CHAPTER1

INTRODUCTION

Oral and maxillofacial surgery, as well as, orthopedics and plastic surgery, frequently encounters large defects in bone due to tumors, trauma or physiologic bone loss. Autologous bone grafting is the standard manner for bone reconstruction. During bone reconstruction, intramembranous and endochondral autogenous bone origin are used clinically for grafting in osseous defects; however, these two types of bones differ in gross morphology, microscopic appearance, cytological and biochemical constituents (McKibbin, 1978; Nah et al., 2000). During bone graft healing the process of endochondral (EC) bone formation was found when the donor came from endochondral bone, while intramembranous (IM) bone formation was noted when the donor came from intramembranous bone. Rabie reported that new bone adopted an endochondral ossification route when they came from endochondral bone origin and through an osteogenic route bypassing a cartilage intermediate stage when came from intramembranous bone (Rabie et al., 1996). In addition, in intramembranous bone, preosteoblasts, osteoblasts and osteocytes were observed, while in endochondral bone chondroblasts and chondrocytes were observed (Chay et al., 2000; Rabie et al., 1996). Many attempts performed to demonstrate the factors that could affect the healing of bone grafts. Hardesty and Marsh postulated that differences in the intramembranous and endochondral graft surface area, volume, and weight are related to three dimension osseous architecture rather than to the embryonic origin of bone (Hardesty and Marsh, 1990). However, in the study of Rabie, fresh autogenous endochondral bone (block) as well as demineralized endochondral bone matrix (powder), produced the identical pattern of healing. In addition, fresh autogenous intramembranous bone, as well as demineralized bone matrix, healed through intramembranous ossification (Rabie et al., 1996). This indicates that the healing must be largely attributable to the biochemical nature of the graft and not only to the gross morphology and architecture of bone grafts.

Demineralized bone matrix or bone morphogenetic protein (BMPs) have been reported to replicate the process of endochondral ossification when implanted into ectopic site. In addition these proteins have different roles at various stages of embryogenesis and in adult animals. Several BMPs produced as recombinant proteins

induce endochondral bone formation when subcutaneously implanted in rats (Sampath et al., 1992). However all studies showed the processes proceeded through endochondral ossification. No attempt has been made to isolate comparable osteoinductive proteins from human intramembranous and endochondral bone to identify the response protein for intramembranous ossification.

Review of literature

1. Embryogenesis; Intramembranous and endochondral ossification

Embryonically, two different types of bone formation can be distinguished, intramembranous and endochondral bone formation (Karsenty, 1999; Yamaguchi et al., 2000). During intramembranous bone formation, mesenchymal stem cells, in a highly vascularized connective tissue, are programmed to differentiate directly into bone-forming cells, synthesizing a collagenous matrix that subsequently will become mineralized. In contrast, during endochondral bone formation, the mesenchymal stem cells differentiate into cartilage-producing cells, that will form a cartilage template of the future bone. Then osteoblasts are differentiated from the surrounding mesenchymal cells immediately after maturation of hypertrophic chondrocytes in the template (Chung et al., 1998). Roach; however, reported very interesting and original concepts regarding the cellular mechanisms involved in the transdifferentiation from hypertrophic chondrocytes to bone-forming cells (Roach et al., 1995). The most significant findings relate to the mechanisms of transdifferentiation. The crucial cellular event is an asymmetric cell division, which result in two different daughter cells, one viable and one dying cell. A viable cell then divides again by mitosis, and another cell undergoes apoptosis. A viable daughter cells then differentiate along the osteogenic pathway (Erenpreisa and Roach, 1996; Roach, 1992; Roach et al., 1995; Roach and Erenpreisa, 1996; Scammell and Roach, 1996). In the other hand ,from recent study, Nah found that normal intramembranous pathway in chicks includes transient chondrogenic phase similar to prechondrogenic mesenchyme (Nah et al., 2000) and that the cells in this phase retain chondrogenic potential that can be expressed in specific *in vitro* and *in vivo* microenvironment (Rabie, 1997). Irrespective if the bone is formed intramembranously or endochondrally, the initially produced bone has a very primitive and

irregular appearance. This woven bone is subsequently remodeled into a bone with a lamellar structure, providing it with added physical strength.

2. Bone cells

2.1 Mesenchymal stem cells

During embryo, embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst that can be propagated indefinitely in an undifferentiated state. ES cells differentiate to all cell lineages *in vivo* and differentiate into many cell types *in vitro* (Thomson et al., 1998). Mesenchymal stem cells or human bone marrow stromal stem cells are defined as multi-potential progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. These primitive progenitors exist postnatally and exhibit stem cell characteristics, namely low incidence and extensive renewal potential.

A large body of evidences demonstrate that stromal tissue derived from adult bone marrow of avian and mammalian species contains clonogenic progenitor cells (CFU-F), a proportion of which have the potential for differentiation along the osteogenic and other stromal lineages (Friedenstein, 1980), some of which are considered to be multi-potent bone marrow stromal stem cells (BMSSCs) with the capacity to differentiate into a range of mesenchymal cell lineages including adipose tissue, bone, cartilage, tendon and ligament (Bruder et al., 1994; Prockop, 1997).

Nakahara studied the osteochondrogenic potential of cultured cells derived from the periosteum (Nakahara et al., 1990). The results suggest that periosteum contains subsets of progenitor cells that possess the potential to differentiate to osteoblasts or chondrocytes which give rise to intramembranous and endochondral bone formation respectively (Nakahara et al., 1990). They also proposed that periosteal progenitor cells are under influence of vascular-derived factors which mesenchymal cells had been reported differentiated directly into osteoblasts (Nakahara et al., 1990).

