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TGFβ as a gatekeeper of BMP action in the developing growth plate

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Abstract

The ligands that comprise the Transforming Growth Factor β superfamily highly govern the development of the embryonic growth plate. Members of this superfamily activate canonical TGFβ and/or BMP (Bone Morphogenetic Protein) signaling pathways. How these pathways interact with one another is an area of active investigation. These two signaling pathways have been described to negatively regulate one another through crosstalk involving Smad proteins, the primary intracellular effectors of canonical signaling. More recently, a mechanism for regulation of the BMP pathway through TGFβ and BMP receptor interactions has been described. Here in this review, we demonstrate examples of how TGFβ is a gatekeeper of BMP action in the developing growth plate at both the receptor and transcriptional levels.

Keywords

Growth plate; cartilage; TGF β; BMP; Receptor interaction

1. Introduction

BMP pathways control nearly every aspect of chondrogenesis [1–6]. Thus, understanding how BMP and TGFβ pathways intersect is fundamental to understanding the mechanisms controlling cartilage formation and maintenance. The Transforming Growth Factor beta (TGFβ) superfamily is comprised of approximately 30 secreted ligands, 7 type I receptors, 5 Type II receptors, and 8 SMAD proteins [7]. In brief, BMP and TGFβ signaling pathways are activated upon ligand binding to their cognate TGFβ/BMP Type I and II kinase receptors [2,7,8]. Ligand binding enables formation of complexes of Type I and II Serine/Threonine kinase receptors on the cell surface and subsequent Type I receptor phosphorylation and activation (Fig. 1). Through phosphorylation, the type I receptor kinase activates R-

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Smads2/3 for TGFβ and R-Smadsi/5/8 for BMP. Whether TGFβ or BMP signaling is activated is determined by the identity of the type I receptor, which exhibits specificity for either Smads 2/3 or Smads 1/5/8. These effectors complex with Smad4 and translocate to the nucleus where they bind directly or indirectly to DNA to modulate expression of target genes [9–11]. This form of activation is known as the canonical signaling pathway. In addition, TGFβs/BMPs signal through a variety of noncanonical or Smad-independent avenues, utilizing MAP kinases, TAK1, RhoA, and mTOR pathways [8,12–17].

2. Growth plate cartilage formation

The skeleton is composed primarily of cartilage and bone. Throughout the axial and appendicular skeleton, with the exception of the skull, the skeleton is formed from a hyaline cartilage template. During development, mesenchymal stem cells from sclerotome, paraxial mesoderm, and neural crest, condense and undergo chondrogenesis to form a cartilage model (anlagen), which is later replaced by mineralized bone [18,19]. Differentiating chondrocytes are organized into growth plates comprised of different layers, including resting, columnar, prehypertrophic, and hypertrophic zones. The pool of resting chondrocytes at the distal ends of developing long bones supplies cells for building growth plate cartilage. Resting chondrocytes differentiate into columnar chondrocytes, which have a high proliferative rate and stack together into columns. Columnar cells further differentiate into prehypertrophic chondrocytes, which cease proliferation and enlarge. These cells further differentiate into hypertrophic chondrocytes, which have a larger cell size and eventually undergo apoptosis or transdifferentiate into osteoblasts [20,21]. Growth plate cartilage supports bone elongation and provides an essential platform for the generation of articular joints [11,18,22,23]. TGFβs/BMPs play critical roles in regulating chondrocyte differentiation from early to terminal stages, including condensation, proliferation and terminal differentiation [1–6,11,24–28].

Both TGFjS and BMP signaling pathways play essential roles for growth plate development, regulating multiple cellular behaviors such as proliferation, self-renewal, migration, mesenchymal condensation, chondrogenic cell commitment, terminal differentiation, maintenance, senescence, and apoptosis [4–6,25,27,29,30]. Through mouse genetic studies, mutations in several TGFβ/BMP family members, receptors, extracellular modulators, and intracellular transducers, have been reported that cause developmental defects of the cartilaginous skeleton. In this review, we focus on inhibitory crosstalk between BMP and TGFβ pathways via a balance of intracellular effectors (inhibitory Smads 6 and 7) and cell membrane mediated antagonism via TGFβ/BMP-receptor interactions (TGFβBRI and ACVRL1). Considering that modulation of TGFβ and BMP signaling is emerging as a promising therapeutic strategy for joint repair, improving bone mass and quality [1,3,14], these new insights into TGFβ/BMP signaling in cartilage may provide new prospects for generating novel therapies against cartilage diseases.

