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THE BMP PATHWAY AND ITS INHIBITORS IN THE SKELETON

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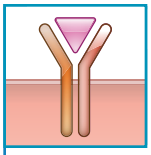

Lowery JW, Rosen V. The BMP Pathway and Its Inhibitors in the Skeleton. *Physiol Rev* 98: 2431–2452, 2018. Published August 29, 2018; doi:10.1152/physrev.00028.2017.—Bone morphogenetic proteins (BMPs) constitute the largest subdivision of the transforming growth factor-β family of ligands. BMPs exhibit widespread utility and pleiotropic, context-dependent effects, and the strength and duration of BMP pathway signaling is tightly regulated at numerous levels via mechanisms operating both inside and outside the cell. Defects in the BMP pathway or its regulation underlie multiple human diseases of different organ systems. Yet much remains to be discovered about the BMP pathway in its original context, i.e., the skeleton. In this review, we provide a comprehensive overview of the intricacies of the BMP pathway and its inhibitors in bone development, homeostasis, and disease. We frame the content of the review around major unanswered questions for which incomplete evidence is available. First, we consider the gene regulatory network downstream of BMP signaling in osteoblastogenesis. Next, we examine why some BMP ligands are more osteogenic than others and what factors limit BMP signaling during osteoblastogenesis. Then we consider whether specific BMP pathway components are required for normal skeletal development, and if the pathway exerts endogenous effects in the aging skeleton. Finally, we propose two major areas of need of future study by the field: greater resolution of the gene regulatory network downstream of BMP signaling in the skeleton, and an expanded repertoire of reagents to reliably and specifically inhibit individual BMP pathway components.

I. BMP ACTIVITY AND PATHWAY MECHANICS

The discovery of the bone morphogenetic protein (BMP) pathway was a direct result of the pioneering experiments of Marshall Urist (247), who was the first to report in 1965 that demineralized bone matrix contained a remarkable osteoinductive activity. Historical accounts of the discovery and progression of research in the BMP pathway have recently been detailed elsewhere (167, 212). Briefly, Urist's initial question was based on an interest in dystrophic calcification, and studies were performed to determine conditions that would allow for mineralization of demineralized substrates in vivo. In the course of experiments on the calcification of tendon, aorta, muscle, and demineralized bone matrix, Urist was astonished to record that, every time demineralized bone matrix was implanted, new bone formed within the connective tissue of the host animal (247). For a time, it was unclear what kind of agent or agents were mediating this new bone formation, but it was later determined to be proteinaceous in nature, and the name "bone morphogenetic protein" was coined (248). Urist et al. continued to systematically explore the biochemical nature of this activity and its interaction with bone extracellular matrix (ECM) for >20 yr, contributing a remarkable wealth of information about the relationship between BMPs and ECM.

Once the existence of BMP as an activity was acknowledged, the focus on the cellular response to BMPs by Reddi and co-workers (167, 212) heightened our understanding of the biological processes involved in endochondral ossification outside of the confines of skeletal development and fracture repair. Painstaking observation of the time course of events that took place after BMP implantation led to description of the specific inductive process, identified potential target cells, and demonstrated the equivalence of BMP-mediated bone formation to the normal process of skeletogenesis (167, 212). Through this work, it became clear that BMPs were the initiators of a biological cascade that involves multiple cell types and signaling events and that culminates in the production of functional bone tissue.

Much effort went into the eventual cloning of the genes responsible for BMP activity in 1988 (259). This information revealed that the dramatic osteoinductive activity discovered by Urist and the cellular responses detailed by Reddi are, in fact, attributable to several related BMP ligands that are deposited in bone matrix (259). Later studies revealed that the same basic BMP pathway, albeit with different cellular outcomes, is conserved across the animal kingdom and participates in myriad biological activities beyond bone development, including gastrulation, embryonic patterning, and organogenesis (113); angiogenesis and vascular integrity (69); iron homeostasis (42); inflammation (72); and sexual reproduction (137).

A generalized schematic of signal transduction in the BMP pathway is presented in **FIGURE 1**. BMPs constitute the largest subdivision of the transforming growth factor-β (TGF-β) family of ligands with nearly 30 distinct human proteins bearing the name. However, important differences exist among BMPs with regard to pathway mechanics and effects on cellular behavior. For this reason, we favor the use of "BMP" for those molecules that elicit activation of the canonical BMP pathway effectors SMADs1, 5, and 8. Using this narrow definition, it is possible to identify approximately 12 bona fide BMP ligands in humans **(TABLE 1)**. BMP ligands undergo multiple processing and posttranslational modifications before forming homodimers of two identical subunits via an intermolecular disulfide bond through a cysteine knot. To date, only homodimeric BMPs have been purified from bone, but in vitro evidence indicates some subunits appear to have the ability to heterodimerize. For instance, heterodimers of BMP2/7, BMP2/6, and BMP4/7 display enhanced functional activity over each respective homodimer (10, 22, 27, 46, 92, 95, 122, 165, 170, 249, 266, 283), although the reason for this remains to be determined.

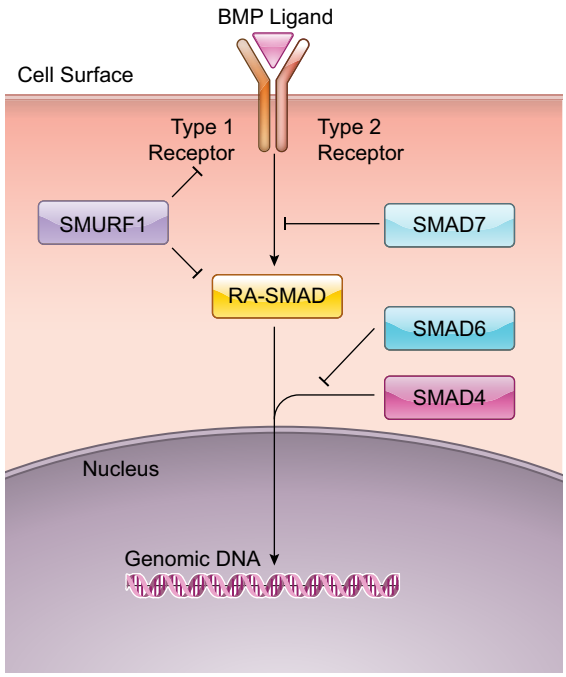

FIGURE 1. Generalized schematic of signal transduction in the canonical bone morphogenetic protein (BMP) pathway. Ligands interact with combinations of type 1 and type 2 receptors, which, in turn, activate effectors called receptor activated (RA)-SMADs. RA-SMADs recruit the transcriptional cofactor SMAD4 and translocate to the nucleus to accomplish gene regulation of genomic DNA. Signal duration and strength are regulated at many levels: Association of ligand with receptors is blocked by secreted antagonists such as noggin or gremlin (not shown); RA-SMAD activation is regulated by SMAD7; recruitment of SMAD4 is regulated by SMAD6; and degradation of receptors and RA-SMADs is promoted by the ubiquitin ligase SMURF1.

BMP ligands undergo complexing with integral transmembrane receptor serine/threonine kinases localized to the cell surface. These receptors are classified into types 1 and 2, of which there are four and three isoforms, respectively **(TABLE 1)** (107). The type 2 receptor is constitutively active and, on ligand binding, is brought into close proximity with the type 1 receptor, thereby allowing trans-phosphorylation to occur (107). In the canonical BMP pathway, activation of the type 1 receptor leads to phosphorylation of the COOH-terminus SMADs1, 5, and 8; renders them active; and increases their association with the transcription factor SMAD4 (107). Notably, SMAD4-independent BMP activities also occur, which is consistent with the finding that several noncanonical signaling pathways, including p38, ERK, and AKT, and micro-RNA (miRNA) processing (47, 279) are also regulated by BMP ligands. It is also likely that BMP pathway activation initiates an extensive signaling cascade, since the phosphorylation status of nearly 400 proteins changes within 30 min of stimulation by BMP2 (111). It is not clear at present how the activation of canonical vs. noncanonical pathways is regulated. One model proposes that differential responses occur downstream of preformed BMP receptor oligomers, as opposed to ligand-induced BMP receptor oligomers (82, 173). More recently, a number of high-resolution microscopy techniques have refined this idea (75). It is important to point out that the exact biological significance of these distinctions in receptor complex formation has yet to be determined in vivo.

Another complex feature of BMPs is the role of the prodomain in modulating osteogenic activity. There appears to be great variability in the interaction of the pro- and mature regions of individual BMPs, and these interactions govern receptor activation, affinity for ECM, and mobility of the secreted proteins once they are released from the cell (8, 41, 218). It has been difficult to predict a priori whether any given interaction between a mature BMP molecule and its prodomain will enhance or diminish activity of individual BMPs (218).

