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Original Paper

Bone Morphogenetic Protein-7 Inhibits EMT-Associated Genes in Breast Cancer

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Key Words
Bone morphogenetic protein-7 (BMP7) • Breast cancer • Transforming growth factor β 1 (TGFβ1) • Epithelial-mesenchymal transition (EMT)

Abstract
Background/Aims: Bone morphogenetic protein-7 (BMP7) has been shown to reduce the severity of injury-induced fibrosis through counteracting the fibrotic effects of transforming growth factor β 1 (TGFβ1). However, this model in the carcinogenesis of breast cancer is unknown. ***Methods:*** We analyzed the effects of BMP7 and TGFβ1 on gene transcripts and protein levels of EMT-related factors in breast cancer cells by RT-qPCR and Western blot, respectively. The effects of BMP7 and TGFβ1 on cell invasiveness and migration were evaluated by scratch wound healing assay and transwell cell migration assay. The cell growth was measured by MTT assay. ***Results:*** BMP7 did not alter the TGFβ1-stimulated phosphorylation of TGFβ receptor, but significantly inhibited the TGFβ1-activated epithelial-mesenchymal transition (EMT)-related genes in breast cancer cells, resulting in a significant reduction in TGFβ1-triggered cell growth and cell metastasis. ***Conclusion:*** Our data suggest that besides being a well-known antagonist for TGFβ1 in fibrosis, BMP7 may also antagonize TGFβ1 in tumorigenesis-associated EMT in breast cancer. Thus, BMP7 may be a promising therapeutic target for treating breast cancer.

Introduction

Breast cancer is a common cancer with high malignancy in women worldwide [1]. In the past decades, the molecular regulation of the development and pathogenesis of breast cancer has been extensively studied, showing involvement of different signaling pathways.

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They are epidermal growth factor receptor signaling pathway [2], Wnt signaling pathway [3], insulin-like growth factor receptor signaling pathway [4], Notch signaling pathway [5] and transforming growth factor β (TGFβ) superfamily receptor signaling pathway [6-9]. Among all these signaling pathways, TGFβ receptor signaling pathway appears to play a critical role in the carcinogenesis of breast cancer.

TGFβ superfamily signaling regulates cell growth, differentiation in the embryogenesis, postnatal growth, and various physiological and pathological events [10-15]. The TGFβ signals are initiated with ligand-binding-induced phosphorylation of receptor kinases, which subsequently phosphorylates the downstream signaling molecules Smad2/3 for the TGFβ/activin pathway, or Smad1/5/8 for the bone morphogenetic protein (BMP) pathway [10-15]. Smads phosphorylation allows them to associate with the common signaling transducer Smad4 to form a SMAD-complex, followed by translocation of the complex into the nucleus [10-15].

TGFβ1 and BMP7 are two key members in the TGFβ superfamily, while they play important but diverse roles in chronic diseases. In line with it, both TGFβ1 and BMP7 share similar downstream Smad signaling pathways, but counter-regulate each other to regulate chronic biological events in various organs, like lung [16], liver [17, 18], kidney [19-22], etc. However, a similar antagonism in breast cancer has not been reported before.

Here, we aimed to address this question by studying several human breast cancer lines *in vitro*. We found that BMP7 did not alter the TGFβ1-stimulated phosphorylation of TGFβ receptor, but significantly inhibited the TGFβ1-induced activation of epithelial-mesenchymal transition (EMT)-associated genes in breast cancer cells, resulting in a significant reduction in TGFβ1-triggered cell growth and cell metastasis.

Materials and Methods

Cell lines and reagents

Human breast cancer cell lines MCF7 [23] and BT474 [24] was chosen to be analyzed in the current study, since they were originated from adenocarcinoma and ductal carcinoma, respectively. Since we got similar results from these two cell lines within the scope of the current study, only data from MCF7 were shown. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Recombinant TGFβ1 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used at 10ng/ml to the cells. Recombinant BMP7 was purchased from Curris Inc. (Hopkinton, MA, USA) and used at 200ng/ml to the cells.

