CHAPTER 3

RESULTS

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

1. Osteoblast culture and osteogenic marker alkaline phosphatase

To establish the bone marrow derived osteoblasts, we utilized three sources of bone origin, mandible which came from intramembranous in origin, iliac crest bone and tibia bone which came from endochondral in origin.

Bone cells achieved from healthy subjects were induced and differentiated to express phenotypes of osteoblasts in cell culture system. At initial seeding, most of the cells had a round shape mixing with small pieces of bone fragment. At 36 hours after seeding, living stromal cells started to attach on the surface of the culture plate while dead cells of bone fragment floated in the culture medium. After 72 hours, the morphological change of attach cells were seen. Cells spread their cytoplasms on flat surface and start to form cell cluster. Number and size of cell clusters increased with the culture time and they reached confluence around 14^{th} -21^{st} days.

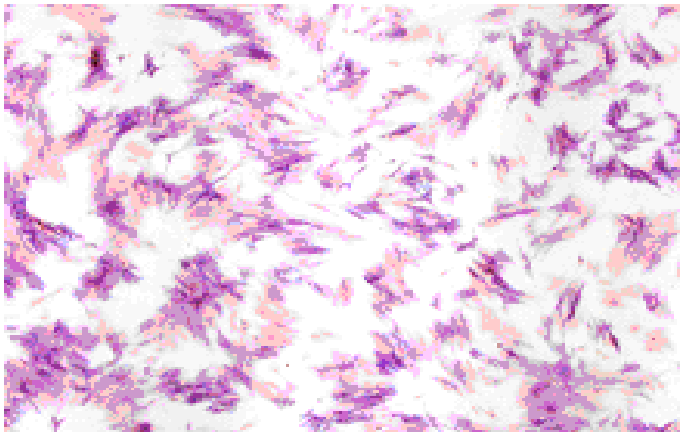
At each culture medium change, cells were observed by inverted microscopy to verify the presence of the osteoblasts. The entire culture of osteoblastic cells appeared to progress to the desired lineage, on the basis of morphology and alkaline phosphatase histochemical staining. All cell cultures contained the adherent spindle-shaped cells and showed 95-99% homogenous. Osteoblastic lineage-specific differentiations of these cells were monitored by measuring alkaline phosphatase activity. The quantitative colorimetric assay revealed an increase in alkaline phosphatase activity, which increased over time (Figure 5A-B and Figure 6A-B). In contrast, cultured normal gingival did not undergo any such differentiation and did not show any alkaline phosphatase activity (Figure 5C and Figure 6C).

2. Identify osteoblast-like cells by Alkaline phosphate (ALP) staining

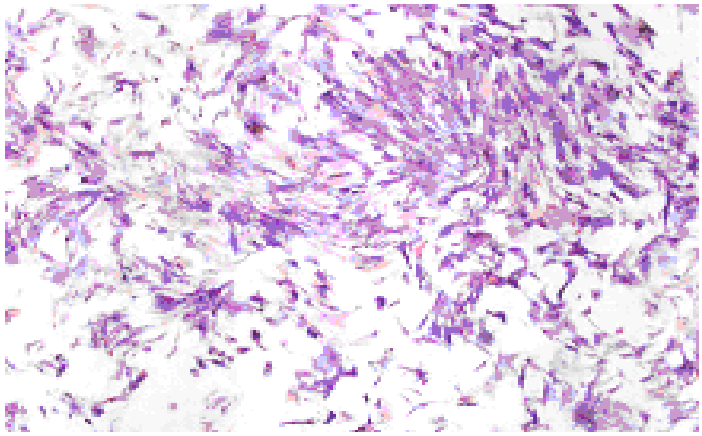
Alkaline phosphatase staining was performed to demonstrate products of the osteoblastic cells, which their expressions associated with mineralization of bone matrices.

Positive staining of ALP was found at the 7 culture-day which the positive stained cells were fibroblast-like cells with elongated cytoplasm, while some spindle-shape cells were negative stained (Figure 5A, Figure 6A). The cytoplasm was positively stained with various intensity of blue staining. The numbers of positive stained cells were markly increased at the 14 culture-days, where the positive stained cells were aggregated and form the cluster of cells (Figure 5B, Figure 6B). The cytoplasm was stained with dark blue to dark purple staining. Moreover, most of the cells in 14 culture-days were fibroblast-like cells with positive staining with ALP.

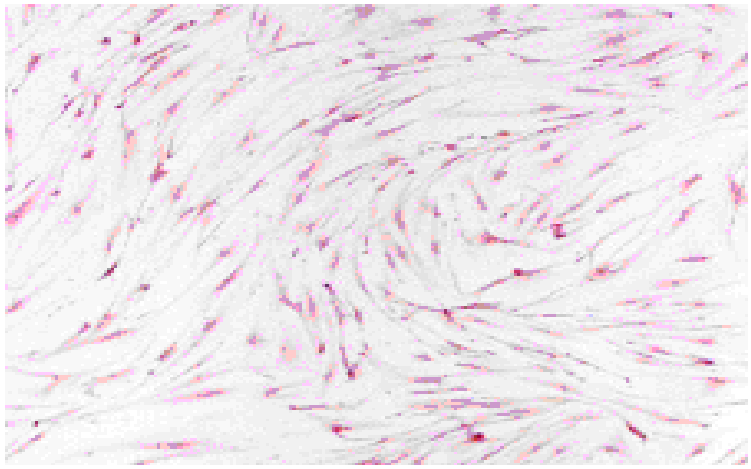
Figure 5 Alkaline phosphatase histochemical staining of human mandibular bone cells (A-B) and control gingival fibroblast (C).



