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Role of bone morphogenetic proteins and their antagonists in healing of bone fracture

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1. ABSTRACT

Recent advances have allowed elucidation of the factors which are important to the healing of bone fracture. The bone morphogenetic proteins (BMPs) of the transforming growth factor-beta (TGF-beta) family have emerged as an effective therapeutic target in the treatment of severe fractures and fracture non-unions which have been resistant to conventional treatment. Treatment with BMP has provided encouraging results, both in animals and humans. Such treatments have reduced the time required for a fracture to heal and have increased the strength of the healed bone. BMP antagonists have been shown to modulate BMP activities in diverse and critical ways in a myriad of tissues and systems during normal vertebrate development. Recent studies have begun investigating the role of BMP antagonists during bone development and in healing of fractures. Better understanding of the effects of such antagonists in the healing of fractures opens the possibility of enhancing those effects which are exerted solely by treatment with BMP.

2. INTRODUCTION

Among the wide spectrum of traumatic injuries, the fracture of bone has undergone treatment refinement for millennia. Stabilization, both with internal and external devices, has long been the mainstay of fracture management. However, our increased understanding of the molecular biology of fracture healing has led to the development of adjunct therapies that increase the speed and efficiency of fracture healing. One of the most significant advances in fracture biology has been the discovery of the bone morphogenetic protein (BMP) family of growth factors. As our understanding of BMP function advances and evidence demonstrates its efficacy in clinical practice, attention is now being directed to those paracrine and autocrine factors that directly or indirectly antagonize BMP activity. These factors may subsequently be utilized as drug targets in the future to create environments in which BMP therapy is even more efficacious.

3. THE MOLECULAR BIOLOGY OF FRACTURE HEALING

3.1. Fracture healing

Depending on the biomechanical environment, there are two main types of healing processes in fractures: primary and secondary. Primary healing consists of bone cortex directly repairing itself across a fracture defect. This occurs as the cortex on each end of the fractured bone re-establishes Haversian systems in discrete units, called cutting cones, which bridge the fracture gap via blood vessels and thus recreate the cortex across the fracture line (1). However, this sort of bone healing appears to only occur in the presence of anatomic reduction and absolute stabilization of fracture fragments with rigid internal fixation. There is minimal periosteal involvement in this form of fracture healing and thus essentially no appreciable callus formation (2).

3.2. Endochondral bone formation

Secondary fracture healing is the more prominent form of fracture healing and is a process that resembles endochondral ossification during bone development (2). The process has five discrete stages. The first stage occurs minutes to hours after fracture and consists of hematoma formation and inflammation. The second stage of fracture healing consists of the formation of capillary buds from periosteal vessels, likely mediated by vascular endothelial growth factor (VEGF) and other pro-angiogenic mediators. Chondrocyte development from pluripotent mesenchymal cells with concurrent significant cartilage formation characterizes the third stage of secondary fracture healing. The fourth stage consists of ossification of the cartilage via endochondral ossification. Mature bone formation and remodeling of the bone are the hallmarks of the fifth and final stage of secondary bone healing. In each of these stages, multiple molecular factors play integral roles in initiating and manipulating the events necessary for successful healing. These factors can be grouped into three general classifications: the pro-inflammatory cytokines, the TGF-beta superfamily and other growth factors, and the angiogenic factors (3).

3.3. Molecular biology of fracture healing

The first stage of endochondral fracture healing consists of hematoma formation and inflammation around the fracture site immediately following the initial insult. Concurrent with this hematoma formation is a spike in the levels of pro-inflammatory cytokines, such as Interleukins 1 and 6 (IL-1 and IL-6), and tumor necrosis factor-alpha (TNF-αlpha) cytokines which play critical roles in initiating the repair cascade (4). Secreted by macrophages as well as mesenchymal cells in the periosteum, these cytokines are potent chemotactic attractors of other inflammatory cells and endogenous fibrogenic cells important for callus matrix synthesis and angiogenesis (4) In addition to initial recruitment roles, these cytokines are also important for chondrocyte apoptosis and resorption of mineralized cartilage, thus a second spike in the levels of these cytokines is seen during the final bone remodeling phase of fracture healing. Angiogenesis is particularly important for optimal bone regeneration. Specific matrix metalloproteinases degrade cartilage and bone to allow for the invasion and penetration of blood vessels into the callus (3-5). Subsequently, it is thought that two separate pathways are at work during fracture healing to regulate angiogenesis, one controlled by vascular endothelial derived growth factor (VEGF), and one by angiopoietins (3).

