Transforming Growth Factor-β family ligands can function as antagonists by competing for type II receptor binding

Senem Aykul, Erik Martinez-Hackert*

From the Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824-1319, USA

*To whom correspondence should be addressed: Erik Martinez-Hackert, Michigan State University, Biochemistry Room 509, 603 Wilson Road, East Lansing, MI 48824-1319, USA. Email: emh@msu.edu, phone: (517) 355 1604

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ABSTRACT

Transforming Growth Factor-β (TGF-β) family ligands are pleiotropic cytokines. Their physiological activities are not determined by a simple coupling of stimulus and response, but depend critically on context, i.e. the interplay of receptors, ligands and regulators that form the TGF-β signal transduction system of a cell or tissue. How these different components combine to regulate signaling activities remains poorly understood. Here we describe a ligand-mediated mechanism of signaling regulation. Based on the observation that the type II TGF-β family receptors ActRIIA, ActRIIB and BMPRII interact with a large group of overlapping ligands at overlapping epitopes, we hypothesized high affinity ligands compete with low affinity ligands for receptor binding and signaling. We show Activin A and other high affinity ligands directly inhibited signaling by the low affinity ligands BMP-2, BMP-7 and BMP-9. We demonstrate Activin A functions as competitive inhibitor that blocks the ligand binding epitope on type II receptors. We propose binding competition and signaling antagonism are integral functions of the TGF-β signal transduction system. These functions could help explain how Activin A modulates physiologic signaling during extraordinary cellular responses, such as injury and wound healing, and how Activin A could elicit disease phenotypes such as cancer related muscle wasting and fibrosis.

INTRODUCTION

Transforming Growth Factor-β (TGF-β) family signaling pathways play fundamentally important roles in stem cell fate determination, embryonic development, organogenesis, immunity, and cancer (1-3). The basic principles underlying TGF-β family action are well established: A dimeric ligand binds two type I and two type II receptors to form a hexameric complex, thus initiating a signaling cascade that leads to phosphorylation of SMAD transcription factors, their translocation to the nucleus, and expression of target genes (4-9). Although this simple mechanism completely describes the molecular basis of signaling and response, it fails to explain the complex, sometimes opposite responses elicited by many TGF-β family ligands. For example, some TGF-β family can both inhibit and promote cell growth, both maintain pluripotency and induce differentiation, and both suppress and activate tumor cells. These paradoxical effects have supported the idea that cellular responses to a TGF-β family ligand depend not only on the ligand induced signaling cascade, but also on the cellular context, i.e. the molecular interplay of all the components that form the TGF-β signal transduction system of a particular cell type or tissue (10-14).
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In humans, the TGF-β family consists of 33 ligand genes (TGF-βs, Activins, Bone Morphogenetic Proteins/BMPs, Growth and Differentiation Factors/GDFs, Nodal, and Lefty), seven type I receptors, (ALK1–7), five type II receptors (ActRIIA, ActRIIB, BMPRII, TGFβRII, and AMHRII), as well as number of co-receptors, regulators, and intracellular SMAD transcription factors (3,15). A distinct feature of the family is the promiscuity of its members. Ligands can bind several different receptors, and receptors can bind multiple ligands. Yet ligand-receptor binding affinities vary greatly. Activin A, Activin B, GDF-8, GDF-11, and BMP-10 bind the type II receptors ActRIIA and ActRIIB with very high affinity (16-18). On the other hand, BMP-2 and BMP-4 bind ActRIIA and ActRIIB with low affinity, but they bind type I receptors with high affinity (19,20). These observations have supported a model of sequential signaling complex assembly where Activins, GDF-8 and GDF-11 first bind type II receptors with high affinity, then recruit low affinity type I receptors (5,21). By contrast, BMPs and GDFs first bind type I receptors with high affinity, then recruit low affinity type II receptors (22). Exceptions include BMP-10, which binds both type I and type II receptors with high affinity (9,23-25).

Significantly, high affinity and low affinity ligands bind the same type II receptors at the same epitope (26,27). This raises the question, what happens to low affinity BMP or GDF signaling when high affinity ligands like Activin A, GDF-11 or BMP-10 are present at the same time? Thus far, it has been suggested low affinity BMP and GDF signaling is independent of high affinity ligands, because they uniquely utilize BMPRII for signaling (4,7,20,27). But recent studies found Nodal, Activin A, Activin B and BMP-10 bind BMPRII with much higher affinity than most BMPs and GDFs (9,18,28,29), indicating low affinity ligands do not have a dedicated type II receptor. Instead, low affinity ligands use the same type II receptors as high affinity ligands. We therefore hypothesized high affinity ligands compete with low affinity ligands for type II receptor binding and antagonize low affinity ligand signaling. In this model, high affinity ligands can function both as signal carriers and as signaling regulators that mediate the biological activities of ligands that bind type II receptors with lower affinities.

To test this hypothesis, we examined ligand-type II receptor binding and ligand signaling. Activins and Activin related ligands like GDF-8 and GDF-11 generally bound type II receptors with higher affinity than most BMPs and signaled via the SMAD2/3 pathway. By contrast, BMPs generally bound type II receptors with lower affinity and signaled via the SMAD1/5/8 pathway, as expected. Notably, high affinity ligands directly inhibited SMAD1/5/8 signaling by low affinity ligands, while they activated their canonical SMAD2/3 pathways. Cross-inhibition was not restricted to low affinity ligands. High affinity ligands also inhibited other high affinity ligands. Significantly, cross-inhibition could be prevented by blocking the Activin A-type II receptor interaction, but not by inhibiting the intracellular signal transduction pathway. These findings thus suggest cross-inhibition is due to competition for type II receptor binding. That ligands can act as antagonists has been suggested for BMP-3 (30-32), Activin A (33), GDF-5 (34) and Inhibin (35-37). We propose ligand antagonism and signal transduction pathway switching is a general mechanism of TGF-β family regulation and an essential program during extraordinary cellular responses, such as wound healing (38). Cross-inhibition may also help explain how increased TGF-β family ligand expression can lead to pathophysiological responses, such as cancer cachexia (39,40).

