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Bone Morphogenetic Proteins an Update and Review

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ABSTRACT

The use of biological factors for bone regeneration has revolutionized the management of fracture repair and spinal fusion. Various biological factors, such as bone morphogenetic proteins (BMP), fibroblast growth factors (FGF), platelet-derived growth factor (PDGF), insulin like growth factors (IGFs) and LIM mineralization protein-1(LMP-1), have been investigated for application in bone regeneration and skeletal repair. Even though autologous bone graft remains the gold standard for most orthopaedic procedures, the procedure suffers from significant disadvantages and hence different and novel approaches are being sought to achieve effective bone regeneration. Bone Morphogenetic proteins (BMPs) as a viable substitute to autologous bone graft have been the subject of intense research in the last few decades and have followed a long and iterative process to provide a burden of proof for clinical use at present time. In this review the focus is on growth and differentiation factors, specifically the bone morphogenetic proteins, which are of major experimental interest, the aim being the clinical use and acceptance of BMPs in the healing of large segmental bone defects.

Introduction

Most often in orthopaedic and reconstruction surgeries there is the need to transplant cancellous or cortical bone to defective sites in order to restore the integrity of fractured bones and enhance healing. The out-come of this surgical procedure is dependent on a number of factors, including the type of graft used, the method of fixation applied and the host species. All bone grafts are initially resorbed however, cancellous grafts generally resorb faster than cortical grafts. [1] The materials applied in bone graft surgeries are broadly divided into autografts, allografts, xenografts, synthetic materials, and a combination of these. [2]

Autogenous graft is superior to allograft, as remodelling and bone healing takes place more slowly in allografts compared to autografts. [1, 3-7] However because there is a limit to the amount of bone available for grafts from the various available graft sites graft surgical procedure causes severe and increased morbidity in the host from which the grafts are harvested. This is why allografts are being used widely. Again, allograft bone has a variant of problems. First, there is the risk of viral disease transmission, such as Human Immuno-deficiency Virus and hepatitis. There is also the risk of causing immune reactions that on the long run may interfere with the bone healing process. Allografts can be processed for long-term preservation and this bone banking ability of allografts allows their wide used in clinical orthopaedics. [8-10] Freezing and freeze-drying are also associated with a reduction in immune reactions, and in case of freeze drying the mechanical strength of the prepared bone graft is often compromised [11-13] Despite the universal usage of banked bones there are many unanswered questions regarding allograft immunology, incorporation and remodeling. [2, 14]

An unlimited supply of bone graft materials could be available from xenografts if they could be processed and made safe enough for transplantation and grafting to a human host. [15]

Even though xenograft bone or xenogenic collagen material have been established experimentally to be a possible alternative source of graft materials the procedure has yet to gain wide acceptance [16-20] The inherent problems of allografts is also seen with xenografts; and being from an entirely different species, there is a likelihood of more pronounced immunological problems. Human allograft materials are considered more effective and more widely available compared to xenografts at present. [2]

The use of demineralized bone matrix (DBM) is therefore an interesting alternative to autologous bone grafts and DBM have been proven to have osteoinductive potentials. [21-25] It has been hypothesized that structural rigidity of non-demineralized or nondecalcified bone does not allow the easy release of osteoinductive substances in them. These proteins however become easily available when bone is decalcified appropriately without interfering with the protein structures of the demineralized or decalcified bone. [26] More so because the process of demineralisation destroys the antigenic properties of bone it is considered an advantage even though there are marked variations in the results obtained from various studies in which DBM was used as bone grafts substitute and compare to autograft bone. [23, 26-28] Most importantly, the methods of processing is very essential and it should be standardized. [29] It has also been proposed that DBM should be bio-assayed appropriately before use due to variations that have been reported in the osteoinductive effect of DBM. [30]

As an alternative to bone grafts materials several synthetic materials have been developed; these include natural coral, hydroxyapatite, tricalcium phosphate, bioactive glasses and synthetic polymers and they have been used as filling material in bone defects in experimental animal studies and clinically. [31-35] However, their use and combination and compartability with the host bone is clearly inferior to autografts. Even though they have been shown to enhance osteoconduction, which is a three-dimensional process of the growth of capillaries, perivascular tissue, and osteoprogenitor cells of the host into the graft. [1] The synthetic materials lack a major characteristic of a good bone graft substitute; osteoinduction.

Osteoinduction was first defined by Huggins [36], who demonstrated that auto implantation of the transitional epithelium of the urinary bladder to abdominal wall muscle in dogs provoked ectopic bone formation [36]. Levander [37] also recognized the phenomenon of osteoinduction when he

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demonstrated the ability of crude alcoholic extracts of bone to induced new bone formation when injected into muscle tissue. [38]

The theory of embryonic induction, a process involving the interaction of two systems: induction and reaction in which hypertrophied cartilage or DBM, transitional epithelium and osteogenic agents was the inducing system, while mesenchymal tissue cells that had the ability to become osteoblasts was the reacting system was defined by Spemann before Marshal R. Urist [21] described DBM ability to form ectopic bone when implanted intramuscularly in rabbits and rats. This was a key seminal discovery. It not only stimulated the search for a bone-inducing substance in the bone matrix (DBM) but also opened frontiers in the investigation science that finally demonstrated that low-molecular weight proteins could be extracted from demineralized bone matrix. [40] These proteins showed more osteogenic activity than DBM, and they were later named the Bone Morphogenetic Proteins (BMPs).

