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by

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A THESIS

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MASTER'S IN CLINICAL DENTISTRY

ABSTRACT

Bisphosphonate is widely used as an effective treatment to prevent bone loss associated with osteoporosis, bone metastasis and multiple myeloma. Bisphosphonate prevents bone loss by blocking osteoclast mediated bone degradation. Bisphosphonate treatment however is linked with osteonecrosis of the jaws but its effects on the PDL and pulp cells of the jaw and teeth remains unknown. To test this, we obtained PDL and pulp tissue from non-infected, impacted third molars of healthy individuals ranging from 17-25 years of age who came to dental school for third molar extraction. When cultured in osteogenic media, PDL and pulp cells showed capacity to differentiate towards osteoblast lineage with a progressive increase in ALP activity, matrix synthesis and mineral deposition. Cells were then cultured in various concentrations of bisphosphonate to evaluate their viability, growth, and differentiation properties. Pulp cells exhibited a 10-fold increased sensitivity to oral forms of bisphosphonate and an early susceptibility to the IV form of bisphosphonate when compared to PDL cells. Growth and proliferation of both PDL and pulp cells was inhibited by bisphosphonate. Surprisingly, sub lethal dose

of bisphosphonate treatment resulted in enhanced osteoblast differentiation of PDL and pulp cells. For a molecular understanding of how bisphosphonate stimulated osteoblast differentiation, we analyzed profile of gene expression. Expression of Runx2 and Osterix, the two essential regulators of osteoblast differentiation were increased by 25-40% in PDL and 0.5-3 fold in pulp cells as determined by western blot analysis. Immunofluorescence studies demonstrated that bisphosphonate treatment enhances nuclear accumulation of Runx2 and Osterix. The upregulation of Runx2 and Osterix coincided with an increased expression of osteoblast marker genes (ALP, osteopontin, and osteocalcin) as monitored by RT-PCR. Taken together these results provide evidence that bisphosphonate activate program of osteoblast differentiation in tooth derived PDL and pulp cells by modulating expression of Runx2 and Osterix.

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LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
ATP	Adenosine triphosphate
BMD	Bone mineral density
BPs	Bisphosphonates
CCD	Cleidocranial Dysplasia
ECM	Extracellular matrix
FGF	Fibroblast growth factor
IF	Immunofluorescence
IGFs	Insulin like growth factors
MSC	Mesenchymal stem cells
ONJ	Osteonecrosis of jaw
PDL	Periodontal ligament
PTH	Parathyroid hormone
RT-PCR	Reverse transcriptase polymerase chain reaction
WB	Western Blot

INTRODUCTION

Osteoporosis is responsible for more than 1.5 million fractures annually in the United States **[1]**. Preventive drug treatments, such as Bisphosphonates (BPs) reduce the risk of fractures in women with postmenopausal osteoporosis by stabilizing or increasing the bone density **[2-4]**. In addition, BPs is used in treatment of other conditions such as osteitis deformans, bone metastasis and multiple myeloma.

Although the BPs are commonly used clinically to treat bone diseases, the mechanism of action of these compounds on bone is not completely understood. BPs can directly inhibit the bone-resorbing activity of osteoclasts at multiple levels including reduction of osteoclast numbers by inhibiting the proliferation and recruitment of osteoclast precursors **[5-9]**, inducing apoptosis in macrophages and mature osteoclast cells **[10-13]**.

In addition to the direct effects on osteoclasts, there is evidence that BPs can act indirectly on the osteoclasts via osteoblasts **[14-16].** It is likely that this indirect effect is due to modulation of osteoblast secretion of soluble paracrine factors that influence osteoclast activity **[17-21]**.

BPs has received attention in the dental and medical scientific literature because of spontaneous necrosis of the jaw following any surgical intervention in the jaw bone subsequent to their use. It is unknown at this time if BPs mediated changes in the

mesenchymal cells contribute to Osteonecrosis of Jaw (ONJ) **[41, 48**]. Therefore the purpose of this study is to test, if the osteogenic capacity of mesenchymal stem cell population is affected by BPs treatment.

Bone Formation and Remodeling

Skeletogenesis begins about the third month of fetal life in humans and is completed by late adolescence. There are two major modes of osteogenesis. A) Intramembranous ossification; where mesenchymal cells are directly converted into bone, such as skull. B) Endochondral ossification; where mesenchymal cells first differentiate into cartilage which is later on replaced by bone. All long bones in mammals are formed by this process.

Common multipotent mesenchymal cells give rise to Fibroblasts, Adipocytes, Chondroblasts, Osteoblast and Myoblasts **[22].** Bone formation during embryonic development and in postnatal life requires coordinated activity of both osteoblast and chondrocyte. Osteoblast and chondrocyte differentiation are regulated by many secreted differentiation factors including TGFßs, bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), FGFs, parathyroid hormone (PTH), PTH–related peptide, thyroid hormone, Indian hedgehog, and retinoic acid **[23].** However, two nuclear transcription factors Runx2 and Osterix provides an essential signal that drives the multipotent mesenchymal cells toward osteoblast / chondroblast lineage by acting both at the early stage of their commitment and continue uptill later stages of differentiation **[24,25].**

Once bone formation has stopped; usually after the acquisition of bone mineral density "BMD", peak bone mass is continued to be maintained by remodeling process. Bone remodeling is a dynamic, lifelong process in which old bone is removed from the skeleton and new bone is added. It consists of two distinct stages – resorption and formation – that involve the activity of osteoclasts and osteoblasts respectively. Usually, the removal and formation of bone are in balance and maintain skeletal strength and integrity. However, imbalance in either bone resorption or formation results in a number of skeletal disorders.

Role of Runx2 in Osteoblastogenesis and Bone Formation

Runx2 (runt-related transcription factor) and Osterix are essential for bone formation and osteo-chondro differentiation **[30, 49].** Similarly, members of Sox family (Sox5, Sox6, Sox9) are critical for chondrocyte differentiation and cartilage development **[26, 27]**. Runx2 plays an important role in both osteoblast and chondrocyte differentiation by directly regulating expression of cartilage and bone specific genes **[28, 29].**

There are three major stages of osteoblastogenesis: proliferation, matrix maturation, and mineralization, which are characterized by sequentially expressed genes that support the progression of osteoblast differentiation **[22].** Runx2 mediates the temporal activation and/or repression of cell growth and phenotypic genes as osteoblasts progress through various stages of differentiation.

The proliferation stage encompasses a wide range of osteogenic cell types, from the earliest MSC through the more committed chondro-osteo-progenitor and pre-osteoblast. Runx2 expressed in early progenitors induce a program of gene expression

required for lineage determination and differentiation of mesenchymal cells **[22].** Interestingly, forced Runx2 expression can redirect a committed pre-muscle cell into the osteoblast lineage **[30].**

The second stage of osteoblast differentiation corresponds to multi-layering of the osteoblasts, production and maturation of collagen matrix. This stage is characterized by the induction of alkaline phosphatase expression, an early marker of the osteoblast phenotype. The accumulation of Type I collagen results in an extracellular matrix (ECM), which contributes to the cessation of proliferation and supports a signaling cascade through cell-matrix and cell-cell interactions to upregulate the expression of osteoblast-related genes. Expression of both alkaline phosphatase and Type I collagen is controlled by Runx2 **[22].**

The final stage of osteoblastogenesis begins with deposition of minerals in the ECM by several mechanisms. The mineralization stage is marked by the upregulation of genes such as bone sialoprotein, osteocalcin, and osteopontin that reflects mature osteoblast phenotype **[22].** Runx2 induce expression of all bone matrix protein genes **[42-44].** Thus Runx2 is a master regulatory switch that exhibit unique properties for mediating the temporal activation and/or repression of phenotypic genes.

Runx2-deficient ($Runx2^{-/-}$) mice completely lacks bone formation owing to the absence of osteoblast **[24, 31].** Runx2 mutation in humans results in cleido-cranial dysplasia (CCD) an autosomal disease characterized by hypoplastic clavicle, open fontanelles, supernumerary teeth, short stature and changes in skeletal patterning **[8]**. In Runx2 null mice, molar odontogenesis does not proceed beyond the late bud stage and hence there is a complete absence of teeth formation **[32].**

Runx2 is also involved in the balance between bone formation and bone resorption. The osteoprotegerin gene, which is expressed in osteoblasts to inhibit osteoclast differentiation, is upregulated by Runx2 **[33]**. Similarly, Runx2 induce expression of the collagenase 3 (MMP13 enzyme) and Macrophage colony stimulating factor genes, products of osteoblasts that contribute to bone matrix remodeling and osteoclastogenesis, respectively. Thus Runx2 mediated osteoblast activity affect osteoclast function during bone resorption **[30]**.