The transforming growth factor-beta (TGF-beta) family of growth and differentiation factors makes up the largest group of mediators with influence on fracture healing. At least 34 members have been identified in the human genome (6). This diverse group of growth factors includes the bone morphogenetic proteins (BMPs), TGF-beta, growth differentiation factors (GDFs), activins, inhibins, and the Mullerian inhibiting substance. Cleaved by proteolytic enzymes from high-molecular weight precursors, these growth factors interact with serine/threonine kinase receptors which activate an intracellular signaling cascade, ultimately affecting gene expression in the nucleus (7). Among this diverse group of mediators the BMPs have undergone the most thorough investigation in musculoskeletal science and eventual application in clinical therapy.

4. THE BONE MORPHOGENETIC PROTEINS

Bone morphogenetic protein (BMP) is a title coined by Dr. Marshall Urist in the 1960s after he isolated protein extracts from demineralized bone matrix that were able to induce bone formation at ectopic sites in rodents (8-9). He further described the formative process initiated by these extracts as closely resembling endochondral fracture healing and bone formation. During the 1980's Reddi *et al*. reported that BMP modulation of bone formation was not species specific and began to elucidate the molecular and cellular events surrounding BMP mediation of fracture healing (9-10). Much research during the past two decades concerning BMP function has centered on the diverse roles these mediators play during vertebrate development. Remarkably, BMPs are found across the entire spectrum of animal life, and in fact, identical or similar molecular pathways appear to control the regulation of coral, sea urchin, fly, worm, frog, chick, mouse, and human growth and development (11). BMPs are cleaved from larger precursor molecules into smaller molecules, which have a highly conserved region consisting of seven cysteines. The three-dimensional structure created by these specific amino acids makes BMPs highly resistant to heat, denaturants, and extremes of pH (12). BMP molecules may directly affect adjacent tissues and cells, be bound up by a host of BMP antagonists, or interact with extracellular matrix proteins (9). An increasing focus of BMP research of late has targeted the powerful effects BMPs have on fracture healing, a process which in many ways recapitulates the bone generation mechanisms observed during pre-natal development (5). Beginning with the earliest stages and continuing throughout fracture healing, BMPs are produced by mesenchymal cells, osteoprogenitor cells, chondrocytes and osteoblasts. Indeed, it has been demonstrated that fracture repair cannot even begin without BMP present (13).

Table 1. A summary of clinic trials

Study	n	BMP	Model	*Results*
Friedlander [24]	122	rhBMP-7	nonunion	No statistical difference between interventions
Dimitriou[3]	25	rhBMP-7	non-unions	92.3% of cases achieved union
Govender[25]	450	rhBMP-2	open fractures	12mg of BMP significantly improved healing
Jones [26]	30	rhBMP-2	cortical defect	No significant difference between interventions
Swiontkowski [27]	510	rhBMP-2	open fractures	BMP significantly improved healing
Kleeman[30]	22	rhBMP-2	lumbar fusion	100% of patients with improvements in fusion

4.1. Molecular biology of BMP

BMP modulates development and healing by binding two different types of serine/threonine kinase receptors, designated as Type I and Type II receptors. Type I receptors are activated by Type II after BMP binding, and it is these Type I receptors that mainly determine intracellular signals (14-15). After these membrane-bound receptors are bound and activated, they phosphorylate intracellular signaling proteins known as Smads. The Smad family includes eight members that are divided into three groups: those that are responsible for signal transduction and are regulated by receptors (Smads 1, 2, 3, 5, and 8), the common mediator Smad (Smad 4), and the inhibitory Smads (Smads 6 and 7). BMP receptor activation leads to phosphorylation of Smads 1, 5, or 8 which then interact with Smad 4. The resultant heteromeric complex then translocates to the nucleus where the expression of specific target genes is modified. (12-15). This mediation of gene expression is accomplished by binding to specific DNA sequences, interacting with other DNA-binding proteins, and recruitment of transcriptional co-activators or co-repressors (12-15).