2.2 Osteoprogenitor cells

The sequence of osteogenic differentiation involves the expression of alkaline phosphatase, the synthesis and deposition of type I collagen, bone matrix protein (osteopontin, osteonectin) and glycosaminoglycans (biglycan, decorin), followed by the

expression of osteocalcin and bone sialoprotein at the onset of mineralization. At the end of bone formation, most osteoblasts become flattened lining cells, some become osteocytes and others undergo apoptosis.

After appropriate stimuli of differentiation of mesenchymal stem cells, in this case towards the osteoblastic pathway, the cells are referred to as committed osteoblastic, or osteoprogenitor cells. These cells are in the first stage of bone cell recruitment. The later stages include preosteoblasts and transitory osteoblasts, both are capable of division. Finally, the functional bone-forming osteoblasts that are post-miotic and secretion of mineralize matrix.

2.3 Osteoblasts

The ultimate stage of bone cell differentiation is the osteoblast, which has lost its ability to proliferate. The osteoblast is a bone-lining cell. The main function of which is to synthesize bone matrix and regulates in subsequent mineralization. Mature osteoblasts always act in groups of 100-400 cells, and they can be active for up to some 12 weeks. These cells are characterized at the light microscopic level by a basophilic cytoplasm, a basally located and round nucleus, and a prominent Golgi apparatus in between.

The fate of an osteoblast is to become either an osteocyte (approximate 10-20% of the osteoblast), covered by its own produced matrix, or become a flattened bone-lining cell. Recent data suggest that some osteoblasts may undergo apoptosis.

There is genetic evidence indicating that the early commitment of mesenchymal stem cells into osteoblasts requires expression of the transcription factor; Core binding factor A1 (Cbfa1), which also called Runx2. Cbfa1 is a member of the runt homology family of transcription factors, which regulates several osteoblast phenotypic genes that carry consensus sequences in their promoter regions, such as genes encoding the (α1) collagen chain, bone sialoprotein, osteopontin, transforming growth factor-β (TGF-β) and osteocalcin (Ducy et al., 1997; Ducy and Karsenty, 1998). This transcription factor is an important modulator of osteoblast commitment and it required for maintainace of osteoblast differentiation in postnatal life.

2.4 Osteocytes

As soon as the osteoblast is surrounded by the mineralized bone matrix, it becomes an osteocyte. The space in which the osteocyte is located is referred to as a lacuna.

The osteocyte is a stellate-shape cell, characterized by numerous cytoplasmic processes that make contact with neighboring osteocytes within the bone, and presumable also with lining the cells at the bone surface.

Age and activity regulate the morphological appearance of the osteocytes. In a young osteocyte, the cytoplasmic volume is somewhat decreased in comparison to the osteoblast. Also the organelles involved in protein synthesis, i.e. the endoplasmic reticulum and the Golgi complex, display decreased volumes. The cell and organelle reduction increases with increasing age, and glycogen is accumulated in granules within the cytoplasm.

The functions of the osteocytes are proposed to involve 1) a maintainace of communication between cells, although within a distance of less than 800 μm; 2) to increase the area of mineral in contact with the extracellular fluid; 3) a minor synthetic capacity within the interior of the bone; and, finally, 4) some bone resorptive capacity has also been suggested. The eventual fate of an osteocyte is to be phagocytized by either an osteoclast or a macrophage.

2.5 Osteoclasts

Osteoclasts are multinucleated cells responsible for bone resorption. The most characteristic feature of osteoclasts is the presence of ruffled borders and clear zone (Zhao et al., 2002). Vacuolar H^+-ATPase exists in the ruffled border membrane of osteoclasts, and acidifies resorbing area under the ruffled border. The ruffled border is surrounded by a clear zone, which serves for the attachment of osteoclasts to the bone surface to maintain a microenvironment favourable for bone resorption. Osteoclasts are differentiated from hemopoietic cells of the monocyte/macrophage lineage under the control of bone microenvironments. Osteoblasts or bone marrow stromal cells have been shown to regulate osteoclast differentiation providing the microenvironment similar to bone. The recent discovery of new members of the TNF receptor-ligand family, terms osteoprotegerin (OPG) (Simonet et al., 1997), has clarified the molecular mechanism of osteoclast differentiation regulated by osteoblasts/stromal cells. OPG strongly inhibits osteoclast formation induced by 1,25(OH)2D3, PTH, PGE2 or IL-11 in co-cultures (Yasuda et al., 1998).The OPG-binding molecule (OPGL) is a type II transmembrane protein of the TNF ligand family, and its expression in osteoblasts/stromal cells is up-regulated by osteotropic factors including 1,25(OH)2D3, PGE2, PTH and IL-11.

3. Bone turnover and remodeling

Althought the main function of osteoblasts is to synthesize, organize and mineralize the bone matrix, cells of the osteoblast lineage also play a central role in the regulation of bone turnover. Bone mass remains nearly constant from the end of puberty to the arrest of gonadal function by a complex process termed bone remodelling (Karsenty, 1999). During modeling, bone is formed in two different ways, intramembranous ossification and endochondral ossification. In intramembranous ossification, condensing cells differentiate into osteoblasts, whereas in endochondral ossification, condensing cells differentiate into chondrocytes to form cartilage, later replaced by bone. It has been suggested that osteoblasts, chondrocytes, adipocytes, and myoblasts are derived from common precursor cells (Fang and Hall, 1997), which are multipotential mesenchymal stem cells derived from mesoderm. The differentiation of multipotential mesenchymal stem cells into a distinct cell lineage is termed ″commitment,″ and their commitment to the osteoblast lineage as well as their differentiation and maturation that follow are necessary for both bone modeling and remodeling. These sequential events are regulated both systemically and locally by several hormones, growth factors, and cytokines. Moreover, transcription factors, such as Runx2/cbfa1 (Runx2), Fra-1, and Osx, are important for osteoblastic differentiation (Jochum et al., 2000; Komori et al., 1997; Nakashima et al., 2002; Otto et al., 1997)and modulate the expression of osteoblast-specific genes, such as type I collagen (COLI) (Fang and Hall, 1997) alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN). However, it has long been believed that bone mass remains unchanged through local (autocrine, paracrine) mechanisms (Manolagas, 2000; Rodan and Martin, 1981).

