APPENDIX

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

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

1. Luria Bertani(LB) broth 1000 ml

Yeast extract	10	g
Bacto-Tryptone	10	g
Sodium Chloride	5	g
Distilled water	1000	ml

2. Luria Bertani(LB) agar (1.8%) 1000ml

Yeast extract	10	g
Bacto-Tryptone	10	g
Bacto-agar	18	g
Sodium Chloride	5	g
Distilled water	1000	ml

3. RNase A 10 mg/ml

RNase A	100	mg
10mM Tris HCl pH7.5 and 15 mM NaCl	10	ml

Boil at100°C for 15 minutes, leave to cold down at RT

Keep at -20°C

4. Antibiotic preparation

4.1 Ampicillin (100mg/ml)

Ampicillin sodium salt	100	mg
Nuclease-free water	1	ml

Keep at -20°C

4.2 Tetracyclin(10mg/ml)

Tetracyclin	10	mg
Nuclease-free water:ethanol (1:1)	1	ml
Keep at -20°C avoid from light		

4.3 10X T4 ligase buffer (300mM Tris-HCl, pH 7.8)

100mM $MgCl_2$

100mM DTT

10mM ATP

5. Electrophoresis buffer

5.1 Tris-Borate(10X TBE)

Tris base	108	g
Boric acid	55	g
Sterile distilled water	800	ml
0.5M EDTA, pH 8.0	40	ml
Sterile distilled water to	1000	ml

5.2 Tris-Acetate(50X TAE)

Tris base	242	g
Sterile distilled water	800	ml
Glacial acetic acid	57.1	ml
0.5M EDTA, pH 8.0	100	ml
Sterile distilled water to	1000	ml

6. ß-Glycerophosphate 1 M, 50 ml

ß-Glycerophosphate	10.8	g
Sterile deionized water	50	ml
Sterile filter		

7. Ascorbic acid 5 mg/ml, 50 ml

Ascorbic acid	0.25	g
Sterile deionized water	50	ml
Aliquot 1000 μl at -20°C		

8. Dexamethasone 500 μM (200 ng/μl)

Dexamethasone	1	mg
Absolute ethanol	5	ml

9. Fetal calf serum (FCS); Inactivate

Stand at room temperature until completely thaw

Place in water bath at 65 °C for 30 min

Keep at -20 °C

10.EDTA 0.5 M pH 8.0, 50 ml

EDTA	186.12 g (o.5 mol)
Sterile distilled water	30 ml

Under hood with continue stirring. Adjust pH to 8.0 using 10 N NaOH

Sterile distilled water to 50 ml

11.Stock culture medium

DMEM	1	pack
Sterile distilled water	800	ml

Adjust pH to 7.1-7.2 using 1 N HCl with continue stirring

Sterile distilled water to 1000 ml

Under hood filtration through sterile membrane filter.

Keep at 4 °C

***12.*Culture medium; proliferating medium 100 ml**

Stock DMEM	90	ml
Fetal bovine serum	10	ml
Penicillin-streptomycin (10,000 μg/ml)	2	ml
Fungizone (250 μg/ml)	1	ml

Under hood filtration through sterile membrane filter

Keep at 4°C

***13.*Culture medium; Mineralized medium 100 ml**

Proliferation medium	100	ml

ß-Glycerophosphate 1M	100	µl
Ascorbic Acid 5 mg/ml	100	µl
Dexamethasone 500 µM	2	µl

