Receptor binding competition: A paradigm for regulating TGF-β family action

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ABSTRACT

The transforming growth factor (TGF)-β family is a group of structurally related, multifunctional growth factors, or ligands that are crucially involved in the development, regulation, and maintenance of animal tissues. In humans, the family counts over 33 members. These secreted ligands typically form multimeric complexes with two type I and two type II receptors to activate one of two distinct signal transduction branches. A striking feature of the family is its promiscuity, *i.e.*, many ligands bind the same receptors and compete with each other for binding to these receptors. Although several explanations for this feature have been considered, its functional significance has remained puzzling. However, several recent reports have promoted the idea that ligand-receptor binding promiscuity and competition are critical features of the TGF-β family that provide an essential regulating function. Namely, they allow a cell to read and process multi-ligand inputs. This capability may be necessary for producing subtle, distinctive, or adaptive responses and, possibly, for facilitating developmental plasticity. Here, we review the molecular basis for ligand competition, with emphasis on molecular structures and binding affinities. We give an overview of methods that were used to establish experimentally ligand competition. Finally, we discuss how the concept of ligand competition may be fundamentally tied to human physiology, disease, and therapy.

1. Introduction

Transforming growth factor (TGF)-β family ligands are a group of structurally related, multifunctional cytokines that are found in all animals. They orchestrate many fundamental embryonic and adult developmental processes, including gastrulation and neurulation in the developing embryo, as well as expansion, homeostasis, and regeneration of adult tissues. Their central importance for animal cell physiology is underscored by their essentiality in both vertebrates and invertebrates [1].

In mammals, the family counts 33 different ligand genes, including TGF-β1-3, after which the family is named, bone morphogenetic proteins (BMP), growth differentiation factors (GDF), activins, nodal, anti-Müllerian hormone (AMH), inhibins and leftys [2]. Their extraordinary degree of molecular conservation and functional preservation across species indicates that there is significant selective pressure to maintain a diversity of ligands and suggest that each ligand has unique and critical biological functions [3].

In general, TGF-β family ligands exert their function by forming a hexameric signaling complex that comprises two type I and two type II TGF-β family receptors. Formation of such a complex results in the activation of SMAD signal transduction pathways [1] (Fig. 1). In this framework, type II receptors activate type I receptors by phosphorylating a glycine/serine-rich "GS domain" near their cytoplasmic kinase domain, which leads to recruitment and phosphorylation of the receptor-activated SMAD transcription factors (R-SMAD) by the type I receptor. Phosphorylated R-SMADs subsequently hetero-oligomerize with SMAD4 and translocate to the nucleus to regulate gene expression. R-SMADs belong to one of two signaling branches: the SMAD1/5/8 branch or the SMAD2/3 branch (Fig. 1A). While SMAD pathways are the principal (canonical) mode by which TGF-β family ligand signals are transduced, activation of non-SMAD pathways by TGF-β family ligands

is also well established. As this subject has recently been reviewed [4], we will only discuss SMAD mediated TGF-β family signaling here.

Although the mechanisms of pathway activation and signal transduction appear to be straightforward and direct, real-life implementation and interpretation of TGF-β family signaling is very complex and subject to many regulatory checkpoints and competing inputs. Thus, whether the SMAD1/5/8 or the SMAD2/3 branch is activated in a cell can depend on many factors (collectively defined as 'context'), including receptor expression and ligand accessibility. For example, when receptor availability is limited, all present ligands that can bind the expressed receptors must compete to form a signaling complex. The binding affinity of ligands to receptors, therefore, will help determine in a complex environment which ligand will signal and, thus, which SMAD branch will be activated.

The large number of ligands stands in stark contrast to the relatively limited number of receptors and an even smaller number of SMAD pathways that mediate their signals in cells. In humans, over 40 ligands (including homodimers and heterodimers) interact with combinations of 7 type I and 5 type II receptors to activate one of two signaling branches mediated by the transcription factors SMAD2/3 or SMAD1/5/8 (Fig. 1B). The difference between ligand number (input) and SMAD transcription factors (output) has long presented a conundrum as to why so many ligands are needed. One hypothesis is that ligands compete for receptor binding and thereby regulate the activity of each other, thus enabling cells to interpret complex information that is perceived as combinations of ligands rather than a single input. This line of thinking is not new but has gained interest through several recently published studies [5–10]. Here, we summarize what is known about ligand binding
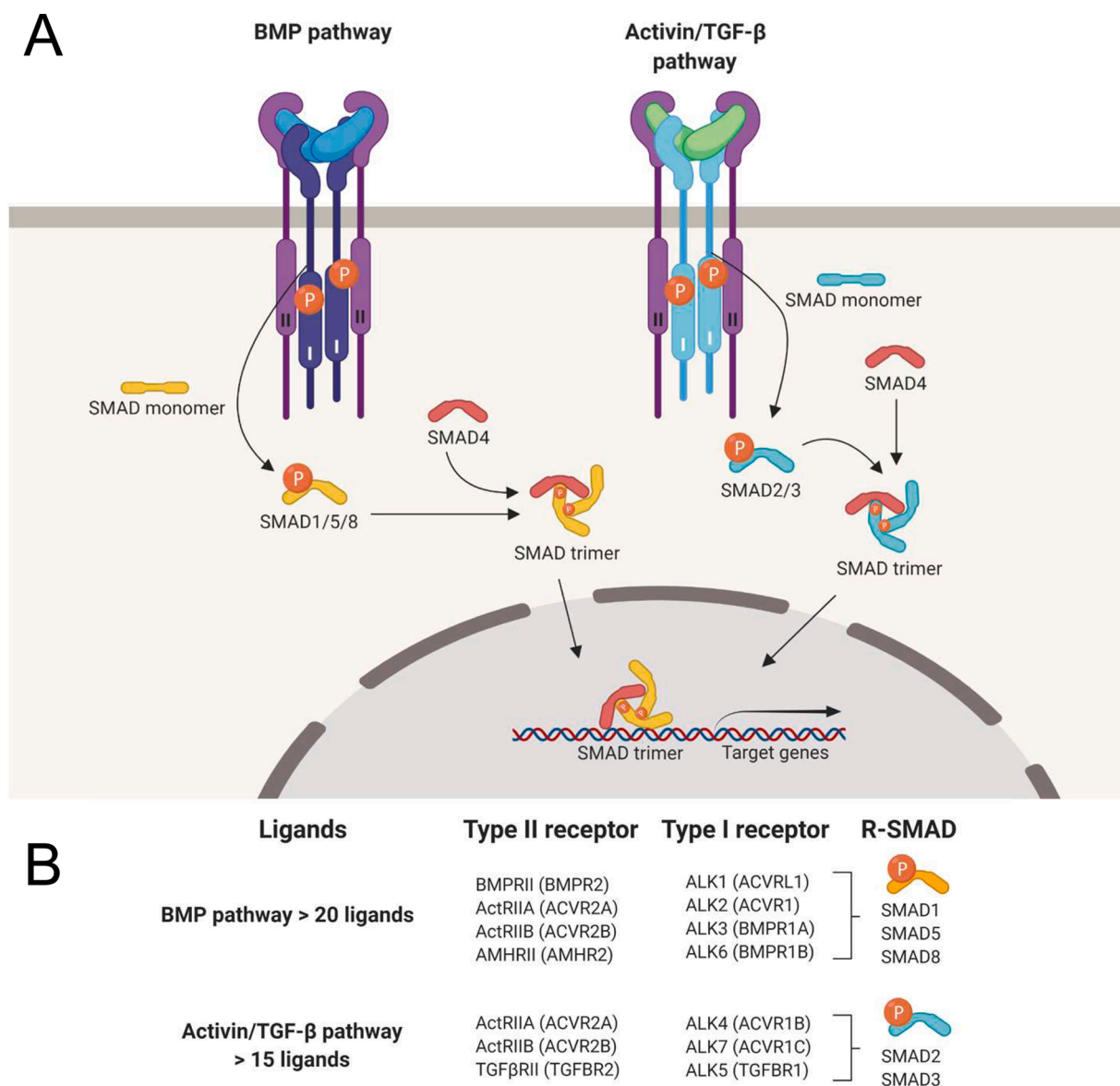

Fig. 1. The TGF-β signaling pathway.
A. Schematic representation of TGF-β family pathway activation and action. Dimeric ligand binding induces assembly of two type I- and two type II-receptors into a hexameric signaling complex, allowing the type II receptors to activate the type I receptors by phosphorylation. The active type I receptors in turn phosphorylate R-SMADs, which then form trimeric complexes with SMAD4, translocate to the nucleus and regulate gene expression. R-SMADs are divided in two functionally different branches, the SMAD1/5/8 branch (a.k.a. "the BMP-pathway"), and the SMAD2/3 branch (a.k.a. "the Activin/TGF-β-pathway"). Ligands on the left side are colored blue, on the right side they are colored green to represent their pathway selectivity. Downstream gene regulation, therefore, depends on which type I receptor forms part of the active signaling complexes. B. The TGF-β/activin and the BMP subfamilies each share a subset of type I receptors and downstream effector SMADs. The family has over 33 ligand genes in humans but only five type II and seven type I receptors, indicating that there is substantial overlap in receptor utilization.

and signaling through specific receptors and we review the current literature on competition between ligands as one out of several ways to regulate physiological and pathological signaling in the TGF-β family. We outline the molecular basis for ligand competition. We review how ligand competition was determined experimentally. Finally, we discuss the relevance of ligand competition for human physiology, disease, and therapy.