3. Overview of TGFβ/BMP proteins and receptors

Ligands that activate TGFβ pathways include TGFβs (1, 2, and 3), Activin (A and B), Nodals, GDFs (1, 8, 9, 10 and 11), and Mullerian inhibiting hormone. The bone

morphogenetic protein (BMP) subfamily of ligands consists of BMPs 2, 4–10, and the growth and differentiation factors (GDFs 5, 6 and 7) [1,8,10,24,31–33]. (Fig. 1).

Seven type I receptors have been described [9,31,34]. The BMP subfamily proteins bind ACVRL1 (ALK1), ACVR1 (ALK2), BMPR1A (ALK3), and BMPR1B (ALK6) and signal via the Smads1/5/8 (BMP signaling) [2,10]. The TGFβ subfamily ligands bind to ACVR1B (ALK4), TGFβRI (ALk5), ACVR1C (ALK7) and signal through Smads2/3 (TGFβ signaling) [9,31,35,36]. A closer analysis of receptor structure has revealed that receptors ACVRL1 and ACVR1 are structurally more similar to each other than to BMPRiA and BMPRiB, while BMPRiA and BMPRiB are highly similar to each other [37–39]. The majority of BMP ligands activate multiple receptors, but with varied affinities. BMPs 9 and 10 are among the most specific, in that they bind only to ACVRLi and ACVRi at physiologically relevant concentrations. BMPs 2, 4, and 7 bind and signal through BMPRiA, BMPRiB, and ACVRi, but not ACVRLi [40–43]. The TGFβ receptor TGFβRI, on the other hand, is activated by TGFβs i-3, and GDFs 8-ii; ACVRiB and ACVRiC, are activated by Activins, GDFs 8 and ii [9,31,35,36]. Distinct BMP/ TGFβ ligands thus exert distinct effects via activation of combinations of type I receptors in a concentration- dependent manner, and for this reason, pathway activation is dependent on the levels expression and ratios of both ligands and receptors [44].

There are five type II receptors: TGFβRII, ACTRIIA, ACTRIIB, BMPRII, and MISRII [2,8,10,31,36]. TGFβs i-3 trigger complex formation betweeen TGFβRII and TGFβRI to activate Smad 2/3 signaling. However, TGFβRII/TGFβRI complexes can also interact with ACVRLi and ACVRi in some cell types, enabling activation of BMP Smads 1,5,8 by TGFβ [45,46]. BMPRII complexes with ACVRi, BMPRiA and BMPRiB to activate BMP signaling. ACTRIIA and ACTRIIB are unique in that they can complex with type I TGFβ/ activin receptors TGFβRI, ACVRiB and ACVRiC to activate TGFβ signaling, or with BMP receptors ACVRLi, ACVRi, BMPRiA and BMPRiB to activate BMP signaling [10,29].

4. The function of TGFβ pathways in the growth plate

4.1 TGFβ subfamily proteins

In vitro data demonstrate that TGFβs play important roles in early chondrogenesis to induce mesenchymal cell condensation [47–51]. However, the in vivo roles of TGFβs in chondrogenesis and cartilage development are unclear. TGFβs i, 2 and 3 are expressed in mesenchymal condensations. Levels of expression of all of these ligands are reduced at later stages in cartilage [52,53]. In the perichondrium, TGFβ3 is expressed at higher levels than other TGFβs [52,53]. In appendicular growth plates, TGFβ1 and TGFβ3 are expressed mainly in the proliferative and hypertrophic zones, whereas TGFβ2 is expressed in all zones, but at its highest levels in the hypertrophic zone [54–57].

The expression of TGFβi and 3 in cartilage suggests they may have a role there. However, knockouts for either TGFβi or 3 do not exhibit phenotypes that support an essential role in cartilage. *Tgfbi* null mice that survive to birth do not exhibit any skeletal defects, but instead die from diffuse inflammation [58]. Similarly, while loss of TGFβ3 leads to perinatal lethality and defects in palate formation, defects in chondrogenesis are not observed [59,60].

However, mice lacking TGFβ2 present with generalized chondrodysplasia that appears to have an onset at late gestation stages [61]. The evidence thus suggests that TGFβ2 may be the predominant TGFβ ligand impacting chondrogenesis in vivo. However, the chondrocyte-autonomous function of TGFβ2 is still not known, and a cartilage specific knock-out of *Tgffi2* would be essential to understand its role in cartilage development. Furthermore, cartilage-specific deletion of TGFβs i-3 alone or in combination in adults might reveal functions for these ligands in articular cartilage.

