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BMP signaling in mesenchymal stem cell differentiation and bone formation*

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Abstract

Bone morphogenetic proteins (BMPs) are members of the TGF- β superfamily and have diverse functions during development and organogenesis. BMPs play a major role in skeletal development and bone formation, and disruptions in BMP signaling cause a variety of skeletal and extraskeletal anomalies. Several knockout models have provided insight into the mechanisms responsible for these phenotypes. Proper bone formation requires the differentiation of osteoblasts from mesenchymal stem cell (MSC) precursors, a process mediated in part by BMP signaling. Multiple BMPs, including BMP2, BMP6, BMP7 and BMP9, promote osteoblastic differentiation of MSCs both *in vitro* and *in vivo*. BMP9 is one of the most osteogenic BMPs yet is a poorly characterized member of the BMP family. Several studies demonstrate that the mechanisms controlling BMP9-mediated osteogenesis differ from other osteogenic BMPs, but little is known about these specific mechanisms. Several pathways critical to BMP9-mediated osteogenesis are also important in the differentiation of other cell lineages, including adipocytes and chondrocytes. BMP9 has also demonstrated translational promise in spinal fusion and bone fracture repair. This review will summarize our current knowledge of BMP-mediated osteogenesis, with a focus on BMP9, by presenting recently completed work which may help us to further elucidate these pathways.

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Keywords

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1. Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming group factor-beta (TGF- β) superfamily. This group of homologous signaling proteins has a diverse number of functions and plays an important role in embryogenesis, organogenesis, cell proliferation and stem cell differentiation [1-5]. For example, BMP7 is involved in proper kidney, eye, and limb development; BMPs 4, 7 and 15 are important for proper reproductive tissue development; BMPs 2, 3 and 7 contribute to cartilage regeneration; and BMPs 12 and 13 are required for normal tendon healing [6-12]. BMPs also play a major role in skeletal development, bone formation and mesenchymal stem cell (MSC) differentiation [13,14].

MSCs are adult stem cells found in the bone marrow and like all other types of stem cells, have the unique ability to self-renew and to differentiate into various mesodermal cell lineages, osteoblastic, chondrocytic, myocytic and adipocytic, as well as non-mesodermal tissues, such as cardiac muscle and skin [11,13,15-18] (Figure 1). The differentiation of these multipotent stem cells towards an osteoblastic fate depends on numerous signaling pathways, including BMP transduction. The osteoinductive ability of BMPs was discovered when it was found that demineralized bone could induce *de novo* bone formation and that BMPs were responsible for this observed osteogenesis [19,20]. Disruptions in BMP signaling have subsequently been shown to result in a variety of skeletal and extraskelatal anomalies [21,22]. At least 15 different BMPs have been identified in humans to date.

BMP9, also known as growth differentiation factor 2 or GDF-2, is a relatively poorly characterized member of the BMP family first isolated from fetal mouse liver cDNA libraries. BMP9 is expressed at high levels within the developing mouse liver and acts to stimulate hepatocyte proliferation [23]. It also acts to induce and maintain the cholinergic phenotype within basal forebrain neurons, inhibit hepatic glucose production, inhibit enzymes of lipid metabolism, maintain metabolic homeostasis of iron and synergize in the generation of hematopoietic progenitor cells [24-26].

BMP9 is among the most osteogenic BMPs and promotes the osteoblastic differentiation of mesenchymal stem cells (MSCs) both *in vitro* and *in vivo* [11,13,27-30]. We have demonstrated that BMP9 regulates a distinct set of downstream targets likely playing a role in osteoinduction, and these targets will be discussed later in this review [11,27-30]. While BMP9 has been demonstrated as one of the most osteogenic BMPs, little is known about the detailed mechanisms responsible for its functions. This review aims to summarize our current knowledge of BMP9-mediated osteogenesis, which may help us to further elucidate these pathways.

2. Axial Skeletal Development and MSCs

Mesenchymal stem cells undergo several stages of maturation during their proliferation and differentiation along the osteoblastic lineage. MSCs initially form preosteoblasts, which proliferate near the surface of bone and secrete alkaline phosphatase, an early marker of osteogenesis [11,31-33]. Preosteoblasts further mature into osteoblasts, which are involved in initial extracellular matrix maturation and mineralization. Osteoblasts ultimately form osteocytes, which are mature, terminally differentiated cells embedded in an extracellular matrix responsible for mechanical support and regulating the mineralization of bone [34,35]. These stages of osteogenic proliferation and differentiation are characterized by the expression of various markers, including cell-cycle associated genes during the proliferative phase, the early osteoblastic marker alkaline phosphatase and late markers osteocalcin and osteopontin [36].

From MSCs, bone can form in one of two ways, either by endochondral or intramembranous ossification [36]. The majority of bones in the human skeleton are formed via endochondral ossification, whereby MSCs first differentiate into chondrocytes and secrete a cartilaginous matrix. This matrix subsequently undergoes osteoblast-facilitated ossification to form bone [37-39]. Flat bones, which mainly comprise the axial skeleton, are formed by intramembranous ossification and do not have a cartilaginous precursor scaffold. Instead, MSCs differentiate directly into osteoblasts, which secrete an osteoid matrix to form bone [37,38]. The bone formed by both of these processes is a highly vascularized tissue which undergoes constant remodeling, necessitating a balance between hematopoietic-derived osteoclasts, which break down bone, and mesenchymal-derived osteoblasts, which rebuild bone [36,40,41]. Thus, bone maintenance and remodeling depends, in part, on the proper formation of osteoblasts from MSCs, a highly regulated and complex process in which the BMP signaling pathway plays a critical role.

3. BMP Knockout Phenotypes

The BMP signaling pathway plays many crucial roles in bone formation and is involved in multiple stages of the developmental process, including osteoblast differentiation, mesoderm patterning, bone formation, and craniofacial and limb development. Knockout of specific BMPs or mediators of BMP signal transduction often leads to phenotypes which demonstrate the critical importance of BMP signaling in skeletal development.

