataviewer software from Skyscan. Series of tibial longitudinal and transversal plane sections were analyzed. Tibial cross-sections were quantified by binary image analysis and presented as the percentage cone comprising area in a given image.

Immuno- and histochemical analysis

Bone tumor sections were imbedded in paraffin and sectioned at 5-6 μm. Histological Masson’s-Goldner staining was performed as previously described [20, 30]. The relative tumor burden and area of osteolytic bone resorption were quantified by image analysis with ImageJ software.

Statistical analysis

All results are expressed as the mean ± S.E.M unless otherwise stated. One-way ANOVA followed by Bonferronis multiple comparison test and two-tailed Students t-test was used where applicable. P≤0.05 was considered to be statistically significant.

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References


Supplemental material

Figure 4.7: Figure S1. BMP secretion in conditioned medium from BMP-7 over-expressing cells. (A) Culture medium was collected from BMP-7 over-expressing cells two days after stimulation and added to MDA-BO2 cells transfected with BRE-luc reporter. A positive control was treated with 200 ng/ml BMP-7 (black bar). Data is presented at mean ± S.D of triplicate samples.

Figure 4.8: Figure S2. Tumor cell proliferation in vitro. Cell viability of stable MDA-BO2 cell lines measured for four consecutive days. The relative growth rates of GFP control (black square), BMP-7 (grey triangle), and caALK2 over-expressing cells (grey open circle) is presented. Error bars indicate mean ± S.D. of four measurements, a representative experiment is shown.
Figure 4.9: Figure S3. Continuous over-expression of BMP-7 or caALK2 in vivo. The relative expression level of BMP-7 (A) or ALK2 (B) compared to GAPDH determined by quantitative real-time PCR in MDA-BO2 cell clones (cl) established from bone tumors and metastases. Error bars indicate mean ± S.D.
Constitutive Activation of Activin Receptor-like Kinase 2 in Breast Cancer Cells inhibits Metastatic Progression and Osteolytic Bone Lesions