In addition, the TGFβ subfamily proteins GDF 1, 10 and 11 are expressed in both condensed cartilage and mature growth plate cartilage [62,63]. Among them, GDF10 is expressed at the highest level; GDF 1 and 11 are detected at low levels. GDF11 was found to inhibit chondrogenesis in the developing chick limb in ex vivo assays [64]. However, little is known of the roles of Gdf 1, 10 and 11 in growth plate chondrocytes in vivo.

4.2 TGFβRI function in the growth plate

The TGFβ receptor TGFβRI (ALK5) is the only receptor known to be activated by both TGFβs and GDFs. Conditional ablation of TGFβRI using Dermo1-Cre, which is expressed in skeletal progenitor cells prior to condensation, results in cartilage malformation and short limbs [65]. In these mice, progenitor cells condense and chondrocytes proliferate and differentiate, but ectopic cartilaginous tissues protrude into the perichondrium. These protrusions are related to the abnormally thin perichondrial layer and to increased chondrocyte proliferation in the protruded cartilage. However, because Dermo1-Cre is expressed in many mesodermal-derived tissues, whether TGFβRI had a direct function in chondrocytes could not be ascertained. Recently, a cartilage specifically knockout of TGFβRI using Col2-Cre was generated to address this unknown [29].

Loss of TGFβRI *(*$Tgfbri^{Co12}$*)* in committed chondrocytes leads to a lethal chondrodysplasia. The precise cause of death is unknown, but mutants exhibit major defects in both axial and appendicular skeletal elements. Interestingly, these defects are distinct from those seen in *Tgfb2*$^{-/-}$ mice. At E13.5 and E14.5, there are no patterning defects in $Tgfbri^{Co12}$ mice, but mutants have smaller vertebrae and occipital bones. At E16.5, mutants develop abnormal spinal curvature and kinks in the ribs, shortened sternebrae, and shorter limbs. At E18.5, mutants have bent scapulae, kinks at the distal ends of humeri, dislocation of elbow joints, and smaller condyles and entheses of tibiae. The axial phenotype appears to be similar to that in $Tgfbri^{Dermo1}$ mice [65]. Col2Cre is expressed in the sclerotome before the specification of separate lineages for vertebral cartilage and intervertebral discs. Since both Col2Cre and Dermo1Cre target this tissue [66,67],, the axial defects in $Tgfbri^{Co12}$ and $Tgfbri^{Dermo1}$ mice most likely arise through the same mechanism.

$Tgfbri^{Co12}$ mice also develop severe defects in appendicular growth plates at E16.5. TGFβRI expression is low in the growth plate until E16.5. Expression is strong at E16.5 and E18.5 in the columnar zone but is absent from the hypertrophic zone [29]. The appendicular growth plate phenotype of $Tgfbri^{Co12}$ is correlated with increased proliferation of chondrocytes in columnar and resting zones [29], suggesting that loss of TGFβRI leads to shorter limbs by premature conversion of slowly dividing resting cells into rapidly proliferating columnar cells and depletion of the resting zone. The above findings demonstrate an essential function

for TGFβRI, but raise questions regarding the identity of the ligands that mediate the effects of TGFβRI in the growth plate. If TGFβs 1–3 are the ligands that activate TGFβRI in cartilage, the same defects seen in TGFβRI mutants should be observed in the mice lacking these ligands or TGFβRII in cartilage *(Tgfbr2^{Cot2}),* because TGFβRII is the type II receptor for TGFβs 1–3. However, similar defects were not seen in mice lacking any of the TGFβ ligands, or in *Tgfbr2^{Co12}* mice [58–61,68].

Conditional deletion of TGFβRII in committed CoZ2ai-expressing chondrocytes did not lead to obvious defects in appendicular elements [68]. In axial elements, *Tgfbr2^{Co12}* mice develop defective segmentation and malformation of intervertebral discs [68]. Although *Tgfbri^{Co12}* mice exhibit smaller vertebral bodies, they do not exhibit defective segmentation [29]. Other differences between *Tgfbri^{Co12}* and *Tgfbr2^{Co12}* mice can be seen in the condyles of appendicular elements. In *Tgfbri^{Co12}* mice, the posterior tibia condyle is smaller than the anterior tibia condyle. These findings establish TGFβRI as a gene that regulates the differential development of condyles. In *Tgfbr2^{Prx1}* mice, the tibial medial condyle and deltoid tuberosity do not form [69]. However, *Tgfbr2^{Co12}* mice do not show such phenotypes [68] suggesting that the defects in *Tgfbr2^{Prx1}* mice reflect a role for TGFβRII in condensing cells prior to their commitment to chondrocytes. Furthermore, the condyle defects in *Tgfbri^{0012}* mice are distinct from those in *Tgfbr2^{Prx1}* mice [29]. This suggests that TGFβRI regulates posterior and anterior condyle formation in cartilage, but not through TGFβRII mediated TGFβ signaling. Scx+;Sox9+ cells in the TGFβRI-expressing regions of the condyles give rise to ligaments and the ligamentous junction [70]. Future studies would thus be of interest to elucidate the role of TGFβRI in the differential development of condyles, ligaments, and associated structures, and to understand the basis for the differing condylar phenotypes in *Tgfbri^{Co12}* and *Tgfbr2^{Co12}* mice. In summary, given that the same Col2-Cre alleles was used in both studies, these phenotypic differences suggest that TGFβRI and TGFβRII function to some extent in independent signaling complexes.

