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SIGNAL TRANSDUCTION AND BIOLOGICAL FUNCTIONS OF BONE MORPHOGENETIC PROTEINS

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1. ABSTRACT

Bone morphogenetic proteins (BMPs) are multi-functional growth factors and belong to the transforming growth factor β (TGFβ) superfamily. The roles of BMPs in embryonic development and cellular functions in postnatal animals have been extensively studied in recent years. Signal transduction studies have revealed that Smad1 and 5 are the immediate downstream molecules of BMP receptor and play a central role in BMP signal transduction. Using transgenic and knockout approaches and animal models with naturally occurring mutations in BMP genes, it has been shown that BMPs play critical roles in mesoderm formation, heart development, cartilage development and postnatal bone formation. Recombinant BMP-2 and 7 have been used clinically for several different clinical interventions such as non-union fractures and spinal fusions. Tissue-specific knockout of a specific BMP ligand, a subtype of BMP receptors or a specific signaling molecule is required to further determine the specific role of a particular BMP ligand, receptor or signaling molecule.

2. INTRODUCTION

BMPs are members of the TGFβ superfamily. BMPs were originally identified from bone matrix using an ectopic bone formation assay (1). The activity of BMPs was first identified in the 1960s (2), but the proteins responsible for bone induction remained unknown until the purification and sequence of bovine BMP-3 (osteogenin) and cloning of human BMP-2 and -4 in the late 1980s (3,4).

The purification of BMPs was completed using the rat ectopic bone formation assay. This assay involves combining the sample containing the unknown protein to be assayed with demineralized rat bone matrix, which has been treated with dissociative agents such as guanidine and urea to remove all of the endogenous BMP activity. This combination is then implanted subcutaneously in rats, and after 1-2 week, formation of new cartilage and bone was detected by the histological method (4). Using this bioassay, BMPs were purified and sequenced. Purifying growth regulatory factors present in trace amounts in bone tissue to homogeneity using a slow and cumbersome *in vivo* bioassay was an amazing feat.

To determine the biological activities of each individual BMP member, a cloning approach was taken to obtain molecular clones corresponding to each of the proteins present in the purified extract. Based on the BMP sequence information, oligonucleotide probes were synthesized, and used to screen bovine genomic libraries. Once bovine genes corresponding to the proteins were obtained, they were then used to obtain human cDNA clones encoding each protein within the extract. A large number of clones were identified by this cloning strategy (1). All of the BMPs have amino acid sequence homology to each other. To date, around 20 BMP family members have been identified and characterized.

BMPs are 30-38 kDa homodimers that are synthesized as prepropeptides of approximately 400-525 amino acids (1). The mature C-terminal region of 100-140 amino acid residues is released from a propeptide region by cleavage at an Arg-X-X-Arg sequence. The secretion of the C-terminal mature segment is as a dimer, in many cases with one disulfide-link (1). Although homodimers are considered the standard form, there are natural heterodimers with similar bioactivity. It has been reported that a heterodimer composed of BMP-4 and BMP-7 is a potent inducer of mesoderm (5). BMPs have similar sequences including seven similarly-spaced cysteine residues located in the mature region of the proteins. Six of seven cysteine residues on each BMP subunit form three

intrachain disulfide bonds, with the remaining one forming an interchain bridge to create the dimer (1,6). Many of the BMPs are glycosylated in both the mature and propeptide regions, as determined by both biochemical characterization and the presence of appropriate carbohydrate addition sites in the presumed amino acid sequence (1Unlike TGFβ, secreted BMP proforms apparently do not form latent complexes with their mature counterparts. There is a considerable cross-species bioactivity for the BMPs. The BMPs have been grouped into subsets based on amino acid sequence homology. The groupings are suggested to be 1) BMP-2 and BMP-4; 2) BMP-3 and BMP-3b; 3) BMP-5, BMP-6, BMP-7 and BMP-8; 4) BMP-9 and BMP-10; 5) BMP-12, BMP-13 and BMP-14; and 6) BMP-11 and growth/differentiation factor 8 (GDF-8) (6,7). The production of BMPs by recombinant methods has allowed the activities of each molecule to be investigated using *in vitro* and *in vivo* assays. These recombinant BMPs have been produced using a mammalian cell expression system (8,9), an Escherichia coli (E. coli)-expression system (10,11) and a baculovirus expression system.