Osteoporosis and Bisphosphonates

Osteoporosis, a disease associated with bone fragility and increased risk of fracture. In osteoporosis the bone mineral density is reduced, amount and variety of non-collagenous proteins are altered and the bone microarchitecture is disrupted. Major factors that contribute to osteoporosis are use of steroids, old age and estrogen deficiency especially in women. **[34].** Hormone replacement therapy, Bisphosphonates (BPs), and other drugs (calcitonin, raloxifene) are commonly used for treatment of osteoporosis **[1].**

BPs is a family of pyrophosphate analogues in which the oxygen linking the phosphates has been replaced by carbon. These compounds have high affinity for hydroxyapatite crystals **[35, 36].** BPs can be taken by either oral or intravenous route. Up to 50% of the absorbed oral dose or the intravenously administered dose is adsorbed to the skeletal system **[34].**

BPs can be divided into two distinct classes with different molecular mechanism of action

- Nitrogen – containing Bisphosphonate.

- Non Nitrogen – containing Bisphosphonate.

BPs' has strong affinity for bone and not for other tissues which makes them a potent inhibitor of bone resorption and bone remodeling activity, with limited potential for side-effects in non-skeletal tissues. Current pharmacokinetic studies indicate that approximately half of any BP dose reaches the skeleton, with an early half-life of ten days, and a terminal half-life of about ten years **[37].**

Non-nitrogen-containing BPs are metabolized by osteoclasts into toxic adenosine triphosphate (ATP) analogues which accumulate intracellularly in osteoclasts and may cause direct osteoclast apoptosis via inhibition of the mitochondrial ADP/ATP translocator **[38].**

The novel and more potent nitrogen-containing bisphosphontes have been shown to inhibit farnesyl pyrophosphate synthase, an enzyme in the cholesterol-synthesis pathway, and consequently inhibit prenylation of small GTP-binding proteins which eventually leads to the indirect, secondary osteoclast apoptosis **[38].**

Bisposphonates directly regulate cell proliferation, differentiation and gene expression in transformed human osteoblasts, stimulate the formation of osteoblast precursors in marrow, promote early osteoblastogenesis, and regulate osteoblastic synthesis of cytokines and growth factors **[39, 40].**

Currently effect of BP on cells derived from the jaw bone is not known. PDL and Pulp tissue in the jaw bone provide an excellent source of mesenchymal cells. Therefore for these studies we used PDL and Pulp tissue was isolated extracted third molars.

OBJECTIVE

Bisphosphonate is commonly used for the prevention of bone loss associated with osteoporosis and various other diseases and bone cancers. However accumulating data suggests that BPs treatment is associated with osteonecrosis of jaw, especially in patients undergoing oral surgical intervention. The molecular reason for this paradoxical effect of BPs is not known. We hypothesize that BPs exerts a differential effect on different cells and tissue types. Therefore this study is focused on identifying the specific action of BPs on PDL and pulp tissue derived cells.

SPECIFIC AIMS

1. Isolation and establishment of primary cultures from healthy human PDL and Pulp tissue.

2. Effects of BPs on proliferation of PDL and Pulp derived mesenchymal cells.

- BP effect on mitotic duplication of the primary cells.

3. Determination of BPs mediated changes on osteogenic differentiation of PDL and Pulp cells.

- Examination of profile of gene expression by RT-PCR as well as ALP cytochemistry and alcian blue and VonKossa staining.

4. Does BPs effects are mediated through modulation of Runx2 and Osterix expression.

- Determination of Runx2 and Osterix mRNA and protein levels by RT-PCR and western blot analysis.

5. Test BPs action on cellular distribution of Runx2 and Osterix.

- Insitu immunostaining to check localization of Runx2 and Osterix with other nuclear and cytoplasmic marker.

MATERIAL AND METHODS

Isolation of PDL and Pulp Tissue

The study protocol was approved by the Institutional Review Board for human use at the University of Alabama at Birmingham. Total 8 donors (2-3 teeth from each patient) were obtained from young healthy individuals who came to School of Dentistry (UAB) in the department of Oral and Maxillofacial Surgery. Informed consent was taken. Pulp and Periodontal tissue was obtained from freshly extracted impacted caries and restoration free human third molars. Teeth were extracted using aseptic conditions. They were split vertically using micro motor hand piece and pulp tissue was obtained. PDL tissue was scraped from the roots of teeth. Tissue was treated with penicillin/streptomycin and chopped into 1-3 mm small pieces (to increase the surface area for emigration of cells out of the tissue). These chunks of tissue were then placed in a 10-cm dish containing growth media (αMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine).

PDL and Pulp Cell Culture

Cells were allowed to migrate out from the Pulp and PDL tissue in 10cm plate in growth media (αMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine). After three weeks when cells reach subconfluency, they were trypsinized

and expanded in additional plates. At passage three cells were either frozen and/or plated for subsequent differentiation or gene expression analysis. Cells were washed with filtered PBS buffer. Cells were then trysinized with 1ml trypsin and then mixed with growth media to stop the reaction. Cells were then plated in 10-cm dish for future experiments.

Osteogenic Differentiation of Pulp and PDL Cells

Pulp and PDL cells were cultured in 6 well plates in the absence or presence of osteogenic media for 21 days. Subconfluent cells (3 days after plating) were induced to differentiate along osteoblast lineage by culturing them in osteogenic media (Growth media supplemented with 10mM β-glycerol phosphate and 50μg/ml of ascorbic acid).

ALP, von Kossa and alcian blue staining was performed each week to check the amount of Alkaline phosphatase activity, matrix formation and mineralization.

Alkaline Phosphatase Cytochemistry

Cells were washed twice with 2ml/well 0.1M cacodylic buffer and fixed with 4% formaldehyde in cacodylic buffer for 10 minute at room temperature. Cells were washed again with 0.1M cacodylic buffer and stained with ALP histological stain solution. Plates were incubated at 37ºC for 12 hours.

ALP histological stain solution contained Napthol AS-Mx phosphate disodium salt 25mg, NN dimethyl formamide 1.4ml, 0.2M Tris maleate buffer 25ml, Fast red TR salt, 50mg and ddH_2O to a final volume of 50ml. All reagents were purchased from Sigma Aldrich corporation.

Cacodylic buffer (0.1M) was prepared by adding 6.9g of cacodylic acid in 500ml ddH_2O. Fixative (4% formaldehyde) was prepared by adding 10.8ml of 37% formaldehyde in 90.1ml of 0.1M cacodylic buffer. The Tris maleate buffer (0.2M) was prepared by dissolving 4.74g of Tris maleate in 100ml of double distilled water.

Von Kossa Staining

Cells were washed twice with 0.1M cacodylic buffer and fixed with 4% formaldehyde in cacodylic buffer for 10 minute at room temperature. Cells were washed again with 0.1M cacodylic buffer. After washing cells, 3% silver nitrate was added (1ml per well of 6 well plate). Cells were then exposed to UV cross linker for 30 minute at 1200X100μJ/cm2.

Alcian Blue Staining

Cells were washed twice with 0.1M cacodylic buffer and fixed with 4% formaldehyde in cacodylic buffer for 10 minute at room temperature. Cells were washed again with 0.1M cacodylic buffer. After washing 0.1% Alcian Blue in 0.1N HCl (1ml per well of 6 well plate) was added and plates were incubated at 37ºC overnight for 12 hours. Changes in intensity of color were recorded by digital photomicrographs.

Progression towards osteoblast differentiation was monitored by ALP, Von Kossa and Alcian Blue cytochemistry.

Establishment of Sublethal Concentration of Bisphosphonate

PDL and pulp cells were plated in a 6 well tissue culture dishes. Upon reaching confluency treatment with Zoledronic Acid (IV form) was started. Growth media mixed with different concentrations of drug ranging form 0.2 μM, 0.4μM, 0.6μM, 0.8μM and

1µM. Zoledronic Acid was obtained from fresh vial of Zometa stored at 4°C. Another plate of PDL and pulp cells was fed with Risedronate tablet form of drug with different concentrations ranging from 2.5ng, 25ng, 250ng, 2.5µg, and 25µg.

Effect of drug on the morphology and proliferation of cells was observed every day till cell death was noticed under microscope. First feeding of cells was carried with indicated concentration of BP and media was not removed until cell death was noticed. Cells were fed with 150µl of fresh FBS every other day. Cell morphology was recorded everyday by photo micrograph.

Cell Growth Analysis

PDL and pulp cell were trypsinized and obtained from 10-cm plate and counted on a haemocytometer. Cell plate were washed with PBS following which 1ml of 0.25% trypsin was added to the wells and incubated at room temperature for 3 minute for the cells to detach from the bottom. Reaction was stopped by adding 4ml of media. Cells were then counted on the haemocytometer. For growth curve 20,000 cells/well were seeded in 12 well plates and allowed to settle down for 24 hours. After 24 hours they were treated with different concentrations of BP (IV Zoledronic acid 0.5, 1, 2µM) and cultured for 10 days. Cells were counted every 24 hours. Number of cells would tell us cell growth and proliferation under the effect of the drug. Growth curve was plotted with Microsoft excel software.

