New insights on the roles of BMP signaling in bone—A review of recent mouse genetic studies

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Abstract.

It is well known that Bone morphogenetic proteins (BMPs) induce bone formation and that some BMPs, including BMP2 and BMP7, are clinically used in orthopedics. Signaling by BMPs plays an important role in a variety of cell-types in bone such as osteoblasts, chondrocytes, and osteoclasts. It is recently reported using an osteoblast-targeted deletion of BMP signaling that BMP signaling in osteoblasts physiologically induces bone resorption by enhancing osteoclastogenesis via the RANKL-OPG pathway and reduces bone mass. In this review, The physiological function of BMP signaling in bone will be focused, and the current outcomes from mouse genetic studies will be discuss.

1. Introduction

Bone morphogenetic proteins (BMPs) were discovered and named in 1965 by Marshall Urist, who initially identified the ability of an unknown factor in bone to induce ectopic bones in muscle [1]. In the last 45 years, the osteogenic function of BMPs has been extensively examined, mainly using osteoblasts in culture with exogenous treatments of BMPs [2]. Based on their potent osteogenic abilities, clinical trials have been initiated to use BMP2 and BMP7 to improve fracture repair [2]. After successful completion of the trials, the FDA has approved BMP2 and BMP7 for clinical use in long bone open-fractures, nonunion fractures, and spinal fusion. Similarly to osteogenic BMPs *in vitro*, studies of human mutations also suggested the importance of BMP signaling for skeletogenesis and bone-related diseases such as chondrodysplasia and fibrodysplasia ossificans progressive [3,4]. Mutations in genes involving BMP signaling associated with skeletal abnormalities in humans are summarized in Table 1 [5–12].

These facts indicate that BMP signaling is involved in the proper development of many components of the skeletomuscular system including bone, cartilage, and soft tissues such as muscle, fat, and tendons. Among them, bone and cartilage are the major components in the skeletal system, and the osteoblast and chondrocyte are the responsible cell types for formation and functions of these tissues, respectively. The osteogenic function of BMPs and BMP signaling has been further investigated over the last decade using a gene targeting technology. This article focuses on the physiological effects of BMP signaling on bone formation, bone resorption, and bone mass, specifically via its action on osteoblasts or chondrocytes by reviewing mouse genetic studies of skeletal development and bone remodeling.

2. BMP signaling and kinase

BMPs belong to the transforming growth factor-β (TGF-β) gene superfamily [13,14], and this family of BMPs comprises ~30 structurally related members. Similar to TGF-β, BMPs signal through transmembrane serine/threonine kinase receptors such as BMP type I and type II receptors. Upon ligand binding, type I and II receptors form heteromultimers [15], and a type II receptor phosphorylates a short stretch of amino acids called a GS box (the glycine- and serine-rich domain between the transmembrane and kinase domains) in the type I receptor to activate its kinase activity. Activated BMP type I receptors relay the signal to the cytoplasm by phosphorylating their immediate downstream targets, Smad1, Smad5, and Smad8 proteins, which then interact with Smad4 and translocate into the nucleus [16]. There are three type I receptors (BMPRIA, BMPRIB, and ACVRI) and three type II receptors (BMPRII, ActRIIA, and ActRIIB) that bind to BMP ligands to signal. The type I receptor ACVRI was originally found as an activin receptor, but it is now believed to be a receptor for BMPs. The specificity of

Table 1
Skeletal abnormalities associated with BMP signaling

Gene	Disease	Ref.
BMP2 regulatory element	brachydactyly type A2	5
BMP4	poly/syndactyly	6
CDMP1/GDF5	acromesomelic chondrodysplasia	3
	brachydactyly type C	7
GDF6	hemi-vertebrae, poly dactyly, Klippel-Feil rib malformation, spondylotheracis dysostosis	8
GDF3	scoliosis, Klippel-Feil, vertebral fusion	9
BMPR1B	brachydactyly type A2	10
	acromesomelic chondrodysplasia	11
ALK2/ACVR1	fibrodysplasia ossificans progressiva	4
NOGGIN	brachydactyly type B	12

signaling is primarily determined by type I receptors [17]; however, the specificity of ligand binding is altered by the combination of type I and II receptors [18].