Table 1. *Principal components of the canonical BMP signaling pathway*

Ligands
BMP-2 (BMP-2A BDA-2A)
BMP-4 (BMP-2B, BMP-2B1, MCOPS6, OFC11, ZYME)
BMP-5
BMP-6 (VGR, VGR1)
BMP-7 (OP-1)
BMP-8A
BMP-8B (OP-2)
BMP-9 (GDF-2, HHT5)
BMP-10
GDF-5 (BMP-14, OS5, LAP4, BDA1C, CDMP1, SYM1B, SYNS2)
GDF-6 (BMP-13, KFM, KFS, KFS1, KFSL, SGM1, CDMP2, LCA17, MCOP4, SCD04, MCOPCB6)
GDF-7 (BMP-12)
Extracellular antagonists
BMPER (CRIM3, CV-2, CV2)
Chordin (CHRD)
Chordin-like 1 (CHRDL1, CHL, MGC1, MGCN, NRLN1, VOPT, dA141H5.1)
Coco (DAND5, SP1; CER2; CRL2; CERL2; DANTE; GREM3; CKTSF1B3)
DAN (D1S1733E, DAN, DAND1, NB, NBL1, NO3)
Gremlin1 (C15DUPq, CKTSF1B1, CRAC1, CRCS4, DAND2, DRM, DUP15q, GREM1, GREMLIN, HMPS, HMPS1, IHG-2, MPSH, PIG2)
Noggin (NOG, SYM1, SYNS1, SYNS1A)
NOV (CCN3, IBP-9, IGFBP-9, IGFBP9 h)
PRDC (GREM2, CKTSF1B2, DAND3, PRDC, STHAG9)
SOSTDC1 (CDAO19, DAND7, ECTODIN, USAG1)
TWSG1 (TSG)
Type 1 receptors
ALK-1 (ACVRL1)
ALK-2 (ACVR1, ActRI)
ALK-3 (BMPRIA)
ALK-6 (BMPRIB)
Type 2 receptors
BMPRII (BMPR3, BMR2, BRK-3, POVD1, PPH1, T-ALK)
ACVR2A (ActRII, ActRIIA, ACVR2)
ACVR2B (ActRIIB)
Coreceptors
Betaglycan (BGCAN, TGFBR3)
DRAGON (RGMb)
Endoglin (ENG, END, HHT1, ORW1)
Hemojuvelin (RGMc, HFE2, HFE2A, HJV, JH)
RGMa (RGM)
Pseudoreceptor
BAMBI (NMA)
Receptor-activated SMADs
SMAD1 (BSP-1, BSP1, JV4–1, JV41, MADH1, MADR1)
SMAD5 (DWFC, JV5–1, MADH5)
SMAD8 (MADH6, MADH9, PPH2, SMAD9, SMAD8/9, SMAD8A, SMAD8B)
Co-SMAD
SMAD4 (DPC4, JIP, MADH4, MYHRS)
Inhibitory SMADs
SMAD6 (A0VD2, HsT17432, MADH6, MADH7)
SMAD7 (CRCS3, MADH7, MADH8)

Alternative names or abbreviations in humans are in parentheses. For a comprehensive review of bone morphogenetic protein (BMP) signaling mechanics, please see Katagiri and Watabe (107).

Strength and duration of BMP pathway signals is tightly regulated at numerous levels via mechanisms operating both inside and outside the cell (89, 251). And, given the widespread utility and pleiotropic, context-dependent effects of the BMP pathway, it is not surprising that defects in the BMP pathway or its regulation underlie multiple human diseases of different organ systems (146). Yet much remains to be discovered about the BMP pathway in its original context, i.e., the skeleton. In this review, we wish to provide a comprehensive overview of the BMP pathway and its inhibitors in bone development, homeostasis, and disease. The discussion is framed around major unknowns in the field as follows: detailing the gene regulatory network downstream of BMP signaling in osteoblastogenesis; examining why some BMP ligands are more osteogenic than others; discussing factors limiting BMP signaling during osteoblastogenesis; considering whether specific BMP pathway components are required for normal skeletal development and if the pathway exerts endogenous effects in the aging skeleton; and discussing therapeutic modulation of the BMP pathway in bone healing. We conclude by proposing two major areas of future study that, we feel, are of significant importance for the field.

II. WHAT IS THE GENE REGULATORY NETWORK DOWNSTREAM OF BMP SIGNALING IN OSTEOBLASTOGENESIS?

Studies referenced later in this review provide strong evidence that BMP signaling, and in some instances a particular pathway component, is required to initiate some or all of the early events in endochondral ossification. By way of introduction, we wish to first focus on the question: what cellular events are initiated downstream of the canonical BMP effectors in skeletal progenitor cells? A large body of experimental evidence unequivocally demonstrates that BMP signaling causes multipotent mesenchymal cells to differentiate into the osteochondral lineage. In in vitro assays, this is functionally demonstrated by alkaline phosphate expression and activity and subsequent mineralization of ECM. However, information gained at the gene expression level indicates that these changes in cellular physiology are

best viewed as resulting from a layered cascade of transcriptional events downstream of BMP signaling rather than a single response. The reader is directed to several helpful reviews describing the BMP-induced osteogenic gene profile (151, 171, 207).

Some of the earliest evidence of the complex gene network activated in response to BMP signaling came from a study in 2001 that utilized microarray technology to analyze transcripts from a human bone marrow stromal cell (BMSC) line treated with BMP2 (138). Locklin et al. (138) examined early events occurring minutes to hours poststimulation and those occurring after 3 days of sustained BMP2 stimulation. This experiment revealed a dramatic induction of *inhibitor of differentiation 1 (ID1)*, *ID2*, and *ID3* within 1 h of BMP2 treatment that declines to nearly baseline levels over several days; *ID4* was regulated in a similar fashion but less strongly than *ID1*, *2*, and *3* (80). These results are remarkably consistent with the nearly 15 yr of additional data, including more recent gene array studies in BMSCs (128, 148, 285) that establish the ID family of basic helix-loop-helix (bHLH) proteins as direct, early targets of the BMP pathway. Moreover, ID proteins, especially ID1 and ID3, are critical effectors of BMP-induced osteoblastogenesis (150). Given that ID proteins are atypical bHLH proteins in that they do not bind to DNA but instead inhibit other bHLH transcription factors via heterodimerization (205), these data indicate that an essential aspect of BMP-induced osteoblastogenesis involves repression of gene transcription rather than simple activation of osteoblast-specific genes. The entire set of genes regulated by ID proteins during osteoblastogenesis is not well established, but it is known that the muscle-specific bHLH transcription factor MyoD is targeted by ID proteins (274). This may be relevant to osteoblastogenesis under certain circumstances, such as heterotopic ossification as the mouse myoblast cell line C2C12 expresses MyoD and undergoes robust transdifferentiation to the osteoblast lineage with concomitant upregulation of *ID* genes after BMP treatment (15, 48, 85, 184). As discussed in a later section, ID1 may also play an important role in regulating osteoclastogenesis (37).

Other early-response genes activated downstream of BMP signaling in primary BMSCs include *distal-less homeobox (DLX) 2* and *DLX5* (128, 138, 191, 285), consistent with findings in the mouse osteoblast-like cell line 2T3, where *Dlx2* and *Dlx5* are induced within 30 min of BMP2 treatment and control a large cadre of transcriptional events (79). A related gene, *Dlx3*, is also upregulated by BMP signaling in C2C12 cells and in the mouse osteoblast-like cell line MC3T3 (81). Detailed in vitro analyses have demonstrated that DLX proteins regulate the expression of several osteoblast-lineage genes, including *Runx2, Osterix, Osteoactivin*, and *Mepe* (36, 40, 81, 131, 224). *Runx2* and *Osterix* are particularly well-established BMP-target genes (36, 116, 129, 130, 193, 216, 238, 265), supporting the idea that several hierarchical relationships are likely to exist during BMP-induced osteoblastogenesis.