Isolation of Nuclear Extracts

PDL and pulp cells were cultured in 2 to 3 (10-cm) plates till they reached confluency. Cells were scraped in 10ml ice cold PBS mixed with protease cocktail inhibitor and MG132. Cell pellet was obtained by centrifugation and cells were resuspended in 400µl of NP 40 lysis buffer. Cells were incubated on ice for 10 minute. Cells were then centrifuged again at 1500rpm for 30 seconds at 4°C and resuspended in 400µl ice cold hypotonic buffer to obtain nuclei. Nuclei were pelleted by centrifugation at 7000rpm for one minute followed by addition of 150-300µl extraction buffer. Tubes with cells were then vigorously rocked in cold room for one hour and centrifuged at 10,000rpm for 5 minute at 4°C. The supernatant containing the nuclear extract/proteins was then transferred to a clean eppendorf tube.

Bradford protein assay was done to determine the concentration of protein in the nuclear extract. Nuclear extract was stored at -20°C.

SDS PAGE and Western Blot Analysis

SDS resolving gel (8%) was prepared by adding Protogel 1.2ml, 4X resolving buffer 1.1ml, ddH_2O 2.1ml, Ammonium persulfate (APS; 10%) 45µl and TEMED 4.5µl. Stacking gel was prepared by mixing Protogel 0.4ml, stacking buffer 0.75ml, ddH_2O 1.8ml, APS (10%) 20µl and Temed 2µl. All the solutions were prepared on ice. Wells in the stacking gel were made using one mm comb.

Equal concentrations of protein sample (40µg) were mixed with 10X SDS lysis buffer. Samples were boiled for 5 minute and then immediately loaded on the gel with the molecular weight marker. Gel electrophoresis was done at 100 mA for 90 minutes.

Proteins were then transferred to Immobilon membrane (Millipore) by using electroblotter at 500mA for 25 minutes.

The blots were blocked in 0.5% nonfat dry milk in phosphate buffered saline with 0.02% Tween (PBST) for 14 hrs at 4ºC. Blots were probed with mouse monoclonal Runx2 (dilution 1:1,000) antibody for one hour at room temperature in PBST containing 1% non fat milk and in case of rabbit polyclonal Osterix (Dilution 1:500) and mouse monoclonal Lamin A/C (Dilution 1:500) antibody, overnight at 4ºC. The blots were then washed four times with the PBS-T solution and again incubated with 1:5,000 dilution of the horseradish peroxidase conjugated secondary antibody for one hour at room temperature. Blots were then again washed 4-time with PBST solution and one time with PBS. Membrane was then stained with 600µl of Chemiglove solution for 5 min, dried and packed in serene wrap to obtain signal.

The immunoreactive bands were detected with AlphaInnotech software. Same membrane was stripped again with mild stripping buffer and blocked again in 5% non fat milk in PBST overnight at 4ºC and probed with Osterix and Lamin A/C antibody.

Cells were plated in 10-cm culture dish in growth media with the presence and absence of IV Zoledronic acid 1µM. Drug treatment was started in the cells when the cells reached subconfluency. Cells were subjected to drug treatment for 4 days. Nuclear extract was obtained with the above steps and level of Runx2, Osterix and Lamin A/C were checked in the cells.

Antibodies

Various antibodies used for western blot (WB) and immunofluorescence (IF) are as follows - mouse monoclonal and rabbit polyclonal Runx2 (1:1000 for WB and 1:200

for IF), mouse monoclonal SC35 (1:150 for IF), mouse monoclonal β-tubulin (1:150 for IF), and mouse monoclonal Lamin A/C (1:1000 for WB). Antibodies were obtained from Santa Cruz CA. The rabbit polyclonal Osterix antibody (1:1000 for WB and 1:150 for IF) was obtained from Abcam Cambridge MA.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total cellular RNA (at day1 and day 4) was obtained from control and drug treated cells cultured for 4 days in growth media and in BPs (1μM of IV Zoledronate). RNA was obtained by Gibco's Trizol method (Invitrogen Inc CA). Briefly, homogenization of cells was done by adding one ml of trizol per well and cells lysate were collected in an eppendorf and were incubated for 5min at room temperature. Then 0.2ml of chloroform was added to the eppendorf and samples were vigorously shaken for 15 seconds and incubated for 2-3 minute at room temperature. Centrifuged at 12,000 x g for 15 minutes at 4 ºC to collect the aqueous phase.

RNA precipitation was done by transferring the aqueous phase to a fresh tube. RNA was precipitated by mixing the aqueous phase with 0.5ml of isopropyl alcohol reagent and incubated at room temperature for 10 minute. Tubes were centrifuged at 12,000 x g for 15 minute at 4 ºC. Supernatant was removed and RNA pellet was washed with on ml of 75% ethanol. Sample was vortexed and centrifuged at 7,500 x g for 5 minute at 4ºC. RNA was dissolved in 150-200μl of DEPC treated H_2O.

RNA (1μg) was reverse transcribed using advantage RT-for-PCR kit following the manufacturer's protocol. Master mix was prepared with following ingredients.

(5X Buffer - 5µl,MgCl2 (25mM) - 2µl, dNTPs – 0.5µl, Taq DNA Polymerase – 0.125µl, Template cDNA - 1µl, Forward and Reverse Primer (100ng/µl) - 1µl, ddH_2O – 14.3 µl). PCR cycle condition used were 94°C for 2 minutes, 94°C for 30 seconds, 58°C (for Osterix, osteocalcin, ALP and osteopontin and 60°C for β-actin) for 30 seconds, 70°C for 30 seconds, (30 cycle for ALP and osteopontin, 28 cycles for osterix and osteocalcin and 25 cycles for β-actin) and 72°C for 10 minute. Sequence of both primers used for RT-PCR and the product sizes are as follow:

Osterix Forward: 5' CCTGGCTGCGGCAAGGTGT 3' ;

Osterix Reverse: 5' GATCTCCAGCAAGTTGCTCTGC 3'

Product size: 398bp

ALP Forward: 5' GCCTGGCTACAAGGTGGTG 3'

ALP Reverse: 5' GGCCAGAGCGAGCAGC 3’

Product size: 293bp

Osteocalcin Forward: 5' GGTGCAGCCTTTGTGTCCAAGC 3'

Osteocalcin Reverse: 5' GGCAAGGGGAAGAGGAAAGAAGG 3'

Product size: 284bp

Osteopontin Forward: F 5’ CCCTTCCAAGTAAGTCCAACGAAAGC 3’

Osteopontin Reverse: R 5’ CTGGATGTCAGGTCTGCGAAACTTC 3’

Product size: 323bp

β-actin Forward. : 5' CGTGATGGTGGGCATGGGTC 3'

β-actin Reverse : 5' ACACGCAGCTCATTGTA 3'

Product size: 162 bp

For analysis of amplified products, a 2% agarose gel (1.2g of agarose in 60ml of Tris borate buffer) containing 6µl of ethidium bromide was poured and allowed to set for 20 minutes. 10µl of master mix was added to the gel along with 100bp DNA ladder. Gel was electrophoresed in TBE buffer in gel electrophoresis for 30 minutes. The gel was then removed and pictures were taken under UV light using Alpha Innotech software. Gel was run to obtain the desired level of separation of band.

Insitu Immunofluorescence

PDL and Pulp cells were plated at a density of one million cells per 6 well on gelatin-coated cover slips in the presence and absence of Zoledronic acid IV form (Control, 0.5µM, and 1µM) and incubated in humidified incubator at 37ºC. Gelatin coated cover slips required for these experiments were autoclaved. After 24hrs, cells were washed twice with ice-cold PBS. Fixed with WC preparation on ice for 10 minutes by adding 2ml of WC fixative (2.5ml of 37% formaldehyde and 22.5ml filtered PBS) per well. Cells were washed again once with PBS. To facilitate antibody staining of WC preparations, Cells were permeabilized with one ml of permeablilzing solution (0.37ml of 10% Triton X-100 with 14.6ml of filtered PBS) on ice for 20minutes. Permeabilizing solution was aspirated and washed twice with PBS. PBSA (1ml) was then added to the wells till the antibody staining.

Immunostaining of the Samples

Antibodies were diluted to the required concentration in PBSA. Diluted antibody (20µl drop) for each sample was dispensed on parafilm that was already flattened on the lids of plates. Carefully cover slips were placed on the drop so that the cells are in direct

contact with the antibody without creating any air bubble by gently placing the cover slips from one edge on the antibody and incubated for one hour at 37ºC. Cover slips were placed back in respective wells with cells facing upward and washed four times with ice-cold PBSA. Cells were stained with Alexa 488 goat anti mouse and Alexa 594 goat anti rabbit for one hour at 37ºC. Cover slips with cells were washed 4 times with PBSA. Cells were stained with DAPI for 5 minutes on ice followed by washing once with 0.1 % Triton x 100- PBSA and with PBS. Cover slips were immediately mounted in antifade mounting medium and sealed and stored at -20ºC in the dark.