Recent genetic studies have shown that osteoblasts and stromal cells synthesize osteoprotegerin (OPG), a member of the tumor necrosis factor (TNF) receptor family, which is essential for the regulation of osteoclast differentiation (Lacey et al., 1998; Simonet et al., 1997). OPG, a soluble protein that is not osteoblast-specific, inhibits osteoclast formation and activity by binding to osteoclast differentiation factor (ODF), also called receptor activator of nuclear factor kappa B (RANKL), and osteoprotegerin ligand (OPGL). RANKL is a membrane protein of the TNF ligand family that is produced by osteoblasts and stromal cells and acts by binding to its receptor (RANK) to support osteoclast differentiation. The essential role of these molecules in the

control of bone turnover and bone mass is shown by the observation that OPG overexpression in mice induce osteopetrosis due to defective osteoclast differentiation, whereas mice lacking the OPG gene or that overexpression of RANKL mRNA are severely osteoporosis due to increase osteoclast formation (Bucay et al., 1998; Simonet et al., 1997). RANKL-deficient mice display severe osteopetrosis with no osteoclasts, and marked chondrodysplasia, showing that RANKL-mediated regulation of the skeleton impacts both chondrocyte differentiation and osteoclast formation (Kim et al., 2000).

Basic studies have shown that bone formation at local sites depends on both the number and activity of osteoblasts (Fisher and Termine, 1985). Of these two determinants, histomorphometric analysis of bone specimens from non-osteoporotic and osteoporotic subjects revealed that 80% of the bone formation rate is determined by osteoblast cell number (Dudl et al., 1973). Many studies aimed toward identifying the factors that control osteoblast cell proliferation are essential to our understanding of the coupling mechanism. In this regard, the proliferation of osteoblasts are regulated by both systemic hormones (e.g., parathyroid hormone [PTH], 1,25-dihydroxycholecalciferol [vitamin D3], and progesterone) and local growth factors (e.g., insulin-like growth factors [lGFs], transforming growth factor-ß, [TGF-ß], and bone morphogenetic proteins [BMPs]). Of the various known systemic and local regulators of osteoblast cell proliferation, there are several features of the BMPs system which suggest that it may provide a major role in the regulation of osteoblast differentiation and proliferation.

4. Systemic regulation of bone metabolism

4.1 Parathyroid hormone

Parathyroid hormone (PTH) is a protein, synthesized by cells in the parathyroid gland, and has a catabolic effect on bone, both through increase bone resorption and through inhibit bone formation. The secretion of PTH is mainly regulate by the level of calcium in the plasma without interfere with the phosphate balance. PTH acts either directly or via 1,25-dihydroxyvitamin D_3 on the kidney to stimulate resorption of calcium. PTH has a direct effect on both the activation of already existing osteoclasts and on the development of new osteoclasts (Sugimoto et al., 1993). But the resoption of bone is dependent on the presence of osteoblasts, since mature osteoclasts do not have PTH

receptors themselves. The decrease in bone formation due to PTH is mediated through direct action of PTH on the osteoblasts (Weryha and Leclere, 1995).

4.2 Vitamin D_3

Vitamin D_3 (cholecalciferol) is synthesized by the skin under ultraviolet radiation from its precursor 7-dehydrocholesterol. Vitamin D_3 is by itself an inactive substance that must be hydroxylated in the liver and in the kidneys to form 1,25-dihydroxyvitamin D_3, also called calcitriol (Brommage and DeLuca, 1985). 1,25-dihydroxyvitamin D_3 is able to induce bone resoption both *in vitro* and *in vivo*. However, receptors for 1,25-dihydroxyvitamin D_3 have not been identified in osteoclasts, only in osteoblasts (Clemens et al., 1988).

4.3 Estrogen

Estrogen also exert multiple effects on osteoblasts through receptor α and ß (Spelsberg et al., 1999). The role of estrogen receptors in the control of osteogenesis is not fully understood, as the deletion of these genes in mice induces complex effects on bone mass (Vidal et al., 2000; Windahl et al., 1999).