The in vivo observations discussed above suggest that TGFβRI can transduce its effects independently of TGFβRII in growth plate cartilage. However, there is a possibility that TGFβRI transduces TGFβ signaling independently of TGFβRII. Prior evidence for this came from a study showing that in cranial neural crest cells, TGFβs activate non-canonical TGFβ signaling through TAK1/JNK in the absence of TGFβRII [71]. However, this mechanism cannot account for the differences between *Tgfbri^{Co12}* and *Tgfbr2^{Co12}* mice because analysis of the growth plate cartilage showed that loss of TGFβRI does not change pTAKi activation [29]. Additional genetic evidence that TGFβRI does not mediate its effects via this noncanonical pathway is discussed below.

4.3 TGFβ signaling mediators

The above findings raise the possiblity that TGFβRI utilizes ligands other than TGFβs 1–3 to mediate its effects in the growth plate. GDF1, GDF10, and GDF11 can activate TGFβ signaling through TGFβRI in complexes containing ACTRIIA or ACTRIIB [72–74]. At present, there is no direct evidence for a role for these ligands in the growth plate in vivo.

TGFβs 1–3, GDFs 1, 10, 11, and other cytokines in the TGFβ/activin sub-family act through canonical and non-canonical pathways, and canonical signals are transduced via the

transcriptional regulators Smad2 and Smad3. Once activated, Smads 2/3 complex with Smad4, translocate into the nucleus, and recruit coactivators and repressors to regulate the expression of target genes. Thus, examination of the phenotypes of mice lacking Smads 2 and 3 would reveal a potential role for multiple TGFβ/activin subfamily ligands.

Smad2, Smad3 and Smad4 are co-expressed throughout the growth plate [25,75–78]. Mice with cartilage-specific loss of Smad2 *(Smad2^{Col2}),* global loss of Smad3 (*Smad.3*or both (*Smad2^{Co}*X2; *Smad3*$^{-/-}$) develop a subtle cartilage defect at E18.5 and survive after birth [25]. The defect in growth plate cartilage in single *Smad2^{Co12}* or *Smad3*$^{-/-}$ mutants and in double *Smad2/3* mutant mice is thus very different from the perinatal lethal chondrodyspalsia of *Tgfbri^{Co12}* mice [29]. There are no condyle formation defects in *Smad2, Smad3* and *double Smad2/3* mutant mice, as are found in *Tgfbri^{Co1}* mice. There are no apparent defects in columnar zones in *Smad2, Smad3* and *Smad2/3* mutant mice, but there is a two-fold increase in cell proliferation in this region in *Tgfbri^{0012}* mice. There is an increase in the length of the hypertrophic zone in *Smad2, Smad3* and double *Smad2/3* mutant mice, but not in *Tgfbri^{001}* mice [25,29]. Overall, the far more subtle growth plate defects in *Smad2/3* mutants compared to *TgJbri^{Col2}mice* suggest that the main role of TGFBRI in growth plate is not to transduce canonical TGFβ signaling via Smad2/3.