3. BMP RECEPTOR SIGNALING

BMPs signal through serine/threonine kinase receptors, composed of type I and type II subtypes. Three type I receptors have been shown to bind BMP ligands, type IA and IB BMP receptors (BMPR-IA or ALK-3 and BMPR-IB or ALK-6) and type IA activin receptor (ActR-IA or ALK-2) (12,13,14). Three type II receptors for BMPs have also been identified and they are type II BMP receptor (BMPR-II) and type II and IIB activin receptors (ActR-II and ActR-IIB) (7,15,16,17). Whereas BMPR-IA, BMPR-IB, and BMPR-II are specific to BMPs, ActR-IA, ActR-II, and ActR-IIB are also signaling receptors for activins. These receptors are expressed differentially in various tissues. Ligand-receptor and some of the downstream event (ie Smad utilization) have cross-interactions as observed among the BMP, activin and TGFβ receptor family members.

Signal transduction through serine/threonine kinase receptors has been best characterized in the TGFβ receptor system. It is likely that BMPs transduce signals in a similar fashion. Type Iand type II BMP receptors are both indispensable for signal transduction. After ligand binding they form a heterotetrameric-activated receptor complex consisting of two pairs of a type I and type II complex (18). Both type I and II BMP receptors are composed of three parts: a short extracellular domains with 10-12 cysteine residues; a single membrane spanning domains; and a intracellular domains with serine/threonine kinase region (12,13,15,16,17). Preceding the serine/threonine kinase domain, type I BMP receptors, but not type II BMP receptors, have a domain, which contains a characteristic SGSGS motif (GS domain) (12,13). The GS domain, which plays an important role in signal transduction, distinguishes type I serine/threonine kinase receptors from type II receptors. In the TGF-β and activin receptor systems, ligands bind to type II receptors in the absence of type I receptors. Type I receptors can bind ligands only in the presence of type II receptors. However, BMPs bind weakly to the type I receptors in the absence of type II receptors. In the presence of type II receptors, the binding of BMPs to type I receptors is accelerated and increase in strength (12,13,18).

In the TGFβ receptor system, the type II receptor kinase transphosphorylates the GS domain in type I receptor. This leads to activation of the type I receptor kinase (7). Phosphorylation of type I receptor is required for signal transduction, as TGFβ-mediated responses are impaired after mutation of serine and threonine residues in the GS domain of type I receptor, or after mutation in type II receptor rendering it incapable to phosphorylate type I receptor (19,20). These observations indicate that type II receptor is a primary binding protein for ligands, and that type I receptor acts as an effector in the signal transduction, although there are clear domains on BMP2, 4, and 7 ligands that bind specifically to the type I receptor (21). Mutation of the glutamine in GS domain of a type I BMP receptor to aspartic acid results in a receptor with a constitutively activated kinase. In these mutants, signals are transduced from type I BMP receptor in the absence of ligand and type II BMP receptor (20).

The type I BMP receptor substrates include a protein family, the Smad proteins, that play a central role in relaying the BMP signal from the receptor to target genes in the nucleus. Smad1, Smad5 and Smad8 are phosphorylated by BMP receptors in a ligand-dependent manner (22,23,24). After release from the receptor, Smad proteins associate with the related protein Smad4, which acts as a shared partner. This complex translocates into the nucleus and participates in gene transcription with other transcription factors. In vertebrates, Smad proteins consist of three regions: a conserved N-terminal domain (MH1 domain), a conserved C-terminal domain (MH2 domain), and a more divergent linker region. In the transcriptional complex, the Smads contact DNA via their N-terminal domains (25). The C-terminal domain of Smads mediates Smad-receptor interaction (24). Smads appear to go from a monomer state before C-terminal phosphorylation to a heterotrimeric state with two regulated Smad molecules with one Smad 4 molecule. This activation state allows the Smad oligomer to translocate into the nucleus (26). However, in the nucleus these complexes associate with molecules such as Ski and HDAC complexes to keep the gene in the off state until further modifications of the chromatin complex with co-activators of a select gene.

