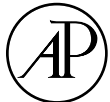
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A kinase-inactive type II TGFβ receptor impairs BMP signaling in human breast cancer cells☆

Nancy Dumont[a] and Carlos L. Arteaga[a,b,c,*]

[a] Department of Cancer Biology, Vanderbilt University School of Medicine, 2220 Pierce Avenue, 777 Preston Research Building, Nashville, TN 37232-6307, USA

[b] Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232-6307, USA

[c] Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN 37232-6307, USA

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Abstract

Dominant negative receptor mutants are often utilized in order to abrogate signaling induced by growth factors. We have previously shown that expression of a dominant negative type II TGFβ receptor (dnTβRII) in MDA-MB-231 breast cancer cells effectively abrogates TGFβ signaling. In this letter, we report that expression of dnTβRII also impairs BMP2-mediated Smad1 phosphorylation as well as BMP2-mediated Smad-dependent transcriptional responses, resulting in an attenuation of BMP-mediated anti-proliferative effects. The fact that dnTβRII not only abrogates TGFβ signaling but BMP signaling as well has important implications for the interpretation of data in which dominant negative mutants are utilized.

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We have shown that expression of the kinase-inactive type II transforming growth factor-β (TGFβ) receptor mutant (TβRII-K277R) in human breast cancer MDA-MB-231 cells impairs the basal migratory potential of these cells, and that this impaired motility is TGFβ type I receptor (TβRI) specific [1]. In addition to associating with TβRI, studies have shown that TβRII can associate with activin and bone morphogenetic protein (BMP) type I receptors following co-transfection of both receptor cDNAs in COS-1 cells [2]. Activins elicit their effects through the activin type IB receptor, ALK4 [3], which is not functional in MDA-MB-231 cells [4]. As a result, these cells do not respond to activins [4,5]. However, MDA-MB-231 cells express BMP type I receptors [6] and have been shown to respond to BMP2 [7]. Therefore, overexpression of TβRII-K277R could potentially result in the blockade of BMP signaling pathways in MDA-MB-231 cells as BMP signal transduction follows the same paradigm established for TGFβ signaling. Like TGFβ, BMPs signal through an interaction with a heteromeric complex of type I (BMPRI) and type II (BMPRII) BMP receptors. Ligand binding results in cross-phosphorylation of BMPRI by BMPRII. BMPRI, in turn, propagates BMP signaling by phosphorylating the receptor-regulated Smads (R-Smads), Smad1, Smad5, and Smad8, which upon phosphorylation, heteromerize with Smad4, translocate to the nucleus, and regulate gene transcription [8]. Thus, by associating with BMPRI, TβRII-K277R could potentially block BMP signaling by preventing the activating phosphorylation of BMPRI by endogenous BMPRII.

BMPs were originally identified as factors capable of inducing ectopic cartilage and bone formation in vivo [9]. However, they are now known to regulate a wide spectrum of biological processes including proliferation, differentiation, and apoptosis in a large variety of cell types [10]. The recent observation that BMPs can inhibit

☆ *Abbreviations:* TGFβ, transforming growth factor-β; BMP, bone morphogenetic protein; R-Smad, receptor-regulated Smad; dnTβRII, dominant negative TGFβ receptor type II; TβRI, TGFβ receptor type I; BMPRI, BMP receptor type I; BMPRII, BMP receptor type II; GFP, green fluorescent protein.

* Corresponding author. Fax: 1-615-936-1790.
E-mail address: carlos.arteaga@vanderbilt.edu (C.L. Arteaga).

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breast cancer cell proliferation in vitro suggests that BMPs may play a role in the control of breast tumor mitogenesis [7,11]. In support of this, BMP2 mRNA is expressed in the mammary gland [12] as well as in breast cancer tissues [13], and some human breast cancer cell lines secrete BMP2 [6], suggesting that BMP2 may function as an autocrine regulator of breast cancer cell proliferation. There is also evidence to suggest that BMPs are involved in the pathogenesis of several cancers including prostate [14], pancreatic [15], and bone [16] cancers. In human prostate cancer, cells often exhibit loss of expression of BMP receptors, and there appears to be an inverse correlation between the levels of BMP receptor expression and tumor grade, suggesting that loss of BMP signaling may contribute to disease progression [14]. Germline mutations in the gene encoding the BMP type IA receptor have also been detected in juvenile polyposis, a syndrome in which patients are at risk for developing gastrointestinal cancers [17]. While little is known about the role of BMPs in breast cancer [11], their involvement in other neoplasias suggests that this may be an avenue worthy of further investigation.

