CHAPTER 4

DISCUSSION

Part I. Illustrate the expression patterns of BMPs family member in primary culture derived from intramembranous and endochondral bone.

From previous reports, it was clear that there were substantial difference in histology, histochemical, biochemical, as well as, chemical compositions of bone matrices, resulting from intramembranous and endochondral bone (Herring, 1968; Moskalewski et al., 1988; Rabie et al., 1996; Scott and Hightower, 1991; Strawich and Glimcher, 1983). The differences stated above strongly suggest that intramembranous and endochondral bones possess the distinction of osteoblasts cells.

Because of the widely used of osteoblastic cell culture for studies of the behaviors and the responses of these cells to exogenous BMPs, we also used the cell culture system to investigate the pattern of BMPs expression in *ex vivo* system.

It is widly accepted that bone marrow cells of fully mature human contained mesenchymal cells, which were able to differentiate into osteoblastic lineage under the influence of dexamethasone and ascorbic acid in culture medium. Mesenchymal cells were identified by an ability to attach, proliferated and form stromal fibroblastic unit or cell nodules (Ashton et al., 1980). As it has been reported that each fibroblastic unit has different osteogenic potential (Kuznetsov et al., 1997) and contains both mesenchymal stem cells and osteoprogenitors cells from single colony or lineage. Therefore, osteoblastic characteristics were identified from multiple clonals of mesenchymal stem cells. Difference in level of osteoblastic differentiation of each colony was not tested. However, osteogenic differentiation of mesenchymal stem cells in bone marrow as a whole was studied. Supplementations of dexamethasone, ascorbic acid and ß-glycerophosphate were aimed to maximize osteogenic differentiation of all colonies in the culture system.

It has been reported that dexamethasone is an essential factor for osteogenic differentiation of bone marrow in cell culture (Cheng et al., 1994; Gundle et al., 1995). Ascorbic acid enhances osteogenic differentiation of undifferentiated cells by inducing a formation of type I collagen (Franceschi and Iyer, 1992; Otsuka et al., 1999). External

phostphate in culture medium promotes mineralization of the extracellular matrix (Franceschi and Iyer, 1992). In this study, bone cells were cultivated in a culture medium supplemented with dexamethasone, ascorbic acid and β-glycerophosphate.

Alkaline phosphatase activity is a widely measured osteoblastic marker during the proliferative stage (Franceschi and Iyer, 1992; Lecoeur and Ouhayoun, 1997). This enzyme begins immediately following the cessation of cell proliferation and reaches a maximum level during the phase of matrix maturation (Risteli and Risteli, 1993). It was found that expression of alkaline phosphatase activity and type I collagen are the markers of osteoblastic differentiation in the early state.

The bone cells, isolated from mandibular, iliac crest bone and tibia bone, characterized the morphology of osteoblast lineage that they exhibited in their capacity to induce alkaline phosphatase activity. Most of the cells in mineralized medium were positive to alkaline phosphatase staining as the osteoblastic marker; in contrast to the fibroblast cells, where no alkaline phosphatase activity was found.

In osteoblastic cells which derived from intramembranous in origin, all BMPs members expressed message in the same pattern as normal human intramembranous bone (Figure 7 and Figure 13). However expressions of BMPs in MO culture were lower than fresh normal human intramembranous bone. BMP7 expression was 75% reduction, as well as, the expression of BMP2, 3, 4, 5, and 8 showed up to 50% reduction. In EO osteoblastic cells, which were endochondral in origin, BMP 2 and BMP5 showed up to 60% reduction; while, BMP 4, 6, 7, 8, 9 also revealed the decrease of message expression range from 16-35%. In contrast to the message for BMP3 in primary osteoblastic cell culture, it was increased by up to nearly 44%, when comparing to the expression in fresh normal human endochondral bone (Figure 15).