For example, activation of specific genes by Smads is conducted by interaction with specific sets DNA-binding proteins. The *Xenopus* protein Fast1 is the prototypic Smad-recruiting DNA-binding factor (25). Fast1, which contains a “winged helix” DNA binding domain, binds to the activin response element (ARE) and is absolutely required for activation of the *Mix.2* gene in response to activin or TGFβ. Fast1 bound to DNA alone does not activate transcription. However, recruitment of an activated Smad2-Smad4 complex to the ARE by Fast1 results in activation of *Mix.2* expression (27). One of the transcription factors that interacts with Smad1 has been

identified as a homeodomain DNA-binding protein, Hoxc-8. Hoxc-8 serves as a transcriptional repressor for osteopontin gene transcription. Interaction of Smad1 with Hoxc-8 relieves the repressive activity of Hoxc-8 and activates osteopontin gene transcription. The interactive regions of Smad1 with Hoxc-8 are in the MH1 domain and linker region (28). Transgenic mice overexpressing Hoxc-8 show abnormal cartilage development, which is characterized by an accumulation of proliferating chondrocytes and reduced maturation (29), suggesting that Hoxc-8 plays a critical role in chondrocyte differentiation *in vivo.* This is very similar to the affect of a constitutive BMP receptor 1a on cartilage development (see Mishina, 2003, FBS). Interestingly, Smad2, a downstream molecule for TGFβ signaling, interacts with another homeodomain DNA-binding protein, TGIF, a repressor of transcription. This occurs when TGIF moves into the nucleus with Smad4. Smad2-Smad4 complex recruits TGIF and histone deacetylases (HDACs) to a Smad target promoter, repressing transcription (30). These findings provide molecular evidences for another functional connection between growth factors such as Activin/BMP/TGFβ and homeodomain DNA-binding transcription factors.

Another protein, which interacts with Smad1 and Smad5 in the nucleus, is core-binding factor α1 (Cbfa1) (31,32,33,34,35). Cbfa1 is an osteoblast/chondrocyte-specific transcription factor and plays a central role in osteoblast differentiation and bone formation. Targeted disruption of Cbfa1 in mice reveals that Cbfa1 expression is absolutely required for bone development *in vivo.* A complete lack of both endochondral and intramembranous ossification, with an absence of mature osteoblasts throughout the body, is observed in homozygous Cbfa1-deficient mice (36,37).

4. MUTATIONS IN BMPS AND BMP RECEPTORS

Studies of naturally occurring mutations of BMPs and BMP receptors have shown that BMPs play important roles in several inherited diseases. Short ear mutations in mice disrupt the gene for BMP-5 and this mutation in the BMP-5 gene is associated with a wide range of skeletal defects, including reductions in long bone width and the size of several vertebral processes and an overall lower body mass (38,39). Mutations in growth/differentiation factor-5 (GDF-5, CDMP-1 and BMP-11) genes result in brachypodism in mice (40) and chondrodysplasia in humans (41,42). Both BMP-5 and GDF-5 genes are localized on chromosome 2 in mice and on chromosome 20 in humans (40). GDF-5 has been shown to bind BMPR-IB specifically (43) and null mutations in the BMPR-IB gene causes a similar skeletal phenotype as that observed in GDF-5 mutant mice (44).

Fibrodysplasia ossificans progressiva (FOP) is an extremely rare and disabling genetic disorder characterized by congenital malformations of the great toes and by progressive heterotopic endochondral ossification in predictable anatomical patterns. Ectopic expression of BMP-4 was found in FOP patients (45,46). Familial primary pulmonary hypertension is a rare autosomal dominant disorder that has been mapped to chromosome 2q33. Monoclonal plexiform lesions of proliferating endothelial cells in pulmonary arterioles are the major phenotype of this disease. These lesions lead to elevated pulmonary artery pressure, right ventricular failure and death. After genotyping multiple families with this disorder, BMPR-II mutations have been found in these patients (47,48,49). Mutations in GDF-9 and GDF-9b genes have been found in patients with premature ovarian failure and polycystic ovary syndrome (50). Overexpression of BMP-2, -4, -5 and BMPR-IA is associated with malignancy of the oral epithelium (51) and overexpression of BMP-3 and BMP2 has been described in prostate cancer cells (52,53). Mutations in the BMPR-IB gene is associated with increased ovulation rates in Booroola Mérino ewes and in sheep (54). Female mice deficient in BMPR-IB are infertile due to a constellation of defects, including irregular estrous cyclicity, impaired pseudopregnancy response, severe defects in cumulus cell expansion, and insufficient uterine endometrial gland development (108).