2. Ligand structure

TGF-β family ligands are synthesized as precursors. They consist of a signal peptide, a prodomain, and a C-terminal mature domain [11]. Furin-like proteases separate the prodomain from the mature domain

through proteolytic cleavage [12]. Most mature domains form disulfide-linked dimers. However, GDF-3, GDF-9, BMP-15, lefty1, and lefty2 likely form non-covalent dimers as they lack the cysteine involved in the intermolecular disulfide bond [1,13]. The mature domains of some ligands remain non-covalently associated with their prodomains [14] and may be kept in a latent form until activated, while the mature domains of other ligands remain associated with but are not inhibited by their prodomains [15,16].

The dimeric ligands resemble two hands that form a butterfly-like shape (Fig. 2A). This architecture is conserved within the family, even as structural details vary. In the hand analogy, the N-terminus represents the 'thumb', the second loop forming the alpha helix is the 'palm', and the first and third loops, which form the beta-sheet are the 'fingers'. The
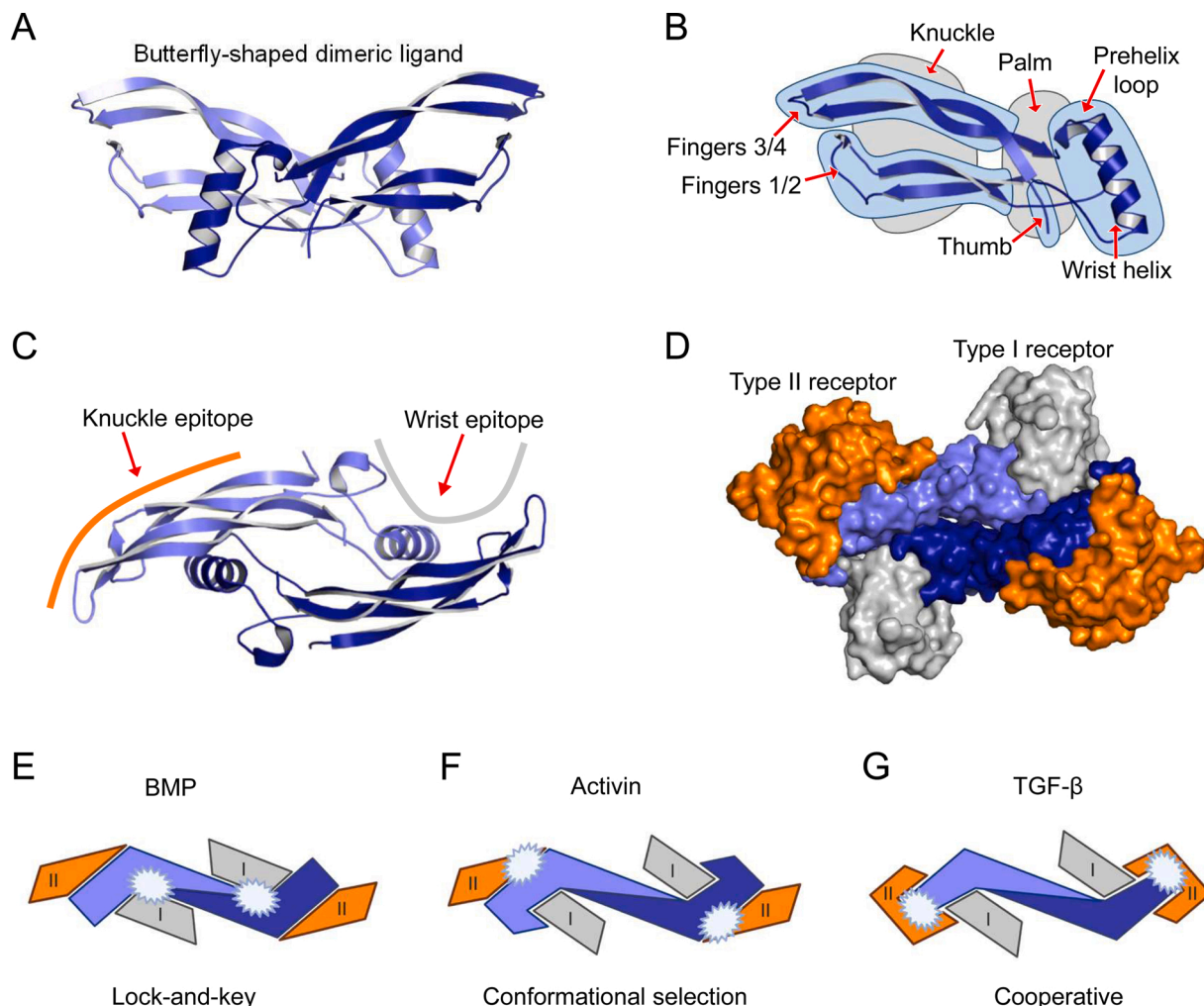

Fig. 2. Ligand architecture exemplified by BMP-9.
A. The butterfly-like structure of mature dimeric BMP-9 is shown. For clarity, each protomer in the mature, dimeric BMP-9 is colored in a different shade of blue. B. The secondary structure of a ligand protomer is frequently described as a left hand. Structural features have been given names that match the left-hand analogy, including knuckle and palm regions (grey highlight), as well as the wrist helix, prehelix loop, thumb, and fingers (blue highlight). C. Mature dimeric BMP-9 is shown rotated by 90 degrees relative to A with a schematic representation of the type I receptor (grey line) and type II receptor (orange line) binding site located, respectively, near the palm and knuckle regions. D. Surface model showing the BMP-9-ALK1-ActRIIB complex. The orientation is the same as C. The palm region in BMP-9 interacts with the type I receptor (ALK1, grey), the knuckle epitope interacts with the type II receptor (ActRIIB, orange). Images were generated using models of BMP-9 (1ZKZ, [153]) and BMP-9 in complex with receptor ectodomains (ECD) (BMP-9/ALK1-ECD/ActRIIB-ECD, 4 FAO [184],). Figures were generated using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.)E-G. Schematic representation of the three proposed modes of ligand-receptor assembly (inspired by [17,18]). The figure illustrates important differences in the contact surface between ligands (monomers, blue and light blue), the type I receptors (grey), and type II receptors (orange) for the three paradigms of ligand-receptor assembly. White stars indicate high-affinity binding surfaces between ligand and receptor. E. BMPs bind receptors via a lock-and-key mechanism where the shapes of the binding interfaces in receptors and ligands fit well. The BMPs bind the type II receptors via the knuckle epitope, whereas binding to the type I receptors happens at the concave dimer interface of the ligand and is largely dependent on the ligand pre-helix loop. F. In contrast to BMPs, activins bind type II receptors with high affinity but still position the type II receptors like BMPs at the ligand knuckle epitope. Also, in this case, the type I receptor binds at the concave dimer interface of the ligand. G. TGF-β utilizes a cooperative mode of ligand-receptor assembly, whereby the type II receptor is bound at the ligand fingertip, enabling binding of the type I receptor, ALK5.

'knuckle' epitope is the region between the fingertips and the palm (Fig. 2B). The loop that precedes the alpha helix or palm is called the 'prehelix loop' (Fig. 2B) and is part of the 'wrist' epitope. Ligands interact with type I receptors via their 'wrist' epitope, and BMPs, GDFs, and activins interact with type II receptors via the 'knuckle' epitope (Fig. 2C, D). [1]

3. Ligand-receptor interaction

Ligands can be divided into three groups based on how they associate with their type I and type II receptors [1,17,18]. Most ligands bind one receptor with high affinity, leading to the widely accepted model of sequential signaling complex assembly. Generally, TGF-βs, activins, and some GDFs bind their type II receptors with high affinity and their type I receptors with low affinity. These ligands are therefore believed to form a complex with their type II receptors first, then engage type I receptors. Members of the BMP/GDF family, including BMP-2 and BMP-4, bind their type I receptors with high affinity and their type II receptors with lower affinity. These ligands therefore presumably interact with their type I receptors first, then engage their type II receptors [19]. Some ligands, including BMP-9 and BMP-10, bind both type I and type II receptors with high affinity [20,21]. These ligands could recruit both types of receptors at the same time. Notably, type I receptors interact with a limited group of ligands, whereas the three type II receptors ActRIIA, ActRIIB, and BMPRII are shared by many ligands of the activin, BMP and GDF families (Table 1).

In the abovementioned butterfly structure model, BMP and activin ligands interact with ActRIIA and ActRIIB via the 'knuckle' epitope (Fig. 2E and F). Two neighboring positions within the knuckle epitope, which contain mostly hydrophobic amino acids, are critical for binding. Although structures of BMPRII-ligand complexes are not yet available, it is suggested that BMPRII binds ligands using a geometry that is similar to ActRIIA and ActRIIB [22]. In contrast to BMPs and activins, TGF-β1-3 have a specific type II receptor, TGFBRII. They do not bind ActRIIA, ActRIIB, or BMPRII as they have large, polar amino acids in key knuckle epitope positions. Instead, TGF-βs bind TGFBRII via their fingertips, which contain a unique structure that is not found in other ligands (Fig. 2G) [23]. The fingertip-binding geometry likely allows TGFBRII and the type I receptor ALK5 to interact cooperatively [23].

4. Pathway activation and intracellular signal transduction

SMAD proteins relay TGF-β family signals inside the cell [24–26]. They are highly conserved among vertebrates and can be divided into three functionally distinct groups: receptor SMADs (R-SMADs: SMAD1/5/8 and SMAD2/3), a common SMAD (SMAD4 in vertebrates), and inhibitory SMADs (I-SMADs: SMAD6 and SMAD7). R-SMADs and SMAD4 have two MAD-homology domains (MH1 and MH2), whereas I-SMADs only have one (MH2) [27]. As SMADs contact DNA with their MH1 domain [28], only R-SMADs and SMAD4 are directly involved in regulating gene expression. However, I-SMADs modulate TGF-β family signaling by, *e.g.*, blocking the R-SMAD interaction with type I receptors [29].