3. Genetic approaches to uncover functions of BMP signaling in mice

Along with the huge advancement in technologies involving mouse genetics over the last decade, many of the BMP signaling related genes have been knocked out in mice. BMP2, BMP4, BMP6, and BMP7 and their receptor BMPRIA and ACVRI are abundantly expressed in bone. It has been reported that BMPRIA is a potent receptor of BMP2 and BMP4 [19,20], and ACVR1 is a receptor of BMP7 [21]. However, conventional knockout mice for these genes result in an early embryonic lethality and thus, it is not possible to investigate bone development and remodeling using these models [22–28]. To avoid the embryonic lethality, a strategy of conditional knockout mice using a Cre-loxP system has been used. A bone-specific conditional deletion of *Bmpr1a* using an *Og2-Cre* mouse, in which a Cre recombination is restricted in differentiated osteoblasts under the osteocalcin promoter, was first reported in 2004 [29]. Interestingly, this study demonstrated that the response of osteoblasts to BMP signaling is age-dependent; in the mutant mice, bone volume decreased in young mice but increased in aged mice. In addition, the activity of osteoclasts was reduced in the aged osteoblast-specific *Bmpr1a*-deficient mice, which may have lead to the complex skeletal phenotype. These facts suggest that the BMP signaling in differentiated osteoblasts can control the balance between bone formation by osteoblasts and resorption by osteoclasts, thereby affecting the final outcome of the amount of bone mass in an age-dependent manner. The increased bone mass in the *Bmpr1a*-deficient mice appeared to be in opposition to the general

concept of BMPs as osteogenic inducers. This leads to a possibility that osteogenic targets of BMPs would be mesenchymal cells or chondrocytes, rather than osteoblasts. It is reasonable to speculate that different cell types exhibit differing responses to BMPs as evidenced by their multifaceted functions *in vivo* [14,30]. The "opposite" outcome in the *Bmpr1a*-deficient mice will be discussed later at a cellular mechanistic point of view in Section 4 and at a molecular mechanistic point of view in Sections 5, 6, and 7.

4. BMP signaling and chondrocytes

During skeletogenesis, bones are formed via two distinct processes: intramembranous and endochondral bone formation [31]. Intramembranous bone formation occurs primarily in flat bones (*e.g.*, calvarial bones) where mesenchymal cells differentiate directly into osteoblasts [32]. Endochondral bone formation occurs primarily in long bones where condensed mesenchymal cells differentiate into chondrocytes to form cartilage templates, and then chondrocytes are replaced by osteoblasts [33]. Recently, many studies have been designed to investigate the difference in the molecular mechanism by which BMP signaling regulates these cell types. A variety of Cre mouse lines have been used to target different cell types including osteoblast, chondrocyte, and mesenchymal cells as summarized in Table 2.

There are several lines of evidence that show that BMP signaling in chondrocytes is required for bone size and the amount of bone mass. BMP signaling through BMPRIA is essential for postnatal maintenance of articular cartilage, using a *Gdf5-Cre* mouse line specific for chondrocytes in joints [37]. Similarly, the critical role of *Bmpr1a* together with *Bmpr1b* in chondrocytes during endochondral bone formation using a *Col2-Cre* mouse line was reported [38]. Moreover, in chondrocytes, a simultaneous deficiency in Smad 1 and Smad 5, which are BMPs' downstream target molecules, reduces bone mass [40]. In parallel, studies focusing on BMP ligands and their antagonists provide further evidence that BMPs are critical for normal development of cartilage. A transgenic mouse line to overexpress *Bmp4* in mesenchymal cells/chondrocytes using a type XI collagen promoter (*Col11a2*) was generated, and bone mass was increased in the mutant mice [39]. Another transgenic mouse line in which *Noggin* was overexpressed in the same cells (*Col11a2-Noggin*) demonstrated a decreased bone mass. As Noggin is an antagonist for BMPs (BMP2, BMP4, BMP5, BMP6, and BMP7) with various degrees of affinity [42], these results suggest that BMP signaling positively controls proliferation and differentiation of chondrocytes.