14.PBS 1000 ml

Na_2HPO_4	1.42	g
$NaH_2Po_42H_2O$	1.7	g
NaCl	8.76	g

Sterile distilled water to 1000 ml

Adjust pH to 7.4 and sterilization by autoclave

15.Alkaline phosphatase staining for histological test

1. Rinse cell suspension once with DMEM and placed in DMEM containging 10% FCS
2. Drawn off the medium and rinsed thoroughly with sterile PBS.
3. Measure 45 ml deionized water and adjust temperature to 18-26 °C
4. Prepare diazonium nitrate solution
5. Add 1 ml Sodium nitrite solution to 1 ml of FBB-Alkaline solution
6. Allow to stand for 2 min
7. Add solution prepared in step 2 to deionized water from step 1
8. Add 1 ml Nephthol AS-BI alkaline solution to diluted diazodium salt solution (step 3). Mix thoroughly and pour into a container.
9. Bring Citrate-Acetone-Formaldehyde fixative solution to room temperature (18-26 °C). Fix the culture plates in fixative solution for 30 sec.
10. Add culture plates to alkaline-dye mixture (step 4) and incubated at 18-25°C for 15 min. Protect immersed culture plates from direct light. Discard alkaline-dye mixture after used.
11. After 15 min incubated, remove culture plates from container and rinse for 2 min in deionized water. Do not allow culture plates to dry.
12. Counterstain for 2 min with Neutral Red solution buffer
13. Rinsed culture plates thoroughly in tab water and air dry. Evaluated microscopy

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

1. Protein determination: Lowry method

Materials

1. Complex-forming reagent: Prepare immediately before use by mixing the following three stock solution A, B and C in proportion 100:1:1 (v:v:v), respectively.

   Solution A: 2% (w/v) Na_2CO_3 in distilled water

   Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water

   Solution C: 2% (w/v) sodium potassium tartrate in distilled water
2. 2 N NaOH
3. Folin reagent: Use at 1 N concentration
4. Standard: Use a stock solution of standard protein (bovine serum albumin fraction V) containing 4 mg/mL protein in distilled water stored frozen at -20°C. Prepare standards by diluting the stock solution with distilled water as follows:

Stock solution (μl)	0	.25	.50	.25	2.5	5.0	2.5	25	50
Water (μl)	500	99	98	94	88	75	38	75	50
Protein conc. (μg/mL)	0	10	20	50	100	200	500	1000	2000

Method

1. To 0.1 mL of sample or standard, add 0.1 mL of 2 N NaOH. Hydrolyze at 100°C for 10 min in a boiling water bath.
2. Cool the hydrolyzate to room temperature and add 1 mL of freshly mixed complexed-forming reagent. Let the solution stand at room temperature for 10 min

3. Add 0.1 mL of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30-60 min.
4. Read the absorbance at 750 mm
5. Plot a standard curve of absorbance as a fuction of initial protein concentration and use it to determine the unknown protein concentrations.

2. SDS-PAGE

2.1 Stock acrylamide solution

Acrylamide	73	g
Bis-acrylamide	2	g
Sterile distilled water	200	ml

Sterring until completely dissolved

Filter through Whatman No. 1 filter paper and store at 4°C

2.2 Stock separating gel buffer(4X): 250 ml

(0.1% SDS, 0.375 M Tris-HCl: pH 8.8)

SDS	1.0	g
Tris-HCl	45.5	g
Sterile distilled water	200	ml

Adjust pH to 8.8 using 1 N HCl with continue stirring

Sterile distilled water to 250 ml. Keep at 4°C, avoid from light.

2.3 Stock ammonium persulfate (10 g%): 10 ml

Ammonium persulfate	1.0	g
Sterile distilled water	10	ml

Keep at 4°C, avoid from light

2.4 Stock stacking gel buffer (6.6X): 250 ml

(0.1% SDS, 0.125 Tris-HCl : pH 6.8)

SDS	1.0	g
Tris-HCl	15.1	g
Sterile distilled water	200	ml

Adjust pH to 6.8 using 1 N HCl with continue stirring

Sterile distilled water to 250 ml. Keep at 4°C, avoid from light.

2.5 Working electrophoresis (Running buffer): 1000 ml

(0.192 M Glycine, 0.025 M Tris-HCl, 0.1w/v SDS)

Glycine	14.4	g
Tris-HCl	3.0	g
SDS	1.0	g

2.6 Bromophenol Blue (0.1%w/v): 10 ml

Bromophenol blue	10	mg
Sterile distilled water	10	ml

2.7 Stock sample solvent (2X)

SDS	0.92	g
ß-mercaptoethanol	2	ml
Glycerol	3.2	ml
Tris-HCl	0.3	g
0.1%bromophenol blue	2	ml
Sterile distilled water	10	ml

Adjust pH to 6.8 using 1N HCl

Sterile distilled water to 20 ml. Keep at 4°C

Sample : Solvent = 1:1(v/v)

3. Protein staining: Coomassie Brilliant Blue

0.1 Coomassie Brilliant Blue R250	0.25	g
Methanol	125	ml
Glacial acetic acid	25	ml
Sterile distilled water	100	ml

Filter through Whatman No. 1 filter paper.

