Opinion

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# Engineering TGF-β superfamily ligands for clinical applications

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**TGF-β superfamily ligands govern normal tissue development and homeostasis, and their dysfunction is a hallmark of many diseases. These ligands are also well defined both structurally and functionally. This review focuses on TGF-β superfamily ligand engineering for therapeutic purposes, in particular for regenerative medicine and musculoskeletal disorders. We describe the key discovery that structure-guided mutation of receptor-binding epitopes, especially swapping of these epitopes between ligands, results in new ligands with unique functional properties that can be harnessed clinically. Given the promising results with prototypical engineered TGF-β superfamily ligands, and the vast number of such molecules that remain to be produced and tested, this strategy is likely to hold great promise for the development of new biologics.**

## TGF-β superfamily ligands as biologics

The pharmaceutical industry is rapidly changing as the cost of developing small molecule drugs increases and new opportunities for the use of biomolecules emerge. It is forecast by the IMS Institute for Healthcare Informatics that pharmaceutical manufacturers will experience slow growth through 2016, while biologic manufacturers will expand and account for 17% of total global spending on medicines by 2016 [1]. This rapid development of biotherapies, including the use of biosimilars and biobetters, represents a paradigm shift in the medical field and reflects recent and rapid advances in our understanding of the structure and function of key biological molecules and signaling pathways involved in diseases and disorders.

The TGF-β superfamily consists of ligands that are found in a diverse range of cell and tissue types where they play ubiquitous roles in a plethora of fundamental cellular events during developmental processes, tissue homeostasis, and disease [2–7]. The TGF-β superfamily consists of 33 members that can be generally divided into TGF-β, bone morphogenetic protein (BMP), growth and differentiation factor (GDF), activin and inhibin, nodal, Müllerian inhibiting substance (MIS), and glial cell line-derived neurotrophic factor (GDNF) subfamilies [3,7]. Effective development of biologics to exploit these natural functionalities depends on a detailed understanding of the relationship between protein structure and function. In the case of the TGF-β superfamily, numerous studies have demonstrated that these ligands share a canonical 'butterfly-shaped' structure (Figure 1A). Although heterodimers have been reported, TGF-β superfamily ligands are generally homodimers in which the two subunits are related by twofold rotational symmetry around the intermolecular disulfide bond through a cystine knot, a hallmark of this ligand family [8–22]. Each subunit consists of a flat, elongated β-sheet structure. Significant structural variations exist between ligands of different subfamilies, as illustrated by the fact that BMPs are more rigid with two spread wings whereas activins are more compact with two flexible wings and a disordered type I receptor-binding loop [9,16]. The functional significance of these variations is currently not well established, although the flexibility of activin appears to play a key role in determining its low affinity for type I receptors and cooperative mode of type I receptor binding in the presence of its high-affinity type II receptors [13].

The TGF-β subfamily has been implicated in many developmental processes [23]. The BMP/GDF subfamily regulates osteo-, chondro-, and tenogenesis, and several other processes [24,25]. Activins and inhibins have been shown to be important in multiple biological processes including testis [26,27] and skin [28] development, wound repair [29,30], and the maintenance of stem cell pluripotency [31]. Dysfunction of TGF-β superfamily ligand signaling can result in multiple pathologies including those related to musculoskeletal and cardiovascular systems [4]. Importantly, aberrant TGF-β signaling plays a major role in various cancers such as colorectal [32], prostate [33,34], breast [35–38], ovarian [39], and melanoma [40]. Given the broad significance of TGF-β signaling, directed targeting of this pathway is likely to have broad therapeutic potential in what are generally referred to as target-directed therapeutics.

In this review we discuss structure-guided design and potential therapeutic applications of engineered TGF-β superfamily ligands. We highlight recent progress with specific designer chimeras of TGF-β superfamily ligands that may be particularly useful in regenerative medicine approaches and the treatment of musculoskeletal disorders. However, this approach provides a means to design and create a vast number of designer chimeras with wide-ranging signaling activities that can function as biobetters