Digital microscopic analyses were carried out using NIS Imaging software and pictures were taken by Nikon camera at 60X magnifications.

RESULTS

Establishment of Primary Cultures of Pulp and PDL cells

Periodontal ligament (PDL) and pulp tissues were obtained from impacted, caries and restoration free human third molars freshly extracted in the UAB dental clinic. Only those teeth with completely formed root were used to obtain PDL tissue. PDL tissue was obtained by scraping the root surface and pulp tissue obtained by vertically splitting the tooth (Fig 1B, C). Tissues from extracted teeth were pooled and treated with penicillin/streptomycin to prevent any residual bacterial growth. Tissue was then chopped into small pieces of 1-3 mm diameter (to increase the surface area for cell emigration) and placed in a 10-cm plate with growth media. Usually, cells started to emigrate from the tissue and formed individual colonies within 15-20 days. Cells reached confluency by day 25-30 and were passed and/or frozen for subsequent experiments. The isolated cells showed elongated morphology typical of fibroblast (Fig 1D, E).

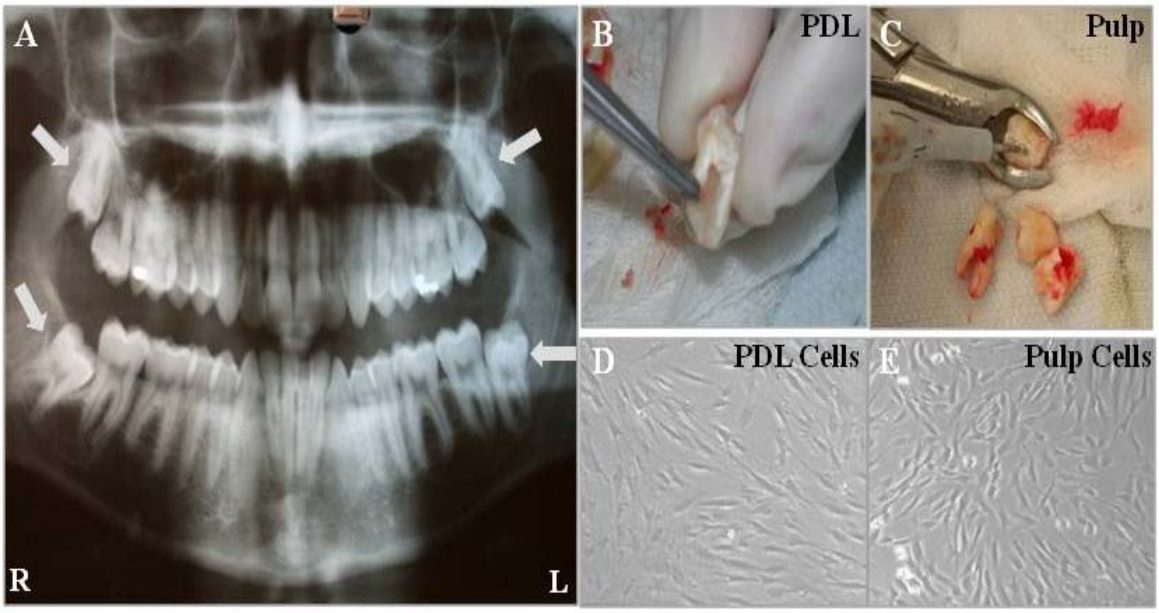

Figure 1: Isolation of PDL and pulp tissues and cells. A) Panoramic radiograph showing impacted third molars with fully developed root. B) PDL tissue being scraped from the root surface of tooth. C) Tooth being split vertically with micro motor hand piece to obtain pulp tissue. D, E) Pictures of emigrated PDL and Pulp cells at day 25.

PDL and Pulp cells Exhibit Capacity of Osteogenic Differentiation

To test if these cells can differentiate towards osteoblast lineage, PDL and pulp cells at passages 4 and 15 were cultured in 6-well plates. Upon reaching subconfluency (2-3 days after plating), cells were exposed to osteogenic media and cultured for 3 weeks (Fig 2). At the end of each week cells were harvested to monitor osteoblast differentiation by

ALP, alcian blue and vonKossa staining. The intensity of the stain reflects the level of ALP activity, proteoglycan (alcian blue) and calcium and phosphate deposition (vonKossa). Both PDL and pulp cells grown in osteogenic media shows a robust ALP activity compared to control cells (Fig 2). Similarly, an increased mineral deposition (VonKossa stain) and extracellular matrix synthesis (Alcian blue) was noted in cells in osteogenic media (Fig 2). A week activity of ALP, an early marker of osteoblast was noted in control cultures. Progressive increase in ALP activity, matrix formation and mineral deposition was noted through out 3 week time period with osteogenic media. Thus our PDL and pulp cells have the capacity to differentiate towards osteoblast when stimulated by osteogenic media.

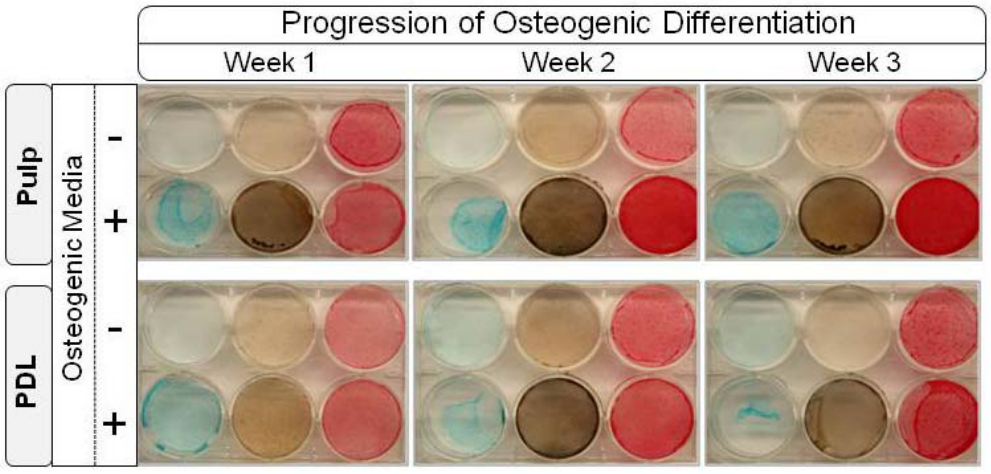

Figure 2: Osteogenic differentiation of PDL and pulp cells. PDL and pulp cells were cultured for 3 weeks in control and osteogenic media (10mM β-glycerol phosphate, and 50µg/ml of ascorbic acid) as indicated. Cells were fixed and stained for ALP (Red color), vonKossa (Black color) and alcian blue (Blue color) as described in the method section.

Digital photographs of the stained plates at different time points of culture are shown. With increased extracellular matrix formation at later time point (2, 3 weeks), cell layers occasionally flipped during staining process.

Osteoblast Master Regulators Osterix and Runx2 are expressed in PDL and Pulp Tissue Derived Cells

Runx2 and Osterix transcription factors are obligatory for osteoblast differentiation and bone formation. To test if these transcription factors are expressed in PDL and pulp cells, we performed insitu immunofluorescence and western blot analysis. PDL and Pulp cells from 8 patients were cultured to confluency in 2-3 10cm plates in growth media to obtain nuclear extracts. Isolated nuclear proteins were then tested for the presence of Runx2 and Osterix by SDS PAGE (Fig 3A). Variable levels of Runx2 and Osterix proteins were observed from cells of individual patients. In general, when data are normalized with Lamin A/C protein, PDL cells exhibited 2 fold increased level of Runx2 compared with pulp cells. We further confirmed the cellular distribution of both Runx2 and Osterix by insitu immunofluorescence microscopy (Fig 3B). PDL and pulp cells were plated on gelatin coated cover slips and processed for insitu immunofluorescence. SC35 nuclear antigen and cytoskeletal β-tubulin protein were detected along with Runx2 and Osterix. Both PDL and pulp cells showed exclusively nuclear but non nucleolar signal for Runx2 and Osterix proteins. Although splicing factor SC35 showed a punctate staining in nucleus but no association was seen with Runx2 protein. Taken together these observations demonstrate that PDL and pulp cells express

Runx2 and Osterix proteins and presence of these factors may play role in their osteogenic differentiation.