To aid in regulating this large gene expression network, BMP signaling also exerts broad translational control via miRNAs. In 2008, Li et al. (132) reported an early miRNA signature in C2C12 cells that had been stimulated to undergo BMP-induced osteoblastogenesis. Here, BMPs rapidly upregulated a handful of miRNAs that target muscle-related genes, such as *MyoD*, while downregulating at least 22 miRNAs, many of which target osteogenesis-related transcripts, such as *Dlx3*, *Runx2*, and the canonical Wnt effector β-catenin (*Ctnnb1*). Li et al. (132) went on to validate two of these miRNAs, miR-133 and miR-135, and showed that overexpression of these blunted BMP2-induced osteoblastogenesis. A more narrowly designed study by Kang et al. (105) using MC3T3 cells identified two additional miRNAs, miR-194 and miR-302a, that are induced by BMP2. It should be noted that these miRNAs were not reported by Li et al. to be upregulated, but these two studies differ in several important ways, including cell type, differentiation stage, and timing of analysis post-BMP treatment. BMP2-mediated regulation of over 100 long noncoding RNAs during osteoblastogenesis has also been reported (286), but the significance of these transcripts in differentiation to an osteoblast phenotype is unclear at present.

It is also likely that, in addition to transcriptional regulation downstream of BMP signaling, nontranscriptional events elicited by the BMP pathway also participate in regulating osteoblastogenesis. As mentioned earlier, BMP2 stimulation alters the phosphorylation status of hundreds of proteins (77, 111) and other BMP2-dependent posttranslational modifications have also been reported, such as increased acetylation and decreased ubiquitination of RUNX2 (97).

In our opinion, several intriguing questions arise from these and other studies regarding gene expression during osteoblastogenesis. First, do specific BMP ligands regulate distinct gene sets in multipotent mesenchymal cells, or is there a common BMP response downstream of all bona fide BMP ligands? This is a deceptively complicated question to answer, since most gene expression studies examine only a single ligand. Perhaps the most reductionist approach is to examine the effects downstream of BMP receptor activation because nearly all osteogenic BMP ligands utilize the type 1 receptors activin receptor-like kinase (ALK) 2, 3, or 6 for signaling. From this perspective, one line of evidence generated by Korchynskyi et al. (120) supports the idea that the response mounted by each of these receptors is the same. These investigators transfected cDNA encoding constitutively active versions of ALK2, 3, or 6 into C2C12 cells and then examined the resulting gene expression profile. Remarkably, each receptor elicited an identical transcriptional response that included upregulation of the aforementioned

targets *Id1*, *Id2*, and *Runx2* among nearly 100 others (120). That said, this artificial approach is not without limitations, and additional work is required to determine the applicability of the findings to endogenous BMP signaling.

III. WHY ARE SOME BMP LIGANDS MORE OSTEOGENIC THAN OTHERS?

With the above information in mind, we now consider the possibility that individual BMPs produce ligand-specific responses in skeletal target cells. In 2003, Peng et al. (184) reported a head-to-head comparison of the effects of BMP2, 6, or 9 on C2C12 cells and found that, in general, the resulting effect on cellular physiology and gene expression responses for each ligand were the same. These results are consistent with the findings of Lorda-Diez et al. (140), who observed that BMP2, 4, 5, and 7 regulate similar genes in limb bud progenitor cells. Subtle differences in gene regulation were noted, however, that align BMP2, 4, and 9 more closely with each other than with BMP5, 6, and 7 (140, 184), a finding consistent with the phylogenetic relationships between these ligands (143).

These data beg the question, then, of why are some BMP ligands more osteogenic than others? For instance, several studies on skeletal progenitor cell types, including BMSCs, adipose-derived mesenchymal stem cells (MSCs), C2C12, and the multipotent mesenchymal line C3H10T1/2, indicate that the concentration of some BMPs required to induce osteoblastogenesis is severalfold lower than that required for other BMPs (2, 21, 39, 49, 106, 116, 140, 149, 166, 182, 185, 200, 256). One ligand that is particularly potent is BMP9, and its heightened activity is likely attributable to the fact that it is not inhibited by the extracellular antagonist noggin (202, 226, 256). These ligand-specific differences are highly relevant when considering utilizing BMPs for tissue engineering approaches. However, we suggest caution should be exercised in interpreting how results relate to the underlying biology of each endogenous BMP in skeletal homeostasis.

One very interesting result from these studies, though, is the consistent finding that some ligands, such as growth differentiation factor (GDF) 5, 6, and 7 (also called BMP14, 13, and 12, respectively) are not robustly osteogenic. For example, work from the He group using adenoviral-based expression in C2C12 and C3H10T1/2 cells indicates that these GDF ligands do not induce robust alkaline phosphatase activity in vivo (39, 106, 149), an intriguing result since GDF5 (BMP14), GDF6 (BMP13), and GDF7 (BMP12) activate the canonical BMP effectors SMAD 1, 5, and 8 and induce osteogenic markers in C2C12 cells and limb bud micromass cultures (21, 140). Deficiency of GDF5 (BMP14) and GDF6 (BMP13) expression in humans and mice leads to short stature, symphalangism, and brachydactyly, as do mutations that result in increased BMP activity of these molecules during skeletal development. As GDF5 (BMP14), GDF6 (BMP13), and GDF7 (BMP12) behave more like osteogenic BMPs in committed osteoprogenitor cultures (39, 116), it is possible that the actions of these ligands are dependent on cell context and/or differentiation stage and may be highly influenced by the specific complement of BMP type 1 receptors present on target cells and/or by the presence of specific antagonists in the local microenvironment. It is interesting, therefore, to speculate that the amount of BMP signaling at specific anatomical sites during skeletal development may be controlled through competition between GDFs and BMPs for common receptors. If this were to be the case, it would provide an additional mechanism for generating highly nuanced signals using a common set of receptors, ligands, and extracellular antagonists. Indeed, major inroads into understanding the integrated cellular response that occurs in the presence of multiple BMP ligands has recently been provided by Antebi et al. (9). Using quantitative reporter assays and mathematical modeling, these authors report that some ligand combinations exert additive effects, while others cause cells to integrate ratiometric or imbalanced inputs, and these events are largely controlled by receptor availability and/or expression profile (9). Future studies are required to determine the applicability of these findings to complex in vivo settings.

IV. WHAT LIMITS BMP SIGNALING DURING OSTEOBLASTOGENESIS?

It is also relevant to ask: what factors limit BMP signaling during osteoblastogenesis? In addition to interaction with other widely studied signaling pathways such as fibroblast growth factor (24, 94, 123, 168, 227, 250, 252), several studies indicate that BMP-dependent osteoblast differentiation involves a negative feedback loop, whereby BMP signaling induces expression of its own inhibitors. For instance, induction of mRNA coding for the inhibitory SMAD6 is observed at 24 h post-BMP2 treatment in C2C12 cells and occurs via RUNX2 (255). It is important to note that these data were obtained from populations of cells and are, therefore, unable to inform us if each cell upregulates SMAD6 expression to the same degree, if at all. We raise this point because SMAD6 is an intracellular protein and is only capable of inhibiting receptor-activated (RA)-SMAD stimulation in the cell that expresses it. Thus it is possible that the strength of BMP signal transduction is different from one cell to the next within a given population, which could lead to diverse cellular responses that may depend on the local availability of the signaling molecule and its cognate receptor. However, this is not the only method of negative feedback employed by BMP signaling in osteoblastogenesis: Canalis' group demonstrated that *noggin* mRNA is induced in a dose-dependent manner within 2 h of BMP2 stimulation in primary rat osteoprogenitors (64), while upregulation of *gremlin* mRNA occurs slightly later (187). Since noggin and gremlin are secreted antagonists that se-

quester BMP ligands upstream of receptor binding, these data suggest the possibility that there is a general dampening of the BMP pathway rapidly following initial stimulation, i.e., a short-duration pulse of pathway activation. This idea is reminiscent of the model put forward by Zohar et al. (284) wherein pulsatile administration of exogenous BMP ligand to late-stage rat calvarial osteoprogenitors promotes osteogenesis, while, in contrast, the continuous presence of exogenous BMP ligand promotes chondrogenesis of these cells. We are unaware of data evaluating this model in vivo, and further experimental evidence is required to determine its broad applicability.

V. ARE SPECIFIC BMP PATHWAY COMPONENTS REQUIRED OR IS THE BMP PATHWAY IN GENERAL REQUIRED FOR SKELETAL DEVELOPMENT?

Bone organs develop via two processes: intramembranous ossification, where bone is formed directly within mesenchymal tissue, and endochondral ossification, where a cartilage template is formed first and later remodeled to bone (271). One well-studied example of endochondral ossification is the embryonic limb skeleton. Here, mesenchymal progenitors resident in the limb bud aggregate in a location-specific manner, creating symmetrical condensations, each bordered by a boundary layer, the perichondrium, that separates condensing and noncondensing mesenchyme (271). Cells within mesenchymal condensations differentiate into chondrocytes and execute a complex maturation program whose end result is the formation of the growth plate, a signaling center that drives linear growth through cell division and hypertrophy (115, 271). Osteoblasts differentiate from mesenchymal precursors resident in the perichondrium and produce a bone collar that will become the future cortical bone (115). Blood vessels invade cartilage at the bone collar, bringing along osteoblast progenitors from the perichondrium that produce trabecular bone (115).