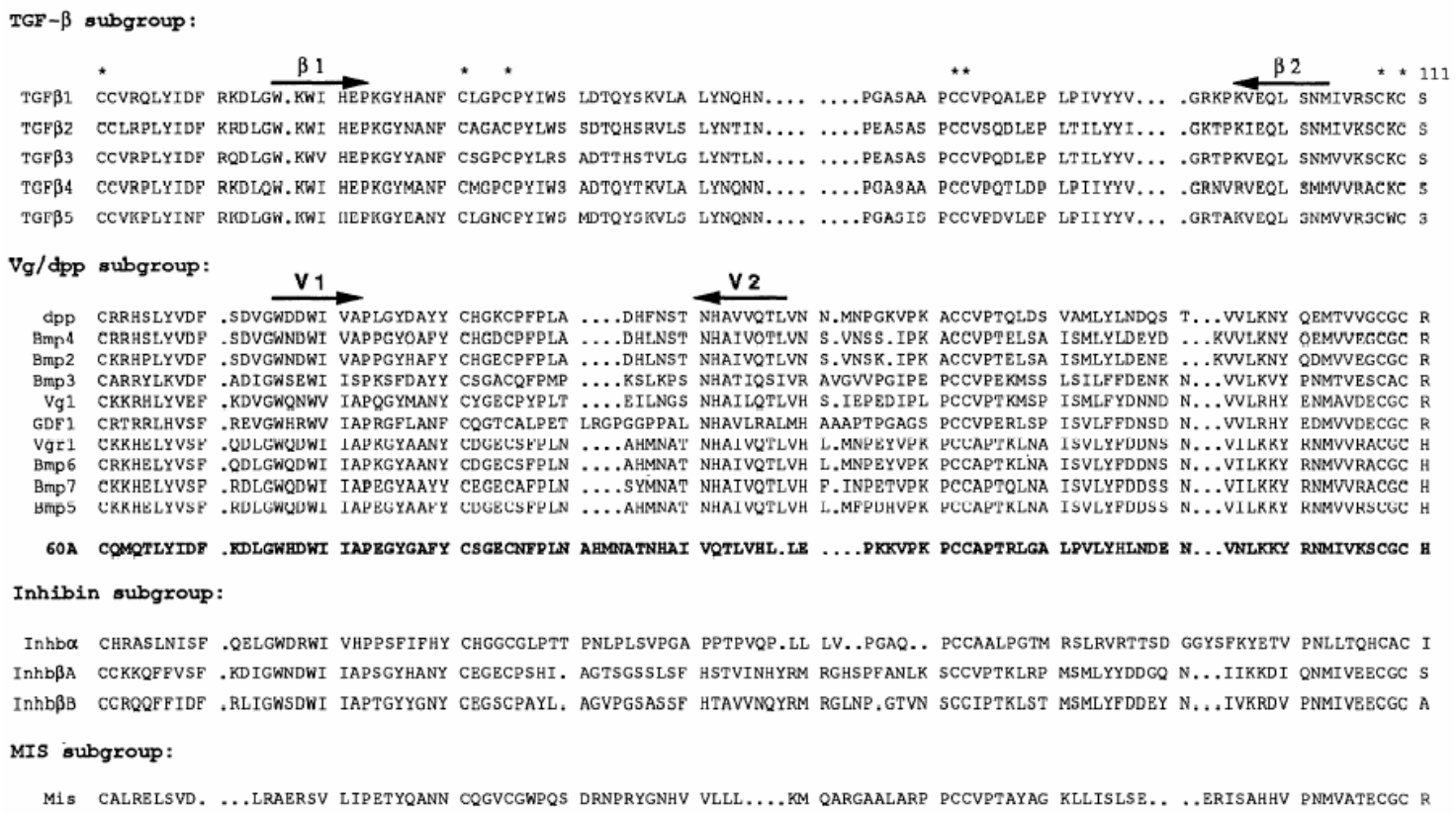
5. Bone morphogenetic protein (BMP) as the local regulation of bone metabolism

The discovery of a family of bone morphogenetic proteins (BMPs), which identified as a inducers of bone and cartilage formation in ectopic skeletal sites *in vivo* (Luyten et al., 1989; Sampath et al., 1990; Urist et al., 1983; Wozney, 1989),suggested that BMP inductive signals may be intimately involved in both development and regeneration of the skeleton. Subsequently, BMPs have been shown to regulate growth and differentiation of several cell types. The BMP family is the largest family of the TGF-ß -like signaling molecules with at least 17 diverse family members. TGF-ß family ligands are characterized by a cysteine knot, seven highly conserved cysteine that give structural support to protein folding and dimerization (Shimasaki et al., 2004). Six cystienes form three disulfide bonds in a knot-like formation to provide structural support (Isaacs, 1995). The seventh cystiene forms a disulfide bond with another BMP molecule upon ligand dimerization.

Certain structural features are shared by all members of the TGF-ß superfamily. The mature bioactive protein is secreted as a dimer (Derynck et al., 1985;

Mason et al., 1985). The precursor polypeptide from which a mature subunit is released is composed of 1) an amino-terminal secretory signal sequence 2) an extended propeptide region 3) proteolytic cleavage sites, that will be cleaving after Arg-X-X-Arg site (Chang et al., 1994), and 4) a carboxyl-terminal region (typically ~110 amino acids), which represents the mature peptide. The mature region contains the most conserved sequence, which is seven highly conserved cysteines, among different superfamily member. Based on degree of sequence conservation seen between the different carboxyl-terminal regions, the superfamily can be split into at least four subgroups (Wharton et al., 1991) (**Error! Reference source not found.**). TGF-ß subgroup consists of human TGF-$ß_1$,human TGF-$ß_2$,human TGF-$ß_3$, chicken TGF-$ß_4$ and *Xenopus* TGF-$ß_5$.Vg/dpp subgroup consists of *Drosophila dpp* ,human BMP-2,-3,-4,-5,-6 and -7/OP-1, *Xenopus* Vg-1, mouse Vgr-1, mouse growth and differentiation factor 1(GDF-1), and *Drosophila* 60A. The inhibin subgroup consists of porcine inhibinα, $ß_A$, and, $ß_B$. The MIS subgroup contains a single member, human MIS.

Figure 1 Amino acid alignment of the mature regions of 20 TGF-β-like proteins beginning at the first invariant cysteine residue.