Scratch wound healing assay

Scratch wound healing assay was performed as has been described previously [25]. Cells were seeded in 24-well plates at a density of 10^4 cells/well in complete DMEM and cultured to confluence. The cell monolayer was serum starved overnight in DMEM prior to initiating of the experiment. Confluent cell monolayer were then scraped with a yellow pipette tip to generate scratch wounds and washed twice with media to remove cell debris. Cells were incubated at 37 °C for 24 hours with the conditioned media containing either TGFβ1, or BMP7, or both, or null control. Time lapse images were captured after 12 hours using a Nikon Eclipse TE2000-5 microscope. Images were captured from five randomly selected fields in each sample, and the wound areas are calculated by NIH ImageJ software.

Transwell cell migration assay

The transwell cell migration assay was performed using a Fluorometric Cell Migration Assay kit with polycarbonate membrane inserts (5-μm pore size; Cell Biolabs, San Diego, CA, USA). Cells were serum-starved overnight in DMEM prior to initiation of the experiment. The lower chambers were filled with 1 ml of conditioned media containing either TGFβ1, or BMP7, or both, or null control. Cells (4×10^4) were resuspended in 200μl of DMEM and added to the upper chamber. Cells were then incubated at 37 °C for 24 hours to allow cell migration through the membrane. Migratory cells were detached from the underside

of the membrane and subsequently lysed and detected by CyQuant GR dye (Invitrogen). Fluorescence measurement was performed in a FluoStar Optima fluorescence plate reader with a 485/520 nm filter set.

Cell growth assay

For assay of cell growth, the cells were seeded into 96 well-plate at 4000 cells per well and subjected to a Cell Proliferation Kit (MTT, Roche, USA), according to the manufacturer's instruction.

RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was extracted from the cultured cells using RNeasy kit (Invitrogen, St. Louis, MO, USA), according to the manufacturer's instruction. For mRNA analysis, complementary DNA (cDNA) was randomly primed from 2.0μg of total RNA using the Omniscript reverse transcription kit (Qiagen, Hilden, Germany). Quantitative Real-time PCR (RT-qPCR) was subsequently performed in duplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen) on a Rotorgene 6000 series PCR machine. Data were collected and analyzed using the Rotorgene software accompanying the PCR machine. Relative expression levels were determined using the comparative quantification feature of the Rotorgene software. Levels of gene transcripts were normalized to α-tubulin, and then compared to controls.

Western blot

Protein was extracted from the cultured cells by RIPA buffer (Sigma-Aldrich) for Western Blot. The supernatants were collected after centrifugation at 12000×g at 4 °C for 20min. Protein concentration was determined using BCA protein assay, and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti-phosphorylated- TGFβ receptor I (pTBR1), anti-phosphorylated-BMP receptor I (pBMPR1) and anti-α-tubulin (all purchased from Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson Labs, Bar Harbor, ME, USA). Images shown in the figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software.

Statistical analysis

Each experiment condition contains 5 repeats. All values are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. Statistic method: one-way ANONA with a Bonferoni correction, followed by fisher's exact test to compare two groups.

Results

TGFβ1 and BMP7 activate different receptors in breast cancer cells

In order to evaluate whether BMP7 may antagonize the effects of TGFβ1 on the growth and invasion of breast cancer cells, we used two human breast cancer cell lines, MCF7 [23] and BT474 [24] in our study. Since these two lines were originated from adenocarcinoma and ductal carcinoma, respectively, they may represent phenotypes for breast cancer cells with different origin. Since we got similar results from these two cell lines within the scope of the current study, only data from MCF7 were shown here.

MCF7 cells were treated with either TGFβ1, or BMP7, or both, or null control. We found that TGFβ1 induced significant phosphorylation of its receptor, TGFβ receptor I (TBR1), without altering the phosphorylation of the receptor for BMP7, BMP receptor I (BMPR1), by representative Western blot images (Fig. 1A), and by quantification (Fig. 1B). Similarly, BMP7 induced significant phosphorylation of its receptor BMPR1, without altering the phosphorylation of the receptor for TGFβ1 (TBR1), by representative Western blot images (Fig. 1A), and by quantification (Fig. 1C). These data suggest that either TGFβ1 or BMP7