There are numerous non-canonical mechanisms for transduction of TGFβ signals, including various MAPK, Rho-like GTPase and phosphatidylinositol-3-kinase (PI3K)/AKT pathways [15–17,79]. There is solid evidence that these pathways are important for chondrogenesis, but the extent to which TGFβs mediate their effects through these pathways in cartilage in vivo is unknown [11]. The most extensively studied noncanonical pathways are those mediated by TGFβ activating kinase 1 (TAK1), a member of the MAPKKK family. TAK1 is activated by type I BMP and TGFβ receptors, and subsequently activates several MAP kinases (MAPKs), including p38, JNK, and ERK. TGFβ receptors also activate PI3K and its downstream target AKT. If TGFβRI transduces its effects through these non-canonical pathways, genetic deletion of TAK1 or AKT in cartilage will lead to similar defects as in *TgfbrI* mutant mice. One of the main defects in *Tgfbri^{Co12}* mice is a significant increase of cell proliferation in the columnar zone. However, this defect is not seen in mutant mice with deletion of TAK1 [80], JNKs [81], ERK1 [82] or AKT1 [83]. Furthermore, as discussed above, *Tgfbri*^{Co12}mice do not exhibit obvious changes in levels of activation of TAK1 [29]. These findings suggest that altered signaling through these noncanoical pathways is not responsible for the effects of TGFβRI in the growth plate.

TGFβRI is unique as a type I receptor in that it not only activates canonical TGFβ pathways, but it also enables TGFβs to activate BMP signaling through a mechanism involving association of the BMP receptor ACVRL1 with TGFβRI/TGFβRII complexes [45,84]. TGFβ signaling through ACVRL1/TGFβRI/TGFβRn complexes increases BMP signaling in vascular cells [45]. According to this model, loss of TGFβRI should lead to decreased BMP signaling. Surprisingly, there is dramatically increased BMP signaling in *Tgfbri^{Co12}* growth plates as evidenced by increased pSmad1/5 levels. This suggests that a major function for TGFβRI is to block BMP signaling in the growth plate. Genetic evidence for this is provided by the observation that simultaneous loss of TGFβRI and the BMP receptor ACVRL1 restores BMP signaling to normal and rescues the cell proliferation defect seen in *Tgfbri^{Co12}*

mutant growth plates [29]. This suggests that the main role of TGFβRI in growth plate is not to transduce TGFβ signaling but rather to block BMP signaling. These studies thus identify a novel form of crosstalk and provide genetic evidence for a pathological role for ACVRL1 in the growth plate.

5. The function of BMP pathways in the growth plate

5.1 BMP subfamily proteins

There are more than 15 structurally related BMPs. They are categorized into subgroups based on amino acid or nucleotide similarity, including BMP2/4, BMP5/6/7/8, BMP9/BMP10, and BMP12/13/14 (GDF5/6/7) [85]. In developing limb cartilage, BMP 2, 4, and 7 are expressed in the perichondrium, whereas BMP6 is detected in prehypertrophic and hypertrophic chondrocytes [86–89]. In addition, BMP7 is expressed in chick sternal prehypertrophic and mouse metatarsal proliferating chondrocytes [89,90].

BMPs 2, 4, 5, 6, and 7 promote chondrogenesis [91,92]. In addition, BMPs have a strong effect on chondrocyte proliferation and matrix synthesis. In growth plate chondrocytes BMPs 2, 4 and 5 upregulate cell proliferation and matrix production [93,94]. Little is known about chondrocyte-intrinsic roles for BMP ligands, but chondrocyte-specific deletion of Bmp2 and Bmp4 showed that BMP2, but not BMP4, is a main player for chondrocyte proliferation and maturation during endochondral bone development [95].

BMP9 is among the most osteogenic BMPs, promoting osteoblastic differentiation of mesenchymal stem cells (MSCs) both in vitro and in vivo [96–98]. Its role in growth plate cartilage is not clear. BMP9 is not expressed in cartilage, but is expressed in the liver and the protein is detected in the circulation. BMP9 protein can be detected in the growth plate, indicating that circulating BMP9 penetrates cartilage [29]. In addition, in vitro assays demonstrated that loss of TGFβRI in chondrocytes increases BMP9 activity through ACVRL1, indicating BMP9 activity is negatively regulated by TGFβRI [29]. However, little is known about the role of BMP9 in cartilage development.

GDF5, 6 and 7 activate BMP signaling in vitro [99], but they do not have robust osteogenic activity compared with other BMPs [97,98]. Studies of GDF5 and GDF6 knockout mice and humans bearing mutations in these genes show that they have variety of defects in joint and cartilage formation [24]. GDF5 stimulates mesenchymal condensation and cartilage formation, and organizes joint formation and segmentation events across developing skeletal structures [100,101]. GDF5 promotes cartilage growth in vivo; transgenic mice expressing Gdf5 under the control of a *Coliia2* promoter show extensive cartilage overgrowth and complete absence of joints [102]. GDF6 is not critical for early chondrogenesis, but it promotes cell proliferation in surface cartilage [103]. Moreover, *Gdfs/Gdfô* double knock-out mice develop a more severe skeletal phenotype [103]. Interestingly, mice lacking GDF7 exhibited a defect in hypertophic phase duration opposite to that reported for other GDF5 mutants [104]. While the basis for the differing phenotypes remains unclear, GDF5, 6, and 7 mutant phenotypes confirm an important role for GDF molecules in cartilage growth and joint development.