6. Expression on BMP in tissue and organ

The expressions of BMPs are reported primarily in embryonic, abnormal bone formation or bone disease samples. Many studies reported the expression of BMPs during different stages of development. BMP activity is required for the formation of primordial germ cells (Ying et al., 2000) and regulates the differentiation of thymocytes (Graf et al., 2002; Hager-Theodorides et al., 2002). BMPs are involved in the formation and patterning of various tissues, including the central nervous system (CNS), skeleton, heart, kidney, gut, lung, liver, teeth and eyes as shown in Table 1 (Hogan, 1996; Wozney and Rosen, 1998; Zhao, 2003). The localization studies in both human and mouse tissue have demonstrated high levels of mRNA expression and protein synthesis for various BMPs in kidney (BMP 3, 4, and 7), lung (BMP 3, 4, 5, 6), small intestine (BMP 3, and 7), heart (BMP 2, 4, 6, and 7), limb bud (BMP 2, 4, 5, 7), and teeth (BMP 3, 4, and 7). In head and neck area, Ashique found that endogenous BMPs have several important roles in the fusion of the lip by BMP signaling which is required to stimulate mesenchymal cell proliferation and directed outgrowth of the facial prominences, and the decrease of BMP activity in the mesenchyme regulates cell survival in the epithelium and consequently increases epithelium thickness (Ashique et al., 2002). In cranial suture development, BMPs are key signaling molecules in the initiation of bone and cartilage formation, and that they regulate the early commitment of mesenchymal cells to the chondrogenic and osteogenic lineages (Wozney, 1992). The composite expression patterns of different BMPs are likely to control the basic form and pattern of the vertebrate skeleton (Katagiri et al., 1994; Kingsley, 1994). Kim suggested that the BMPs may be involved in regulating the balance between the undifferentiated and differentiated states of osteogenic cells (Kim et al., 1998). For osteoblasts composing in bone tissue, BMP2, 4, 7 were found expressing in MC3T3-T1 cell line (Xiao et al., 2002). Mutations in components of the BMP signaling pathway have been associated with several human diseases, such as nonfamilial pulmonary hypertension and cancer syndromes, including juvenile polyposis and Cowden syndrome (Waite and Eng, 2003). The studies in exophytic bone formation have shown that each BMP has a distinct pattern of distribution; BMP2 was observed in fibrous tissue matrix as well as in osteoblasts; BMP3 was mainly present in osteoblasts; BMP6 was restricted to young osteocytes and bone matrix; BMP7 was observed in hypertrophic

chondrocytes, osteoblasts and young osteocytes; BMP3 and 6 were found in osteoclasts at the sites of bone resorption (Zoricic et al., 2003).

However, until now, no study has been performed to postulate the expression of BMP family in normal mandible and maxilla which is intramembranous in origin, and normal endochondral bone such as iliac bone. These factors may be involved in the normal functioning of the bone tissues involved in stem cells differentiation, and alterations in their expression may be associated with the development of specific pathway of bone formation.

Table 1 Properties, roles, and location of bone morphogenetic proteins; BMPs

BMP	Properties, roles and locations
BMP1	se (member of the astacin family); may function as a procollagen C-proteinase responsible for removing carboxy propeptides from procollagen I, II, and III; activates BMPs. Not osteoinductive; may be involved with Langer-Giedion syndrome (rare inheritable disorder involving skull and long bones); Drosophila tolloid gene homologue: dorsoventral patterning(Finelli et al., 1995; Shore et al., 1995; Suzuki et al., 1996)
BMP2	nductive; embryogenesis; apical ectodermal ridge; pattern formation; differentiation: osteoblasts, adipocytes, chondrocytes; influence osteoclast activity; neuronal differentiation; repair and remoldeling of long bone, alveolar clefts, spine fusion; augmentation of maxillary sinus; located in bone, spleen, liver, brain, kidney, heart, placenta.(Ahrens et al., 1993; Engstrand et al., 2000; Gugala et al., 2003; Inoda et al., 2004; Iwasaki et al., 1996; Kanatani et al., 1995; Wozney et al., 1988)
BMP3 (osteogenin)	tor of osteogenic BMPs; modulator of the activity of osteogenic BMPs in vivo; most abundant BMP in demineralized bone; located in lung, kidney, brain, intestine.(Bahamonde and Lyons, 2001; Daluiski et al., 2001; Vukicevic et al., 1994)
BMP4	nductive; embryogenesis: gastrulation and mesoderm formation (mouse); produced by dorsal aorta (direct sympathetic neuron differentiation); fracture rapair; overexpression associated with ectopic ossification of fibrodysplasia ossificans progressive; located in apical ectodermal ridge, muscle, meninges, lung, kidney, liver. (Bostrom et al., 1995; Reissmann et al., 1996; Wright et al., 2002)
BMP5	nductive; embryogenesis (short ear); located in lung, kidney, liver. (Kingsley et al., 1992; Kingsley, 1994; Wozney, 1992)

Table 1 Properties, roles, and location of bone morphogenetic proteins; BMPs (continue)