Binding of a ligand to two type I and type II receptors initiates TGF-β family signaling [30,31]. Specifically, formation of a ligand-receptor signaling complex triggers activation of the type I receptors, as type II receptors phosphorylate a regulatory glycine/serine-rich "GS domain" on type I receptors [32]. This alters the binding specificity of the GS domain, reducing its affinity for the inhibitory protein FKBP12, and enhancing recruitment of R-SMADs, which in turn leads to their carboxy-terminal phosphorylation [33–35]. Once C-terminally phosphorylated, R-SMADs dissociate from the receptor complex and associate with SMAD4. The heteromeric R-SMAD-SMAD4 complex then translocates to the nucleus to regulate gene expression [36].

A structure in type I receptors (specifically, the 'L45' loop) likely determines which R-SMADs are recruited to an active signaling complex

Table 1

Interactions between ligands and receptors in the TGF-β family.

Ligand (protein/ gene)	Type II receptors	Type I receptors	References
Inhibin[a]/*INHA*	ActRIIA, ActRIIB	No type 1 receptor	[48]
Activin A[b]/*INHBA*	BMPRII, ActRIIA, ActRIIB	ALK2, ALK4, ALK5, ALK7	[5,59,128,129,130, 131,132,133,134]
Activin B[b]/*INHBB*	BMPRII, ActRIIA, ActRIIB	ALK2, ALK4, ALK5, ALK7	[5,122,131,132,133, 135,136]
Activin C[b]/*INHBC*	ActRIIA, ActRIIB	No type 1 receptor	[49]
Activin E[b]/*INHBE*	N.D.	N.D.	
TGF-β1/*TGFB1*	TGFβRII	ALK5, ALK1	[14,59,129,137,138, 139,140,141]
TGF-β2/*TGFB2*	TGFβRII	ALK5	[14,139,142]
TGF-β3/*TGFB3*	TGFβRII	ALK5, ALK1	[14,139,140,141]
BMP-2/*BMP2*	BMPRII, ActRIIA, ActRIIB	ALK2, ALK3, ALK6	[134,143,144,145, 146]
BMP-3/*BMP3*	ActRIIB	No type 1 receptor	[51,147]
BMP-4/*BMP4*	BMPRII, ActRIIB	ALK3, ALK6	[5,146,148,149]
BMP-5/*BMP5*	N.D.	ALK2, ALK3, ALK6	[120,150,151]
BMP-6 (VGR1)/ *BMP6*	BMPRII, ActRIIA, ActRIIB	ALK2, ALK3, ALK6	[5,143,147]
BMP-7 (OP-1)/ *BMP7*	BMPRII, ActRIIA, ActRIIB	ALK2, ALK3, ALK6	[5,134,144,145,148, 149,152]
BMP-8A (OP-2)/ *BMP8A*	N.D.	N.D.	
BMP-8B (OP-3)/ *BMP8B*	N.D.	N.D.	
BMP-9/*GDF2*	BMPRII, ActRIIA, ActRIIB	ALK1, ALK2	[5,6,20,21,121,153, 154,155]
BMP-10/*BMP10*	BMPRII, ActRIIA, ActRIIB	ALK1-3, ALK6	[5,21,154,155,156, 157]
GDF-1/*GDF1*	ActRIIA, ActRIIB	ALK4, ALK7	[158,159,160]
GDF-3 (VGR2)/ *GDF3*	ActRIIA, ActRIIB	ALK4, ALK7	[158,161,162]
GDF-5 (BMP-14)/ *GDF5*	BMPRII, ActRIIA	ALK3, ALK6	[134,163]
GDF-6 (BMP-13)/ *GDF6*	BMPRII, ActRIIA	ALK3, ALK6	[157,163]
GDF-7 (BMP12)/ *GDF7*	BMPRII, ActRIIA	ALK3, ALK6	[157]
GDF-8 (Myostatin)/ *MSTN*	ActRIIA, ActRIIB	ALK4, ALK5, ALK7	[5,10,131,133,164]
GDF-9/*GDF9*	BMPRII	ALK5, ALK6	[165,166,167]
GDF-9B/*BMP15*	BMPRII	ALK3, ALK6	[168,169,170]
GDF-10 (BMP-3b)/*GDF10*	TGFβRII, ActRIIA	ALK4, ALK5	[171,172]
GDF-11 (BMP-11)/*GDF11*	ActRIIA, ActRIIB	ALK4, ALK5, ALK7	[5,131,133,173,174]
GDF-15 [c]/*GDF15*	No type II receptor[d]	No type I receptor[d]	[126,127]
MIS/*AMH*	AMHRII	ALK2, ALK3, ALK6	[175]
Nodal/*NODAL*	BMPRII, ActRIIA, ActRIIB	ALK4	[5,52,176]
Lefty1/*LEFTY1*	ActRIIA, ActRIIB	No type 1 receptor	[52]
Lefty2/*LEFTY2*	ActRIIA, ActRIIB	No type 1 receptor	[52]

N.D.: not determined, [a]Inhibin A and inhibin B are heterodimers of the inhibin α (*INHA*) monomer and an inhibin β monomer, βA (*INHBA*) or βB (*INHBB*), respectively. [b]The activins are homodimers of inhibin β monomers, but heterodimers of the inhibin β monomers also exist. [c]GDF-15 was recently shown to

signal via GFRAL, an orphan receptor of the glial-derived neurotrophic factor (GDNF) receptor α family and previous reports indicating GDF-15 signaling through TGF-β receptors was likely a result of contaminated protein batches [126,127].

and, thus, are activated by a particular ligand (Fig. 3) [37–39]. Based on this specificity, R-SMADs are grouped into two branches: the SMAD1/5/8 branch (a.k.a. "the BMP-pathway"), which is activated by the type I receptors activin receptor-like kinase (ALK)1, ALK2, ALK3, and ALK6, and the SMAD2/3 branch (a.k.a. "the Activin/TGF-β-pathway"), which is activated by the type I receptors ALK4, ALK5, or ALK7. Significantly, SMAD2/3 and SMAD1/5/8 regulate different target gene sets [24,40]. Which SMAD branch is activated therefore has fundamental consequences for a cell. For example, in myeloma cells, activation of SMAD1/5/8 inhibits cell proliferation and, in many cases lead to cell death. By contrast, activation of SMAD2/3 has little or no effect on myeloma cell survival [6,41,42]. Similarly, SMAD1/5/8 primes 3T3-L1 cells to become adipocytes, whereas SMAD2/3 signaling suppresses 3T3-L1 adipogenesis, possibly by reprogramming these cells toward a different fate [43].

5. Ligand competition

TGF-β family receptors are promiscuous, *i.e.*, they bind two or more different ligands with varying affinities using the same recognition site (Fig. 4A) [1,5,14,19,44,45]. Among type II receptors, ActRIIA and ActRIIB bind the greatest number of ligands, with BMPRII following closely behind (reported ligand-receptor interactions are summarized in Table 1) [5]. As the number of available receptors per cell is likely limited [46,47], ligands that engage the same receptors have to compete with each other for binding to these receptors. One consequence of this condition is that high-affinity ligands can block low-affinity ligands from interacting with a particular receptor and from inducing signaling. Established ligand-receptor-competition examples are listed in Table 2.

At the same time, the competing ligand may activate its specific R-SMAD branch. For example, activin A suppressed SMAD1/5/8 signaling by BMP-2 and BMP-7 while it activated SMAD2/3 signaling, causing a gradual, concentration dependent switch in signal transduction pathway utilization [5]. However, if competing ligands activate the same SMAD branch, they can produce a spectrum of responses that depend on specific ligand groupings, their relative concentrations, and receptor expression levels of a particular cell. For example, BMP-4 exhibited both antagonistic and synergistic interactions with BMP-family ligands. The precise effect depended on the specific ligand with which BMP-4 was

paired [8]. This observation led the authors to propose that the ability to form many competing ligand-receptor complexes with distinct affinities and activities likely enables a cell to process complex information encoded in ligand combinations [8]. We further speculate that the ability to respond to multiple ligands simultaneously or in sequence enables a cell to exhibit input-output plasticity in a fluctuating environment.

6. Antagonistic ligand competition

While ligand binding competition or cross-inhibition is an inherent molecular property of the TGF-β family, the outcomes of this competition vary depending on the specific ligands involved. Some TGF-β family ligands bind receptors but do not activate SMADs. They include inhibin, activin C, BMP-3, lefty1 and lefty2 [48–52]. These ligands may be regarded as true antagonists, as they regulate SMAD signaling either by forming heterodimers with agonistic ligands to produce growth factors that are devoid of signaling activity 50], or by competing with agonistic ligands for receptor binding to block SMAD pathway activation (Fig. 4B, C).

For example, inhibin α and inhibin β are true antagonists. They consist of one inhibin α subunit (encoded by the INHA gene) and one inhibin βA, or βB subunit (encoded by the INHBA and INHBB gene, respectively). Inhibin A, like activin A, binds ActRIIA and ActRIIB. They, therefore, compete for binding to these receptors. But in contrast to activin A, inhibin A does not recruit the type I receptor ALK4 into the ligand-receptor complex, thus acting as a true antagonist of activin A signaling [53]. The antagonistic relationship between inhibins and activins is essential to the control of pituitary follicle-stimulating hormone release and for normal gonadal function [48]. Inhibin A also suppressed signaling by several BMPs/GDFs. This inhibition was associated with its interaction with ActRIIA, ActRIIB, or BMPRII [54].

Activin C is another antagonistic ligand as it blunted the growth inhibitory effect of activin A in LNCaP prostate cancer cells [55], and suppressed the growth-promoting effect of both activin A and activin B in COV434 granulosa cells. Its antagonistic effect was proposed to be dependent on type 2 receptor binding [49]. Supporting this conclusion, SMAD2 activity decreased when activin C was overexpressed *in vivo* [55].