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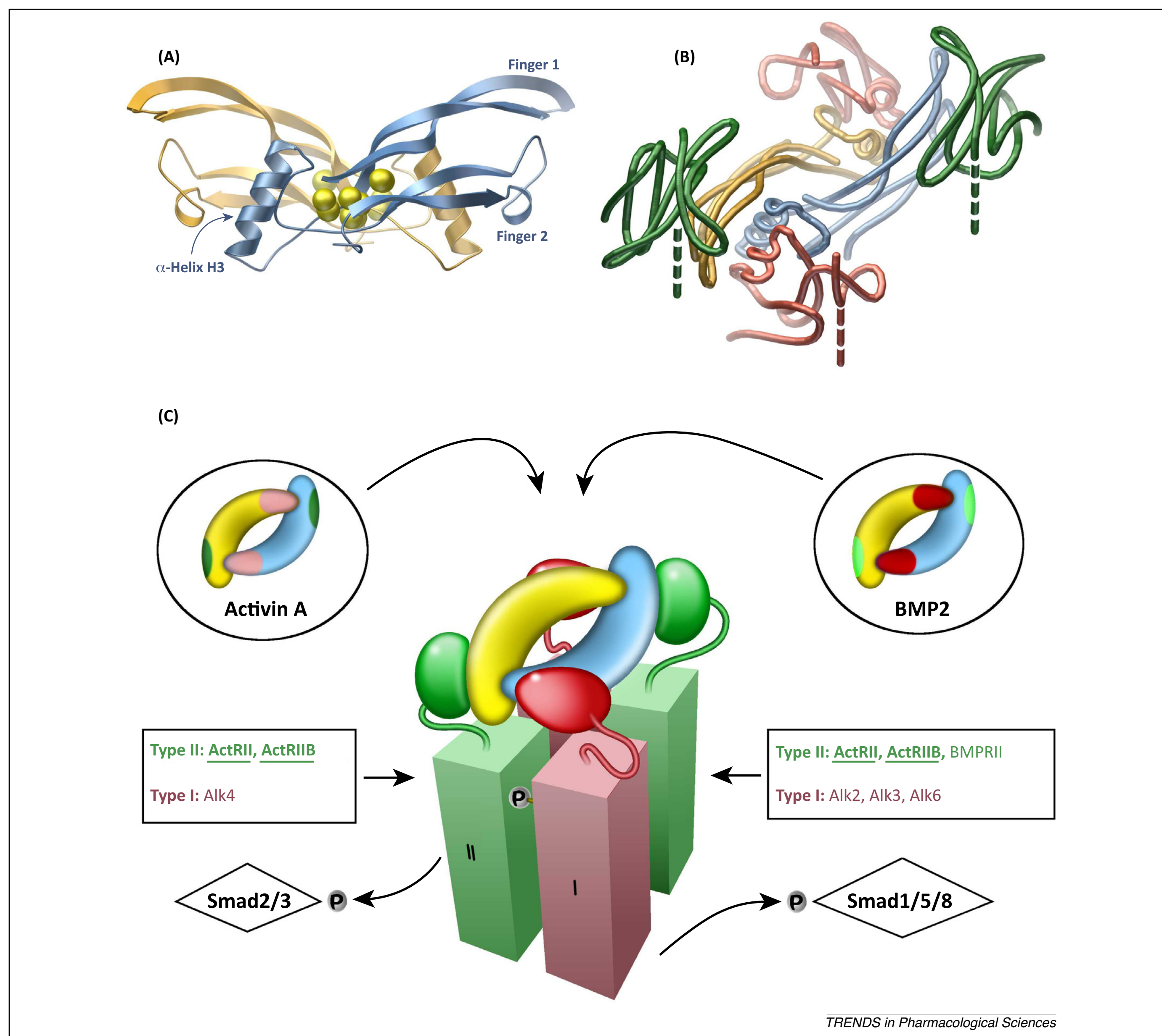


**Figure 1**. TGF-β superfamily ligand structure and mode of receptor assembly. **(A)** Ribbon diagram of BMP2 illustrating that TGF-β superfamily ligands have a 'butterfly'-like structure. One monomer of the BMP2 homodimer is shaded yellow and the other is shaded blue; the cysteines forming the disulfide bonds linking the monomers are depicted by yellow spheres. **(B)** The structure of BMP2 (yellow and blue monomers) bound to its type I (BMPRIa/ALK3, red) and type II (ActRII, green) extracellular domains. As with other TGF-β superfamily ligands, the BMP2 dimer assembles two type I and two type II signaling receptors. **(C)** BMP2 and the related TGF-β superfamily ligand, activin A, assemble type I and type II receptors in a similar manner and share type II signaling receptors (i.e., ActRII and ActRIIB). However, BMP2 utilizes the type I receptors ALK2, 3, and 6 and signals via Smads 1, 5, and 8, while activin A signals via the type I receptor ALK4 and Smads 2 and 3. BMP2 and activin A are depicted in cartoon form with one monomer being yellow and the other monomer blue, and with the type I receptor-binding epitopes shaded red and the type II receptor-binding epitopes shaded green (light shading, lower affinity; dark shading, higher affinity).

for the treatment of human diseases including fibrosis, diabetes, and cancer.

## TGF-β superfamily ligands are amenable to therapeutic protein engineering

In addition to their biological importance and functional variety, the chemical stability and relatively easy production of TGF-β superfamily ligands make them attractive candidates as biologics. However, to control the activity of engineered TGF-β superfamily ligands they must be carefully tailored in a manner that will require a detailed understanding of the structure/function relationships of the natural ligands from which they are derived.

TGF-β superfamily ligands signal by assembling two type II and two type I transmembrane serine kinase receptors into hexameric ligand–receptor signaling complexes (Figure 1B,C). Ligand-induced receptor assembly allows the type II receptor kinase to phosphorylate and activate the type I receptor kinase, which in turn initiates downstream signaling by phosphorylating cytoplasmic Smad (from SMA, small body size; and MAD, mothers against decapentaplegic) proteins (Figure 1C) [2,41–43]. In general, TGF-β superfamily ligands can be divided into two major groups defined by the two separate Smad pathways. Activins, nodal, and TGF-βs signal predominantly through Smad2 and Smad3 [44,45] (Figure 1C left),

whereas BMPs and GDFs generally signal via Smads 1, 5, and 8 [42] (Figure 1C right). This signaling is also regulated in a ligand-dependent manner by a variety of secreted ligand traps such as follistatin and noggin, and by coreceptors including betaglycan (TGF-βRIII) and cripto [43].

TGF-β superfamily ligands share a common structural scaffold but are distinguished by their various affinities for type I and type II signaling receptors. There are disproportionately more TGF-β superfamily ligands (33 in human) than receptors (seven type I and five type II). Therefore, individual affinities between ligands and receptors are as crucial as their binding specificities for appropriate receptor assembly and signaling outcome. An H3 helix and pre-helix loop of the ligand constitute the type I receptor-binding epitope, while the wing tips constitute the type II receptor-binding epitopes [8] (Figure 1). The mechanism of receptor assembly by BMP/GDF subfamily ligands can be inferred from the ternary complex of BMP2 with the extracellular domains (ECDs) of its type II and type I receptors (Figure 1B). This shows that BMP2 does not undergo significant conformational changes upon binding to its receptors, and that the receptor ECDs do not interact with each other [46]. Conversely, the X-ray crystal structure of TGF-β3 bound to its type I receptor Alk5 (TGF-βRI) and type II receptor TGF-βRII ECDs indicates a different mode of receptor assembly by the ligand in which the two types of receptor ECDs do interact [47]. The BMP7/ActRII complex structure [48] shows that BMPs can bind to type I and type II receptor ECDs independently without a change in the shape of the ligand. This again differs from two activin A/ActRIIB ECD structures [12,13] which show that activin is flexible and can undergo significant conformational change upon binding to its high-affinity type II receptor. This flexibility may be necessary for subsequent activin binding to its type I receptor ALK4 [13]; atomic structures of ternary activin/ActRII/ALK4 complex would be instrumental to better understand how activin may interact with receptors during formation of its complex. Even less is known about receptor assembly by nodal and related ligands that require a coreceptor from the epidermal growth factor-cripto/FRL1/cryptic (EGF-CFC) protein family for signaling [49,50]. It is clear that TGF-β superfamily ligands assemble their signaling receptors into active signaling complexes in a manner that highly depends not only on their receptor specificities but also on their respective affinities for these receptors and associated coreceptors.