Arguably, the most striking evidence that the canonical BMP pathway is required for endochondral ossification was provided by Retting et al. (198), who deleted *Smad1* and *Smad5* in chondrocytes, resulting in severe chondrodysplasia and a subsequent lack of ossification. A considerable degree of functional overlap was noted, as *Smad1* and *Smad5* single mutants displayed only minor phenotypes. Thus, while it is not clear at present if SMAD1 or SMAD5 are preferred in vivo, it is clear that an adequate level of canonical BMP pathway activity is essential to endochondral ossification. Interestingly, this study also suggested that endogenous SMAD8 plays a less critical role in this process than SMADs 1 and/or 5 since global loss of *Smad8* does not significantly modify either the *Smad1* mutant or *Smad1/Smad5* double-mutant phenotypes (198). This differential effector requirement is somewhat surprising, given that SMADs 1, 5, and 8 are often viewed as a single response. But, it should be noted that specific effects downstream of individual SMADs have been observed previously (143). Additionally, work from Tsukamoto et al. (244) indicates that SMAD8 exerts weaker transcriptional control than SMADs 1 or 5 and, in some instances, might act as a negative regulator of the BMP-induced gene regulation.

Several lines of evidence indicate that the pool of activated SMAD1 and 5 molecules is tightly regulated during endochondral ossification, and that skeletal defects can result from elevated or abnormally persistent signaling. For instance, SMAD6 and SMAD7, which inhibit receptor-activated (RA)-SMAD activation and promote their degradation (162), are expressed in developing cartilage and homozygous, global deficiency of either factor and lead to impaired terminal differentiation of chondrocytes and delayed mineralization (57, 58). In vitro experiments indicate that deficient levels of SMAD6 or 7 leads to higher basal BMP signaling and increased responsiveness to BMP ligands.

Deletion of *Smad4* using *Prx1*-Cre results in mice born with essentially no forelimbs and only hindlimb rudiments (19, 133), and these mice also lack parietal and interparietal bones, which are all skeletal regions targeted by *Prx1*-Cre (139), consistent with the idea that SMAD4 is a central component of the canonical BMP pathway. It is intriguing that the skeletogenesis phenotype of the *Smad1/Smad5* col2-cre double mutant is more severe than loss of *Smad4* in the same cell type (278). Zhang et al. (278) demonstrated that deletion of *Smad4* in chondrocytes causes drastic defects in the growth plate that lead to dwarfism, but chondrogenesis is indeed initiated, and ossification subsequently occurs. We will discuss the centrality of SMAD4 in canonical BMP signaling, or the possible lack thereof, at greater length later in this review, but wish to briefly state here that the combined findings of Retting et al. (198) and Zhang et al. (278) provide evidence that SMAD1 and/or SMAD5 have additional SMAD4-independent actions that are essential in endochondral ossification.

Genetic models have also been utilized to interrogate BMP receptor involvement in skeletogenesis. In general, these have revealed that the type 1 receptors ALK2, ALK3, and ALK6 participate in endochondral ossification, but that single loss of either receptor leads to only mild skeletal phenotypes: loss of *Alk2* in chondrocytes leads to craniofacial and axial defects and progressive kyphosis (199); loss of *Alk3* in limb bud mesenchyme or chondrocytes leads to growth plate defects and shortened limbs (275); and global loss of *Alk6* leads to impaired metacarpal/metatarsal chondrocyte differentiation defects (275). We are unaware of data demonstrating major skeletogenesis-related defects in *Alk1* single-mutant animals. Given the somewhat overlapping expression domains, combining deletion of multiple type 1

receptors may be useful to ask if a certain level of BMP pathway activity vs. action of particular receptors is required for endochondral ossification. Indeed, Rigueur et al. (199) demonstrated that combined loss of both *Alk2* and *Alk3* in chondrocytes or loss of *Alk2* in chondrocytes on a global knockout *Alk6* background leads to generalized chondrodysplasia that is more profound than each single mutation alone. Moreover, by combining loss of *Alk3* in chondrocytes and global loss of *Alk6*, Yoon et al. (275) demonstrated that these receptors are likely redundant since, like the *Smad1/Smad5* double mutant, *Alk3/Alk6* double mutants display severe chondrodysplasia and a lack of endochondral ossification.

Similar to type 1 receptors, the available data indicate that no single type 2 BMP receptor (BMPR2) is preferred or required for endochondral ossification. For instance, while BMPR2 is commonly thought of as the major type II receptor, Gamer et al. (62) demonstrated that limb patterning and development are normal when *Bmpr2* is deleted in limb mesenchyme. Similarly, global loss of ACVR2A or ACVR2B expression leads to only minor skeletal patterning defects (153, 175). Importantly, Gamer et al. demonstrated that the expression level of *Acvr2a* and *Acvr2b* does not change upon loss of *Bmpr2*, indicating that type 2 receptor expression level is not limiting to BMP signaling during endochondral ossification. When taken together, the findings available at present suggest that loss of a single type 2 receptor can be compensated by one or more of the remaining type 2 receptors without impairing endochondral ossification; we will draw distinction with this topic in postnatal bone remodeling, as discussed later in this review.

Numerous BMP ligands are expressed in the skeleton, including BMP2, 3, 4, 5, 6, 7, GDF5 (BMP14), and GDF6 (BMP13) (209). However, we are not aware of data showing that any particular BMP ligand is dominant in skeletal development. For instance, loss of individual BMP ligands leads to either no discernible skeletal phenotype at birth or to relatively mild patterning defects (209). It is only when loss of multiple BMP ligands is combined, such as BMP2 and BMP4, as demonstrated by Bandyopadhyay et al. (16), that severe defects become apparent. This is supportive of the idea that a threshold of BMP pathway activation, irrespective of specific ligands, is required to initiate endochondral ossification. It is important to point out, though, that this study also demonstrated that BMP7 is a less important partner for BMP2 or BMP4 in endochondral ossification (16), although all three ligands are known to activate SMADs 1 and 5 in chondrocytes with similar kinetics and robustness. This difference could be explained by BMP7 having a distinct expression from BMP2 and BMP4, but, as will be discussed below, somewhat differential gene regulation downstream of BMP7 vs. BMP2/BMP4 in osteochondroprogenitors has been reported (39).

These data establish that BMP signaling promotes endochondral ossification during normal skeletal development. Numerous studies though reveal that this pathway is also a potent inducer of endochondral ossification in extraskeletal locations (214). In fact, ectopic bone-forming ability is a hallmark of BMP ligands discovered years before the pathway components that participate in the response. It follows then that aberrant BMP signaling is implicated in the heterotopic bone formation that can follow soft tissue trauma or central nervous system injury. Additionally, mutations that cause ALK2 to misinterpret activin, a related molecule in the TGF-β superfamily, as a BMP ligand are strongly linked to a heritable form of heterotopic ossification, fibrodysplasia ossificans progressiva (FOP) (83, 87) **(TABLE 2)**. Overactivation of the BMP pathway is also observed in vascular calcification which, at the molecular level, recapitulates osteoblastogenesis to a striking degree (143). Collectively, these findings suggest that proper regulation of BMP signal strength, duration, and location is crucial to normal development and homeostasis.

VI. WHAT ARE THE EFFECTS OF THE BMP PATHWAY IN POSTNATAL AND AGING BONE?

Given that proper BMP signaling is required for normal skeletal development and patterning, numerous investigators have examined whether BMP signaling has a role in the postnatal skeleton and, in particular, in the regulation of bone mass. This is an important goal, since more than 50 million people in the United States alone have low bone mass, and this number is expected to rise in the coming decades (260). However, despite the extensive functional data documenting the embryonic role of BMP signaling in skeletogenesis, relatively little is known about the role the BMP pathway plays in the maintenance of bone mass in adults. For example, BMP signaling levels reportedly correlate with bone mineral density (74, 124, 169, 220, 234, 268), and BMP signaling ability is reduced in BMSCs obtained from aged (163) or osteoporotic (76, 192) subjects. However, it is possible these findings are associative rather than causative. Additionally, other data, such as genome-wide association studies, are controversial and/or yield inconsistent results with regard to the influence of BMPs on adult bone mass (146).