BMP-3 is also considered to be an antagonistic ligand, as it has no known type I receptor but can compete with activin and BMP-4 for binding to ActRIIB [51,56]. BMP-3 may also act as a general BMP antagonist as it also inhibits signaling by other BMPs [8].

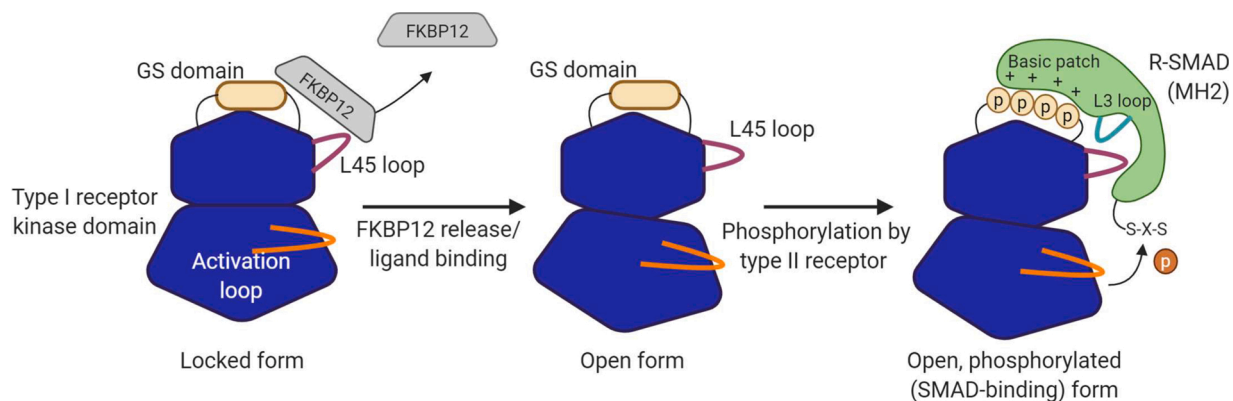Lefty1 and lefty2 are unusual ligands in that they share a uniquely

Fig. 3. Activation of type I receptor kinase and R-SMADs.
Type I receptor activation is inhibited by the protein FKBP12 (left panel). FKBP12 binds the GS domain of type I receptors and prevents its phosphorylation and activation. FKBP12 is released upon ligand binding, or by the macrolide antibiotic FK506. FKBP12 release makes the GS domain available for phosphorylation by type II receptors (middle panel). The phosphorylated GS domain interacts with a basic patch near the L3 loop in the R-SMAD (right panel). The interaction between the L45 loop in the type I receptor and the L3 loop in R-SMAD mediates specificity. The C-terminal S-X-S motif in the R-SMAD is then phosphorylated by the activated type I receptor kinase.
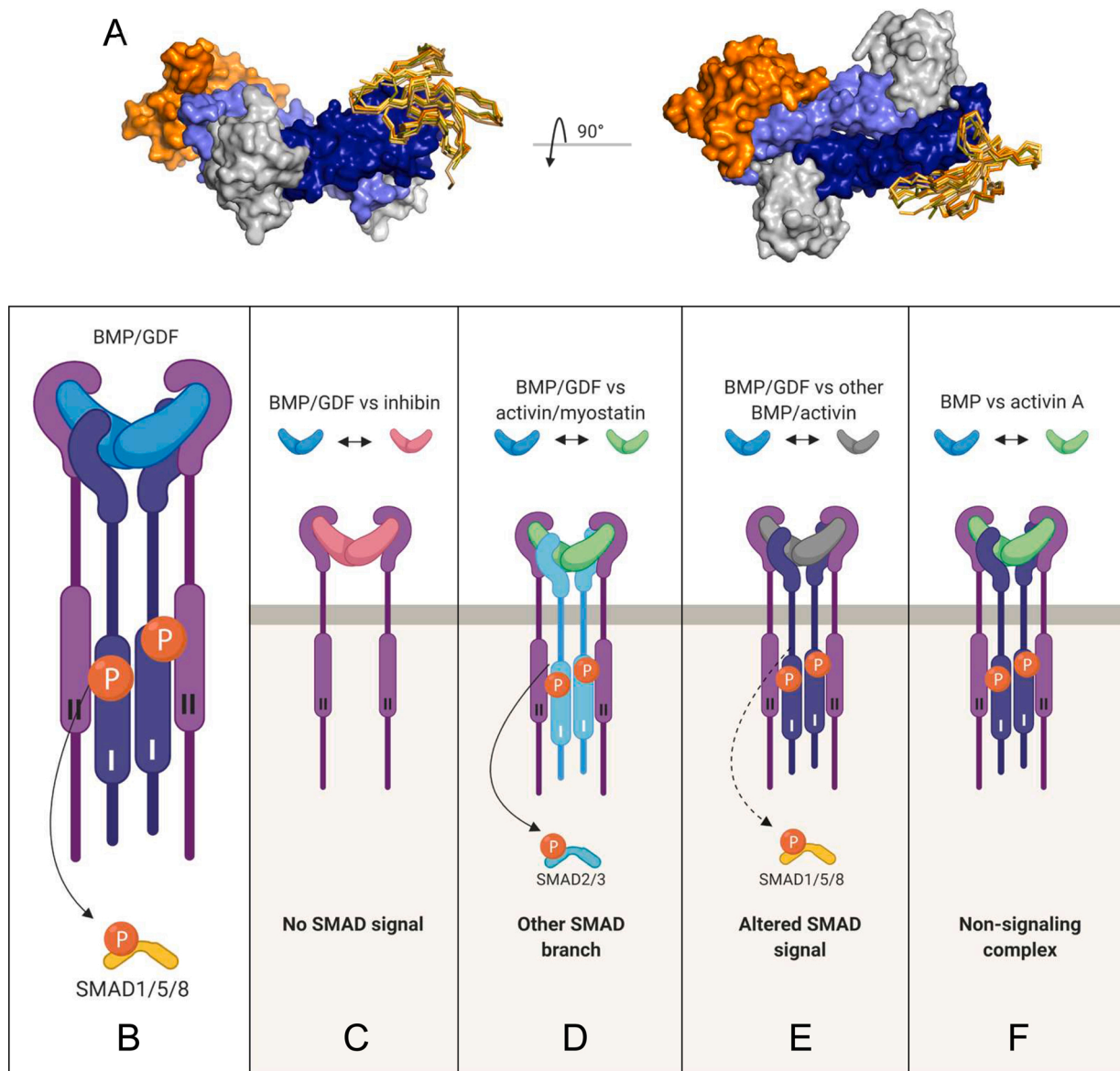
Fig. 4. TGF-β family receptor promiscuity and functional outcomes of ligand competition.

The promiscuous nature of TGF-β ligand-receptor interactions can be visualized by superimposing ligand-receptor complexes. Specifically, the knuckle region of BMP-9 (4FAO [184]) was used as template and other ligand-ActRIIA/B complexes were superimposed, including GDF11 (6MAC, beige [18]), BMP-2 (2H62, bright orange, [44]), Activin A (1NYU, yellow, [18]), and BMP-7 (1LX5, olive, [185]). A. The BMP-9 complex is shown as surface model with BMP-9 colored blue, ALK1 colored grey, and ActRIIB colored orange. The superimposed type II receptor ECDs including ActRIIB in the BMP-9 complex are shown for one protomer as Cα-traces/sticks. The figure to the right represents an orthogonal view of the left image. Ligand competition in the TGF-β family and various possible consequences for R-SMAD-mediated signaling are shown. B. Schematic of canonical BMP/GDF signaling. BMP/GDF (blue) bind type II and type I receptors (light and dark blue, respectively) and activate the SMAD1/5/8 pathway. C. An antagonistic ligand, such as inhibin (red), competes with BMP/GDF for binding to type II receptors but does not recruit type I receptors or activate R-SMAD signaling. Inhibin is an antagonist, as it suppresses BMP/GDF mediated SMAD1/5/8. D. A high affinity ligand, such as activin or myostatin (green), competes for binding to type II receptors and recruits a different type I receptor. Activin/myostatin suppress BMP/GDF mediated SMAD1/5/8 signaling and activate the SMAD2/3 branch causing a switch in signaling pathway use. E. A BMP/GDF ligand (grey) competes with another BMP/GDF (blue) for type II receptor binding and activates SMAD1/5/8 signaling. Different combinations of BMP/GDFs will activate SMAD1/5/8 signaling with distinctive potencies that can be additive, subtractive, or other. F. A high affinity ligand (green), such as activin A, forms a non-signaling complex (NSC) with type II receptors (ActRIIA/B) and the type I receptor ALK2 to inhibit BMP/GDF. Activin A blunts BMP/GDF mediated SMAD1/5/8 signaling in this context.

extended C-terminal sequence, and do not form covalent dimers as they lack the cysteine residue required for dimerization. Nevertheless, they competed with nodal for binding to ActRIIA and ActRIIB and inhibited Nodal signaling [52].

A special example of antagonistic competition may occur when a cell lacks the cognate type I receptor for an agonistic ligand. For example, BMP-10 competes with BMP-2 and BMP-7 for binding to shared type II receptors [5]. However, BMP-10 would fail to activate SMAD1/5/8 signaling in cells that lack its cognate receptor, ALK1. As many cells have

restricted receptor expression, this special example of antagonism could be a prevalent mode of regulation.

7. Agonistic ligand competition

Agonistic ligands, *i.e.* ligands that can activate SMAD pathways, may also compete with other agonistic ligands. Thus, a SMAD2/3-activating ligand could antagonize a SMAD1/5/8-activating ligand (Fig. 4D) to inhibit SMAD1/5/8 signaling. Two examples are GDF-11 and GDF-8

Table 2
Competition for receptor binding between TGF-β family ligands.