The type I and type II receptor-binding epitopes on TGF-β superfamily ligands are well characterized and, as mentioned above, many ligand/receptor complexes have been studied in detail structurally. In a simplified view, one can hypothesize that the functionality of a ligand is strongly correlated with its type I receptor-binding epitope because the type I receptor phosphorylates Smad proteins and determines which Smad pathway, Smad2/3 or Smad1/5/8, will be activated. However, manipulating affinities to both type I and type II receptors can change the potency of chimeric ligands. Therefore, by mixing and matching the receptor-binding epitopes for type I and type II, one can design a chimeric ligand for a specific purpose.

## Redesigning TGF-β superfamily ligands opens new therapeutic opportunities

In principle, the formation of heterodimers rather than homodimers represents the simplest way to alter the functionality of TGF-β superfamily ligands, and the rationale for this approach is based on the observation that several ligands that naturally occur as heterodimers have unique properties. For example, BMP4/7, BMP2/7, and BMP2/6 are each more potent in bone and cartilage induction than are the corresponding homodimers [51–54]. It has also been shown that the BMP2/6 heterodimer is a more potent inducer of human embryonic stem cells than is either homodimer [55].

Importantly, structural and functional experiments demonstrate that the binding epitopes for type I and type II receptors within each monomer of TGF-β superfamily ligands are distinct and that they can function independently from one another. Targeting the binding epitopes is therefore an obvious way to alter ligand function. For example, mutations in the binding epitopes created specific agonists or antagonists of BMP2 [56]. In another example, site-directed mutagenesis experiments led to the identification of activin A point mutants with disrupted type I receptor binding but with type II receptor binding indistinguishable from that of wild type activin A [57]. These mutants lacked signaling activity and acted in a dominant negative manner to antagonize activins and other ligands that signal via activin type II receptors [57]. Activin type II receptor antagonists were also generated by replacing a large portion of the type I receptor-binding epitope of activin A with the corresponding region of the biologically inactive activin-C [58]. Chimeric ligands were also generated by swapping type I and type II receptor-binding epitopes between activin A and either BMP2 or BMP7 [59]. Once again, the type II receptor-binding epitope of activin A was unaffected by changes in the type I receptor-binding region. In this case, however, fusing the type I receptor-binding epitopes of BMP2 or BMP7 to the type II binding epitope of activin A caused signaling specificity to switch from Smad2/3 to Smad1/5/8 [59].

In general, the therapeutic potential of these mutant TGF-β ligands remains to be determined using animal models. Nevertheless, the approach provides an important proof of concept that the functional properties of TGF-β superfamily ligands can be altered in a predictable manner to yield mutants with unique properties such as receptor antagonism or switched receptor specificity. Importantly, these studies paved the way for more systematic chimera studies as described below.

## Rationale for random assembly of segmental chimera and heteromers (RASCH)

As mentioned above, the available crystal structures of TGF-β superfamily ligands reveal that they share a similar overall architecture. Each of the two fingers consists of two β- strands that, together with the pre-helical loop and the α-helix H3, make up a total of six structurally distinct segments per monomer. Each ligand can be divided into six segments according to its structure providing the basis for the RASCH strategy (Figure 2) [60]. Through a process of segmental recombination an enormous number of chimeric