BMP signaling is required for the differentiation of multipotent mesenchymal cells into osteochondroprogenitor cells, which are capable of forming both chondrocytes and osteoblasts. BMP signal transduction is also necessary for the proper functioning of differentiated osteoblasts, enabling them to appropriately secrete the matrix upon which bone formation occurs [31]. This is especially important during development, when the axial skeleton forms from cellular condensations of mesenchymal cells, which proceed to form bone via the previously described process of endochondral ossification. The first genetic evidence that BMPs have a role in bone morphogenesis was natural loss-of-function mutations of BMP5, resulting in *short ear* phenotype and brachypodism [22,42]. Subsequent

studies have supported the crucial role of BMP signaling in both cartilage and bone formation during endochondral ossification [43-46].

To investigate the functions of BMP2 and BMP4 in the growth plate, chondrocyte-specific BMP2 and BMP4 conditional knockout mice and BMP2/BMP4 conditional double knockout mice were developed [47]. Deletion of BMP2 and BMP4 or BMP2 alone resulted in a severe chondrodysplastic phenotype, while deletion of BMP4 alone had minor effects on cartilage development. Double knockout and BMP2 knockouts demonstrated disorganization of chondrocytes within the growth plate, decreased cell proliferation, poor differentiation and increased apoptosis. BMP2 up-regulated protein expression of the essential osteogenic regulator Runx2. These findings demonstrate that BMP2, but not BMP4, is critical in chondrocytic differentiation and hypertrophy within the growth plate during endochondral bone formation.

BMPs have been shown to regulate endochondral ossification by promoting chondrocyte proliferation and inducing chondrocyte hypertrophy [48-50]. Loss of the inhibitory Smad, Smad6, in mice causes defects in axial and appendicular skeletal development [48]. Smad6 double knockouts demonstrate posterior transformation of the cervical vertebrae, ossification centers within lumbar vertebrae and incomplete sternal band fusion. These mice feature delayed hypertrophic differentiation and mineralization during gestation. However, by late gestation, the hypertrophic zone within the growth plate demonstrated an increased pool of proliferating, hypertrophic chondrocytes likely caused by increased BMP responsiveness in the Smad6 knockout mutants. Smad6 thus appears to be necessary to limit BMP signaling during endochondral bone formation.

Smad1 is a critical immediate downstream mediator of BMP receptor transduction [51,52]. Chondrocyte-specific and osteoblast-specific Smad1 conditional knockout mice were bred to assess postnatal bone formation [51]. Chondrocyte-specific deletion of the Smad1 gene resulted in delayed calvarial bone development. Osteoblast-specific deletion resulted in partial inhibition of BMP signaling and an osteopenic phenotype, with impaired osteoblastic proliferation and differentiation. These findings demonstrate the critical role of Smad1 and BMP signal transduction in postnatal bone formation.

Several studies have also investigated mediators and inhibitors of BMP signal transduction. Noggin is a well-established antagonist of BMPs, and overexpression of noggin results in osteopenia [53-56]. Other studies have also shown that bone and skeletal development are decreased when BMP antagonists are overexpressed in osteoblasts [45,57]. While complete inactivation of noggin results in death *in utero*, noggin conditional knockout mice demonstrate decreased weight, shortened femoral length and osteopenia [53]. Although bone formation was found to be increased in 3-month-old mice, adult female mice did not exhibit increased bone formation or remodeling. Noggin-deficient mice also exhibit enlarged growth plates and joint fusions [48,58]. These findings indicate that either BMP excess can have a detrimental effect on bone or that noggin has BMP-independent roles in skeletal homeostasis.

In mice with conditional knockout of BMP receptor type IA (BMPRIA), an unexpected increase in bone mass was observed in mouse embryos, neonates and adults [59-61]. Adult bone demonstrated severely decreased resorption due to reduced RANKL-OPG-induced osteoclastogenesis [53]. Expression levels of both bone formation and resorption makers were decreased. In another study, mice with osteoblasts expressing a dominant-negative, non-functional BMP receptor (BMPRIB) exhibited a decrease in bone mineral density, bone volume, and bone formation [62]. Thus, BMP signaling is important both for the initial formation of chondrocytes and cartilage, as well as the subsequent formation of bone. These findings demonstrate the importance of BMP signaling in regulating the balance between bone formation and resorption.

Unlike the axial skeleton, which forms from cellular condensations of mesenchymal cells, the craniofacial skeleton and its associated cartilaginous elements are formed from the neural crest. This group of pluripotent cells forms in the dorsal neural tube and subsequently migrates to various areas of the developing embryo, including the skull, differentiating into bone, cartilage, and connective tissue of the head and neck. BMP signaling has been found to play a role in proper neural crest cell formation, migration, and differentiation, with BMP2, 4, and 7 specifically expressed during craniofacial skeletal development [63,64]. The importance of BMP signaling in craniofacial development is highlighted by multiple studies [65,66]. In one study, when Alk2, a type I BMP receptor, was conditionally deleted from a murine neural crest population, subsequent craniofacial malformations occurred, including cleft palate, hypotrophic mandible, and reduced ossification of the frontal bone [67].

BMPs are also expressed in the developing limb bud, where they play a role in proper limb formation and contribute to limb patterning along three different axes: anterior-posterior, dorsal-ventral, and proximal-distal. The apical ectodermal ridge is a structure located at the distal ectodermal tip of the limb bud and is especially important for proximal-distal limb patterning. It has been shown that BMP2, 4, and 7 are expressed in the AER, as well as in underlying mesenchymal cells, where these signaling cascades are likely involved in proper limb development [2,22]. Since BMP2 and BMP4 are embryonic lethal, conditional knockouts were created to remove BMP2 and BMP4 from the limb bud mesenchyme [68]. A threshold level of BMP signaling was found to be required for the onset of chondrogenesis, and chondrogenic condensations were not formed in limbs deficient in both BMP2 and BMP4. When condensations did form, however, chondrogenic differentiation proceeded normally in the absence of BMP2 and BMP7 or BMP2 and BMP4. Additionally, the combined knockdown of BMP2 and BMP4 from the limb bud resulted in lack of bone marrow cavity formation, as well as inability to form trabecular or cortical bone. Decreased expression of osterix, an osteoblast-specific gene, was also observed, showing that osteoblast differentiation was also decreased in this knockout model [68]. These findings demonstrate the importance of BMP2 and BMP4 in proper limb formation.