Similar to chondrocytes, a few studies demonstrated a requirement of BMP signaling in mesenchymal cells for proper bone development and remodeling. In a *Prx1-Cre* mouse line, Cre is active in mesenchymal cells as early as embryonic day 9.5 [43]. Using the *Prx1-Cre* mouse, the simultaneously conditional deletions of *Bmp2* and *Bmp4* in mesenchymal cells resulted in an impairment of osteogenesis during late embryogenesis [34,35]. In contrast, the

Table 2
Bone mass observed in genetically engineered mutant mice for BMP signaling

Target cell type/gene	Promoter	BMP signal	Stage	Bone mass	Ref.
Mesenchymal cells					
Double knockout of BMP2 and BMP4	Prx1-Cre	down	E10.5–3M	Reduced	34
Bmp2 cKO	Prx1-Cre	down	5 M	Reduced	35
Bmp7 cKO	Prx1-Cre	down	E10.5–13M	No change	36
Chondrocytes					
Bmpr1a cKO	Gdf5-Cre	down	E12.5–16.5	Reduced	37
Double knockout of Bmpr1a and Bmpr1b	Col2-Cre	down	E12.5–16.5	Reduced	38
Bmp4 overexpression	Col11a2	up	E18.5	Increased	39
Noggin overexpression	Col11a2	down	E18.5	Reduced	39
Double knockout of Smad1 and Smad5	Col2-Cre	down	E12.5-Newborn	Reduced	40
Osteoblasts					
Bmpr1a cKO	Og2-Cre	down	3M	Reduced	29
			10 M	Increased	29
Bmp4 overexpression	2.3 kb Col1	up	E18.5	Reduced	41
Noggin overexpression	2.3 kb Col1	down	E17.5, 3w	Reduced	41
Bmpr1a cKO	3.2 kb Col1	down	E18.5, 3w, 5M	Reduced	47, 48 and 68

conditional deletion of *Bmp2* in mesenchymal cells does not show overt developmental abnormalities, suggesting a compensation of BMP2 function by other BMPs such as BMP4. Interestingly, the *Bmp2*-deficient mice lack an initiation of fracture healing [34,35]. Interestingly, *Bmp7*-deficiency in mesenchymal cells did not affect bone mass probably due to the compensation by Bmp4 [36]. Taken together, it is possible that the defects in the BMP signaling in chondrocytes largely contribute to the phenotypes described above because chondrocytes are derived from mesenchymal cells and play an important role in the process of fracture repair.

Recent histological findings suggest that endochondral bone formation plays a critical role in the process of ectopic bone formation [44]. The origin of precursor cells for the ectopic bone is under investigation [45,46]; however, it is possible that formation of ectopic bones by BMPs [1] is largely due to the stimulation of chondrocytes or mesenchymal cells in soft tissue, which results in an expansion of ectopic cartilage subsequently replaced by osteoblasts. There is another possibility that the BMP signaling directly affects osteoblasts to form ectopic bone. However, this possibility is less likely based on recent evidence that reduced BMP signaling in osteoblasts results in an increase in bone mass.

5. BMP signaling and osteoblasts

As aforementioned, a differentiated osteoblast-specific deletion of *Bmpr1a* caused an increase in bone mass in aged mice [29]. Similar to this finding, an overexpression of a BMP antagonist, Noggin, in osteoblasts increases bone volume with a reduced osteoclast number and osteoclastogenesis both at embryonic day 17.5 (E17.5) and at 3 weeks [41]. In parallel, the overexpression of *Bmp4* in osteoblasts reduced bone mass presumably due to the increase in the osteoclast number at E18.5 [41]. Recently, *Bmpr1a* was conditionally disrupted in immature osteoblasts using a tamoxifen