In this report, we show that expression of TβRII-K277R impairs BMP2 signaling in MDA-MB-231 breast cancer cells, resulting in an attenuation of the anti-proliferative effects of BMP2 on these cells.

Materials and methods

Cell culture and reagents. The MDA-MB-231 breast cancer cell line was purchased from the American Type Culture Collection (Rockville, MD) and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. MDA-MB-231 cells stably expressing TβRII-K277R and green fluorescent protein (GFP) or GFP alone were generated and characterized previously [1]. The goat polyclonal antibodies against Smad1/5/8 (Cat #sc-6031) and Smad2 (Cat #sc-6200) were from Santa Cruz Biotechnology (Santa Cruz, CA). The C-terminal phospho-Smad2 antibody (Cat #06-829) was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). The C-terminal phospho-Smad1 antibody (Cat #9511) was from Cell Signaling Technology (Beverly, MA).

Immunoprecipitation and immunoblot analysis. Cells were washed twice with ice-cold D-PBS and lysed with 50 mM Tris/150 mM NaCl buffer containing 1% NP-40, 0.25% deoxycholate, 1 mM EDTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml soybean trypsin inhibitor. Protein content was quantitated utilizing the BCA Protein Assay Reagent (Pierce, Rockford, IL). For immunoprecipitation experiments, protein extracts were incubated with 1 μg of Smad2 antibody overnight at 4 °C and subsequently incubated with protein G–Sepharose for 2 h at 4 °C. The beads were pelleted by centrifugation and washed thoroughly with D-PBS containing 0.2% Triton X-100. Immune complexes were resuspended in sample buffer, boiled for 5 min, separated by 10% SDS–PAGE, and transferred to nitrocellulose membranes at 100 V for 2 h. Membranes were blocked with 5% nonfat dried milk in TBS-T (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20 [v/v]) for 1 h at room temperature and incubated with primary antibodies diluted in TBS-T plus 2.5% nonfat dried milk overnight at 4 °C. The membranes were then washed four times for 10 min with TBS-T, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, and rewashed four times for 10 min with TBS-T. Immunoreactive bands were visualized by chemiluminescence (Pierce).

Transcription reporter assays. Cells were transiently tranfected with 1 μg per 35-mm dish of the Smad-dependent heterologous promoter reporter construct $(SBE)_4$-Luciferase [18] provided by Dr. Wiebe Kruijer (Groningen Biomolecular Sciences and Biotechnology Institute, The Netherlands) along with 0.005 μg per 35-mm dish pCMV-Renilla using FuGENE6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. The following day, cells were split into 24-well plates, and approximately 30-h post transfection, cells were either left unstimulated or were stimulated with 4 nM BMP2 or 40 pM TGFβ1 for 24 h. All cells were then washed with D-PBS and lysed. Firefly and *Renilla reniformis* luciferase activities were measured using Promega's Dual Luciferase Reporter Assay System according to the manufacturer's protocol. Luciferase activity was normalized utilizing the ratio of Firefly to *R. reniformis* luciferase activity and presented as fold induction. All assays were done in triplicate wells and each experiment was repeated at least twice.

Proliferation assays. Cells were seeded into 12-well plates at 3.5×10^4 cells/well in complete growth media containing serum. The following day, cells were treated with 4 nM BMP2 or an equivalent volume of 0.1% BSA/D-PBS vehicle control. After 24, 48, and 72 h of treatment, cells were washed with PBS, harvested with 500 μl trypsin, transferred to diluent, and counted using the Coulter Counter Model ZF Analyzer (Coulter Electronics, Hialeah, FL). Data are presented as percent growth inhibition relative to control and each data point represents the mean ± SD of four wells.

