MATERIALS AND METHODS

3.1 Chemicals used

All the chemicals used were of analytical or molecular biology grade obtained from commercial sources.

Dulbecco's Modified Eagle Media: Ham's media (DMEM:Ham's F12) medium, Alizarin Red S stain, 1-bromo,2-chloro,3-propane, Tri-reagent, 5-Aza-2′-deoxycytidine, Thapsigargin, and MH132 were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Charcoal-dextran-treated fetal bovine serum (FBS) trypsin/EDTA solution, were purchased from Invitrogen, CA, USA.

rhPTH was provided in kind by Ranbaxy, India, and ZOL was kindly provided by Novartis Pharma AG.

Acrylamide, Ammonium persulphate (APS), Bis-acrylamide, Comassie brilliant blue G-250, Comassie brilliant blue R-250, molecular biology grade, Big-dye terminator cycle sequencing kit were purchased from Biorad, Hercules, CA, USA.

Agar powder, bacteriological Agarose, Ammonium hydroxide, AMP buffer (2-amino,2-methyl,1-propanol), Antibiotics solution, Ethidium bromide (EtBr) solution (10mg/ ml), Ethylenediaminetetra-acetic acid (EDTA) disodium salt, dihydrate Glycine, Magnesium chloride hexahydrate, Dimethyl suphoxide (DMSO), Phenol were purchased from Himedia, Mumbai, India.

Bovine serum albumin (BSA), Bradford reagent, Bromocresol purple, Formalin, Disodium paranitrophenyl phosphate, Methanol, para-nitrophenyl-phosphate, Paranitrophenol, Phenylmethylsulphonyl fluoride (PMSF), Proteinase K, Sodium carbonate, Sodium dodecyl sulphate, Transforming growth factor beta (TGF-β) 10ng/ml, dNTP, $Mgcl_2$, Taq polymerase PCR , DNase, Restriction enzyme EcoRI, HinDII and SalI were purchased from Fermentas, Life Science, MA, USA.

Trichloroaceiticacid, Trisbase, TrisHCl, AR, Triton-X100, Trypsin, Trypsin-EDTA solution, Ethanol, Glycerol, Real time PCR reagents were purchased from Thermo Fisher Scientific, MA, USA.

Kits: cDNA kit (Fermentas, Life Sciences, USA), BCA for protein estimation from Pierce (Thermo Fisher Scientific, MA, USA), Site direct mutagenesis (Agilent, CA, USA), Nucview 488 caspase-3 assay kit (Biotium, Hayward, CA), PCR purification kit from Qiagen (Hilden, Germany).

Table 2a. Oligonucleotide primers of the COL1A1 gene

S No.		Primer sequence	Length (bp)	Exon	Tm
1	1-F:	5 GGAGCAGACGGGAGTTTC 3	349	Exon 1	55
	1-R:	5 TCATCATCTCCCTTCCATTCC 3			
2	2-F:	5 TTGAGGAAAGAAGAACGGG 3	1000	Exon 2-5	57
	2-R:	5 AAACTGTGAAGGGTATGTGAGA 3			
3	3-F:	5 GCCGTCTTCTGCCTTTCA 3	524	Exon 6-7	55
	3-R:	5 GTCCCTGTCAACCTTCTCCAAT 3			
4	4-F:	5 GGAAGACTGGGATGAGGG 3	460	Exon 8-9	57
	4-R:	5 CAAATGTGGTGGAGTGGA 3			
5	5-F:	5 GAGGCACTCAGATTTCACC 3	492	Exon 10-11	57
	5-R:	5 CTTTTGGGGAAGAGGTTG 3			
6	6-F:	5 CTTTTGGGGAAGAGGTTG 3	784	Exon 12-15	57
	6-R:	5 AACATAACCTGCTCCCATT 3			
7	7-F:	5 CCATCTCTTCCTTCTCGC 3	522	Exon 16-17	57
	7-R:	5 GAGTGTCAGCAACAGGCA 3			
8	8-F:	5 CCTTTGCCACTTTCTAACCT 3	602	Exon 18-20	57
	8-R:	5 GGACTCCCCCAGAAGACTA 3			
9	9-F:	5 CAGAAAGGAAGAGGAGCC 3	704	Exon 21-23	57
	9-R:	5 GAACGCCTCATCCCAGAC 3			
10	10-F:	5 CTGGGATGAGGCGTTCTG 3	515	Exon 24-25	57
	10-R:	5 CGCCGAGAAGTCTTTCATTT 3			
11	11-F:	5 GCCCTGGCTTCTCACTTC 3	738	Exon 26-29	57

S No.		Primer sequence	Length (bp)	Exon	Tm
	11-R:	5 CGGCGTCTAACCTCAATC 3			
12	12-F:	5 CCAGACCCCAGGAGGAAG 3	799	Exon 30-32	60
	12-R:	5 GAGAGATTCAAAGCAGGCAGA 3			
13	13-F:	5 GAAACCCAGACACAAGCAGA 3	750	Exon 33-36	53
	13-R:	5 TTGTCCTCATTCCGTCCC 3			
14	14-F:	5 GTTTCCCACCCAAGCATCT 3	441	Exon 37-38	54
	14-R:	5 AGCCAACTCATCCGACCC 3			
15	15-F:	5 GCTGGGTCGGATGAGTTG 3	505	Exon 39-40	57
	15-R:	5 AAACAGGGGTGAGGTGCC 3			
16	16-F:	5 TGCTGATGGTCCTGCTGTA 3	561	Exon 41-42	57
	16-R:	5 GGGAAGAGGGCTTAGGCA 3			
17	17-F:	5 GCCTTGCCTAAGCCCTCT 3	412	Exon 43-44	58
	17-R:	5 AAGTCCGACACCCATCCC 3			
18	18-F:	5 GGGAGTTGGGAGAGATGGC 3	481	Exon 45-46	61
	18-R:	5 GGGAAACTGAGGCGAAGC 3			
19	19-F:	5 ACATTTGACGCTCACTGGG 3	626	Exon 47-48	62
	19-R:	5 CAAGTGCTTTGGGGGCTG 3			
20	20-F:	5 CTCATCCCCTCTGCTCAT 3	794	Exon 49-50	57
	20-R:	5 GCTCTGTCCATCACCCTTA 3			
21	21-F:	5 TAGTGGTTCAGACACAGGC 3	749	Exon 51-52	58
	21-R:	5 GGTTGCTTGTCTGTTTCC 3			
22	22-F:	5 CCACCCAACCAACTTTCC 3	770	Exon 52	57
	22-R:	5 AACGAGGTAGTCTTTCAGCA 3			
23	23-F:	5 CACTGGGTTCGGAGGAGA 3	857	Exon 52	57
	23-R:	5 AAAGGGCAGGAATGGAAG 3			

