CHAPTER 2

MATERIALS AND METHODS

Materials

1. Bone

Human intramembranous bone (mandible; MAND and maxilla; MAX) and human endochondral bone (iliac bone; EC) weighing approximately 20-30 mg per tissue were prepared from healthy adult patients (ages 20 to 58 years), who had torectomy, alveoloplasty, orthognathic surgery (bilateral sagital split osteotomy, and/or segmental osteotomy), or iliac crest bone graft for reconstruction oral and maxillofacial defects, performed in the Dental Hospital, Faculty of Dentistry, Prince of Songkla University. Informed consent, according to guidelines from the Faculty of Dentistry, Prince of Songkla University Committee on the Use of Human Subjects in Research, was obtained from the bone donor.

2. Bacteria Strain

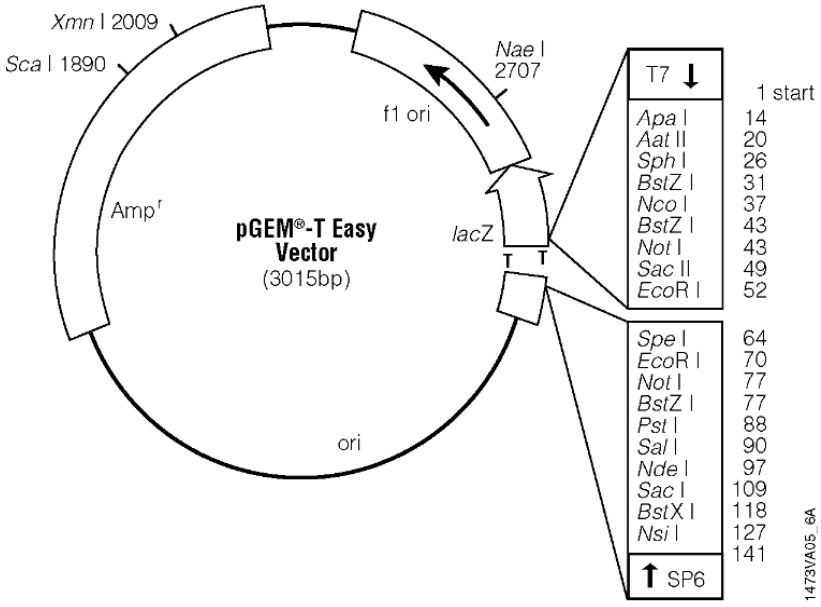
To amplify the PCR product achieved from RT-PCR, the *E.Coli* TOP 10F' (Invitrogen, Netherlands) was used as a host for plasmid propagation.

Genotype: F'[lac I^q Tn10(Tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ 80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 *gal*U *gal*K rpsL(Str^R) endA1 nupG

3. Plasmid vector

pGEM T easy (Promega) was used for recovery of PCR products after ligation, and was used for sequencing analysis. The physical map of plasmid pGEM-T easy was shown in Figure 4.

Figure 4 Physical map of plasmid pGEM-T easy. The figure illustrates plasmid pGEM-T easy containing lacZ gene, multiple cloning sites, origin of replication of E.coli, amplicillin resistant marker gene.



4. Primer for RT-PCR amplification

The nucleotide primers were synthesized by Gibco-BRL, USA and are shown in Table 2. The first set represented four degenerate oligonucleotide primers (S1, AS1; S2, AS2) corresponding to highly conserved motifs in the carboxyl-terminal region of the BMPs were used for amplification of the BMPs family in fresh intramembranous bone samples. The second sets of RT-PCR primer (BMP2 to BMP9) were used as specific primers for amplification of the specific fragments of BMPs. The third sets of PCR primers were used for sequencing of the fragments of BMPs gene amplifying from the second sets primers. The last set of PCR primers was used for amplification of the GAPDH fragment which is the house keeping gene.

5. Chemicals

All chemicals used were analytical grade.

ß-Glygerophosphate, Cat. No. 9891, Sigma, USA
ß-Mercaptoethanol
6-aminohexanoic acid
Absolute ethanol, Cat. No. A1613, Sigma, USA
Acetic acid 100%
Acetone
Acrylamide
Agarose
Alkaline phosphatase histochemical staining assay; Sigma
Ammonium persulfate
Ampicillin
Ascorbic acid, Cat. No. 1.00127, 100 g, Merck, USA
Bezamidine HCl
Bis-acrylamide
Bovine serum albumin standard
Calcium Chloride ($CaCl_2$)
Cellophane paper
Concert Rapid Gel Extraction System; Gibco

Coomassie Brilliant Blue R-250

Culture medium DMEM, Cat. No. 12100-046, 10x1L, GIBCO

De-ionize water

Dexamethasone, Cat. No. D1756, Sigma, USA

Dithiothreitol (DTT)

Ethanol

Ethidium bromide (EtBr), Cat No. H5041

Ethylene diamine tetraacetic acid ; EDTA

Fetal calf serum, Cat No. 10270-098, 100 ml, GIBCO)

Folin reagent

Formaldehyde 37% Cat. No.