Bone Morphogenetic Proteins (BMP):

Since Huggins reported in 1931 that bone growth was evident in surgically implanted fascia to bridge gaps within the bladder, [41] and subsequent identification of the proteins by Urist; [21] there is extensive evidence in support of their role as regulators of bone induction, skeletal tissue maintenance and repair. [42, 43] Bone marrow stromal cells and perivascular mesenchymal cells form an important source of pluripotential progenitors that are capable of differentiating into osteoblast and chondroblast under appropriate conditions. [43, 44]

Osteoinduction can be defined as a process whereby one tissue, or its product, causes an undifferentiated tissue to differentiate into bone. Thus, it is clear that skeletal tissue regeneration requires the interaction of cells, growth and differentiation factors and an appropriate or suitable matrix scaffold. [45.46] These three basic elements are necessary for successful bone regeneration; several studies have demonstrated the effectiveness of combining these three essential elements in bone regeneration and repair. [47-51]

This review focuses on the growth and differentiation factors, specifically the BMPs, which are of major experimental interest, the aim being the clinical use of BMPs in the healing of large segmental bone defects.

Discovery of BMPs:

The fact that bone has a potential for regeneration and repair has been evident since the time of Hippocrates; Pierre Lacroix proposed that in bone, there might be a hypothetical substance, osteogenin that might initiate bone growth.[52] The bio-science behind bone morphogenesis was demonstrated by Marshall R. Urist when he made the seminal discovery that demineralized, lyophilized segments of bone induced new bone formation when implanted in muscle pouches in rabbits and thereafter; Urist proposed the name "Bone Morphogenetic Protein" in the scientific literature in the Journal of Dental Research in 1971.[21, 53]

Osteoinduction was later shown to be a sequential multistep event. The major steps leading to the final event in osteoinduction were chemotaxis, mitosis, and differentiation this was revealed in studies involving bone matrix-induced bone morphogenesis.[54] On the basis of Urists' work, it seemed likely that morphogenes were present in the bone matrix. Using a battery of bioassays for bone formation, a systematic study was undertaken to isolate and purify putative bone morphogenetic proteins. [21, 54]

One major difficulty encountered by scientist in the process of purification of Bone mophogenes was the insolubility of demineralized bone matrix. To overcome this hurdle, Hari Reddi and Kuber Sampath used dissociative extractants, such as 4M guanidine HCl, 8M Urea, or 1% SDS. [55] However, the soluble extracts and insoluble residues obtained were incapable of inducing new bone formation independently. This therefore suggested that optimal osteogenic activity requires a synergy between soluble extract and the insoluble collagenous substratum.[56, 57] This presented a dynamic advancement towards final isolation and purification of BMPs by the Reddi laboratory,[56, 57] and also enabled the initial cloning of BMPs by John Wozney and colleagues at the Genetics Institute.[58]

The use of biological factors for new bone formation and skeletal tissue regeneration has brought a revolutionary turn around in the management of fracture and spinal fusion. A number of biological factors, such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), insulin like growth factors (IGFs) and LIM mineralization protein-1(LMP-1), have undergone a series of investigative research as possible application in bone regeneration and skeletal repair and as viable substitute for autologous bone grafts.[59-63]

Even though autologous bone grafts still remains the gold standard in most clinical orthopaedic procedures; the significant disadvantage of autologous graft procedures cannot be overlooked hence different novel approaches are being tried to achieve sound bone regeneration and enhance large segmental skeletal tissue repair. BMPs as a viable alternative for autologous bone graft have therefore been in the fore front of skeletal tissue engineering research in the last few decades and have followed a long and iterative process to provide a burden of proof for clinical use at present time. Bone Morphogenetic proteins are by far the most widely reseached orthobiologic product in recent times with over 1000 peer-reviewed publications in worldwide literature. The approvals for use of BMPs clinically and commercially beyond research have only recently been granted. Further research in the usage and application of BMPs is being pursued vigorously and studies on their mechanism of action, optimal formulations, and alternative use continues. Since newer insights into the nature of bone biology and the breakthrough in the recombinant technology has made commercial availability of BMP products a reality. These proteins have been isolated from the bones of a variety of mammals: mouse, rats, bovine, monkey and man. [64-69] and also from clonal osteogenic sarcoma lines. [70, 71] In 1979, Urist *et al* showed that BMP can be extracted from animal cortical bones by digesting the demineralized bone matrix with bacterial collagenase and solubilization of the digest in a neutral ethylene glycol and a salt mixture. [72] The extracted BMP was found to induce bone formation in not only the same species but also in other species. The human BMP was later extracted by Bauer and Urist using a 4M guanidine hydrochloride solution, this extracted substance was shown to induce bone formation in thigh muscles of athymic nude mice. [73]

In 1980's bone inductive preparations were purified from bovine bone in sufficient quantity and purity to provide amino acid sequence data. Using these sequences, nucleic acid probes were generated and used for the identification and characterization of DNA sequence encoding these proteins. [58] With advent of better isolation techniques and the research leading to recombinant cloning techniques, a large number of molecules that form part of the BMP family have been described and have provided a vital impetus to research in this field. The availability of recombinant human BMP (rhBMP) created an opportunity to assess the material properties devoid of impurities and without the potential risk of xenograft reaction during human use. All except BMP-3 have shown to be osteoinductive. BMP-3 has in fact shown to be an inhibitor of osteoinductive activity in the rat assay; this is interesting given the fact that the BMP-3 is the most abundant BMP in bone. [37]

Types of BMPs:

BMPs are a group of growth factors and cytokines known for their ability to induce bone and cartilage formation. Originally, seven such proteins were discovered. Of these, six (BMP-2 through BMP-7) belong to the Transforming growth factor β superfamily of proteins. Since then, thirteen more BMPs have been discovered, bringing the total to twenty.