It was interested that the *ex vivo* osteoblastic cell, deriving from human intramembranous and endochondral in origin, even though the most pattern of expression were in the same style, the message of all BMPs expressions were lower approximately by half. However we found the expression of BMP3 in EO osteoblastic cell was two-times higher than in normal human endochondral bone. It is unclear whether these data result from the changes in both autocrine/paracrine of cytokines or neighborhood cells present in bone tissue. A number of local and systemic factors have shown to influence BMP-dependent bone formation. Local factors have been shown to modulate the effect of BMP including growth and differentiation factors, transforming growth factor-beta. These growth

factors are in the transforming growth factor beta (TGF-ß) superfamily, signalling occurs through the activation of the same transmembrane receptor complex formed by type-I and II serine/threonine kinase receptors (Kawabata et al., 1998; Massague, 1996). Several hormones, including parathyroid hormone (PTH)(Cosman et al., 2001), vitamin D (Eisman, 2001), glucoticoids (Canalis and Delany, 2002) and sex steroids hormones, are also known to act on osteoblasts and modulate bone formation and resorption. Moreover, the total RNA deriving from fresh bones might combine of more than one type of cell such as osteoblasts, osteocytes and osteoclasts; in contrast to total RNA of *ex vivo* osteoblastic cells which were mostly osteoblast cells. Our results indicated the distinctions between *in vivo* and *ex vivo* environment of osteoblasts that may have resulted in the changing of intracellular function. Due to the lower level of BMPs expression of osteoblastic cell in *ex vivo* condition comparing with osteoblastic cell *in vivo*, this should be notified for further BMPs member investigation using cell culture system.

Part II. Identify and compare the member of bone morphogenetic proteins (BMPs) family expression in human intramembranous and endochondral bone.

Intramembranous and endochondral ossification are two types of bone formation that differ in both cells component and process of new bone formation. Human intramembranous and endochondral bone are different in pattern of BMPs expression. In addition, this study reported the first finding of the expression of two BMPs in normal human bone; BMP9 and BMP15. This preliminary investigation also appears to be the first report of the expression of BMPs, comparing between fresh human intramembranous and endochondral bone.

Bone morphogenetic protein were initially identified as components of bone extracts that induced ectopic bone and cartilage formation (Urist, 1965; Wozney et al., 1988). In addition to bone and cartilage formation, these proteins have now been shown to regulate many fundamental biological processes, including cell proliferation, differentiation, apoptosis, cell migration, cell adhesion, and embryonic development (Hogan, 1996). More than 15 BMP family members are expressed in mammals and *Drosphilia* (Kawabata et al., 1998; Yamashita et al., 1996).

With degenerate primers encoding for conserved regions of known BMPs, we amplified reverse transcribed mRNA from normal human intramembranous bone and

identified two BMPs, BMP9 and BMP15, which were first reported in human bone. BMP9 is a new member of transforming growth factor-beta superfamily, which has recently been identified and shown to be expressed in developing mouse liver (Celeste et al., 1994). Miller demonstrated the expression of BMP9 using ribonuclease protection assay (RPA) among organs and tissues of adult rats. They reported the BMP9 transcription occurs predominantly in the liver; while in the bone, none of the expression was observed (Miller et al., 2000). There are still a small number of studies concerning the osteoinductive activity of BMP9. Celeste demonstrated that BMP9 on a biological carrier could stimulate ectopic bone formation, but only when applied at very high concentrations (Celeste et al., 1994). Helm first reported the used of BMP9 gene therapy to induce endochondral bone formation in rodent. They proposed that BMP9 adenoviral vector (Ad-BMP9) transduced the muscle fibers, which expressed and secreted BMP9. The extracellular BMP9 promoted the chemotaxis and proliferation of mesenchymal cells that form cartilage and ultimately normal lamellar bone (Helm et al., 2000a). While BMP15, a recently discovered TGF-beta family member (Dube et al., 1998; Laitinen et al., 1998) is an oocyte specific growth factor, which expresses during folliculogenesis and acts directly on follicular granulose cells(GCs) to regulate their proliferation and cytodifferentiation (Otsuka et al., 2000). Until now no study reported the osteoinductive activity of BMP15 both *in vivo* and *in vitro*. Since this study reported the first finding of BMP9 and BMP15 transcription messages during normal bone maintenance, further study to elucidate the exacted function of these two genes in bone homeostatis is required.

To evaluate the different of BMPs member expression between intramembranous and endochondral bone, mandibular and iliac bone which were most commonly were used for bone grafting in oral and maxillofacial reconstruction. With 12 fresh normal human bones samples from 11 different subjects, one pair of sample, one from the mandible (intramembranous) and one from the iliac (endochondral), came from one subject (Table 9 ; sample number 1). This sample eliminated the effect of intra-individual variation; on the other hand, sample number 2-6 came from different subjects, which confirmed the result of the existing data.