Table 2b. Oligonucleotide primers of the COL1A2 gene

S No.		Primer sequence	Length (bp)	Exon	Tm
1	1-F:	5 CCTTATTATTTTAGCACCACGGC 3	309	Exon 1	59
	1-R:	5 CAGGTTACAGGTTTTCCTTTTCAG 3			
2	2-F:	5 CCCTGCCATACTTTTGACCTG 3	365	Exon 2	55
	2-R:	5 TTATTAGCCCCGCCTATTTTG 3			
3	3-F:	5 GCGAATCTTTTCACTCCTTTG 3	557	Exon 3	53
	3-R:	5 GCGAATCACCAGTTTGTATCA 3			
4	4-F:	5 TTATTCTGGAGATGGAAGGCA 3	742	Exon 4	54
	4-R:	5 CGAGGACTGTGGTGGTAGGTA 3			
5	5-F:	5 GGATACGATGTAAGTCCTTGTGC 3	594	Exon 5	54
	5-R:	5 GGGTAAAGCAGAATCTTGAGTGA 3			
6	6-F:	5 GGTAAAAGGTGGGAGTGGA 3	749	Exon 6	54
	6-R:	5 GGTATGCCTCTAAGTGTTGCT 3			
7	7-F:	5 GAACCTATCTGCCCCGTCTAA 3	858	Exon 7-9	54
	7-R:	5 CGCCTTCTTCTTGGTAAACTATC 3			
8	8-F:	5 GCGATAACTTTCTCCCCTTGC 3	899	Exon 10-11	51
	8-R:	5 CGATGATTTTCAGACGGTTGT 3			
9	9-F:	5 TGCCAAAGGGAAGAAAGAGTT 3	746	Exon 12	52
	9-R:	5 GCGGAATAATAGAGGGAGGAA 3			
10	10-F:	5 GCCACTCAAATAACCACATCT 3	861	Exon 13-15	52
	10-R:	5 GCCGTGCTATTTCGTAAGAG 3			
11	11-F:	5 AGTTGAGAATGTGGGGTGGGT 3	765	Exon 16	56
	11-R:	5 GGGGTTTCCTATTTTCCTTGC 3			

S No.		Primer sequence	Length (bp)	Exon	Tm
12	12-F:	5GGAGACAGAAGGAGAGGGAAGGT 3	845	Exon 17-19	57
	12-R:	5GGGCTAAAAATGCTAAGGAAGAGA 3			
13	13-F:	5 GGAATACCACCTCTGCCATCAT 3	937	Exon 20-21	56
	13-R:	5 TCAAGACACAAACCAAGCCAAA 3			
14	14-F:	5 CGTCTGTATCTCCCCTGTAA 3	595	Exon 22-23	50
	14-R:	5 GGAACCTCATAGCCATTGTA 3			
15	15-F:	5 GGGGCAGAACTCTTTTCACACT 3	977	Exon 24-25	54
	15-R:	5 CGATGGATTTTTAGCAGGGATA 3			
16	16-F:	5 ATCCCTGCTAAAAATCCATCTC 3	523	Exon 26	55
	16-R:	5 GGGGTAAAATAAAAGCCAAGTG 3			
17	17-F:	5 AGTATTTGGGCTTTCGTGG 3	802	Exon 27-29	51
	17-R:	5 GTGGGGTAGTAAACAGGGG 3			
18	18-F:	5 GCCAAGTTCTGGGATGGATAC 3	634	Exon 30	55
	18-R:	5 CGGCTTTAGGCTTTGAACATC 3			
19	19-F:	5 CCCCCGAATACTACACATAAC 3	644	Exon 31	52
	19-R:	5 CCCCACAGTTTGTTGCTAT 3			
20	20-F:	5 ACTCCCACTACCCTCATCTCTT 3	1143	Exon 32-33	55
	20-R:	5 CCCCCAATAAAGTGTGTGTAGT 3			
21	21-F:	5TTTCGGACCTGCTATGACTTACAC 3	557	Exon 34	57
	21-R:	5GCGTCAGTGCATTAGGATATTGTT 3			
22	22-F:	5 CATACGATGTCCTCCTCCTGGT 3	812	Exon 35-37	56
	22-R:	5 GGGCAAATGTTAATCCTTCCG 3			

S No.		Primer sequence	Length (bp)	Exon	Tm
23	23-F:	5 CGGTGGAAAATAACGGAAGGAT 3	609	Exon 38	58
	23-R:	5 ATAGTCAGGCAGAAGAAGGGGC 3			
24	24-F:	5 ACTCAGCCAGGTGGTAAAGAT 3	553	Exon 39	52
	24-R:	5 GCCCGATAACTTGTGATGAA 3			
25	25-F:	5 CCCTTCTACATAGTTCTGATTCCA 3	759	Exon 40	54
	25-R:	5 TCAGCAATGGGACTCTGTTCTA 3			
26	26-F:	5GCGAGGATGAATCAGATACAAAAGT 3	554	Exon 41	53
	26-R:	5 AGTGCGTGGAAAGAACCAAAAA 3			
27	27-F:	5 CCTGGGATACTGAATGACACGA 3	617	Exon 42	54
	27-R:	5 GGGATAAAAACCTGCCAACCTA 3			
28	28-F:	5 CGGCTCCATAGTATCTACACCT 3	955	Exon 43-45	53
	28-R:	5 TGCTGGTAATCTGCTTTAGTTG 3			
29	29-F:	5 CCCACTAAAGCAGATTACCAGCT 3	425	Exon 46	54
	29-R:	5 CCGAAATAGCATTACCTCCAAG 3			
30	30-F:	5 CGGGCATAATGGGAGAAAAGAG 3	665	Exon 47-48	59
	30-R:	5CGGGGGTAATCAAGTTCACATAAA 3			
31	31-F:	5 TCGCCCCATTTCAATATATCCTC 3	1327	Exon 49-50	59
	31-R:	5 CCCGCTTTTTCAATGATTACCA 3			
32	32-F:	5CCCCGATAACTTTTTTAGATGACC 3	795	Exon 51	58
	32-R:	5 CCTGGGCACACATTACCATTTA 3			
33	33-F:	5 GGCAAGACTACATACCCACCCA 3	755	Exon 52	56
	33-R:	5 GCCCACATTTTCCATAACAGGT 3			