5.2 BMP Receptors

There are fewer BMP receptors than ligands, and therefore studies of BMP receptor knockout mice could provide a broader picture of the role of BMP signaling. The results suggest that BMP receptors promote chondrocyte proliferation and differentiation. Loss of type 1 receptors ACVRL1, ACVR1, BMPR1A, or BMPR1B leads to no or mild cartilage phenotypes during embryonic development, but combining deletion of two of these type I receptors in cartilage leads to more profound chondrodysplasia. Loss of BMPR1A in chondrocytes leads to decreased cell proliferation, delayed hypertrophy in growth plate cartilage and shortened limbs [6]; global loss of BMPRiB leads to delayed hypertrophy of metacarpal/metatarsal chondrocytes [6]. However, BMPR1A/BMPR1B double mutants display a very severe chondrodysplasia and a lack of endochondral ossification [6]. Loss of ACVRi in cartilage impacts development of axial skeletal elements, but has little consequence in appendicular growth plate cartilage development at embryonic stages [4]. However, loss of both ACVRi and BMPRiA in chondrocytes or loss of ACVRi in chondrocytes on a global BMPRiB knockout background leads to a generalized chondrodysplasia that is more severe than each single mutation alone [4]. This suggests that ACVRi, BMPRiA and BMPRiB have some redundant roles. Recently, it was shown that depletion of ACVRLi in cartilage does not affect embryonic cartilage development [29]. However, since ACVRi is structurally related to ACVRLi, it is possible that ACVRLi and ACVRi have overlapping functions in cartilage such that ACVRi can compensate for the loss of ACVRLi. A combined deletion of ACVRLi and ACVRi in cartilage may help to further understand their roles.

6. TGFβ limits BMP signaling

6.1 TGFβ limits BMP signaling through extracellular and intracellular mechanisms

BMP signaling pathways regulate multiple aspects of endochondral bone formation. The intensity and duration of BMP signaling in the growth plate is regulated extracellularly and intracellularly. One example of extracellular regulation occurs via binding of antagonists, such as noggin, follistatin and chordin, to BMPs, thereby preventing them from binding to or enabling formation of BMP receptor complexes [2,105–109]. Extracellular regulation by the BMP antagonist noggin is required, as noggin-deficient mice exhibit massively enlarged growth plates and joint fusions [105,110]. TGFβ1 induces noggin expression in the growth plate, although not as potently as the BMPs [111]. Noggin functions to limit BMP signaling during the recruitment of progenitor cells into cartilage elements. At later stages Noggin is essential to insulate articular cartilage from BMP signaling that would otherwise promote hypertrophy in this tissue [110].

Activation of both TGFβ and BMP signaling pathways can lead to intracellular antagonism via crosstalk between the R-Smad mediators of TGF-P and BMP signaling. In vivo data demonstrated that <Smad3 can repress Smadi/5/8 activation to prevent chondrocyte hypertrophy [112]. In accordance, there is an increase in the level of pSmadi/5/8 activity with the loss of Smad3. *Smad3*$^{-/-}$ chondrocytes were more responsive to BMP2, exhibiting increased pSmadi/5/8 levels, and BMP-responsive luciferase reporter activity [112]. In vitro assays using MDA-MB-231 breast cancer cell lines showed that TGFβ can also inhibit BMP

responses by inducing the formation of pSmad3-pSmadi/5 complexes, which bind to BMP-responsive elements and mediate TGFβ-induced transcriptional repression [113]. In ATDC5 chondrocytic cells, TGFβ suppresses BMP signaling and chondrocyte hypertrophy via SnoN, a transcriptional corepressor [114]. SnoN is induced by TGFβ signaling in maturing chondrocytes and suppresses the BMP-Smad signaling pathway to inhibit hypertrophic maturation of chondrocytes [114].

Intracellular regulation also occurs, in part, through the actions of inhibitory Smads (I-Smads) 6 and 7. Smad7 can inhibit multiple pathways, including TGFβ/activin and BMP signaling, while Smad6 has been known to inhibit BMP signaling [U5-U7]. I-Smads block the phosphorylation of R-Smads by forming stable associations with activated type I receptors [118–120]. In addition, I-Smads can recruit E3 ubiquitin ligases to type I receptors, leading to ubiquitination and subsequent degradation of these receptors [121–123]. I-Smads can also bind to Smad1, thereby interfering with Smad1-Smad4 complex formation [123–125], Furthermore, I-Smads directly regulate transcription of TGFβ family signaling in the nucleus [126–128]. The expression of I-Smads is directly induced by TGFβ and BMP signaling, thus forming a negative feedback loop [129–131].