(A) Osteoblasts from intramembranous bone 1 week

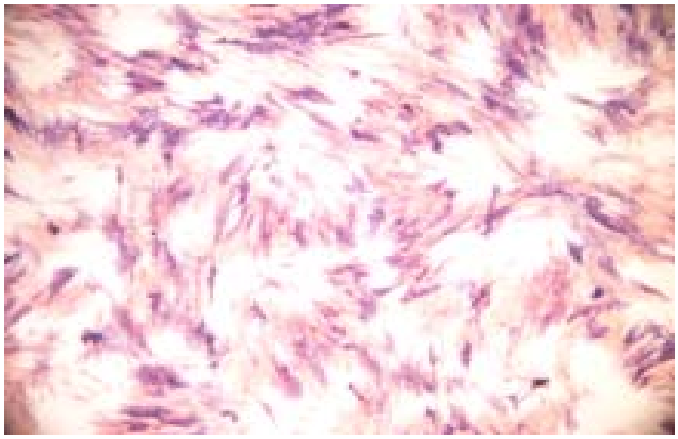


(B) Osteoblasts from intramembranous bone 2 week

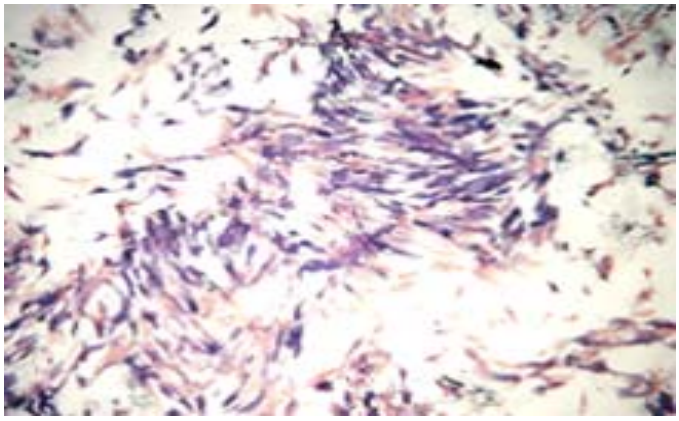


(C) Control gingival fibroblast 2week

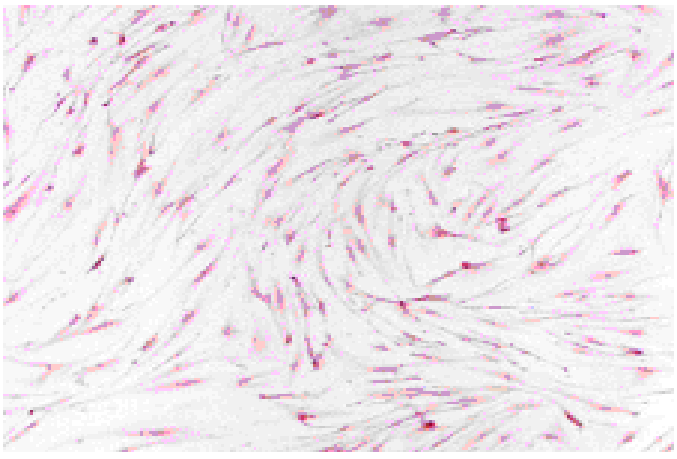
Figure 6 Alkaline phosphatase histochemical staining of human iliac crest bone cell (A, B) and control gingival fibroblast (C).



(A) Osteoblasts from endochondral bone 1week



(B) Osteoblasts from endochondra bone 2 week



(C) Control gingival fibroblast 2week

3. Expression of specific BMPs mRNA members in primary culture of human intramembranous and endochondral bone

To evaluate the diversity of BMPs member expression in *ex vivo* human osteoblasts from three different sources, we utilized the primary culture osteoblasts as starting materials. Amplification products of expected size for BMP2-BMP9 primer pairs in primary culture of human intramembranous and endochondral bone were shown in Figure 7 and Table 7. All BMPs members expressed message for BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8 and BMP9. However, message for BMPs expression were different among various BMPs members.

In primary culture osteoblast derived from human intramembranous bone, as shown in Figure 8 and Figure 9, BMP6 showed the highest level of BMP expression (26.6%), while BMP3 showed the lowest expression level (6.7%). Comparing with primary osteoblastic cell culture originated from human endochondral bone, BMP6 stilled showed the highest level of BMP expression (26.9%). BMP2, 5 each was observed 15-19% of total BMPs. Whereas BMP4 was found only 2.8%.

Figure 7 The expression of specific BMPs members comparing between primary cell culture derived from intramembranous (mandible) and endochondral (iliac and tibia) bone. First lane of each panel showed from intramembranous bone and the second one showed from endochondral bone, and GAPDH as internal control.

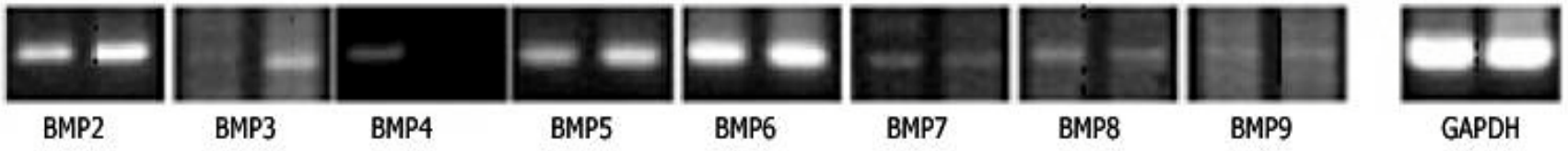


Table 7 Relative abundance of RT-PCR amplified products comparing between intramembranous osteoblastic cell line and endochondral osteoblastic cell line. Images such as those generated in Figure 7 were digitized and band intensity were converted into mean pixel per area using Scion Image Analysis software. MO; Mandibular osteoblastic cell line, EO; Iliac crest and Tibia osteoblastic cell line ** statistically significant p<0.05, *statistically significant p<0.10