The most convenient approaches to study differential expression and coexpression are by using specific mRNA measurements. Although mRNA is not the ultimate product of a gene, and that mature protein levels in a cell do not show perfect correlation with the abundance of mRNA (Anderson et al., 1997; Gygi et al., 1999),

transcriptional activity and its variation are useful indicators of the involvement of a given gene in a given physiological process. Reverse transcription-polymerase chain reaction (RT-PCR) is a highly sensitive and specific method for the detection of rare transcripts or for the analysis of samples available in limited amounts. With specific design primers, we amplified and quantified the BMPs transcription messages. Using fresh normal human intramembranous bone as starting material, the messages for BMP3, BMP4, BMP7 and BMP8 were increased significantly; while, using endochondral bone, the messages for BMP2, BMP5 were raised.

BMP2-9 were previously reported and widely accepted as the osteogenic proteins (Celeste et al., 1990; Helm et al., 2000b; Jane, Jr. et al., 2002a; Marden et al., 1994). Nevertheless, current information on the role of BMP3 in bone was contradictory. The recent studies shown that recombinant BMP3 had no osteogenic activity and had antagonist effect to osteogenic BMP2, 4, 6, 7, 9 through a TGF-beta/activin pathway (Bahamonde and Lyons, 2001; Cheng et al., 2003; Daluiski et al., 2001). Consistent with our study, BMP3 expressed higher in intramembranous bone than in endochondral bone, which may elucidate the negative effect of BMP3 to endochondral ossification of BMP2. Cheng performed a comparative analysis of distinct osteogenic activity of fourteen human BMPs and found that BMP2, 6, and 9 induced osteoblast differentiation of mesenchymal stem cells (Cheng et al., 2003). Compatible with our study, BMP2, 6, 9 also have highly expression level in both endochondral and intramembranous bone which may relate to the maintainance of normal homeostasis of bone remodeling.

While most of the studies reported the exogenous BMPs in de novo bone formation, only small numbers of studies have established to find the pathway that new bone is formed. To study the effect of BMPs and the process of bone formation, all of the studies introduced the exogenous BMPs or delivered the recombinant DNA transcribed BMPs proteins (Alden et al., 1999; Gitelman et al., 1994; Helm et al., 2000b; Jane, Jr. et al., 2002a). However, all of the studies mentioned above were associated with the overexpression of BMP proteins. None of the studies indicates the normal homeostasis condition of two different types of bone, especially in human bone. Our study carried out a comparative analysis of distinct BMPs member expression of intramembranous and endochondral bone in normal condition (steady stage). We also confirmed the differences between osteoblasts of different osseous tissue origins in the transcription levels of BMPs, ones of the most important cytokines that modulates the function of the bone cells.

Part III. Identify the osteoinductive factors extracted from human intramembranous and endochondral bone.

To evaluate the osteoinductive factors in intramembranous and endochondral bone, mandibular, maxillary and iliac bone were used to be subjected for sequential extraction and partial purified. Until now most of known bone-derived osteoinductive factors have been isolated from endochondral bone and all initiate bone induction via endochondral ossification (Bentz et al., 1989; Luyten et al., 1989; Sampath et al., 1987; Urist et al., 1984; Wang et al., 1988; Wozney et al., 1988). Only the study by Scott in 1944 demonstrated the heparin separose binding osteoinductive factor which was extracted and partial purified from bovine bone matrix. The majority of known molecules associated with chondro- and osteo-inductive have apparent molecular weights of 30 kDa or less (Luyten et al., 1989; Sampath et al., 1990; Wang et al., 1988; Wozney et al., 1988). Many studies described 16, 18 and 30 kDa polypeptides associated with osteoinductive activity (Luyten et al., 1989; Sampath et al., 1990; Wang et al., 1988). Recombinant human bone morphogenetic protein 2 (rhBMP 2), the most well known and widely study molecule possessed complete cartilage and bone inductive activity, has a molecular weight 30 kDa and is composed of two disulfide-linked 16 to 18 kDa subunits (Wang et al., 1990). Consistent with purification of bovine osteogenic protein shown that it also migrated at an apparent molecular weight of 30 kDa and upon reduction yielded two subunits that migrated at molecular weight of 16 and 18 kDa (Sampath et al., 1990). The molecular weights of protein profile from SDS-PAGE were summarized inTable 11.