Applications and Role of BMPs:

With the advent of recombinant DNA technology, the BMPs are now easily cloned and produced and these formulations have found useful applications in several disciplines in surgery. Today the orthopaedic surgeons, oral and maxillofacial surgeons have benefited immensely from commercially available BMP formulations.

BMPs have been found to play a largely important role in embryogenesis and early prenatal skeletal formation and development, disruption of BMP signalling can affect the body plan of the developing embryo. BMP-4 has been found to be associated with a number of human skeletal disorders.

Several BMPs are also named 'cartilage-derived morphogenetic proteins' (CDMPs), while others are referred to as 'growth differentiation factors' (GDFs).

Bone Morphogenetic Protein Classification, Character and Properties:

BMPs are members of the TGF–β super family; this super family compromises of proteins that are coded for by a 45-gene sequence that has a highly characteristic conserved 7 Cysteine motifs in their mature domain. [74]. This super family of proteins contains: five isoforms of TGF – β (TGF - β 1 through TGF - β 5), the BMPs, Growth Differentiation Factors (GDFs), activins, inhibins and Mullerian inhibiting substance and the superfamily has impact on a wide array of cellular activities including growth, differentiation and extracellular matrix formation. [74]

BMP is the largest sub-group belonging to the TGF-β superfamily; they are synthesized and stored as large dimeric proteins in the cytoplasm and cleaved by proteases during secretion. [74] The structure of BMPs that have been most extensively studied OP-1 is one of a polypeptide containing 431 amino acids with a crystal structure consists of a "hand shaped structure" comprising two fingers of anti-parallel β strand and an alpha helical region at the heel of the palm. [74]

BMP Signaling Pathway:

Bone morphogenetic protein (BMP) induces ectopic bone formation, ligand binding to its receptor induces the formation of a complex in which the Type II BMP receptor phosphorylates and activates the Type I BMP receptor (Figure 1). The Type I BMP receptor then propagates the signal by phosphorylating a family of signal transducers, the Smad proteins. Upon phosphorylation by the BMP Type I receptor, Smad1 can interact with either Smad4 or Smad6. The Smad1-Smad6 complex is inactive; however, the Smad1-Smad4 complex triggers the expression of BMP responsive genes. The ratio between Smad4 and Smad6 in the cell can modulate the strength of the signal transduced by BMP (Figure 1).

BMPs exert their effect through activation of transmembrane heteromeric receptor complex formed by type I and type II serine/threonine kinase polypeptides, also known as the BMP receptor (BMPR) type IA and IB and BMPR Type II. [75, 76] The activated receptor kinases in turn phosphorylate the transcription factors Smad 1, 5, and 8. The phosphorylated Smads then form a heterodimeric complex with Smad 4 in the nucleus and activate the expression of target genes in concert with co-activators. [75-77]

BMP localization:

Traditionally BMP were considered localized to bone but subsequent studies have shown that BMPs are expressed in most tissues and throughout the embryonic development. [78] Some members of BMP family have also been mapped to different chromosomes loci's: BMP 2 (Chromosome 20), BMP 3 (Chromosome 4), BMP 4 (Chromosome 14), BMP 6 (Chromosome 6), BMP 7 (Chromosome 20), BMP 8 (Chromosome 1), and BMP 15 (chromosome X). [78]