Sample Number	Band Intensity in Mean pixel per area after standardized with GAPDH															
	BMP2		BMP3		BMP4		BMP5		BMP6		BMP7		BMP8		BMP9	
	MO	EO	MO	EO	MO	EO	MO	EO	MO	EO	MO	EO	MO	EO	MO	EO
1	12.48	16.43	5.33	10	6.26	2.51	9.42	12.81	18.82	20	7.01	4.71	7.13	6.32	9.57	9.69
2	18.59	24.37	7.98	14.82	8.54	2.92	12.03	16.59	28.02	29.66	7.00	5.48	9.96	8.96	10.41	10.56
3	9.17	11.62	3.87	9.93	11.75	2.21	9.52	12.64	21.2	23.09	6.53	4.29	7.15	4.94	5.66	7.44
SD	4.78	6.44	2.08	2.80	2.76	0.36	1.48	2.23	4.77	4.93	0.27	0.60	1.63	2.04	2.53	1.61
Average	13.41	17.47	5.72	11.58**	8.85**	2.55	10.32	14.01**	22.68	24.25**	6.85	4.83	8.08	6.47	8.55	9.23

Figure8 Relative abundance of RT-PCR amplified products comparing between intramembranous and endochondral osteoblastic cell line.

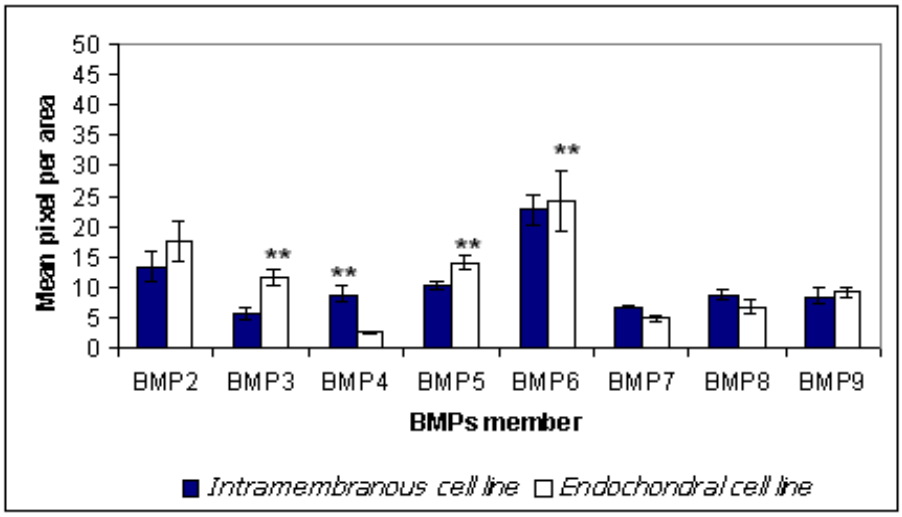
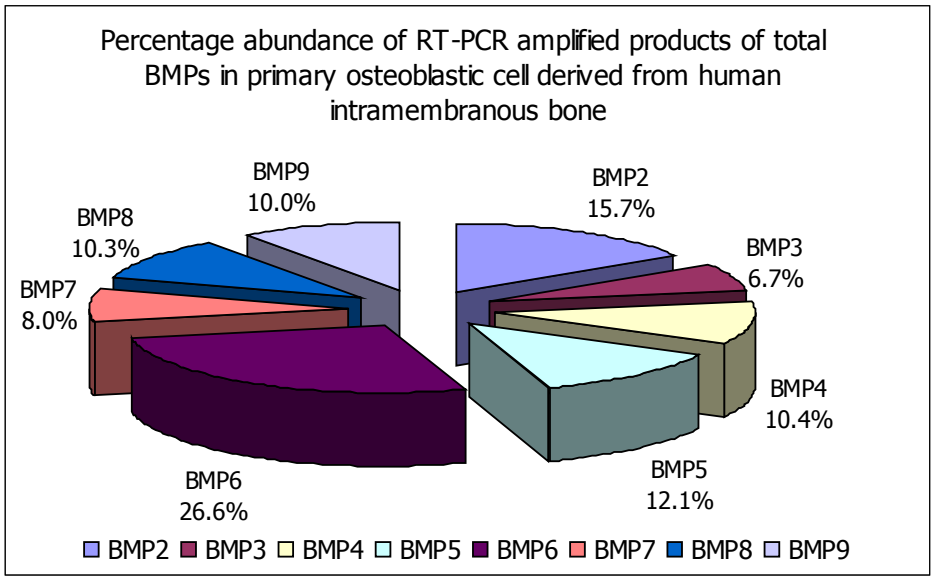
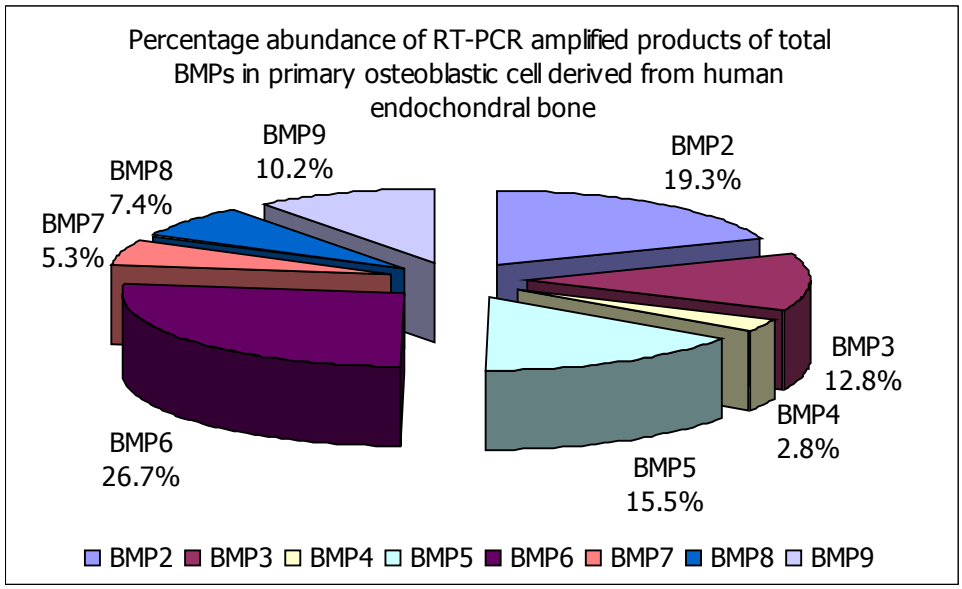


Figure 9 Percentage abundance of RT-PCR amplified products of total BMPs in primary osteoblastic cell culture of (A) intramembranous and (B) endochondral bone.