METHODS

Ligands. Human Activin B (Q53T31), GDF-8 (O08689), TGF-β1 (P01137), TGF-β2 (P61812), TGF-β3 (P10600), Nodal (Q96S42), GDF-1 (NP_001483), BMP-2 (P12643), BMP-3 (P12645), BMP-4 (P12644), BMP-6 (P22004), BMP-7 (P18075), BMP-9 (Q9UK05), and BMP-10 (O95393) were obtained from R&D Systems or PROMOCES. Activin A (P08476) was produced in-house. Shown in parenthesis are NCBI-protein accession numbers.

Receptor-Fc constructs. Synthetic genes of human ActRIIA (P27037) and ActRIIB (Q13705) fused to human IgG1-Fc genes were obtained from GeneArt. Human BMPRII (Q13873) and TGFβRII (P37173) were cloned from cDNA (Open
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Biosystems) and fused to IgG1-Fc by PCR. Receptor-Fc fusion constructs included signal peptide (SP) and extracellular domains (ECD) of human ActRIIA (1-120), ActRIIB (1-120), TGFβRII (1-166), or BMPRII (1-136). ECDs were linked at the C-terminus to human IgG1-Fc via a 22 amino acid long linker containing a TEV cleavage site. NCBI-protein accession numbers are shown in parenthesis.

**Receptor-Fc purification.** Proteins were expressed using Chinese Hamster Ovary (CHO) cells. Secreted receptor-Fc fusion proteins were captured from condition medium using Protein A affinity chromatography, eluted with 100 mM Glycine, pH 3.0, and directly neutralized by adding 10% v/v 2 M Tris/HCl pH 9.0. Purified proteins were either dialyzed directly into phosphate-buffered saline (Amresco), pH 7.5 and stored at -80 °C, or further purified by size exclusion chromatography in phosphate-buffered saline, pH 7.5 and stored at -80 °C. Purity of receptor-Fc fusion proteins was determined by SDS-PAGE under reducing and non-reducing conditions.

**Surface Plasmon Resonance.** All experiments were performed using a Biacore 2000, and carried out at 25 °C using HBS-EP (0.01 M HEPES, 0.5 M NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20, pH 7.4) containing 0.1% BSA as running buffer. Experimental flow rate was 50 µl/min. For receptor capture, anti-human IgG (Fc) antibody was immobilized on four channels of a CM5 chip using amine-coupling chemistry. 250 response units (RU) of purified ActRIIA-Fc, ActRIIB-Fc, BMPRII-Fc, or TGFβRII-Fc were loaded on the experimental flow channels. A reference channel was monitored to account for nonspecific binding, drift, and bulk shifts. For ligand binding studies, different ligands at a concentration of 80 nM were injected, including Activin A, Activin B, GDF-1, GDF-8, GDF-11, TGF-β1, TGF-β2, TGF-β3, BMP-2, BMP-3, BMP-4, BMP-6, BMP-7, BMP-9, BMP-10 and Nodal. After each binding cycle, the antibody-BMP-7 surface was regenerated to base line by injecting MgCl₂.

**Cell lines.** A-204 rhabdomyosarcoma cells (HTB-82) and HepG2 hepatocellular carcinoma cells (HB-8065) were obtained from ATCC (American Type Culture Collection). Cells were maintained according to standard ATCC culture conditions. Briefly, A-204 cells were grown in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptavidin (P/S). HepG2 cells were grown in Eagle’s Minimum Essential Medium supplemented with 10% FBS and 1% P/S. Cells were grown at 37 °C under humidified, 5% CO₂ atmosphere. Freshly thawed cells were passaged at least three times before performing assays. A 40 µm cell strainer (Greiner Bio-one) was used to obtain a uniform, single-cell suspension of HepG2 cells for plating.

**Reporter assays.** ~ 50,000 A-204 or ~ 10,000 HepG2 cells in complete medium were seeded in each well of a 96-well plate and grown overnight. For transfection, solutions containing 24 µl lipofectamine 2000, assay medium (960 µl growth medium supplemented with 0.1% BSA), 192 ng pGL4.74 [Luc2P/hRluc/TK] vector (control luciferase reporter plasmid, Promega) and 19.2 µg of the SMAD3 responsive reporter plasmid pGL4.48 [Luc2P/SBE] or the SMAD1/5/8 responsive reporter plasmid pGL3 [Luc2P/BRE] were prepared and incubated at room temperature for 30 minutes. After incubation, 3840 µl assay medium was added to the transfection solutions, and 50 µl of this mixture were added to each well. Transfection medium was removed the following day, and medium was replaced with assay medium, which contained test proteins, including ligands (1-10 nM) and/or the receptor-Fc constructs (0-250 nM). Assay medium containing test proteins was incubated at 37 °C for 1 h before adding to cells. After addition of medium, A-204 cells were incubated for 6 h and HepG2 cells were incubated for 16 h at 37 °C, luciferase activity was detected using a homemade dual-glow luciferase assay (41). Luminescence was determined using a FluoStar Omega plate reader. Relative luciferase units were calculated by dividing firefly luciferase units (FLU) with renilla luciferase units (RLU). Data are expressed as mean of four independent
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measurements. Error bars correspond to SE of four independent measurements.

**Immunoblotting.** ~ 8.0x10⁴ HepG2 cells were plated in 12-well plates and grown to 80% confluence in complete medium, washed with 1X PBS, starved overnight in serum free medium supplemented with 0.1% BSA (assay medium) and grown for additional 16 h in assay medium with or without treatment. After 16 h incubation at 37 °C, protein lysate was prepared by using ice-cold RIPA lysis buffer (150mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 50mM Tris pH 8.0, 1X ‘Recom Protease Arrest’ protease inhibitor cocktail (G-Biosciences) and 2X ‘Phosphatase Arrest’ phosphastase inhibitor cocktail (G-Biosciences). Cell lysates were stored at -80 °C. Protein concentration of lysates was determined using Bradford. For Western blot, equal amounts of protein (10µg) were separated under reducing conditions on TGS-polyacrylamide gels (Bio-Rad) and transferred to Hybond-P membranes (GE Healthcare). Membranes were blocked with 5% BSA and incubated with primary antibodies from Cell Signaling at 1:1000 dilution, including anti-phospho-SMAD2 (138D4), anti-SMAD2 (D43B4), anti-phospho-SMAD1/5 (41D10), anti-SMAD4 (9515), and anti-β-Actin (8H10D10), followed by incubation with Horseradish peroxidase conjugated secondary antibody at 1:2000 dilution (7074). WesternBright ECL HRP substrate was used for detection (Advanta). Western blots were visualized by exposing the gel to autoradiography film.

