CHAPTER X

BONE MORPHOGENETIC PROTEINS IN BONE FORMATION AND DEVELOPMENT

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The discovery, purification, and recombinant synthesis of bone morphogenetic proteins (BMPs) constitute a major milestone in the understanding of bone physiology, and this chapter discusses the history of BMPs from Senn's accidental discovery in the 1880s to Urist's monumental discoveries in the 1960s through to the present day and the FDAs decision on the use of BMPs in mainstream medicine, their classification and functions. The role of BMPs in the development and formation of bone in the embryo and in the adult, their clinical applications in orthopaedics, dentistry, gene therapy and in other medical fields, the dosage, carriers for BMPs, and the potential risks that accompany the use of BMPs, are all reviewed and discussed.

1. The history and classification of BMPs

During 1889, Senn[1] noted while he was treating osteomyelitis defects in bone using a decalcified residue of ox bone and iodoform that the decalcified bone induced healing in the bone defect. In the 1930's Levander[2, 3] noted that crude alcohol extracts of bone induced new bone formation when injected into muscle

tissue. In 1961 Sharrard and Collins[4] reported the use of ethylenediaminetetraacetic acid (EDTA) decalcified allograft of bone for spinal fusion in children. The idea was supported by laboratory studies carried out by Ray and Holloway.[5] Probably one of the most significant discoveries in this field was made by Marshall Urist. In 1965 Urist[6] showed the ability of bone matrix to induce bone formation. Urist did this by implanting HCL-decalcified homogenous diaphyseal bone from animal donors into ectopic sites, e.g. a pouch in the belly of the rectus abdominus, quadriceps, or erector spinae muscles. Urist found that the implanted bone extracts induced new bone formation and he named the active ingredient "bone morphogenetic protein" or "osteogenic protein". However, this research was hampered by the fact that there was no reproducible assay for the protein and that it was not conclusively determined that the putative protein was responsible for new bone induction at the ectopic sites. In 1983 Reddi and Sampath created a crude but highly reproducible assay for ectopic bone formation, and they showed that when the protein component was isolated from the rest of the matrix the remaining matrix did not induce new bone formation. However when the protein was reconstituted with the matrix it was as effective at inducing new bone formation as the original matrix responsible for ectopic bone formation.[7]

The first clinical study was by Johnson et al in 1992, who studied purified human BMP[8]. Intense competition followed in gene sequencing for BMP during the 1990s. The final landmark in this saga is the FDA approval in 2002 for OP-1 (BMP-7) for long bone defects treatment and rhBMP-2 in a collagen carrier within a cage for anterior lumbar interbody fusions.

BMPs are members of the TGF-beta superfamily, as classified on the basis of similarities in the amino acid sequence, which includes TGF-beta activins and inhibins, and Müllerian inhibiting substance (MIS). [9, 10]

Table 2: Bone morphogenetic protein family

BMP	Main Functions
BMP-1	Release of BMPs from bone matrices
BMP-2	Osteoinductive, osteoblast differentiation
BMP-3 (Osteogenin)	Most abundant BMP in bone, inhibits osteogenesis
BMP-4	Osteoinductive, lung and eye development
BMP-5	Chondrogenesis
BMP-6	Osteoblast differentiation, Chondrogenesis
BMP-7 (OP-1)	Osteoinductive, development of kidney and eye
BMP-8 (OP-1)	Osteoinductive
BMP-9	Nervous system, hepatogenesis
BMP-10	Cardiac system development
BMP-11 (GDF-8)	Mesodermal and neuronal tissues patterning
BMP-12 (GDF-7)	Tendon and ligament formation
BMP-13 (GDF-6)	Tendon and ligament formation
BMP-14 (GDF-5)	Chondrogenesis, enhancing tendon and bone healing
BMP-15	Modifies follicle-stimulating hormone activity

GDF: Growth Differentiation Factor.