Table 3. Oligonucleotide primers for gene expression analysis

S No.	Gene name	Primer sequence 5' to 3'	Length (bp)	Tm
1	18S-F	AAACGGCTACCACATCCAAG	230	58
	18S-R	CGCTCCCAAGATCCAACTAC		
2	COL1A1-F	CCCCAGCCACAAAGAGTCTACA	301	58
	COL1A1-R	CTCGGGGACTTCGGCGCCGG		
3	ALP-F	CCTCTCCAAGACGTACAACAC	126	58
	ALP-R	CGGGAACGCTCAGTGGCTGCGC		
4	IGF1-F	GAGGATTTTCTCTAAATCCCTC	247	54
	IGF1-R	CAGCACTTAAATAATTGGGTTGG		
5	Osteocalcein F	GCCCAGCGCAGCCACCGAGAC	150	62
	Osteocalcein R	GCCCTCCTGCTTGGACACAAAGG		
6	Runx2-F	GCCTCACAAACAACCACAGAAC	244	55
	Runx2-R	GAAGTTTTGCTGACATGGTGTC		
7	IHF-F	CCCCGGCCATGTCTCCCGC	269	59
	IHF-R	GCTCCTTGAAGCGCTCGGAG		
8	TGFβ-F	GGCCATCTCCCTCCCACCTCCC	232	62
	TGFβ-R	CTCCCGCTCCTCCTCCCCCT		
9	BMP4-F	GGGAAGGAGTGTGGTGGTGG	269	56
	BMP4-R	TTCGGTTACCAGGAATCATGGTG		

Table 4. Oligonucleotide primers for cloning of HSP47 gene

S. No.	Gene name	Primer sequence 5' to 3'	Length(bp)	Tm
1	HSP47 F	TAAAGCTTATGCGCTCCCTCCTGCTTCTC	1247	62
	HSP47 R	CAGAATTCCTATAACTCGTCTCGCATCTT		

Table 5: Composition of various reagents /buffers

S No.	Assay	Composition.
1	**Alizarin stain**	
	Chemicals	70% alcohol, 0.5% NH_4OH, 1XPBS
	Alizarin Red stain	40 mM ARS, pH 4.1-4.3
	CPC (Cetylpyridinium chloride) buffer	10% CPC buffer
2.	**MTT assay**	3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) 5mg/mL in 1XPBS, 100% DMSO
3.	**ALP assay**	
	Chemicals	Sodium hydroxide 0.25 M,
	AMP buffer	2-amino, 2-methyl, 1-propanol (AMP) buffer pH 10.5.
	pNPP	2 mg/mL of para-nitrophenyl phosphate.
4.	**SDS-PAGE**	
	Chemicals	SDS, Acrylamide, Bis-acrylamide, TEMED, APS, Protein ladder, Glycine, Tris-base,
	2XSDS loading dye	100mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM BME.
	Tris- Glycine Buffer	25mM Tris-Cl, 250mM Glycine, 0.1% SDS.

Table 6: Composition of various reagents /buffers

Reagents/Buffers	Composition
Phosphate buffered saline	NaCI 138 mM, KCI 2.7 mM, Na_2HPO_4 4.3 mM, KH_2PO_4 1.5mM(pH7.4)
Culture medium	DMEM(High/ Low- Glucose) with $NaHCO_3$, 2mM glutamine, 10%(v/v) FBS, 100 units/ml penicillin and 100µg/ml streptomycin
RIPA Buffer	150 mM sodium chloride, 1.0% (v/v) NP-40 or Triton X-100, 0.5% (w/v) Sodium deoxycholate, 0.1% SDS (w/v) (sodium dodecyl sulphate) 50Mm Tris, pH 8.0, 1mM PMSF and protease inhibitor cocktail (1 Ul/100µL of RIPA buffer)
Laemmili buffer(2 X)	4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol ,20% (v/v) glycerol and 0.004 % (w/v) bromophenol blue
SDS- Running Buffer (1X)	Tris glycerol Buffer which contains 25mM Tris base , 250 mM glycerol (electrophoresis grade) (pH 8.3), 0.1% SDS
10% Buffered formaldehyde	10% (w/v) formaldehyde in 1X PBS
4 % PFA (fixative)	4 % paraformaldehyde (v/v) in 1 X PBS**.**
Staining solution	0.25 % (w/v) coommasie R 250 in 50% (v/v) methanol and 10 % (v/v) Glacial acetic acid
Destining solution	30 % (v/v) methanol and 10% (v/v) acetic acid
Transfer buffer	Tris 25 mM ; Glycine 192 mM; methanol 20 %; SDS 0.1%
Blocking buffer	5 % (w/v) dry non- fat milk or BSA in 1X TBST
Ponceau S staining solution	2 % Ponceau S in 30 % trichloroacetic acid
TBST	Tris 25mM, NaC1 15mM, tween 20(0.1%)
Plasmid DNA isolation from *E. coli* by Alkaline lysis	Solution I: 50mM Tris pH 8.0 with HCl, 10mM EDTA Solution II[b]: 200mM NaOH, 1%SDS Solution III: 3.0M Potassium Acetate, pH 5.5
LB	10g/L of Bacto-tryptone, 5g/L of Bacto-yeast extract, 10g/L of NaCl were added. The pH of the medium adjusted to 7.2
LB agar	LB medium with 15g/L Bacto agar
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	20mg/mL dissolved in N, N-dimethyformamide

Table 7: Resolving gel composition (for 10 ml)

Components	**For 10% gel (mL)**	**For 20% gel (ml)**	**For 15% gel (mL)**
Water	4.0	3.3	2.3
30% Acrylamide solution	3.3	4.0	5.0
1.5 M Tris (pH 8.8)	2.5	2.5	2.5
10% Ammonium per sulphate	0.1	0.1	0.1
10% SDS	0.1	0.2	0.1
TEMED	0.004	0.004	0.004

Table 8: Stacking gel composition (5%)

Components	**Stacking gel 3% (ml)**
Water	2.1
30% Acrylamide solution	0.5
1.0 M Tris (pH 6.8)	0.38
10% Ammonium per sulphate	0.03
10% SDS	0.03
TEMED	0.003

Table 9: Detailed Summary of drug treatment protocol

Details of drug treatment on U2OS cells	**First 24h**	**Next 24h**
Vehicle alone for the entire 48h	Control	Control
rhPTH alone for the entire 48h	rhPTH	rhPTH
ZOL alone for the entire 48h	ZOL	ZOL
ZOL for the first 24h followed by rhPTH for the next 24h	ZOL	rhPTH
rhPTH for the first 24h followed by ZOL for the next 24h	rhPTH	ZOL

3.2 Cell lines and cell culture maintenance

Osteoblastic like cell lines U2OS (human bone osteosarcoma epithelial cells) were used in this study. These cell lines were cultured in standard tissue culture flasks in DMEM: F12 Ham's media (Dulbecco's Modified Eagle Media, from Sigma-Aldrich, USA) containing 10% charcoal-dextran-Treated FBS in the presence of antibiotics at $37^{o}C$ and 5% CO_2**.** The research proposal has been approved by the Institutional Ethics Committee (Ref. no **PGI/IEC/2014/2248).**

3.2.1 KO (WT, KO13) cell line

It is a fibroblast cell line with either SERPINH1$^{-/-}$ KO or WT phenotype and was a generous gift from Prof Kazuhiro Nagata, Dr Shinya Ito, Department of Molecular Biosciences, Faculty of Life Sciences, Kyoto Sangyo University, Japan.