Other BMP knockout models also highlight the importance of this signaling cascade in proper bone and skeletal development. BMP7 mutants have multiple skeletal defects, as well as kidney and eye defects [69,70]. However, BMP6 mutations only result in minor sterna defects [71]. BMP11 mutations result in defects of anterior-posterior axial skeletal patterning [72]. Meanwhile, BMP3 inactivation results in increased bone density [73].

Although BMP2 and -4 mutant embryos die in utero before limb phenotypes can be determined, deletion of BMP7 embryos display polydactyly with incomplete penetrance and otherwise normal limbs [5,69,70,74]. These many studies highlight the critical importance of BMPs and mediators of BMP signal transduction in skeletal development and homeostasis (Table 1).

4. BMPs and Osteogenesis

While the specific molecular mechanisms underlying BMP-mediated osteogenesis are not well-characterized, studies have demonstrated that BMPs play a critical role in osteogenic differentiation; overexpression of osteogenic BMPs with adenoviral, retroviral and recombinant systems have induced bone formation in animal studies [75-95] (Table 2). Exposure of MSCs to osteogenic BMPs results in increased expression of osteoblast-specific markers, including the early osteogenic marker alkaline phosphatase (ALP), the late osteogenic markers osteocalcin and osteopontin, connective tissue growth factor (CTGF), inhibitor of DNA binding (Id) and Cbfa1/Runx2 [11,18,28-30,96-98].

Among the osteogenic BMPs, BMP2 and BMP7 were the first to be studied in depth. Adenoviral-mediated delivery of BMP2 to MSCs and other nonosteogenic cells increases osteogenic activity *in vitro*, lending support to the role of BMP2 in osteoblastic differentiation [76,86, 99,100]. C3H10T1/2 cells overexpressing AdBMP2 exhibited increased ALP activity, mineralization and mRNA expression of bone-specific proteins including type I collagen, osteopontin and osteocalcin [86,99]. AdBMP2-transfected bone marrow osteoprogenitor cells formed bone when seeded onto biodegradable polymer scaffolds as assessed by increased ALP activity, type I collagen production and mineralization [100]. BMP7 has also demonstrated the ability to induce osteogenesis. AdBMP7-transduced C2C12 myoblasts and muscle-derived progenitor cells differentiated into osteoblasts as assessed by ALP activity and matrix mineralization [81]. Furthermore, adipose-derived adult stem cells transduced with AdBMP7 differentiated into osteoblasts, eventually formed bone [101].

Both BMP2 and BMP7 have the ability to induce osteogenesis *in vivo*. Several studies have shown that MSCs and other progenitor cell types transduced with BMP2 or BMP7 induces bone formation in various animal models [75,76,78,79,81,83-86,88,89,91,99-102]. AdBMP2 injected directly into the thighs of rats led to bone formation at the injection sites as observed with CT, digital radiography and planar radionuclide scintigraphy [102]. When AdBMP7-transduced human and rat fibroblasts were subcutaneously implanted in mice, these normally non-osteogenic cells differentiated into osteoblasts and induced bone formation [83].

Non-adenoviral vectors, including recombinant BMP2 (rhBMP2) and rhBMP7, also induce bone formation [82, 95,103-108]. Treatment of C3H10T1/2 cells with rhBMP2 increased ALP activity [105]. In a canine ulnar segmental defect model, rhBMP7 treatment initiated new bone formation and led to defect repair and complete bony union [103]. rhBMP2 has shown a similar ability to repair bony defects in animal models [73].

The results from these studies demonstrating the osteoinductive properties of BMP2 and BMP7 have increased the clinical utilization of recombinant BMPs. In degenerative lumbar disc disease patients, use of rhBMP2/collagen within a lumbar spine interbody fusion cage achieved better fusion rates than use of autologous bone graft [109]. Treatment with rhBMP2 at the fracture site improved healing of patients with open tibial fractures [110]. rhBMP2 and rhBMP7 have subsequently been used to augment bone healing with improved fusion rates compared to autografts and fewer associated complications [83,91,93,94,99,100,102,104,111-114].

While BMP2 and BMP7 were initially identified by their ability to promote osteogenesis, it was unknown then if they were the most osteogenic BMPs. Subsequent studies have shown that osteogenic BMPs include 2, 4, 6, 7 and 9 [1,3,11,16,18,37,39,115-117]. Previously, the recombinant form of each BMP was unavailable and the osteogenic activity of all BMPs could not be analyzed. However, using adenoviral-mediated gene delivery into MSCs, we conducted a comprehensive analysis of the *in vitro* and *in vivo* osteogenic activity of 14 BMPs [11,13, 15-18]. We demonstrated BMP2, BMP6 and BMP9 as the most osteogenic BMPs, with BMP9 inducing the most potent osteogenic activity both *in vitro* and *in vivo* [11,13,18].

The osteogenic potential of BMP6 has been described both *in vitro* and *in vivo* [118-128]. AdBMP6-transduced equine bone marrow MSCs demonstrated enhanced osteoblastic differentiation with increased ALP activity, matrix mineralization and expression of osteogenic marker genes similar to AdBMP2 [128]. In a rabbit model, autologous bone marrow-derived osteoprogenitor cells exposed to an rhBMP6-containing extracellular matrix induced bone formation and enhanced spinal fusion [125]. In a rabbit ulnar osteotomy model, AdBMP6 accelerated bone formation and mineralization, leading to orthotopic bone formation when injected into the calf muscles of mice [118,124].

BMP9 has demonstrated potent osteoinduction in many studies including our comprehensive analysis of the osteogenic capacity of BMPs [13,18], but it remains one of the least studied and most poorly characterized BMPs. Further investigation may show that BMP9 provides a better therapeutic avenue for the augmentation of bone regeneration than the BMPs currently used in the clinical setting.

5. BMP9 Induces Osteogenic Differentiation

Many investigations have described the osteogenic properties of BMP9, implicating its role in osteoblastic differentiation and bone regeneration. Adenoviral-mediated overexpression of BMP9 in C3H10T1/2 cells increased ALP activity and calcium deposition at significantly higher levels than BMP2 or Bmp7 treatment groups. [11,45,66]. BMP3, a known inhibitor of BMP2 and BMP7-mediated osteogenesis, did not inhibit BMP9-mediated bone formation, suggesting that BMP9-mediated osteogenesis may occur via a distinct mechanism from other osteogenic BMPs [13].