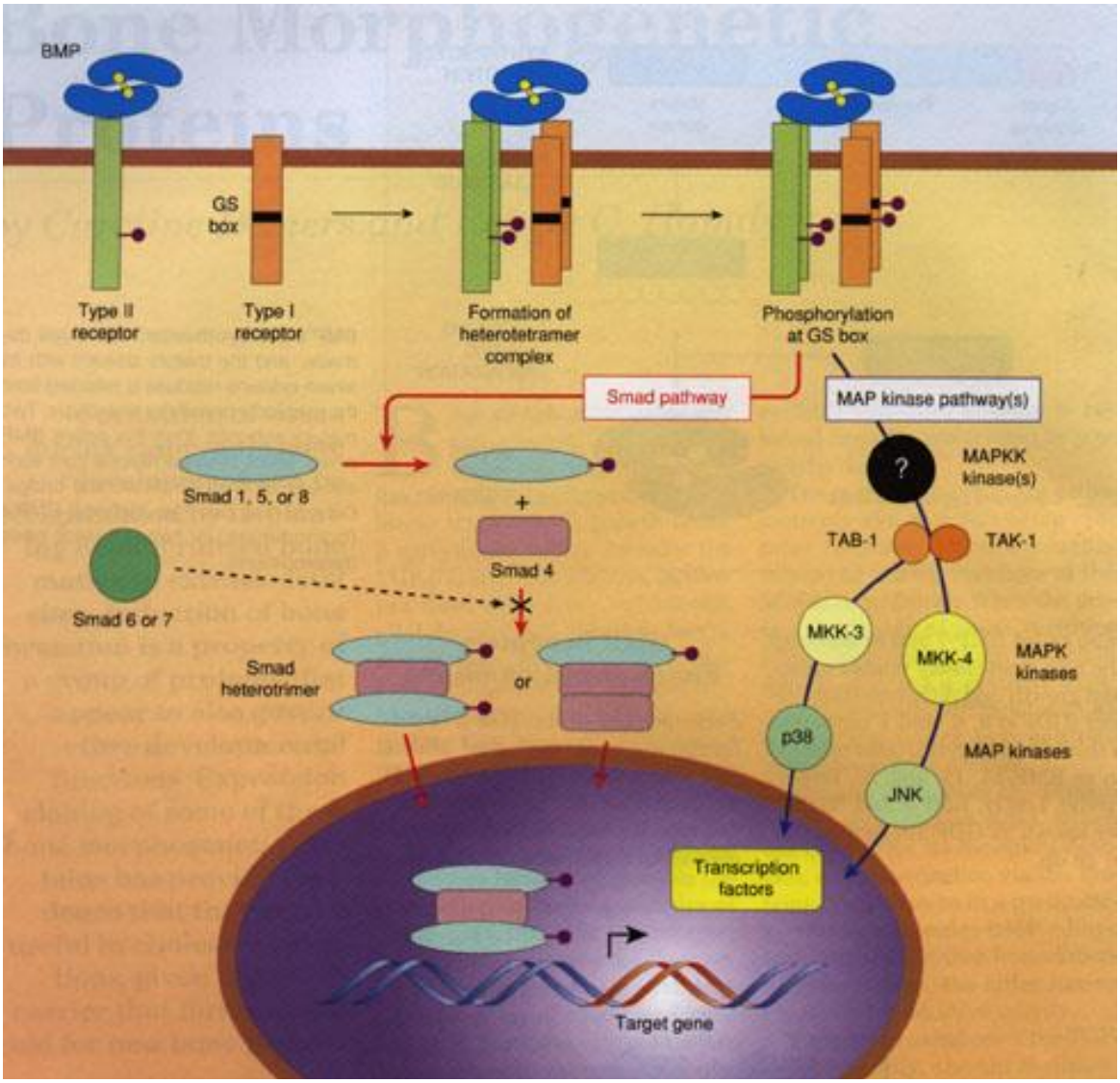

Figure 1: Binding of a BMP dimer to its type II receptor recruits type I receptors, so that a heterotetramer is formed with two receptors of each type. The proximity of the receptors allows the type II receptor to phosphorylate the type I receptor. One of two identified downstream pathways, the Smad cascade, is initiated by phosphorylation of certain Smad proteins by type I receptors, and the other pathway involves two mitogen-activated protein kinase (MAPK) cascades. In either case, the consequence is regulation of gene transcription. [77]

Biological Activity of BMPs:

BMP are pleiotropic regulators orchestrating various sequential cellular responses: chemotaxis of cells, mitosis and proliferation of progenitor cells, differentiation into chondroblasts, cartilage calcification, vascular invasion, bone formation, remodelling and bone marrow differentiation. BMP also stimulates extra cellular matrix formation. [79-85] Besides its osteogenic potential the BMPs have also been shown to have an effect on the development of other organs and tissues particularly those formed through the mesenchymal-epithelial interactions. [86-88] Different BMPs have being indicated for varying functions (Table 1).

Implantation of purified recombinant BMP with bone collagen matrix in subcutaneous sites in rats has been shown to induce a sequence of cellular event leading to formation of new bone with all its elements. [89] The implanted BMP triggers a biological reaction in which pluripotent stem cells are stimulated to proliferate and differentiate into chondrocytes. This bio transformative process takes about 5 to 7 days, after which the capillary invasion takes place. The differentiated chondrocytes under goes a process of hypertrophy and calcification, and osteoblasts appear at the implant site. Hence new bone formation is seen at 9-12 days and remodeling, ossicles formation and bone formation takes place in the following 14 –21 days. [89, 69] The biological process which is similar to physiologically occurring endochondral ossification and intramembranous ossification wherein pluripotent progenitor cells directly differentiate into the osteoblasts has been seen with BMP in in vitro studies. However, this effect may be seen only at a higher concentration of BMPs. [90]

Development and production of BMPs:

BMPs extracted and purified from cortical bone is not a commercially viable option as cost of extraction is high and the process is cumbersome. [91] This prevented the exploitation of BMP technologies in the 80's and 90's, but with the advent of recombinant technology, commercial development of BMPs has taken the centre stage of mainstream research in orthopaedics. The method offers extreme pure preparations of single BMPs. The recombinant technology used to develop and manufacture BMP involves; identification and cloning the human gene for BMP and subsequent production of recombinant human BMP (rhBMP). The Specific genes responsible for carrying the code for making BMP in humans were identified at Genetics institute. [43] Once this gene was identified and isolated, it was spliced and recombined into the DNA of a commonly used production cell. This insertion or 'recombination' of gene results in formation of a "recombinant". The recombinant cell grows and multiplies, a process called 'cloning'.

Table 1: Bone morphogenetic proteins and their functions.