2. Roles of BMPs in bone development and formation

2.1 Ectopic bone formation and BMPs

Cells located in periosteum, bone marrow, and other extraskeletal sites, have the capacity for bone formation.[11, 12, 13] The differentiation of an unspecialized mesenchymal cell population into bone tissue is initiated by a process known as bone induction. Histologically, formation of bone from a transplanted bone chip (which contain BMPs) resembles the classic picture of endochondral ossification. The initial phase is characterized by attraction of mesenchymal stem cells to the site of implantation. These stem cells surround the chip and within 1–3 days there is a powerful wave of mitogenic activity followed by differentiation into cartilage around the bone fragment. The cartilage becomes calcified and new bone forms. It has been accepted that this

process demonstrates the cartilage model system for bone formation, but closer inspection of the temporal events has revealed otherwise. Caplan[14] reports that there is a layer of osteogenic cells that form a sheet covering the bone chip and that this layer of cells, in intimate contact with invading capillaries, forms the first osteoid, which is mineralized onto the surface of the bone fragment. The hypertrophic cartilage is, however, replaced by marrow, and there are accounts of marrow formation associated with these bone chips.[15] Ectopic bone formation is usually used a functional assay of the true bone induction capacity of BMPs.

2.2 BMPs and the embryonic skeleton

Over the last number of years BMPs have been localized in developing skeletal structures. This has provided evidence that the role of BMPs can be linked to the patterning and differentiation of skeletal cells. *In situ* hybridization has confirmed that BMP-2 to BMP-7 and GDF-5 to GDF-7 transcripts are present in the developing embryo. This is of particular relevance as the various transcripts are present at times and sites within the embryo that are consistent with their participation in mesenchymal condensation and cartilage differentiation.[16] Although many of the upstream signals of BMP expression at specific sites are unknown, studies suggest that BMPs, fibroblast growth factors (FGFs) and sonic hedgehog (SHH) interact in a hierarchical way to pattern skeletal elements.[17, 18, 19]

BMP-2 and 4 were found in the apical ectodermal ridge and the zone of polarizing activity which are two important signalling centres involved in defining limb patterning,[20] however mice carrying the null mutations for BMP-2/4 die at a stage before limb patterning occurs in embryogenesis. This means that there is little know about the specific roles played by BMP-2/4 in early limb development except that mice deficient for BMP-2 are non-viable and have defects in amnion /chorion and cardiac development. [21, 22] It is likely that unlike other BMPs the role of BMP-2 cannot be

compensated for, as BMP-2/4 null mice die before birth but BMP-7 null mice only had mild skeletal deformities. This maybe related in part to the role of BMP-4 in the development of lung tissue; however as the full spectrum of BMP-2/4s functions is unknown, other possibilities may exist. BMP-2 has been proven to stimulate bone and cartilage growth in numerous clinical trials and therefore we can hypothesize that BMP-2 should have a major role to play in bone induction and mesenchymal cell differentiation during embryogenesis if indeed other BMPs cannot compensate for the role it plays. It is important to note that without a cartilage precursor for bone induction life could not occur, as the majority of our vital organs are protected either by bone or by cartilaginous tissues at birth. Without these structural protectors our vital organs would not be able to withstand any form of trauma and numerous birth defects could occur, but this is not sufficient to explain why BMP-2/4 null mice die early during embryogenesis.

BMP-3 is the most abundant BMP in bone matrix and is thought to be an antagonist to BMP-2 activity.[23] The mechanism by which this occurs may relate to the activins receptor pathway. BMP-3 null mice showed increased bone density and increased trabecular volume, therefore BMP-3 maybe responsible for halting bone growth at appropriate sites and times. BMP-5 plays a central role in the formation of cartilaginous structures in the outer ear, sternum and ribs, mice with non-functioning BMP-5 genes showed defects in these structures.[23] BMP-5 was found in other sites but there was no apparent abnormality when BMP-5 was not present, presumably due to other BMPs compensating effects for its absence.[24] BMP-6 is involved in chondrocyte hypertrophy and replacement by bone,[25] however in BMP-6 null mice there was no apparent abnormalities at birth.[26] BMP-7 is present in the early limb bud, however mice lacking BMP-7 still survive until birth but there were mild skeletal abnormalities present. BMP-7 is also an inducer of nephrogenesis, and is required for eye development and skeletal patterning, [27, 28] and it may be compensated by other BMPs