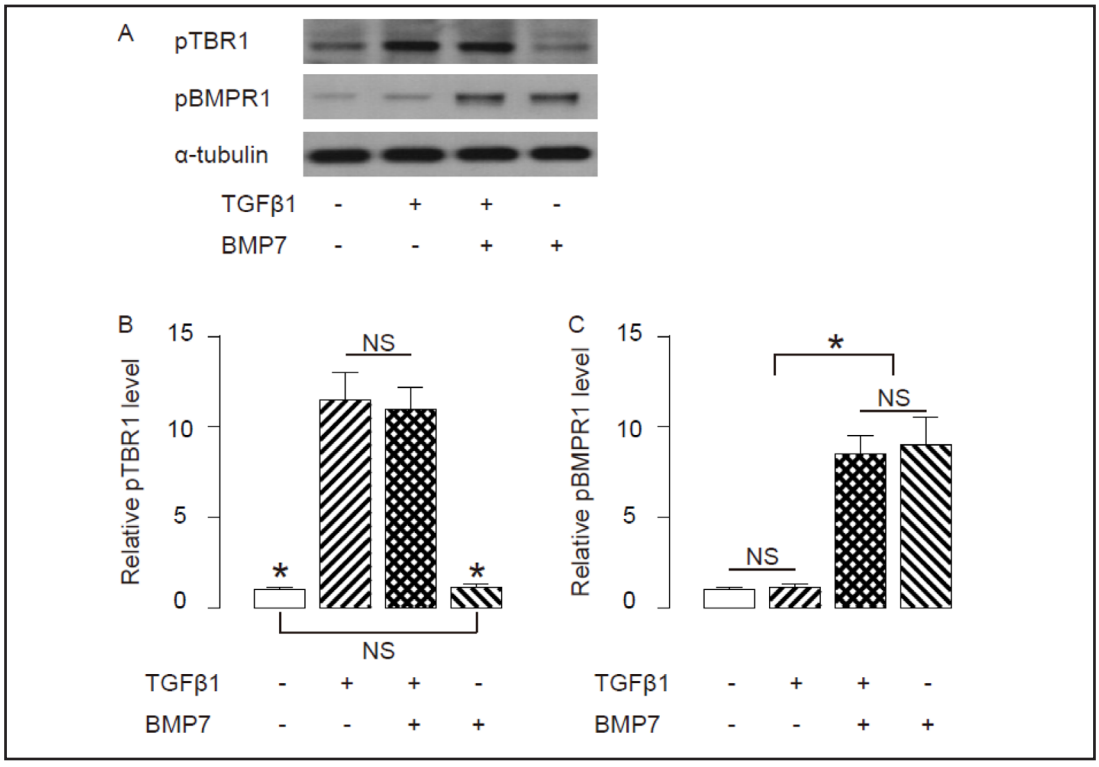

Fig. 1. TGFβ1 and BMP7 activate different receptors in breast cancer cells. (A-C) MCF7 cells were treated with either TGFβ1, or BMP7, or both, or null control. Western blot was done for phosphorylated TGFβ receptor I (pTBR1), phosphorylated BMP receptor I (pBMPR1), and α-tubulin (loading control), shown by representative images (A), and by quantification (B-C). *p<0.05. NS: non-significance. n=5. Statistics: one-way ANOVA with a Bonferoni correction, followed by fisher's exact test for comparison of two groups.