To gain clarity in this discussion, we wish to first make a distinction between the role that BMP signaling can play in postnatal bone remodeling vs. the role that it does play. The former is arguably easier to examine due to the ability to activate the pathway via exogenous means. Several studies demonstrate that systemic administration of recombinant BMP2, BMP6, or BMP7, or alleviating inhibition of the BMP receptor ALK3 using a synthetic peptide, improves bone mass and associated parameters (4, 53, 223, 245). In light of the studies using isolated MSCs described earlier, these

Table 2. *Summary of skeletal phenotypes of relevant genetically modified mice*

Gene(s)	Strategy	Ref. No.	Notable Phenotype(s)
Bmp2	Prx1-Cre	239	Defective periosteal apposition; spontaneous fractures and defective fracture repair
	Col2-Cre	157	Defective fracture repair
	Col2-CreER	222	Chondrodysplasia
	Col1(3.6kb)-Cre	155, 270	Low bone mass; normal fracture repair
	Osx1-Cre	156	Low bone mass
	Col1(2.3kb)-Cre	157	Normal bone mass; normal fracture repair
Bmp4	Prx1-Cre	16	Normal bone mass; normal fracture repair
	Col2-CreER	222	Minor chondrocyte differentiation defect
Bmp5	Global null	159	Abnormal bone geometry
Bmp6	Global null	225	Mildly delayed ossification
Bmp7	Prx1-Cre	16, 242	Normal skeletogenesis or mild defects; normal fracture repair
Bmp2/4 double mutant	Prx1-Cre	16	Severely defective osteogenesis
	Col2-CreER	222	Defective chondrocyte proliferation and differentiation
Bmp8a	Global null	281	Presumed normal skeletogenesis
Bmp8b	Global null	280	Presumed normal skeletogenesis
Gdf5	Global null	230, 231	Defective limb development and growth plate function; shortened limbs
Gdf6	Global null	219	Site-specific cartilage/tendon defects; patterning defects
Gdf7	Global null	158	Impaired longitudinal bone growth
Alk2	Col2-Cre	199	Axial skeleton hypoplasia and patterning defects; kyphosis
Alk3	Prx1-Cre	179	Shortened limbs; patterning defects
	Col2-Cre	275	Shortened limbs, delayed ossification
	Col1(3.2kb)-CreER	100–103	High bone mass due to decreased resorption
	Og2-Cre	160	High bone mass due to decreased resorption
Alk6	Global null	273	Metacarpal/metatarsal chondrocyte differentiation defects
Alk2/3 double mutant	Prx1-Cre	133	Significant limb development defects; mild patterning defects
	Col2-Cre	199	Severe chondrodysplasia
Alk2/6 double mutant	Col2-Cre for *Alk2*; Global null for *Alk6*	199	Severe chondrodysplasia
Alk3/6 double mutant	Col2-Cre for *Alk3*; Global null for *Alk6*	275	Severe chondrodysplasia, lack of endochondral ossification
Alk2/3/6 triple mutant	Prx1-Cre for *Alk2/3*; Global het for *Alk6*	133	Significant limb development defects
Bmpr2	Global het hypomorph	51	Delayed ossification, mild vertebral patterning defects
	Prx1-Cre	62, 144	Normal skeletogenesis; high bone mass with increased bone formation rate
Acvr2a	Global null	153	Mandibular hypoplasia, cleft palate, mild patterning defects
	Ocn-Cre	67	High bone mass
Acvr2b	Global null	175	Mild vertebral patterning defects
	Ocn-Cre	67	Normal skeletogenesis and bone mass
Acvr2a/b double mutant	Ocn-Cre	67	Similar phenotype as *Acvr2a* cKO using Ocn-Cre
Smad1	Col2-Cre	109, 198, 253	Normal skeletogenesis
	Col1(2.3kb)-Cre	253	Low bone mass due to reduced bone formation rate

Continued

Table 2.—*Continued*

Gene(s)	Strategy	Ref. No.	Notable Phenotype(s)
Smad1/5 double mutant	Col2-Cre	198	Severe chondrodysplasia, lack of endochondral ossification
Smad8	Global null	198	Normal skeletogenesis
Smad1/5/8 triple mutant	Col2-Cre	198	Same as *Smad1/5* double cKO
Smad4	Col2-Cre, Ocn-Cre	235, 278	Dwarfism; growth plate defects
	Prx1-Cre	19, 133	Significant patterning and osteogenesis defects
	Osx1-Cre	210	Dwarfism; brittle bones
	Osx1-CreER	211	Altered osteoprogenitor proliferation rate
Smad6	Global null	57	Impaired terminal differentiation of chondrocytes and delayed mineralization
Smad7	Global null	58	Impaired terminal differentiation of chondrocytes and delayed mineralization
Bmp3	Global null	44	High bone mass
Noggin	Global null or het	68, 257	Patterning defects; kyphosis
	Ocn-Cre	32	Low bone mass with increased bone resorption rate
Gremlin	Global null	110	Patterning defects
	Ocn-Cre	66	High bone mass
Noggin/Gremlin double mutant	β-Actin-Cre	229	Severe axial skeletogenesis defects
Nov	Global null	34	Normal skeletogenesis

potent effects are likely due to promoting osteoblastogenesis and/or increasing bone formation rate in vivo. This idea is supported by a study from Cao's group using transgenic mice in which canonical BMP signaling is constitutively activated in osteoblasts, which leads to high bone mass by four months of age due to increased osteoblastogenesis and elevated bone formation rate (277).

We find these studies highly informative and potentially clinically important; however, they cannot answer the question of whether the endogenous BMP pathway does in fact regulate postnatal bone remodeling. The first line of evidence we will discuss in this regard comes from several approaches that lead to inhibition of the BMP pathway in vivo upstream of RA-SMAD activation. One such approach taken independently by Chen's group and Cao's group is the transgenic expression of dominant negative BMP receptor mutants in osteoblasts, which leads to decreased bone mass by 2 mo of age (269, 282). Chen's group used static and dynamic histomorphometry to demonstrate that inhibition of the BMP pathway in osteoblasts causes a substantial decrease in the bone formation rate that is not accounted for by decreased osteoblastogenesis, raising the possibility that BMP signaling exerts dual cell-autonomous effects on osteoblast differentiation and function (282). These findings are corroborated by several studies utilizing a complementary approach whereby transgenic expression of the extracellular antagonists noggin, gremlin, or nephroblastoma overexpressed (Nov) from osteoblasts decreases bone mass, osteoblastogenesis, and osteoblast activity (52, 65, 206, 262). Given that each of these approaches inhibit endogenous BMP signaling, albeit by artificial means, we find this to be convincing evidence that the BMP pathway promotes osteoblast function in vivo.

It should be noted, though, that this point of view is not without important caveats. First, Mishina's group has demonstrated that BMP signaling in osteoblasts upregulates the expression of several Wnt pathway inhibitors, such as sclerostin and Dickkopf-1 (DKK1) (98, 177, 276). Since Wnt signaling generally promotes osteoblast function, BMP signaling might indirectly provide negative feedback to restrict osteoblast activity. Additional support for this model comes from Baud'huin et al. (18), who systemically treated 3-mo-old mice with a soluble ALK3 decoy and observed a robust increase in bone mass and bone formation rate with decreased serum DKK1 levels. That said, the Wnt and BMP pathways interact at many levels and in a context-dependent manner, making it difficult to predict a priori how osteoblasts integrate these inputs to mount a coordinated response. Indeed, sclerostin was initially thought to antagonize BMP signaling (125, 127, 258), but detailed biochemical and molecular studies have revealed that any effects of sclerostin on BMP signaling are indirect and most likely due to modulating the interaction between the BMP and Wnt signaling pathways (236). Furthermore, even though Wnt inhibitors are reportedly decreased in osteoblast-specific *Alk3* conditional knockout mice, bone formation rate is severely reduced rather than increased (160, 177), and interpretation of these studies may be complicated by BMP-dependent effects on bone resorption (see below). That said, several recent review articles have attempted to assemble

these data into cohesive models that explain the context-dependent interactions between the BMP and Wnt pathways, and the reader is encouraged to utilize these resources (134, 147, 151, 195, 261).

The second caveat we must discuss is the fact that osteoblast-specific *noggin* conditional knockout mice display low bone mass (32). However, bone formation rate is actually increased in male *noggin* conditional knockouts, and low bone mass in these animals is most likely due to the observed increase in osteoclast number and/or activity. These interesting findings add an important dimension to the present discussion in that they indicate an appropriate level of BMP signaling is required for normal skeletal homeostasis.