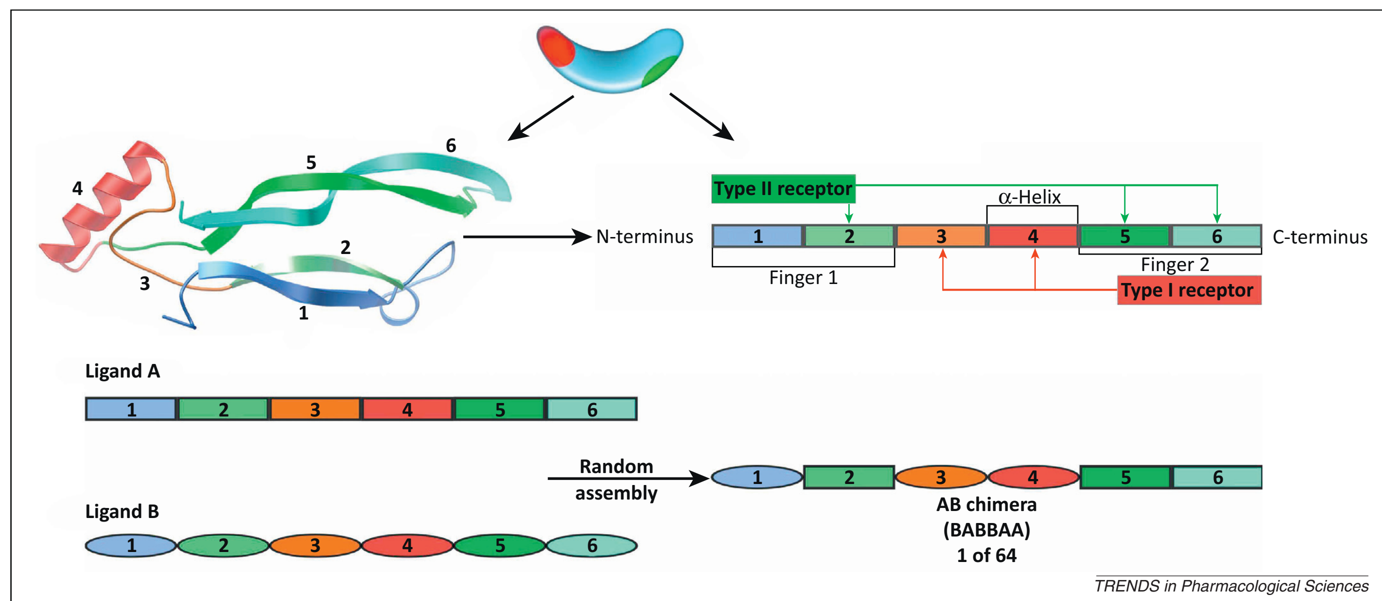


**Figure 2**. Structure-guided generation of TGF-β superfamily ligand chimeras. Based on their crystal structures, TGF-β superfamily ligand sequences can be divided into six structural segments and then mixed in a systematic way as is shown for a BMP2 monomer. This segment-swapping strategy is termed random assembly of segmental chimera and heteromers (RASCH). Segments 2, 5, and 6 constitute the type II receptor-binding epitope of the ligand, while segments 3 and 4 make up the type I receptor-binding site. For two ligands A and B, random assembly of segments yields a total of $2^6 = 64$ distinct AB chimeras, and one of them (BABBAA) is depicted.

ligands can be created when segments are randomly incorporated from all 33 TGF-β superfamily ligands. For instance, $64 = 2^6$ chimeric ligands would be generated if six segments were swapped even between any two ligands. We focus here on two studies in which only pairs of ligands were used for segment swapping.

## Activin A/BMP2 chimeras

Activin A and BMP2 share type II signaling receptors but utilize distinct type I receptors and Smad proteins. The overlapping but divergent characteristics of these ligands prompted us to combine them in a search for novel chimeras with interesting and useful signaling properties. A library of activin A/BMP2 (AB2) chimeras is denoted by the code (BXXXXX) where X is either A (activin A) or B (BMP2). Segments were mixed systematically resulting in $32 = 2^5$ AB2 library chimeras [60]. The first segment is always from BMP2 because chimeras with the first segment derived from activin A cannot be efficiently refolded. There are many interesting properties of such chimeras that can be generalized. As it was hypothesized, the type I receptor-binding epitope defines Smad specificity and thereby determines signaling outcome. Segments 3 and 4 are crucial, therefore, because they define the type I epitope (Figure 2). We find that segmental swapping can produce two key outcomes: (i) when segments 3 and 4 are both from activin (AA) or BMP2 (BB) the resulting chimera will be endowed with activin or BMP2 Smad specificity, respectively (Figure 3D); and (ii) when segments 3 and 4 are mixed (AB or BA) the resulting chimeras will lack type I receptor binding and likely function as antagonists (Figure 3B,C).

In addition, diversity arises from the fact that both parental ligands can bind to the same type II receptors, ActRII/IIB, but with different affinities (Figure 3A). Activin A is known to possess very high affinity for its type II receptors while BMP2 affinity for these receptors is much weaker [60–62]. Therefore, both the activity and antagonistic effect resulting from loss of type I receptor binding are graded depending on the type II receptor-binding affinity. One interesting outcome we observed is the creation of mimics of the parental ligands. One of the chimeras, AB208 (BAAAAA), is an activin A mimic; the fact that AB208 is easy to produce is significant because the parental activin A ligand is hard to make in large quantities as a recombinant protein. The first segment does not participate in receptor binding but rather is crucial for ligand refolding. AB208 contains segments 3 and 4 of activin A and, therefore, signals via Smad2/3. In addition, the affinity of AB208 for activin type II receptors is similar to that of activin A [60], implying that AB208 will be functionally indistinguishable from activin A [60].

## Nodal/BMP2 chimeras

Similarly to activin A, nodal also shares type II receptors with BMP2, suggesting that it would be amenable to the generation of nodal/BMP2 chimeras. Nodal is unusual, however, because it requires a coreceptor such as cripto to bind and assemble its signaling receptors, and it was unclear how this difference would affect chimera behavior. Similarly to the AB2 library, NB2 library chimeras are denoted by the code (XXXXXX), where X is either N (nodal) or B (BMP2), and all $2^6 = 64$ were generated [63]. The basis for the evaluation of proper refolding is the formation of dimeric ligands, and 19 of 32 AB2 ligands and 23 of 64 NB2 chimeras met this criterion. The first BMP2 segment was generally essential for proper refolding of both AB2 and NB2 library chimeras. Only two of 32 chimeras with a nodal segment in their first section produced a dimer, whereas 21 of 32 NB2 chimeras with BMP2 segment in their first section were refolded correctly. Similarly to activin A, recombinant nodal is very hard to produce