(A)



(B)

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

1. BMPs members expression in human intramembranous bone

In order to identify the diversity of BMPs members expression in normal human intramembranous bone, RT-PCR with total RNA from normal human intramembranous bone as the template were performed using degenerated oligonucleotide primers (Degen BMPs). The expression levels of BMPs in intramembranous (mandibular and maxillary bone) and endochondral (iliac bone) were different as shown in Figure 10. Start with 500 μg of RNA per lane, the RT-PCR was performed in the same condition. GAPDH which was used as the internal control showed in all samples. However, the expression level of GAPDH in endochondral bone seemed to be slightly stronger than those in intramembranous bone. When compare the expression of BMPs family in intramembranous and endochondral bone using degenerated primer (the expected sized was 120 bp), the massage in intramembranous (mandible and maxilla) bone was notably higher than endochondral (iliac) bone. It revealed that the BMP members consisted in intramembranous and endochondral bone were dissimilar in types or in volume.

The expected size of the product obtained form the RT-PCR reaction was 120 base pair. After purification using Concert Rapid Gel Extraction System (Gibco) or Wizard® PCR Preps DNA Purification System (Promega), the fragment was purified and cloned into E.*coli* using pGEM T-easy. E.*coli* transformants were then screened on LB plates containing 80 μg/ml of ampicillin. A total of twenty-three clones transformants containing the BMPs inserted fragment were obtained and sequenced on an automated sequencer (ABI PRISM™ 377 DNA sequencer). From twenty-three selected clones, 16 clones showed 84-100% homology to human BMP2, BMP4, BMP5, BMP6, BMP7, BMP8 and TGF-beta genes (Table 8). One clone showed 93% homology to human BMP9 gene (Figure 11) and four clones showed 94-100% homology to human BMP15 gene (Figure 12).

Figure 10 Amplification products from RT-PCR using degenerated primer showed unequal expression massages of BMPs family.

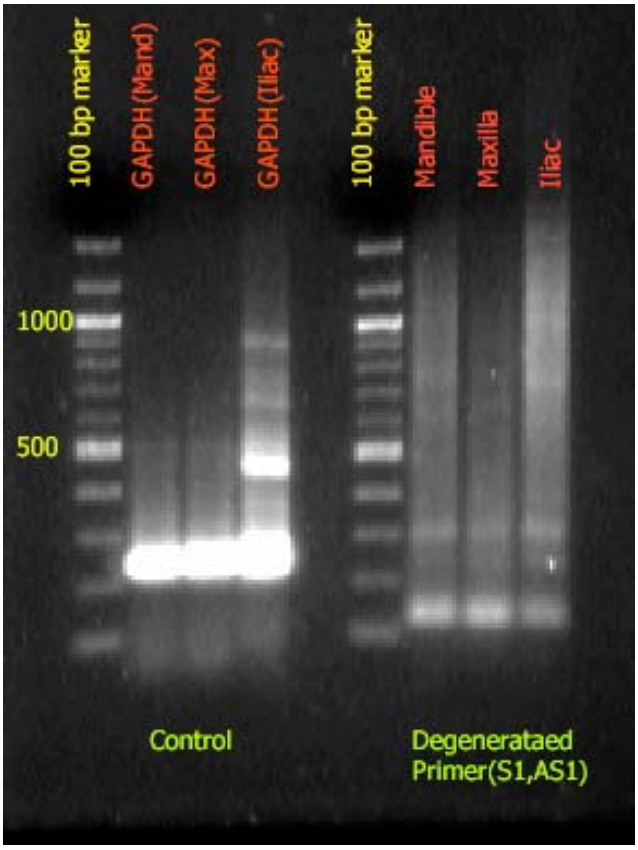


Table 8 Amplification DNA fragments from degenerated oligonucleotide primers and number of cognate clone which matched by BLAST program.

RNA Samples	Size	Number of clone	E(BLASTX)	%I(BLASTX)	E(BLASTN)	%I(BLASTN)	RESULT
MAND	120	1	3E-18	95%	2E-35	96%	TGF-beta
MAX	120	7	9E-16	100%	6E-38	96%	BMP2
MAND	120	2	7E-19	95%	2E-35	96%	BMP4
MAND	120	1	2E-17	92%	7E-04	86%	BMP5
MAX	120	1	3E-18	95%	2E-35	96%	BMP6
MAX	120	2	1E-15	82%	3E-09	84%	BMP7 (OP1)
MAND	120	2	5E-18	92%	2E-12	94%	BMP8 (OP2)
MAND	120	1	3E-16	87%	1E-36	93%	BMP9 (GDF2)
MAND	119	4	1E-11	96%	2E-32	100%	BMP15

Figure 11 The graphic showed the alignment of acquired sequence (A1Mand) from intramembranous bone and the known sequence (BMP9).