Despite the highly conserved nature of their core architecture, BMPs have been subdivided into four separate groups based upon subtle differences in their molecular structure. Group 1 consists of BMP-2 and BMP-4; group 2 includes BMP-5, BMP-6, and BMP-7; group 3 is composed of BMP-12 (or GDF-7), BMP-13 (or GDF-6), BMP-14 (or GDF-5); finally, group 4 consists of BMP-3 (or osteogenin) and BMP-3b (or GDF-10) (7). BMPs-2, -6, and -9 have been shown to be the most potent inducers of osteoblast differentiation of mesenchymal precursor cells (16). BMPs appear to have distinct patterns of expression and effects on bone formation and repair. BMP knock-out mice demonstrate an inability to initiate fracture healing, despite demonstrating the ability to form intact bone during initial embryonic development (13). Conversely, mice similarly lacking BMP-4 activity are able to initiate and complete fracture repair in a normal fashion, in addition to forming normal bone during embryonic development (17). Most studies in both humans and animals investigating the clinical efficacy of BMPs, in terms of increasing the speed and quality of fracture healing, have involved the use of BMPs-2 and -7.

4.2. BMP enhancing fracture healing

Multiple studies have shown that exogenous application of BMP to long bone fractures in animal models leads to faster and more robust fracture healing. Fracture callus size is 20-60% greater in a rabbit ulnar model two weeks after fracture when treated with recombinant human BMP-2 (rhBMP-2) compared to untreated controls (18). In a monkey fibula model, there is a significantly greater callus area in those fractures treated with rhBMP-2 compared to untreated controls (19). This effect is amplified when the rhBMP-2 is administered one week after fracture instead of within hours after the fractures. Presumably this is because the cellular and matrix framework required for successful healing is already present at the time of rhBMP-2 administration. BMP-7 administration results in larger callus volume in a closed goat tibia fracture model compared to untreated controls (20). Finally, the speed of fracture callus formation is significantly increased in a rat femoral fracture model injected with rhBMP-2 compared to uninjected controls (21). BMP has additionally been shown to increase the biophysical strength of healing bone. Significantly increased torsional stiffness and bending strength has been demonstrated in rabbit ulnar fracture models treated with rhBMP-2 and goat tibial fracture models treated with BMP-7 (18-20). Utilization of gene therapy to deliver BMP has resulted in significant improvements in fracture healing time and efficiency (22).

4.3. Clinical application of BMP

Studies investigating the clinical effects of BMP administration in human long bone fractures have also been numerous during the past decade (Table 1). The majority of these studies investigated BMP modulation of fracture healing in the context of non-unions, a problem frequently encountered by orthopedic surgeons. As the physiologic role of BMP has been increasingly appreciated, it has been assumed that non-unions may be due, at least in part, to a deficiency or malfunction of BMP activity. However, there are preliminary indications that BMP function is normal even in the context of fracture non-unions (23). Compared to studies involving fracture non-unions, fewer studies have specifically addressed BMP efficacy in acute, sometimes open, long bone fractures in humans.

Friedlaender *et al*. (24) followed 122 patients with tibial non-unions who were randomized into one of two groups. One group received rhBMP-7 in a Type I collagen carrier at the non-union site and the other group received fresh bone autograft at the non-union site. No statistical significance was found between the two groups in terms of time to union, complications, and pain out to 24 months post-operatively. The authors suggest this demonstrates BMP-7 is a safe, effective, and less morbid alternative to the use of autograft in tibial non-unions. Dimitriou *et al*. (3) evaluated the safety and efficacy of rhBMP-7 administration in non-unions involving tibial, femoral, humeral, ulnar, patellar, and clavicular fractures. They reported a 92% union rate across all fracture types with no complications or apparent adverse effects. There have been two randomized control trials comparing the treatment of acute tibial fractures both with and without

rhBMP-2. Govender *et al*. (25) randomized 450 patients to have tibial fractures treated with intramedullary nailing alone, intramedullary nailing plus administration of 6 mg of rhBMP-2 in a collagen sponge or 12 mg of rhBMP-2 in a collagen sponge. The patients who were administered 12 mg of rhBMP-2 at the time of initial surgery showed significantly fewer subsequent invasive interventions, significantly faster healing time, and significantly fewer hardware failures compared to the group that did not receive rhBMP-2. Similarly, Jones *et al*. (26) randomized 30 patients to receive either autogenous bone graft or allograft with rhBMP-2 during treatment of acute diaphyseal tibial fractures with cortical defects averaging 4 cm. Thirteen patients in the rhBMP-2 group went on to heal without further intervention compared to only ten patients in the autogenous graft group. Finally, Swiontkowski (27) performed subgroup analyses on the data from the previous two studies, and was able to demonstrate that patients with Gustillo Type IIIA or IIIB open tibial fractures had significantly lower bone-grafting procedures and significantly lower incidence of invasive secondary interventions, as well as significantly lower rates of infection compared to controls.