inducible Cre driven by a 3.2-kb alpha1(I) collagen chain gene (*Col1a1*) promoter. In the mutant mice, bone mass was dramatically increased during the bone remodeling stage at 22 weeks as well as the bone developmental stages at E18.5 and 3 weeks (Fig. 1) [47,48]. This result is an interesting contrast to previous works that disruption of *Bmpr1a* in differentiated osteoblasts results in decrease of bone mass in young adult stages (3–4 weeks). The increased bone mass in the *Bmpr1a*-deficient mice resulted from severely suppressed bone resorption due to reduced osteoclastogenesis, despite a simultaneous small reduction in the rate of bone formation [48]. Levels of RANK ligand (RANKL) and osteoprotegerin (OPG) (see Section 6 for details) are changed in the *Bmpr1a*-deficient osteoblasts and fail to support osteoclastogenesis [47,48]. In addition, the conditional disruption of *Acvr1* in osteoblasts also demonstrated a dramatic increase in bone mass, similar to the bone phenotype of *Bmpr1a*-deficient mice (unpublished data). These findings suggest that BMP signaling has dual roles in osteoblasts; to stimulate both bone formation by osteoblasts and bone resorption supporting osteoclastogenesis. Disruption of BMP signaling in immature osteoblasts alters the balance of bone turn over to increase the bone mass, which is opposite to what people have expected for the past 4 decades.

6. BMP signaling in osteoblasts that regulates osteoclastogenesis

Bone mass is determined by the balance between bone formation and bone resorption. Osteoclasts are multinuclear cells derived from hematopoietic stem cells to secrete enzymes for bone resorption [49]. It is expected that BMPs play roles in osteoclastogenesis and their functions, because receptors for BMPs are expressed in these cells [50]. In addition, osteoblasts also play critical roles in bone
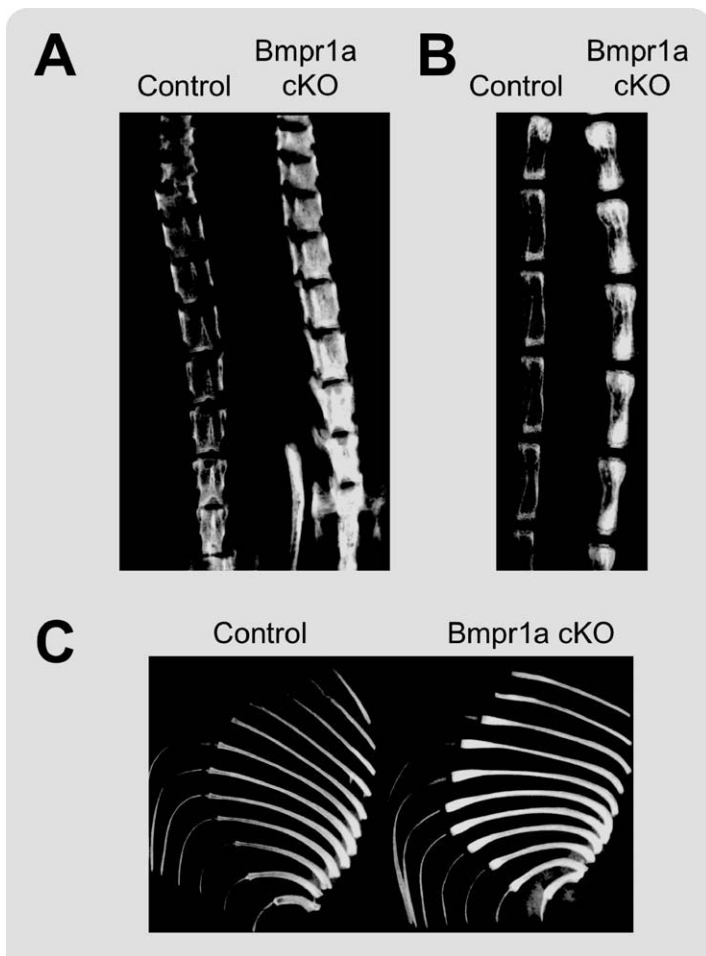
Fig. 1. Increased bone mass in the osteoblast-specific *Bmpr1a* **conditional knockout (cKO) mouse at the adult stage.** *Bmpr1a* **cKO mouse was generated by crossing a floxed** *Bmpr1a* **mouse line with a transgenic mouse line harboring a tamoxifen–inducible Cre driven by a 3.2 kb mouse** *procollagen α1I* **promoter. The Cre recombination was induced specifically in the osteoblasts by 10 weeks of tamoxifen administration from 10 weeks after birth, and bones were removed at 22 weeks. Radiodensity of the spine (A), tail (B) and rib bones (C) was dramatically increased in the** *Bmpr1a* **cKO mice (Cre+,** *Bmpr1a***fx/fx) compared with controls (***Cre−,*** *Bmpr1a***fx/fx) when assessed by X-ray imaging.**