Results

TβRII-K277R impairs BMP2-mediated smad1 phosphorylation

In order to determine whether expression of TβRII-K277R had any effect on BMP signaling, we first examined its effect on BMP2-induced phosphorylation of Smad1 by immunoblot analysis using a phospho-specific Smad1 antibody. Following 60 min of stimulation with BMP2, a robust increase in phosphorylation of Smad1 was observed in control cells expressing GFP alone, but not in cells expressing GFP and TβRII-K277R (Fig. 1A). The lack of phosphorylation of Smad1 in TβRII-K277R cells was not due to a decrease in total Smad1 protein, as reprobing with an antibody that recognizes the unphosphorylated forms of Smad1, -5, and -8 did not reveal any significant change in total Smad protein. In order to rule out the possibility that the phospho-specific Smad1 antibody might be cross-reacting with the phosphorylated form of Smad2, which is normally activated by TGFβ, cells were stimulated with TGFβ1 for 60 min, immunoprecipitated with an antibody directed against Smad2, and subjected to immunoblot analysis utilizing phospho-specific Smad1 or Smad2 antibodies. Smad2 immunoprecipitates were detected only by the phospho-specific Smad2 antibody. This indicates that the phospho-specific Smad1 antibody is indeed specific for Smad1, as it failed to recognize immunoprecipitated Smad2, but did recognize Smad1 in

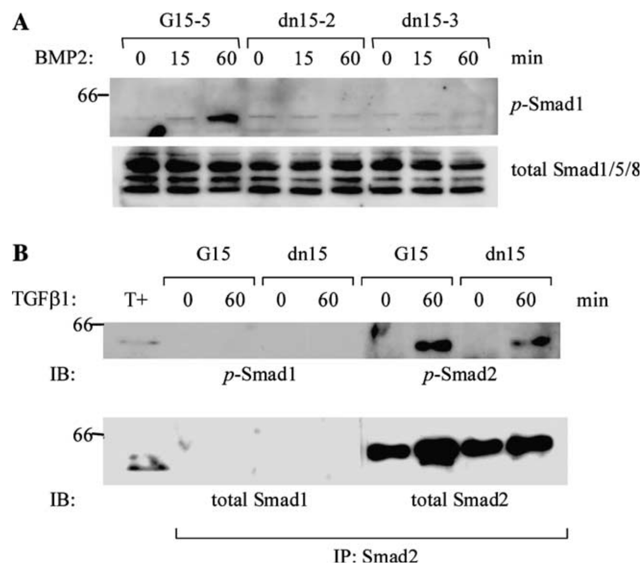

Fig. 1. TβRII-K277R impairs BMP2-mediated Smad1 phosphorylation in MDA-MB-231 cells. (A) Near confluent clones expressing GFP alone (G15-5) or GFP and TβRII-K277R (dn 15-2 and dn 15-3) were grown to near confluence in 100-mm dishes, incubated overnight under serum-free conditions, and stimulated with 400 pM BMP2 for the times indicated. Cells were then washed and lysed, and protein extracts (100 μg/lane) were separated by 10% SDS–PAGE followed by immunoblot analysis for phospho-Smad1 (*p*-Smad1) and total Smad1/5/8. (B) Pools of cells expressing GFP alone (G15) or GFP and TβRII-K277R (dn 15) were grown to near confluence in 100-mm dishes, serum starved overnight, and stimulated with 80 pM TGFβ1 for 60 min. Cells were then washed, lysed, and incubated with a goat polyclonal antibody directed against Smad2 overnight for immunoprecipitation of Smad2. Smad2 immunoprecipitates as well as total G15 cell lysates stimulated with 80 pM TGFβ1 for 60 min (T^+) were resolved by 10% SDS–PAGE followed by immunoblot analysis utilizing phospho-specific antibodies against Smad1 (*p*-Smad1) and Smad2 (*p*-Smad2). Blots were then stripped and reprobed with antibodies directed against the unphosphorylated forms of Smad1 (total Smad1) and Smad2 (total Smad2).