All of these studies consistently show that therapy with BMP in humans for the treatment of both acute and non-united fractures significantly decreases healing time as well as the rates of infection and complications, even in severely open tibial fractures (Table 1). Additionally, it has been demonstrated that therapy with rhBMP-7 in non-unions is significantly more cost-effective than traditional treatment modalities (28). Multiple studies have also demonstrated that BMP-2 and -7 are safe, effective, and at times superior alternatives to autologous bone grafting during interbody spinal fusion procedures (29, 30)

Although these clinical data are promising, BMP therapy is still in the beginning stage. A thorough understanding of these antagonists and agonists is critical to maximize the efficacy of clinical BMP therapy, and to help fashion treatment protocols which are most appropriate. Additionally, an understanding of these associated factors should reveal potential new sources of drug targets. Of particular interest are those factors which antagonize BMP activity at the level of the callus, as an ability to manipulate the activity of these antagonists would substantially maximize BMP efficacy in fracture therapy.

5. BMP ANTAGONISTS

Factors which inhibit BMP activity generally elicit their effects through negative feedback loops or crosstalk mechanisms. They may interfere with signaling pathways by decreasing the amount of available signaling factor (i.e. BMP), direct receptor interaction, intervention of downstream cytoplasmic pathways, or by a direct effect of the genome in the nucleus. These ubiquitous factors have been found to modulate a diverse array of BMP activities, including neural development (31), prostate development (32), inflammation of cardiac tissues (33), and bone development (34). Factors antagonistic to BMP activity can be grouped into those which modulate by means of influencing BMP extracellularly and those which act intracellularly (Figure 1).

5.1. Noggin

Noggin is a potent inhibitor of BMP activity that directly binds BMP in the extracellular environment. Noggin binds with differing affinities to BMP-2, -4, -5, -6, and -7, with the highest affinities observed between noggin and BMP-2 and -4 and the relatively weakest affinity between noggin and BMP-7 (35-36). Noggin is essential for normal skeletal and joint development (37-38). A single dose of noggin in rats significantly decreases the rate of membranous ossification as measured by a bone in-growth chamber technique. Interestingly, the total amount of tissue in-growth was similar between controls and the noggin groups, but ossification was significantly reduced in the noggin group (35). Noggin expression is clearly upregulated by BMP as a protective feedback mechanism to prevent bone overgrowth in BMP stimulated osteoblasts (39). An investigation into the supraphysiologic effects of noggin demonstrated the over-expression of noggin in transgenic mice results in the development of osteopenia, spontaneous fractures during the first month of life, and decreased bone formation as determined by micro-CT scanning (40).

A landmark study by Warren *et al*. (41) clearly demonstrated the essential role of noggin in maintaining the patency of cranial sutures in growing mouse calvaria. Exogenous noggin prevented normal cranial suture fusion, and sutures placed in an organ culture and exposed to noggin did not fuse, while those without noggin reliably fused. Finally, it was shown that fibroblast growth factor-2 (FGF2) downregulates noggin expression in a dose-dependent manner, thus potentially explaining the mechanism responsible for premature craniosynostosis seen in syndromes characterized by FGF receptor mutations such as Apert's syndrome. Another study by Lories *et al*. (42) examined the potential therapeutic effects of noggin gene transfer into a mouse ankylosing enthesitis and spondyloarthopathy model. Male mice that demonstrated clinical signs consistent with ankylosing enthesitis were transfected with noggin cDNA or an empty vector. Noggin was transfected both preventatively and therapeutically (gene transfer performed at the first sign of arthritic symptoms) and significantly decreased the initiation and progression of ankylosis. These important findings further allude to potential therapeutic roles for noggin in relatively common and frequently morbid human diseases.

Despite the wealth of evidence describing the potent antagonistic effects of noggin on BMP function during growth and development, fewer studies have investigated the dynamics of noggin modulation of BMP function during repair of fractured mature bone. In one of the first studies to examine noggin dynamics during fracture healing, Yoshimura *et al*. (43) performed Northern blot analysis and *in situ* hybridization in tissue samples obtained at different time-points from experimentally induced mouse rib fractures. There was a clear similarity observed between the temporal expression of BMP-4 and