resorption by regulating osteoclastogenesis because they produce RANK ligand (RANKL), essential to promote osteo-clastogenesis, and its decoy receptor, OPG [51,52]. A balance between RANKL and OPG is important to determine the degree of osteoclastogenesis, that is, more RANKL production by osteoblasts leads to more osteoclasts; thus more bone resorption is expected. As RANKL is an osteoblastic product and BMPs induce osteoblast maturation, BMPs indi-rectly stimulate osteoclastogenesis and thus, osteoclasto-genesis is impaired when osteoblastogenesis is blocked with BMP antagonists in culture [53]. The physiological effects of BMP signaling in osteoblasts on osteoclastogenesis were

determined later using an osteoblast-specific gain-of-function or loss-of-function mouse model. For the cases of the osteo-blast-specific deletion of *Bmpr1a* and osteoblast-specific over-expression of *Noggin*, osteoclastogenesis is highly compro-mised leading to an increase of bone mass [29,41]. In con-trast, osteoblast-specific overexpression of *Bmp4* increased osteoclastogenesis [41]. The regulation of RANKL by BMPs was suggested based on an *in vitro* study [54]. This concept was recently proven in mouse studies, as *Bmpr1a*-deficient osteoblasts were not able to support osteoclastogenesis due to an imbalance between RANKL and OPG [47,48].

There is accumulating evidence that Wnt signaling also plays a critical role in osteoclastogenesis regulated by osteoblasts through the RANKL-OPG pathway. As discussed in Section 7, how BMP and Wnt signaling interact with each other is an interesting topic. Recently, two *in vivo* studies have suggested that the canonical Wnt signaling is impor-tant in the regulation of osteoclastogenesis by osteoblasts. One study provided evidence that the Wnt pathway posi-tively regulates the expression of *Opg* in osteoblasts [55]. Overexpression of stabilized β-catenin in osteoblasts, which results in an increase of canonical Wnt signaling level, decreases osteoclast differentiation leading to increased bone volume in mice [55]. Another study showed that an osteoblast-specific deletion of β-catenin leads to an impaired maturation and mineralization of bones in mice due to the elevated expression of RANKL and diminished OPG [56]. These facts suggest that the canonical Wnt pathway nega-tively regulates osteoblasts in their supporting function in osteoclastogenesis, and thus upregulation of Wnt signaling in osteoblasts can suppress osteoclast-mediated bone resorption [56].