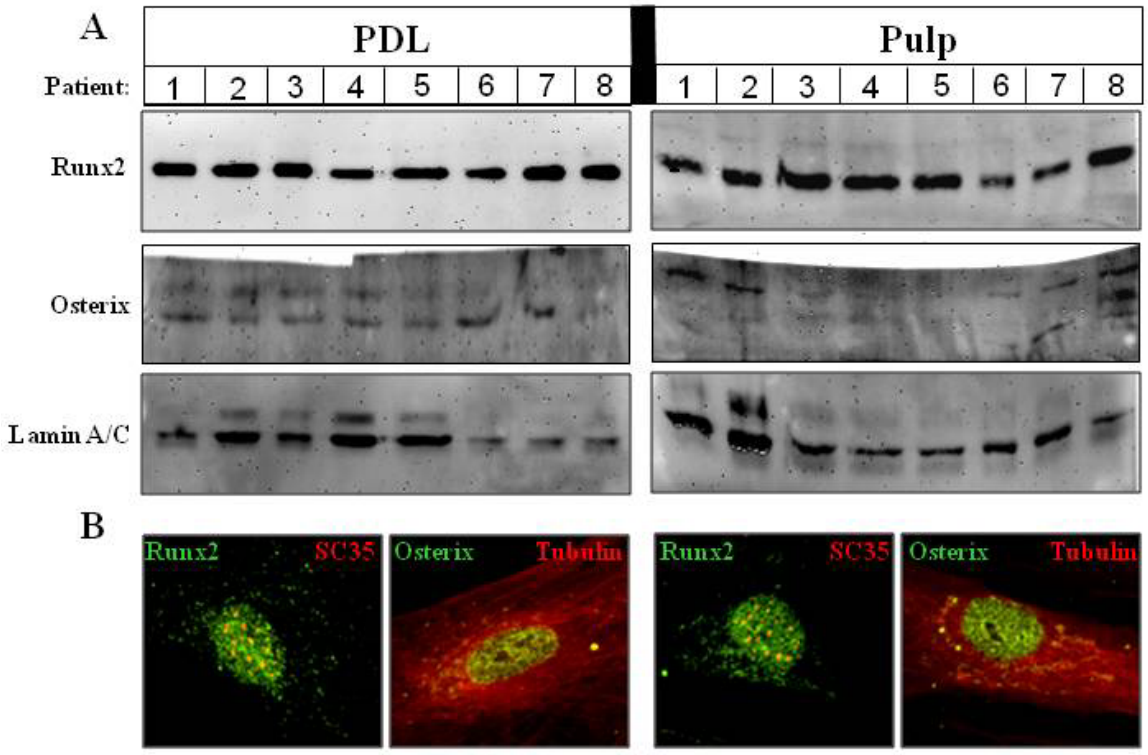

Figure 3: Expression and nuclear localization of Runx2 and Osterix proteins in PDL and pulp cells. Cells from indicated patients were cultured to confluency in growth media. Cells were harvested to obtain nuclei which were subjected to biochemical extraction to isolate nuclear proteins. A) Equal amount (40µg) of nuclear proteins was separated on SDS PAGE and blots were probed with Runx2 and Osterix antibodies as described in material and methods. Blots were stripped and reprobed with Lamin A/C antibody, used as a loading control. B) Cellular distribution of Runx2 and Osterix protein

by insitu immunofluorescence analysis. PDL and pulp cells were stained with polyclonal antibodies against Runx2 and Osterix and monoclonal antibodies SC35 and β-tubulin respectively. β-tubulin signal is localized to cytoplasm where as Runx2 and Osterix are exclusively nuclear.

Growth and Proliferation of PDL and Pulp Cells is Inhibited by Bisphosphontes

To check the effect of BPs on growth and proliferation of PDL and pulp cells, sub lethal dose of BPs was determined. BPs drug is administered either orally or by intravenously to patients for the treatment of Osteoporosis and other bone related illness. We tested the effect of both IV (Zoledronic Acid) and oral form (Risedronate) of BPs. PDL and Pulp cells were seeded in a 6 well plate and upon reaching subconfluency were treated with different concentrations of Risedronate (2.5ng-25μg) and Zoledronic Acid (0.2μM -1μM) in growth media (Fig 4). Risedronate Sodium tablet was dissolved in PBS completely to obtain a suspension of 3.5mg/ml. In these experiments a single treatment of approximately one million fold diluted concentration (relative to human dosage) were used for both IV and tablet forms of BPs. PDL and pulp cells treated with 0.2-0.8 μM of Zoledronic acid showed no difference than untreated control cultures indicating these dosage do not affect cell growth and survival. Pulp cells treated with 1μM dosage show changes in cell morphology and increased cell death by day 10. However, PDL cells showed no changes at this time but started dying by day 15 (Fig 4). Similarly when oral form of BPs was tested, neither cell showed any response to 2.5ng-25ng dosage. Death was noted in pulp cells with 250ng of BPs by day 7 and all the higher concentrations tested. PDL cells however showed no cell death with 250ng dosage but cytotoxicity was

noted by 2.5µg and 25µg dosages. Taken together our results indicate that pulp cells exhibit 10 fold more sensitivity to BPs as compared to PDL cells.

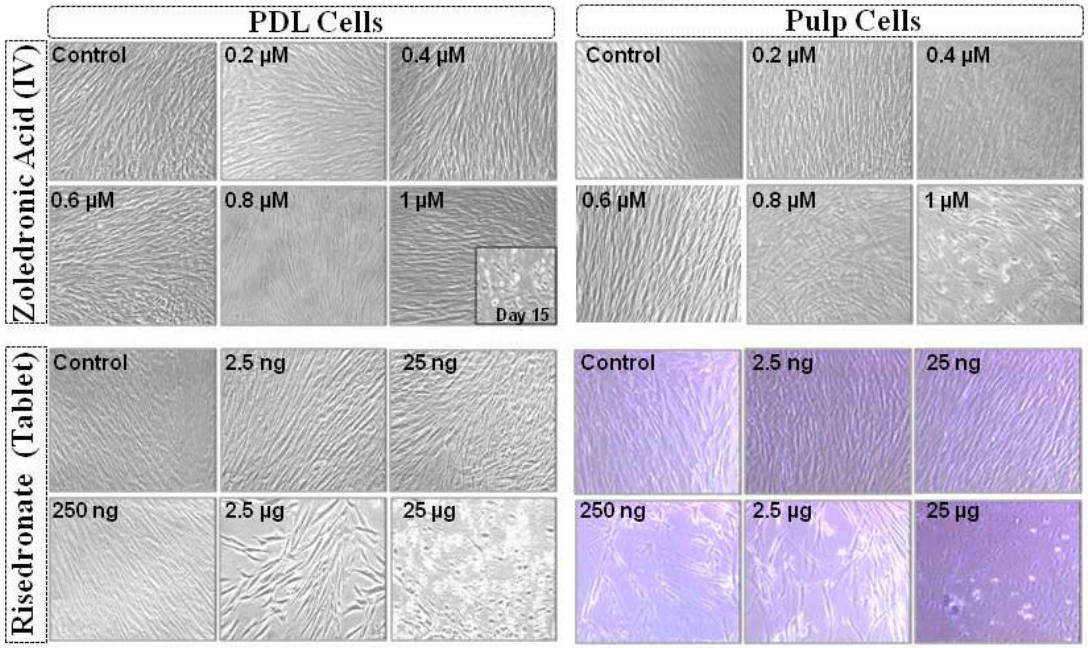

Figure 4: Pulp cells exhibit higher sensitivity to BPs. PDL and pulp cells were seeded in 6 well plate and fed with media containing BPs (IV and tablet form) 24 hours later. Culture medium was not changed till the completion of experiment but was supplemented with fresh FBS every other day. Changes in morphology and cell death were observed under microscope every day. Representative pictures of cell morphology at day 10 (Zoledronic acid) and at day 7 (Risedronate) are shown. The inset picture (1µM PDL) shows the same PDL cell layer at day 15.

Having established the sub lethal concentration of BPs for PDL and pulp cells, we next tested the effect of BPs on growth and proliferation of cells. Both lethal and sublethal dosages of BPs were used to monitor cell proliferation. Equal number of PDL

and pulp cells (20,000/well) were plated in 12 well dish and one day later fed with media containing indicated dosage of BPs. Cells were then cultured for 5 days. Three independent samples of control and treated cells (3 wells) were harvested and counted every day.

PDL cells showed a progressive doubling until 3 days of culture in all treatment groups. Dose dependant decrease in cell number was observed at day 4 and 5 (Fig 5A). Pulp cells started showing inhibition of cell proliferation by day 3. Similar to PDL cells, a dose dependant decrease in cell number was noted for all concentrations throughout later time points (Fig 5B). It is important to note that by day 5 these concentrations do not show any sign of cytotoxicity and cell death and confirmed earlier observation (Fig 4). Thus the effect of BPs on the PDL and pulp cell proliferation is due to inhibition of cell cycle and is not related to cell death.