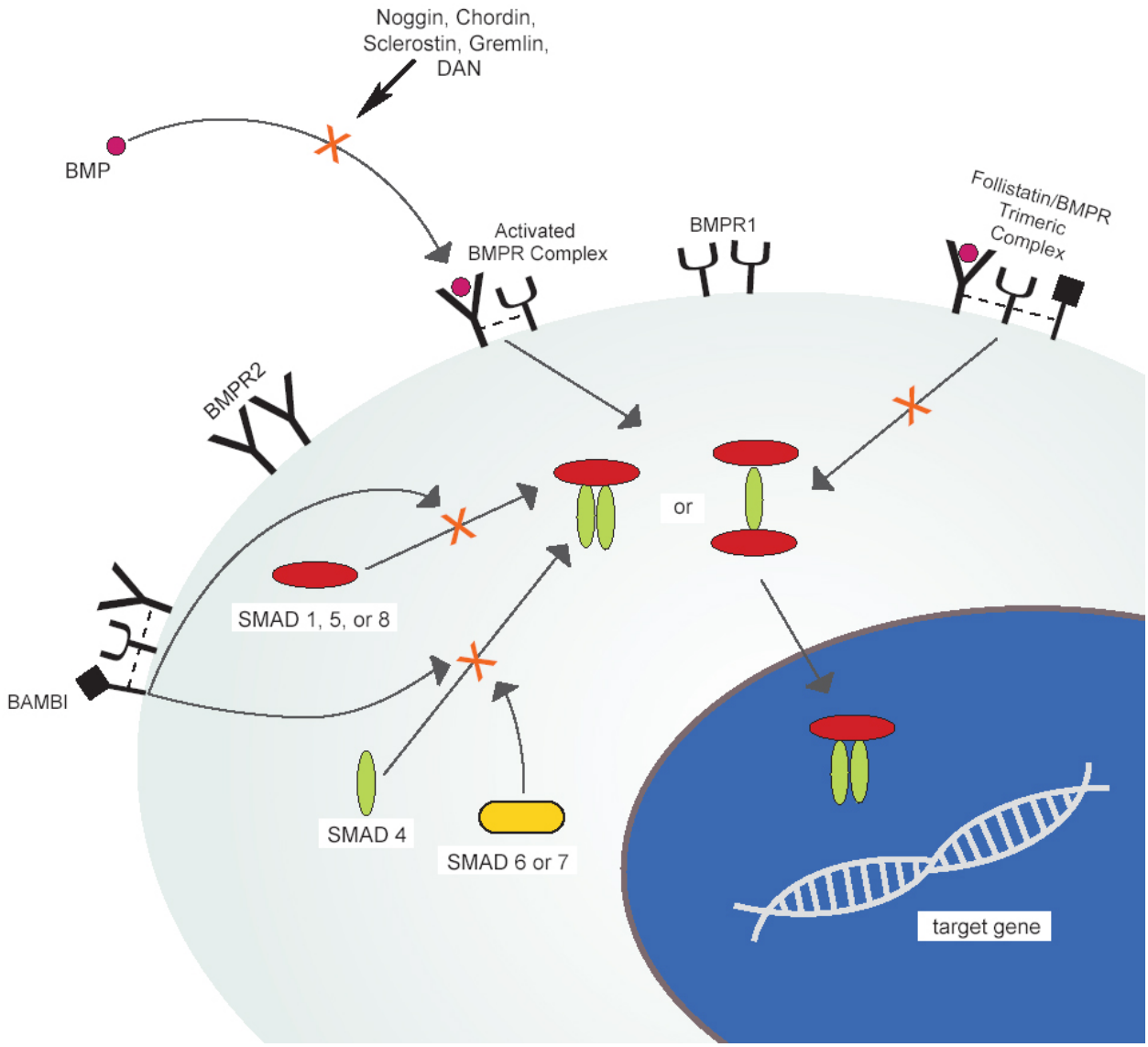

Figure 1. A diagram shows BMP antagonists blocking BMP signals at various levels of the pathways. While noggin, chordin, sclerostin, DAN, and gremlin interact with BMP receptors extracellularly, BAMBI, and smad6 and 7 inhibit BMP signals intracellularly. Follitatin forms a trimeric complex with BMP and BMP receptor to antagonize BMP signals.

noggin mRNA, with both demonstrating a peak at two days after fracture, followed by a gradual decline out to the final measured time-point of 21 days. Additionally, *in situ* hybridization revealed essentially identical spatial expression of BMP-4 and noggin mRNA in the proliferating periosteal layer, osteoblasts in newly formed bone, and differentiating chondrocytes. Presumably this association between the temporal and spatial expressions of BMP-4 and noggin may be attributed to the aforementioned upregulation of noggin by BMP.