	BMP-2	BMP-4	BMP-5	BMP-6	BMP-7	BMP-9	BMP-10	Activin A	Activin B	Nodal	GDF-5
Activin A	ActRII [5,57]			ActRII [6]	ActRII [5, 9]	ActRII [5,6]	ActRII [5]				
Activin B	ActRII [5]				ActRII [5]						
Activin C								ActRII [49,55]	ActRII [49]		
GDF-8	ActRII [5]				ActRIIB [10]						
GDF-11	ActRII [5]	ND [8]	ND [8]	ND [8]	ND [8]						
GDF-3		ND [177,178]									
GDF-5	ALK3 [179], ND [8]	ND [8]			ND [8]						
GDF-6		ND [8]	ND [8]				ND [8]				
GDF-7		ND [8]									
BMP-2		ND [180], ActRIIB [56]				ActRII [5]	ActRII [5], ND [8]	ActRII [5,57]			ALK3 [179], ND [8]
BMP-3	ND [8,181]	ActRIIB [51, 56], ND [8]	ND [8]	ND [8]	ND [8]		ND [8]	ActRIIB [51]			
BMP-3b	ND [172,181]	ND [172]		ND [172]	ND [172]						
BMP-7								ActRIIA [9]			
BMP-8a			ND [8]	ND [8]							
BMP-9	ActRII [5]				ActRII [5]						
BMP-10	ActRII [5], ND [8]	ND [8]			ActRII [5]			ActRII [5]			
Inhibin	ActRII, BMPRII [54]				ActRII [54]	ActRII [54]		ActRII [48,53, 182,183]			ActRII [54]
Lefty1										ActRII [52]	
Lefty2										ActRII [52]	

The table provides an overview of reported competition between TGF-β family ligands, including which receptor(s) the ligands supposedly compete for in cases where this has been investigated. The ligands listed in the first row indicate agonist ligands, whereas the ligands listed in the first column indicate ligands that compete with the agonist ligands by one of our proposed modes of competition illustrated in Fig. 4. In the cells, we have indicated the receptor for which the ligands compete. ActRII: indicates ActRIIA and ActRIIB, ND: Not Determined, indicates competition for an unknown receptor. The table is likely not complete since no single literature search will cover this information.

(myostatin), which activate SMAD2/3 via the type I receptors ALK4, ALK5, or ALK7. Both antagonized the low-affinity ligands BMP-2 and BMP-7 by competing for ActRIIA and ActRIIB and inhibited BMP-2 and BMP-7 mediated SMAD1/5/8 signaling in HepG2 liver carcinoma cells [5]. Likewise, activin A inhibited signaling of several BMPs while activating its canonical SMAD2/3 pathway [5,6,9,57]. Agonistic ligands that bind receptors with high affinity can, therefore, broadly act as antagonists of low-affinity ligands and inhibit their signaling.

In a more subtle scenario, two agonistic ligands may bind the same receptors and activate the same pathway but bind receptors with different affinities and activate signaling with different potencies (Fig. 4E). In that case, ligands pairings could result in stronger or weaker signaling than expected given the responses of the individual ligands. For example, BMP-7 and BMP-4 exhibited synergistic or antagonistic signaling depending on the ligand with which they were paired [8]. Another example is seen in multiple myeloma cells where activin A weakly activates SMAD1/5/8 via ALK2, but at the same time antagonizes a strong BMP-mediated SMAD1/5/8 signaling due to its stronger binding affinity for type II receptors [6,58].

Finally, agonistic ligands could form 'non-signaling' complexes (NSCs) with a particular receptor (Fig. 4F). For example, activin A activates the SMAD2/3 branch via the type I receptor ALK4 [59]. However, activin A may also form an inhibitory NSC with ALK2, as it inhibited BMP-6 and BMP-7 signaling in cells where ALK2 is the main type I receptor [60,61]. Competition in this case involved the ligand interaction with ALK2 and type II receptors. The authors of this work suggested that the NSC could restrict ALK2 and type II receptor

accessibility or prevent engagement of receptors with BMPs to inhibit ALK2-mediated BMP signaling.

In conclusion, the cross-inhibition or receptor binding-competition paradigm offers a unifying molecular mechanism that explains the broadly inhibitory or regulatory action of both agonistic and antagonistic ligands, as well as their role in signaling and non-signaling complexes.

8. Experimental determination of ligand competition

Several approaches have been used to study competition between ligands for receptor binding and downstream signaling. Direct approaches, such as surface plasmon resonance (SPR) or enzyme-linked immunosorbent assay (ELISA), can detect physical interactions between ligands and receptors, providing a molecular readout. Indirect approaches, such as cell-based assays, can be used to examine the biological effects of ligand combinations on signaling, offering a functional readout. Here we discuss how different approaches were used to establish competition between TGF-β family ligands.

In a typical ELISA format, ligands are captured on a plate coated with a ligand-specific antibody. After washing away any unbound substances, ligand binding is detected with an enzyme-conjugated secondary antibody that recognizes a different epitope on the ligand. To investigate receptor binding competition, this format is adjusted so that a ligand is captured with a recombinant Fc-fusion receptor instead of an antibody (Fig. 5A). Importantly, the secondary antibody must recognize an epitope on the ligand that is different from the receptor-binding site. As
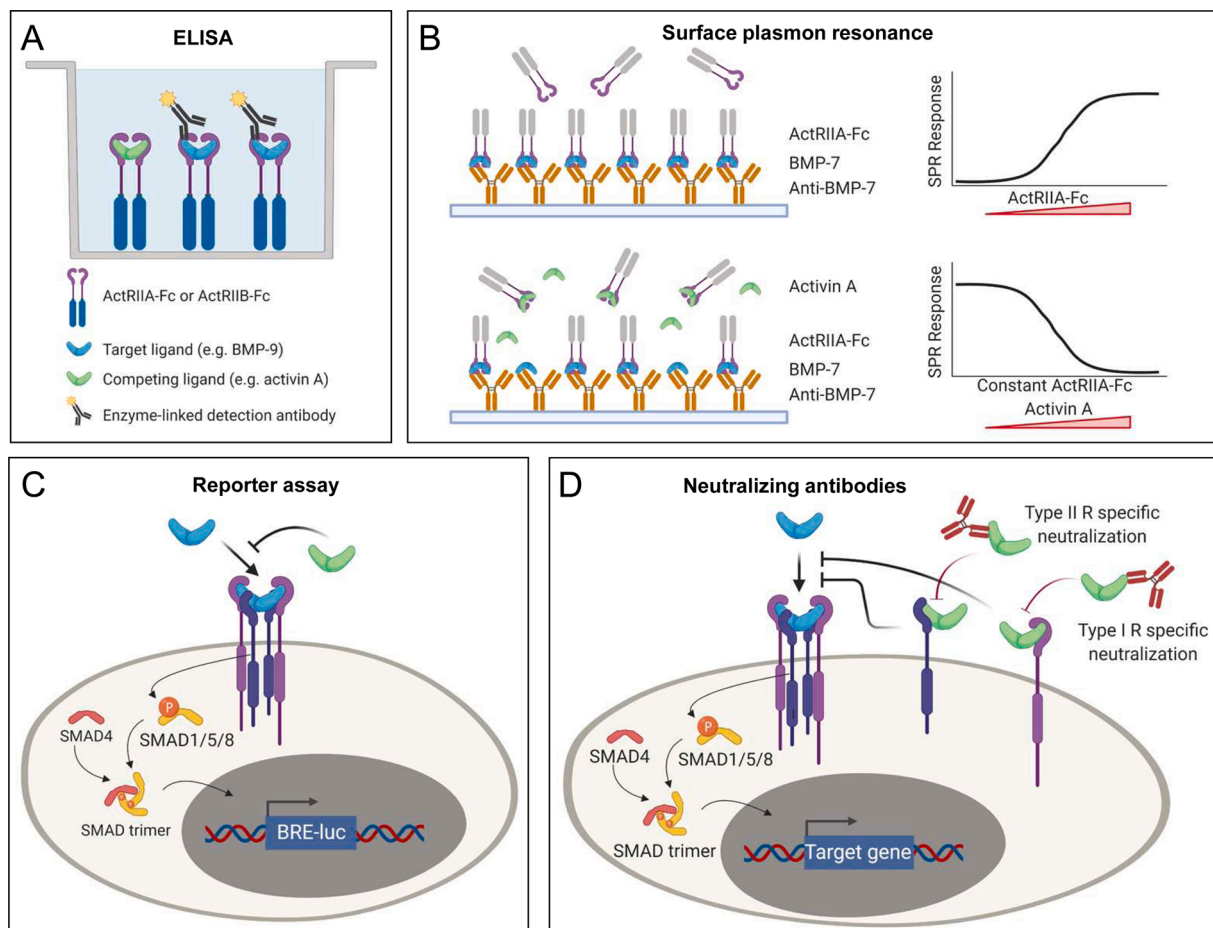
Fig. 5. Methods to measure ligand competition.
A. ELISA measures levels of ligand captured using Fc-fusion receptors (ActRIIA-Fc, ActRIIB-Fc). Binding of ligand (here BMP-9) is detected with a target-ligand specific, enzyme-conjugated antibody (here anti-BMP-9). A competing ligand (here activin A) is not detected by the that antibody. Competition between activin A and BMP-9 therefore presents as an activin A concentration dependent reduction in the target-specific antibody signal. B. Surface plasmon resonance (SPR) can directly measure competition between ligands for binding to a receptor. Here, a BMP-7 antibody that targets the type I receptor binding site is immobilized and BMP-7 is captured by this antibody. ActRIIA-Fc binds to the captured BMP-7. ActRIIA-Fc is incubated with varying concentrations of activin A. Competition between BMP-7 and activin A presents as an activin A concentration dependent decrease in ActRIIA-Fc binding to captured BMP-7. C. Cell-based assays measure the effect of competition on SMAD signaling via SMAD responsive luciferase reporters. Luciferase activity correlates with SMAD activity. Here, competition between BMP-7 and activin A presents as an activin A concentration dependent decrease in the BMP-7 mediated SMAD1/5/8 signal. D. Antibodies and inhibitors that specifically block the type II or type I receptor binding sites help elucidate the molecular basis for ligand competition.

ligand capture levels can be estimated from the intensity of the substrate signal, the changing intensities of the substrate signal can be used to deduce the degree of competition between two ligands or molecules. Of note, this approach is limited to ligands that bind the recombinant Fc-fusion receptor with sufficient affinity to remain bound after washing of the plates. Thus, BMP-9 was captured on an ELISA plate coated with the Fc-fusion receptors ActRIIA-Fc and ActRIIB-Fc. Activin A prevented BMP-9 binding to these receptors in a dose-dependent manner. This inhibition could be blunted by adding the activin A trap Follistatin. Together, these findings showed that activin A competes with BMP-9 for binding to its type II receptors ActRIIA and ActRIIB [6].