3.2.2 U2OS cell line

Osteosarcoma cell line was derived from moderately differentiating osteoblastic cells isolated from tibia of a 15 year old girl. J. Ponten by E. Saksela (originally 2T) in 1964. U2OS cell line was a generous gift from Dr M. Subramanium, Mayo Clinic, Rochester, Minnesota, United States.

3.3 Subjects and Method

Osteogenesis imperfecta patients were enrolled from Endocrinology, Orthopedic and Genetics department of APC, PGIMER, Chandigarh were included in this study. Fifty OI patients and twenty-five control were screened for COL1A1 and COL1A2 gene as well as quantitative expression of COL1A1 and COL1A2 gene. The patients associated with other diseases were excluded from the study. The research proposal has been approved by the Institutional Ethics Committee (Ref No. **PGI/IEC/2013/536-37)**. Blood sample collection was done for mutational analysis and analysis of circulating precursor osteoclasts for apoptosis.

Inclusion Criteria: diagnosis of OI is based on clinical (blue sclera, dentinogenesis imperfecta, pathological fracture), and radiological investigations. The diagnosis was confirmed by genetic analysis.

Exclusion criteria: patients associated with other metabolic bone diseases were excluded from the study.

3.3.1 Biochemical and hormonal assays

Serum levels of total calcium (8.7-10.2mg/dl), phosphate (2.7-4.5mg/dl), Alkaline phosphatase (40-129 IU/L), by standard methods (Auto Analyzer Modular P 800; Roche Diagnostics). Plasma levels of 25 (OH) Vitamin D (25(OH) D, 11.1-42.9 ng/ml) and intact parathyroid hormone (iPTH, pg/ml) were measured using Electrochemiluminescence based immunoassays as per manufacturer's instructions (ELECYS 2010, Roche Diagnostics). Serum bone turnover markers: C terminal telopeptide (CTx) and Procollagen type 1 amino-terminal propeptide (P1NP) levels were also measured by Elecsys and Cobas e-immunoassay analyzers (ECLIA). The measuring range for CTx as per the package insert was 10-6000 pg/mL with an analytical sensitivity of 10 pg/mL and an intraassay coefficient of variation of 17.9 %. The measuring range for P1NP as per the package insert was 5-1200 ng/mL with an analytical sensitivity of 5 ng/ml and an intraassay coefficient of variation of 4.1%.

3.3.2 Radiological (X-ray and DXA)

X-ray was performed to check the bone deformity and other clinical features such as scoliosis. BMD at spine and hip measured by Dual-energy X-ray absorptiometry (Hologic viewer 6.0 with WHO standards for T score calculation).

3.4 To screen the known and unknown mutations in Type I Collagen (COLIA1 and COLIA2) and its interacting partner Hsp47 gene in Osteogenesis imperfecta patients

3.4.1 Nucleic acid extraction

3.4.1.1 DNA was isolated from blood samples of the OI patients and unrelated healthy people, using Phenol: Chloroform extraction followed by purification and elution in $T_{10mM}E_{0.2mM}$buffer. COL1A1 and COL1A2 were chosen as the target genes. Primers were designed for conventional PCR (COL1A1, COL1A2 and SERPINH1 genes) and Real-Time PCR (18S, COL1, OCN, ALP, IHH, TGF-β and RunX2 genes) using primer3 blast. Nucleotide sequence was taken from NCBI and forward and reverse

primers were picked with the help of primer3 blast and finally, blast was done to check homology with other sequences.

3.4.1.2 RNA was isolated from the cell line (U2OS, WT and KO 13) using tri-reagent. To the cell pellet 300 µL of tri-reagent was added and then spun down for 10 minutes at 10,000 rpm at 4°C. The supernatant was transferred to a fresh tube and 1/5 volume of tri-reagent 1-bromo, 2-chloro, 3-propanol was added. The solution then turned milky and tubes were kept on ice for 10 to 15 minutes. Phases were then separated by centrifugation at 10,000 rpm for 20 minutes. The aqueous phase was transferred to a fresh tube and isopropanol (same volume as tri-reagent) was added to precipitate the RNA. Tube were kept on ice and centrifuged for 15 minutes. The pellet was washed with 70% alcohol (prepared in DEPC water). After drying, the pellet was dissolved in 20 µL of RNase free DEPC water and stored at -80°C after spectrophotometric quantification. The quality of RNA was checked on 1.5% agarose gel. The DNase treatment was performed to denature any contaminating DNA before cDNA synthesis.

3.4.1.3 Complementary DNA synthesis (cDNA synthesis).

The cDNA synthesis was carried out using kit based protocol. Briefly, one µg of template RNA, 0.5 µg of random primers were added and the volume was made up to 12 µL using with deionized nuclease-free water. This mixture after spinning down was incubated at 70^0C for 5 minutes and chilled on ice and centrifuged. To this 4 µL of 5 X reaction buffer, 20U of Ribonuclease inhibitor and 2 µL of 10 mM dNTP mix were added and centrifuged. After a pre-incubation step at 37^0C for 5 minutes, 200 U of Revert Aid H Minus M-MulV reverse transcriptase was added to the mixture and incubated at 42°C for 60 minutes. The reaction was stopped by heating at 70^0C for 10 minutes, chilled on ice and stored for subsequent use in PCR.

3.4.2 Amplification of the COL1A1 and COL1A2 gene

In our subjects known mutations present in type I collagen (COL1A1 Exon 1, 18-20 and 47-48 and COL1A2 Exon3) were screened along with unknown mutation of COL1A1 and COL1A2 exons (Table 2 a&b).

Amplified products of COL1A1 and COL1A2 genes were screened for mutation using 25µl reaction mixture with 0.8 µg DNA template, 1 µL (10 mM) of each primer, 0.25 µL 2X Taq PCR. The amplified products were visualized on agarose gel.

3.4.3 Amplified PCR product purification:

All amplified products were visualized on 1.5 % agarose gel and bands having amplified products of interest were cut out from the gel. Amplified products were extracted from the gel using gel purification kit according to manufacturer's protocol. The purified PCR products were used for sequence analysis.

3.4.4 Sequencing of amplified PCR products

The concentration and quality of purified products was checked before doing sequencing PCR.