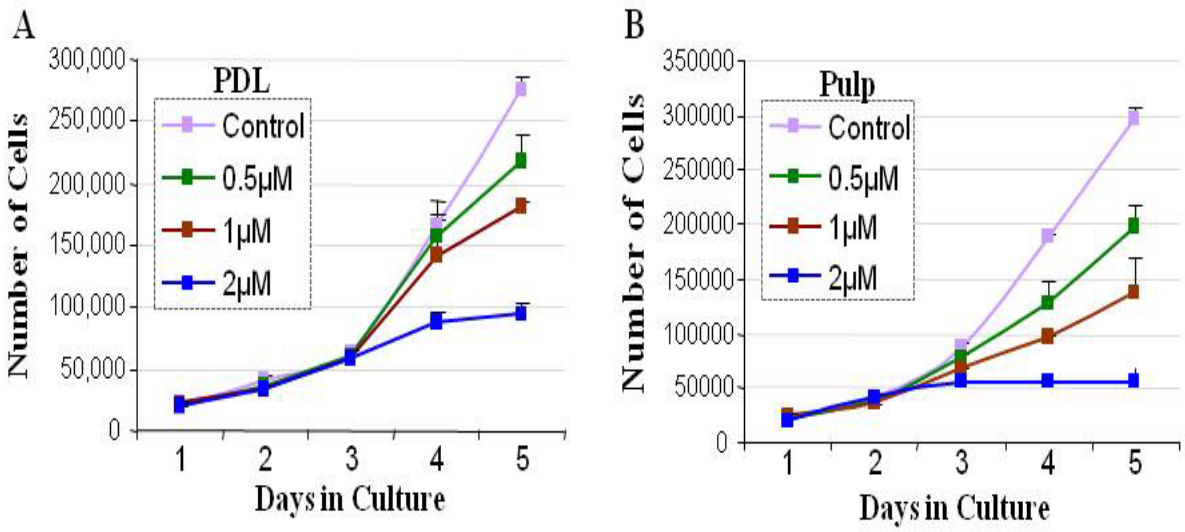

Figure 5: BPs reduced cell growth of PDL and pulp cells. PDL and Pulp cells were counted and 20,000 cells were plated per well in a 12 well plate. Cells were either fed

with control media or media containing 0.5μM, 1μM and 2μM of Zoledronic acid. Proliferation was monitored by counting cells at indicated days. For each time point and condition 3 independent determinations were made. Data are presented as mean value with standard deviation.

Bisphosphonate Promotes Osteogenic Differentiation of Pulp and PDL Cells by Upregulation of Runx2 and Osterix Expression

Exit from cell cycle is essential for lineage commitment and cell differentiation. Lack of cell death and inhibition of cell proliferation by BPs suggested that PDL and pulp cells may have exited the cell cycle. To define if this property of BPs will enhance osteogenic differentiation of PDL and pulp cells we performed biochemical, insitu and molecular analysis.

We first tested the effect of BPs on expression of the osteoblast master regulator Runx2 and Osterix. PDL and pulp cells from 3 independent patients were cultured for 4 days either in the presence or absence of 1μM BPs. Equal amount of nuclear extracts from these cells was fractionated by SDS PAGE. We observed an enhanced expression of Runx2 and Osterix in both PDL and pulp cells treated with BPs. The degree of increased Runx2 levels ranged from 20% -42% in PDL and 0.5-2.6 fold in pulp cells. Similarly BPs treatment elevated Osterix expression that ranged from 11%- 30% in PDL and 25% to 2 fold in pulp cells. Thus BPs stimulates expression of key osteoblast transcription factor in both PDL and pulp cells.

We further confirmed the BPs mediated increased expression of Runx2 and Osterix is associated with increased nuclear accumulation by IF. Control and BPs treated

cells (ten randomly selected individual nuclei) were analyzed by integrated signal intensity and line graph profile (Fig 7, 8). BPs treatment caused a 33% increase in Runx2 nuclear signal in PDL and 25% in pulp cells. Similarly Osterix nuclear signal was enhanced by 25% in PDL and 29% in pulp cells treated with BPs. Intensity profile across individual cells monitored by line graph analysis further confirmed these observations.

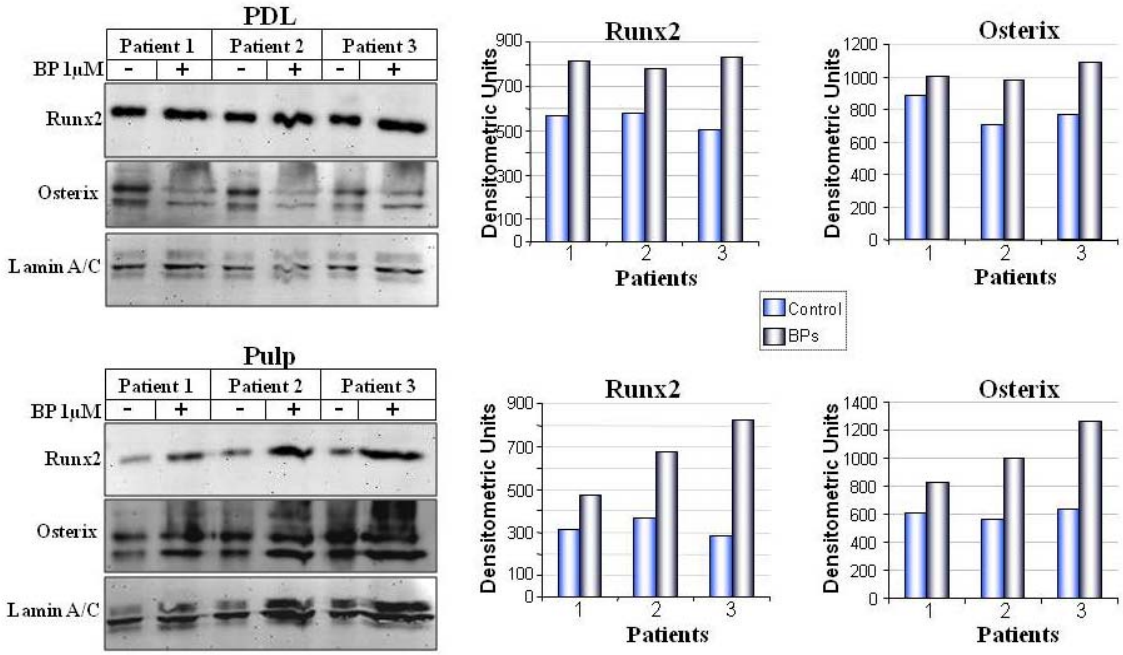

Figure 6: BPs enhances expression of Runx2 and Osterix. A) Cells from patients 1, 2 and 3 were cultured to confluency and then exposed to growth media containing 1μM Zoledronic acid for 4 days. Nuclear extract was isolated from these cells and equal amount (40μg) of protein was used to perform western blots analysis. Antibodies and blotting conditions are essentially as described earlier. Lamin A/C was used as an internal control. Panels on right represent densitometric graph of Runx2 and Osterix in PDL and pulp cells. Osterix protein is detected as two closely migrating bands representing post

translationally modified species. Signal from both Osterix bands were pooled for densitometric analysis. Alpha innotech software was used to calculate density of bands and signal intensity in the western blots.