BMP	FUNCTION
BMP-2	Osteoinduction, Osteoblast differentiation and apostosis
BMP-3	Most abundant BMP in bones, inhibits osteogenesis
BMP-4	Osteoinductive, lungs and eye development
BMP-5	Chondrogenesis
BMP-6	Osteoblast development, chondrogenesis
BMP-7 (OP-1)	Osteogenesis development of kidney and eyes
BMP-8 (OP-1)	Osteoinductive
BMP-9	Nervous system, hepatic reticuloendothelial system, hepatogenesis
BMP-10	Cardiac development
BMP-11 (GDF-8) Myostatin	Patterning mesodermal and neuronal tissues
BMP-12 (GDF-7)	Induces tendon-iliac tissue formation
BMP-13 (GDF-6)	Induces tendon-iliac tissue formation
BMP-14 (GDF-5)	Induces tendon and ligament – like tissue formation
BMP-15	Modifies follicle stimulating hormones activity

GDF = Growth, differentiation factor

Source: Neurosurg focus © 2002 American Association of Neurological Surgeons. Medscape ® www.medscape.com

This results in development of a homogenous population of cells producing a recombinant human bone morphogenetic protein. This batch of recombinant cells is preserved and maintained at -135^{0}C for future production in several small vials known as cell bank. [45] The recombinant cells when cultured in optimal media produce the BMP that are purified and made available for commercial use.

The commercially available BMPs currently approved by Food and Drug Administration (FDA) includes rh BMP-2 (Medtronics Sofamor Danek, Memphis, Tenesse) and OP-1 (Stryker Biotech, Hopkinton, MA). Other BMP products that are being currently evaluated for commercial use include BMP–X (Sulzer Biologics, Wheat Ridge, Colarado), BMP–9, combinations of animal and human BMP implants, etc.

In spinal fusion surgeries BMP family members have been found to be potentially useful for therapeutics. BMP-2 and BMP-7 in clinical studies have proven to be beneficial in the treatment and management of a series of bone-related conditions including delayed union and non-union. BMP-2 and BMP-7 have received Food and Drug Administration (FDA) approval for human clinical uses. At between $6000 and $10,000 for a typical treatment, the cost of BMP treatment is high apparently when compared with bone grafting. However, this cost is often becoming far lesser than the final costs required with orthopaedic revision in multiple graft surgeries.

Dosage and Toxicity of BMP:

The application of growth factors for tissue repair and reconstruction is increasingly popular and gaining favourable acceptance as clinicians, scientists, and patients search for less invasive therapies for dental, maxillofacial, craniofacial and orthopaedic indications.[92-97] Currently, most bone repair and regenerative approach using growth factors have focused on BMP-2, a potent osteoinductive molecule.[99, 100] However, concerns about the physiologic doses required for effective bone regeneration, as well as adverse effects such as heterotopic bone formation are raised when high doses are used.[101-104]
Many preclinical toxicity studies evaluating acute and systemic toxicity, bio-distribution, reproductive toxicity and carcinogenetic effects of BMPs; [105, 106, 107, 108] have been carried out and the BMPs have demonstrated excellent safety profile in most of these studies. [105] There is no evidence that the BMP is carcinogenic and conversely, it has shown anti proliferative effect in vitro on human breast, ovary, lung and prostate cells.[106] Pre clinical safety studies have shown BMP to have inhibitory effect on the human osteosarcoma, prostate, lung, breast and tongue carcinoma lines.[107, 108]

Studies have documented presence of up to 0.7% antibodies to rh BMP 2 in titres of patients treated with rh BMP 2 collagen sponge in tapered cages for anterior spinal fusions[109] raising the concern and suspicion that its use may not be effective in all group of patients.

The ability of BMPs to form ectopic bone at implantation sites is also a potential concern that is being investigated. Paramore *et al* [110] evaluated the toxicity of OP-1 by placing OP-1 into the epidural space after laminectomy and posterolateral fusion in a dog model. They demonstrated that animals with OP –1 implantation demonstrated bone formation adjacent to spinal cord that caused mild spinal cord compression. The spinal cord histology however, showed no evidence of spinal cord inflammation or neuronal cell death. [110] Some other animal studies however did not find any bony encroachment on the exposed thecal sac after laminectomy and intertransverse arthrodesis with the use of rh BMP-2 in non-human primate model.[111] The direct application of BMP on nerve tissue has not been shown to have any adverse physiologic or permanent histologic effects.[111]

The signalling cascade of bone morphogenetic proteins:

Osteogenesis is a sequential multistep cascade with three phases: first it involves the movement and division of mesenchymal cells and the subsequent differentiation of mesenchymal cells into chondroblasts, the second involves cartilage formation and finally, substitution of cartilage by bone.[112] These sequence is triggered by the binding of plasma fibronectin to the demineralized bone matrix, enhancing adhesion and proliferation of mesenchymal cells at 3 days after implantation[112] Chondrogenesis is observed after 5 days, reaching its peak at 7-8 days. Cartilage hypertrophy and mineralization are observed after 9 days.[112] Osteoblast differentiation depends on angiogenesis and the highest level occurs after 10-11 days.[112] The sequence of morphogenetic events in response to the demineralized bone matrix simulates the initial embryonic

skeletal morphogenesis and is also similar to the process of bone repair in adults. [112]