SPR can be used to demonstrate the ligand competition directly using several different experimental formats (Fig. 5B). Using the format described in Fig. 5B, the BMP-7 was captured on the sensor chip with a BMP-7-specific monoclonal antibody. ActRIIA-Fc bound the captured BMP-7 in the absence of Activin A. However, ActRIIA-Fc binding to BMP-7 decreased with increasing concentrations of activin A, and was completely suppressed by excess activin A. This experiment directly showed that activin A blocks BMP-7 binding to its type II receptor ActRIIA [5]. Variations of this experimental format could be used to investigate competitive inhibition for an expanded range of examples

[62].

Like ELISA and SPR, cell-based assays can also be used to measure competition directly, as ligands associate with receptors on the surface of cells. Such measurements could be achieved using radiolabeled ligands along with immunoprecipitation of the receptor [10], or with ligand-specific antibodies and flow cytometry [63]. For example, COS-1 cells transfected with BMP-7 receptors were radiolabeled with [^{125}I] BMP-7. Increasing concentrations of myostatin (GDF-8) reduced [^{125}I] BMP-7 binding to these cells [10], indicating that competition can be measured in a whole-cell context. However, this example probes competition for binding to the cell surface but cannot identify the specific cell-surface molecule for which BMP-7 and GDF-8 competed.

While cell-based assays can directly probe competition, they are best suited to evaluate ligand competition indirectly by detecting SMAD activation, or by analyzing downstream effects that are based on transcriptional responses, including SMAD target gene transcription, reporter gene expression assays (*e.g.*, BRE-Luc for SMAD1/5/8 [64] or CAGA-Luc for SMAD2/3 [65], Fig. 5C), cell viability, proliferation, or differentiation. These approaches support a direct comparison of signal or biological response intensities, enabling examination of the balance in signaling between the two main SMAD branches, a key factor

controlling cell fate decisions. Notably, when measuring effects on transcriptional responses, the read-out could be affected by several independent factors, such as expression levels of the co-transcription factor SMAD4, the inhibitors SMAD6 and SMAD7, or R-SMADs [66].

At the most basic level, indirect cell-based assays can show how the signaling response of one ligand is affected by another ligand. As most activins, BMPs and GDFs use the same type II receptors and frequently share type I receptors for signaling, high-affinity ligands like activin A may be expected to inhibit signaling by ligands that interact with the same receptors with lower affinity. Thus, activin A antagonized BMP-2- and BMP-7 dependent SMAD1/5/8 signaling in HepG2 cells carrying the SMAD1/5/8 responsive BRE reporter [5]. Critically, activin A also activated its canonical SMAD2/3 signaling pathway, revealing the profound biological consequences of the ligand competition paradigm, which can result in both SMAD signaling antagonism and SMAD pathway switching. In another example, activin A blunted BMP-9 induced apoptosis in INA-6 and IH-1 multiple myeloma cells and inhibited BMP-9-induced phosphorylation of SMAD1/5/8 [6], showing both biological and molecular consequences of ligand competition. Activin A also inhibited BMP-6 dependent SMAD1/5/8 phosphorylation, highlighting the broad applicability of ligand competition. A subsequent analysis using reporter cells delineated cellular response profiles to combinations of BMP ligands. This work showed how specific ligand combinations produced distinct, cell-dependent outcomes, thus supporting the conclusion that promiscuous BMP receptor-ligand interactions enable cells to process multi-ligand inputs to produce complex responses that include additive, subtractive, and multiplicative signaling outputs [8]. Notably, receptor expression levels also shaped responses to ligand combinations, indicating how ligands could have distinct roles in different cell types.

A powerful tool to study ligand competition at the cellular level uses receptor or growth factor specific monoclonal antibodies or other inhibitors (Fig. 5D). One example of this approach exploited critical functionalities of the three activin A inhibitors ActRIIA-Fc, Follistatin, and SB-431542 [5]. ActRIIA-Fc and Follistatin trap activin A and block its binding to receptors. By contrast, SB-431542 is a small molecule kinase inhibitor that blocks activin A signaling but does not prevent activin A from binding its receptors [67]. Both ActRIIA-Fc and Follistatin rescued BMP-2 and/or BMP-7 signaling in the presence of activin A whereas SB-431542 did not. Similarly, Follistatin blunted activin A antagonism of BMP-6 and BMP-9 [6]. Collectively, these results support the conclusion that activin A antagonism is a direct consequence of competition for type II (and possibly also type I) receptor binding.

A particularly striking and therapeutically important example of competition and its blockade with monoclonal antibodies was reported in a group of studies that discovered activin A as a key driver in the pathology of Fibrodysplasia Ossificans Progressiva (FOP) [60,61,68]. FOP is a genetic disorder characterized by episodic formation and growth of heterotopic bone in connective tissue. It arises from missense mutations in the kinase domain of the type I receptor ALK2 (which is encoded by ACVR1). In cells that harbor wild-type receptors, activin A antagonizes BMP-6, BMP-7, and BMP-9 signaling by forming an inhibitory, non-signaling complex with the type I receptor ALK2 and the type II receptors ActRIIA and ActRIIB. However, FOP-causing ALK2 variants perceive activin A as an agonist that aberrantly activates the SMAD1/5/8 pathway [60]. To elucidate the molecular basis of activin A antagonism in the context of ALK2 non-signaling complexes, these studies exploited monoclonal antibodies that target the receptor-interacting epitopes of activin A, monoclonal antibodies that target the ligand-binding epitopes of receptors, and the small molecule kinase inhibitor SD208 [6,60,61]. Both REGN2476, an anti-activin A monoclonal antibody that blocks its type II receptor binding site, and H4H10442, an anti-activin A antibody that blocks its type I receptor binding site, blunted activin A antagonism of BMP-7 as evidenced by the rescue of BMP-7 signaling in cells co-treated with activin A and either antibody. By contrast, the anti-ALK4 antibody MAB222 and the small

molecule ALK4/ALK5 inhibitor SD208 inhibited activin A-mediated SMAD2/3 signaling as expected but did not blunt activin A antagonism of BMP-6. Together, these results indicated that extracellular competition for ligand-receptor binding was the key step in activin A dependent antagonism of BMP-6/BMP-7 [61]. Notably, REGN2476 inhibited activin dependent SMAD1/5/8 signaling mediated by ALK2 variants found in FOP and suppressed heterotopic ossification in an animal model of FOP. These insights were effectively translated into the clinic, as data from a phase II trial by Regeneron Pharmaceuticals (NCT03188666) indicates that the anti-activin A antibody REGN2477, which has similar properties to H4H10442, could markedly reduce new abnormal bone formation and flare-ups in FOP patients.

In conclusion, combinations of monoclonal antibodies, extracellular antagonists, and small molecule kinase inhibitors have helped elucidate the molecular logic and biological function of the competition paradigm, firmly establishing cross-inhibition or competition as a common mechanism for modulating TGF-β family signaling activation and regulation (Fig. 5D). These critical insights helped drive the development of a novel, disease-modifying therapy for FOP patients.

9. Physiological and pathological roles of ligand competition

In addition to the now well-established roles for activin A as competitive BMP-6/BMP-7 antagonist in the context of ActRIIA/ActRIIB-ALK2 dependent signaling and its gain of function in ALK2 variants that cause FOP [69], competition between TGF-β family ligands may also have critical physiologic and clinical relevance for bone and muscle development, osteoporosis, sarcopenia, cachexia, pulmonary arterial hypertension, tissue fibrosis, and cancers (recently reviewed [70]) (Fig. 6).

For instance, BMPRII knock-out in bone-forming cells resulted in a high bone mass phenotype [7]. Strikingly, BMPRII ablation blunted activin dependent SMAD2/3 signaling but did not affect BMP dependent SMAD1/5/8 signaling. This observation led the authors to propose that BMPs outcompete activins for binding to the remaining type II receptors ActRIIA and ActRIIB to suppress activin A signaling and increase bone mass in this genetic background. Similarly, several studies showed that BMP signaling controls muscle mass, as BMP-mediated SMAD1/5/8 activation induced muscle growth, whereas its inhibition resulted in muscle atrophy [71,72]. Extracellular myostatin and activin inhibitors both induced BMP dependent SMAD1/5/8 signaling in skeletal muscle and increased muscle mass, supporting the conclusion that myostatin and activin antagonize BMPs in muscles by competing for the same receptors to negatively regulate BMP signaling and muscle mass [73,74]. Collectively, these findings indicate that cross-regulation/crosstalk of BMP and activin/myostatin pathways is fundamentally important for bone and muscle homeostasis and that proper re-balancing of these signals using extracellular antagonists could be exploited to regulate bone and muscle mass.