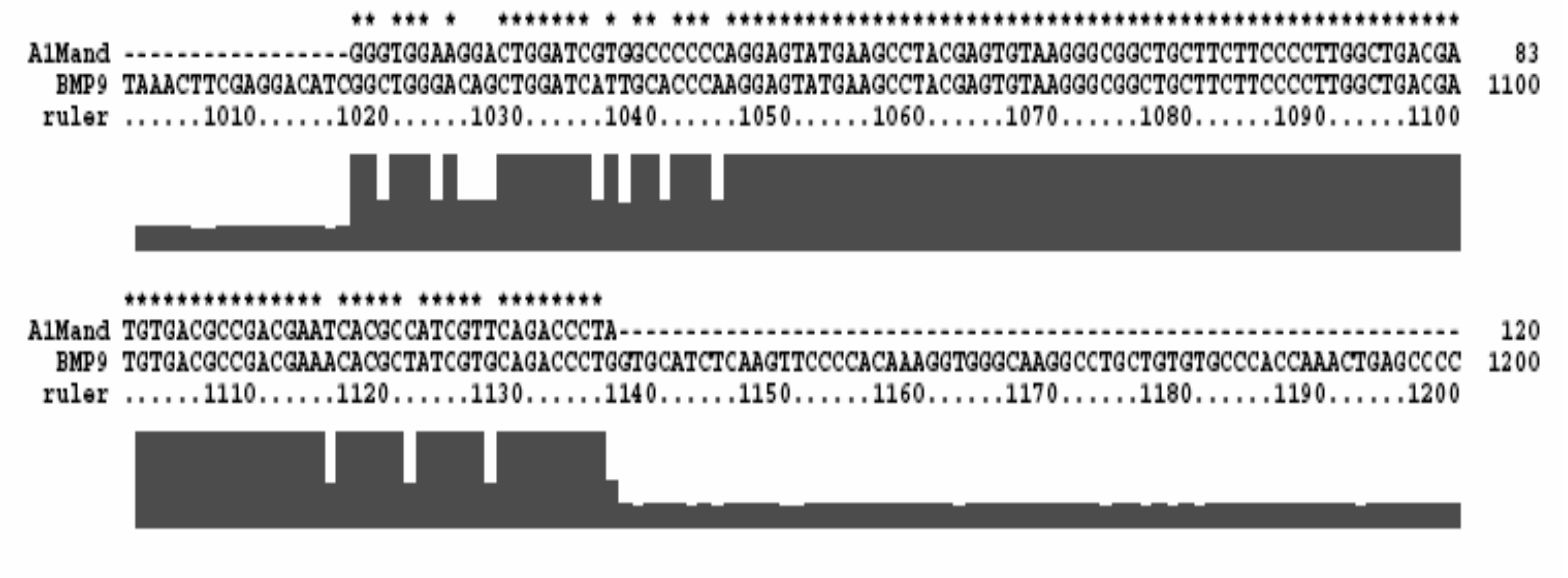
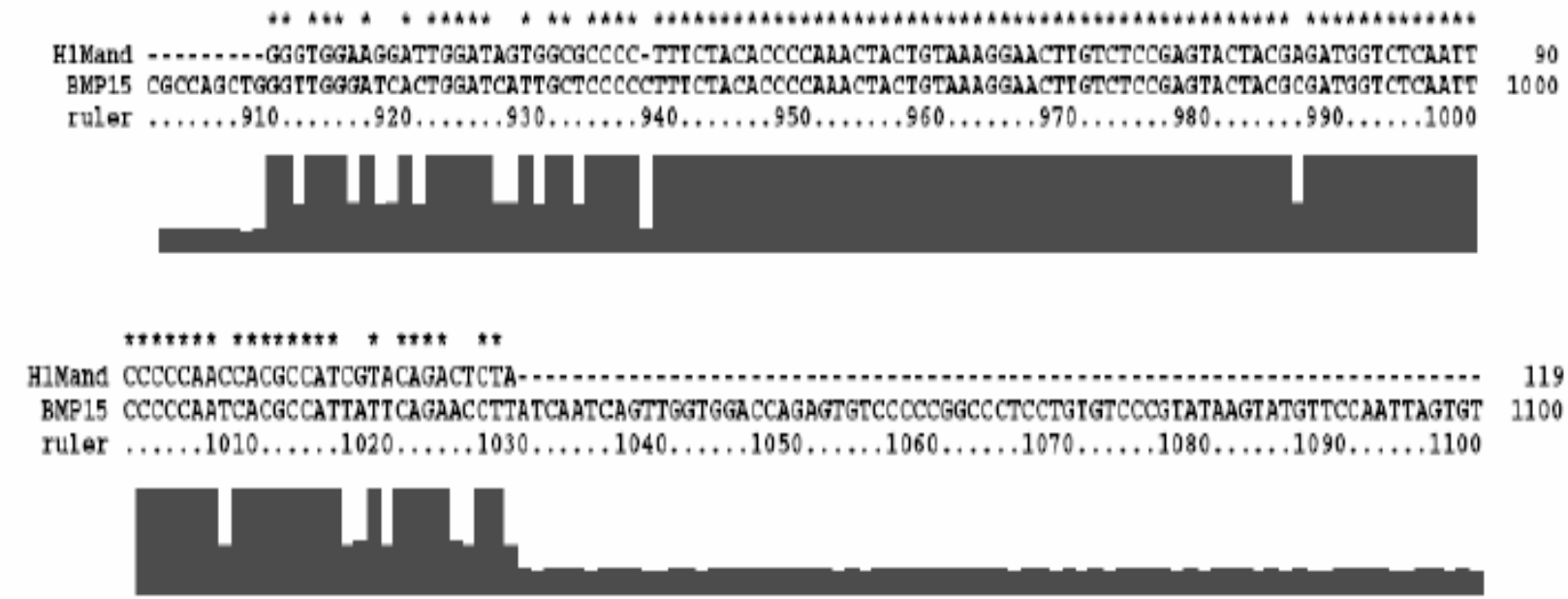


Figure 12 The graphic showed the alignment of acquired sequence (H1Mand) from intramembranous and known sequence (BMP15).