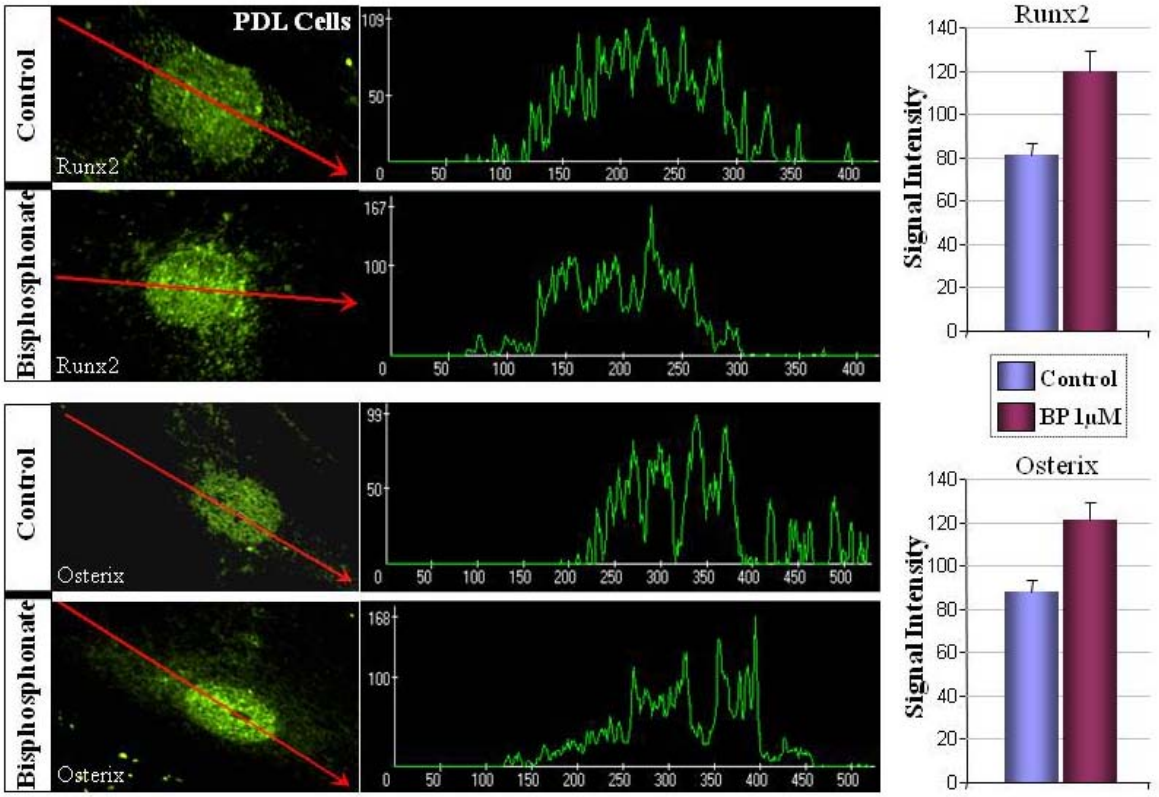

Figure 7: BPs treatment is associated with increased nuclear accumulation of Runx2 and Osterix in PDL cells. Insitu immunofluorescence analysis was performed on control and 1μM BPs treated PDL cells to detect endogenous Runx2 and Osterix proteins. Left panels show a representative cell image showing Runx2 and Osterix signal. An arrow was drawn across the long axis of the cell to monitor intensity peaks for Runx2 and Osterix signal (middle panels). Cumulative signal values from ten randomly selected

control and BPs treated cells are plotted as bar graph. Error bar represent mean standard deviations. Increased total intensity signal and line graph peaks were consistently noted in BPs treated cells.

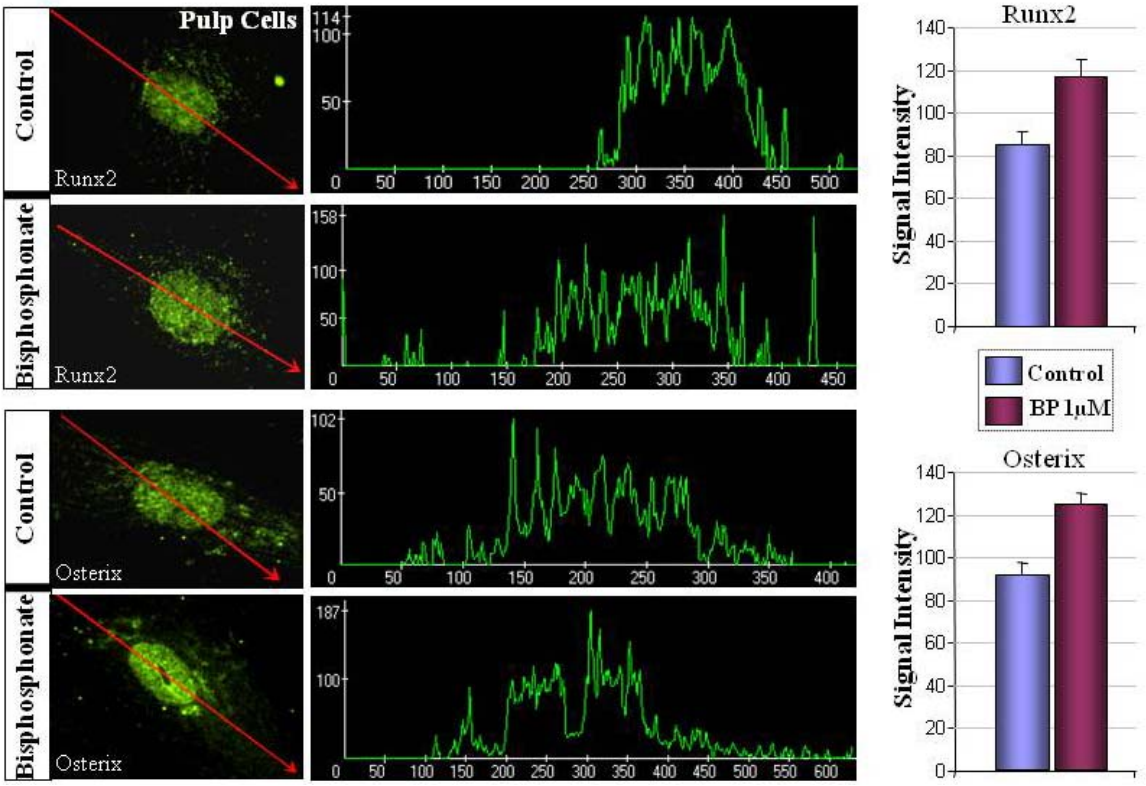

Figure 8: BPs treatment is associated with increased nuclear accumulation of Runx2 and Osterix in pulp cells. Insitu immunofluorescence analysis was performed on control and 1μM BPs treated pulp cells to detect endogenous Runx2 and Osterix proteins. All analyses were performed essentially as described in figure 7.

Thus BPs treatment results in induction of both Runx2 and Osterix. To better define if increased Runx2 and Osterix can provides the essential signal that drives mesenchymal

cells toward osteoblast lineage, we monitored expression of marker genes that characterize early and late differentiation stages. Cells were grown in the presence and absence of 1μM BPs for 4 days. Total RNA was harvested at day 1 and day 4 of differentiation. RT-PCR was used to examine the expression of osteoblast genes such as Osterix, ALP, OP and OC (Fig 9). Consistent with earlier observations we find an increase in relative levels of Osterix at both day 1 and 4 upon BPs treatment. Increased expression of early marker ALP, osteopontin and maturation marker osteocalcin was evident in BPs treated PDL and pulp cells. Equal loading was confirmed by equivalent expression of β-actin in all samples.

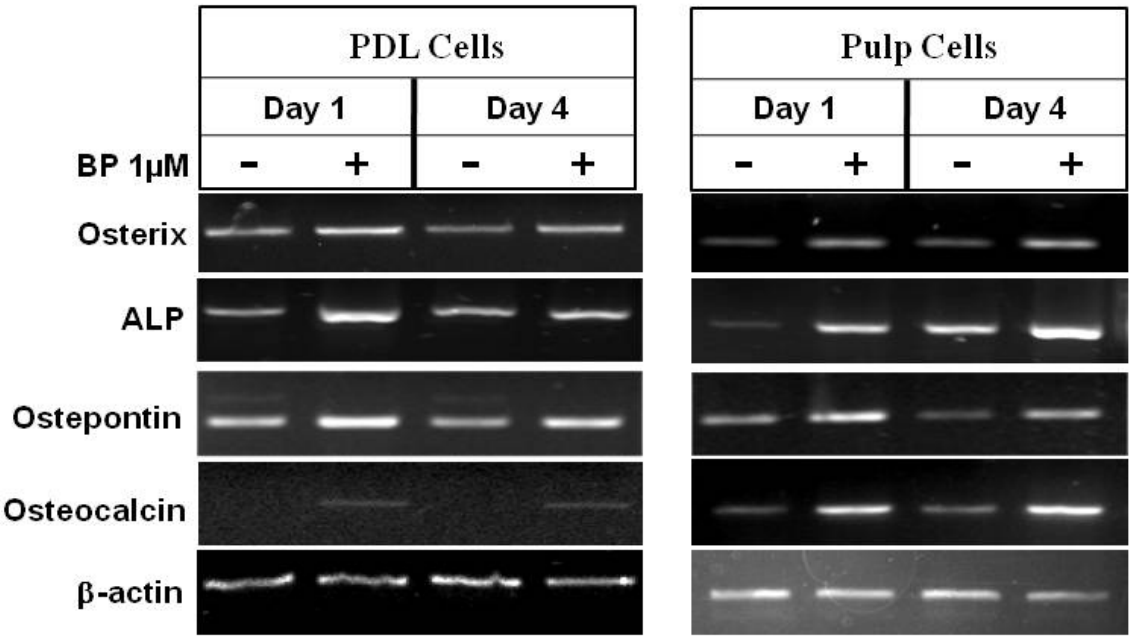

Figure 9: **Osteogenic differentiation of PDL and pulp cells is enhanced by BPs.** Confluent PDL and pulp cells were treated with 1μM BPs and total RNA isolated from day 1 and day 4 cultures. Purified RNA (1μg) was used to generate cDNA by RT-PCR.