Fungizone, 20 ml, Cat. No. 15295-071, GIBCO)

Glutaraldehyde 25%

Glycerol

Glycine

Guanidine HCl

Isopropanol

Liquid nitrogen

Luria-Bertani (LB)

Methanol

Na_2HPO_4

NaCl

NaH_2PO_4

NaOH

Penicillin/Streptomycin

Phenylmethanesulfonyl fluoride (PMSF)

Prestained low range molecular weight protein standard

QIAprep Miniprep System; Qiagen

Rnase-free water

RNeasy Kit; Qiagen Inc., Valencia, CA

Silver nitrate

Sodium carbonate

Sodium citrate dihydrate

Sodium dodecyl sulfate (SDS)

TEMED

Tetracycline

Tris-HCl

Tritron x-100

Trypsin-EDTA(0.5%trysin,5.3mMEDTA, Cat.No.15400-054)

Whatman filter paper No.1

Wizard PCR Prep DNA Purification System; Promega

Urea

6. Restriction enzyme

To screen the inserted-DNA fragments in competent cells, the recombinan EcoRI t DNA were cut in the specific sequence with restriction enzyme, in this study EcoRI, to confirmation of the size of inserted-DNA

Enzymes were purchased from Biolabs, USA; Boehringer Mannheim, Germany; Promega, USA; QIAGEN, Germany and Stratagene, USA.

EcoRI is the restriction endonuclease, that binds and cleave double strand DNA at specific sequences G↓AATTC

7. Equipment and instruments

ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems)

Automated DNA Thermal Cycler; Perkin-Elmer model 2400

Amicon YM-10 filters, M_r =10,000 cutoff

BioLogic, BIO-RAD, USA

Electrophoresis Unit

Freeze-dried

Gel Doc Model 1000, BIO-RAD, USA

Polytron

Spectapor I acetylated dialysis tubing

Spectrapor I acetylated dialysis tubing (M_r=6,000-8,000 cut-off)

Thermal cycler (Hybaid)

Ultrafitration (Amicon YM-10 filters, M_r =10,000 cutoff

Vortex mixer

Water bath

Table 2 Reverse transcription polymerase chain reaction (RT-PCR) oligonucleotide primer sequences and expected amplification product sizes.

Primer	Sequence of primer(5'-3')	Corresponding to amino acid	Size (bp)	Annealing temp (°C)
First set: Degenerated primers				
S1	GGITGG(C/A)AIGA(C/T)TGGAT(A/C/T)(A/G)TI GC(A/C/G/T)CCCC	GW(Q/N)DWI(I/V)AP	120	58
AS1	A(A/G)(A/G)GT(C/T)TG(A/C/G/T)AC(A/G)AT(A/G)GC(A/G)TG (A/G)TT	NHAIVQTL		53
S2	GGITGG(A/T)(G/C)(I)GA(G/A)TGGAT(T/C/A)ATI(A/T)G(A/C/G/T)CC	GWSEWIISP	280	48
AS2	CAI(C/G)C(A/G)CAI(G/C)(A/C/T)I(C/T)(C/G/T)IACIA(C/T)CAT	M(V/I)V(E/R)(G/S/A)C(G/A)C		47

Table 2 Reverse transcription polymerase chain reaction (RT-PCR) oligonucleotide primer sequences and expected amplification product sizes. (continue)

Primer	Sequence of primer(5′-3′)		Size(bp)	Annealing temp (°C)
	Forward primers	Reverse primers		
Second set: Sequencing primers				
BMP2	GGAAGAACTACCAGAAACGAG	AGATGATCAGCCAGAGGAAAA	657	55
BMP3	TCTTTCTGTGGCTGGGCTGCTT	GGCGGCATCATTGGCATATA	722	58
BMP4	GATTCCTGGTAACCGAATGCT	TCTCGTGTCCAGTAGTCGTGTGAT	606	55
BMP5	AAGAGGACAAGAAGGACTAAAAATAT	GTAGAGATCCAGCATAAAGAGAGGT	303	55
BMP6	CGGGTCTCCAGTGCTTCAGATT	GCATCCACAAGCTCTTACAACC	351	55
BMP7	CGGATCAGCGTTTATCAGGTGC	GGTGGCGTTCATGTAGGAGTTCAG	552	55
BMP8	GCTCTTCATGCTGGACCTGTACC	GGGACTGCGTTTGGCTTCAT	867	55
BMP9	GGACGGTTCCTTCAGAGCAA	AATGGTACTTGAGGGTGGGC	1376	52