4. Destaining solution for Coomassie Brilliant Blue

Methanol	100	ml
Glacial acetic acid	100	ml
Sterile distilled water	800	ml

Destaining for 24 hours with gentle agitation

5. Protein staining: Silver nitrate staining

5.1 Destaining I (40% methanol, 7% acetic acid)

Methanol	400	ml
Acetic acid	70	ml

Sterile distilled water to 1000 ml.

5.2 Destaining II (7% acetic acid, 5% methanol)

Methanol	500	ml
Acetic acid	700	ml

Sterile distilled water to 10 L

5.3 Cross-linking solution (10% Glutaraldehyde)

25% glutaraldehyde	40	ml

Sterile distilled water to 100 ml.

5.4 Dithiothreitol (DTT) solution (5µg/ml)

DTT	0.0005	g

Sterile distilled water to 100 ml.

5.5 Silver nitrate solution (0.1% w/v silver nitrate)

Silver nitrate	1	g

Sterile distilled water to 1000 ml.

5.6 Sodium carbonate (3% w/v)

Sodium carbonate	60	g

Sterile distilled water to 2000 ml. Store in glass container.

5.7 Developing solution (3% sodium carbonate, 0.019% formaldehyde)

3% Sodium carbonate	200	ml
37% Formaldehyde	100	µl

Freshly prepare before use.

5.8 Stop solution (2.3 M Sodium citrate)

Sodium citrate dehydrate	67.64	g

Sterile distilled water to final volume of 100 ml.

6. Silver stain protocol

1. Place gel in Destain I (100 ml) for 30 minutes to overnight with gentle shaking
2. Replace with 100 ml of Destain II, shake slowly for 30 minute
3. Discard Destain II and replace with 100 ml cross-linking solution (10% glutaraldehyde), shake for 30 minutes. For small peptide increase the incubation period to overninght.
4. Pour off the glutaraldehyde solution and wash gel with several changes of water over 2 hours. Alternatively, the gel can be removed from the glutaraldehyde and place into 2 liters of water for overnight storage. The next morning, wash 30 minutes in fresh water. Discard the water. Failure to remove completely glutaraldehyde will induce high background.
5. Add DTT solution and incubate with slow shaking for 30 minutes.
6. Remove DTT solution. Drain well, but do not rinse the gel. Add 100 ml of silver nitrate solution. Shake slowly for 30 minutes.
7. Place the staining tray under running deionized water, swirl for a few seconds, and then dump the rinse water.
8. Add 50 ml of Developing solution, swirl brifly, and then discard the solution. Repeat once.
9. Add 100 ml of Developing solution, and shake slowly. Stain occurs slowly at first but then rapidly progresses.
10. when the bands look slightly lighter than the desired staining level, remove developer, rinse quickly with water, and add Destain II as the stop the solution. Alternatively, 5 ml of citric acid can be added directly to the developer to stop the development. In case, the development does not stop immediately but continues for 5 minutes after add stop solution.
11. Wash the gel several times in Destain II and finally with water. Store in water.

7. Heparin affinity column buffer for 250 ml

Urea		90.09 mg
Tris-HCl		1.52 mg
NaCl	0.15 M	2.2 mg
	0.5 M	7.3 mg
	1.5 M	22.0 mg
6-aminohexanoic acid		1.65 mg
Benzamidine HCl		0.045 mg
PMSF		0.2 mg

Sterile distilled water to 250 ml

(6 M urea, 50 mM Tris-HCl, 0.15-1.5 M NaCl with protease inhibitor; 50 mM 6-aminohexanoic acid, 5 mM Benzamidine HCl, 1 mM PMSF)