Total Reaction mixture = 10 µl
Big Dye Terminator Enzyme = 5 µl
Buffer = 1.5 µl
DNA = 1 to 2 µl
Water = 6 – 7 µl

96° C = 1 min
96° C = 10 sec
50 ° C = 5 sec } 25 cycles
60 ° C = 4 min
Hold 4 ° C

3.4.5 Sodium Acetate Method for precipitation:

After brief spun to all PCR products 3M sodium acetate pH – 5.2, 125mM EDTA, 10 µl MilliQ water was added followed by mixing with gentle tapping. Two volumes of absolute alcohol were added and spun for 30 min at 4°C. The pellet was washed by adding 70% ethanol followed by drying of the pellet at RT.

3.4.6 Sequencing

Dried pellet was resuspended in 10 µl of HiDi and incubated at 37 °C for 15 min. The reaction mixture was incubated at 95 °C for 5 min followed by the quick chill in ice for 10 minutes. The sequencing protocol was performed according to the manufacturer's instruction ABI. The chromatogram was analyzed for sequencing results.

3.4.7 *In-silico* analysis of variants in OI patients

Sequences obtained were then aligned to the human reference genome (GRCh37/hg19) using BWA program and analyzed using Picard and GATK-Lite toolkit to identify variants in each exon relevant to clinical indication. Clinically relevant mutations were annotated using published variants in the literature and variant database such as http://www.le.ac.uk/ge/collagen/. Only non-synonymous and splice site variant found in the targeted genes were used for clinical interpretation. For *in silico* analysis, CHIMERA 1.12 Dunbrack rotamer library and I-Mutant3 server, software's were used.

3.5 Functional characterization of novel mutations in Type I Collagen (COL1A1 and COL1A2) and Hsp47 gene is required to prevent the premature fibril formation in OI patients

Cloning

3.5.1 Plasmid DNA Isolation

Plasmid DNA from *E. coli* cultures was isolated by alkaline lysis protocol. 3mL of Luria-Bertani (LB) broth containing the selective antibiotic at a suitable concentration (50µg ampicillin per mL) was inoculated along with a single bacterial colony from LB agar plate. The culture was kept at 37°C for 12-14 hrs at 200 rpm. Solution I, II, II was prepared (composition mentioned in table 5). Cells from this culture were harvested at 6,000rpm for 5min, resuspended in 200µL of solution I by vortexing, and incubated on ice for 5min; then 400µL of solution II (freshly prepared) was added and mixed gently. To this 300µL of solution III was added and mixed gently. The tubes were then kept on ice for 5min and centrifuged at 12,000rpm for 5min. The supernatant was transferred to a fresh 1.5ml MCT and extracted with equal volume of phenol: chloroform (1:1) solution. The aqueous layer was transferred to a fresh tube and the plasmid DNA was precipitated by adding two volumes of absolute alcohol and incubating at RT for 15min. The plasmid DNA was pelleted at 10,000 rpm for 5min and washed with 70% alcohol. The final plasmid DNA pellet was dried and dissolved in 25µL of 1X $T_{10mM}E_{0.2mM}$ containing 20µg/mL of RNase A.

3.5.2 Transformation

The host and antibiotics were selected. The cells were subjected to artificial heat shock method of transformation as it gives a high yield of transformed colonies. For good transformation efficiency, the host in log phase of their growth cycle cells were used. 50µL aliquot of overnight culture was added to 5mL of fresh LB broth in 1:100 ratio and incubated at 37°C with shaking for 2-3hrs until the culture reached the O. D_{600} 0.4-0.6. Bacterial culture (1.5mL) was then taken in a microfuge tube and pelleted down by centrifugation (8,000rpm, 2min). The supernatant was then discarded and cells were washed in 1mL of $CaCl_2$-Tris buffer and resuspended in 100µL chilled $CaCl_2$-Tris buffer and kept on ice for 2 hrs. About 2-4µL of the ligation mixture (along with the appropriate controls) was added to the competent cells prepared above, mixed carefully and kept on ice for 30min. The cells were then subjected to heat shock (42°C for 90sec) and immediately chilled on ice for 1min and then at RT for 2-3min. 900µl of fresh LB was then added and the cells were incubated at 37°C for 1hr to allow the expression of the antibiotic resistance genes in the transformed cells. The cells were plated on LB agar plate containing the respective antibiotics for selection of clone and kept overnight at 37˚C.

3.5.3 Digestion of DNA with restriction enzymes

The vector DNA and insert DNA were prepared by digestion using appropriate restriction enzyme(s) in separate tubes and purified.

Digestion of DNA samples with restriction enzymes was carried out as shown below:

Required components	**10µL reaction mix**
NEB cut smart 10X buffer	1µL
Vector DNA (4kb)	50ng
Restriction enzyme	1µL (5U)
Nuclease-free water	Up to 10µL

3.5.4 DNA ligation

For ligation, purified sample (digested vector and insert) was taken in 1:2-5 ratios (vector: insert), in a microfuge tube and the following ligation reaction was set up e.g.: For 1:3 vector insert ratio

Required components	**Reaction 10**μL
10X T_4 DNA Ligase Buffer	1μL
Vector DNA (4kb)	50ng
Insert DNA (1kb)	37.5ng
Nuclease-free water	Up to 10μL
T4 DNA Ligase	1μL

Reaction mixture was briefly spun down. For sticky end ligations, the tubes were incubated at 16°C for overnight or at RT for 10 min. For blunt end, ligation tubes were kept at 16°C for 2hrs. Both cohesive ends and blunt end ligations were heat inactivated by incubating them at 65°C for 10min. 1-5μL of the ligation reaction was used for transformation of 50μL competent cells.

3.5.5 PCR amplification and cloning of Hsp 47 in pEGFP-C1 vector:

Hsp47 gene-specific primer was designed by the Primer Premier 5.0 software. PCR reaction was performed as per the standard protocol using high fidelity Taq polymerase. Amplified HSP47 gene (wild type) was cloned in pEGFP-C1 vector by sequencing confirmation of Hsp 47 gene inserted into a cloning vector also restriction digestion by EcoR I and Hind II.

3.5.6 Site-directed mutagenesis:

Primer were designed for site-directed mutagenesis. Amplification of Hsp 47 gene from mutated primers with pfu turbo elongase enzyme as per the manufacturer's instructions. Treated with dpn I enzyme to cleave the parent strand. Transformation

into electrocompetent cell of *E.coli* (DHα). Confirmation of site-directed mutagenesis through sequencing also restriction digestion by EcoR I and Hind II.