BMP	Properties, roles and locations
BMP6	nductive; embryogenesis; neural maturation; regulates chondrocyte differentiation; found in olfactory system, lung, brain, kidney, uterus, muscle, skin.(Ferguson et al., 2004; Gajavelli et al., 2004; Hughes et al., 1995; Jane, Jr. et al., 2002b; Kim et al., 2001; Nonner et al., 2001; Peretto et al., 2002; Somi et al., 2004)
BMP7 (osteogenic protein1)	nductive; embryogenesis; repair of long bone, alveolar bone, spine fusion; differentiation of osteoblasts, chondroblasts, adipocytes; located in adrenal glands, lachrymal gland, bladder, brain, eye, heart, kidney, lung, placenta, spleen, skeletal muscle.(Dean et al., 2004; Franceschi et al., 2000; Kim et al., 2001; Liem, Jr. et al., 1995; Macias et al., 1997; Nonner et al., 2001; Ozkaynak et al., 1990; Sampath et al., 1990; Sampath et al., 1992; Wagner and Mullins, 2002; Zhu et al., 2004)
BMP8 (osteogenic protein2)	nductive; embryogenesis; spermatogenesis (mouse).(Kingsley, 1994; Ozkaynak et al., 1992; Zhao et al., 1996; Zhao and Hogan, 1996)
BMP8B (osteogenic protein3)	on and maintenance of spermatosis (mouse)(Ying et al., 2000; Zhao and Hogan, 1996)
BMP9	nductive; stimulates hepatocyte proliferation; hepatocyte growth and function.(Celeste et al., 1994; Helm et al., 2000b; Helm et al., 2000a; Song et al., 1995)
BMP12 and BMP13	on of terminal differentiation of myoblasts; locate in tendon.(Chuen et al., 2004; Fu et al., 2003; Furuya et al., 1999)
BMP15	n folliculogenesis; human fertility control; maintenance of fertility and normal ovarian physiology. (Bodensteiner et al., 1999; Dube et al., 1998; Eckery et al., 2002; Galloway et al., 2000; McGrath et al., 1995; Yan et al., 2001)

7. Intracellular signaling of BMPs

BMPs are synthesized as large precursor proteins. During dimerization, the molecules are proteolytically cleaved within the cell to yield carboxy-terminal mature proteins. Once secreted, the BMP dimers initiate signaling by binding cooperatively to serine/threonine kinase receptors, and determine the specificity of intracellular signals.

The first intracellular mediator of TGF-ß signaling was identified in the fruitfly *Drosophila melanogaster* and the locus was called mothers against decapentaplegic (MAD), decapentaplegic (Dpp) being the *D.m.* TGF-ß protein. Subsequently related proteins were found in other invertebrates (the worm genes were called sma) and in vertebrates. Using an amalgam of the worm and fly nomenclature the proteins were named Smad proteins (Miyazono et al., 2000).

Like other members of the TGF-ß family, BMPs elicit their effects through activation of serine/threonine kinase receptors (Figure 2). There are two types of serine/threonine kinase receptors creatively named: type I and type II. Type I receptors can be further divided into type IA or activin receptor like kinase (ALK) -2 and-3 and type IB (ALK) -6. ALK3 and ALK6 are activated by BMP2, BMP4 and BMP7, whereas ALK2 binds BMP6 and BMP7 but neither BMP2 nor BMP4. While type II receptors can be divided into three distinct groups, BMP receptor type II (BMPRII) and activin type IIA and IIB receptors (ActRIIA and ActRIIB) (Canalis et al., 2003). Before ligand binding, BMPR-I and BMPR-II become homodimerization. The activated signaling complex which is formed upon ligand binding consists of a receptor tetramer made up of the type I homodimer and the type II homodimer. The method of complex formation is still and to date there are two competing theories based on the knowledge that BMPs have low affinity for their type II receptor and high affinity for their type I receptors (Kirsch et al., 2000). One theory is the ligand binds first to the type I receptor and the complex then recruits the type II receptor. The second theory is that the tetramer is preformed for ligand binding making the tetramer high affinity (Canalis et al., 2003; Shimasaki et al., 2004).