7. Interplays between BMP and Wnt signaling in bone

Both BMP and Wnt signaling regulate development and remodeling of many tissues and organs. Numerous studies have reported functions of each signaling [57–62]. Results from these studies suggest that these two signals regulate one another synergistically or antagonistically in context-dependent and age-dependent manners. In bone, experi-ments using pluripotent mesenchymal cell lines to test the interaction between BMP and Wnt signaling in osteoblasts have yielded both synergistic and antagonistic results: BMP2 induces both Wnt3a and Wnt/β-catenin signaling [63,64], while Wnt3a in turn enhances the BMP4 expression [65], suggesting a positive autocrine loop [66,67]. However, inhibition of BMP signaling by treatment of osteoblasts with dorsomorphin, a selective inhibitor for BMP type I receptors, increases the canonical Wnt signaling [68]. Further, Wnt3a is reported to repress BMP2-dependent *Id1* expression [69], suggesting a negative feedback loop. *In vivo*, the BMP signaling in osteoblasts downregulates the canonical Wnt signaling during embryonic and postnatal bone development [47,68]. This is due to the fact that Wnt inhibitors Sost (sclerostin) and Dkk1 are direct targets of the BMP signaling
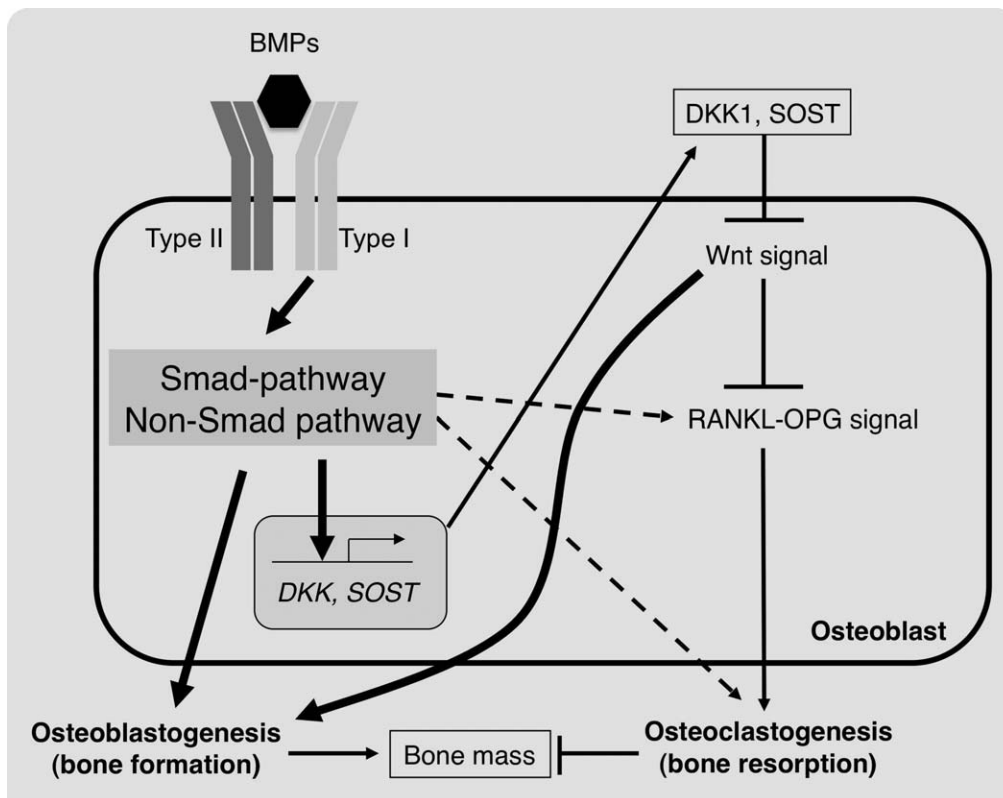
Fig. 2. A proposed model of the relationship between the BMP signaling via BMPRIA and the canonical Wnt signaling in osteoblasts. Both Dkk1 and sclerostin/Sost are downstream targets of the BMP signaling. The BMP signaling upregulates the Sost expression primarily through the Smad-dependent signaling while it upregulates the Dkk1 expression through both the Smad and non-Smad signaling (p38 MAPK). As Dkk1 and sclerostin/Sost act as Wnt signaling inhibitors, BMP signaling in osteoblasts, in turn, leads to a decrease in bone mass through regulating expressions of RANKL and OPG to suppress osteoclastogenesis. Dkk1 and sclerostin/Sost play an important role in regulating bone mass as downstream effectors of BMPRIA signaling in bone taking balances between BMP signaling and Wnt signaling, as well as bone formation and bone resorption.