5. MUTATIONS IN BMP ANTAGONISTS

Several mutations of the BMP antagonist now underscore how important it is to control the levels of BMP activity in a given system. For example, proximal symphalangism is an autosomal-dominant disorder with ankylosis of the proximal interphalangeal joints, carpal and tarsal bone fusion, and conductive deafness. These symptoms are shared by another disorder of joint morphogenesis, multiple synostoses syndrome. Recently, it was reported that both disorders were caused by heterozygous mutations of the human noggin gene. To date, seven mutations of noggin gene have been identified from unrelated families affected with joint morphogenesis (55,56). Noggin is a secreted polypeptide, which binds and inactivates BMP-2, 4 and 7. Recent co-crystal structures of noggin and BMP-7 gave clear insight into how noggin functions. It is similar in structure to a BMP ligand and can form a dimer and contains a cysteine knot. Most interesting aspect of the co-crystal structure was that the type I receptor binding domain and the type II receptor binding domain on each BMP7 monomer interacted with a specific clip section from each monomer of the dimeric noggin complex (21,57). This structure clearly show how noggin is so specific in inhibiting BMP 2, 4, and 7.

Sclerostosis is a recessively inherited osteosclerotic disorder caused by mutations in a protein called sclerostin (58). The disease was initially considered to be a variant of osteopetrosis (59), but subsequent metabolic studies revealed that the disorder is primarily due to increased bone formation, rather than defective bone resorption (60). Recently it has been reported that sclerostin is related in sequence to a family of secreted BMP antagonists, which includes Gremlin, Cerberus and Dan. Sclerostin is expressed in osteoblasts and osteoclasts and binds BMP-5, 6 and 7 with high affinity. Expression of sclerostin in C3H10T½ cells blocks acquisition of the osteoblast phenotype, and transgenic expression sclerostin in osteoblasts under the control of the osteocalcin promoter causes osteoporosis (58). These findings suggest that

BMPs play an important role in controlling bone formation and bone mass.

6. GENE ALTERATIONS FOR BMPS AND BMP RECEPTORS

To understand the roles of BMP ligands and BMP receptor signaling in embryonic development and in postnatal life, null mutations of BMP ligands and BMP receptors have been created and phenotypic changes in these animal models have been extensively studied. Mice deficient for BMP-2 and BMP-4 are nonviable. Homozygous BMP-2 mutant embryos die between day 7.5 and 10.5 of gestation and have defects in cardiac development, manifested by the abnormal development of the heart in the exocoelomic cavity (61). Homozygous BMP-4 mutant embryos die between day 6.5 and 9.5 and show little or no mesodermal differentiation (62). For more details, see review by Mishina in this issue of FBS.

BMP-7-deficient mice die shortly after birth because of poor kidney development. Histological analysis of mutant embryos at several stages of development reveals that metanephric mesenchymal cells fail to differentiate, resulting in a virtual absence of glomerulus in newborn kidneys. In addition, BMP-7-deficient mice have eye defects that appear to originate during lens induction (63,64). BMP-7 deficient mice have minor defects in the skeleton. BMP-6-deficient mice are viable and fertile, and show no overt defects in tissues known to express BMP-6 mRNA (65). BMP-6 is mainly expressed in hypertrophic cartilage. Since BMP-2 and BMP-6 are co-expressed in this tissue, BMP-2 may functionally compensate in BMP-6 null mice. Targeted null mutations of BMP-2 with BMP-6 null mice will be required to answer this question.