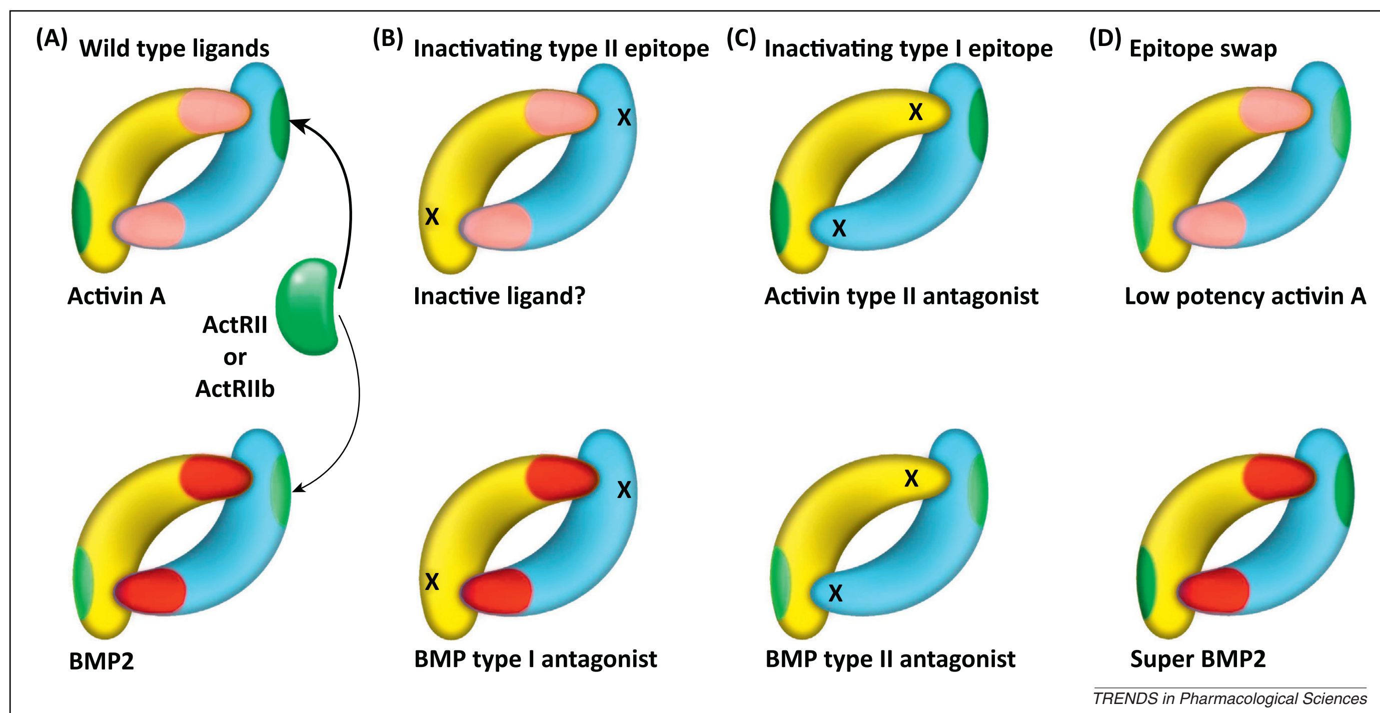


**Figure 3**. Disrupting or swapping receptor-binding epitopes yields TGF-β superfamily ligand chimeras with novel functions. **(A)** Activin A and BMP2 are depicted in cartoon form with their type I receptor and type II receptor epitopes being indicated by red and green shading, respectively (dark shading, higher affinity; light shading, lower affinity). Activin A (high affinity) and BMP2 (low affinity) each bind to the type II receptors ActRII and ActRIIB as indicated. **(B)** Disruption of the type II receptor-binding epitope is predicted to yield an inactive activin A mutant (because activin A lacks type I receptor binding in the absence of type II receptor binding) and a BMP2 mutant that binds to type I receptors but fails to recruit type II receptors, resulting in a dominant negative type I receptor antagonist. **(C)** Selective disruption of the type I receptor-binding epitope in activin A results in a potent inhibitor of activin type II receptors, while loss of the BMP2 type I receptor-binding epitope is similarly predicted to result in lower-potency antagonism of activin type II receptors as well as of BMPRII. **(D)** Swapping the type II receptor-binding epitopes between activin A and BMP2 is predicted to result in a lower-potency activin A and, as we have shown, a BMP2-like ligand with enhanced signaling potency (e.g., AB204).

and purify; we therefore sought a nodal mimic among NB2 ligands. As with AB2 chimeras, we predicted that segments 3 and 4 would dictate type I receptor binding and the signaling specificity of the chimera. However, this could be complicated in the case of nodal and nodal-like NB2 chimeras because a cell surface coreceptor such as cripto is required for their signaling and must also be considered. Given the importance of segments 3 and 4, we tested NB264 (BNNNNN) and NB263 (BBNNNN). As expected, NB264 was indistinguishable from nodal and caused induction of a Smad2/3-dependent luciferase reporter and Smad2 phosphorylation in the presence but not in the absence of cripto [63]. While NB263 also resembled nodal in the luciferase assay (16 h ligand treatment), it differed from nodal in that it did not cause detectable Smad2 phosphorylation as measured by western blot (30 min ligand treatment) [63]. Therefore, segment 2 may play a role in controlling the signaling pattern of nodal. Of all the successfully refolded NB2 chimeras, only NB250 (BNNNBB), NB260 (BNNNNB), and NB264 (BNNNNN) had a desired pattern of XNNNXX, and all mimicked nodal in the signaling assays [63].

NB250 represents a 'minimalistic' nodal-like chimera in the sense that the three nodal segments it contains (2, 3, and 4) appear to be the minimum number of nodal segments required for nodal-like activity. NB250 and nodal displayed a nearly identical dose–response relationship in a Smad2-responsive luciferase assay [63], and it was found that NB250 induces ectopic expression of *Pitx2* and alters heart looping during the establishment of vertebrate embryonic left–right asymmetry in a manner very similar to that of nodal [63,64]. Interestingly, NB250 has rigid structure and, because it appears to be functionally indistinguishable from nodal, it was hypothesized that nodal itself adopts a BMP2-like fold in complex with signaling receptors and cripto. Structural rigidity of NB250 will likely facilitate structural studies of its various complexes, including the NB250/cripto/ActRII/ALK4 complex [63].