Non-adenoviral delivery of BMP9 has also demonstrated potent osteoinduction of MSCs [129-131]. Direct sonoporation of rhBMP9 into mouse quadriceps muscles caused the formation of ectopic bone [131]. Nucleofection of human MSCs (hMSCs) with BMP9

caused bone formation at four weeks post-injection *in vivo* and significantly increased calcium deposition *in vitro* [129]. Treatment of MC3T3-E1 preosteoblastic cells with a peptide derived from BMP9 (pBMP-9) induced downstream phosphorylation of Smad uninhibited by noggin, a known extracellular antagonist of BMP2 and caused a dose-dependent increase in ALP activity, Runx2, Osterix, type 1 collagen and osteocalcin [132].

In vivo studies have confirmed BMP9 as a potent inducer of bone formation. BMP9-transduced C2C12 cells injected into the quadriceps muscles of mice demonstrated significant orthotopic bone formation [13,14,115]. AdBMP9 directly injected into the quadriceps muscles of mice and rats increased osteoid and mature lamellar bone formation in BMP9-treated group compared to BMP2- and 7-treated groups, demonstrating that skeletal muscle may harbor multipotent MSCs or osteoblastic progenitor cells [79,84,133]. Furthermore, the BMP9-induced ectopic bone was histologically determined to be the result of normal physiologic endochondral mechanisms.

BMP9 has demonstrated efficacy in non-union bone fracture repair and inducing spinal fusion in animal models [134,135]. Percutaneous paraspinal injection of AdBMP9-transduced hMSCs resulted in successful spinal fusion [134]. Non-union fracture was created in the radii of mice and filled with a collagen sponge electroporated with BMP9 plasmid; new bone was formed bridging the defect gap [135]. A defect in the radius of rabbits filled with an implant consisting of bone cement and AdBMP9 demonstrated more rapid callus and more bone formation compared to control and BMP2 treatment groups [136].

BMP9-induced bone formation demonstrates a distinct ossification pattern from other BMPs. In mice injected with AdBMP2 and 9-transduced C2C12 cells, increased bone maturation and marrow elements were seen in the BMP9-treated group [13]. When AdBMP9 was injected into the quadriceps muscles of mice and rats, primitive chondroblasts secreting a loose extracellular matrix was seen by six days, chondroblasts by nine days, areas of hypertrophic chondrocytes histologically similar to the epiphyseal end plate by 12 days, woven bone between days 12 and 19 and mature lamellar bone by three months [94]. These studies illustrate that the process of BMP9-induced osteogenesis resembles the sequential physiologic phases of endochondral ossification which occur during the repair of bony fractures. While the specific mechanisms of BMP9-mediated osteogenesis remain to be defined, it appears that the BMP9-mediated osteogenic pathway is unique from that of other members of the BMP family and may provide a more effective, physiologic therapy for bone regeneration.

6. Canonical BMP Signaling Pathway

While the specific mechanisms governing for BMP9-mediated osteogenesis are largely unknown, much work has been done to elucidate the signaling pathways of the BMP family. And while many characteristics of BMP9 signaling are similar to other members of the BMP family, identification of the unique aspects of BMP9 signal transduction will allow us to better understand and utilize its potent osteogenic properties.

6.1. BMP Ligands, BMP Type I and Type II Receptors Are Required for BMP Signaling

Like other members of the TGF- β superfamily, mature BMPs are secreted as a long precursor protein containing N-terminal pro-region that is cleaved prior to secretion. However, the mature BMP9 protein retains this N-terminal pro-region, which does not inhibit the function of BMP9 and may actually stabilize it following secretion [5,21,22, 137-142].

The BMP signaling pathway is initiated upon BMP ligand binding to a heterodimeric Type I/Type II BMP transmembrane serine/threonine kinase receptor (BMPRI and BMPRII); both must be active for signal transduction (Figure 2) [143,144]. Type II receptors are constitutively active and serve to phosphorylate and activate BMPRI upon BMP ligand binding [145]. The phosphorylation site on Type I receptors is located near the C-terminal intracellular domain in a glycine-serine rich region termed the GS domain [3,143].

While seven different type I receptors have been identified, only three type I receptors, ALK1, ALK5 and endoglin, are considered potential BMP9 type I receptors [137,146-148]. In fact, BMP9 has a poor affinity for BMPRI, a receptor that generally transduces BMP signaling [137]. Dominant negative mutations of the seven type I receptors demonstrated that only ALK1 and ALK2 mutants effectively inhibit BMP9-mediated osteogenic differentiation and bone formation [66]. ALK1 and 2 directly interacted with BMP9, and silencing of ALK1 and 2 inhibited BMP9-induced osteogenic differentiation of MSCs both *in vitro* and *in vivo*. These results strongly suggest that ALK1 and ALK2 are the type I BMPRI responsible for BMP9-mediated osteogenic signaling.

Type II BMP receptors are thought to be largely responsible for the osteogenic activity of BMPs, and four have been identified [45]. Dominant negative (DN) mutations introduced in two of these receptors, BMPRII and ActRII, decreased BMP9-induced ALP activity, expression of downstream Smad 6 and Smad 7, bone mineralization *in vitro* and ectopic bone formation *in vivo* [45]. These results strongly suggest that BMPRII and ActRII are the type II BMP receptors responsible for BMP9-mediated osteogenic signaling.

6.2. Smad-Dependent Signaling Is Required for BMP Osteogenic Signaling

BMP binding to receptor heterodimers triggers one of two signaling pathways: Smad-dependent and Smad-independent. For the purpose of understanding the role of BMPs in osteogenesis, we will specifically focus on the Smad-dependent signaling pathway. There are 3 classes of Smads, all of which are important for BMP signal transduction [149]. Receptor-Smads (R-Smads) interact directly with activated type I receptors, while Common-Smads (co-Smads) form complexes with activated R-Smads to then regulate gene expression in the nucleus. Inhibitory Smads (I-Smads) negatively regulate BMP signaling, inhibiting signal transduction at several points in the pathway.