Table 2 Reverse transcription polymerase chain reaction (RT-PCR) oligonucleotide primer sequences and expected amplification product sizes. (continue)

Primer	Sequence of primer(5′-3′)		Size(bp)	Annealing temp (°C)
	Forward primers	Reverse primers		
Third set: Sequencing primers				
SP6	TATTTAGGTGACACTATAG	-	-	-
T7	TAATACGACTCACTATAGGG	-	-	-
Forth set: Positive control (house keeping gene)				
GAPDH	ACGCATTTGGTCGTATTGGG	TGA TTT TGG AGG GAT CTC GC	231	55

Methods

The study was composed of three parts. The first part was performed to illustrate the expression patterns of BMPs family member in primary culture derived from intramembranous and endochondral bone. The second part aimed to identify and compare the member of bone morphogenic proteins (BMPs) family expression in fresh human intramembranous and endochondral bone. The last was to identify the osteoinductive factors which may be compiled as essential factors function as bone formation in intramembranous and endochondral bone.

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

1. Culture of human osteoblastic cells

Primary cell culture of intramembranous cell (MO), primary cell culture of endochondral-1,2 bone (EO-1, EO-2) derived from trabecular section of bone specimens from mandibular (MO), iliac crest bone (EO-1), and tibia bone (EO-2), obtained form patients undergoing necessary surgery, were dissected, and rinsed several times with phosphate buffer saline (PBS).

Bone fragments were digested at 37^{o}C for 20 min with an enzymatic solution containing 0.1% collagenase (Sigma) and 0.05% trypsin in DMEM according to the method of Cohn and Wong (Cohn and Wong, 1979). The insoluble fragment were collected by centrifugation at 1,000 rpm for 5 min and were then resuspended in proliferating medium; DMEM containing 10% FBS and antibiotics (penicillin, 100 unit/ml and streptomycin, 100 μg/ml, all from Gibco, Invitrogen Co., USA). The cells of each fraction were collected and seeded in T-75 vented flasks (Becton-Dickinson Labware, NJ, USA) at 1x10^{5} cells/flask. The cells were incubated at 37^{o}C in humidified 5% CO_2 environment. On the third day of culture, the non adherent cells were removed along with culture media. The culture media was changed every 2 days and passages between days 7-10 by releasing the cells with 0.05% trypsin-EDTA exposure for 5 min. The cells were replated at 1x10^{6} cells/flask and the medium was changed to mineralized

medium (proliferating medium containing ß-Glycerophosphate; 10mM, Ascorbic acid; 50ug/ml and Dexamethasone; 500uM) for further experiment.

Human normal gingival cells obtained from the patients undergoing gingivectomy for crownlengthening were used as control. The tissues were cut in about 2 mm^2 and place in 25 cm^2 plastic culture flasks containing 15 ml of proliferation medium and replated as describe above. On days 7-10, they were replated at $1x10^6$ cells/flask and the medium was changed to mineralized medium. Both bone and gingival cells in passage 3^{rd}-5^{th} were used for further experiment.

2. Histochemical staining of alkaline phosphatase activity (ALP acitivity)

Exponentially growing primary human osteoblastic cells culture from mandible, iliac and tibia bone (MO, EO-1 and EO-2) were seeded in 35 mm culture dishes. The induction of alkaline phosphatase expression was detected at five days after seeding with use of histochemical staining assays (Sigma) according to the manufacturer's instructions. The cells were fixed with Citrate-Acetone-Formaldehyde Fixative solution at room temperature for 30 sec. After being washed with deionized water, cells were stained with use of a mixture of 0.1 mg/ml naphthol AS-BI Alkaline solution for 15 min and counterstained with neutral red solution for 2 min. Histochemical staining was recorded using of bright-field microscopy.

3. Analysis the expression patterns of BMPs family member in primary culture derived from intramembranous and endochondral bone.

3.1 Total RNA extraction

Approximate $1x10^6$ cells of culture osteoblastic cells were used for extracting total cellular RNA. The medium were removed and the cells were washed with PBS, befored releasing with 0.05% trypsin-EDTA exposure for 5 minutes. After cells detached from the flask, the culture medium containing 10% FBS was added and transfer to RNAse-free polypropylene centrifuge tube. Cells were pelleted by centrifugation at 300 g for 5 minutes. The supernatant was completely removed and cells were disrupted by addition of Buffer RLT(RNeasy, Qiagen).Then, total cellular RNA was prepared from

primary culture derived from human intramembranous and endochondral bone cell using RNeasy Kit, according the instruction manual.