## The role of TGF-β superfamily chimeras in osteogenesis

Osteogenesis plays a key role in bone formation and bone repair, and TGF-β superfamily ligands, especially BMPs, are essential inducers of this process [65,66]. Two BMPs are currently available for clinical use for bone healing: BMP2 (rhBMP2, Medtronic, USA) and BMP7 (osteogenic protein-1, Stryker, UK). Numerous studies have reported the use of BMP2 in craniofacial, periodontal [67,68], and orthopedic procedures in humans [69,70]. BMP2 has been approved for clinical use; however, its off-label use in a variety of bone repair processes has raised concerns and side effects have been reported [71]. To heal bone defects, BMP2 is administered in high quantities [70,72], but such doses of BMP2 are at the same time associated with undesirable side effects such as cyst-like bone formation and abnormal soft tissue swelling [73,74]. Therefore, BMP2 mimics with higher therapeutic potency are needed.

AB204 (BABBAA) has segments 3 and 4 of BMP2 and retains BMP2-like signaling through the Smad1/5/8

pathway [60,75]. AB204 has a much higher signaling potency than BMP2 because it possesses the high-affinity type II receptor-binding epitope from activin A (Figure 3D). Consistently, both the level and duration of Smad1/5/8 phosphorylation are greater when pre-osteoblasts are treated with AB204 than with BMP2 [75]. AB204 is also superior to BMP2 in causing mineral calcium nodule formation, an important and pivotal step in bone apatite formation [75]. The activin A sequence in AB204 was predicted to render this chimera insensitive to the BMP antagonist noggin [60], and this was reflected by the fact that while noggin strongly downregulates the mineralization function of BMP2 it had no effect on AB204 function in this capacity [75]. Unlike BMP2, AB204 also potently inhibits activin signaling owing to competition for activin type II receptor binding [75]. Because activin antagonism can stimulate bone formation [76–80], this provides yet another possible mechanism whereby AB204 may promote bone growth and healing. Indeed, we find that AB204 heals critical size defects (CSD) in mice tibia and calvaria, and does so much more effectively than BMP2. Strikingly, 1 month of AB204 treatment completely heals tibial and calvarial defects of critical size in mice at a concentration 10-fold lower than a dose of rhBMP2 that only partially heals the defect [75].

The crystal structure of AB204 indicates that its overall architecture is similar to that of BMPs, despite the fact that AB204 shares half of its amino acid sequence with activin A, including parts of finger 1 and finger 2 [75]. The sequence of the AB204 type I receptor-binding epitope is mostly, although not entirely, derived from BMP2. This is predicted to affect AB204 function because the structure of the BMP2/BMPRIa/ActRII complex shows that there is an important contact between finger 2 of BMP2 and the BMPRIa ECD that is lacking between AB204 and BMPRIa. Specifically, Tyr103 of BMP2 within finger 2 creates a strong hydrogen bond to Asp84 of BMPRIa that is aided by additional Π stacking interactions between Tyr103 of BMP2 and Phe85 of BMPRIa. AB204 possesses an activin A-derived Ile at position 103, and therefore lacks these interactions with BMPR1a. However, we find that the affinity of AB204 for BMPRIa is increased to BMP2-like levels when the Ile at position 103 is replaced with a Tyr [AB204, $K_D$ = 170 nM; AB204 (I103Y), $K_D$ = 6.6 nM; BMP2, $K_D$ = 5 nM], while its affinity for ActRII remains unchanged [75]. Furthermore, the potency of this site-directed point mutant, AB204 (I103Y), also referred to as AB204.1, is significantly higher than that of AB204 in a BMP-responsive luciferase assay with $EC_{50}$s of 2 nM and 9 nM, respectively. Importantly, AB204.1 is significantly more potent than AB204 (and BMP2) in promoting osteogenesis *in vitro* [75].

## The role of TGF-β superfamily chimeras in chondrogenesis

Chondrogenesis is an essential process in cartilage formation. However, cartilage has a very limited regeneration capability because, unlike bone, cartilage is not vascularized [81]. Nevertheless, similarly to osteogenesis, chondrogenesis is largely governed by TGF-β superfamily ligands (Figure 4) [82–87]. Both osteocytes and chondrocytes can be differentiated from pluripotent stem cells (PSC) *in vitro* using a two-step process [88]. In the first step, PSCs differentiate toward a mesenchymal stem cell (MSC) phenotype following treatment with decreasing concentrations of activin A for the first three days and then subsequent blockade of activin A signaling with its antagonist follistatin from days 4–7. In the second step, the MSCs differentiate to chondrocytes [88].

In an approach that measures differentiation of adipose-derived MSCs into a 3D pellet of chondrocytes in a single step [89], we find that the activin A-mimicking AB208 chimera is not effective, whereas the AB208 mutant AB235, nodal, and the nodal-mimicking NB250 are all strongly chondrogenic [63,90]. AB235 is a derivative of