The R-Smads, Smad1, Smad5 and Smad8, are activated upon binding and phosphorylation by the Type I receptor at a conserved sequence in the C-terminus termed the Ser-Ser-Val/Met-Ser sequence, or SSXS motif. Along with this conserved sequence, R-Smads have two additional regions of homology at the N and C-terminal ends, the Mad homology (MH) 1 and MH2 domains, both of which are crucial for proper signal transduction [3,150]. The

MH1 domain directly interacts with DNA sequences, while the MH2 domain interacts with BMPR1. Additionally, the MH2 domain can bind to other Smads and has a role in transcriptional activation. These domains are conserved across co-Smads and I-Smads. Upon activation, the phosphorylated R-Smad dissociates from BMPR1 and forms a complex with Smad 4 [143,151,152]. Smad 4 is the sole Co-Smad and is common to all BMP signaling pathways. In the nucleus, the R-Smad/Co-Smad heterodimer complexes with various transcription factors, co-activators and corepressors to modulate gene expression.

The BMP signaling pathway is negatively regulated by I-Smads, Smad 6 and Smad 7. These Smads typically reside in the nucleus and migrate to the cytoplasm and plasma membrane upon BMP activation, acting at various points along the BMP pathway to inhibit signal transduction [1,151,153-157]. Smad7 binds to activated BMPR1, preventing R-Smads from becoming activated. Smad7 also interacts with E3 ubiquitin ligase proteins, Smurf1 and Smurf2, targeting the BMP receptor for proteasomal degradation. Unlike Smad 7, Smad 6 can bind directly to R-Smads, thus competing with Smad 4 and ultimately preventing proper heterodimer complex formation.

Activation of Smads was found to be necessary for BMP9-mediated osteogenic differentiation of MSCs [158]. Phosphorylated Smad 1/5/8 levels were simultaneously increased in BMP9-treated MSCs, while knockdown of Smad 4 resulted in reduced formation of Smad heterodimers and nuclear translocation of Smad 1/5/8; knockdown of Smad4 also inhibited BMP9-induced ALP activity and calcium deposition. The p38 inhibitor SB203580 decreased BMP9-induced Smad signaling in MSCs, while the ERK1/2 inhibitor PD98059 stimulated Smad signaling. Together, these findings suggest that activation of the Smads pathway is critical in BMP9-induced osteogenesis.

7. Mediators of BMP9-Induced Osteogenic Signaling

Recent studies have identified various mediators thought to contribute to the potent osteogenic effects of BMP9. Among these mediators are Id genes, connective tissue growth factor (CTGF) and Hey1. Each of these three genes is among the most up-regulated following BMP9 stimulation of MSCs [24,26,139].

Balanced regulation of Id expression is important in lineage-specific MSC differentiation

Id genes are inhibitors of the binding of basic helix-loop-helix (bHLH) transcription factors [159-161] and function by dimerizing with bHLH proteins; these heterodimers are unable to bind DNA and regulate transcription. Id-1, -2 and -3 are among the most significantly up-regulated genes upon BMP9 stimulation [30]. However, both knockdown and overexpression of these three Id genes diminished BMP9-induced osteogenic differentiation. BMP9-mediated Id expression was also shown to be dependent on Smad4 signaling.

Balanced regulation of CTGF expression is important in BMP9-induced osteogenic differentiation

Connective tissue growth factor (CTGF) is a member of the CCN (Cyr61, CTGF and Nov) family of secreted cysteine-rich multimodular proteins [162-167] and has a major role in bone formation and embryogenesis [168]. Upon BMP9-stimulation of MSCs, CTGF was

among the most up-regulated genes, especially during early stages of differentiation [28]. Similar to Id genes, both knockdown and overexpression diminished BMP9-mediated osteogenic differentiation.

Hey 1 Expression Enhances BMP9-induced osteogenic differentiation via Runx2

Hey 1 (also known as Hesr1, HRT1, CHF2 and HERP2) is a nuclear protein of the Hairy/Enhancer of split-Related (HERP) family of basic helix-loop-helix transcriptional repressors and is a direct target of the Notch pathway [14]. Constitutive Hey 1 expression synergized with BMP9-induced osteogenic differentiation *in vitro* and *in vivo* [169], while Hey 1 silencing decreased osteogenic differentiation. Hey1 and the essential osteogenic transcription factor Runx2 synergized in BMP9-induced osteogenic differentiation, whereas Hey 1 silencing decreased Runx2 expression. Following knockdown of Hey 1, exogenous Runx2 expression rescued defective osteogenic signaling, strongly suggesting that Runx2 is a downstream mediator of Hey 1 signaling.

8. Major Signaling Pathways Crosstalk With BMP9 Signaling

Several major signaling pathways with wide-ranging functions participate in BMP9-mediated osteogenesis, and many of these pathways are critical in the differentiation of other cell lineages. The following section describes recent studies illustrating the crosstalk between BMP9 signaling and these other important pathways. A brief description of each of these signaling pathways will be followed by a summary of results from recent studies.

TGF- β 1 is one of the most abundant members of the TGF- β superfamily, regulating bone formation, osteoblast proliferation and mineralization while increasing the strength and flexibility of bone [170-173]. TGF- β 1 and BMPs both regulate the late phases of differentiation and mineralization of bone [138,174-176].

Growth hormone (GH) plays a critical role in postnatal growth [177-184]. GH signaling pathway begins when GH binds GHR, triggering receptor tyrosine kinase activity and activation of JAK/STAT and other pathways [178,179,184]. IGF-2 is a member of the IGF signaling system, playing a critical role in prenatal growth and development [185]. IGF-2 signaling activates the phosphatidylinositol-3-kinase (PI3K)/AKT pathway or the mitogen-activated protein kinase pathway (MAPK) [186].

Wnts are a family of secreted proteins critical in osteoblastic differentiation and skeletal development [16, 116,187-193]. Upon Wnt binding the Frizzled (Frz) and LRP-5/6 co-receptors, distinct signaling pathways including the canonical Wnt pathway are activated [194]; mutations in LRP-5 adversely affect skeletal development and bone mass deposition [195].

MAPKs are protein kinases critical in regulation of gene expression, mitosis, metabolism, motility, survival, apoptosis and differentiation [21,158,196-199]. Members of the MAPK family become activated by BMPs in response to a variety of extracellular stimuli leading to diverse effects in cellular responses [198-201]. Hypoxia inducible factor 1 Alpha (HIF1 α) is

a regulator of angiogenesis during many developmental processes, including skeletal development [64,202].