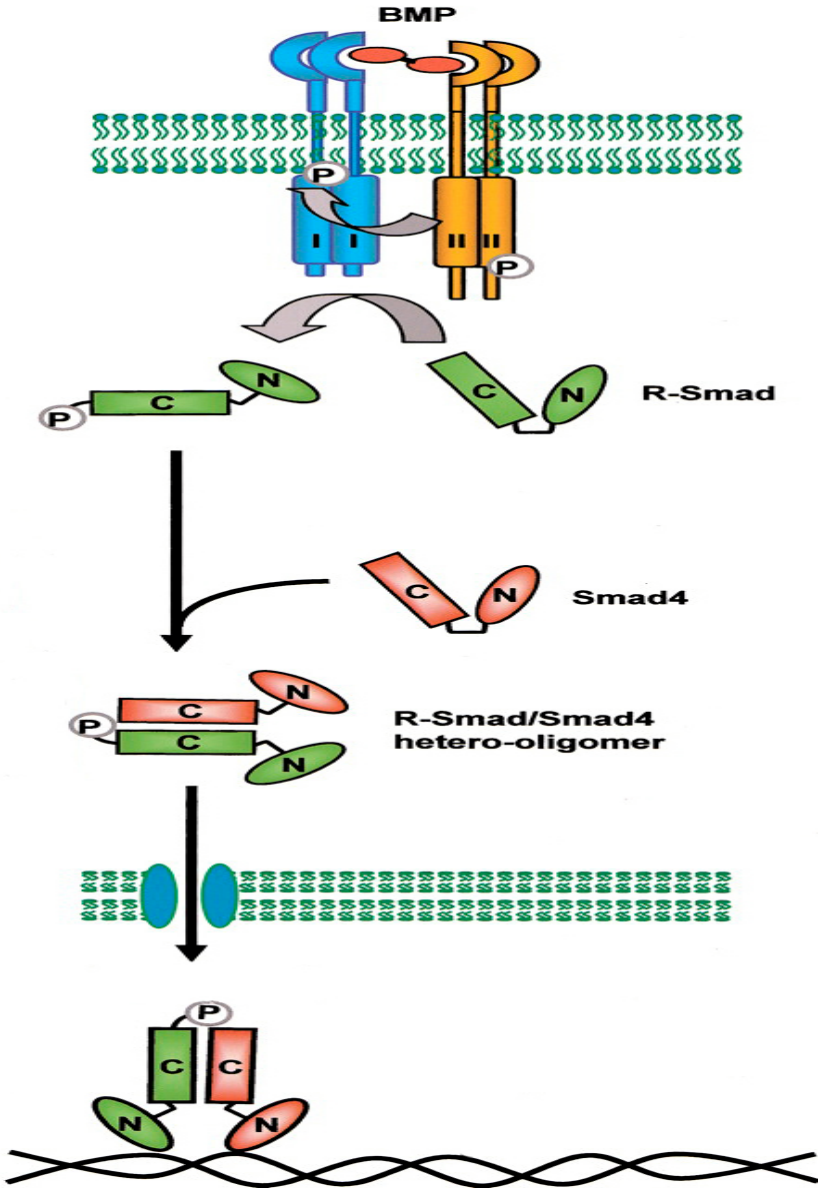


Figure 2 BMP is recognized by cell surface receptor serine/threonine kinases, of which the type II receptor phosphorylates and activates the type I receptor kinase, which phosphorylates the C-terminus (C) of a receptor-activated Smad (R-Smad) protein, leading to conformational changes. Phosphorylated Smads hetero-oligomerize with the nonphosphorylated Smad4, translocate to the nucleus, and engage in target gene expression.

The type I receptors within the tetrameric receptor complex initiate signal propagation by phosphorylation of the receptor-activated Smads (R-Smads). In addition to the R-Smads, two other types of Smad have been identified, the common mediator Smads (co-Smads, Smad4, or Smad4α and 4ß in *Xenopus laevis*) and the inhibitory Smads (I-Smads, Smad6 and Smad7). BMP receptors activate Smad1, Smad5 and Smad8), whereas Smad2 and Smad3 are phosphorylated by the activin or TGF-ß receptors. Remarkably, ALK3 and ALK6 can activate Smad1, Smad5 and Smad8, but ALK2 only phosphorylates Smad1 and Smad5 (Aoki et al., 2001). Smad1, Smad5 and Smad8 can however also transduce TGF-ß signals from the ALK1 type I receptor (for TGF-ß) in endothelium and hematopoietic cells (Goumans et al., 2002). Similarly, Müllerian inhibiting substance (MIS; or anti-Müllerian hormone, AMH) acts through MISRII (AMHRII) with ALK1 or ALK6 (Clarke et al., 2001; Visser et al., 2001), and some BMP-like growth/differentiation factors (GDF5, 6 and 7) act through activated ALK6 (Baur et al., 2000). For many ligands (e.g. BMP10 and 11, GDF1), it is still unclear through which receptors and R-Smads they signal, particularly *in vivo*.

Smad proteins have three domains, the N-terminal Mad homologydomain (MH1), a divergent proline-rich linker domain, and the C-terminal MH2 domain (Heldin et al., 1997). The MH2 domain, which is highly conserved among all Smads, is in R-Smads involved in type I receptor recognition and becomes directly phosphorylated in its C-terminal SSXS motif by type I receptors. This domain is also required for Smad oligomerization and interaction with Smad4, and is shown to interact with cytoplasmic adapters and nuclear transcription factors. The MH1 domain is conserved among R- and co-Smads, binds to several cytoplasmic partners, and is required for nuclear import of Smads through its N-terminally located nuclear localization signal.

8. Purification of osteoinductive factors in bone tissue

In 1965, Urist reported the ectopic bone formation can be formed by the implantation of demineralized bone matrix at subcutaneous tissue or intramuscular sites in the rat or mouse (Urist, 1965). The induction process involves recruitment and differentiation of mesenchymal progenitor cells into chondroblasts, followed by calcification and formation of bone (Reddi, 1981; Reddi and Huggins, 1972). It has been evident that

there are component(s) present in bone matrix responsible for this biolological activity (Guterman et al., 1988; Reddi, 1983; Sampath et al., 1982; Thielemann et al., 1982).

The purification processes which used to extract osteoinductive factors in bone tissue have been reported since early 1980 and further developed by many investigators. The scheme of purify includes: 1) the acid demineralized and extraction active component; 2) isolation the bone-inductive proteins by heparin affinity chromatography; 3) determine the biological activity and 4) analytic the amino acid composition.

Many chemicals were reported effectively to extract the active components in bone tissue such as guanidine HCl (Bentz et al., 1989; Goldberg et al., 1988a; Hanamura et al., 1980; Katz and Reddi, 1988; Sampath et al., 1987; Schwartz et al., 2000; Scott et al., 1994; Takaoka et al., 1982; Termine et al., 1980; Termine et al., 1981; Uchida, 1994; Wang et al., 1988; Wendel et al., 1998) which was believed to solubilize proteins and other macromolecules that are noncovalently associated with the unmineralized collagen matrix ; Ethylene diamine tetraacetic acid; (EDTA) (Domenicucci et al., 1988; Goldberg et al., 1988a; Hill et al., 1994; Suzuki, 1990; Thielemann et al., 1982) which reported to remove the mineral-associated proteins; acetic acid (Hou et al., 2000) and urea (Luyten et al., 1989; Syftestad et al., 1984; Urist et al., 1983) which has the advantage over guanidine HCl extraction in less time-consuming buffer exchange from guanidine to urea.

Affinity chromatography involves the covalent attachment of a ligand to a solid support. Heparin is a linear and highly sulfated glycosaminoglycan. Due to its polyanionic nature heparin binds to many cationic proteins and enzymes including osteoinductive proteins (Chang et al., 1994; Sampath et al., 1987).