Accordingly, the key signals for bone morphogenesis have been identified. The BMP, as a signaling molecule, binds to a type II specific receptor present on the cell membrane and recruits a type I receptor, forming a complex. (Figure 1) These receptors are transmembrane serine/threonine kinase proteins that self-phosphorylate after the formation of the BMP-receptor II-receptor I complex. They subsequently acquire the ability to phosphorylate Smad proteins, a family of TGF-ß transducers (Figure 1). Smads are a family of signaling mediators of BMP receptors in vertebrates homologous of Mad (mothers against decapentaplegic, in *Drosophila*) and Sma (related to Mad in *C. elegans*) and can be classified into three subtypes by structure and function, i.e., receptor-regulated Smads (R-Smads), common-mediator Smads, and inhibitory Smads. R-Smads are phosphorylated by activated serine/threonine kinase receptors (BMP-receptor II-receptor I complex). R-Smads interact with common-mediator Smads to form hetero-oligomeric complexes, which then translocate into the nucleus and regulate the transcription of various target genes. [113] It is not clear whether Smads can recognize specific binding sites and bind to DNA by themselves (Figure 1). Recent studies have identified specific BMP antagonists (i.e., noggin and chordin) and members of the DAN family (i.e., gremlin). Such antagonists bind to BMP with the same affinity as their specific receptors, blocking signal transduction and thus decreasing bone formation. Therefore, these antagonists may be used therapeutically in pathological conditions characterized by excessive bone formation. [114]

Bahamonde and Lyons demonstrated that BMP-3 has an inhibitory effect on osteogenesis, presenting a signalling pathway similar to TGF-ß/activin. The ability of BMP-3 to inhibit the activity of BMP-2 seems to result from competition for common signalling components of the TGF-ß/activin and BMPs pathways. Since BMP-3 is by far the most abundant BMP in demineralized bone, it probably plays a fundamental role as a modulator of the osteogenic activity of other BMPs *in vivo*. [115]

These findings are of great clinical relevance because of the need to quantitate the amount of BMP-3 when products composed of exogenous BMPs are used to accelerate bone regeneration. The osteogenic potential of BMPs is increased when the antagonists are eliminated. Nevertheless, BMP-3 could be used in the treatment of diseases characterized by bone hypermineralization, such as osteopetrosis [115].

Even though current BMPs delivery vehicles show promising results, optimization of the site-specific binding and controlled release remains a challenge. [116]

BMP Carriers:

To enhance osteoinduction, bone morphogenetic proteins must be used with an appropriate carrier substance, since the proteins are soluble within biologic fluids. Although there is no absolute need for a delivery system, if a sufficient amount of bone morphogenetic protein is applied, bone formation can be observed.[58, 117] An appropriate delivery system such as a carrier is essentially required to enable the optimization of osteogenic activity of BMPs in the localize sites.[118,119] It has also been shown that the carrier material may eventually have an effect on the pharmacokinetics of BMP on the basis of its ability to release the protein as desired.[120, 121]

Overall, the development and production of these osteoconductive carriers has not progressed as rapidly when compared to the isolation, purification and synthesis of these novel growth factors. This search for an effective carrier has significantly slowed down the pace of development of clinically successful biosynthetic composite implants.[122] Theoretically, the carrier material will have to meet the following requirements; relative insolubility in physiological conditions, biodegradability, protection against proteolytic activities, substrate for cell adhesion and proliferation, immunologically inertness, slow release of BMPs through controlled biological degradation and lastly, mechanical stability in bridging segmental bone defect.[123] Many different carrier materials have been used in a variety of animal models, in which bone morphogenetic proteins have been tested [58, 94, 121, 124-126] but the optimal carrier material for BMPs still remains to be found. The optimal type of carrier material used will probably depend on the clinical indication to which the morphogenetic protein is being applied.

In recent years, the greatest interest has focused on resorbable synthetic polymers, such as polylactide (PLA) and polyglycolide (PGA), which are members of a large family of poly-alpha-hydroxy-acids. Polylactide is a synthetic thermoplastic polymer of cyclic diesters of lactic acid. Polylactic acid has two optically active stereoisomers, poly-L-lactic acid (PLLA) and poly-D-lactic acid (PDLA). [127] The physical properties of the copolymers of L-lactic acid and D-lactic acid (PDLLA) are dependent on the relative amounts of L- and D-monomers. Their advantages include the synthetic nature of the system and the accumulated clinical and regulatory ability of PLA. [128]

Calcium phosphate materials, including coralline, hydroxyapatite, tricalcium phosphate and their composites, have been proposed as potential carrier materials for BMP; they resemble bone tissue structurally and are usually biocompatible, but their variable and often extremely slow biodegradation makes them suboptimal as carriers. [129]

Hydroxyapatite (HA) is a material that has been used widely in animal studies as a carrier material for various BMPs. It has been used in ectopic muscle implantation, in a skull defect model, under the periosteum of parietal bone and in mandibular bone defects, and a combination HA-BMP proved to be more effective than HA alone in all these studies.[69, 71, 126, 130, 131, 132, 133, 134, 135] The effect of a HA-BMP combination in spinal fusion was clearly demonstrated by Boden *et al.* [136] The addition of collagen or bone marrow has further enhanced the osteogenic potential of the HA-BMP composite.[137, 138] It has been suggested that the geometrical configuration of hydroxyapatite may be an important factor in osteogenesis.[139,140]

Natural coral has been used in animal bone defect models with good results; [141, 142] although there was obviously an immunological reaction to natural bovine BMP in the former, which impaired healing at the later stages of the study.[141] A study conducted using natural coral with BMP in rat cranioplasty revealed that; natural coral with BMP was superior to natural coral alone.[51]

Food and Drug Administration (FDA) Status on BMPs:

On October 17, 2001, the FDA granted Stryker Biotech approval for its OP-1 Implant under a humanitarian device exemption (HDE). The HDE is similar to a premarket approval (PMA) application, but is exempt from the effectiveness requirements of a PMA. The application must allow the FDA to determine that the device does not pose a significant risk of illness or injury. Product labelling must state that the device is a humanitarian use device and that effectiveness of the device for the specific indication has not been demonstrated. [143]

The OP-1 Implant is approved as an alternative to autograft in recalcitrant long bone nonunion where use of autograft is not feasible and alternative treatments have failed. On July 2, 2002, the FDA granted Pre-Market Approval for Medtronic Sofamer Danek's InFUSE® Bone Graft and LT-CAGE® Lumbar Tapered Fusion Device system. The device is classified as Protein, collagen scaffold with metal prosthesis, osteoinduction. InFUSE® Bone Graft and LT-CAGE® Lumbar Tapered Fusion Device system is FDA indicated for patients with degenerative disc disease at one level from L4-S1. Degenerative disc disease is defined as discogenic back pain with degeneration of the disc confirmed by patient history, function deficit, or neurological deficit and radiographic studies. Patients could also have had Grade I Spondylolisthesis. The device is to be implanted via an anterior open or anterior laparoscopic approach. [143]

Clinical Applications of BMPs:

BMP have been used in various clinical applications including canine ulnar defect, treatment of recalcitrant non unions and large segmental defects some are discussed below.

Canine ulnar segmental defects:

Canine ulnar segmental defect is a well-established model. The dog ulna is not directly a weight-bearing bone, as the radius gives some support to the ulna, and there has been some controversy about the fixation methods. The ulnar defect model has been used with no fixation at all. With BMP implants, these defects have been found to heal effectively rather than being a permanent non-union or a pseudo arthrodesis. [144, 145, 146, 147] with an intramedullary Steinmann pin [148,149] or plate fixation [150,151]

Treatment of a segmental bone defect with BMPs:

Segmental long bone defects have been used as models for bone reconstruction to evaluate different implant materials as well as the efficacy of BMP. This model is valid in studying osteoconductive agents when the defect (large enough) does not heal spontaneously. Animal studies with bone defects treated with bone substitute materials or BMP

include dog radius, [152,153] dog femur, [154] dog fibula, [155,156] sheep and goat tibia, [147, 157] rabbit ulna, [27] rabbit radius, [158-160] rat femur, [161, 162] and dog ulna. [145, 147, 149, 151] In evaluating the results, various methods of analysis have been used, the principal methods being radiography, histology and torsion testing.

Purified versus Recombinant BMPs:

As with every growth factor, BMPs act at very low doses in the tissues, existing in nanograms or micrograms. However, in order to isolate a couple of micrograms of BMPs, kilograms of demineralized bone matrix are needed. Having been isolated, different BMPs may be identified by their amino acid sequences. Purification of BMPs from the demineralized bone matrix can be carried out by four distinct methods:

1) Enzymatic digestion, since they resist collagenase.
2) Ethylene glycol extraction, due to the hydrophobic nature of the BMP molecule.
3) 6M Urea plus 0.5 M $CaCl_2$, since BMPs can be dissociated from other non-collagen proteins in chaotropic solvents.
4) Concanavalin A affinity chromatography due to their hydrophobic nature and to carbohydrates present in their structure.

In spite of these methods, purification of BMPs is an extremely laborious process and the yields are low. Preparations must be initiated with a minimum of 100 kg of washed fresh cortical bone free of bone marrow. [141, 69] Isolation of a particular native BMP yields even smaller amounts of the order of µg/kg tissue. The small amounts of BMPs resulting from such a laborious purification process have stimulated the application of molecular biology techniques for the cloning and expression of these proteins. [58, 69, 141, 163]

The molecular cloning of the first genes encoding BMPs took place at the end of the 1980's and more than 30 members of the BMP family have been described [163] The study of different BMPs revealed that their expression pattern and their biological functions are not restricted to skeletal development. Other functions have been identified, such as cell proliferation and differentiation, apoptosis, morphogenesis of various organs, including the skeleton, and organogenesis (Table 1).

Alternatively, many researchers have isolated bioactive proteins which induce cartilage and/or bone formation at the sites implanted, but the yields were low and the purification process was very laborious. [164] In addition, the potential risk implicated in their origin from allogeneic donor bone reduced their clinical application. [165] cDNAs for different BMPs have been identified and cloned. The sequences deduced from these cDNAs have indicated that these proteins are members of the TGF-ß superfamily, except for BMP-1, which has been identified as procollagen C proteinase. [166] The molecular cloning of BMP-encoding genes and their identification as TGF-ß relatives has enhanced the interest in these proteins and has permitted their expression and functional studies.

In view of the osteoinductive properties of BMP-2 and BMP-7, scientists set out to isolate the human cDNA counterparts of these molecules to clone them into appropriate transducing vectors in order to produce and purify these recombinant proteins using heterologous bacterial, mammalian and baculovirus expression systems [166, 167]. The recombinant proteins obtained are being used in blind cDNA cloning strategies to identify and characterize novel potential regulators of the osteoblast differentiation process, to better understand the molecular mechanisms involved in bone formation and to gain new therapeutic insights. [116, 167]

The osteoinductive properties of recombinant BMPs are reduced compared to purified BMPs and require the characterization of BMPs by genetic engineering techniques.[167] Bessho *et al.*, [167] have analyzed in detail the effects of purified versus recombinant bovine BMP. On the basis of Ca^{2+} content and radiographic aspects, they observed that maturation of bone tissue ectopically formed in rat muscle was as much as 10 times greater when bovine BMP was used.