We wish to now turn our attention to a discussion of which BMP pathway components are involved in postnatal bone homeostasis. As with endochondral ossification, determining which BMP pathway components regulate osteoblast function in vivo has been complicated due to a high degree of compensation and functional redundancy of pathway components. For example, Wang et al. (253) demonstrated that loss of SMAD1 expression in osteoblasts leads to reduced bone formation rate and osteopenia by 2 mo of age. The direct interpretation of these findings is that signaling through SMAD1 is required for appropriate BMP signaling in the postnatal skeleton, which stands in contrast to the effector redundancy observed in endochondral ossification, where SMAD5 seemingly compensates for loss of SMAD1 (see above). However, it is formally possible that the osteopenia in *Smad1* conditional knockout mice is due to a reduction in BMP effector availability rather than a strict requirement for SMAD1 per se; we are unaware of a study where SMAD5 expression has been removed in osteoblasts but suggest that this experiment might help clarify the postnatal effector requirement. Regardless, the data from Wang et al. indicate that RA-SMAD signaling is required for normal postnatal bone homeostasis.

Findings regarding the requirement for BMP effector activity in postnatal bone homeostasis are strikingly consistent with the fact that loss of the type 1 receptor ALK3 expression in osteoblasts leads to severely reduced bone formation rate by 8 wk of age (102, 160, 177). The accompanying phenotype of these mice, however, is remarkably complex. For instance, it is unclear if ALK3 deficiency causes osteoblastogenesis defects, as the data are decidedly mixed on this point. And, although the expression of several Wnt inhibitors is reduced in these mice (100, 103), this is apparently insufficient to increase osteoblast activity levels. Furthermore, while loss of ALK3 expression in pre-osteoblasts impairs BMP signaling responsiveness in vitro (180) and its loss in late osteoblasts in vivo leads to reduced bone volume at 3 mo of age (160), which is consistent with the reduced bone formation rate, these animals develop high bone mass by 10 mo of age (160). This surprising outcome is seemingly due to defective osteoblast-mediated regulation of osteoclastogenesis, causing secondary defects in bone resorption that tilt the remodeling balance toward bone mass accrual (102, 103, 160, 177). While not specifically addressed, we suspect this is also the mechanism leading to high bone mass in mice lacking ALK2 expression in osteoblasts (98).

As stated earlier, skeletal development is essentially normal, with single loss of any type 2 BMP receptor (62, 153, 175), which likely indicates functional compensation between these molecules. All three type 2 receptors for BMPs are expressed by adult osteoblast lineage cells (136), thus making it difficult to predict which, if any, is the preferred type 2 receptor for BMPs in the postnatal skeleton. That said, conditional knockout strategies have recently provided the first insight into this open question. For instance, loss of BMPR2 in limb bud mesenchyme leads to increased bone mass by 9 wk of age (144). In striking contrast to *Alk3* mutant mice, high bone mass in *Bmpr2* mutants is due to increased bone formation rate with no observable alteration in osteoclast activity or differentiation. This finding is quite surprising, since the standard model of BMP pathway activation predicts that loss of type 1 or type 2 receptors should have a similar effect on downstream signaling, since both are required for pathway activation. Resolution of this apparent contradiction may come from the fact that the level of BMP signaling is unchanged in the bones of *Bmpr2* mutant mice, indicating that, as in the developing skeleton, ACVR2A and/or ACVR2B can compensate for the loss of BMPR2 (144). However, *Bmpr2* mutant mice display reduced activation of the canonical activin/TGF-β effectors SMAD2 and SMAD3 in their skeletons and in primary osteoblasts (144), which is consistent with previous reports detailing the negative effect of activin/TGF-β signaling on bone formation and matrix mineralization (7, 23, 54, 55, 59, 91, 119, 141, 183, 188, 204, 221). Although it is possible that BMPR2 may act as a low-affinity receptor for activin ligands (84, 197), activin A responsiveness is preserved in primary osteoblasts lacking BMPR2 expression (144), which suggests that the decrease in basal SMAD2 and SMAD3 activation is not due to a strict requirement for BMPR2 in activin signaling. This point of view is supported by the available kinetic data indicating that the high-affinity type 2 receptors for activin ligands are ACVR2A and ACVR2B, which, in the absence of BMPR2, must also bind BMP ligands. This leads us to favor the idea that increased utilization of ACVR2A and/or ACVR2B by BMP ligands comes at the expense of activin signaling, and that the availability of BMPR2 alleviates this receptor-level competition, thus identifying a previously unknown function for BMPR2 in segregating the BMP pathway from the activin pathway **(FIGURE 2)**. Support for this model may be found in earlier work demonstrating that BMP7 directly and effectively competes with activin A and the activin-like ligand GDF8

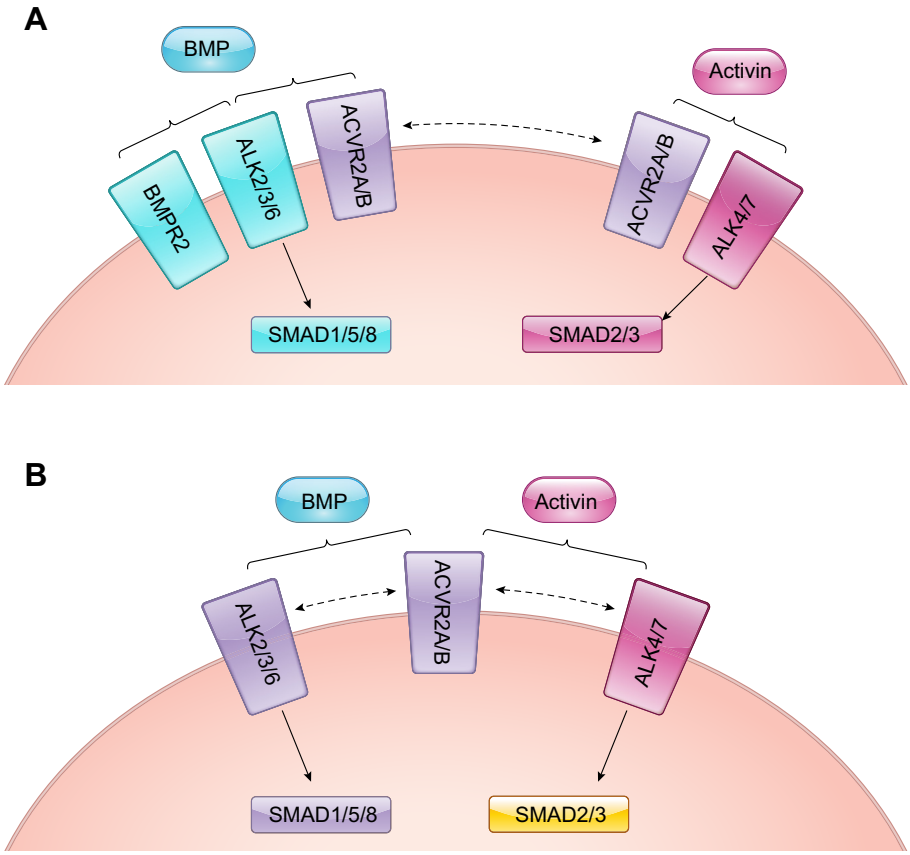

FIGURE 2. ACVR2A and ACVR2B are shared between bone morphogenetic protein (BMP) and activin signaling pathways. *A*: transforming growth factor-β (TGF-β) superfamily signaling requires the interaction of type 1 and type 2 receptors. The type 1 BMP receptors [activin receptor-like kinase (ALK) 2/3/6], in combination with the type 2 receptor, BMPR2, specifically transduce signals from BMP ligands to activate SMADs 1, 5 and 8; the type 1 activin receptors (ALK4/7) specifically transduce signals from activin ligands to activate SMADs 2 and 3. The type 2 receptors ACVR2A and ACVR2B (ACVR2A/B) complex with BMP type 1 receptors or activin type 1 receptors to elicit activation of SMAD1/5/8 or SMAD2/3 in response to BMP or activin ligands, respectively. *B*: In the absence of BMPR2, signals from both BMP and activin ligands are predicted to be transduced via ACVR2A and/or ACVR2B.

for utilization of ACVR2A and/or ACVR2B (190, 196), and also the recent work of Goh et al. (67), who report that deletion of *Acvr2a* in osteoblasts leads to high postnatal bone mass by 6 wk of age due to an apparent selective decrease in activin signaling. Interestingly, ACVR2B does not appear to be essential for activin signaling in osteoblasts, since loss of *Acvr2b* in these cells has no impact on in vitro osteoblastogenesis or postnatal bone mass and does not enhance the high bone mass phenotype of *Acvr2a* mutant mice.