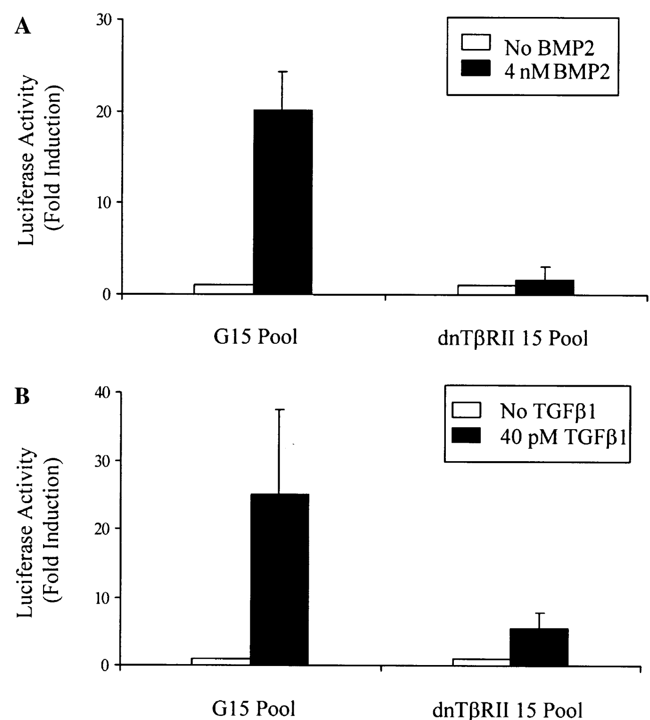

Fig. 2. TβRII-K277R impairs BMP2-mediated transcription in MDA-MB-231 cells. Pools expressing GFP alone (G15 Pool) or GFP and TβRII-K277R (dnTβRII 15 Pool) were transiently transfected with $p(SBE)_4$-Luciferase along with pCMV-Renilla. The following day, cells were split to 6 wells of a 24-well plate, stimulated with either (A) 4 nM BMP2 or (B) 40 pM TGFβ1 for 24 h, washed, and lysed. Firefly and *Renilla* luciferase activities were measured using Promega's Dual Luciferase Reporter Assay System. Fold induction of luciferase activity (*Y* axis) is based on the ratio of firefly to *Renilla* luciferase activities. Each data point represents the mean ± SD of three wells.

the total protein lysate (Fig. 1B, top panel). The specificity of the immunoprecipitation was confirmed by reprobing the blot with antibodies directed against the unphosphorylated forms of Smad1 and Smad2. Again, only the Smad2 antibody recognized immunoprecipitated Smad2 (Fig. 1B, bottom panel).

TβRII-K277R impairs BMP2-mediated transcription

Having observed that expression of TβRII-K277R impaired BMP2-induced Smad1 phosphorylation, we next examined its effect on BMP2-mediated transcription utilizing a reporter construct consisting of four repeats of the sequence CAGACA, $(SBE)_4$-Luciferase, which has been shown to be strongly activated by BMP2 [18]. Twenty-four hours of stimulation with 4 nM BMP2 resulted in strong induction of luciferase activity in control cells, but little or no luciferase activity was observed in cells expressing TβRII-K277R (Fig. 2A). Cells stimulated with TGFβ were used as a positive control and similar results were obtained (Fig. 2B). These data indicate that expression of TβRII-K277R impairs both TGFβ1- and BMP2-mediated signaling in MDA-MB-231 cells.

The anti-proliferative effects of BMP2 are attenuated by TβRII-K277R

In order to determine whether the impaired BMP2 signaling observed in cells expressing TβRII-K277R was physiologically relevant, we examined the effect of TβRII-K277R in cell proliferation assays, as previous studies have shown that BMP2 can inhibit the proliferation of MDA-MB-231 cells [7]. Sparse cell cultures were allowed to proliferate in the absence or presence of 4 nM BMP2 and total cell numbers were determined sequentially after 24, 48, and 72 h. Although BMP2 had little or no effect on the proliferation of cells after 24 h, a 30–40% decrease in proliferation was observed in cells expressing GFP alone after 48 and 72 h (Fig. 3). Expression of TβRII-K277R almost completely abrogated this anti-proliferative effect at 48 h and significantly attenuated it at 72 h. These data suggest that the blockade