3.5.7 Colony PCR for identification for positive clones

After transformation, the colonies were analyzed initially by colony PCR to identify those recombinant plasmids. Cell mass equivalent to the size of a small colony was picked up with a sterile pipette tip and suspended in 24µL of sterile water in a PCR tube. The cells were completely lysed by incubating at 95°C for 5min in a thermal cycler. The cell debris were pelleted down by centrifuging at 12,000 rpm for 3min and 2µL of the lysate was used as template for amplification in a 25µL reaction containing 1X PCR buffer (20mM Tris HCl pH 9.2, 10mM KCl, 10mM ammonium sulphate, 0.1% Triton X-100) 2mM $MgCl_2$, 0.2mM dNTP, 0.4µM primers and 0.5U of Taq DNA polymerase). The PCR profile included initial denaturation at 95°C for 5min followed by 30 cycles of denaturation at 95°Cfor 30sec, annealing at 50°C for 30sec, extension at 72°C for 30sec and a final extension at 72°C for 5min.

3.5.8 Transfection of a human osteoblastic cell line with expression vector through lipofectamine.

SERPINH1-/- cell line KO 13 Fibroblast cell line was cultured in Dulbecco Modified Eagle medium (DMEM) supplemented with 10% FBS with 1X antibiotics. Confirmed clone was transfected in cultured cells using Lipofectamine. Cells were harvested after 36-60 hrs of transfection. Newly synthesized protein was analyzed by western blotting and immunocytochemistry.

3.5.8.1 Protein Expression studies by Western Blot:

Mutant and wild-type, fibroblast cells were lysed. Cell lysate was mixed with ice cold RIPA buffer (150mM sodium chloride, 1.0% NP-40 or triton-X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 50 mM Tris, pH 8.0) and homogenized for every sample and 1µl protease inhibitor was added. Cell lysate was separated by spinning at 12000 rpm for 15 min at 4°C. Supernatants were collected and spun again at 12000 rpm for 15 min at 4°C. Isolated proteins from cell lysate

were stored at -80°C until analysis. Protein concentration was estimated with Bicinchonic assay, using bovine serum albumin as standard. After 30 min incubation at 37°C, optical density was read at 660nm and was compared with standards. The protein concentration was calculated from standard curve. The protein concentration was expressed in mg/ml or µg/ml. Samples were separated on 12.5% of SDS-PAGE using Mini protein gel electrophoresis system (Bio-rad). Proteins were denatured by adding 2X laemmelli buffer (156) and heated in heat plate at 100°C for 5min. Denatured protein samples were loaded equally 40µg in each well of the gel in one well loaded with protein ladder. For transfer semidry method was used on Polyvinylidene fluoride (PVDF) membrane transfer apparatus. The membrane was blocked with 5% (w/v) non-fat dry milk (in TBST) for 2 hours at RT on a shaking apparatus and then incubated overnight at 4°C with primary antibody for HSP47 (1:1000 in 2.5% (w/v) BSA), anti β-actin (1:1000 in TBST containing 2.5% (w/v) BSA.

After five washes of 5 min each with 1X TBST, membrane was incubated with HRP labeled secondary antibody (1:1000) containing 2.5% (w/v) BSA for 2 hours in shaker apparatus at RT. After two hour membrane was washed with 1X TBST for Five times Five min each. The protein bands were visualized by enhanced chemiluminiscence using ECL reagent (Bio-rad) and image were acquired in gel documentation system (ProteinSimple, San Jose, USA).

3.5.8.2 Protein expression by Immunocytochemistry:

Wild-type cell line fibroblast and mutant KO13 SERPINHI -/-cell line was seeded at 2000 cells /cm^2 in chamber slide and incubated at 37℃ for 24 h in DMEM medium with 10% FBS and 1X antibiotics for anti-HSP47 protein expression. The medium was then replaced with fresh medium containing ZOL or rhPTH alone or in sequence as described above (table 8) for 48 h. The protein expression was determined under an inverted microscope. The cells were fixed in 4% PFA for 15 min followed by washing with 1X PBS with 100x triton X. Again washing with 1X PBS two times after that blocking with 2.5% BSA for overnight at 4°C.Next day washing with

1XPBS for 3 times at 5min interval. The cells were then incubated with primary antibody for overnight at 4°C.After overnightincubation first rinsed with 1XPBS for 3 times at 5min interval. The cells were then incubated with secondary antibody for 2 hours and fixed followed by washing with 1XPBS for 2 times. The cells were then incubated with DAB for 2-3 minutes counterstaining with hematoxylin then followed by washing with 1XPBS then slide was mounted with DPX. The cells were viewed under a microscope for HSP47 protein expression.

3.6 To study the effect of bisphosphonates along with rhPTH (1-34 amino acid) on PBMC of OI.

3.6.1 PBMC isolation:

Fresh peripheral blood in EDTA vacutainers was diluted with 1X PBS (pH7.4) at ratio 1:1. The diluted blood was slowly overlaid at 2:1 ratio of parts diluted blood to parts Ficoll-Hypaque (1.077g/L) (Sigma Aldrich, St. Louis, USA) in the 15 or 50 ml sterile conical tubes (BD Falcon, San Jose, USA). The blood was layered carefully to avoid mixing of the interface. All the reagent and diluted blood were at room temperature (RT). The tubes were centrifuged at 450 g for 30 min at RT with brakes off in swinging bucket rotor of Eppendorf Centrifuge 5702 (Eppendorf, Hamburg, Germany). The upper layer containing plasma and platelets was carefully drawn off using a sterile pipette, leaving the mononuclear cell layer undisturbed at the interface. The cloudy interface (PBMC layer) was then carefully collected into appropriately labelled 15 or 50 ml sterile tubes. PBMCs were washed with an equal volume of 1X PBS (pH7.4) and centrifuged at 250 g for 10 minutes. Platelets were removed by second washing with 1X PBS (pH7.4) and centrifugation at 100 g for 10 min at RT. The PBMCs were re-suspended with complete RPMI [RPMI-1640, supplemented with 0.1% penicillin, streptomycin and 10% fetal bovine serum (Life Technologies, Carlsbad, USA)] or appropriate buffer for counting on a haemocytometer and further use in downstream applications

Apoptosis was induced by the desired method and an untreated cell sample was included as a control. Cells were re-suspended at a density of 1000000 cells/mL in medium or buffer. 200 µl cell suspension was added to a flow cytometry test tube. Now, only inhibitor control samples were treated with Ac-DEVD-CHO. In order to obtain a final NucView 488 substrate concentration of 5µM, 5µl of 0.2mM NucView 488 substrate solution was added. Cells were incubated at room temperature for 15-30 minutes away from light. 300 µl medium or PBS was added to each tube and analysed by measuring fluorescence in the green detection channel (excitation/emission: 485/515) of the flow cytometer.