A 260/280 values was used to estimate RNA concentration and purity. The extracted RNA was stored in Rnase-free water at -80° C until be used.

3.2 Reverse transcription polymerase chain reaction (RT-PCR) using specific primer for BMP2-BMP9

The primer pairs, expected product sizes, and annealing temperatures were listed in Table 2. All primer pairs were designed so that amplification of potentially contaminated genomic DNA sequences would produce mRNA PCR products that would be substantially larger than expected, because intron sequences that were excised during RNA processing would be included in genomic DNA (Wordinger et al., 2002).

The standard procedure for RT-PCR reaction was performed in a 50 μl mixture containing 0.2 mM dNTPs; 1X RT-PCR buffer (2.5 mM $MgCl_2$; Tris-HCl; KCl; $(NH_4)_2SO_4$; dithiothreitol (DTT); pH 8.7); 500 nM each of the primers; 50 ng of total RNA or water blank (no RNA) and 2 μl of Enzyme mix(1 mM DTT; 0.1 mM EDTA; 0.5% (v/v) Nonidet® P-40; 0.5% (v/v) Tween® 20; 50% glycerol (v/v), stabilizer; pH 9.0). The reaction was performed in an automated DNA thermal cycler (Hybaid, UK) according to conditions show in Table 3. The GAPDH PCR primers were used as the positive control. The standard cycling PCR program comprised 1) reverse transcription reaction 50°C for 30 min, 2) initial PCR activation step at 95°C for 15 min, 3) PCR amplification cycled between 94°, 55°, 72°C (30 seconds each) for 35 rounds, and 4) final extension at 72°C for 10 min.

Table 3 RT- PCR parameters for automated DNA thermal cycler

Segment	Step	Temperature (°C)	Time (minutes)	Number of cycles
1	Reverse transcription	50	30	1
2	Initial PCR activation	95	15	1
4	Denaturing Annealing Extension	94 55 72	1 1 1	26
5	Final extension	72	10	1
6	Holding	4	∞	

4. Agarose gel electrophoresis

Gel electrophoresis was conducted for determining the size of the product of RT-PCR and the DNA insertion. A 1.0% (w/v) agarose solution was prepared using 1X TAE buffer (Appendix), melted, and poured on a plastic tray. A plastic comb was then placed in the gel. After the agarose gel was completely set (30-45 min at room temperature), the comb was removed and the gel was installed on the platform in the electrophoresis tank containing 1X TAE buffer. The one-tenth of reaction product of the RT-PCR or the DNA samples were mixed with 30% (v/v) gel loading buffer (Appendix) and slowly loaded into the slots of the submerged gel using an automatic micropipette. Electrophoresis was carried out at a constant 100 V for 25-30 min, or until the running dyes had migrated the necessary distance. Next, the gel was stained with 2.5 μg/ml of ethidium bromide (EtBr) solution for 5 min and destained with water for 10-15 min. The DNA patterns were observed using a UV light box (Gel Doc model 1000, BIO RAD, USA) and the photograph was taken and printed out on the paper.

5. Purify the DNA fragments from the gel

The PCR-amplification fragments were purified by agarose gel electrophoresis. 40 μl of cDNA product was electrophoresed on 1.5% agarose gels run at 100 V simultaneously with the DNA marker and 5 μl of cDNA product as reference. After

25-30 min, the two reference lanes were excised and stained with ethidium bromide and were used as the landmark for corresponding bands in the unstained part.

The gel containing interested band was excite and was purifed using Concert Rapid Gel Extraction System (Gibco) or Wizard® PCR Preps DNA Purification System (Promega) according the instruction manual. The fragments of cDNA were confirmed using 1.5% agarose gel electrophoresis and ethidium bromide staining.

6. Ligation of cDNA fragment to pGEM- T Easy

The purified DNA fragment of PCR product was ligated by T4 DNA ligase into plasmid pGEM-T-Easy vector system (Promege) as described by Technical manual. The ligation mixture was performed in a final volume of 10 μl. Set up a ligation reaction using 1: 1 molar ratio of insert cDNA to the pGEM T-Easy vector, 1 μl of T4 DNA ligase (3 Weiss units/μl),5 μl of 2X ligation buffer (50 mM Tris-HCl; pH7.6; 10 mM $MgCl_2$; 1 mM ATP; 1 mM DTT) and deionized water to a final volume of 10 μl. The reaction will be incubated overnight at 4°C to produce the maximum number of transformants.