2. Expression of specific BMPs mRNA members in fresh normal human intramembranous and endochondral bone

To elucidate the proportion of BMPs member message expression in normal human intramembranous and endochondral bone, twelve bone sample form 11 individual subjects, one pair of sample, one from the mandible (intramembranous) and one from the iliac (endochondral), came from one subject were used.

Amplification products of expected size for BMP2-BMP9 primer pairs in normal human intramembranous and endochondral bone were shown in Figure 13 and Table 9. All BMPs members expressed message for BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8 and BMP9. However, message for BMPs expression were different among various BMPs members. In two sample of normal human endochondral bone, message for BMP4 were undetectable from visual analysis (Figure 13).

In normal human intramembranous bone, BMP6 showed the highest level of BMP expression (18.1%). In addition BMP2, 5, 6 and 7 composed more than 60% of total BMPs; while, BMP3, 4, 8, 9 each was found only 6-10% of total BMPs (Figure 14). In contrast to normal human endochondral bone, BMP2 showed the highest level of BMP expression (26.9%). BMP2, 5, 6 each was observed 24-26% of total BMPs. Whereas BMP4 was found only 2.2%, BMP3, 7, 8, 9 were found range from 3.6-9.9% of total BMPs.

Figure 13 The expression of specific BMPs members comparing between fresh normal intramembranous and endochondral bone. Figure 10 presented the band intensity from RT-PCR products from specific BMPs. First lane of each panel showed from intramembranous bone and the second one showed from endochondral bone, and GAPDH as internal control.

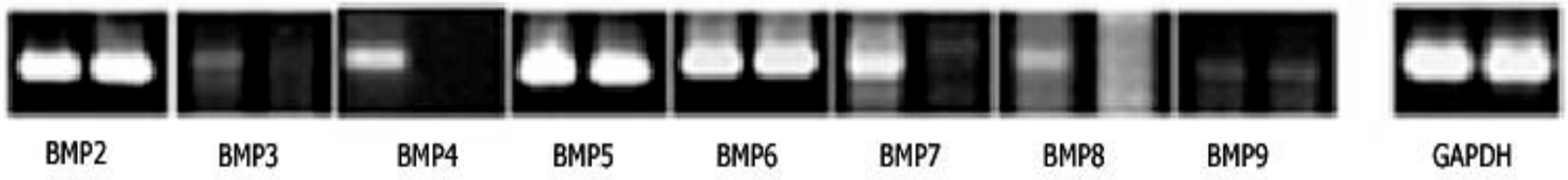
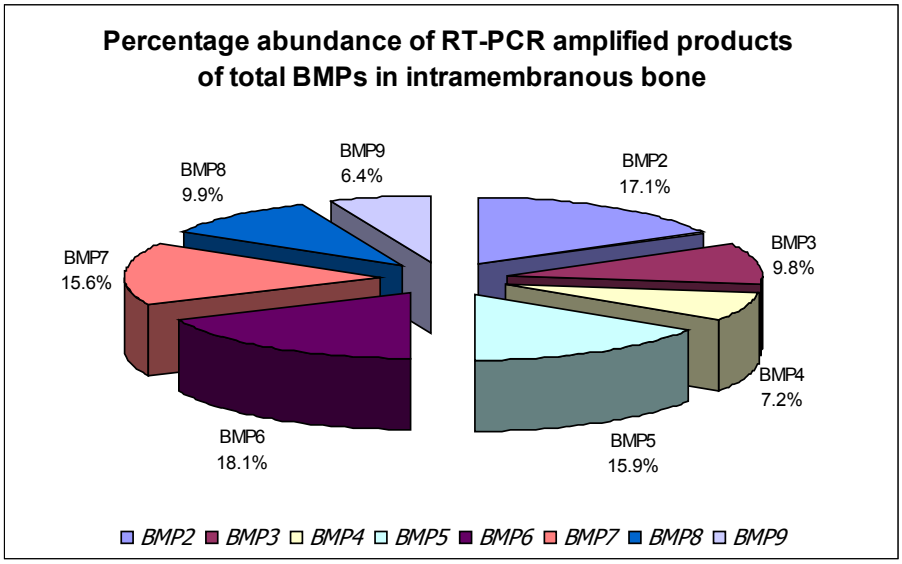


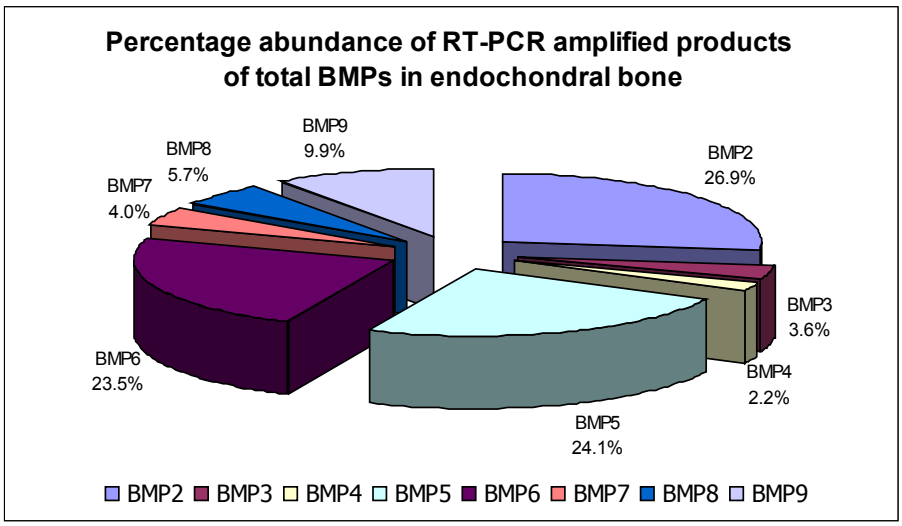
Table 9 Relative abundance of RT-PCR amplified products comparing between normal intramembranous and endochondral bone. Images such as those generated in Figure 13 were digitized and band intensity was converted into mean pixel per area using Scion Image Analysis software. M; Intramembranous bone, E; Endochondral bone, ** Statistically significant $p<0.05$, *Statistically significant $p<0.10$