e.g. BMP-2/4,[29] suggesting that the roles and functions of BMPs may overlap in skeletal development.[30] In the GDF-5 (BMP-14) null mouse there was a clear shortening of the long bones, a reduction in the number of digits in the paws and misshapen bones in the front and hind feet.[31, 32] GDF-5 expression normally occurs at the sites where these malformations and abnormalities where present. Therefore it is most probable that GDF-5 is responsible for joint morphogenesis between individual bones and for maintaining regularity in the size and shape of mesenchymal condensations. These can be observed in humans with GDF-5 gene mutations where joint dysmorphogenesis occurs.[33, 34, 35] GDF-11 is thought to be linked to the development of the axial skeleton and in palate development, mice lacking functional GDF-11 had developed additional thoracic and lumbar vertebrae and the complete absence of a tail.[36] GDF-11 may be a negative regulator in skeletal planning and inhibiting chondrongenesis and myogenesis, as ectopic application of GDF-11 in the developing limbs of chicks resulted in shortening of the limbs.[37]

2.3 BMPs in bone remodelling

The size, shape and location of various skeletal elements are determined during embryogenesis; however the adult skeleton undergoes a continuous turnover, bone remodelling, which occurs in response to various systemic and local signals and to mechanical stimulus. Bone remodelling requires the differentiation of osteoblasts and osteoclasts from bone marrow and other precursors.[38] BMPs are local signals which are thought to induce the differentiation of mesenchymal stem cells into osteoprogenitors and osteoblast.[39, 40] Osteoblasts synthesise and secrete BMP both *in vivo* and *in vitro* suggesting that BMPs initiate mesenchymal cell differentiation and create a positive feedback loop allowing the production of additional BMP signals. When recombinant human bone morphogenetic protein 2 (rhBMP-2) was used in segmental bone defect models in rats and in rabbits, it induced endochondral bone formation. Antagonists to BMPs exist, noggin and gremlin

are also present in bone and are made by osteoblasts, suggesting that there maybe local control over mesenchymal cell activation and differentiation.[41, 42] BMP-1 is thought to relate to the release of BMPs from collagenous matrix providing an addition source of exogenous BMPs for site specific remodelling.[43] Bone formation and resorption in remodelling cycle maybe linked through BMPs as BMPs regulate the transcription of several osteoblast specific transcription factors.[44]

3. Therapeutic applications of BMPs

3.1 Matrices and carriers for BMPs

Matrices and carriers are usually needed for BMPs delivery to a particular site. The purpose of using a carrier is not only to control the distribution of BMPs to a specific site but also to retain BMPs long enough for a cellular response to occur. Matrices and carriers are also helpful as they can be used to define the shape and volume of the bone being induced and their physical form can be manipulated and optimized for particular therapeutic applications. Matrices or carriers can be made out of numerous materials such as collagen, hyaluronic acid, allograft of bone, calcium phosphates, hydroxyapatite/tricalcium-phosphate or synthetic materials like polylactide.[45, 46. 47. 48]