7. Transformation of inserted-DNA to competent cells

7.1 E. coli Top 10F' competent cells preparation (Hanahan, 1983).

The *E. coli* Top10F' were streaked in Luria-Bertani (LB) medium with tetracycline 10 μg/μl and were incubated overnight at 37°C. The single colony of *E. coli* was picked to 25 ml of LB broth with tetracycline 10 μg/μl and incubated overnight under gently shaking at 37°C. The bacteria was inoculated to 225 ml of LB broth with tetracycline 10 μg/μl and incubated under gently shaking at 37°C until the OD_{600} reached 0.5-0.6. The cells pellet was harvested by centrifugation at 4,500X for 5 min at 4°C and washed with 40 ml of freeze-cold 0.1M $CaCl_2$. The pellet was resuspended in 20ml freeze-cold 0.1M $CaCl_2$, incubated the resuspension mixture on ice for 30 minutes. The pellet was collected by centrifugation at 4,500X g at 4°C for 10 min. The pellets were resuspended in 8 ml freeze-cold 0.1M $CaCl_2$ with 15% glycerol. The cell suspension was aliquoted in a volume of 200 μl per tube and kept frozen at -70°C until use.

7.2 Transformation of recombinant DNA to competent cells.

A volume of 200 μl of E. coli competent cells was mixed gently with 5 μl of plasmid DNA. The mixture was left on ice for 30 minutes to give higher transformation frequency, then transform the recombinant DNA to the competent cells by heat shock; placed at 42°C for 90 seconds, and finally put on ice for an additional 5 minutes. Transformed cells were mixed with 800 μl of LB broth prewarm at 37°C, and incubated at 37°C for 16-18 hours. The confirmation of inserted DNA in transformants was performed by plasmid screening.

7.3 Screening of transformants.

A single bacterial colony was inoculated into 3 ml of LB broth with 80 μg/ml of ampicillin in a loosely capped 15 ml tube and incubated overnight at 37°C with gently shaking. The bacteria were transferred to 1.5-ml microcentrifuge tube and were collected by centrifugation at 10,000X g for 1 minute at room temperature. The supernatant was discard and the bacterial pellet was resuspended in 100 μl of STET solution (8% glucose; 5% (v/v) Tritron X-100; 50 mM EDTA; pH 8.0; 50 mM Tris-HCl; pH 8.0). The suspension was mixed with vortex and incubated at room temperature for 10 minutes. The mixture was incubated in boiling water for 40 second before immediately placed in ice box for 10 minutes, and centrifuged at 12,000X g for 10 minutes at room temperature. The coalescence was removed and the supernatant was mixed with the equal volume of cold isopropanol. Then the mixture was incubated at -80°C for 60 minutes. After centrifuged at 12,000X g for 10 minutes at 4°C, the supernatant was discarded. The pellet was dried at the room temperature for 1-2 hours by place in the reversed position on a paper towel to allow the remnant fluid to drain off. The pellet was then resuspended in 30 μl of sterile distilled water and was electrophoresed on 1.5% agarose gels with self ligation product as the marker run at 100V for 25-30 minutes. The screenings of inserted-DNA fragments with ethidium bromide staining was determined. The cut-check clone was performed with the ECO*R*I restriction enzyme to confirmation of the size of inserted-DNA and was electrophoresed on 1.5% agarose gels with 100 bp marker run at 100V for 25-30 minutes.

8. Purify the plasmid containing inserted-DNA

All of the plasmids containing expected size DNA fragment were purified using QIAprep Miniprep System (Qiagen) as described in technical manual. The clone were selected and grown in 6 ml LB broth with 80 μg/ml of ampicillin under gently shaking at 37°C overnight. The pellet was collected by centrifuging 10,000 X g for 1 minute.

The confirmation of DNA fragment existing was performed by cut-check using *EcoR*I restriction enzyme (Promega) and was electrophoresed on 1.5% agarose gels run at 100V for 25-30 min. The detection of DNA fragments with ethidium bromide staining was determined using GelDoc 1000(Bio-Rad)

9. DNA Sequencing

The inserts presented in the clones obtained in this study were sequenced using ABI PRISM 377 DNA sequencer (model 377, Perkin-Elmer Applied Biosystems, USA) based on PCR amplification of DNA in the presence of the fluorescent-labeles terminator (Prober et al., 1987; Smith et al., 1986). The protocol has been optimized for an automated DNA Thermal Cycler (Perkin-Elmer) model 2400 and completed in 25 cycles in 3 hours. Each reaction was carried out in a labeled 0.2 ml microcentrifuge tube containing 8 μl of Terminator Premix, 0.5 μg of double-stranded DNA, 3.2 pmol of primer and double-distilled water to bring the final volume to 20 μl. The tube was placed in the thermal cycler preheated to 96°C. After that the thermal cycler proceeded as follows (Table 4).