Equal volume (1µl) of converted cDNA was subjected for PCR amplification of indicated genes. Initially, PCR cycle conditions were established that did not resulted in saturation of amplified product. Electrophoresis was performed using 2% agarose gels. Ethidium bromide stained gels were photographed and are presented as individual panel for each gene.

DISCUSSION

Bisphosphonates is the most potent class of drug used in the treatment of osteoporosis. The fact that this drug has high affinity to bind with hydroxyl apatite crystals helps them to be released over a period of time. The major cellular actions of BPs are known particularly for osteoclast [**45, 46, 47]**. BPs reduces the function of osteoclast and increases their apoptosis. In addition to the direct effects on osteoclasts, there is evidence that BPs can act indirectly on the osteoclasts via osteoblasts **[14-16].** It is likely that this indirect effect is due to modulation of osteoblast secretion of soluble paracrine factors that influence osteoclast activity **[17-21]**. Bisphosphonates regulate cell proliferation and differentiation of transformed human osteoblasts, and regulate osteoblastic synthesis of cytokines and growth factors **[39, 40].** Some patients on bisphosphonate drug therapy suffer from Osteonecrosis of jaw especially if they undergo any surgical intervention in the jaw. The reason and molecular mechanism for this selective behavior of BPs in oral tissue are not known.

In this study we explored the effect of BPs on human pulp and PDL tissue derived cells. We find that cells from both tissues are capable of osteogenic differentiation when provided appropriate stimuli and culture conditions. Both cells express Runx2 and Osterix, two factors genetically required for bone synthesis and development. Our data shows varying levels of these factors among eight patients. Theses inherent differences

may be related to parameters which were not studied in this project: such as age, genetics, race, habits and unknown disease status of individual donors.

Pulp cells appeared to be more sensitive to the cytotoxic effects of BPs. This property may reflect larger proportion of immature cells in pulp compared to dominant fibroblastic population in PDL tissue. However we did not observed adipocytic differentiation of the PDL or pulp cells in our hand (data not shown), suggesting these cells may be limited in their capacity to commit to other lineages. Interestingly, BPs treatment causes cessation of cell proliferation. This finding is consistent with published observation of osteoblast and osteoclast and may be related to exit from cell cycle.

BPs treatment stimulated osteogenic differentiation of both PDL and pulp cells. Markers of both early and late stages of osteoblast were induced. Induction of master osteogenic regulators Runx2 and Osterix may explain the activation of the osteoblast markers gene. These finding correlates well with the known invivo actions of BPs of prevention of bone loss by osteoclast suppression and activation of osteoblast function. However these data are inconsistent, with the selective bone loss in BPs associated ONJ. It is possible that increased osteoblast differentiation and bone formation, coupled with decreased osteoclastic bone resorption will deregulate the remodeling process. The “denser bone” may be a target of hypoxic necrosis as it will be devoid of vasculature. Other factors such as constant loading and unloading, which is ten times higher in jaw bone can contribute to ONJ along with the BPs.

CONCLUSION

PDL and pulp derived mesenchymal cells exhibit osteogenic capacity and pulp cells are comparatively more sensitive to bisphosphonate then PDL cells. Furthermore, our study reveals BPs promotes osteogenic differentiation of PDL and pulp cells by several mechanisms. Firstly BPs decreases cell proliferation and perhaps causes the cell to exit cell cycle. Secondly, BPs directly upregulates expression of major osteoblast regulator Runx2 and Osterix. The increased levels of Runx2 and Osterix are sufficient to induce expression of genes considered hallmark of early and mature osteoblast.

FUTURE STUDIES

PDL and pulp cells have the inherent capacity to differentiate towards osteoblastic lineage when cultured in osteogenic media. However our results do not distinguish if this is due to a transdifferentiation of PDL and pulp fibroblast or undifferentiated mesenchymal cells. In such case the presence of undifferentiated mesenchymal cells will be tested by activity of cells to undergo differentiation into other lineages such as adipocytes and chondrocytes.

BPs treatment resulted in an increase in Runx2 and Osterix mRNA and protein synthesis. Transcription factor must bind specific DNA sequence in target gene promoters to execute their transcriptional function. To test if BPs mediated enhanced Runx2 and Osterix protein is also associated with increase DNA binding we will perform EMSA (Electrophoretic mobility shift assay) studies.

We have seen an increase in osteogenic differentiation by BPs treatment as monitored by ALP, OC and OP gene expression. Levels of Runx2 and Osterix are similarly enhanced in these samples, however their increased expression does not prove direct involvement in activation of target genes. To establish a direct role of Runx2 and Osterix transcriptional activation, BPs stimulated cells will be transfected with RNAi oligos to knock down Runx2 and Osterix expression. These future experiments will confirm if BPs promote osteogenic differentiation through Runx2 and Osterix activity.

BPs mediates osteogenic changes in the PDL and pulp cells. However it is not clear if this is accomplished by A) incorporation of BPs into the cell or by B) BPs action with the cell surface receptor only and transduce its signal through other pathways. We will label the BPs either chemically or by radioactive compounds to test if BPs remains membrane bound or internalized by the PDL and pulp cells.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and expires on October 26, 2010. The Assurance number is FWA00005960.

Principal Investigator: SITTITAVORNWONG, SOMSAK

Co-Investigator(s):

Protocol Number: **X060705005**

Protocol Title: *Transcriptional Regulation of Runt-Related Transcription Factor-2 (Runx2) in the Pulp and Periodontal Ligament Fibroblasts during Osteogenic and Cell Differentiation*

The IRB reviewed and approved the above named project on 6-18-08. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 6-18-08

Date IRB Approval Issued: 6-18-08

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

470 Administration Building
701 20th Street South
205.934.3789
Fax 205.934.1301
irb@uab.edu

The University of
Alabama at Birmingham
Mailing Address:
AB 470
1530 3RD AVE S
BIRMINGHAM AL 35294-0104

Project Revision/Amendment Form

(PLEASE TYPE: In MS Word, highlight the shaded, underlined box and replace with your text; double-click checkboxes to check/uncheck.)

- Federal regulations require IRB approval before implementing proposed changes.
- Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.).
- Complete this form and attach the changed research documents.

Today's Date: *08/26/2008* 8/26/08

1. Contact Information

Principal Investigator's Name: Somsak Sittitavornwong BlazerID: **sjade**
E-mail: **sjade@uab.edu**
Contact Person's Name: Somsak Sittitavornwong BlazerID: **sjade**
E-mail: **sjade@uab.edu**
Telephone: 996-2456 Fax: 975-6671
Campus Address: **SDB 419, Oral and Maxillofacial Surgery, School of Dentistry, Birmingham, AL 35294-0007**

2. Protocol Identification

Protocol Title: **Transcriptional Regulation of Runt-Related Transcription Factor-2 (Runx2) in the Pulp and periodontal Ligament Fibroblasts during Osteogenic and Cell Differentiation.**
IRB Protocol Number: **X060705005**

Current Status of Project (check only one):

- ☒ Currently in Progress (Number of participants entered:_____)
- ☐ Study has not yet begun (No participants entered)
- ☐ Closed to participant enrollment (remains active)—
  Number of participants on therapy/intervention:_____
  Number of participants in long-term follow-up only:_____
- ☐ Closed to participant enrollment (data analysis only)—
  Total number of participants enrolled:_____

This submission changes the status of this study in the following manner (check all that apply):

- ☐ Protocol Revision
- ☐ Protocol Amendment
- ☐ Study Closed to participant entry
- ☐ Study Closure
- ☐ Revised Consent Form
- ☐ Addendum (new) consent form
- ☐ Enrollment temporarily suspended by sponsor
- ☐ Change in protocol personnel
- ☒ Other, (specify) *I need to add another investigator to the protocol . His information is as follows:*

Name- Nachiket Saoji
E-mail- nachiket@uab.edu
Blazer ID – nachiket
Telephone – 996-5767

3. Reason for change

Briefly describe, and explain the reason for, the change. If normal, healthy controls are included, describe in detail how this change will affect those participants.
Include a copy of the protocol and any other documents affected by this change (e.g., consent form, questionnaire) with all the changes highlighted.

_____

4. Does this change revise or add a genetic or storage of samples component?

☐Yes ☒No

If yes, please see the Guidebook to assist you in revising or preparing your submission, or call the IRB office at 934-3789.

5. Does the change affect subject participation (e.g. procedures, risks, costs, etc.)?

☐Yes ☒No

6. Does the change affect the consent document(s)? ☐Yes ☒No

If yes, briefly discuss the changes. _____

Include the revised consent document with the changes highlighted.

Will any participants need to be reconsented as a result of the changes? ☐Yes ☒No

If yes, when will participants be reconsented?_____

Signature of Principal Investigator Sarah Silverman Date 8/26/08

DOLA 6/18/08

APPROVED
Marilyn Doss 8-29-08
MARILYN DOSS, M.A.
Vice Chair – IRB