Growth/differentiation factor-8 (GDF-8, myostatin) is expressed specifically in developing and adult skeletal muscle. During early stages of embryogenesis, GDF-8 expression is restricted to the myotome compartment of developing somites. At later stages and in adult animals, GDF-8 is expressed in many different muscles throughout the body. GDF-8 null mutant mice are significantly larger than wild-type mice and show a large and widespread increase in skeletal muscle mass (66).

Null mutation of the BMPR-IA gene causes embryonic lethality in mice. Animals die at embryonic day 9.5. Homozygous mutants with morphological defects are first detected at day 7.0. No mesoderm forms in the mutant embryos, suggesting that BMPR-IA is essential for the inductive events that lead to the formation of mesoderm during gastrulation (67). Mice lacking BMPR-IB are viable and exhibit defects in the appendicular skeleton. In BMPR-IB-deficient mice, proliferation of prechondrogenic cells and chondrocyte differentiation in the phalangeal region are markedly reduced. In adult mutant mice, the proximal interphalangeal joint is absent, and the phalanges are replaced by a single rudimentary element, while the distal phalanges are unaffected. The lengths of the radius, ulna and tibia are normal, but the metacarpals/metatarsals are reduced (44). The appendicular defects in BMPR-IB mutant mice resemble those seen in mice homozygous for the GDF-5^{bp-j} null allele of the GDF-5 locus. Since GDF-5 has been shown to play a critical role in cartilage formation and binds BMPR-IB with high affinity (43), these results suggest that BMPR-IB plays a non-redundant role in cartilage formation *in vivo.* BMP ligands may utilize multiple type I receptors to mediate their signaling during bone formation. This hypothesis is supported by observations in BMPR-IB and BMP-7 double mutant mice. In the double mutant, severe appendicular skeletal defects have been observed in the forelimbs and hind limbs. The ulna is nearly absent and the radius is shortened (44). Since BMP-7 binds efficiently to both BMPR-IB and ActR-IA(Alk2) (14), it is conceivable that BMPR-IB and ActR-IA(Alk2) play important synergistic or overlapping roles in bone formation *in vivo.*

7. REGULATION OF BMP GENES

The transcription and expression of BMP genes are controlled by many growth factors and transcription factors. To understand the regulatory mechanisms of BMP-2 and BMP-4 genes, mouse and human BMP-2 and BMP-4 genomic DNA and promoters have been cloned (68,69,70,71) and BMP-2, 3 and 4 cDNA from different animal species has been isolated (53,72,73,74,75). Alternative promoters for BMP-2 and BMP-4 genes have been identified (70,75,76). All-trans and 9-cis retinoic acid activate mouse and human BMP-2 and BMP-4 promoters in F9 embryonic carcinoma cells and in human and murine osteoblastic cell lines (69,76). BMP-4 gene transcription is also regulated by an orphan receptor COUP-TFI (70).

BMP-2 up-regulates BMP-2 and BMP-4 mRNA expression in primary osteoblastic cells (74,77,109), suggesting that BMP-2 acts as an autocrine and paracrine factor during osteoblast differentiation. The autocrine effect of BMP-2 may be mediated in part through transcription factors, Dlx2 and Dlx5, as BMP-2 stimulates Dlx2 and Dlx5 mRNA expression and Dlx2 binds and activates BMP-2 gene transcription (78,79). Recently we found that BMP-2 gene transcription is controlled by transcription factors NF-kB and C-terminal truncated Gli3 in chondrocytes and in osteoblast precursor C2C12 and 2T3 cells. The responsive elements for these transcription factors have been mapped. Mutations in these responsive elements of BMP-2 gene alter BMP-2 promoter activities and cause losses of the responsiveness of BMP-2 gene to these transcription factors (80,81). It has also been reported that Gli1 and Gli3 stimulated BMP-4 and BMP-7 promoter activities (82).

BMP-6 is mainly expressed in chondrocytes and plays an important role in chondrocyte differentiation and endochondral bone formation. Glucocorticoid, estrogen and BMP-7 selectively upregulate BMP-6 expression (83,84,85) and PTHrP, a key factor in chondrocyte differentiation, inhibits BMP-6 expression in chondrocytes (86).