Peroxisome proliferator-activated receptor gamma (PPAR- γ) is a critical regulator of adipogenesis and osteogenesis [203,204]. PPAR- γ binds fatty acid derivatives to induce differentiation of preadipocytes into terminal adipocytes, and PPAR- γ 2 is the predominant iso-form expressed in adipose tissue [81,159,161]. Retinoic acids (RAs) play a crucial role during embryonic development and in the maintenance of vital organs [38,39]. RAs are ligands for two families of receptors, the RA receptors (RAR) that bind all-trans-RA (ATRA) and the Retinoid X Receptors (RXR) that bind 9-cis-RA (9CRA) [205,206]. RA binding to RAR/RXR causes heterodimerization and eventual transcriptional regulation [206].

Effects of Crosstalk Pathways on BMP9-Induced Osteogenic Differentiation

TGF- β 1 has a biphasic effect on BMP9-induced osteogenic differentiation of MSCs—In BMP9-transduced MSCs, low concentrations of recombinant TGF- β 1 (rhTGF- β 1) synergistically induced expression of ALP and matrix mineralization, while high concentrations of TGF- β 1 inhibited BMP9-induced osteogenic activity [175].

GH synergizes with BMP9 via activation of the JAK/STAT/IGF1 pathway to induce osteogenesis—After over-expression of BMP9 in MSCs, GH was one of the most up-regulated transcripts [158]. Exogenous GH synergized with BMP9 to induce early and late osteogenic markers. Co-stimulation of long-bone explants with GH and BMP9 resulted in significant expansion of the growth plate, and co-stimulation of MSCs with BMP9 and GH formed mature ectopic bone masses; these synergistic effects of BMP9 and GH were inhibited by JAK/STAT inhibitors.

BMP9 signaling crosstalks with IGF-2 via PI3K/AKT signaling—While endogenous IGF-2 levels are relatively low in MSCs, exogenous expression of IGF-2 potentiates BMP9-induced expression of early and late osteogenic markers [207]. Conversely, PI3K inhibition diminished IGF-2 potentiation on BMP9-mediated osteogenesis. IGF-2 augmented BMP9-induced ectopic bone formation and BMP9-mediated endochondral ossification in perinatal limb explants.

The canonical Wnt/ β -catenin pathway interacts with Runx2 as a critical mediator of BMP9-mediated osteogenic signaling—Wnt3a and BMP9 synergized to induce ALP activity in MSCs, while the Wnt antagonist FrzB inhibited BMP9-induced ALP activity [194]. BMP9 stimulation of MSCs recruits β -catenin and Runx2 to the osteocalcin promoter, whereas knockdown of β -catenin decreased expression of early and late osteogenic markers [194]. BMP9-induced ectopic bone formation and matrix mineralization *in vivo* were inhibited by FrzB overexpression or β -catenin knockdown [194].

p38 and ERK1/2 have opposing regulatory effects in BMP9-induced osteogenic differentiation of MSCs via Smad signaling—BMP9 simultaneously promotes phosphorylation and thus activation of Smads, p38 and ERK1/2 [158]. p38 and ERK1/2 acted in opposition to regulate BMP9-mediated osteogenic differentiation via

interactions with Smads. *In vivo*, inhibition of p38 significantly decreased BMP9-induced osteogenic differentiation, while inhibition of ERK1/2 significantly increased BMP9-induced osteogenic differentiation [208].

HIF1 α synergizes with BMP9-induced osteogenic differentiation of MSCs—

Exogenous overexpression of HIF1 α synergistically increased BMP9-induced osteogenic differentiation of MSCs. Conversely, inhibition of HIF1 α diminished BMP9-induced osteogenic signaling. BMP9 directly induced HIF1 α expression in MSCs via Smad1/5/8 signaling [209]. HIF1 α activated both angiogenic and osteogenic signaling pathways in MSCs. Osteogenic factors, including BMP9, may induce the convergence of osteogenic and angiogenic signaling in MSC differentiation, thereby enhancing the efficiency of bone formation and development.

PPAR- γ is an important regulator of BMP9-mediated osteogenesis—

Overexpression of PPAR- γ 2 in BMP9-stimulated MSCs promoted both osteogenic and adipogenic differentiation, with mutually exclusive commitment to either lineage [18]. Conversely, knockdown of PPAR- γ 2 in BMP9-stimulated MSCs showed significant decreases in osteogenic differentiation and matrix mineralization.

Retinoic acids synergistically enhance BMP9-mediated osteoinduction of MSCs—

Both ATRA and 9CRA induced expression of BMP9, activated BMP9 transcription activity and increased expression of early and late osteogenic markers; these effects were synergistic when combined with overexpression of BMP9 [210]. RAs combined with BMP9 promoted expansion of the hypertrophic chondrocyte zone in neonatal mouse limb explants, and RARs synergized with BMP9 to induce trabecular bone formation and osteoid matrix production *in vivo*.

9. Other Functions Of BMP9

BMP9 is known to be a potent osteogenic factor, but it also influences several other pathways including cancer development and angiogenesis. While some studies have shown BMP9 to restrict tumor growth through diverse mechanisms [211-213], other studies have shown BMP9 to promote cancer progression [214]. BMP9 inhibited cell migration and induce apoptosis of osteosarcoma via the PI3K/ALT pathway [212] slowed tumor growth of colon adenocarcinoma via inhibitory effects on angiogenesis via the ALK1 receptor and endoglin coreceptor [211]. Conversely, BMP9 induced *in vivo* angiogenesis within pancreatic tumors [214]. The effects of BMP9 on angiogenesis remain controversial as well, with some studies demonstrating a pro-angiogenic effect [215,216] and others an anti-angiogenic effect [146,217-219]. It is evident that the effects of BMP9 on angiogenesis and cancer progression remain to be fully elucidated.