In vitro and in vivo studies reveal that (I-Smads 6 and 7 regulate BMP-mediated effects in chondrocytes. Both gain of function and loss of function studies in mice show that they limit BMP signaling in cartilage and are required for proper skeletal development [116,117]. In particular, Smad6, induced by TGFβ1 and BMPs [132] is required for inhibition of endochondral bone formation; *Smad6*$^{-/-}$ mice have abnormal growth plates exhibiting an expanded hypertrophic zone and enhanced expression of Ihh, a factor shown by Seki et al. to be a direct target of canonical BMP pathways [133]. I-Smad7 inhibits both BMP and TGFβ signaling. Smad7 is induced by TGFβ ligands [129,130]. Studies in which Smad7 was overexpressed in chondrocytes demonstrated that Smad7 impacts chondrogenesis by inhibiting BMP signaling [134]. Smad7 is required for both axial and appendicular skeletal development in mice [117]. Loss of Smad7 in chondrocytes resulted in cell cycle impairments and defects in terminal maturation. This phenotype was attributed to upregulation of both BMP and TGFβ signaling.

6.2 TGFβ limits BMP signaling at the receptor level

As discussed above, loss of the TGFβ type I receptor TGFβRI leads to enhanced BMP signaling in the growth plate [29]. Biochemical and genetic studies indicate that this crosstalk occurs at the level of receptor complex formation. Genetic evidence for receptor-level crosstalk came from the finding that growth plate defects in *Tgfbri*^{Co12} mice were rescued by depletion of ACVRL1 in the *Tgfbri* mutant background, indicating that TGFβRI blocks BMP signaling transduced by ACVRL1. Evidence that this rescue is due to alterations in receptor complex formation came from co-immunoprecipitation assays, which confirmed that ACVRL1 complexes with ACTRIIA but not with ACTRIIB in normal chondrocytes. However, loss of TGFβRI increases ACVRL1/ACTRIIB complex formation in cartilage. This is potentially signficiant because BMP9, a ligand that preferentially binds to ACVRL1, is detected in growth plate cartilage, and ACTRIIB has about 300-fold higher affinity for BMP9 than to ACTRIIA [135]. In accordance, co- immunoprecipitation assays

showed that TGFβRI associates with ACTRIIB but not with ACTRIIA in normal chondrocytes. These results suggest that TGFβRI blocks ACVRL1/ACTRIIB complex formation, thereby preventing formation of these high affinity BMP9-binding complexes (Fig. 2). It is not clear why TGFβRI is required to inhibit ACVRL1-mediated BMP signaling in cartilage; one possibility is that because BMP9 is in circulation and cannot be inhibited by Noggin [2,136,137], this alternative method is utilized.

7. Summary and Perspectives

Genetic models have provided a better understanding of the physiological functions of TGFβ and BMP signaling network components and their crosstalk during cartilage development. However, many questions remain regarding the relative importance of various pathways downstream of TGFβ and BMP signaling, the mechanisms and consequences of many types of TGFβ/BMP ligands competing for a more limited set of receptors, the mechanisms determining type I and type II receptor interactions, and how TGFβ/BMP-regulated canonical and noncanonical pathways intersect. It is clear that the absolute and relative output of TGFβ/BMP signaling, and crosstalk between these signaling pathways, determines the final cellular response. Not every signaling component, however, is well understood with regard to in vivo functions at different developmental stages. For example, the functions of ACTRIIA and ACTRIIB in mediating TGFβ and BMP actions in cartilage development are still not clear. Given that ACTRIIA/B can transduce both TGFβ and BMP signals that have fundamentally different and usually opposing effects in cartilage, understanding the extent to which these receptors utilize TGFβ/BMP pathways in vivo is an important goal for the future. Similarly, it will be of interest to assess the importance of genetic and physical interactions between ACVR1L and TGFβRI in multiple skeletal tissues, and whether TGFβRI can limit the signaling of similar BMP type I receptors, like ACVR1.