3.2 Orthopaedic applications

The main application of BMPs in relation to orthopaedics is in fracture repair, where BMPs could be used when insufficient repair has occurred or simply to accelerate the rate of fracture repair. They may also be used to treat large segmental bone defects and in spinal fusions where large amounts of bone are needed. Many animal studies have demonstrated the ability of BMP-2 to accelerate fracture repair in rabbit, goat, and dog models.[45, 46, 47, 48] These studies showed that BMP-2 was able to reduce the fracture

healing time by 30-50% and that addition of BMP-2 increased the amount of fracture callus formed and accelerated the maturation of the callus. Several clinical studies have also shown that BMP-2 was useful in treating tibial non-unions and acute tibial fractures.[49] BMP-2 has been shown to heal large segmental bone defects in rats, dogs, rabbits and monkeys.[50] rhBMP-2 has been tested successfully in preclinical animal models of spinal fusion (interbody and intertransverse process) by radiographic, mechanic and histologic criteria.[51, 52, 53, 54, 55] These studies have demonstrated that rhBMP performance in spinal fusion is equal to or better than the current autograft procedure.[56]

3.3 Dental applications

In dentistry there is a need for bone regeneration to fill tooth extraction sockets, for bone lost to periodontal disease, and to augment the alveolar bone that has decreased with age for dental implants and restoration. Preclinical studies have demonstrated BMPs ability to induce bone formation in segmental defects in the jaw in animal models.[57, 58] Augmentation of maxillary bone and mandibular bone by rhBMP-2 has been shown in goat, dog, monkey and human models respectively,[59, 60, 61, 62] and in all scenarios the newly formed bone behaves like the native bone, are capable of supporting dental implants.

3.4 Gene therapy

This involves vectoring of a gene into cells, which will then synthesize the BMPs of interest. Major problems arise from gene therapy relating to BMPs as they are only needed for a short period of time to heal the fracture/instigate bone induction and finding a mechanism by which the expression of BMPs can be “switched off” will be the largest challenge. However adenoviral vectors carrying a BMP-2 gene have been shown in animal models to enhance fracture healing and enhance spinal fusions.[63, 64, 65, 66, 67] BMP carrying adenoviral vectors inserted into bone marrow

mesenchymal cells have been shown to induce new bone formation and repair bone and cartilage defects.[68, 69, 70, 71] Direct application of DNA containing a BMP gene construct has also been shown to enhance long bone repair in a rat model.[72]

3.5 Other uses of BMPs

rhBMP-2 has been shown to inhibit proliferation of vascular smooth muscle cells without stimulating extra cellular matrix synthesis, and this suggested the possibility of therapeutic application of rhBMP-2 for the treatment and prevention of vascular proliferative disorders.[73] BMP-6 is believed to be a brain and muscle protective agent, in an ischemic rat model, BMP-6 has been shown to reduce the size of the infarct in hear and brain.[74] It is an intriguing possibility that in the future BMP may be useful as a protective agent in severe head trauma and stroke. In patients with chronic renal disease levels of BMPs are lower because kidneys are their primary source in the human adult. Renal osteodystrophy syndrome occurs in patients undergoing long-term dialysis in cases of end stage kidney disease. It is possible that systemic administration of BMPs may restore some of the renal functions in patients with chronic renal failure.[74]

4. Potential risks of using BMPs

As BMPs occur naturally in the body we already know that their presence is tolerated, however they are only present in small quantities and therefore the dosage of BMPs must be considered. Toxicity studies in rat and rabbit models using 1,000 times the human rhBMP-2 dose have not found any systemic effects. However, there was evidence that BMP-4 over-expression was associated with heterotopic ossification in fibrodysplasia ossificans progressiva.[75] Recent studies have indicated that the increased levels of BMP-4 mRNA in fibrodysplasia ossificans progressiva cells are attributable to an increased rate of transcription of the