Urist reported a 18-kDa protein derived from bovine called bone morphogenetic protein (BMPs) (Urist et al., 1984) which is most famous osteoinductive factor in stimulating new bone formation. Other osteoinductive factors include, a 22-28-kDa Osteoinductive factor (OIF) which induced new bone formation when implanted with extracted bone matrix at a subcutaneous site (Sampath et al., 1987), a 22-kDa Osteogenin which was purified from bovine bone matrix (Luyten et al., 1989). However, all known bone-derived osteoinductive factors have been isolated from endochondral bone, and all initiate bone induction via endochondral ossification (Carlevaro et al., 2000; Carrington et al., 1988; Duneas et al., 1998; Gitelman et al., 1994; Long et al., 2001; Ripamonti et

al., 1997; Ripamonti et al., 2000; Suda, 1997; Suzuki, 1996; Yu et al., 1993). BMPs which belonging to the TGF-ß superfamily is widely accepted in inducing endochondral bone formation in adult animal (Duneas et al., 1998; Hiraki et al., 1991; Ripamonti et al., 2001; Urist et al., 1983; Volek-Smith and Urist, 1996). As well as OIF and Osteogenin, they both are reported to induced de novo bone through endochondral ossification (Bentz et al., 1989; Luyten et al., 1992; Reddi et al., 1989; Reddi and Cunningham, 1990; Ripamonti et al., 1992). Recently, Scott isolated the osteoinductive factor from bovine intramembranous bone which have a SDS-PAGE banding pattern different from that of a comparable osteoinductive-chondroinductive factor isolated from endochondral bone (Figure 3) (Scott et al., 1994). This partial purified intramembranous bone matrix extract induced the direct bone formation which was claimed as intramembranous ossification; however, this fact is unequivocal because the author did not examine histological protein implant sites at early and intermediate stage of bone development.

Since intramembranous ossification bypasses the cartilage stage, the shorter period of healing is achieved. This is benefit in clinical cases where earlier loading is needed, such as reconstruction in mandible and lower extremities. Moreover, in some clinical setting, it would be more appropriate to replace traumatized or diseased osseous tissue with intramembranous than endochondral bone (Scott and Hightower, 1991). Since intramembranous bone grafts are often superior to endochondral bone grafts (Sullivan and Szwajkun, 1991; Wong and Rabie, 1999; Zins and Whitaker, 1983), the component(s) that could be extracted from human intramembranous bone will be potentially more beneficial in oral and maxillofacial reconstructive surgery. To date, no study has been performed to identify the response component for intramembranous ossification in human. Cells and bone matrix derived from human intramembranous bone may be committed to retained their phenotype and, may be, are source of unidentified osteoinductive factor(s) that stimulate the recipient mesenchymal stem cells to progress further though the same lineage as themselves. The molecular basis for such a disparity in the phenotypic potential of these different cell types remains unclear and requires further investigation.

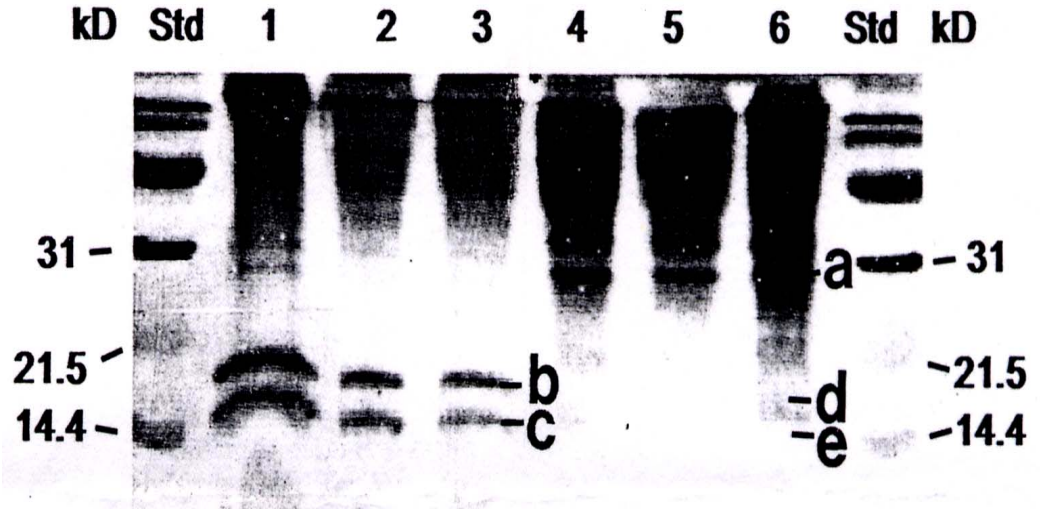


Figure 3 Heparin-Separose bound polypeptides extracted from intramembranous (lane 1-3) and endochondral (lane 4-6) tissues. (Scott et al., 1994).

Aims of the study

1. BMPs are ones of the most importance cytokines responses in bone remodeling and new bone formation. Until now no study postulate the BMPs component in normal bone. This study aims to identify the member of bone morphogenetic protein (BMP) family expressing in human bone.

2. Because the previous knownledge showed differences between two sources of bone origin from many aspects; such as gross morphology, cytological and biochemical constituents. This study means to compare the member of bone morphogenetic protein (BMP); as well as, the proportion of each bone morphogenetic protein (BMP) expressing in human intramembranous and endochondral bone to maintain normal homeostasis.

3. Since in vitro systems have been particularly informative for investigating the expression of BMP. The system can generate the almost stages of bone formation from earliest stages of osteo-chondrogenesis to the complete mineralization. However, in culture system can only mimics the true event in normal condition; many conditions are obviously different such as systemic regulation of endogenous hormones and other cytokines. The comparative study between in vivo and in vitro system may be very valuable as a baseline for further study in BMP expressions. This study aims to compare the expression pattern of bone morphogenetic protein (BMP) between normal human bone and ex vivo osteoblastic cell culture in human intramembranous and endochondral.