	Band Intensity in Mean pixel per area after standardized with GAPDH															
	BMP2		BMP3		BMP4		BMP5		BMP6		BMP7		BMP8		BMP9	
	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M	E
1	24.52	29.6	7.04	4.14	5.05	2.58	28.32	32.26	33.28	38.4	17.4	5	13.16	10.4	5.42	4.82
2	31.42	32.88	6.88	2.62	3.72	0.04	26.46	33.98	34.08	34.68	30.02	6.42	15.48	8.82	5.94	6.96
3	30.79	40.65	24.9	8.23	20.13	5.63	30.01	42.36	35.62	37.45	28.2	7.43	15.41	9.6	17.5	20.36
4	29.9	44.12	20.49	4.7	18.8	3.74	33.93	45.67	31.93	32.41	28.52	5.33	25.83	6.99	14.68	22.73
5	31.68	41.56	29.3	5.36	15.47	5.58	19.55	21.2	33.48	34.23	33.35	8.43	19.37	7.735	13.74	15.11
6	33.67	42.44	15.44	5.61	12.99	1.41	31.15	32.34	24.06	25.54	28.47	2.01	15.96	5.83	10.89	15.47
SD	3.108	5.86	9.26	1.86	6.91	2.26	4.95	8.64	4.10	4.59	5.38	2.24	4.52	1.70	4.88	7.12
Avg	30.33*	38.54	17.34**	5.11	12.69**	3.16	28.23*	34.63	32.07	33.78	27.66**	5.77	17.53**	8.22	11.36	14.24

Figure 14 Percentage abundance of RT-PCR amplified products of total BMPs in fresh normal intramembranous (A) and endochondral (B) bone.



(A)

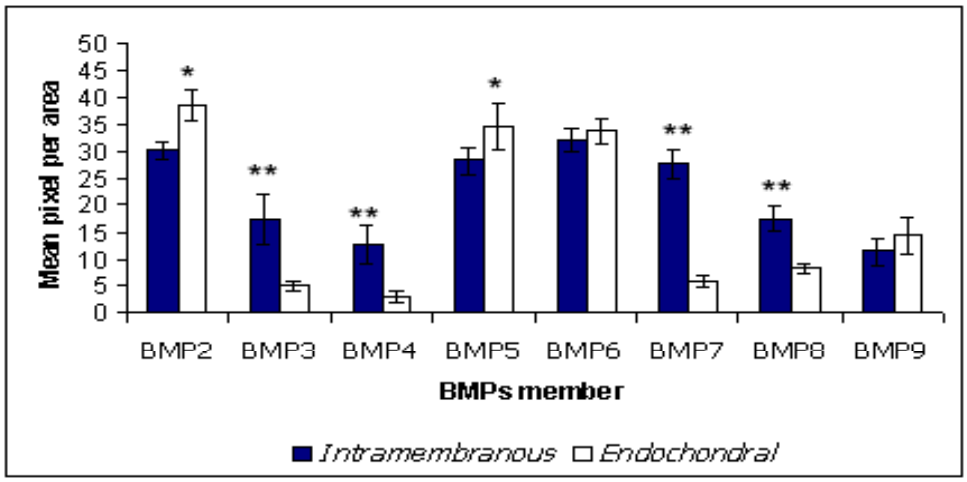


(B)

3. Distinct expressed message from fresh human intramembranous and endochondral bone

To identify the specific pattern of BMPs, comparing between fresh normal human intramembranous and endochondral bone tissues, we used 11 individual subjects. One pairs of the experimental sample (sample pair number 1 in Table 9) both bone types came from same subject; while, other 5 pairs of sample came from 10 different subjects. When compared the expression of BMPs members in human intramembranous and endochondral bone, the message for BMP3, BMP4, BMP7, and BMP8 were statistically significant with $p<0.05$ higher expressed in intramembranous bone. However, BMP2 and BMP5 were highly expressed in endochondral bone ($p<0.10$). A 70-80% reduction was noted when compared BMP3, BMP4, BMP7, and BMP8 of endochondral bone with intramembranous bone (Figure 14 and Figure 15).

Figure 15 Relative abundance of RT-PCR amplified products comparing between fresh normal intramembranous and endochondral bone.



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

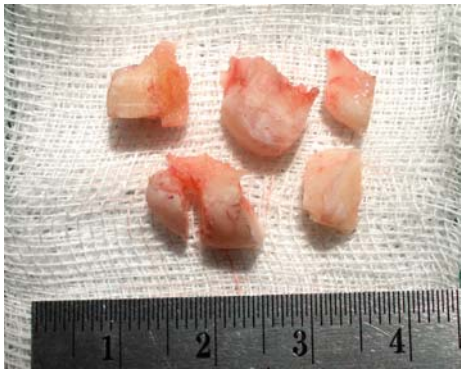
1. Tissue preparation and sequential extraction

Bone samples were achieved from the 21 patients; 17 from intramembranous in origin, and 4 from the endochondral in origin. The information associated with bone samples were summarized in Table 10. The bones, both cortex and cancellous part, were removed the attached soft tissue and periosteum and were cut in to small pieces (approximate 5x5 mm.) as shown in Figure 16. After that, bone samples were washed extensively in cold ($4^{\circ}C$) sterile PBS containing protease inhibitors, and they were subjected to sequential extraction to remove mineral content from their tissues.