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Highlights

- TGFβ and BMP signaling negatively regulate one another during cartilage development
- TGFβ inhibits the BMP pathway through receptor interactions and transcriptional regulation
- TGFβRI blocks BMP signaling through restricting ability of ACVRL1 to complex with ACTRIIB

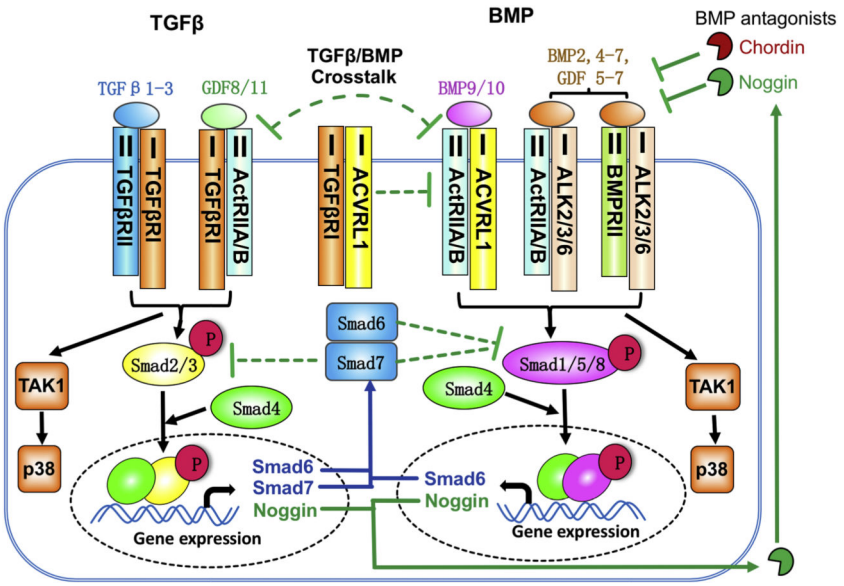

Figure. 1.

Crosstalk between TGFβ and BMP signaling. TGFB ligands bind and activate the type II TGF-B receptor and the type I receptor TGFβRI (ALK5), triggering canonical and noncanonical pathways. In the canonical pathway, transcription factors Smad2 and Smad3 are phosphorylated and associate with Smad4. This complex enters the nucleus and regulates gene expression. BMP ligands activate Smadsi, 5 and 8, which regulate a distinct set of genes. BMP ligands bind to type I BMP receptors ACVRLi (ALKi), ACVRi (ALK2), BMPRiA (ALK3), and BMPRiB (ALK6). BMPs 9/i0 are the only ligands that activate ALKi. However, the BMP type II receptors ACTRIIA and ACTRIIB can associate with either TGFβRI or with type I BMP receptors ALKi/2/3/6 to transduce either a TGFB or a BMP pathway. In addition, TGFβRI can complex with ALKi. The competition for ACTRIIA/B and binding of TGFβRI/ALKi creates a negative crosstalk between TGFβ and BMP signaling at the receptor level. Moreover, TGFβ signaling can inhitit BMP signaling by increasing Smad6, Smad7 and Noggin expression. Smad6 and Smad7 inhibit the phosphorylation and activation of Smadi/5/8. Noggin is a BMP antagonist that blocks the binding of BMPs(2,4–7) and GDFs(5–7) to receptors, but it can not antagonize BMP9 and BMP10.

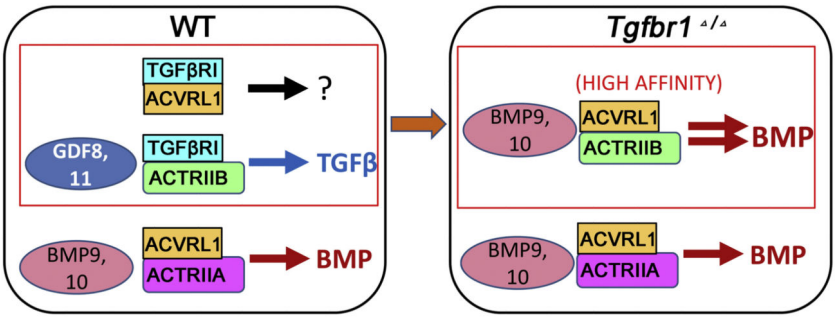

Figure 2.

Proposed model for ACVRL1/TGFβRI interaction. In WT chondrocytes, a significant proportion of ACVRLi and ACTRIIB receptors are contained in complexes with TGFβRI, and TGFβRI blocks ACVRLi complexing with ACTRIIB. In TGFβRI mutant cells, loss of TGFβRI releases both ACTRIIB and ACVRLi receptors, enabling formation of ACVRLi/ACTRIIB complexes that have high binding affinity with BMP9.