**Statistics.** Reporter gene assays were performed in quadruplicates and were repeated two to three different times. Statistical analysis was done with GraphPad Prism 6. To determine statistical significance for induction experiments (Fig. 2), one-way analysis of variance (ANOVA) and Dunnett’s post hoc test were used. For inhibition experiments (Figs. 3-7), two-way ANOVA and Tukey’s post hoc test were used. P values < 0.05 were considered statistically significant. A bar marks statistically significant differences in figure 2, horizontal lines between experimental pairs mark statistically significant differences in figures 3-6. Light grey lines correspond to SMAD1/5/8 signaling experiments, dark grey lines correspond to SMAD2/3 signaling experiments.

**RESULTS**

**Type II receptors bind multiple ligands with high affinity.** Most type II TGF-β receptors bind multiple ligands and many ligands bind multiple type II receptors. Because of this promiscuity, ligand-receptor interactions in the TGF-β family remain poorly defined. To characterize the ligand-binding specificity of the four major type II TGF-β receptors, ActRIIA, ActRIIB, BMPRII and TGFβRII, we used a high throughput, SPR-based binding assay (Fig. 1). We captured purified receptor-Fc fusion proteins on a Biacore sensor chip that was cross-linked with an anti-human Fc antibody and injected 16 different ligands at concentrations that exceeded physiological levels (80 nM) (42-44). As anticipated, ActRIIA-Fc and ActRIIB-Fc had similar ligand binding profiles (Fig. 1). Both receptors bound Activin A and Activin B with very high affinity as seen in the very fast association and very slow dissociation rates (Fig. 1 A, and B, red and cyan curves). Other ligands that bound both ActRIIA-Fc and ActRIIB-Fc with high affinity included GDF-8, GDF-11, BMP-6, and BMP-10 (Fig. 1 A, and B, green, blue, magenta, and maroon curves). However, either their association rates were slower or their dissociation rates were faster than those seen for Activin A or Activin B, resulting overall in a weaker interaction. Important specificity differences between these two highly homologous receptors were seen with BMP-7 and BMP-9, which bound ActRIIA-Fc and ActRIIB-Fc, respectively, with relatively high affinity.

TGFβRII and BMPRII had very distinctive binding profiles. TGFβRII only bound TGF-β1 and TGF-β3 with very high affinity, but not TGF-β2 (Fig. 1D, light and dark gray curves) (45,46). The BMPRII ligand profile was unexpected, as we found it also has high affinity ligands, namely Nodal, Activin B and, to a lesser degree, BMP-10 (Fig. 1C, olive green, red, and maroon curves). BMP-9, Activin A, and BMP-6 also associate with BMPRII, but these complexes are much weaker. Notably, some ligands, including GDF-1, BMP-2, BMP-3, and TGF-β2, did not bind any one of these type II receptors with high affinity. We estimated $K_d$ values based on single injections (Table S1-S4). These are comparable to published $K_d$ values determined from multiple injections (9,16,45,46).
Ligands have distinctive, cell-line specific signaling profiles. TGF-β family ligands activate intracellular SMAD signal transduction pathways. Type I receptors determine which SMAD pathway is activated. It is suggested SMAD2/3 pathways are activated by the type I receptors ALK4, ALK5 and ALK7, while SMAD1/5/8 pathways are activated by ALK1, ALK2, ALK3, and ALK6 (47). To characterize the signaling activities of the tested ligands, we used a luciferase reporter gene assay and two different cell lines of human origin, A-204 rhabdomyosarcoma and HepG2 hepatocellular carcinoma cells (Fig. 2). Both cell lines expressed the principal TGF-β family receptors (Figs. 2A, R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl, dataset id GSK950). Cells were transfected with control plasmid pGL4.74 [hRluc] and the SMAD3 responsive reporter plasmid pGL4.48 [luc2P/SBE] or the SMAD1/5/8 responsive reporter plasmid pGL3 [luc2P/BRE] (48,49). Transfected cells were treated with 1 or 10 nM ligand.

A-204 cells responded to Activin A, Activin B, GDF-8 and GDF-11, inducing a strong SMAD2/3 mediated response (Fig. 2B). By contrast, TGF-βs and BMPs did not induce luciferase gene expression in this cell line. Strikingly, HepG2 cells showed a completely different behavior. Both TGF-βs and BMPs induced a very strong luciferase signal (Fig. 2C). Among BMPs that signal via the type I receptor kinases ALK2, ALK3 or ALK6, BMP-7 mediated SMAD1/5/8 signaling was strongest (165-fold over background) and BMP-2 signaling was weaker (40-fold over background). BMP-3 did not elicit a meaningful luciferase signal (Figs. 2B, and 2C). Among BMPs that signal via ALK1, 1 nM BMP-10 elicited a small SMAD1/5/8 signal (6-fold over background), whereas BMP-9 required a 10 nM concentration. However, at this concentration, the BMP-9 signal was very strong (160-fold over background). Both TGF-β2 and TGF-β3 induced a strong SMAD2/3 mediated signal at 1 nM concentration. This signal was one order of magnitude greater than the response to TGF-β1 (Fig. 2C, approximately 4-fold over background for TGF-β1 compared with 45- and 50-fold over background for TGF-β2 and TGF-β3, respectively). Intriguingly, TGF-β2 induced a statistically significant SMAD1/5/8 mediated luciferase signal. This is consistent with previous findings that TGF-β ligands (i.e. TGF-β1, -β2 and/or -β3) can activate SMAD1/5/8 via ALK5 and/or ALK2/ALK3 (50-52). Differences in TGF-β isoform signaling activities may result from differences in type II receptor binding affinities or co-receptor expression.