BMP9 also modulates neurogenesis, hepatocellular regeneration, adipogenesis, chondrogenesis and myogenesis. Several studies have demonstrated BMP9 to promote the cholinergic phenotype neurologically [220-223]. BMP9 also acts as a hepatic insulin-sensitizing substance and may play a role in hepatocellular regeneration [224,225]. BMP9 promotes adipogenesis [18] and also upregulates Sox9 expression to induce chondrogenic

differentiation [226,227]. While BMP9 promotes MSC differentiation along osteogenic, adipogenic and chondrogenic lines to varying degrees, it inhibits the myogenic phenotype [29]. Overall, BMP9 has diverse effects beyond osteogenesis.

10. Concluding Remarks and Future Directions

The investigations discussed here demonstrate the critical role of BMPs, particularly BMP9, in the osteogenic differentiation of MSCs. The findings discussed here strongly support the notion that BMP9 may provide a more effective clinical strategy for the augmentation of bone regeneration and healing than other BMPs. Furthermore, studies demonstrating that BMP9-mediated osteogenesis resembles the physiologic phases of bone healing occurring during fracture repair make the prospect of clinical translation quite promising. With several diverse signaling pathways enhancing BMP9-mediated osteogenesis, further elucidation of these specific pathways will allow for the development of improved therapies. Over the last decade, there has been a substantial increase in the therapeutic use of recombinant proteins and medications targeting small molecules acting in various signaling pathways. With this, it is imperative that the mechanisms underlying BMP9-mediated osteogenesis become fully elucidated to allow for the development of much-needed clinical therapies.

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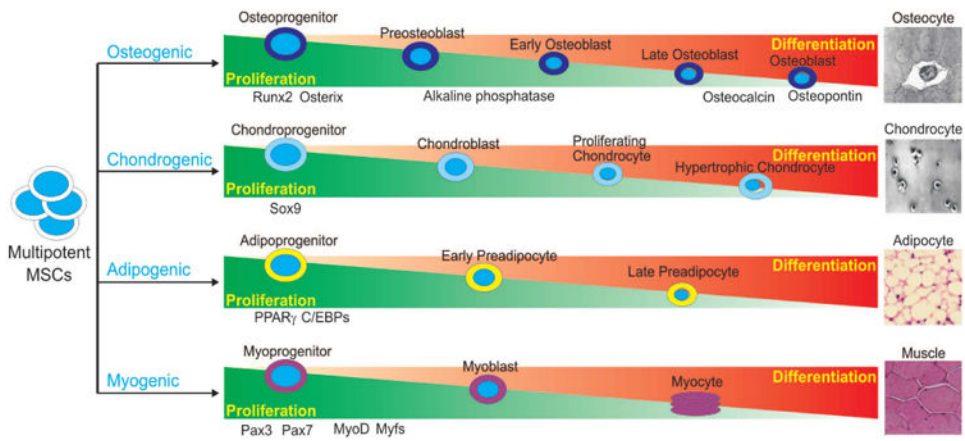


Figure 1. Schematic representation of lineage-specific differentiation of mesenchymal stem cells (MSCs). MSCs are pluripotent progenitor cells with the ability to differentiate along multiple lineages, including osteogenic, chondrogenic, adipogenic and myogenic lineages. Differentiation of MSCs along these unique lineages is an exquisitely coordinated process with critical regulators responsible for each lineage. Regulators and indicators of lineage-specific differentiation are depicted.

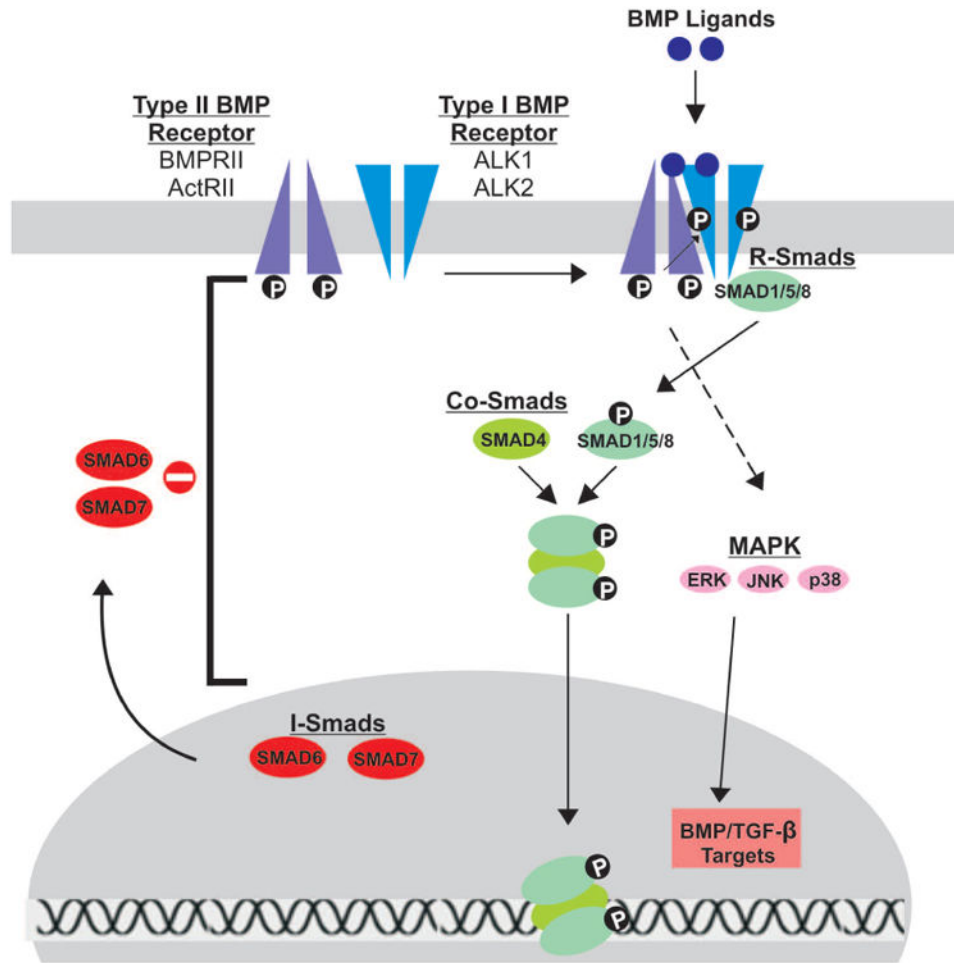


Figure 2. Schematic representation of BMP signal transduction. Upon BMP ligand binding to Type II BMP receptors, Type I BMP receptors are cross-phosphorylated, recruiting R-Smads (Smads 1/5/8) to the intracellular domain of the Type I receptor and initiating signal transduction via phosphorylation. Activated R-Smads then form a heteromeric complex with Co-Smads (Smad 4) before translocating to the nucleus to regulate gene expression. Inhibitory Smads (Smads 6/7) reside in the nucleus, migrate to the cytoplasm and negatively regulate BMP signaling by inhibiting signal transduction at several points along the pathway. The Type I and Type II receptors thought to be involved in BMP9 signal transduction are highlighted.