In addition to these physiologically relevant examples, three examples stand out where TGF-β family ligand binding competition may be a pathology driving factor and/or a critical point for therapeutic intervention, including cachexia, pulmonary arterial hypertension, and multiple myeloma. Cachexia is a wasting syndrome characterized by the progressive loss of adipose and skeletal muscle tissues that affects most advanced cancer patients, and that may be responsible for approximately 20 % of all cancer deaths [75]. Recent findings have implicated activin A in its development and progression, as serum levels correlate with the levels of muscle wasting and mortality in patients with pancreatic ductal adenocarcinoma [76], malignant pleural mesothelioma [77], colorectal, and lung cancers [78]. Also, many well-established animal models of cachexia present elevated activin A levels, and activin A or myostatin overexpression leads to muscle wasting in mice [79–81]. These findings have indicated that activin/myostatin antagonism could have therapeutic potential for treating cancer cachexia and other types of muscle wasting. Indeed, extracellular activin/myostatin inhibitors,
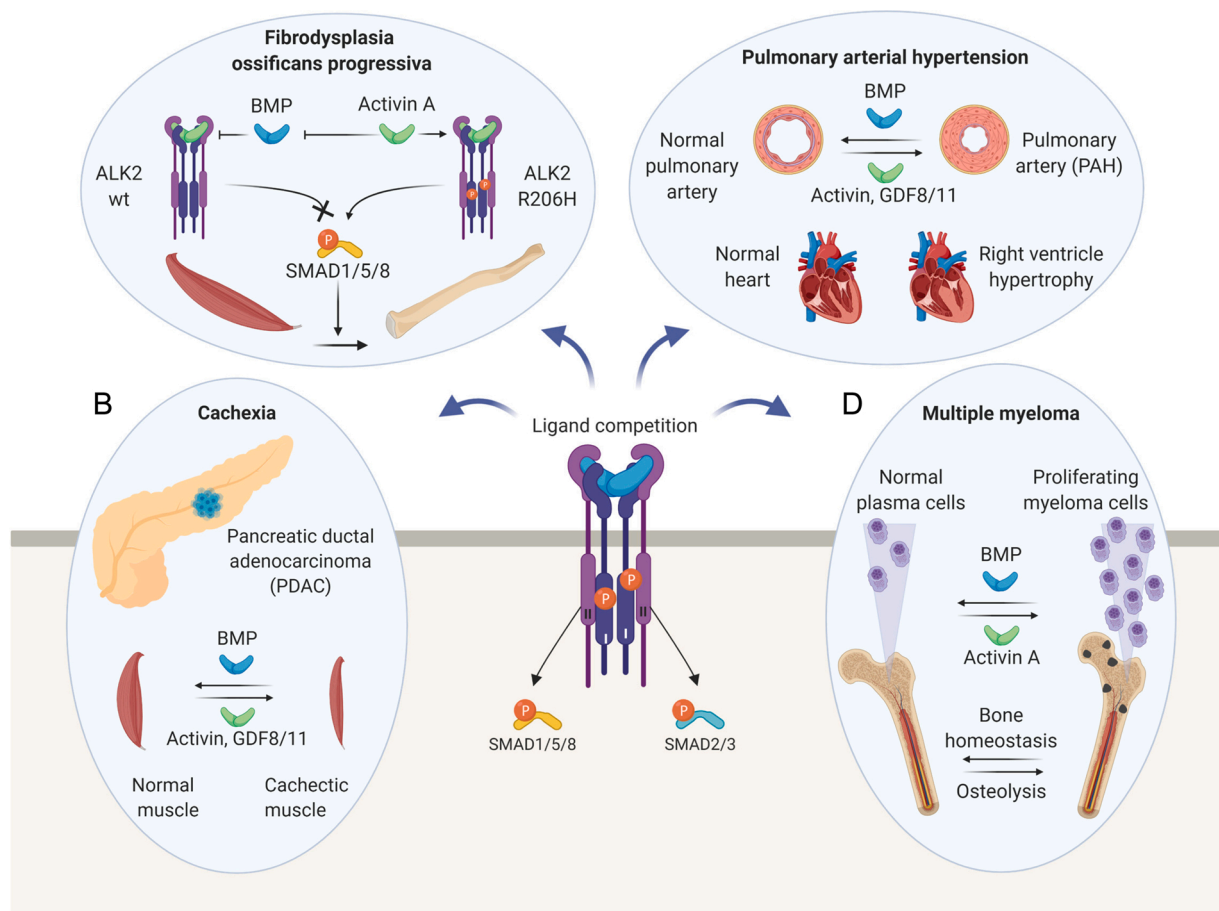
Fig. 6. Proposed pathological roles of ligand competition.
A. Fibrodysplasia Ossificans Progressiva (FOP). In wild-type cells, activin A antagonizes BMP signaling by forming an inhibitory, non-signaling complex with ALK2 and type II receptors. In FOP, disease-causing ALK2 variants perceive activin A as an agonist that aberrantly activates the SMAD1/5/8 pathway, and, thus, induces osteogenic differentiation of fibroadipogenic progenitors to form ectopic bone. B. Wasting Syndrome/Cachexia. BMP signaling is vital for muscle and adipose tissue formation and maintenance. Elevated activin/GDF levels in patients with pancreatic cancers may antagonize BMP-SMAD1/5/8 activity in these tissues, contributing to cancer cachexia. C. Pulmonary Arterial Hypertension (PAH). Deficient BMP-SMAD1/5/8 and increased activin-SMAD2/3 signaling may contribute to endothelial arterial cell proliferation in PAH patients, and, thus, to the narrowing of pulmonary arteries. Increased activin/GDF-11 levels could promote PAH by shifting the balance from SMAD1/5/8 to SMAD2/3 signaling. D. Multiple Myeloma (MM). BMP signaling helps maintain MM cells in a slow-proliferating state. Activin A expression is induced in the bone marrow of MM patients and may contribute to tumor cell expansion by antagonizing BMP activity. Also, activin A may alter bone homeostasis and contribute to MM bone disease by suppressing osteoblastic bone formation and inducing osteoclastic bone destruction.

such as the ligand traps ActRIIA-Fc and ActRIIB-Fc, the antagonist Follistatin, and the ActRIIB neutralizing antibody Bimagrumab, reversed the cachectic phenotype in several animal models, including inhibin-α knockout mice, which develop gonadal tumors, and mice bearing C26 colon carcinoma, G361 melanoma, Lewis lung carcinoma, or TOV21 G ovarian carcinoma xenografts [74,82–86].

Although these results firmly establish that extracellular activin/myostatin antagonism prevents cachexia-induced muscle wasting, thus implicating SMAD2/3 signaling as a key effector of muscle atrophy, recent findings indicate that the mechanism of cachexia pathogenesis and therapeutic action may critically include reduced SMAD1/5/8 signaling and ligand binding competition [87]. For example, studies found that SMAD1/5/8 activation is a fundamental hypertrophic signal in mouse muscles [71,72], that BMP signaling antagonism with the kinase inhibitor LDN-193189 caused significant atrophy of tibialis anterior myofibers [72] and injured shoulder muscles [88], and that overexpression of the extracellular BMP antagonist noggin led to severe muscle wasting in both hyper-muscular $Mstn^{-/-}$ and wild-type mice [72]. These findings reveal the importance of BMP-SMAD1/5/8 signaling in muscle mass maintenance [87]. Strikingly, extracellular activin/myostatin antagonism with Follistatin inhibited SMAD2/3 signaling, as expected, but also activated the SMAD1/5/8 pathway in

muscles of C57Bl/6 mice [73,89]. Also, activin/myostatin antagonism with their pro-domains both inhibited SMAD2/3 and activated SMAD1/5/8 signaling in muscles of C26 tumor-bearing BALB/c mice, leading the authors of this work to speculate that multiple TGF-β family ligands may cooperate to regulate myostatin activity in muscle-wasting pathologies [73,74]. Together, these findings support a model where extracellular ligand competition could be a key principle driving the cachectic phenotype, as elevated levels of the high-affinity ligands activin or myostatin could both activate their canonical SMAD2/3 signaling pathway and suppress SMAD1/5/8 signaling by blocking the binding of BMPs to their cognate receptors and, thus, promote muscle atrophy. Significantly, at least one example showed that extracellular inhibition of these high-affinity ligands rebalanced SMAD pathway activation in mice and reversed the cachectic phenotype [74], an observation that was recapitulated using Follistatin in healthy animals and culture [5,[73]. By contrast, the intracellular activin signaling inhibitor SB431542 did not prevent muscle loss in C26 cachectic animals [90], or rebalance SMAD pathway signaling *in vitro* [5], highlighting a fundamental functional difference between intracellular and extracellular TGF-β family antagonism.

Like cachexia, Pulmonary Arterial Hypertension (PAH) is also driven by a perturbation in SMAD signaling that could be attributed to ligand-

binding competition. PAH is a progressive disorder characterized by elevated blood pressure in the lung arteries [91]. It is caused by the abnormal remodeling of the small peripheral lung vasculature through proliferation, migration, and survival of vascular cells within the pulmonary arterial wall. The resulting vasoconstriction and small lung artery obstruction lead to increased pulmonary arterial pressure, progressive right heart functional decline, and, eventually, right heart failure [92].