Other Applications under development:

Fracture healing may be one of the major applications of bone morphogenetic proteins in the future. Several growth-promoting substances have been identified at the site of skeletal injury that appear to play a physiologic role in fracture healing. [168] BMPs may be capable of healing the cases of delayed union or non-union, which represent 5–10 % of all fractures. [169] It has been suggested that BMP-2 and BMP-4 are important regulators of cell differentiation during fracture healing and bone tissue repair. [170] On the basis of the in situ hybridization technique, BMP 4 seems to be one of the local contributing factors in callus formation in the early phases of fracture healing.[171] Bax *et al.*, [172] used rhBMP-2 in fractures of the rabbit tibia. In a series of mechanically unstable fractures, those treated with BMP gained union more rapidly, while in stable fractures the effect of BMP was minimal. It was argued that mechanical factors influence the size of the callus of normally healing fractures, and although BMP-2 accelerates the rate of development of the callus and cortical union, it does not affect the amounts of bone and cartilage produced. Welch *et al.*, [173] treated goat tibial fractures with rhBMP-2. Callus formation was increased significantly in BMP-treated fractures, but strength and stiffness were only moderately increased.

Most of the studies with BMPs deals with the bridging of critical-sized defects and very few with fracture repair. Although some animal studies have had promising results, the therapeutic efficacy of bone morphogenetic protein in fracture healing remains uncertain and has to be determined in future studies.

The repair of articular cartilage defects is another possible future application of BMPs. So far, very little information is available in this area. It has been reported that articular cartilage defects fill with repair tissue and show good healing with well-organized and intact cartilage at early time points postoperatively, but the repair tissue eventually degenerates due to its inability to withstand the biomechanical forces in the joint.[174] In in vitro studies, rhOP-1 has been shown to stimulate the synthesis of cartilage-specific molecules by human articular chondrocytes[175] and the differentiation of cartilage from perichondrium tissue. [176] Gregic *et al.*, [177] demonstrated rabbit articular cartilage regeneration in drill holes treated with BMP-7. Sailor *et al.*, [178] showed that rhBMP-2 maintains the articular chondrocyte phenotype in long-term cell culture. In a study by Lietman *et al.*, [179] BMP-7 stimulated the proteoglycan synthesis in porcine articular cartilage, implicating that BMP-7 may play a role in the process of cartilage repair. In a rabbit femoral cartilage defect model, Sellers *et al.*, [180] showed that rhBMP-2 applied to the defect resulted in an improvement in the histological appearance and composition of the extracellular matrix at one year postoperatively.

Conclusion:

After decades of intense research BMPs have finally moved from the realm of *in vitro* to *in vivo*. Researchers have demonstrated beyond doubt; the role of BMPs as superior alternative to autologous bone graft and their medical and therapeutic efficacy. Research has also instilled hopes of its use in different and varied musculoskeletal conditions such as; spinal disc regeneration, bone cartilage repair, osteonecrosis of the femoral head, general osteonecrosis, segmental bone repairs, spinal fusion and hip arthroplasties. Progress in identification of suitable delivery materials and appropriate delivery methods may further its use and overall acceptance in surgical procedures that would be minimally invasive. This will decrease operative time and reduced number of surgeries, with a significant decrease in the overall morbidity, patient recovery time and duration of hospital stay. Discovery and progress in the field of BMPs has highlighted the facts that its use as an alternative to autogenous bone grafts has high potential. This excitement is however reduced with the knowledge that a majority of the generated scientific evidence on the successful use of BMPs as a substitute to bone grafts are generated from animal studies. Care must be taken in extrapolations of data to humans. Even though animal studies are key to providing baseline data; further clinical studies in humans is important. There is an inverse proportionality in the rate of bone repair relative to the phylogenetic position of the species. There is therefore a decreased potential of bone formation in humans and higher primates when compared to lower animals like mice, moreover the quadrupeds have different biomechanics compared to upright humans. A host of other factors linked with human behaviour and activities such as cigarette smoking, use of certain steroidal drugs, age and osteoporosis, nutritional deficiencies and prevalence and severity of disease play a major role in determining the physiology of bone regeneration in humans. Additional concerns that may need to be addressed include the fact that BMPs may be degraded more rapidly in humans, the biology of the receptor ligand interaction may be different and the pharmacokinetics of the activity of these growth factors may also be different in humans. Therefore, the actual efficacy, pharmacokinetics and

safety levels of these agents at different level and species interactions must be established by the use of well-designed randomized prospective clinical studies before they are eventually embraced for clinical and therapeutic use in humans. . The search for a suitable and appropriate carrier for BMPs remains and as a priority a search for the perfect carrier continues.

Another concern regarding the use of BMPs is its cost; currently the estimated cost of BMP at $3000 to $5000 limits their clinical use to recalcitrant non-unions where several surgical interventions have failed and revised fusion surgeries. It is however hoped that the cost drops and BMPs eventually becomes readily and easily available and as affordable as other recombinant products like recombinant insulin or recombinant vaccines, enabling and facilitating its use and final embrace in majority of indicated patient population.

In a nut shell, it is time for orthopaedic surgeons to look beyond just the autologous bone grafts and metallic fixation devices and embrace novel discoveries and technologies that involve manipulation of cellular environment to achieve the desired bone formation.

Conflict of Interests:
The authors declare no conflict of interest.

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