The extension products were purified by ethanol precipitation. The entire contents of the reaction tube was transferred to the 1.5 ml microcentrifuge tube containing 2.0 μl of 3 M sodium acetate, pH 4.6 and 50 μl of 95% ethanol, vortexed and placed on ice for 10 minutes. the mixture was centrifuged at maximum speed for 15-30 min. The ethanol solution was removed as completely as possible, and the pellet was washed by adding 250 μl of 70% isopropanol. The pellet was then dried in a vacuum centrifuge and resuspended in 4 μl of loading dye (Appendix). The samples were then loaded on a 6% polyacrylamide gel.

Table 4 PCR parameters for sequencing

Segment	Step	Temperature (°C)	Time	Number of cycles
1	Denaturing	96	5 min	1
2	Denaturing	96	10 sec	25
	Annealing	50	5 sec	
	Extension	60	4 min	
3	Holding	4	∞	1

10. Analysis of nucleotide sequencing

The inserts were sequenced on both strands. To determine specificity, all sequences were compared with the Genbank using BLAST searches of National Center for Biotechnology Information (NCBI) GenBank/EBI databases.

11. Verify the specificity of the RT-PCR product

To confirm the specificity of the RT-PCT experiment, the PCR products were purified and were cloned using the pGEM T Easy System (Promega, Madison, WI). Plasmid DNA was purified with the QIAprep Spin Miniprep (Qiagen, Valencia, CA) as describe above. Sequencing was performed by dideoxy DNA sequencing using ABI PRISM 377 DNA sequencer (version 3.2). Then, the acquired-sequences were determined the specificity by sequencing and were compared with the Genbank using BLAST searches of National Center for Biotechnology Information (NCBI) GenBank/EBI databases.

12. Semi-quantitative RT-PCR product

Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a GelDoc1000 (BioRad) and quantification of the bands was performed by Scion Image Analysis (Scion Corporation). Band intensity was expressed as mean pixels per area. The ratio between the sample RNA to be determined and GAPDH was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization to GAPDH.

13.Statistic Analysis

The expressions of BMP2 to BMP9, as measured by semi-quantitative RT-PCR from these two different bone sources, were compared using the Mann-Whitney Test. Differences level at $p<0.05$ was considered significant.

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

1. Tissue sample

Fresh human intramembranous bone (mandible; MAND) and human endochondral bone (iliac bone; EC) weighing approximately 20-30 mg per tissue were prepared from 11 healthy adult patients (ages 21 to 42 years), who had orthognathic surgery (bilateral sagital split osteotomy, and/or segmental osteotomy), or iliac crest bone graft for reconstruction oral and maxillofacial defects, performed in the Dental Hospital, Faculty of Dentistry, Prince of Songkla University. Informed consent, according to guidelines from the Faculty of Dentistry, Prince of Songkla University Committee on the Use of Human Subjects in Research, was obtained from the bone donor. The attached soft tissue were removed from the bone samples before being rinsed with cold RNAase-free PBS and flash-frozen with liquid nitrogen and stored at $-80^{\circ}C$ until be extracted.

2. Total RNA extraction

Bone samples from $-80^{\circ}C$ were weight prior to process for RNA extraction. Bone sample (20-30mg per reaction) was crashed with hammer in custom-made plastic bag with liquid nitrogen before finely minced and homogenized with a Polytron (top speed, 2x30 sec) in 600 μl of RLT buffer (RNeasy Kit; Qiagen Inc., Valencia, CA). Total RNA from human intramembranous or endochondral bone was conducted with RNeasy Kit, according the instruction manual. A 260/280 values was used to estimate RNA concentration and purity. The extracted RNA was stored in Rnase-free water at -80° C until be used.

3. RT-PCR Using Degenerate Oligonucleotide primers.

For effective amplification, the appropriate size of the primer was judged to be 20-30 bases. In this study, two pair of degenerate oligonucleotide primers were synthesized corresponding to highly conserved motifs in the carboxyl-terminal region of the BMPs(GW(Q/N)DWI(I/V)AP; 5'-GGITGG(C/A)AIGA(C/T)TGGAT(A/C/T)(A/G)TIGC(A/C/G/T)CCCC-3' and GWSEWIISP; 5'-GGITGG(A/T)(G/C)(I)GA(G/A)TGGAT (T/C/A)ATI(A/T)G (A/C/G/T)CC-3') (Table 2; S1, AS1; S2, AS2). And oligonucleotide primers of GAPDH were used as internal control.