BMP-4 gene.[75] The increased activation of BMP-4 in fibrodysplasia ossificans progressiva cells may be attributable to a mutation within the BMP-4 gene itself or to a mutation in another genetic locus that causes over-expression of BMP-4 in the cells of fibrodysplasia ossificans progressiva patients. Therefore, over expression of BMP-4 is related to a disabling disease and that it is entirely possible that large doses of BMPs may elicit a similar response even if it is on a smaller scale. At present a dose of between 6 mg and 12 mg of rhBMP-2 is recommended for treatment of open tibial fractures.[49] It has been estimated that normal bone contains approximately 0.002 mg of BMPs per kilogram of pulverized bone, although at a fracture site, the BMPs may be at a higher concentration as a result of release from the injured bone and inflammatory cells, but the exact concentration of the BMPs at the fracture site as opposed to physiological concentration in the normal bone is unknown. However, this means the recommended dosage is at least a magnitude of over 1000 times greater than the amount of BMP present during normal fracture healing. At a cost of approximately £1000/mg of rhBMP-2 (at 2003), the dosage being recommended raises the question of whether or not the use of rhBMPs is economically viable.

Although BMPs are human proteins, there is still a risk of human body developing an immune reaction to the recombinant proteins. This risk increases if rhBMPs are administered repeatedly. Although the magnitude of this risk remains unknown, the FDA has concerns on the potential immune response, which may cause adverse effects on embryogenesis, and maternal immune response. One study has shown that the level of anti-BMP antibody in the serum significantly increased in the animals implanted with BMPs, but returned to normal after 6 weeks.[76] Previous research work have found that there are several other molecules which can inhibit osteoinduction by BMPs in addition to noggin and gremlin. TNP-470, a synthetic analog of fumagillin, is an antiangiogenic agent that strongly inhibits neovascular formation *in vivo*. TNP-470 reversibly inhibited the biological activity of rhBMP-2 in the early

stage of bone induction, suggesting that angiogenesis may play an essential role in the recruitment of BMP-receptor-positive cells that can respond to rhBMP-2 and differentiate into chondrocytes and/or osteoblasts.[77] The major implication is that TNP-470 like molecule may have a transient inhibitory effect on BMPs and thereby lessen the effectiveness of BMPs, increases the uncertainty of the delayed effects of rhBMP-2 on osteogenesis.[77]

The other major concern is that BMPs may initiate tumors as they were found at higher concentrations in osteosarcomas.[74] Although some tumours express BMP-2 and have BMP-2 receptors, tumour biology studies have found no evidence that rhBMP-2 would initiate tumour. No cytotoxic or mutagenic activity has been found *in vitro*, and no evidence of abnormal cell biology has been found in implant toxicity studies of rhBMP-2. *In vitro* testing of 51 tumour cell lines resulted in growth promotion only in 3 lines (2 pancreas, 1 prostate) and no effect on the remaining cell lines. The preclinical evaluations on carcinogenicity of BMPs, however, are not sufficient to reveal the proteins' effect on tumorigenesis.[74] Another concern is that without appropriate containment of BMPs within carriers, the BMPs may "leak" into inappropriate areas and stimulate bone growth. This may lead to malignancy or a loss of function due to ectopic bone formation which may impede joint mobility if bone is laid down within a joint capsule or more severely disturb metabolic and renal processes if it is laid down in the liver or kidneys. However the systemic availability of rhBMP-2 is low and minimal exposure to the protein occurs outside the implantation site as rhBMP-2 is rapidly cleared from the body through a renal pathway.[74]

5. Conclusion

BMPs are still relatively new to us despite their accidental discovery over 100 years ago. Many of their functions are still unknown, but at present we have the capabilities to hypothesize and assess the positive and negative effects of BMP use in

transgenic animal models and animal/human trails. More research still needed before we can safely recommend rhBMPs use in clinical situations. Not only are there the potentials for some severe negative effects may occur, but the use of rhBMPs may be severely restricted by the fact that they are too costly as the current recommended dosage for most procedures is extremely high. However, many research works continue to evaluate the signalling pathways, which BMPs are involved in, in the hope that a smaller but more potent molecule involved in the pathway that is cheaper to produce, and easier to use maybe found. As with all clinical procedures it will come down to a battle between the pros and cons, whether or not the benefits outweigh the risks. Although BMPs have promising therapeutic potentials in many clinical aspects, the long-term effects of rhBMPs usage on human body and health are yet to be defined.

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