A similar study by Nakamura *et al.* (44) investigated the expression and regulation of BMP receptors and noggin in a mouse ectopic bone formation model. The paraspinal muscles of three groups of study mice were impregnated with collagen pellets containing either rhBMP-2 (Group 1), containing no BMP (Group 2), or underwent a sham surgery with no implantation (Group 3). At 21 days after implantation of the BMP impregnated pellet, a mature ectopic bone mass was present in Group 1 mice with complete resorption of the implanted pellet. At no time during the study did Groups 2 or 3 demonstrate any cartilage or bone formation. Upregulation of BMP receptors, as well as increased noggin gene expression, was first detected at the 2-day time-point and peaked at the 4-day time-point. Interestingly, a progression was observed in the spatial distribution of both noggin and BMP receptor mRNA during the endochondral ossification and bone maturation process observed in the Group 1 mice. Both mRNA types were seen in undifferentiated mesenchymal

cells and regenerating muscle fibers on day 4, were additionally localized to cartilage cells one week after implantation, and were finally observed in the osteoblastic cells around the newly formed bone by the two-week time-point.

Most recently, Wan *et al*. (45) used siRNA silencing noggin activity to evaluate the resultant effects seen both *in vitro* and *in vivo*. This study utilized multiple avenues of evidence to demonstrate the powerful modulatory effects of noggin on the BMP signaling pathway. Pre-osteoblasts were infected with specific siRNA constructs that were shown to effectively silence noggin activity. In these infected cells, expression of Smad 1 and 5, and rate of osteoblast differentiation were all significantly increased compared to sham infected controls. Expression of osteoblastic differentiation markers *Runx2, osteocalcin,* and *osteopontin* all peaked approximately three days earlier in the siRNA infected cells compared to sham infected controls. Finally, critical sized defects were created in mouse calvaria and the cortical defects were seeded with either siRNA infected osteoblasts or sham infected osteoblasts. At two and four weeks, the defects seeded with siRNA infected osteoblasts demonstrated significantly more bone formation as measured by histomorphometric analysis and micro-CT scanning. Interestingly, at eight weeks there were similar levels of bone formation with no appreciable difference between siRNA infected and sham infected control groups. These data clearly indicate that it may be possible to manipulate noggin modulation of BMP activity in such a way to enhance and speed bone regeneration and, potentially, fracture healing. Studies examining the effects of noggin silencing on long bone fracture healing have yet to be performed, but are necessary to further the possibility of therapeutic algorithms utilizing the manipulation of noggin.

There have been few investigations into the dynamics of noggin expression during fracture healing. It appears that noggin expression in the fracture callus peaks approximately 10 days after fracture and that expression is depressed during all stages of healing in experimentally created non-unions compared to normally healing fractures (46).

5.2. Gremlin

Gremlin similarly binds BMP-2, -4, and -7 in the extracellular realm and effectively blocks BMP activity by not allowing receptor activation. It is part of the large differential screening-selected gene aberrative in the neuroblastoma (DAN) protein family (7). Gremlin modulation of BMP activity has classically been described as occurring primarily in the extracellular environment, but there is evidence that it may additionally interfere with BMP-4 precursor formation intracellularly (47). Much research has been conducted investigating the role of gremlin in vertebrate development, as well as its role in multiple human diseases.

Gremlin has been shown to have no direct effect on Smad activity, while clearly antagonizing BMP-2 mediated Smad activation in murine stromal cell lines (48). In terms of appendicular skeleton development, gremlin is secreted at the limb bud and effectively inhibits the activity of locally secreted BMPs in a paracrine fashion, thus promoting proximodistal limb outgrowth and development (49). In transgenic mice with gremlin overexpression, bone mineral density is 20% lower at ages 4.5-12 weeks compared to controls, and the transgenics displayed spontaneous fractures of tibiae and humeri, but interestingly no other bones. Additionally, the transgenic mice had a 35% lower body weight compared to wild-type controls, and this body weight differential persisted throughout the 12-week observation period (50). Conditional deletion of mouse genes allowing for specific inactivation of gremlin in the post-natal environment resulted in a 40% increase in trabecular bone volume compared to controls between ages one and three months (30). Similarly RNAi downregulation of gremlin activity *in vitro* demonstrated a 2 to 7 fold increase in BMP-2 activity in cultured cells (30). Gremlin expression is significantly lower in the proliferative and hypertrophic zones of ossification compared to the resting zone in 7-day-old rat tibial physes, suggesting a possible link between gremlin expression and activation of early endochondral ossification (51). Beyond its actions in the skeletal system, gremlin has been shown to be critical for mouse embryonic renal development, as unchecked BMP-4 activity causes catastrophic failure of kidney branching morphogenesis (52). In contrast to its antagonistic effects on BMP activity, gremlin is a pro-angiogenic factor as demonstrated both *in vitro* and *in vivo* in mouse aorta endothelial cells and chick chorioallantoic membranes, respectively (53). These studies clearly indicate an important physiologic function for gremlin during skeletogenesis and other important physiological functions. However, there have been few studies to date investigating the role of gremlin in adult skeleton fracture repair and remodeling. It has been shown that gremlin expression gradually increases in a nearly linear fashion out to four weeks after an experimentally created fracture in rats (46).