Responses to Activin A, Activin B, GDF-8 and GDF-11 diverged in HepG2 cells. The SMAD2/3 mediated Activin B response remained constant in both A-204 and HepG2 cells (8-fold over background at 1 nM Activin B). In contrast to A-204 cells, the Activin A response in HepG2 cells was smaller at 1 nM concentration, but could be induced strongly at 10 nM concentration (Figs. 2B, and 2C). Interestingly, GDF-8 signaling was reduced relative to A-204 cells, whereas GDF-11 signaling increased significantly (from 16-fold over background in A-204 cells to approximately 40-fold over background in HepG2 cells) (Figs. 2B, and 2C). Notably, 1 nM Activin B induced a strong SMAD1/5/8 mediated response in HepG2 cells, which exceeded the canonical SMAD2/3 response (Fig. 2C, 18-fold compared with 8-fold over background).

To explain the differences in signaling, we analyzed expression of TGF-β family components in both cell lines (Figs. 2A, http://r2.amc.nl, dataset id GSK950). Levels of ActRIIB and TGFβRII are low in A-204 cells. By contrast, HepG2 cells express type II receptors at much higher levels. Notably, TGFβRII expression is so high in HepG2 cells that they rank in the top 8 percentile of 470 cell lines when comparing TGFβRII levels (dataset id gse57083). We suggest differences in ActRIIB and TGFβRII expression could account for significant differences in signaling. But HepG2 cells also express high levels of betaglycan, which helps increase cellular sensitivity to BMPs (53) and enhance responsiveness to TGF-βs (54). We speculate betaglycan could contribute significantly to the divergent signaling activities.

Activin A antagonizes BMP-2 and BMP-7 signaling. As BMPs, GDFs and Activins utilize the same type II receptors for signaling and bind these receptors at the same site (27), we hypothesized high affinity ligands like Activin A could directly inhibit low affinity ligand signaling.
To test this hypothesis we first determined by titration the limits of Activin A signaling in HepG2 cells (Fig. 3A). The greatest increase in Activin A signaling occurred between 0 and 5 nM concentrations. Beyond that, signaling reached a plateau. Even a 10-fold higher concentration of Activin A (50 nM) did not increase the SMAD2/3 luciferase signal further (Fig. 3A). To evaluate cross-inhibition, we incubated Activin A with BMP-2 or BMP-7. Activin A strongly inhibited BMP-2 and BMP-7 mediated SMAD1/5/8 signaling (Figs. 3B, and C). At a 5-fold excess, Activin A reduced the SMAD1/5/8 signal approximately 7-fold for BMP-2 and 10-fold for BMP-7. Even at equimolar concentrations, Activin A attenuated the BMP-2 and BMP-7 mediated SMAD1/5/8 responses 4-fold. Notably, Activin A also activated its canonical SMAD2/3 signaling pathway. The SMAD2/3 signal was the same, whether BMP-2 or BMP-7 were present or not (Figs. 3A-C). Yet a small, statistically significant residual SMAD1/5/8 signaling activity remained for both BMP-2 and BMP-7 at the highest Activin A concentrations tested (Figs. 3B, and 3C).

We performed a similar inhibition experiment with TGF-β2 or TGF-β3 (Fig. 3D). Activin A did not inhibit the SMAD2/3 mediated TGF-β2 and TGF-β3 signals, as expected. Instead, the activities of Activin A and TGF-β2 or TGF-β3 were additive (Fig. 3D). We also examined if Activin A could inhibit ligands that bind the same type II receptors with high affinity, including BMP-9 and BMP-10 (Figs. 3E, and 3F). As we show for BMP-2 and BMP-7, Activin A antagonized signaling by these two ligands, reducing their peak response approximately 2.5-fold for BMP-10 and 25-fold for BMP-9 at equimolar concentrations. Higher concentrations of Activin A further reduced BMP-9 and BMP-10 signaling, but a small SMAD1/5/8 signal remained.

**BMP-2 and BMP-7 signaling is antagonized by high affinity ligands.** To demonstrate ligand antagonism is not specific to Activin A, we examined BMP-2 and BMP-7 inhibition by other high affinity ligands, including Activin B, GDF-8, GDF-11, BMP-9 and BMP-10 (Fig. 4). These ligands also bind ActRIIA-Fc, ActRIIB-Fc and/or BMPRII-Fc to varying degrees: Activin B binds all three receptors, BMP-9 predominantly binds ActRIIB, GDF-8 and GDF-11 bind ActRIIA and ActRIIB, but not BMPRII (Fig. 1). Using HepG2 cells and 10 nM ligand concentrations, we confirmed GDF-8 and GDF-11 exclusively signaled via SMAD2/3. Notably, the GDF-11 signal was approximately 10 times greater than the GDF-8 signal (28.9 RLU vs. 3.0 RLU) (Fig. 4A). Activin B induced both SMAD2/3 and SMAD1/5/8 pathways more or less equivalently (18.9 RLU vs. 22.2 RLU) (Fig. 4A). BMP-9 and BMP-10 exclusively induced SMAD1/5/8 signaling (Fig. 4B).

GDF-8 reduced the BMP-2 signal approximately 2-fold (from 24.7 to 11.5 RLU for BMP-2 and from 145.6 to 70.1 RLU for BMP-7). GDF-11 was more effective and reduced the SMAD1/5/8 signal approximately 4- to 6-fold (from 24.7 to 6.6 RLU for BMP-2 and from 145.6 to 24.4 RLU for BMP-7) (Fig. 4A). However, neither ligand inhibited signaling as powerfully as Activin A. The behavior of Activin B was more complex, as it also induced SMAD1/5/8 signaling in HepG2 cells (Fig. 4A). The SMAD1/5/8 response was similar whether cells were treated with BMP-2 alone or whether cells were treated with BMP-2 and Activin B. The effect of Activin B on BMP-7 signaling was clearer ActivinB reduced the BMP-7 mediated SMAD1/5/8 signal approximately 6-fold (from 145.6 to 27.3 RLU) (Fig. 4A). Notably, the SMAD1/5/8 signal intensity obtained with Activin B alone was virtually the same whether BMP-2 or BMP-7 were present (22.7, 27.3 and 22.2 RLU, respectively). We propose the residual SMAD1/5/8 activity is due to Activin B. In conclusion, GDF-8, GDF-11 and Activin B antagonize BMP-2 and BMP-7 signaling, although they inhibit less effectively than Activin A.