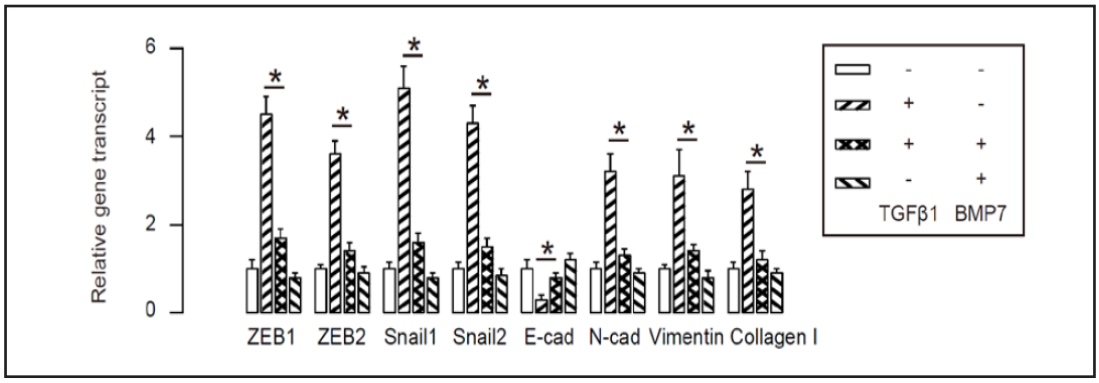

Fig. 2. BMP7 inhibits TGFβ1-activated EMT-related genes in breast cancer cells. MCF7 cells were treated with either TGFβ1, or BMP7, or both, or null control. RT-qPCR for key EMT-associated genes ZEB1, ZEB2, Snail1, Snail2, E-cadherin (E-cad), N-cadherin (N-cad), Vimentin and Collagen I. *p<0.05. n=5. Statistics: one-way ANOVA with a Bonferoni correction, followed by fisher's exact test for comparison of two groups.

only activated its own receptor, without cross-activating the receptor for the other. Thus, in case BMP7 may affect the effect of TGFβ1, it should function through signaling cascades downstream of TGFβ receptor activation, rather than directly through modulation of TGFβ receptor activation.

BMP7 inhibits TGFβ1-activated EMT-related genes in breast cancer cells

Since activated TGFβ/SMAD2/3 receptor signaling by TGFβ1 has been well known for its role in promoting EMT-regulated tumor growth and invasion, we examined levels of

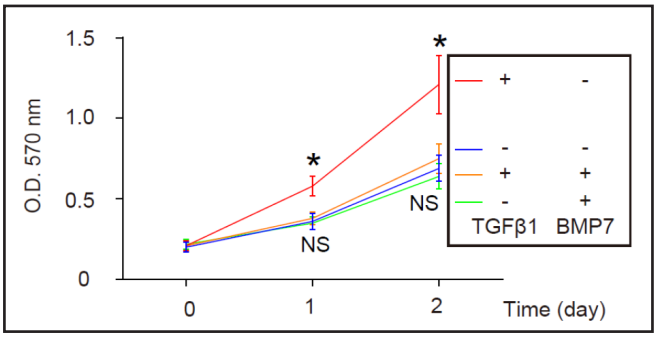

Fig. 3. BMP7 inhibits TGFβ1-stimulated growth of breast cancer cells. MCF7 cells were treated with either TGFβ1, or BMP7, or both, or null control. Cell growth was quantified in a MTT assay. *p<0.05. NS: non-significance. n=5. Statistics: one-way ANOVA with a Bonferoni correction, followed by fisher's exact test for comparison of two groups.