There may be clinical situation in which it would be more appropriate to replace traumatized or diseased osseous tissue with intramembranous bone rather than endochondral bone (Scott and Hightower, 1991). Since intramembranous bone grafts are often superior to endochondral bone graft (Smith and Abramson, 1974; Zins and Whitaker, 1983), the factors that could be extracted from intramembranous bone could be potentially more beneficial in the clinical repair of bony defects than factors extracted from endochondral bone. From this knowledge arose the interest in isolating a factor from human intramembranous bone which could induce direct bone formation.

BMPs composed in the very small proportion in bone matrix. Starting from 10 kg of bovine bone, the yield of protein was approximately 20 μg (Wang et al., 1988). This obstacle was the main factor relate to the lack of knowledge of human intramembranous bone extraction and purification. In this study, human intramembranous bone (approximate 500 g) extracted with guadinine hydrochloride with EDTA and SDS-PAGE demonstrated the unique band below 21 kDa which were absent or present in much lower amount in comparable endochondral fractions. This result was consistent with the study from Scott et al whom reported the osteoinductive factors extracted and partial purified from bovine intramembranous bone. Moreover, the results also shown the similar pattern of SDS-PAGE at 30 kDa which was consistent with BMPs in previous studies (Sampath et al., 1990; Wang et al., 1990).

Table 11 Molecular weight of osteoinductive factors and bone related component from various species.

Component	Molecular weight (kDa)	Source	Reference
BMP	18	Bovine	(Urist et al., 1984)
BMP	17	Human (femoral neck)	(Bessho et al., 1999)
BMP2	12.9	Bovine	(Celeste et al., 1990)
BMP3	14.5	Bovine	(Celeste et al., 1990)
BMP4	13.1	Bovine	(Celeste et al., 1990)
BMP5	15.6	Bovine	(Celeste et al., 1990)
BMP6	15.7	Bovine	(Celeste et al., 1990)
BMP7	15.7	Bovine	(Celeste et al., 1990)
Osteogenin	22	Bovine	(Sampath et al., 1987)
Osteogenin	28-43	Bovine	(Paralkar et al., 1990)
rhBMP 2	30 (16 and 18 subunit)	Bovine	(Wang et al., 1990)
Osteoinductive factor (OIF)	22-28 12 (after deglycosylation)	Bovine	(Bentz et al., 1989)
Osteogenic protein	30 (16 and 18 subunit)	Bovine	(Sampath et al., 1990)
Hyaluronate binding proteoglycans	63 and 74	Rat (mandible)	(Lee et al., 1998)
Vitronectin	73	Rat (mandible)	(Kumagai et al., 1998)
Type I procollagen	28	Porcine (calvarial)	(Goldberg et al., 1988b)
Type I procollagen	28	Rabbit (mandible)	(Maeno et al., 1993)

Because the fact that osteoinductive factors, as well as, BMP activity bind to heparin and its complete elutes by 0.7 M NaCl (Luyten et al., 1989; Wang et al., 1988). This finding co responses with the study from Ruppert who representing that BMP2 contained a heparin-binding site at N-terminal domains which modulated BMP's activity (Ruppert et al., 1996). This study used heparin affinity chromatography as the tool to purify osteoinductive factor activity from other proteins. However after heparin affinity chromatography the binding proteins were too low for further investigation.

To modify the protocol used to extract and purify the human intramembranous osteoinductive factor, the increased volume of starting bone material should be achieved. The high performance liquid chromatography (HPLC) which required small number of starting material may be useful for purify the gel-eluted interested bands from SDS-PAGE.

Since the question of adult bone reconstruction and repair remains of utmost importance in the field of oral and maxillofacial reconstruction, the pattern of BMP expression in normal human intramembranous and endochondral bone that maintains the matrix integrity and source of BMP autocrine would be almost valuable. Our study indicates the distinct pattern of BMPs member expression which confirms the different in two type of human bone cell function. There are the numerous different in BMPs expression between normal human intramembranous and endochondral bone. BMP3, BMP4, BMP7 and BMP8 are highly expressed and may associate with intramembranous ossification. In contrast, BMP2 and BMP5 have increase messaged in endochondral bone. The more that we learn of the complexities of the BMPs in normal condition of bone, the more likely that the use of "cocktails", rather than single BMP proteins, will provide the most effective means of promoting specific pathways of new bone formation.