Recently it was shown that estrogens can activate the BMP-2 transcription throught an estrogen response

element. ERalpha seems to be selective in the context of an osteoblast precursor (87). This also may help explain the bone inducing qualities of many estrogenic ligands, both synthetic and natural.

8. BIOLOGICAL FUNCTIONS OF BMPS

BMPs have been implicated in a variety of functions. BMPs induce the formation of both cartilage and bone. During the process of bone formation, BMPs create a rudimentary environment that is conducive to the development of functional bone marrow (88). In addition, BMPs play a role in a number of non-osteogenic developmental processes. Neural induction represents the earliest step in the determination of ectodermal cell fates. In vertebrates, BMPs act as signals of epidermal induction (89). BMP-2 can direct the development of neural crest cells into neuronal phenotypes (90), while BMP-4 and BMP-7 specifically induce a sympathetic adrenergic phenotype. BMPs give direction to somite development by inhibiting the process of myogenesis. BMPs also appear to be responsible for normal dorsal/ventral patterning. BMP-4 specifies the development of ventral structures (*e.g*., skin from ectoderm and connective tissue/blood from mesoderm). Dorsal structures (nervous system and muscle) apparently appear when BMP-4 signals are interrupted through the activities of binding proteins (92). In the limb bud, and as part of the fibroblast growth factor 4 (FGF-4) and sonic hedgehog (Shh) interaction, BMP-2 apparently inhibits limb bud expansion and induces the formation of chondrocyte and osteoblast precursors (93,94). The list can go on and on. As one biologist recently said, Wnts maybe the architects of developments but BMPs are both architects and general contractors.

Physiological roles of BMPs and BMP receptor signaling on normal bone formation have also been studied. Injection of BMP-2 locally over the surface of calvariae of mice can be used to induce periosteal new bone formation on the surface of calvariae without a prior cartilage phase (74). Systemic administration of BMP-6 increases trabecular bone volume and bone formation rates in mice (95). Overexpression of dominant-negative type IB BMP receptors (BMPR-IB) in osteoblast precursor 2T3 cells inhibits osteoblast-specific gene expression and mineralized bone matrix formation (96). Overexpression of a dominant negative BMPR-1A actually slowed growth but accelerated differentiation. In the transgenic mice which express a dominant-negative truncated BMPR-IB driven by the osteoblast-specific type I collagen promoter, the bone mineral density, static bone volume and dynamic bone formation rates are decreased in one-month-old transgenic mice (97), demonstrating that BMP receptor signaling plays a necessary role in normal postnatal bone formation. Future bone and cartilage specific activation of constitutive BMP receptors and tissue specific knock-outs of the BMP-2 and BMP-4 ligands should begin to clarify the roles of individual BMP ligands and receptors in the formation of bone by endocondral as well as intramembraneous mechanisms.

9. CLINICAL APPLICATIONS OF BMPS

The osteoinductive capacity of BMPs has been demonstrated in preclinical models, and the efficacy of BMPs for the treatment of orthopedic and dental patients is now being evaluated in clinical trials. The clinical applications of recombinant BMP-2 and BMP-7 are being studied most extensively. Many of the animal models used to evaluate the capacity of BMPs to heal bone defects have utilized critical-sized defects. In these animal models, bone defects are large enough that they will not heal without a therapeutic intervention. This setting facilitates analysis of the ability of a BMP to induce bone. Recombinant human BMP-2 combined with a variety of matrices has been shown to heal defects in a canine mandibular defect model (98). Healing of long bone critical-sized defects by BMP-2 has been demonstrated in species including rats, rabbits, dogs, sheep and non-human primates (99,100). The implantation of BMP-7 containing bovine collagen matrix preparations restored large diaphyseal segmental defects in monkeys, rabbits, and dogs, leading to the regeneration of new bone that is fully functional biologically and biomechanically (101,102). Osteochondral defects are the common end point for several types of joint diseases, such as degenerative arthritis, infection, and trauma. Preparations of BMP-7 in conjunction with a type I collagen carrier induces autologous cells and repairs osteochondral defects in rabbits (103). These results suggest that BMP-7 may be useful in articular cartilage repair. Both BMP-2 and BMP-7 have been shown to induce new dentine formation and have a potential application as a substitute for root canal surgery (104). BMP2 has also been shown to be an effective bone inducer around dental implants for periodontal reconstruction (105).