5.3. Sclerostin

Sclerosteosis is a congenital dysplasia characterized by generalized massive bone overgrowth that leads to a tall stature, facial distortion, entrapment of cranial nerves, and often sudden death due to elevated intracranial pressures (54). *SOST*, the gene encoding the peptide molecule sclerostin, has been implicated as the site of the mutation responsible for the development of this disease. Sclerostin, like gremlin, has been characterized as a member of the DAN family of BMP antagonists. Unlike most BMP antagonists, which are found in a myriad of tissues, it has been definitively demonstrated that sclerostin is only expressed in skeletal tissues, specifically only in mature osteocytes within the mature mineralized matrix, osteoblasts, and hypertrophic chondrocytes (54-55). Because sclerostin binds BMP-2, -4, -5, -6, and -7 with only weak affinity, the exact effects on BMP activity modulation specifically by sclerostin are controversial (56). However, targeted deletion of the sclerostin gene in mice clearly results in a significant increase in bone formation compared to controls (57).

A flurry of recent studies have described the role of the Wnt/beta-catenin signaling pathway in regulating bone mass in both mice and humans, and the antagonistic effect sclerostin exerts on this pathway (58). While the Wnt family of proteins exerts their effects by multiple signaling pathways, the most well-understood is the beta-catenin or canonical pathway. Briefly, Wnt binds to the cell surface low-density lipoprotein receptor related protein 5 (LRP5) which prevents phosphorylation of the cytoplasmic protein beta-catenin. Eventual accumulation of beta-catenin in the cytoplasm results in translocation to the nucleus with subsequent activation of Wnt targeted genes. Loss-of-function mutations in the LRP5 gene have been identified in humans with osteoporosis pseudo-glioma syndrome (OPPG), a disease characterized by extremely low bone mass and frequent fractures. Similarly, a gain-of-function mutation of the LRP5 gene has been identified in humans with unusually high bone mass. Additionally, during embryonic bone development the Wnt/beta-catenin pathway appears to dictate whether bi-potential mesenchymal cells will differentiate into chondrocytes (thus initiating endochondral ossification) or osteoblasts (thus initiating intramembranous ossification) (59). This ubiquitous pathway is also important for bone regeneration in experimentally created mandibular defects in mice (60). The Wnt pathway clearly plays a crucial role in the regulation bone mass and bone regeneration, and the antagonistic effects of sclerostin presumably play a role in modulating this pathway. Indeed, recent evidence seems to indicate that sclerostin modulation of BMP-stimulated bone formation is potentiated via the Wnt pathway rather than through the BMP signaling cascade (61). Most recently it has been shown that osteocyte expression of sclerostin is significantly reduced when bone is mechanically loaded, possibly explaining, in part, the mechanism by which bone density increases in response to increased loading demands (62). Sclerostin expression is similarly downregulated by parathyroid hormone (PTH) (63). There have been few studies investigating the temporal dynamics of sclerostin in fracture healing; however, there appears to be a gradual increase in sclerostin expression within the fracture callus during the first four weeks of normal fracture healing (46).

5.4. Chordin

Chordin appears to bind BMP-2, -4, and -7 in the extracellular environment similar to noggin and thus prevents BMP activity by preventing it from binding to receptors (64-7). Chordin is particularly important for embryological development, especially skeletogenesis, during which it is predominately expressed in the epiphysis (65-7). When cells over-expressing chordin were experimentally implanted into the developing wing buds of chicks, the resultant phenotype demonstrated an average of 20% loss of limb length and blockage of ulnar bone mineralization compared to controls. Additionally, chordin expression during development was shown to be inversely related to the stages of chondrocyte maturation, indicating that chordin is likely an inhibitor to this critical developmental process (65). In a similar finding, chordin expression is significantly lower in the proliferative and hypertrophic zones versus the resting zone in 7-day old rat tibial physes (51). Despite conclusive evidence that chordin is an inhibitor of BMP during normal development, few studies to date have specifically investigated whether chordin plays any role during fracture healing, or if it is in fact involved at all in the normal bone healing process in adults. One investigation demonstrated a peak of chordin expression within callus 10 days after fracture in normally healing fractures and a consistent level of chordin expression in experimentally produced non-unions (46).