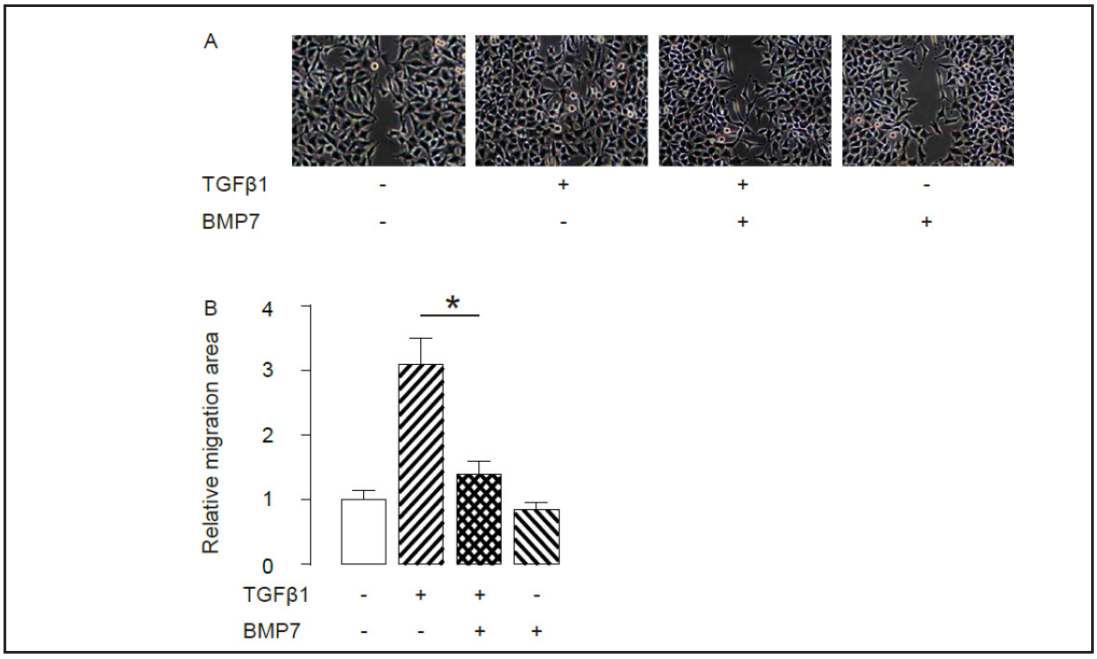

Fig. 4. BMP7 inhibits TGFβ1-stimulated cell invasiveness of breast cancer cells in a scratch wound healing assay. MCF7 cells were treated with either TGFβ1, or BMP7, or both, or null control. (A-B) Cell invasiveness was quantified in a scratch wound healing assay, shown by representative images (A), and by quantification (B). *p<0.05. n=5. Statistics: one-way ANOVA with a Bonferoni correction, followed by fisher's exact test for comparison of two groups.

key EMT-related genes ZEB1, ZEB2, Snail1, Snail2, E-cadherin (E-cad), N-cadherin (N-cad), Vimentin and Collagen I. We found that BMP7 completely abolished TGFβ1-induced activation of ZEB1, ZEB2, Snail1, Snail2, N-cad, Vimentin and Collagen I, and significantly abolished TGFβ1-induced suppression of E-cad (Fig. 2), suggesting a possible antagonizing effect of BMP7 against TGFβ-mediated EMT in breast cancer cells.

BMP7 inhibits TGFβ1-stimulated growth of breast cancer cells

Moreover, we found that the inhibition of TGFβ1-mediated EMT by BMP7 in breast cancer cells resulted in a significant decrease in cell growth, in a MTT assay (Fig. 3). These data suggest that BMP7 may inhibit TGFβ1-stimulated growth of breast cancer cells.

BMP7 inhibits TGFβ1-stimulated cell invasiveness of breast cancer cells

In addition, we found that the inhibition of TGFβ1-mediated EMT by BMP7 in breast cancer cells also resulted in a significant decrease in cancer cell invasiveness, in either a scratch wound healing assay (Fig. 4), or a transwell cell migration assay (Fig. 5). These data suggest that BMP7 may inhibit TGFβ1-stimulated cell invasiveness of breast cancer cells.