The behavior of BMP-9 and BMP-10 was intriguing. Their ability to induce a response was nearly indistinguishable whether BMP-2 was present or not (118.7 vs 103.5 RLU for BMP-9 ± BMP-2 and 42.5 vs 42.2 RLU for BMP-10 ± BMP-2) (Fig. 4B). By contrast, both attenuated a BMP-7 mediated SMAD1/5/8 response. Significantly, the response converged (from 145.6 to 27.3 RLU for BMP-9 and from 145.6 to 26.7 RLU for BMP-10) (Fig. 4B). We speculate BMP-9 and BMP-10 override BMP-2 signaling, but remain in a competitive equilibrium with BMP-7 that leads to an attenuated, but defined response.
ActRIIA-Fc and Follistatin, but not SB-431542, inhibit Activin A antagonism. To demonstrate that cross-inhibition is due to ligand-receptor binding-competition, we examined how the extracellular inhibitors ActRIIA-Fc, Follistatin, and the intracellular kinase inhibitor SB-431542 affected Activin A antagonism (Figs. 5-7). We hypothesized ActRIIA-Fc and Follistatin would block Activin A antagonism, as both prevent binding of Activin A to type II receptors and preclude Activin A from competing with BMP-2 or BMP-7 (55). By contrast, SB-431542 would not prevent Activin A antagonism (56,57), as Activin A would still bind type II receptors and compete with BMP-2 and BMP-7 for type II receptor binding. To test this model, we examined BMP-7 mediated reporter gene expression in A-204 and HepG2 cells (Figs. 5A, and B), BMP-2 mediated reporter gene expression in HepG2 cells (Fig. 6A), and BMP-2 mediated SMAD phosphorylation in HepG2 cells (Fig. 6B).

ActRIIA-Fc inhibited SMAD2/3 mediated Activin A signaling, but not SMAD1/5/8 mediated BMP-2 or BMP-7 signaling (Figs. 5A, 5B, and 6A). Significantly, Activin A strongly inhibited BMP-7 and BMP-2 signaling when alone, but not when ActRIIA-Fc was also present. (Figs. 5A, 5B, and 6A) Likewise, Follistatin inhibited SMAD2/3 mediated Activin A signaling and rescued BMP-7 signaling in the presence Activin A (Fig. 7A). But Follistatin also inhibited BMP-7 signaling at high concentrations (58,59). In sharp contrast to ActRIIA-Fc and Follistatin, SB-431542 did not prevent Activin A antagonism. Activin A reduced BMP-7 and BMP-2 signaling equally whether SB-431542 was added or not (Figs. 5A, 5B, and 6A). Importantly, these findings were paralleled by SMAD phosphorylation (Fig. 6B), indicating that the response measured by reporter gene expression is directly linked to SMAD mediated signal transduction pathways. Thus, our findings show inhibition of BMP-7 and BMP-2 signaling by Activin A can be rescued by trapping Activin A with ActRIIA-Fc or Follistatin and preventing Activin A-receptor binding. But suppressing signal transduction pathway activation with intracellular kinase inhibitors does not block the Activin A antagonist activity.

Activin A inhibits BMP-7 – ActRIIA binding. To demonstrate directly that Activin A inhibits binding of BMP-7 to type II receptors, we developed a competition SPR binding assay (Fig. 7B). We captured BMP-7 on the sensorchip using a cross-linked, BMP-7 specific monoclonal antibody and flowed ActRIIA-Fc preincubated with different concentrations of Activin A. ActRIIA-Fc (12 nM) bound captured BMP-7 with an association rate that was nearly the same as that determined using our standard setup (Table S5). ActRIIA-Fc binding to BMP-7 decreased with increasing Activin A concentrations and a 4-fold molar excess of Activin A (48 nM) completely inhibited the ActRIIA-Fc – BMP-7 interaction. Thus, Activin A can function as competitive inhibitor of BMP-7 binding to ActRIIA.

BMP-10 is a weak Activin A antagonist. As the antagonist activity of TGF-β family ligands was not limited to Activin A (Fig. 3), we hypothesized some ligands may antagonize Activin A signaling. Previous studies have indicated BMP-3 inhibits Activin A signaling (30-32), and we speculated BMP-10 could potentially antagonize Activin A as it is a high affinity ligand of the three type II Activin A receptors (Fig. 1). To test this hypothesis, we examined Activin A mediated reporter gene expression in HepG2 cells and antagonism by BMP-3 and BMP-10 (Fig. 6C). Notably, 50 nM BMP-3 did not inhibit signaling by 5 nM Activin A, even after we tested three different BMP-3 samples. On the other hand, a 10-fold excess of BMP-10 reduced the Activin A mediated SMAD2/3 signal about 2-fold (from 37.4 to 21.7 RLU), while Activin A attenuated the BMP-10 mediated SMAD1/5/8 response as expected (from 45.3 to 16.6 RLU). Although BMP-10 antagonism was not as potent as Activin A antagonism, this finding shows Activin A signaling could also be inhibited by some TGF-β family ligands.

DISCUSSION

Our goal was to test the hypothesis that TGF-β family ligands can function as signaling antagonists by competing for type II receptor binding. We showed several TGF-β family ligands directly inhibited signaling by other members of the family. The antagonist activity could be suppressed by blocking the type II receptor binding-site on antagonistic ligands, but not by inhibiting their signal transduction cascade.
Moreover, we demonstrated ligand antagonism is mediated by direct competition for receptor binding. We propose ligand antagonism is a common mechanism that can be used to modulate TGF-β family signaling. We speculate the precise combination of ligands available to a cell or tissue will profoundly affects how they read a particular TGF-β family signal.