The model of receptor-level competition between BMPs and activins predicts that changes in either receptor or ligand availability could modulate the balance of signaling in a given cell. While it is not known if the expression levels of type 2 BMP receptors change with age, the ligands available to interact with shared type 2 BMP/activin receptors do change: BMP levels decline with age, and this decline correlates with reduced ability to form new bone (163, 233). There is also a significant increase in circulating activin levels in adults of both sexes, especially in the last decades of life (13, 25, 90). Serum immune-reactive activin is further elevated in several disease states (50, 78, 189, 246). When taken together with the fact that activin ligands typically have better affinity than BMPs for ACVR2A and ACVR2B (6, 20, 45, 70, 71, 84, 88, 92, 112, 114, 119, 203, 208, 217), these data suggest that the increased activin availability makes it more likely for shared type 2 receptors to be utilized for activin signaling than BMP signaling in the aging skeleton. Hence, it is somewhat surprising that BMPs are able to effectively utilize ACVR2A and ACVR2B in vivo in the absence of BMPR2 (62, 144). We contend that insight into this may come from the fact that, whereas activins must first bind to a type 2 receptor to initiate a functional signaling complex, BMPs possess a flexible mode of signaling complex assembly by either binding to a type 2 receptor and recruiting a type 1 receptor, or vice versa (11, 73, 84, 86, 117, 135, 152, 172, 174, 186, 203, 267) **(FIGURE 3)**. Thus it is possible that BMPs with lower affinity for ACVR2A and/or ACVR2B may indirectly compete with high-affinity activin ligands via type 1 receptor engagement **(FIGURE 3)**.

An additional complication to the topic of type 2 receptor utilization comes from the fact that BMP3, which is a nonsignaling decoy ligand, binds specifically to ACVR2B molecules (5) and renders them unavailable for use by other ligands (61). Based on amino acid similarity and abundance in bone ECM, it is surprising to find that BMP3 acts as an endogenous antagonist of osteogenic BMP signaling. *Bmp3* knockout mice have a unique skeletal phenotype as they attain the same level of peak bone mass as wild-type mice, but do not lose bone as they age, leading to a high bone mass phenotype (44). By 45–50 wk of age, compared with wild-type mice, *Bmp3* nulls have significantly increased bone volume that correlates with greater numbers of trabeculae (44). Moreover, *Bmp3* null bones show a greatly

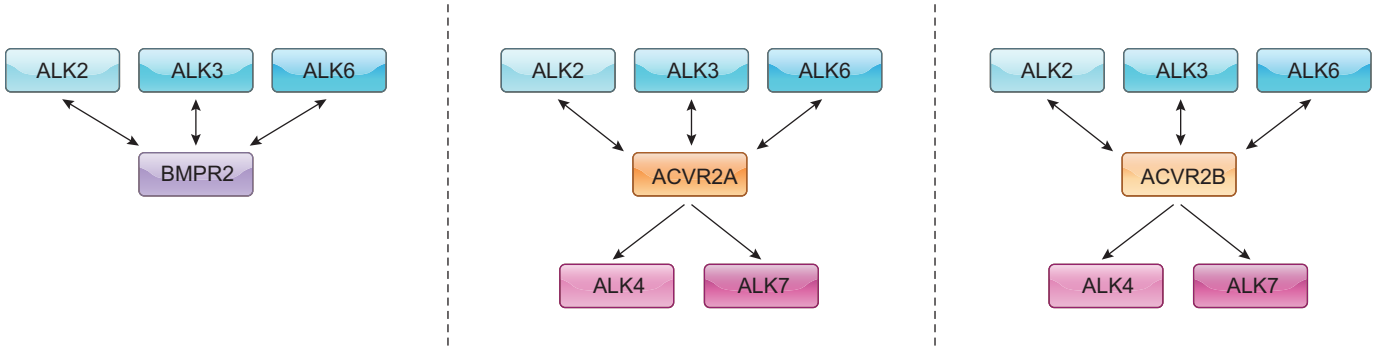

FIGURE 3. Schematic of ligand-induced receptor complex assembly. In the transforming growth factor (TGF)-β superfamily, ligands must interact with both type 1 and type 2 receptors for signaling to occur. Bone morphogenetic protein (BMP) ligands may initiate signaling complex assembly via binding to any of six receptors expressed by bone cells (ALK2/3/6, ACVR2A/B, or BMPR2), as indicated by bidirectional arrows. In contrast, activin ligands must initiate assembly by binding to two receptors, ACVR2A/B, indicated by unidirectional arrows. Thus, although activin ligands display higher direct affinity for ACVR2A/B than most BMP ligands, the flexible mode of BMP signaling complex assembly allows BMPs with moderate affinity for ACVR2A/B to bind and then recruit a type 1 receptor (ALK2/3/6), or BMPs with high affinity for ALK2/3/6 may recruit ACVR2A/B into ligand/type 1 receptor complexes. The latter assembly mechanism allows BMPs with lower affinity for ACVR2A/B to indirectly compete with high-affinity activin ligands for type 2 receptor utilization.

reduced presence of fat cells in the marrow space, consistent with the idea that bone formation is occurring at the expense of marrow adipogenesis (44). Recent data identify highly conserved *cis*-regulatory control of *Bmp3* expression and implicate osteoblasts and osteocytes as the source of BMP3 found in bone, and the frequency of spontaneous osteoblast differentiation of BMSCs is increased in the absence of BMP3 production (118, 145). Collectively, these observations establish BMP3 as a negative regulator of osteoblast differentiation in the bone microenvironment and suggest that type 2 BMP receptor availability may be limited in the aging skeleton.

Additionally, studies examining extracellular antagonists of the BMP pathway demonstrate that the level of BMP signaling in osteoblasts is also regulated upstream of receptor binding. Multiple secreted proteins serve as BMP antagonists by binding directly to BMPs and inhibiting BMP interactions with BMP receptors (63, 201, 209). The developmental functions of these antagonists have been extensively studied, while the ability of the same proteins to modulate postnatal bone formation and function requires further consideration. Early in vitro studies established that MSC, osteoprogenitors, and osteoblasts secrete BMP antagonists when activated by BMPs, suggestive of the existence of a negative feedback loop that serves to limit excessive BMP activity (64, 187). Addition of exogenous antagonists to these same cultures was also shown to be an effective way to block BMP signaling (1, 202). Examination of the role of BMP antagonists in vivo has taken two routes: creating mice with overexpression of antagonist using a bone-specific promoter, or developing mice with conditional deletions of antagonists in bone lineage cells. The most well-studied BMP antagonists present within bone matrix or made by osteoblast lineage cells are noggin and gremlin. Transgenic mice overexpressing noggin exhibit severe osteopenia, inhibition of bone formation through a decrease in osteoblast function without a significant effect on osteoblast number, and spontaneous fractures (33, 52, 262). In comparison, overexpression of gremlin using the same osteoblast-targeted promoter results in a more severe phenotype that includes alterations in the structure of both trabecular and cortical bone. Noticeably, *gremlin* transgenic mice show a transient decrease in osteoclast numbers, which, when coupled to changes in osteoblast parameters, indicates a generalized decrease in bone remodeling (65, 66). The reasons for the different phenotypes that are observed when noggin and gremlin are modulated are not immediately apparent, as both antagonists inhibit the actions of BMPs 2, 4, and 7 to similar degrees (38).

Another less-well-known BMP antagonist, Nov, is a member of the connective tissue growth factor family (31). Nov is expressed by osteoblasts and so could play a regulatory role in mediating BMP activity in the skeleton (181, 206). Overexpression of Nov using the osteoblast-specific osteocalcin promoter leads to reduced osteoblast function and a decrease in the bone formation rate in young, actively growing mice (206). However, the physiological relevance of this observation is unclear due to the extremely high levels of Nov that are produced in bone using this experimental approach (206). In fact, when Nov is inactivated in mice, no obvious skeletal abnormalities are observed during development, and Nov null mice have normal femoral length and BMD at 1, 4, 7, and 10 mo of age (34). These data suggest that Nov is dispensable for skeletal homeostasis under normal circumstances.