The standard procedure for RT-PCR reaction was performed in a 50 μl mixture containing 0.2 mM dNTPs; 1X RT-PCR buffer (2.5 mM $MgCl_2$; Tris-HCl; KCl; $(NH_4)_2SO_4$; dithiothreitol (DTT); pH 8.7); 500 nM each of the primers; 50 ng of total RNA or water blank (no RNA) and 2 μl of Enzyme mix(1 mM DTT; 0.1 mM EDTA; 0.5% (v/v) Nonidet® P-40; 0.5% (v/v) Tween® 20; 50% glycerol (v/v), stabilizer; pH 9.0). The reaction was performed in an automated DNA thermal cycler (Hybaid, UK) according to conditions show in Table 5.

Table 5 RT- PCR parameters for automated DNA thermal cycler

Segment	Step	Temperature (°C)	Time (minutes)	Number of cycles
1	Reverse transcription	50°C	30	1
2	Initial PCR activation	95°C	15	1
3	Denaturing Annealing Extension	94°C 44°C 72°C	1 1 1	6
4	Denaturing Annealing Extension	94°C 55°C 72°C	1 1 1	26
5	Final extension	72°C	10	1
6	Holding	4°C	∞	

4. Analysis of nucleotide sequencing

The inserts were sequenced on both strands. To determine specificity, all sequences were compared with the Genbank using BLAST searches of National Center for Biotechnology Information (NCBI) GenBank/EBI databases.

5. Analysis the expression of the Bone morphogenetic protein (BMPs) mRNA in fresh human intramembranous and endochondral bone

The primer pairs, expected product sizes, and annealing temperatures were listed in Table 2. All primer pairs were designed so that amplification of potentially contaminated genomic DNA sequences would produce mRNA PCR products that would be substantially larger than expected, because intron sequences that were excised during RNA processing would be included in genomic DNA (Wordinger et al., 2002).

Total cellular RNA was prepared from human intramembranous and endochondral bone using RNeasy Kit (Qiagen Inc., Valencia, CA), and the first strand cDNA was synthesized using the One-step RT-PCR kit (Qiagen Inc.) from 5 ug of total RNA as template. Primers for the specific BMPs were designed using Vector NTI Suite 8

software (InforMax, Inc) or were designed from published sequences. The regions amplified were all in the mature domain or the carboxy terminal region of the BMPs genes. The GAPDH PCR primers with an annealing temperature of 55° C yielded a PCR product of 231 bp were used as the positive control. The standard cycling PCR program comprised 1) reverse transcription reaction 50°C for 30 min, 2) initial PCR activation step at 95°C for 15 min, 3) PCR amplification cycled between 94°, 55°, 72°C (30 seconds each) for 35 rounds, and 4) final extension at 72°C for 10 min.

The experiments were performed from six different bone subjects to minimize inter-individual variation. One pair of bone sample, mandibular and iliac bone, was achieved from the same subject to minimize intra-individual variation.

6. Verify the specificity of the RT-PCR product.

To confirm the specificity of the RT-PCT experiment, the PCR products were purified and were cloned using the pGEM T Easy System (Promega, Madison, WI). Plasmid DNA was purified with the QIAprep Spin Miniprep (Qiagen, Valencia, CA) as describe in the material and methods in Part I (illustrate the expression patterns of BMPs family member in primary culture derived from intramembranous and endochondral bone). Sequencing was performed by dideoxy DNA sequencing using ABI PRISM 377 DNA sequencer (version 3.2). To determine specificity, all sequences were compared with the Genbank using BLAST searches of National Center for Biotechnology Information (NCBI) GenBank/EBI databases.

7. Statistic Analysis

The expressions of BMP2 to BMP9, as measured by semi-quantitative RT-PCR from these two different bone sources, were compared using the Mann-Whitney Test. Differences level at $p<0.05$ was considered significant.

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

1. Tissue preparation and Extraction

Human intramembranous bone (mandible; MAND) and human endochondral bone (iliac bone; EC) were prepared from healthy adult patients (ages 21 to 42 years), who had torectomy, surgical removal of 3rd molar, orthognathic surgery (genioplasty), or iliac crest bone graft for reconstruction oral and maxillofacial defects, performed in the Dental Hospital, Faculty of Dentistry, Prince of Songkla University. Informed consent, according to guidelines from the Faculty of Dentistry, Prince of Songkla University Committee on the Use of Human Subjects in Research, was obtained from the bone donor. The bones were removed the attached soft tissue and periosteum and were cut in to small pieces (approximate 5x5 mm.) using bone cutter (Ronger forceps). The bone samples were washed extensively in a cold (4°C) sterile PBS containing protease inhibitors (0.5 M NaCl, 0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4, 0.05 M 6-aminohexanoic acid, 0.005 M bezamidine HCl, 0.001 M phenylmethanesulfonyl fluoride (PMSF), pH 7.2) and stored at -80°C.