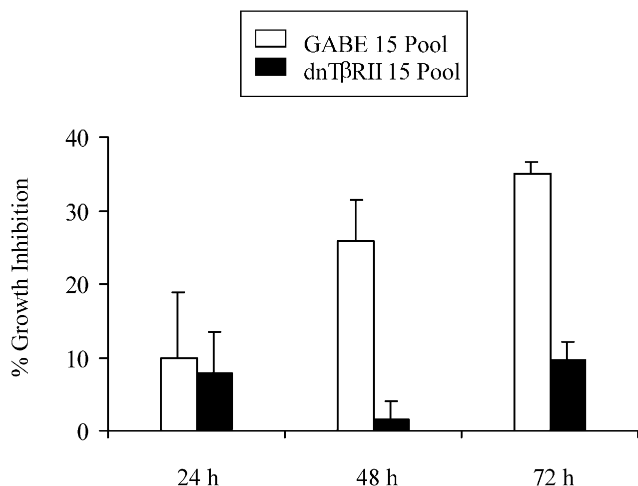

Fig. 3. The anti-proliferative effects of BMP2 are attenuated by TβRII-K277R. Pools expressing GFP alone (GABE 15 Pool) or GFP and TβRII-K277R (dnTβRII 15 Pool) were seeded into four 12-well plates at 3.5×10^4 cells/well in complete media. The following day, cells were treated with 4 nM BMP2 or vehicle control. Cell numbers were determined after 24, 48, and 72 h of treatment. Data are presented as percent growth inhibition relative to control and each data point represents the mean ± SD of four wells.

of BMP2-mediated signaling by TβRII-K277R expression can affect biological responses induced by BMP2.

Discussion

These studies indicate that in addition to blocking TGFβ signaling, expression of TβRII-K277R can also impair BMP signaling. This blockade of signaling appears to be physiologically relevant as it results in attenuation of BMP2-mediated anti-proliferative effects in the MDA-MB-231 breast cancer cells. Although interactions among different TGFβ superfamily type I and type II receptors appear to be limited under physiological conditions, the fact that different receptor types can interact when overexpressed [2] suggests that such an interaction is likely to be the mechanism by which TβRII-K277R inhibits BMP signaling. This has important ramifications for the interpretation of experiments in which reagents such as dominant negative mutant receptors are used.

While the effects observed following expression of dominant negative TβRII (dnTβRII) are likely due to blockade of TGFβ signaling, it is also possible that other related pathways that are blocked as a result of dnTβRII overexpression may be contributing to the observed phenotype. This can be ascertained only if the impact of dnTβRII on these pathways is rigorously assessed. However, this is rarely done. In fact, other than our own [1], we are aware of only two studies in which the impact of dnTβRII expression on other signaling pathways was investigated. In one study, the effect of dnTβRII expression on signaling induced by basic fibroblast growth and platelet-derived growth factor (PDGF) was examined and found to be unaffected [19]. However, signaling through such unrelated tyrosine kinases is less likely to be affected by dnTβRII overexpression than signaling through related serine/threonine kinases [19]. In another study, activin-mediated inhibition of DNA synthesis was unaffected by dnTβRII expression in pancreatic cells, suggesting that the dominant negative mutant did not inactivate signaling by other TGFβ family members [20]. In both of these studies, truncated versions of TβRII lacking the cytoplasmic domain were employed as dominant negative mutants. It is possible that such mutants may not interact as efficiently with other type I receptors as do mutants with point mutations in their kinase domains, such as TβRII-K277R. Alternatively, blockade of other related pathways may occur only with higher levels of expression of dnTβRII. Since different biological effects elicited by TGFβ appear to require different thresholds of signaling [1,21], and different signaling pathways activated by TGFβ require different levels of receptor expression [22], achieving higher levels of expression may be necessary in order to adequately examine the diversify of signals elicited by TGFβ. In such cases, caution must be exercised when interpreting data as the results of this study indicate that other related signaling pathways could be perturbed by dnTβRII overexpression.

In conclusion, these studies highlight the importance of investigating the impact of dominant negative mutant receptor expression on the activity of related signaling pathways. This is particularly important in light of the fact that many TGFβ superfamily members modulate similar biological responses. Consequently, certain functions may erroneously be attributed to one pathway when they are in actual fact shared by multiple pathways. In addition, perturbation of more than one TGFβ superfamily pathway may result in a phenotype that would otherwise not be observed if only one pathway had been perturbed.

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