**Ligand competition regulates signaling.** An enduring conundrum in the TGF-β field is the promiscuity of its ligand-receptor pairings. This promiscuity is particularly relevant to the ‘Activin receptors’ ActRIIA, ActRIIB, and the ‘BMP receptor’ BMPRII. They interact with a large group of overlapping ligands, including Activins, BMPs, GDFs and Nodal (Fig. 1). As these ligands bind receptors at a common site (Fig. 8A), we hypothesized ligands can compete for receptor binding and high affinity ligands can suppress low affinity ligand signaling (Fig. 8B). We show the high affinity ligand Activin A effectively inhibited signaling by the low affinity ligand BMP-2, the medium affinity ligand BMP-7 and the high affinity ligands BMP-9 and BMP-10 (Figs. 3, 5, 6, and 7). The ability to inhibit signaling was not specific to Activin A. GDF-8, GDF-11, BMP-9, BMP-10, and Activin B also inhibited BMP-2 and BMP-7 signaling. However, Activin A was the most powerful BMP-2 and BMP-7 antagonist. Notably, BMP-10 antagonized Activin A signaling with some efficacy (Fig. 6C). The pervasiveness of competition and antagonism between ligands suggests they are a key feature of the TGF-β family. But they depend on shared receptor utilization, as TGF-β1 cannot inhibit BMP-6 or BMP-9 signaling (33). We speculate competition and antagonism could play an important role in the biological activities of ligands that are involved in acute biological responses, such as injury, infection, and inflammation (61-63). Thus, by acting as antagonist, Activin A secreted in wound healing could shift the signaling equilibrium from a state that supports tissue homeostasis to a state that leads to regeneration (64-66). Importantly, while Activin A suppressed BMP-2 and BMP-7 mediated SMAD1/5/8 signaling, it also activated its canonical SMAD2/3 signaling pathway, causing a switch in signal transduction pathway utilization.

**Molecular basis of ligand competition.** ActRIIA-Fc, Follistatin and SB-431542 are potent Activin A signaling inhibitors with distinct modes of action. ActRIIA-Fc binds Activin A and shields its type II receptor binding-site (26). Follistatin binds Activin A and blocks both type I and type II receptor binding-sites (67). SB-431542 inhibits type I receptor kinase activity (57). Activin A trapped by ActRIIA-Fc or Follistatin cannot bind type II receptors. By contrast, Activin A can bind type II receptors when SB-431542 is present. We show ActRIIA-Fc and Follistatin rescued BMP-2 and/or BMP-7 signaling in the presence of Activin A, but SB-431542 did not (Figs. 5-7). The antagonist function of Activin A thus can be suppressed by blocking Activin A binding to type II receptors, but not by inhibiting its signal transduction cascade. As Activin A, BMP-2 and BMP-7 share type II receptors and bind receptors at the same site, we speculated Activin A is a competitive BMP inhibitor that blocks the weak BMP-type II receptor interaction (Figs. 1 and 8). Indeed, Activin A prevented BMP-7 binding to ActRIIA in vitro, providing direct evidence for a competitive inhibitor function (Fig. 7B). Notably, binding competition and signaling antagonism are not new concepts in the TGF-β family. Functional antagonism was described for the Activin A/BMP-7 pairing in human embryonic carcinoma cells (30) and for the GDF-8/BMP-7 pairing in C3H10T1/2 cells (68). Inhibin A, an Activin A related ligand that lacks signaling activity, inhibited BMP responses by competing for type II receptor binding (35-37). Binding competition was also proposed for some BMPs together with type II receptors (69), for Activin A combined with type I receptors (70), and for Activin A with BMP-9 (33). We propose binding-competition and signaling antagonism is a common regulatory mechanism in the TGF-β family enabled by receptor promiscuity and binding site conservation (19).

**A role for ligand competition in human diseases.** TGF-β family ligands play critical roles in many human diseases, including cancer, fibrosis, bone loss, and muscle loss (10). It is suggested elevated ligand expression leads to hyper-activation of their canonical signaling pathways and, consequently, to disease onset and progression (71). However, the ability of a TGF-β family ligand to hyper-
activate its signaling pathways can be limited; TGF-β family ligands can have a ‘signaling ceiling’ (Fig. 3). On the other hand, we found ‘high affinity’ ligands like Activin A, attenuated or antagonized signaling pathways that were activated by ‘lower affinity’ ligands like BMP-2 and BMP-7 (Figs. 3, 5, 6, and 7). Thus, while hyper-activated signaling could occur in the appropriate context and may be the root cause of a number of diseases, we speculate binding-competition and signaling antagonism between ligands could also play a role in the pathobiology of Activin A and other TGF-β family ligands. Significantly, a recent study indicated Activin A mediated progression of fibrodysplasia ossificans progressiva by competing for type I receptor binding (70).

**Differential effect of inhibitors on antagonism.**

Inhibiting Activin A signaling has considerable therapeutic potential. Several approaches have been explored, including blocking the receptor kinase activity with SB-431542, and preventing formation of Activin A-receptor signaling complexes using ActRIIA-Fc (55-57,72). Both SB-431542 and ActRIIA-Fc inhibited Activin A signaling, as determined by SMAD2/3 phosphorylation and SMAD2/3 mediated luciferase expression. But they differed drastically in their ability to prevent Activin A antagonism. Only extracellular Activin A inhibitors rescued low affinity ligand signaling (Figs. 5-7). As Activin A could either hyper-activate its canonical signaling pathway or antagonize low affinity BMP signaling in disease, our findings indicate selection of an appropriate therapeutic approach to inhibit Activin A signaling should depend on the mechanism of pathogenesis. Even so, extracellular inhibitors that target ligands and prevent receptor binding offer a more defined approach for rescuing the effects of excess ligand in disease.

**CONCLUSIONS**

We show high affinity TGF-β family ligands like Activin A compete with low affinity ligands for receptor binding and antagonize low affinity ligand signaling. Several diseases associate with Activin A overexpression, including inflammation, fibrosis and cancer related muscle wasting (39,40,73). How elevated Activin A levels lead to disease is not well understood. It is thought hyper-activated signaling causes pathogenesis. But our findings indicate a second possibility. Namely, ectopic Activin A could antagonize normal signaling, and thus promote disease. Significantly, a recent study indicated Activin A antagonism may be critical for progression of fibrodysplasia ossificans progressiva (70).

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**CONFLICT OF INTERESTS**

The authors declare no competing interests.

**AUTHOR CONTRIBUTIONS**

SA and EMH designed experiments. SA performed experiments. SA and EMH interpreted experiments. EMH wrote manuscript. SA and EMH revised manuscript.
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FIGURE LEGENDS:

Fig. 1. Type II receptors bind a range of TGF-β family ligands with high affinity. 250 response units (RU) of protein-A purified (A) ActRIIA-Fc, (B) ActRIIB-Fc, (C) BMRPII-Fc, and (D) TGFβRII-Fc were captured on an SPR sensor chip, and ligands at 80 nM concentration were injected, including Activin A, Activin B, GDF-1, GDF-8, GDF-11, TGF-β1, TGF-β2, TGF-β3, BMP-2, BMP-3, BMP-4, BMP-6, BMP-7, BMP-9, BMP-10, and Nodal. Ligands that bound receptors as determined by a measurable SPR response are shown in relevant panels. Color of binding curve and ligand name are matched. Curves of ligands that did not bind are colored dark blue, their names are not shown.