(Fig. 2). It is noted that the *Sost* was the most downregulated gene in the *Bmpr1a*-deficient bone as assessed by microarray analysis [47]. Interestingly, both Smad-dependent and Smad-independent pathways appear to contribute to the Dkk1 expression, whereas Sost expression requires only Smad-dependent signaling, suggesting a differential regulation of these genes by the BMP signaling via BMPRIA (Fig. 2) [68].

Both Dkk1 and Sost are expressed by osteoblasts as secreted proteins which inhibit Wnt/β-catenin signaling by binding to co-receptors, low density lipoprotein receptor-related protein 5 and 6 (LRP5 and LRP6) [70]. Conventional knockouts of *Dkk1* die *in utero* from defective head induction and limb formation [71]. Mice heterozygous for *Dkk1* (*Dkk1*$^{+/-}$ mice) exhibit a high bone mass (HBM) phenotype [72], whereas overexpression of *Dkk1* in osteoblasts causes osteopenia [73]. In addition, the increased *DKK1* expression in bone marrow has also been associated with lytic bone lesions in patients with multiple myeloma [74]. Collectively, these results support the hypothesis that Dkk1 functions as a potent negative regulator of bone mass. Conventional knockouts of *Sost* are viable and exhibit increased bone

mass [75], similar to *Dkk1*$^{+/-}$ mice. In humans, loss-of-function and hypomorphic mutations in *SOST* cause sclerosteosis [76,77] and Van Buchem disease [78,79], respectively, with a HBM phenotype. Consistent with these observations, conditional knockouts of *Bmpr1a*, which are deficient in the *Dkk1* and *Sost* expression, show a HBM phenotype [48]. Furthermore, an increased expression of *Dkk1* and *Sost* in osteoblasts by constitutively activated BMPRIA signaling is associated with partial rescue of the bone phenotype of *Bmpr1a*-deficient mice [68]. Therefore, it is possible that Dkk1 and Sost (sclerostin) act physiologically as downstream molecules of BMP signaling to inhibit canonical Wnt signaling and therefore negatively regulate bone mass, at least, in mice as shown in Fig. 2.

8. Clinical application of BMPs

The FDA has approved BMP2 and BMP7 for clinical use in long bone open-fractures, nonunion fractures, and spinal fusion, and BMPs' treatment has shown a clear benefit for patients. However, despite significant evidence of their abilities for bone

regeneration in animal and preclinical studies, some clinical data are unconvincing to support the effectiveness of BMP treatment on fracture healing and spine surgery [80–83]. This is partly because of BMPs' numerous functions by cell type. It is important to understand that BMPs have variable and context-sensitive effects on diverse cell types in bone including chondrocytes, osteoblasts, and osteoclasts. Studies focusing on BMP receptors in chondrocytes including mesenchymal cells suggest that these cells can respond to BMP signaling by increasing bone mass during the endochondral formation process as discussed earlier. In contrast, when the function of osteoblast-dependent BMP signaling is examined with respect to bone mass determination, BMP signals can consistently inhibit Wnt signaling and bone mass while exerting concordant effects on *Dkk1* and *Sost*. The function of the BMP signaling in osteoclasts remains largely unknown and merits future study, although the BMP signaling regulates osteoblast-dependent osteoclastogenesis via the RANKL-OPG pathway. This revision of traditional understanding of the BMP signaling pathway in clinical therapeutics might suggest that in some circumstances, BMP inhibition would be desirable for promoting bone mass.

9. Conclusions

Understanding the complex roles of the BMP signaling pathway in a variety of cell-types in bone including chondrocytes, osteoblasts and osteoclasts, which contribute to bone development, homeostasis, and remodeling will not only help to improve current knowledge of the dynamic processes which are perturbed in the settings of bone fracture, mechanical loading, and congenital and aging-related bone diseases but may provide novel therapeutically useful strategies.

Acknowledgements

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