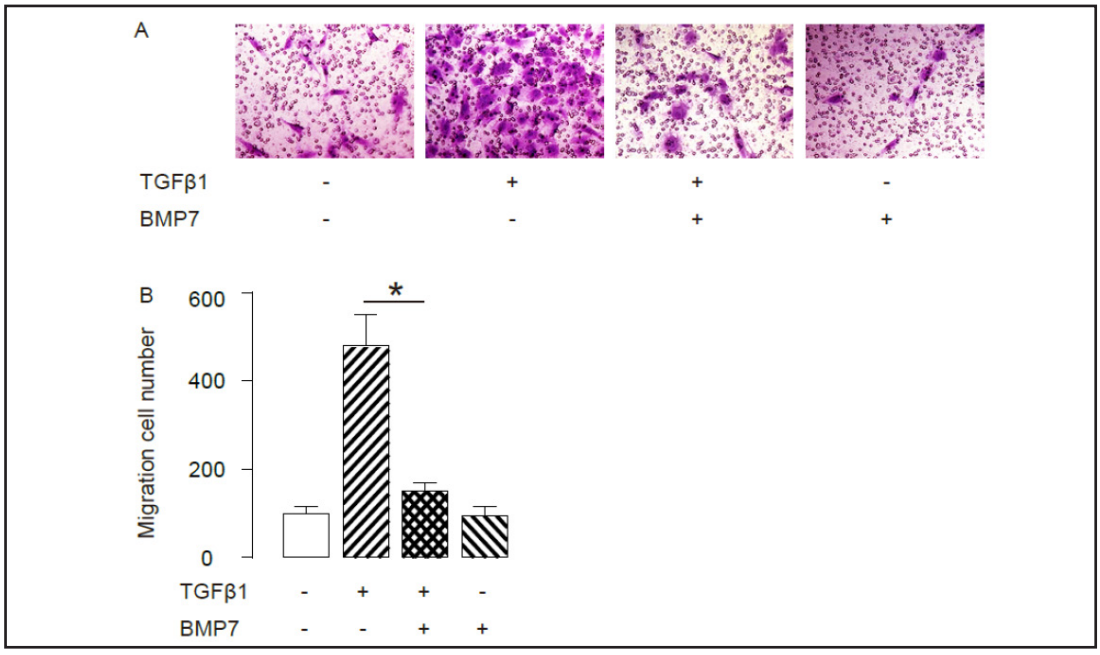

Fig. 5. BMP7 inhibits TGFβ1-stimulated cell invasiveness of breast cancer cells in a transwell cell migration assay. MCF7 cells were treated with either TGFβ1, or BMP7, or both, or null control. (A-B) Cell invasiveness was quantified in a transwell cell migration assay, shown by representative images (A), and by quantification (B). *p<0.05. n=5. Statistics: one-way ANOVA with a Bonferoni correction, followed by fisher's exact test for comparison of two groups.

Discussion

Among all the signaling pathways that regulate carcinogenesis of breast cancer, TGFβ receptor signaling pathway plays a pivotal role, which makes it a focus of the past studies on breast cancer. However, TGFβ receptor signaling pathway belongs to a superfamily called TGFβ superfamily signaling pathway and the members from this superfamily frequently have either agonizing or antagonizing effects to each other to coordinate the balance of specific biological events. TGFβ1 and BMP7 are such two members in the TGFβ superfamily, and they often counter-regulate each other during chronic biological processes in various organs, like lung [16], liver [17, 18], kidney [19-22], etc. Since evidence for a similar antagonism between TGFβ1 and BMP7 in breast cancer is lacking, but could be critical for further elucidation of the tumorigenesis of as well as for efficient development of effective therapies for breast cancer, we aimed to address this question in the current study.

We found that BMP7 significantly inhibited the TGFβ1-induced activation of EMT-related genes in breast cancer cells, resulting in a significant reduction in TGFβ1-triggered cell growth and cell metastasis.

Besides MCF7, we also examined another breast cancer cell line BT474, which has a distinct origin. These approaches largely excluded possibilities of cell-line-dependence of our findings, and particularly of a result dependent on the pathological phenotype of the analyzed breast cancer cells.

Since both TGFβ1 and BMP7 induced phosphorylation of its own receptor, but not the other, these data suggest that there was no cross-activation of receptor by these two TGFβ superfamily members. Thus, BMP7 and TGFβ1 may affect the effect of each other through interaction with downstream signaling, rather than through direct interference with receptor activation of each other. It may be hypothesized that BMP7 activated BMPR1 to induce phosphorylation of SMAD1/5/8, which competed with phosphorylated SMAD2/3 by TGFβ1 stimulation for binding with SMAD4 to form SMAD-complex for nuclear translocation. Such

a competition resulted in an attenuation, or even a complete abolishment of downstream signal transduction for TGFβ1-stimulated TGFβ receptor signaling. Of note, this competition may present from both sides, and may be dose-dependent. Future experiments may address these questions by adapting the dosages of BMP7 in the intervention.

TGFβ1-mediated EMT has been shown as a mechanism for both chronic diseases like fibrosis and carcinogenesis. We confirm the latter in the current model, using several well-established assays. Our data thus suggest that BMP7 may antagonize the tumorigenic effect of TGFβ1, specifically in breast cancer, which suggests that BMP7 may be a promising therapeutic target for controling the tumorigenesis of breast cancer.

Disclosure Statement

The authors have declared that no competing interests exist.

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