The most common causes of hereditary PAH (HPAH) are loss-of-function mutations in the type II receptor BMPRII (*BMPR2*), the type I receptor ALK1 (*ACVRL1*), the co-receptor endoglin (*ENG*), the ligand BMP-9 (*GDF2*), and their intracellular effectors SMAD4 and SMAD8 [93]. The genetics of HPAH, therefore, implicate the BMP-9 signaling axis and deficient SMAD1/5/8 signaling in the pathogenesis of HPAH [94]. However, the penetrance of pathogenic BMPRII variants is low [95], indicating that additional factors are necessary for HPAH onset and progression.

Many studies have also found that inflammation plays a key role in the pathobiology of both HPAH and idiopathic PAH (IPAH) [96,97], potentially linking cytokines secreted by immune cells such as activin A with these conditions [98]. Although activins (and related ligands such as GDF-8 and GDF-11) share type II receptors with BMP-9, they do not intersect directly with the pathway and, thus, have not been considered until recently as drivers of, or targets for treating PAH. Nevertheless, reports showed that activin A levels were increased in the circulation of PAH patients and the lungs of mice with hypoxia-induced pulmonary hypertension (PH) [99]. Also, activin A promoted proliferation of pulmonary artery smooth muscle cells (PASMCs) and its antagonism with a neutralizing antibody inhibited PASMC growth *in vitro* [100].

A recent study further showed that activin A, GDF-8, and GDF-11 levels were elevated in the distal pulmonary arterioles of patients with HPAH and IPAH, as well as in vessels of rat PH models.Activin A and GDF11 also stimulated proliferation in HPAH-patient-derived pulmonary microvascular endothelial cells (PMVECs), and GDF-11 blunted BMP-9 mediated SMAD1/5/8 signaling in these cells [101]. Collectively, these findings indicated that activins and the related GDFs could compete with BMP-9 for binding to surface receptors, causing a signaling imbalance that leads to vascular cell proliferation in PAH [101]. To establish the functional significance of the binding-competition model in PAH, the authors of this work turned to ActRIIA-Fc, an extracellular antagonist that blocks binding of the proposed PAH ligands activin A, GDF-11, and GDF-8 to their type II receptors [101]. ActRIIA-Fc both rescued BMP-9 signaling in the presence of GDF-11, and inhibited activin/GDF-8/-11-mediated proliferation of smooth muscle cells *in vitro* [101], recapitulating a previous finding, which showed that inhibition of BMP-2 and BMP-7 signaling by activin A can be suppressed by trapping activin A with ActRIIA-Fc [5]. Significantly, ActRIIA-Fc blunted pathophysiologic remodeling of the pulmonary vasculature in several experimental models of PH and abrogated the ability of activins, GDF-8 and GDF-11 to compete with BMP-9 for receptor binding to restore BMP-9 signaling *in vivo* [101]. These findings led the authors to propose that activins and/or GDFs are key drivers of PAH, and that receptor binding competition between these ligands and BMP-9 could be a key mechanism underlying PAH pathogenesis [101]. Consistent with a critical role for binding competition, exogenous BMP-9 rescued, while the BMP-9 trap ALK1-Fc exacerbated PAH in animal models [102–104]. Importantly, these new insights have provided a preclinical rationale for the clinical development of ActRIIA-Fc as therapeutic for treating PAH patients. Recent phase II clinical trial results by Acceleron Pharma using ActRIIA-Fc (a.k.a. Sotatercept) now indicate that rebalancing BMPRII signaling by inhibiting activins and GDFs could restore vascular homeostasis in PAH patients (NCT03496207), underscoring the potential impact of the competition paradigm in human diseases and their therapies.

Ligand binding competition also provides a molecular rationale for the role of activin A in multiple myeloma (MM) progression [57]. MM is a malignant disease of the bone marrow that is characterized both by a pathological increase in antibody-producing plasma cells and osteolytic bone destruction [105]. Although a definitive role for activin A in MM has not been firmly established, several groups have reported that elevated activin A blood plasma levels associate with advanced-stage disease, as indicated both by the degrees of plasma cell infiltration of the bone marrow and the levels of osteolysis in patients [106,107]. Mechanistically it is suggested that activin A enhances osteolytic bone destruction in MM patients by magnifying its natural biological role in bone remodeling, which includes activating bone-resorbing osteoclasts and inhibiting differentiation of bone-forming osteoblasts [108]. Supporting this view, ActRIIA-Fc increased bone formation and osteoblast number and reduced osteolytic lesions and the number of tumor cells in two distinct MM mouse models, providing direct evidence that activin antagonism has significant therapeutic potential for treating MM [106, 109].

Most MM cells express TGF-β family receptors and the ligand BMP-6 [63,110,111]. Although these cells do not produce activin A, they induce its secretion from bone marrow stromal cells (BMSC) via adhesion mediated JNK kinase activation [106,112,113]. BMSCs are therefore the likely source of excess activin A in MM patients. Critically, several lines of evidence link activin A and its antagonism of BMP signaling with bone destruction. For example, activin A inhibited osteoblast differentiation *in vitro* as it both activated SMAD2/3 and inhibited SMAD1/5/8 signaling. Also, exposure of BMSCs to MM cell lines or primary cells increased SMAD2/3 phosphorylation and decreased osteoblast differentiation. By contrast, simultaneous treatment with ActRIIA-Fc decreased SMAD2/3 phosphorylation and rescued osteoblast differentiation [106]. Collectively, these findings indicate that activin A could activate its canonical signaling pathway and inhibit BMP mediated SMAD1/5/8 signaling to drive bone destruction in MM patients. Of note, while the role of activin A in MM bone destruction is well-established, a recent study showed that inhibition of the BMP pathway also helped restore bone mass, presumably through a mechanism that is biologically distinct from that seen with activin antagonism [114].

In addition to its role in bone remodeling, activin A may also antagonize BMP signaling in MM cells to promote their proliferation. BMPs are potent inhibitors of the B cell lineage. They regulate B-cell growth and differentiation at multiple stages [115–117] and induce apoptosis via SMAD1/5/8-dependent repression of MYC [42]. Notably, BMP-6 is expressed by many MM cells, and high expression levels predicted improved survival in untreated MM patients [63]. BMP-6 also inhibited the proliferation of MM cell lines and survival of primary MM cells, suggesting that it (and possibly other BMPs) could help keep myeloma cells in a slow or non-proliferative state [63]. Indeed, BMP-4 gene therapy blunted myeloma tumor growth in a humanized myeloma mouse model [118], and several BMPs induced growth arrest and apoptosis in MM cells *in vitro* [42,119–123], underscoring the potential protective role of BMP ligands in MM. As MM cells, BMPs, and activin A are present in the bone marrow, and as activin A is elevated in the bone marrow of MM patients, it was proposed that the growth-inhibitory effect of BMPs on MM cells could be blunted by activin A to promote MM progression. Indeed, activin A antagonized BMP-6 and BMP-9-mediated apoptosis and signaling in INA-6 and IH-1 MM cells and prevented binding of these two ligands to their type II receptors [6]. Similarly, activin A dampened the anti-proliferative activity of wild-type BMP-2 in KMS-12-BM cells, but not of BMP-2 variants that bind type II receptors with activin A-like high affinity [57]. Consequently, these findings indicated that activin A likely promotes MM cell proliferation by competing with BMPs for binding to an overlapping set of cell surface receptors and inhibiting BMP dependent SMAD1/5/8 signaling [6,57].

Thus, activin A could play a dual role in MM progression. On one hand, it may alter bone homeostasis in MM patients by suppressing osteoblastic bone formation and promoting osteoclastic bone

destruction. On the other hand, it may promote MM cell proliferation by blunting the anti-proliferative activities of BMPs. Significantly, both roles are likely rooted in the receptor-binding competition paradigm, as activin A prevents binding of BMPs to their cognate receptors and inhibits activation of BMP mediated SMAD1/5/8 signaling.

10. Concluding remarks

A striking feature of TGF-β signaling pathways is the large number of ligands that can interact promiscuously with a much smaller group of receptors to activate one of two intracellular signal transduction pathways. The biological reason underlying what seems to be a contradictory principle (namely, ligand-receptor binding promiscuity along with signal transduction pathway convergence) has long puzzled. Several models have aimed to explain its purpose. For example, the redundant use of ligands and receptors was suggested to "offer robustness to genetic variation", to permit "subtle forms of control and more flexibility during evolution", or to provide signal processing capabilities that allow a cell to "perceive information encoded in combinations of ligands" [8, 124,125]. In addition to these concepts, recent work has demonstrated that ligand-receptor binding promiscuity, which manifests at the molecular level as competition of ligands for receptor binding, could provide a distinct mode of regulation that includes signaling antagonism or potentiation, and signal transduction pathway switching (see Fig. 4) [5, 6]. The ligand-receptor binding competition (or cross-inhibition) paradigm we discussed here provides a unifying molecular and functional logic that explains the previous models, and the diverse and pleiotropic effects attributed to many ligands in the family. Significantly, the biological impact of competition between ligands is fundamental. To illustrate the potential breadth of this paradigm, we presented several physiological and pathological examples where competition likely takes place, including physiological bone and muscle development, as well as pathological Cancer Cachexia, Pulmonary Arterial Hypertension, Fibrodysplasia Ossificans Progressiva, and Multiple Myeloma. In each one of these examples, the competition paradigm could explain how different ligands balance the activity of each other to produce, modulate, or shift a biological outcome. Critically, receptor-binding competition has been embraced as a possible mechanism underlying FOP and PAH, and its modulation (or rebalancing) has been successfully translated into the clinic with several Phase II trials showing considerable therapeutic potential.

Author contributions

T.H., E.M.-H., and A.S. conceived, wrote and edited the manuscript.

Declaration of Competing Interest

E.M.-H owns shares of Acceleron Pharma and is co-founder of Advertent Biotherapeutics.

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