2. Sequential extraction

The bone samples (approximate 3 g. of frozen tissue preparation/ liter of extracting solution) were first extracted at 4°C for 72 hours in 4.0 M guanidine HCl, 0.05 M Tris, and protease inhibitors (0.1 M 6-aminohexanoic acid, 0.005 M bezamidine HCL, 0.001 M phenylmethanesulfonyl fluoride), pH 7.4 (Extract 1). This initial extract was centrifuged (3000 rpm, 20 min, 4°C) and the residue was extracted further (Extract 2) for 72 hours at 4°C in 1000 ml of guanidine HCl/EDTA (a solution identical with Extract 1 but containing 0.5 M ethylene diamine tetraacetic acid; EDTA). An insoluble residue was removed by centrifugation at 12,000 rpm for 40 min (4°C).

Both the Extract 1 and Extract 2 were concentrated at 4°C by ultrafitration (Amicon YM-10 filters, M_r =10,000 cutoff) and then dialyzed against distilled water at

4°C with spectrapor I acetylated dialysis tubing (M_r =6,000 to 8,000 cutoff) and freeze-dried.

3. The protein determination

The protein was measured according to the method of Lowry (Waterborg and Matthews, 1996). A 10% (W/V) freeze-dried samples solution was prepared using sterile distilled water. To 0.1 ml of sample or standard, 0.1 ml of 2 N NaOH was added and place in boiling water bath (100°C) for 10 min. The mixture was cooled to room temperature before adding 1 ml of freshly mixed complex-forming reagent (appendix). Folin reagent (0.1 ml) was added using vortex mixer and placed at room temperature for 30 min. Protein amount was estimated by UV absorption at 750 nm($OD_{750\ nm}$) using 4 mg/ml bovine serum albumin as the standard to prepare a calibration curve.

4. SDS-Polyacrylamide Gel Electrophoresis and silver staining

The molecular mass of proteins extracted from bone tissues were determined by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli(Laemmli, 1970) (Appendix). The gel solution was prepared as shown in Table 6. Freeze-dried samples were dissolved in loading buffer containing 1% SDS and 0.5%β-mercaptoethanol. 10 μl of the mixture was incubated for 30 min at 37°C, and then loaded into polyacrylamide gel made of 4.5% stacking gel and 15% separating gel and subjected to electrophoresis at constant current of 15 mA/gel using vertical electrophoresis (Bio-Rad). The electrophoresis was carried out in the decending direction in a Tris-glycine buffer (25 mM Tris-HCl; pH 6.8; 192 mM glycine and 0.1% (w/v) SDS). Prestained low range molecular weight protein standards were added to every gel.

After electrophoresis, gels were stained with (a) 0.05% (w/v) Coomassie Brilliant Blue R-250 in 15% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acitic acid as described by Weber and Osborn (Weber and Osborn, 1969) (Appendix); or (b) silver nitrate staining (Appendix). After the background was cleared, the gel was rinsed with copious distilled water and air-dried between 2 sheets of cellophane paper using the technique described by Juang (Juang et al., 1984).

Table 6 Preparation of SDS- Polyacrylamide Gel

Solution	Separating Gel (15%)	Stacking Gel (4.5%)
30% stock acrylamide solution	7.5 ml	750 μl
Stock separating gel buffer (0.1% SDS, 0.375 M Tris-HCl pH 8.8)	3.75 ml	-
Stock stacking gel buffer(0.1% SDS, 0.125 M Tris- HCl, pH 6.8)	-	1.25 ml
Distilled water	3.75 ml	3 ml
10% Ammonium persulfate	15 μl	50 μl
TEMED	15 μl	5 μl

5. Heparin affinity column chromatography

Extract 1 and 2 from sequential extraction was concentrated and exchanged with 6 M urea to reduce the salt concentration. The solution was loaded on 0.5-liter heparin-affinity column connecting to the BioLogic workstation with fraction collector (BIO-RAD, USA). Thereafter, the column was washed with 5 bed volumes of 6 M urea, Tris-HCl, pH7.4 with 0.15 M NaCl and then eluted with 2 volumes of 1.5 M NaCl in the same buffer.