3.6.2 Detection of caspase-3 activity Apoptosis Assay

Caspase-3 activity was detected using the NucView 488 Caspase-3 Assay Kit (Biotium, Hayward, CA) according to the manufacturer's instructions. Signals were observed using an EVOS FL Auto Cell Imaging microscope (Keyence, Osaka, Japan). The same exposure time was used for the acquisition of all images.

3.6.3 Immunostaining of osteoclast cells

A sample of whole blood was layered over Ficoll-Hypaque density gradient and direct immunostaining for PBMCs was performed. Mononuclear cells were stained with monoclonal antihuman RANK-PE (9A725, Thermofisher, scientific) and Caspase-3 NucView 488 antibodies. Cells were incubated for 30 minutes at room temperature, followed by washing using Phosphate buffer saline (PBS) with 2% Fetal bovine serum (FBS). The cells were finally acquired using flow cytometer (BD FACS CANTO-II, Becton Dickinson, CA, USA) and data were analyzed using FACSDiva software. Single colour tubes were used for compensation. Osteoclasts were gated from Peripheral blood mononuclear cells (PBMC) cluster as cells expressing RANK and frequency of Caspase-3 expressing osteoclasts and median fluorescence intensity (MFI) of Caspase-3 were compared among subject groups.

3.7 To study the effect of bisphosphonates along with rhPTH (1-34 amino acid) on the bone specific cell line (U2OS).

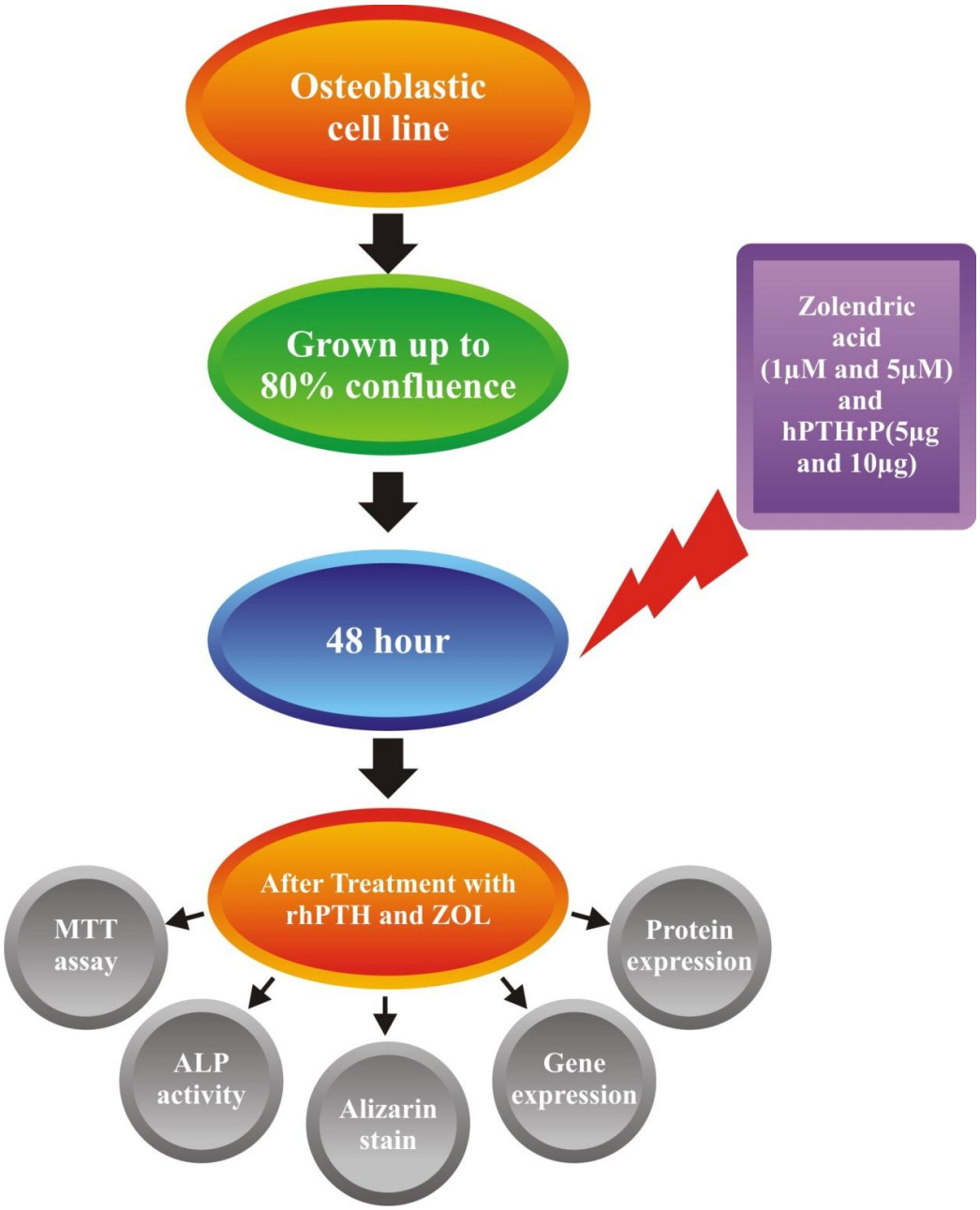

Fig. 8: Experimental Design

3.7.1 Determination of cell number and viability

U2OS cells were seeded at 10,000 cells /cm^2 in a T-25 flask and incubated at 37℃ for culture in DMEM: F12 Ham's media containing 10% csFBS and 100 U/mL of penicillin, 100 µg/mL of streptomycin (Himedia, India). The cells were then incubated under 5 different conditions (Table-9): Control (vehicle alone) for 48h,

rhPTH alone for 48h, ZOL alone for 48h, ZOL for 24h followed by rhPTH for the next 24h, and rhPTH for 24h followed by ZOL for the next 24h at 37^{o}C and in the presence of 5% CO_2. Cells were seeded in 96 well plate with a seeding density of 1.5 x10^4 cells per well. Cell counting was performed using hemocytometer and viability by Trypan blue (Lonza, Cat No# 17945E) dye exclusion principle.

3.7.2 Determination of the effective dose of ZOL and rhPTH:

U2OS cells were seeded at 10,000 cells /cm^2 in a T-25 flask DMEM-F12 medium and incubated at 37℃ for 24 h with 5% CO_2. The medium was then replaced with fresh medium containing ZOL or rhPTH alone for 48 h. Based on previous dose-dependent studies on osteoblast cell viability and gene expressions, we selected 5 and 10 µg/mL doses of rhPTH and 1 and 5 µM doses of ZOL over 48h to determine the most effective dose for subsequent experimental conditions. The relative mRNA expressions of alkaline phosphatase (ALP) and insulin-like growth factor-1 (IGF-1) were analyzed for osteoblastic activity and found that the optimal gene expression by RT-PCR was seen at 5µg of rhPTH and 1µmol of ZOL (**Fig. 50**). Consequently, the following experiments were performed using 5 µg/mL of rhPTH and 1 µM of ZOL to examine cell viability, osteoblastic activity (by ALP activity), mineralization, and osteogenic gene expressions.