Table 1

Examples of BMP-induced osteogenesis in MSCs.

Treatment	Experiment setting	Experiment metric	Experimental results	Reference
BMP2 to BMP15 (14 BMPs)	<i>In vitro</i>	ALP activity	BMP2, 6, and 9 induced ALP activity in C3H10T1/2 pluripotent cells.	Cheng <i>et al.</i> 2003; Kang <i>et al.</i> 2004
		Osteocalcin expression	BMP2, 4, 6, 7, and 9 led to increased ALP activity in C2C12 preosteoblastic cells and TE-85 osteoblastic cells.	
		Alizarin Red S	BMP2, 6, and 9 were able to significantly induce osteocalcin expression and mineralization in C3H10T1/2 cells.	
	Mouse	Bone formation	BMP6 and BMP9-transduced C2C12 cells induced the most osteogenesis was not inhibited by BMP3, a negative regulator of bone formation.	Kang <i>et al.</i> 2004
BMP2	<i>In vitro</i>	ALP activity	Mesenchymal stem cells transduced with AdBMP2 showed increased osteoblastic differentiation and were able to form bone <i>in vitro</i> , with increased ALP activity, Alizarin Red S osteocalcin, COL1a1, and bone sialoprotein	Cheng <i>et al.</i> 2001
		Alizarin Red S		
		Northern blot analysis		
	Rabbit	Bone formation	AdBMP2-transduced cells were able to form bone when injected into the L5/6 intertransverse spinal space	
BMP6	<i>In vitro</i>	ALP activity	AdBMP6-transduced equine mesenchymal stem cells showed increased ALP activity and mineralization.	Zachos <i>et al.</i> 2006
		Von Kossa staining mRNA microarray	AdBMP6 also induced increased expression of genes associated with osteoblastic differentiation.	
			AdBMP6 subcutaneously injected into athymic nude was able to induce rapid ectopic bone formation.	
	Mouse	Bone formation		Jane <i>et al.</i> 2002
BMP7	<i>In vitro</i>	ALP activity	C2C12 murine myoblast cells transduced with AdBMP7 differentiated into osteoblasts, displaying increased ALP activity and osteoblast-like morphology. These cells did not develop a myogenic phenotype.	Francheshi <i>et al.</i> 2000
		Cell morphology		
	Mouse	Bone formation	Subcutaneous and intramuscular injections of AdBMP7 induced ectopic bone ossicle formation in mice.	
BMP9 + TGF- β 1	<i>In vitro</i>	ALP activity	Low concentrations of rhTGF- β 1 synergistically increase ALP activity, matrix mineralization, gene expression and protein expression of osteopontin, osteocalcin and COL1a2 on BMP-9mediated osteogenic differentiation.	Li <i>et al.</i> 2012
		Alizarin Red S		
		RT-PCR & Western blot		
		Smad pathway activation	TGF- β 1 combined with BMP9 exhibits lower BMPR-Smad receptor activity than BMP9 alone.	

Treatment	Experiment setting	Experiment metric	Experimental results	Reference
BMP9 + GH	<i>In vitro</i> and ex vivo	ALP activity	GH potentiates BMP9-induced ALP activity, osteopontin/osteocalcin, expression and calcium deposition in MSCs	Huang <i>et al.</i> 2012
		Osteocalcin/Osteopontin expression	JAK/STAT inhibitors blunt BMP9-GH synergy	
		Alizarin Red S	GH enhances BMP9-induced endochondral ossification in cultured limb explants	
	Mouse	Ectopic bone formation	GH augments BMP9-induced ectopic bone formation with more mature bone	

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Table 2

Skeletal phenotypes of several members of the BMP signaling pathway.

Deleted gene	Cells affected	Skeletal phenotype	Reference
BMP2; BMP2 + 4	Chondrocytes	Chondrodysplasia, disorganized chondrocytes in growth plate, defects in chondrocyte proliferation, differentiation, increased apoptosis	Shu <i>et al.</i> 2011
BMP2 + 4	Limb bud mesenchymal cells	Failure of chondrogenic condensations to form normally, no formation of bone marrow cavity, trabecular or cortical bone	Bandyopadhyay <i>et al.</i> 2006
BMP3	All cells	Increased bone density	Daluiski <i>et al.</i> 2001
BMP5	All cells	<i>Short ear</i> , brachypodism	King <i>et al.</i> 1994
BMP6	All cells	Minor sternal defects	Solloway <i>et al.</i> 1998
BMP7	All cells	Hindlimb polydactyly, defects in rib cage, skull	Luo <i>et al.</i> 1995
BMP11	All cells	Anterior-posterior axial skeletal patterning defects	McPherron <i>et al.</i> 1999
Alk2	Neural crest cells	Craniofacial malformations, including cleft palate, hypotrophic mandible, reduced ossification of frontal bone	Dudas <i>et al.</i> 2004
BMPR1A	Osteoblasts	Increased bone mass, decreased bone resorption, reduced osteoclastogenesis	Kamiya <i>et al.</i> 2008
BMPR1B	Osteoblasts	Decreased bone mineral density, bone volume, and bone formation	Zhao <i>et al.</i> 2002
Smad6	All cells	Axial and appendicular skeletal defects, posterior transformation of the cervical vertebrae, bilateral ossification centers in lumbar vertebrae, incomplete sternal band fusion,	Estrada <i>et al.</i> 2011
Smad1	Chondrocytes	Delayed calvarial bone development	
Smad1	Osteoblasts	Osteopenia, impaired osteoblast proliferation and differentiation	Wang <i>et al.</i> 2011
Noggin	Osteoblasts	Decreased weight, shortened femoral length, osteopenia	Canalis <i>et al.</i> 2012