10. DRUG TARGETING ON BMP SIGNALING

In attempts to identify small molecular weight compounds that stimulate substantive formation of new bone, a cell-based screening assay was utilized using an osteoblast precursor cell line that was derived from transgenic mice, and where the transgene was SV40 large T antigen targeted to the osteoblast lineage (106). These cells were stably transfected with the BMP-2 promoter operatively linked to the firefly luciferase reporter gene. The capacity of the small molecular weight compounds to stimulate the BMP-2 promoter was assessed by the increase in luciferase activity in cells exposed to compounds.

Using this cell-based screening assay, we examined approximately 150,000 compounds,which include chemically synthesized compounds and the natural product extracts for their capacity to activate the BMP-2 gene transcription. From the natural product extracts, we identified a yeast extract, which has BMP-2 stimulating activity. Upon fractionation of this extract, we identified the active constituent as lovastatin, a molecule that was known to inhibit the enzyme hydroxymethylglutaryl-coenzyme (HMG CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Lovastatin, which increased BMP-2 transcription in doses of 1-5 uM, belongs to a class of drugs called statins, which are widely prescribed in clinical medicine for lowering serum cholesterol and reducing the frequency of heart attacks. We then found that other closely related statins, namely simvastatin,

mevastatin, fluvastatin and cerivastatin, had similar effects on the BMP-2 promoter. We examined their effects on endogenous BMP-2 mRNA expression and found that simvastatin increased BMP-2 mRNA expression in human osteoblast MG-63 cells, detected by Northern analysis (107).

To determine if this occurs in conjunction with a biological effect in the osteoblasts, we first cultured the statins with explants of murine calvarial bones. We found that each of the statins caused a marked increase in osteoblast cell accumulation and bone formation in doses of 0.5-5 uM over 4-7 days of culture. We then tested the effects of the statins *in vivo* by local injections over the murine calvaria. We found that there was a 30-60% increase in periosteal new bone formed after only 5 days of exposure to lovastatin or simvastatin. We next determined if they produced a similar effect when administered systemically. These experiments were performed in intact rats, and also in rats that were ovariectomized in order to mimic the situation that occurs in post-menopausal women. Following 35 days of administration by oral gavage of lovastatin or simvastatin in doses of 5-10 mg/kg per day, there was a marked increase in trabecular bone volume in each of these animal models associated with increases in mineral apposition rates. There was also a decrease in osteoclast numbers, suggesting that statins were also decreasing bone resorption rates (107). These data show that statins are capable of stimulating new bone formation.

Using the same cell-based screening assay, we also identified another class of compounds that stimulate BMP-2 gene expression and bone formation. These compounds belong to a class of compounds that have proteasome inhibitor activity. Proteasome inhibitors stimulated BMP-2 gene transcription (promoter assay), mRNA expression (Northern analysis and bDNA assay) and protein expression (Elisa assay). Proteasome inhibitors stimulated periosteal new bone formation in calvarial organ culture assay *in vitro* and in calvarial local injection model *in vivo.* Proteasome inhibitors PS1 and epoxomicin increased trabecular bone volume (more than 50%) and bone formation rates in mice when administered systemically. Using specific inhibitors of different proteasome activities, we further demonstrated that inhibition of chymotryptic activity of the proteasome is responsible for the bone formation effect induced by proteasome inhibitors (81). More recently, we have also found that microtubule inhibitors activate BMP signaling, probably by increasing the dissociation of Smad1 and Smad5 from their binding to microtubule, and stimulate bone formation *in vivo* (110).

The results that small molecular weight compounds stimulated BMP-2 gene transcription and subsequent bone formation provide new evidence that BMP-2 signaling plays a critical role in bone formation and suggesting that targeting on BMP-2 transcription and BMP signaling may lead to the development of new therapeutic agents for the treatment of bone loss associated diseases. Estrogen now appears to belong to this class of small compounds that can stimulate BMP-2 transcription and argue positively for the use of phytoestrogens and estrogenic flavenoids in preventing bone loss.

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