3.7.3 Cell Viability Assay for drug treatment

U2OS cells were seeded at 2000 cells /cm^2 in 96-well plate and incubated at 37℃ for 24 h in the DMEM-F12 medium for cell viability assessment. The medium was then replaced with fresh medium containing ZOL or rhPTH alone or in sequence as described above (table 9) incubated for 48 h at 37℃ with 5% CO_2 for 48 h. The cell viability was assessed by MTT (3-(4, 5- Dimethylthiazol-2-yr)- 2,5-diphenyl tetrazolium bromide assay as previously described(157) Briefly, cells were treated with MTT reagent (Calbiochem, Germany) at a final concentration of 0.5 mg/mL and then incubated for 2 h at 37°C. This was followed by addition of 150 µL of solubility reagent (Dimethyl sulfoxide; Himedia, India) to each well. Absorbance was measured at 570nm and 670nm using microplate reader. All the experiments were performed in triplicates.

3.7.4 Alkaline phosphatase (ALP) activity for treated U2OS cells.

U2OS cells were seeded at 2000 cells /cm^2 in 96-well plate in DMEM-F12 medium and incubated at 37℃ for 24 h with 5% CO_2. The medium was then replaced with fresh medium containing ZOL or rhPTH alone or in sequence as described above (table 8) for 48 h. The treated cells were rinsed thrice with phosphate buffer saline pH 7.4 (PBS), and 0.1 mL of 2-amino, 2-methyl, 1-propanol buffer (pH 10.5) containing substrate (2 mg/mL of para-nitrophenyl phosphate i.e. PNPP) was then added. After mixing the lysate, the cells were incubated at 37ºC for 15 minutes. The ALP activity was inhibited by addition of 0.1 mL of 0.25M NaOH and the absorbance of PNP was read in a microplate reader at 405 nm and compared with standard i.e. PNP (Paranitro phenol). The ALP activity was normalized to the protein content in 0.05 ml of this mixture for protein estimation as previously described.

3.7.5 Mineralization for treated U2OS cells.

U2OS cells were seeded at 2000 cells /cm^2 in 24-well plate DMEM-F12 medium and incubated at 37℃ for 24 h with 5% CO_2 for mineralization. The medium was then replaced with fresh medium containing ZOL or rhPTH alone or in sequence as described above (table 9) for 48 h. The osteoblastic activity was determined by assessing the amount of mineralization after treatment. The cells were first rinsed with PBS and fixed for 1hour in ice-cold 70% alcohol followed by washing with deionized water. The cells were then incubated for 30 minutes in the presence of Alizarin Red S solution in the dark to determine the mineralization activity with each of the five treatment strategies (Table-9). The unbound or non-specifically bound stain was removed by rinsing the cells in water. The bound stain was solubilized in 10 % cetylpyridinium chloride and quantitated at 500 nm.

3.7.6 Osteogenic Gene Expression Studies by RT-PCR

U2OS cells were seeded at 10,000,00 cells /cm^2 in a T25 flask DMEM-F12 medium and incubated at 37℃ for 24 h with 5% CO_2 to isolate the RNA to analyze osteoblast-specific genes. The medium was then replaced with fresh medium containing ZOL or rhPTH alone or in sequence as described above (table 9) for 48h.Briefly, the treated cells were trypsinized and rinsed with sterile PBS followed by addition of TRI-reagent (Sigma) containing phenol, guanidine isothiocyanate and chloroform. The

aqueous phase is then extracted with chloroform and the RNA was precipitated with isopropanol. The RNA pellet was then washed with 75% ethanol, air dried, and then dissolved in sterile deionized water. The quality and quantity of RNA were evaluated by spectrophotometer and agarose gel electrophoresis. For cDNA synthesis**,** total RNA was immediately converted to cDNA using AMV-reverse transcriptase and oligo dT primer.

Gene expression was assessed by quantitative RT-PCR. Specific primer sets were used for ALP, COL-1A1 and OCN genes, and the experiments were performed on Light Cycler-480-II (Roche) as per the manufacturer's recommendations using 18s rRNA as endogenous housekeeping gene. Primers for RT-PCR were designed by using primer blast program (www.ncbi.nlm.nih.gov/tools/primer-blast/) in table 3 were used for amplifying the genes. The reactions were prepared in a 96-well optical reaction plate containing 1x master mix (SYBR ® Green PCR Master Mix; Thermo Fisher Scientific Co.) One µl of template cDNA (equivalent to 500 ng of total RNA) was added to 10 µl of PCR reaction mixture containing 0.25 µM each forward and reverse primers. All experiments were carried out in duplicates with two non-template controls as a negative control and normalized to their corresponding housekeeping gene, 18s rRNA. Analysis of relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method to generate data as fold changes.

3.7.7 Immunocytochemistry of osteocalcin protein

Protocol was followed as described in previous section. The cells were then incubated with DAB for 2-3 minutes counterstaining with hematoxylin followed by washing with 1XPBS. Then slide was mounted with DPX with each of the five treatment strategies **(Table-9).** The cells were viewed under microscope for osteocalcin protein expression.

3.7.8 Protein expression by western blot protein

Protocol was followed as described earlier. The membrane was blocked with 5% (w/v) non-fat dry milk (in TBST) for 2 hours at RT on a shaking apparatus and then incubated overnight at 4°C with primary antibody for anti β-actin (1:1000 in TBST containing 2.5% (w/v) BSA), OCN (1:1000 in TBST containing 2.5% (w/v) BSA), OP (2:1000 in TBST containing 2.5% (w/v) BSA). The protein bands were visualized

by enhanced chemiluminiscence using ECL reagent (Bio-rad) and image were acquired on gel documentation system (ProteinSimple, San Jose, USA).

3.8 Statistical Analysis

First the data was checked for normality using D'Agostino and Pearson omnibus normality test. For normally distributed data unpaired Student's t-test (95% CI) was used to compare means between groups and paired Student's t-test (95% CI) was used to compare means for the same sample and Mann-Whitney U test was used for unequally distributed data to compare single groups. All experiments were carried out in triplicates and the data is presented as mean ± standard deviation (SD) and for flow cytometry experiments mean± standard error of mean (SEM) was considered. ANOVA with Bonferroni correction was used for data derived from cell culture experiments. All statistical analysis was performed using Graph pad prism 7.0 software (Grahpad software, La Jolla, CA) and p values less than 0.05 were considered significant.