Table 10 Bone samples achieve from patients underwent necessary surgery

Bone type	Site	Sex&age(yrs) of donors	Weight of samples (mg)	Note
Intramembranous	Mandible	Male / 21	12	3rd molar
	Mandible	Male / 21	14	3rd molar
	Mandible	Female / 26	29	3rd molar
	Maxilla	Male / 38	56	Torus palatinus
	Mandible	Female / 43	24	Torus mandibularis & buccal exostosis
	Maxilla	Female / 22	21	3rd molar
	Maxilla	Female / 24	21	3rd molar
	Mandible	Male / 58	32	Torus mandibularis
	Mandible	Male / 55	28	Torus mandibularis
	Mandible	Female / 24	22	3rd molar
	Mandible	Female / 29	12	3rd molar
	Mandible	Female / 25	20	3rd molar
	Mandible	Female / 34	29	Chin
	Maxilla	Male / 42	31	Torus palatinus & buccal exostosis
	Mandible	Male / 26	24	3rd molar
	Mandible	Male/ 28	21	3rd molar
	Maxilla	Female / 43	38	Torus palatinus
Endochondral	Iliac	Female / 23	46	-
	Iliac	Female / 20	31	-
	Iliac	Female / 21	19	-
	Iliac	Female / 28	42	-

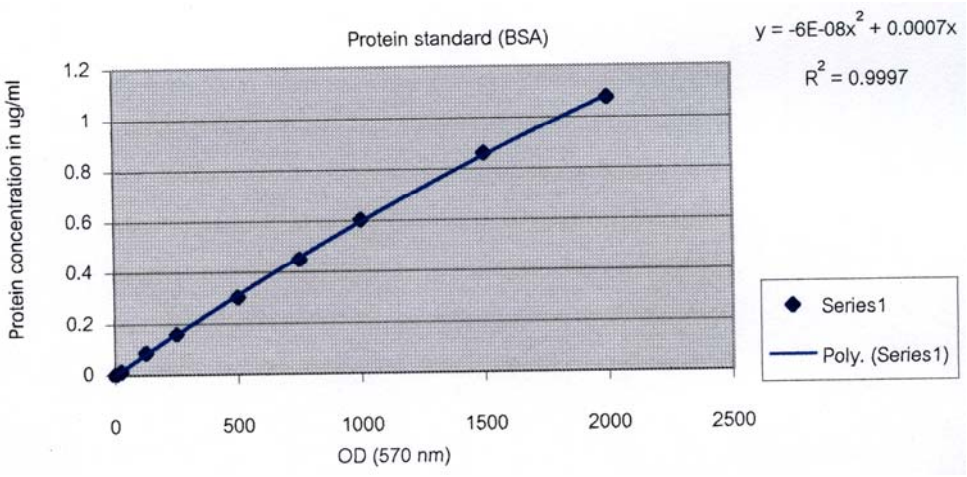
Figure 16 Intramembranous bones achieved from patients undergoing torectomy (torus palatinus) were cut into small pieces. The bones were composed of both cortical and cancellous part.



2. Protein profile of the intramembranous and endochondral bone

After achieved adequate amount of the freeze-dried bone (approximate 3 g.), the samples were dissolved in sample buffer for protein quantitation using Lowry method (appendix) with bovine serum albumin as the standard (Figure 17). An equal volume (500 μg) of proteins was loaded in each lane of SDS-PAGE for analyzing protein mixtures. The protein profile from SDS-PAGE was shown in Figure 18. The SDS-PAGE analysis revealed a number of proteins, the intramembranous fraction contain a distinct band below 43 and 21 kDa (Figure 18(A); lane 1, 2, 3, 4 and Figure 18(B); lane 6, 7, 8) which were absent or present in much lower amount in comparable endochondral fractions (Figure 18(A); lane 5). The analysis by SDS-PAGE also presented the 30 kDa proteins which were presented both in intramembranous and endochondral fraction.

Figure 17 Standart curve of absorbance (OD750) as a function of initial protein concentration.



The 17-20 kDa proteins were most likely corresponded to the osteoinductive proteins found by Scott (Scott et al., 1994). In order to determine whether this protein had the specific biological activity, a heparin affinity chromatography was conducted to isolate the partial purify protein. The heparin affinity chromatography was conducted using running buffer containing 6 M urea, 50 mM Tris-HCl and protease inhibitor (Appendix) and eluting buffer containing 0.15-1.5 M NaCl. However, the purification with heparin affinity chromatography struggle with the very small proportion of interested protein in bone sample. The fraction of proteins binding to heparin was only in microlitter that did not enough for further biological test.

Figure 18 (A) The SDS-PAGE profile of proteins extracted from intramembranous (mandibular and maxilla bone) and endochondral (iliac bone).

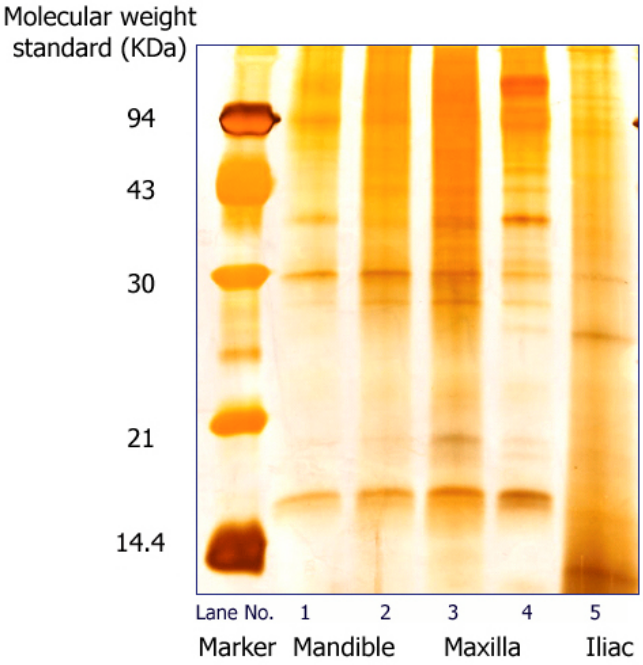


Figure 18 (B) The SDS-PAGE profile of proteins extracted from intramembranous (mandibular and maxilla bone) and endochondral (iliac bone).