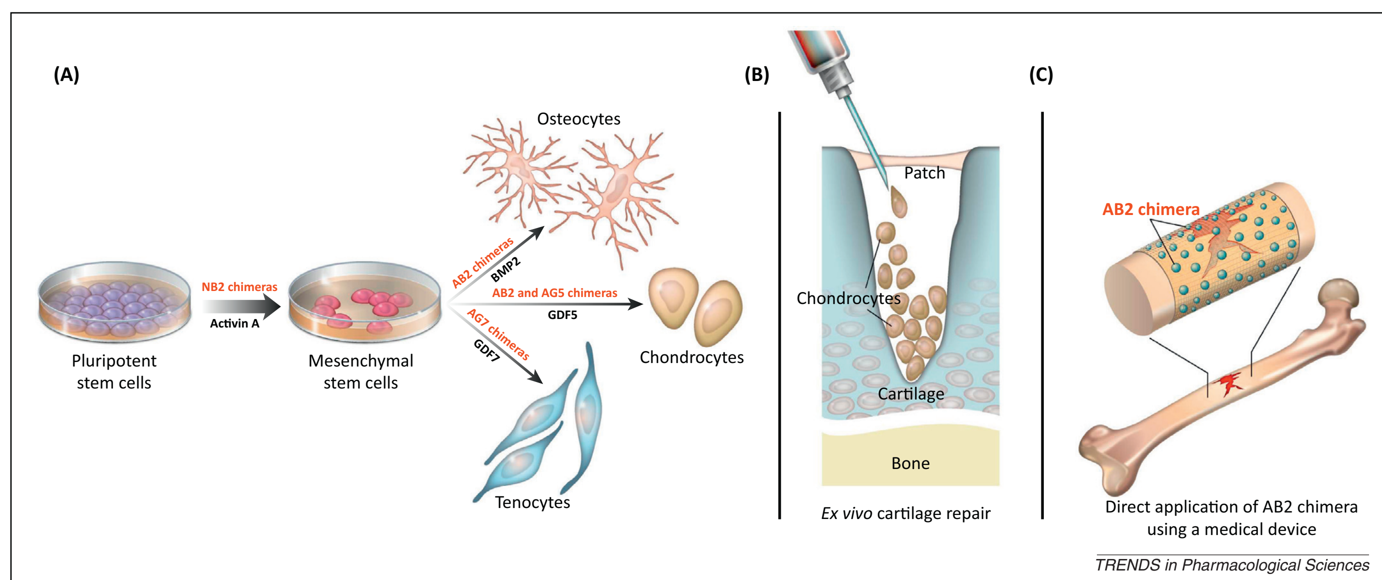


**Figure 4**. Medical applications of chimeric TGF-β superfamily ligands. One potential use of novel chimeric TGF-β superfamily ligands is in the treatment of bone, cartilage, and tendon injuries. **(A)** We propose that replacement of key TGF-β superfamily members commonly used for each stage of *in vitro* differentiation or for *in vivo* therapy (black) with engineered chimeric ligands with superior properties (red) will lead to improved yields of desired cell types and better patient outcomes. Somatic lineages generated in vitro (e.g., chondrocytes) can be used for cell transplantation **(B)** and/or chimeric ligands can be directly delivered *in vivo* to aid in processes including bone healing **(C)**.

AB208 in which the activin A sequence 100-IIKKDIQN-107 in segment 6 that is involved in both type I and type II receptor binding has been replaced with the corresponding BMP2 sequence, 98-VVLKNYQD-105. This mutation was originally designed to reconstitute noggin binding into AB208 [60] but it also includes substitution of lysine at position 102 to leucine that is predicted to disrupt high-affinity AB208 binding to ActRII and thereby diminish activin-like signaling [13,57]. Interestingly, the attenuated activity of AB235 relative to AB208 and activin A more closely resembles nodal and NB250 signaling activity, and this may provide the basis for its increased chondrogenesis.

Notably, despite the broad use of activin A for differentiation of pluripotent cells to mesoderm-derived progenitor cells [88], natural development strongly relies on nodal signaling rather than activin A signaling during mesendodermal commitment across all different vertebrates [49,91,92]. Nodal is a morphogen that not only induces mesendoderm but also appears to pre-specify other somatic lineages derived from the mesoderm and endoderm according to the level of nodal signaling in a cell [49,91]. Moreover, we have observed that the maximal dose-dependent activin A signaling is much stronger than corresponding maximal nodal signaling [93]. The chondrogenesis experiment described above, therefore, supports the hypothesis that nodal-like levels of signaling might be more appropriate for early mesodermal differentiation than higher activin-like levels. Recent experiments also suggest that nodal/GDF1 heterodimers are better than nodal itself in stimulating hESCs into endoderm progenitors [94].

### Therapeutic applications for engineered TGF-β superfamily ligands in regenerative medicine

The incidence of musculoskeletal diseases is sharply rising owing to the fact that individuals are increasingly elderly, sedentary, and obese, particularly in developed countries [95]. Both *in vivo* and *ex vivo* approaches are being used to develop regenerative therapies for the treatment of these diseases, and these include the use of TGF-β superfamily ligands for differentiation of PSCs into specific musculoskeletal lineages (Figure 4).

Engineered TGF-β superfamily ligands have multiple potential benefits for regenerative medicine, including the production of mesoderm-like progenitor cells with varying differentiation potential ranging from unipotency to multipotency. This can predispose the progenitor cells to specific lineages upon terminal differentiation, generate higher-quality cells upon *in vitro* differentiation, and elicit a more robust tissue-repair response *in vivo*. Engineered TGF-β superfamily ligands may also speed up the kinetics of differentiation, thereby minimizing the risk of unwanted mutations. Finally, they should allow administration of more potent ligands at lower concentrations, therefore representing a safer venue for human treatment (Figure 4).

As outlined above, our data show that simple swapping of receptor-binding epitopes can be an effective step in initial ligand construction for a particular medical purpose. Furthermore, we have provided evidence suggesting that this approach can be complemented by structure-guided mutations of first-generation chimeras to further enhance their properties.

As a general strategy, we propose a two-step process for engineering TGF-β ligands for therapeutic applications. In the first step, swapping receptor-binding epitopes ligands can yield desired properties, as illustrated by AB204. In the second step, incorporation of structure-guided mutations can further improve ligand function, as we have demonstrated with the AB204 (I103Y) mutant that we named AB204.1. Such a two-step approach should be generally applicable to other TGF-β superfamily ligands. For example, we predict that musculoskeletal disorders can also be treated by increasing the chondrogenic properties of GDF5 by replacing its low-affinity type II receptor-binding epitope with that of activin A, thereby maximizing its type II receptor binding (Figure 4A). In addition, the structure of the GDF5/BMPRIa complex [21] indicates that the affinity of GDF5 for BMPRIa and BMPRIb can be switched and adjusted to maximize ligand-induced chondrogenesis or *ex vivo* regenerative medicine directed to cartilage repair (Figure 4B).