5.5. BMP and activinbound protein (BAMBI)

The pseudoreceptor BMP and activin bound protein (BAMBI) is a transmembrane glycoprotein with an extracellular domain similar to that of the BMP receptor. By associating with BMP receptors, BAMBI inhibits the effects of activated receptors without directly interacting with BMP. Few attempts have yet to be made to characterize the temporal or spatial dynamics of this membrane bound protein during normal fracture healing. However, BAMBI expression has been shown to increase in a nearly linear fashion out to four weeks in a normally healing experimental fracture (46).

5.6. Smads

In addition, the Smads are important for intracellular signaling in the BMP activation pathway. There are inhibitory Smads which effectively antagonize BMP action in the cytoplasm. Smads 6 and 7 interfere with the phosphorylation of Smad 1, 5, and 8 and thus prevent subsequent heterodimerization with Smad 4, a step necessary for translocation of the protein complex to the nucleus.

5.7. Follistatin

Follistatin was first described after being isolated from ovarian fluid and was so named because of its ability to suppress follicle-stimulating hormone (FSH) from the pituitary (66). During *Xenopus* development follistatin binds to a complex of BMPs and BMP receptors forming a trimeric complex that inhibits further signaling in the BMP activity cascade (67). Removal of follistatin during *Xenopus* development results in severe abnormalities in dorsal structures (68). Follistatin expression is decreased in human prostate cancer cells which have experimentally decreased BMP-7 activity (and subsequent increase in cell motility), indicating a feedback mechanism at work between endogenous BMP expression and follistatin levels (69). There have been no attempts to date at investigating the temporal and spatial dynamics of follistatin expression during fracture healing or the role that follistatin may play in modulating BMP activity and potency during fracture healing or skeletogenesis.

5.8. DAN

DAN, the vanguard member of the DAN family of proteins, has been shown to bind BMP-2 and -4 with relatively high affinity. Additionally, DAN null mutations demonstrated a subtly different phenotype from controls, which was different from phenotypes observed in the previously discussed extracellular BMP antagonists, possibly indicating that each of the proteins have specific and independent functions (32).

5.9. Cereberus

Cereberus, another member of the DAN family, has been demonstrated to inhibit BMP activity during early development of the anterior head of *Xenopus*, but little evidence has been found for a physiologic role played by this protein in skeletogenesis (32). Finally, protein related to DAN and cereberus (PRDC) has been found to share similar sequence homology as gremlin and binds and inhibits BMP-2 and -4 with significant affinity (32). Additionally, PRDC expression is induced by the Wnt/beta-catenin pathway and the resultant PRDC then negatively feeds back on local BMP-4 activity (70). The physiologic significance of these less well-described BMP antagonists is yet unknown. Many more studies are required to further elucidate whether any of these proteins may demonstrate the potential to function as targets and participants of clinical fracture healing therapy in humans.

6. CONCLUSION

BMPs have revolutionized the treatment of both acute fractures and fracture non-unions, and have played a large role in the advent of the rapidly expanding field of orthobiologics. As new and expanded clinical applications are developed for BMP therapy, the role of BMP activity modifying factors must be taken into account. In addition to maximizing the effects of BMP therapy, these antagonists may prove to be potential targets for manipulation and/or pharmaceuticals that will allow indirect activity modification of native BMP. Many questions concerning the temporal and spatial dynamics of BMP antagonists during fracture healing remain unanswered. By advancing our understanding of the roles these powerful factors play in modulating BMP activity during fracture healing, we may in time develop the ability to exploit their inherent functions in order to significantly enhance our therapy of fractures and fracture non-unions.

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Abbreviations: BMP: bone morphogenetic protein, VEGF: vascular endothelial growth factor, TGF-ß: transforming growth factor-ß, TNF-alpha: tumor necrosis factor-alpha, GDF: growth differentiation factors, Smads: small mothers against decapentaplegic, DNA: deoxyribonucleic acid

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