With regard to the required ligand(s) in postnatal skeleton homeostasis, the available evidence indicates that endoge-

nous BMP2 is uniquely required for proper osteoblast function. For instance, reduced bone mass is observed when BMP2 expression is lost in limb bud mesenchyme (239) or specifically in osteoblasts (156, 270). This appears to be due to a combination of both impaired osteoblastogenesis and reduced activity of the fewer osteoblasts that are formed. In contrast, BMP4 and BMP7 expression are dispensable for proper postnatal osteoblast differentiation and function (241, 242). Given that BMP2, 4, and 7 are each osteogenic and generally induce similar gene expression profiles, we find it more likely that the in vivo requirement for BMP2 is due to expression domain rather than unique signaling ability. This idea is consistent with data from Pregizer and Mortlock (194), who utilized *LacZ* reporters to detail the distinct expression domains of *Bmp2* and *Bmp4* in the postnatal skeleton.

In comparison to the abundant and largely consistent data regarding BMP signaling in osteoblastogenesis, there are relatively few studies that specifically investigate a role for BMP signaling in osteoclastogenesis or bone resorption. In vitro studies demonstrate that BMP2, 4, 7, and 9 each generally promote mature osteoclast function, as measured by survival in culture, surface area in contact with substrate, resorption pit size, or expression of genes required for bone matrix resorption (26, 60, 104, 283). These data largely agree with the observation that administration of exogenous BMP2 to sites of bone healing or fusion promotes a transient increase in osteoclast activity (14, 215, 237). Data concerning BMP signaling in osteoclast differentiation, however, are less consistent. Using various in vitro monocyte/macrophage cell models, some investigators have reported that exogenous BMP activation increases osteoclastogenesis (96, 182, 263, 264), whereas others report it decreases osteoclastogenesis (154) or has no significant effect (121, 232). It is possible that specific culture conditions or cellular origins might lead to conflicting findings; for instance, BMP ligands have been shown to selectively activate canonical vs. noncanonical pathways, depending on the timing of treatment (26). Regardless, these findings should be balanced with the functional data acquired from mice, wherein global loss of the BMP inhibitor Tsg leads to increased osteoclast number and activity (228), presumably due to increased BMP responsiveness. Also, loss of BMPR2 in the myeloid lineage or loss of ALK3 in differentiated osteoclasts leads to reduced osteoclast activity in vivo (26, 177). Collectively, these in vivo studies suggest that BMP signaling in the osteoclast lineage correlates with osteoclast function.

VII. IS BMP PATHWAY STIMULATION A USEFUL THERAPEUTIC FOR BONE HEALING?

The skeleton has a remarkable intrinsic capacity for repair. In secondary fracture healing, i.e., in the absence of rigid fixation, a hematoma forms at the fracture site soon after injury, signaling the recruitment of skeletal stem/progenitor cells to the injury site. Once present, these cells differentiate into chondrocytes that produce a repair callus at the endosteal surface and into osteoblasts that form new bone directly at the periosteal surface, effectively uniting the ends of the fractured bone. Remodeling of the repair tissue then occurs, reestablishing the bone marrow cavity, the bone vascular supply, and the bone material properties that allow for normal bone function (56). Studies using mouse models in which individual BMPs have been inactivated in a tissue-specific manner provide a compelling demonstration that BMP2 is required for successful bone repair due to its key role in activating skeletal progenitor cells residing in the periosteum (239). Approximately 5–10% of the time, fracture repair is incomplete or severely delayed, resulting in nonunion (287). However small in number, these nonunions are clinically significant due to the increased patient morbidity that accompanies recalcitrant or absent healing (29). BMP therapy has been used successfully in this patient population, with recombinant human BMP (rhBMP) 2 and rhBMP7 approved by the Food and Drug Administration as adjunct therapies for treatment of nonunion fractures, where the benefits of using BMPs are reported to accelerated healing and lower infection rates (142).

Apart from fracture healing, BMP therapy has been widely used in conjunction with bone grafting procedures that are performed more than 500,000 times per year in the U.S. (30). Autograft, bone harvested from the patient's own skeleton, is the optimal choice, but, by nature of being obtained from the patient, autografts are of limited supply and come with the additional trauma that occurs during graft harvest and the subsequent recovery of the skeleton at the harvest site, which are significant clinical obstacles (178). Allograft, bone harvested from cadavers, is a popular alternative to autograft; it is more readily available and provides structural support similar to native bone. However, processing of allografts to prevent disease transmission kills resident skeletal stem cells and removes most of the osteogenic activity present in bone matrix. Placement of an allograft at the repair site effectively creates a suboptimal healing environment that slows down the graft incorporation process and allows for accumulation of microdamage, leading to fatigue weakening within the allograft (12). Allograft failure rate is 20–25% in the first 5 yr after surgery, and escalates to ~60% at 10 yr after surgery (213). Adding BMPs to allografts has been shown to promote healing by initiating new bone formation at the interface between the graft and host bone (28, 272). In so doing, the potential for formation of fibrotic tissue at this site is dramatically reduced. rhBMPs 2 and 7 have shown efficacy, when combined with allograft, in a variety of preclinical animal models, and these data provided the basis for clinical trials of rhBMPs in spine fusion surgeries (146). At present, rhBMPs are approved for specific types of spine fusion; however,

Table 3. *Survey of proposed therapeutic strategies to modulate BMP signaling*

	To Increase BMP Signaling	To Reduce BMP Signaling
Intracellular target or strategy	• Increasing expression of BMP pathway components by gene transfer, pharmacological agent, or RNA-interference-mediated silencing of micro-RNAs • Potentiating BMP receptor activity by CK2.3 peptide or FK506 compound to alleviate BMP receptor inhibition • Stabilizing RA-SMAD degradation kinetics	• RNA interference-mediated silencing of BMP pathway components • Delivering BMP receptor kinase inhibitors
Extracellular target or strategy	• Increasing BMP ligand concentration by gene transfer of delivery of exogenous product • Neutralizing extracellular antagonists with antibodies, decoy ligands, or compounds • RNA interference-mediated silencing of extracellular antagonists or micro-RNAs	• Increasing extracellular antagonist concentration by gene transfer or delivery of exogenous product • Neutralizing BMP ligand with antibodies • RNA interference-mediated silencing of BMP ligand expression

The reader is directed to two recent reviews for an extended discussion of these strategies and specific applications (142, 146). BMP, bone morphogenetic protein; RA-SMAD, receptor-activated-SMAD.

concerns over the supraphysiological doses of recombinant protein required for successful healing remain and continue to limit the potential of BMPs as therapeutic agents. A comprehensive review of available clinical data regarding rh-BMP use during grafting procedures can be found elsewhere (17, 43).

VIII. FINAL PERSPECTIVES

In our opinion, the information discussed above solidly demonstrates the importance of appropriately regulated strength, timing, location, and duration of BMP signaling in skeletal development and homeostasis. That said, there is much yet to be discovered about the endogenous mechanisms underlying these effects and how to best modulate this pathway to treat skeletal disease and improve patient quality of life (146). In conclusion, we wish to highlight two major areas of need for future study.

First, despite significant advancement, there remains relatively poor resolution of the vast gene regulatory network that is downstream of the BMP pathway during skeletal development and remodeling. Clarification of the hierarchical relationships that exist between effectors, transcription factors, and regulators and interaction with other relevant signaling pathways is required for a fully comprehensive understanding of the BMP pathway. The increasing adoption of new and emerging technologies, such as chromatin immunoprecipitation coupled with next generation sequencing (ChIP-Seq) and transcriptome-wide RNA-sequencing, place this goal within reach. That being said, these approaches should ideally be coupled with functional analyses, such as Cre-loxP technology. Unfortunately, even with advances such as CRISPR/Cas9, developing conditional gene knockout models remains laborious and costly, and there are limited Cre recombinase drivers available, especially for the postnatal skeleton.

This underscores a need, then, for reagents to reliably and specifically inhibit individual BMP pathway components (142). One can envision opportunities for inhibition strategies ranging from discovery-driven research to clinically-oriented interventions. At present, however, there is a paucity of tools, such as small-molecule inhibitors or neutralizing antibodies, to accomplish this work. The current strategies designed to modulate BMP pathway activity are summarized in **TABLE 3**. Certainly, the large degree of homology and functional redundancy within the pathway poses a challenge for this area of research. Yet notable exceptions do exist, and these should serve as motivation for extension to other targets. For instance, neutralizing antibodies have been reported for a handful of ligands (BMP2, 4, 6, 7, and 10), antagonists (noggin and gremlin), and receptors (ALK1, ACVR2A, and ACVR2B) (126, 142). Also, the advances achieved in pursuit of small-molecule type 1 receptor inhibitors for the treatment of FOP (142) is remarkable in its rapidity. In silico evidence indicates that compounds may exist for the inhibition of targets such as noggin and SMURF1 as well (3, 35, 108, 176).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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