### Other disease applications

We predict that chimeric TGF-β superfamily ligands will also have applications in diseases including cancer, fibrosis, and diabetes. The roles of TGF-β superfamily ligands and pathway modulation in cancer, and methods of targeting their signaling for medical purposes, have been the subject of intense studies [96–101]. TGF-β superfamily ligand signaling can cause potent tumor-suppressive or tumor-promoting effects depending on the tumor type and the stage of tumor progression [102]. For example, BMP2 is reported to suppress the proliferation of MCF7 breast cancer cells [103]. However, in contrast to this anti-oncogenic effect, BMP2 has also been reported to act as a pro-oncogene in breast cancer by promoting cancer cell invasion [104]. Interestingly, AB215 (BABBBA), a chimera with activin-like affinity for the type II receptor ActRII and augmented BMP2-like signaling [60], is able to exert stronger anti-proliferative effects on estrogen receptor α-positive breast cancer cells than BMP2 [105]. Exploration of other ligands and chimeras has also shown that BMP9 and its analogs have very strong anti-proliferative properties in liver carcinomas.

Abnormal formation of excess fibrous connective tissue is related to many diseases involving single or multiple organs, including idiopathic pulmonary fibrosis (IPF), tubulointerstitial fibrosis (kidney), cirrhosis (liver), cardiac fibrosis, and skin fibrosis. Each of these diseases has a different onset, but in each case the TGF-β pathway, particularly TGF-β1, plays a prominent role in inducing fibrotic change [106–111]. Because of the promiscuity between TGF-β superfamily ligands and their receptors, the level of TGF-β1 signaling can be tempered with other TGF-β superfamily ligands. It has been shown, for example, that attenuation of phosphorylation mediated by BMPs by the BMP antagonist gremlin overexpressed in fibroblasts in lungs of IPF patients may lead to TGF-β1-induced epithelial–mesenchymal transition (EMT) and thus promote fibrosis [112]. It has also been shown that, through activation of the Smad1/5/8 pathway, BMP-7 suppresses

TGF-β1-induced nuclear accumulation of Smad3 [113], leading to the discovery that BMP-7 can antagonize hepatic fibrosis in rats [109]. It is therefore likely that antagonizing TGF-β1 signaling, and possibly doing so by exploiting other TGF-β superfamily ligands such as BMPs, will play an important role in fibrosis treatment. Using natural ligands for this purpose, however, may alter their normal essential physiological processes in a deleterious manner. In this case, engineered ligands designed to have maximal therapeutic activity and minimal side effects may provide a unique therapeutic opportunity in the treatment of fibrosis.

Type 1 diabetes mellitus is caused by autoimmune destruction of insulin-producing β cells in the pancreas. Currently there is no cure for type I diabetes, and patients require constant insulin intake. Insulin independence can be achieved by the transplantation of pancreatic islet-like cell aggregates (ICAs) [114]. However, because donor tissue is not readily available, the production of pancreatic ICAs by differentiating autologous adipose-derived stem cells (ASCs) [115] could be an excellent alternative in *ex vivo* cell replacement therapy. Natural TGF-β superfamily ligands have been employed in this process but often have limited capabilities to promote efficient differentiation of ASCs to ICAs. Alternatively, chimeric TGF-β ligands can be designed with enhanced and directed cell differentiation activity. The potential of using the chimeras to drive cell differentiation was demonstrated in a pilot experiment showing that AB204 and AB211 from the AB2 library are equal to or better than BMP4 in driving cord blood-derived iPS to hemangioblasts. This finding is remarkable because neither activin A nor BMP2 promotes the differentiation of iPS to hemangioblasts, and both AB204 and AB211 are distinct from BMP4.

## Concluding remarks

Although small-molecule inhibitors of TGF-β receptor kinases [97,116], antisense oligonucleotides and antisense RNA [117], monoclonal antibodies [118], and ligand traps [119–122] are in various stages of development [123], therapeutic modalities directly targeting the TGF-β pathway have yet to emerge in the clinic. Because TGF-β signaling is driven by ligand/receptor affinities and specificities, it is not simple to address how a subset of ligands with overlapping use of signaling receptors gives rise to diverse signaling outcomes in a given cell. We also do not currently have a clear quantitative understanding of such mechanisms, and detailed knowledge of the intercellular interplay of ligand signaling and crosstalk with other signaling pathways is lacking. Therefore, using a hit-and-miss approach to target the TGF-β pathway is an oversimplified approach to the overly complicated problem, and will likely result in side effects that outweigh the desired end result.

We contend that engineered TGF-β superfamily ligands have the potential to redirect particular TGF-β pathways for medical purposes. Such designer ligands can be developed to target specified receptors and to be free of complex regulatory and counter-regulatory mechanisms. For example, AB204, AB211, and AB215 augment BMP signaling but are noggin-insensitive [60]. AB215 is also a potent activin inhibitor [105]. Inactivating its BMP-like type I binding site by set of specific point mutations can, therefore, provide a ligand whose only function will be to block the signaling of ligands, such as activin, that require activin type II receptors. Such simplified function might be very useful in pharmacology.

Designer chimeras are also excellent tools for the study of the mechanistic aspects of signaling. They can act as interfering probes that disrupt natural cell-to-cell signaling and help in deciphering the signaling synchronization mechanism during tissue development. As demonstrated above, the use of chimeras is particularly exciting for its potential use *ex vivo* for tissue engineering including bone and cartilage production [63,90,124–127]. Libraries of designer chimeras with various new functions can easily be tested in *ex vivo* settings where side effects are not an impediment to the rational development of regenerative cell therapeutics.

There are many illnesses, cancer and developmental disorders included, that are related to dysfunctional signaling pathways. After almost a century of intense research, the cure for cancer is not a near prospect and new aging-related developmental disorders are rapidly rising. Correcting dysfunctional signaling pathways in disease may require a set of very specific biobetters either as therapeutics or as tools for *ex vivo* conditioning of stem cells. It is in this context that we envisage engineered TGF-β superfamily ligand chimeras having an important place as one arrow in the quiver of future pharmaceuticals.

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