Fig. 2. TGF-β family signaling activity is cell line dependent. (A) Microarray data show expression levels of TGF-β family receptors in A-204 rhabdomyosarcoma and HepG2 hepatocellular carcinoma cells (Genomics Analysis and Visualization Platform (http://r2.amc.nl), dataset id GSK950). The left panel shows expression of type II receptors, the right panel shows expression of type I receptors. Normalized data is displayed as SQRT transformation. Ligand signaling in (B) A-204 and (C) HepG2 cells. Cells were transfected with control plasmid and SMAD2/3 or SMAD1/5/8 responsive reporter plasmids. Dark grey bars and striped dark grey bars correspond to SMAD2/3 mediated reporter gene expression induced with 1 nM ligand and 10 nM ligand, respectively, light grey bars and striped light grey bars correspond to SMAD1/5/8 mediated reporter gene expression induced with 1 nM and 10 nM ligand, respectively. Relative Light Units (RLU) were calculated as the ratio of luminescence from the experimental reporter to luminescence from the control reporter. Data are expressed as mean + SE of four independent measurements. Horizontal bars denote statistically significant differences in luciferase activity relative to the ‘no-ligand’ control (p ≤ 0.05). The base line of the ‘no-ligand’ control is marked by the dotted line.

Fig. 3. Activin A antagonizes BMP signaling. HepG2 were transfected with control plasmid and SMAD2/3 or SMAD1/5/8 responsive reporter plasmids and treated with various ligands. (A) HepG2 cells were treated with 0.1, 1.0, 2.5, 5.0, 10.0, and 50.0 nM Activin A. (B) HepG2 cells were treated with 0.1, 0.5, and 1 nM BMP-2. Signaling induced with 1 nM BMP-2 was then inhibited with 1.0, 5.0, and 10.0 nM Activin A. (C) HepG2 cells were treated with 0.1, 0.5, and 1 nM BMP-7. Signaling induced with 1 nM BMP-7 was then inhibited with 0.5, 1.0, 5.0, and 10.0 nM Activin A. (D) HepG2 cells were treated with 1.0 nM TGF-β2, 1.0 nM TGF-β3, and/or 10 nM Activin A. (E) HepG2 cells were treated with 1.0, 5.0, and 10.0 nM BMP-10. Signaling induced with 5.0 nM BMP-10 was then inhibited with 5.0, 10.0, and 50.0 nM Activin A. (F) HepG2 cells were treated with 1.0, 5.0, and 10.0 nM BMP-9. Signaling induced with 10.0 nM BMP-9 was then inhibited with 0.5, 1.0, 5.0, and 10.0 nM Activin A. Dark grey bars correspond to SMAD2/3 mediated reporter gene expression and light grey bars correspond to SMAD1/5/8 mediated reporter gene expression. Relative Light Units (RLU) were calculated as the ratio of luminescence from the experimental reporter to luminescence from the control reporter. Data are expressed as mean + SE of four independent measurements. Horizontal light grey lines indicate statistically significant differences in SMAD1/5/8 signaling and horizontal dark grey lines indicate statistically significant differences in SMAD2/3 signaling (p ≤ 0.05) between relevant experimental pairs.

Fig. 4. Antagonism of low affinity BMP-2 and BMP-7 signaling is common. Inhibition of BMP-2 and BMP-7 signaling with (A) GDF-8, GDF-11 and Activin B, or (B) BMP-9 and BMP-10. HepG2 were transfected with control plasmid and SMAD2/3 or SMAD1/5/8 responsive reporter plasmids. 1 nM BMP-2 or BMP-7 were added to induce signaling, 10 nM GDF-8, GDF-11, Activin B, BMP-9 or BMP-10 were added for inhibition. Dark grey bars correspond to SMAD2/3 mediated reporter gene expression and light grey bars correspond to SMAD1/5/8 mediated reporter gene expression. Relative Light Units (RLU) were calculated as the ratio of luminescence from the experimental reporter to luminescence from the control reporter. Data are expressed as mean + SE of four independent measurements. Horizontal light grey lines indicate statistically significant differences in SMAD1/5/8 signaling and horizontal dark grey lines indicate statistically significant differences in SMAD2/3 signaling (p ≤ 0.05) between relevant experimental pairs.
Fig. 5. The extracellular Activin A signaling inhibitor ActRIIA-Fc rescues BMP-7 activity. Luciferase reporter gene expression was induced with BMP-7 in (A) A-204, and (B) HepG2 cells transfected with SMAD2/3 or SMAD1/5/8 responsive reporter plasmids. 1 nM BMP-7 was added to induce signaling, 10 nM Activin A was added to antagonize BMP-7 signaling. 250 nM ActRIIA-Fc was used to block binding of Activin A to type II receptors, 5 µM SB-431542 was added to block Activin A mediated kinase activation and SMAD2/3 phosphorylation. Dark grey bars correspond to SMAD2/3 mediated reporter gene expression and light grey bars correspond to SMAD1/5/8 mediated reporter gene expression. Data are expressed as mean ± SE of four independent measurements. Horizontal light grey lines indicate statistically significant differences in SMAD1/5/8 signaling and horizontal dark grey lines indicate statistically significant differences in SMAD2/3 signaling (p ≤ 0.05) between relevant experimental pairs.

Fig. 6. Activin A role in BMP-2 signal transduction and inhibition with BMP-10. (A) Luciferase reporter gene expression was induced with BMP-2 in HepG2 cells transfected with SMAD2/3 or SMAD1/5/8 responsive reporter plasmids. 1 nM BMP-2 was added to induce signaling, 10 nM Activin A was added to antagonize BMP-2 signaling. 250 nM ActRIIA-Fc was used to block binding of Activin A to type II receptors, 5 µM SB-431542 was added to block Activin A mediated kinase activation and SMAD2/3 phosphorylation. Dark grey bars correspond to SMAD2/3 mediated reporter gene expression and light grey bars correspond to SMAD1/5/8 mediated reporter gene expression. (B) Western blots showing BMP-2 and Activin A mediated SMAD phosphorylation in HepG2 cells. 1 nM BMP-2 was added to induce SMAD1/5/8 phosphorylation, 10 nM Activin A was added to antagonize BMP-2 mediated SMAD1/5/8 phosphorylation, 250 nM ActRIIA-Fc was used to block binding of Activin A to type II receptors, 5 µM SB-431542 was added to block Activin A mediated kinase activation and SMAD2/3 phosphorylation. (C) HepG2 were transfected with SMAD2/3 or SMAD1/5/8 responsive reporter plasmids. 5 nM Activin A was added to induce signaling, 50 nM BMP-10 or BMP-3 were added to inhibit Activin A mediated SMAD2/3 reporter gene expression. Dark grey bars correspond to SMAD2/3 mediated reporter gene expression. Light grey bars correspond to SMAD1/5/8 mediated reporter gene expression. Data are expressed as mean ± SE of four independent measurements. Horizontal light grey lines indicate statistically significant differences in SMAD1/5/8 signaling and horizontal dark grey lines indicate statistically significant differences in SMAD2/3 signaling (p ≤ 0.05) between relevant experimental pairs.

Fig. 7. Follistatin suppresses Activin A antagonism. (A) Luciferase reporter gene expression was induced with BMP-7 in HepG2 cells transfected with SMAD2/3 or SMAD1/5/8 responsive reporter plasmids. Follistatin inhibited BMP-7 signaling at high concentrations (40 and 100 nM), but less at lower concentrations (10 and 20 nM). Activin A (10 nM) effectively inhibited BMP-7 signaling. Follistatin (10-20 nM) inhibited Activin A signaling and prevented antagonism of BMP-7. Dark grey bars correspond to SMAD2/3 mediated reporter gene expression and light grey bars correspond to SMAD1/5/8 mediated reporter gene expression. Data are expressed as mean ± SE of three independent measurements. Horizontal light grey lines indicate statistically significant differences in SMAD1/5/8 signaling and horizontal dark grey lines indicate statistically significant differences in SMAD2/3 signaling (p ≤ 0.05) between relevant experimental pairs. (B) Activin A inhibits binding of ActRIIA-Fc to BMP-7. BMP-7 was captured on the SPR sensor chip using an anti-BMP-7 monoclonal antibody (top right panel). 12 nM ActRIIA-Fc preincubated with 0 nM (blue), 3 nM (red), 6 nM (magenta), 12 nM (green), 24 nM (maroon), 48 nM (grey) Activin A was injected over the sensor chip. Activin A prevented binding of ActRIIA-Fc to BMP-7 in a concentration dependent manner. The bottom right panel shows a dose response curve. RU values were taken at 290 seconds after starting injection of ActRIIA-Fc.

Fig. 8. Model of ligand antagonism. (A) Molecular structures of Activin A, BMP-2, BMP-9 and BMP-7 in complex with type II receptors ActRIIA or ActRIIB, based on PDB coordinates 1NYU (74), 2H62 (7), 4FAO (9), and 1LX5 (26) displayed in corresponding orientations. Ligands are shown as orange ribbon diagrams. Type II receptors are shown as molecular surface colored blue with surface atoms that contact...
ligands colored grey. The ligand binding-site on the type II receptors ActRIIA and ActRIIB is highly conserved (grey surface area). (B) Signaling in the TGF-β family is induced when dimeric ligands (orange/khaki ovals) bind the extracellular domains of type I Activin receptor-like kinases (grey/brown) and type II receptors (blue). Formation of the hexameric ligand-receptor complex leads to phosphorylation of SMAD transcription factors. Ligands that bind type II receptors with high affinity (orange) out-compete ligands that bind type II receptors with lower affinity (khaki) and block low affinity ligand signaling. For example, low affinity ligands, like BMP-2 and BMP-7, induce SMAD1/5/8 phosphorylation via type I receptors ALK2, ALK3, or ALK6 (brown). High affinity ligands like Activin A block BMP-2 and BMP-7 binding to type II receptors, blocking SMAD1/5/8 phosphorylation whilst activating their canonical signaling pathways, i.e. SMAD2/3 phosphorylation via type I receptors ALK4, and ALK7 (grey). Low affinity ligands therefore likely signal only in the absence of high affinity ligands, or when type II receptors are expressed at such high levels that ligand competition is not possible.
Competition and antagonism in TGF-β family signaling

FIGURE 1

A) ActRIIA-Fc (chip)
- RU
- 140
- 80
- 20
- 0
- 400
- 800
- 1200 s

B) BMPRII-Fc (chip)
- RU
- 120
- 70
- 20
- 0
- 400
- 800
- 1200 s

C) ActRIIB-Fc (chip)
- RU
- 100
- 60
- 20
- 0
- 400
- 800
- 1200 s

D) TGFβRII-Fc (chip)
- RU
- 90
- 50
- 10
- 0
- 400
- 800
- 1200 s

Legend:
- Activin B
- BMP7
- GDF11
- Activin A
- BMP10
- BMP4
- BMP6
- GDF8
- BMP9
- Nodal
- Activin B
- GDF11
- BMP4
- BMP6
- BMP10
- BMP9
- BMP7
- BMP3
- GDF11
- TGF-β3
- TGF-β1
- BMP10
- BMP7
- BMP6
FIGURE 3

A

B

C

D

E

F

Competition and antagonism in TGF-β family signaling
FIGURE 4
FIGURE 5

A

B

FIGURE 5
FIGURE 6

A

B

C

Competition and antagonism in TGF-β family signaling
Competition and antagonism in TGF-β family signaling

FIGURE 8

A

Activin A  BMP-2  BMP-9  BMP-7

B

BMP/GDF/Activin signaling

with competition

without competition

ActRIIA  ActRIIB  BMPRII

Extracellular

Membrane

Intracellular

ALK4  ALK7

Type I  Type II

P

Smad-2/3  Smad1/5/8  Smad-1/5/8  Smad-2/3
Transforming Growth Factor-β family ligands can function as antagonists by competing for type II receptor binding
Senem Aykul and Erik Martinez-Hackert

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