

Static compression regulates OPG expression in periodontal ligament cells via the CAMK II pathway

Jianru YI¹, MeiLe LI¹, Yan YANG¹, Wei ZHENG², Yu LI¹, Zhihe ZHAO¹

1- Sichuan University, West China School and Hospital of Stomatology, State Key Laboratory of Oral Diseases, Department of Orthodontics, Chengdu, China.

2- Sichuan University, West China School and Hospital of Stomatology, State Key Laboratory of Oral Diseases, Department of Oral and Maxillofacial Surgery, Chengdu, China.

Corresponding address: Yu Li - 14#, 3rd Section - South Renmin Road - Chengdu 610041 - PR - China - Phone: 028-85503645 - e-mail: yuli@scu.edu.cn

Submitted: April 21, 2015 - **Modification:** August 31, 2015 - **Accepted:** September 1, 2015

ABSTRACT

Objective: This study aimed to investigate the potential role of CAMK II pathway in the compression-regulated OPG expression in periodontal ligament cells (PDLs). **Material and Methods:** The PDL tissue model was developed by 3-D culturing human PDLs in a thin sheet of poly lactic-co-glycolic acid (PLGA) scaffolds, which was subjected to static compression of 25 g/cm² for 3, 6 and 12 h, with or without treatment of KN-93. After that, the expression of OPG, RANKL and NFATC2 was investigated through real-time PCR and western blot analysis. **Results:** After static compression, the NFATC2 and RANKL expression was significantly up-regulated, while partially suppressed by KN-93 for 6 and 12 h respectively. The OPG expression was significantly down-regulated by compression in 3 h, started to elevate in 6 h, and significantly up-regulated in 12 h. The up-regulation after 12 h was significantly suppressed by KN-93. **Conclusions:** Long-term static compression increases OPG expression in PDLs, at least partially, via the CAMK II pathway.

Keywords: Osteogenesis. Periodontal ligament. Calcium-calmodulin-dependent Protein kinases. Mechanotransduction.

INTRODUCTION

Mechanical loading has long been deciphered to regulate bone metabolism, and plays critical roles in orthodontic tooth movement (OTM)^{4,10,22}. When teeth are subjected to orthodontic force, the remodeling of the adjacent periodontium is initiated. The static compression induces bone resorption at the pressure side, resulting in a "loose" tooth, followed by bone formation to complete a bone remodeling cycle, which restores the periodontium and strengthens the tooth again²⁹. Lack of this recovery process may lead to alveolar bone loss followed by gingival recession and tooth removability.

The molecular mechanisms under bone remodeling in OTM has been extensively investigated. Numerous studies have focused on osteoclastogenesis, the rate-limiting step in OTM^{1,12,16}. Particularly, COX-2 has been shown to induce up-regulation of receptor activator for

nuclear factor- κ B ligand (RANKL), an essential pro-osteoclastogenic factor, in periodontal ligament cells (PDLs) under compression¹⁹. However, little is known about the pathways to regulate the mechano-induced expression of osteoprotegerin (OPG), the decoy receptor of RANKL, which inhibits bone resorption while promoting bone formation⁶.

The Ca²⁺/calmodulin-dependent kinase (CAMK) family has been recognized as a key mediator in living organisms and various biological processes⁹. Recent studies have revealed its critical role in bone development and homeostasis. The CAMK II pathway has been found to regulate the RANKL-induced osteoclast formation via the cAMP-response element binding protein (CREB) pathway³. The involvement of CAMK II in regulating the RANK-MEK-ERK pathway has been detected²¹. Moreover, the CAMK II-CREB pathway was proposed to play an important role in bone homeostasis.

We previously established a periodontal

ligament tissue model (PDLtm) to simulate the bioprocess at the pressure side of PDL¹⁸. Interestingly, through microarray screening, we found up-regulation of the CAMK II pathway in the loaded PDLCs¹⁷, which is known to respond to compression and result in OPG enhancement in osteoblast¹¹. Therefore, in the present study, we aimed to investigate the potential role of CAMK II pathway in the expression of OPG and RANKL in the PDLtm under static compression.

MATERIAL AND METHODS

Preparation of PLGA scaffold

The poly lactic-co-glycolic (PLGA) polymers (10⁵ g/mol) were used for the synthesis of the scaffolds as previously described¹⁷. Briefly, 200 mg mixture of PLGA polymers and sucrose particulates with a volume ratio of 15:85 was applied with compression to form a thin sheet in a square mould (2 cm × 2 cm). Subsequently, the sheets were placed in a CO₂ reaction kettle for 48 h, and the sucrose was then removed by immersing the PLGA sheets in ddH₂O for 48 h. After that, the PLGA sheets (2 cm×2 cm×300 μm) were packaged and sterilized for experimental use.

Establishment of the PDLtm

Five lines on human PDLCs were established following a well-documented method¹⁷. The periodontal tissue was collected from the teeth extracted for orthodontic reasons with the donors signing an informed consent form and the approval of the Institutional Review Board in our hospital. The PDLCs of the 3-6th passage were used for this experiment. The 3-D PDLtm was established by dripping suspension of PDLCs into the PLGA sheet that was put in a 6-well plate, approximate 1×10⁵ cells in 2 ml medium *per* sheet. After 24 h, the PDLtm was displaced to another 6-well plate. Each cell line (n=5) was used for the experiment in three replicates, two of which were pooled together for western blot and one for real-time PCR.

Histological observation

Four days after the establishment of PDLtm, the growth of PDLCs in scaffolds was investigated by microscopic observations. First, the PDLtm was

stained by acridine orange (0.01%) and observed under a fluorescence inverted microscope (Leica DMI6000B, Germany). Second, the PLGA/PDLC construct was prepared for scanning of electron microscopy (SEM, Inspect F, FEI, USA)¹⁷.

Application of compressive force

The PDLtms were randomly assigned into three groups, i.e., the control group, the compression group (Cg) and the compression+KN-93 (an inhibitor of the CAMK II pathway) group (CKg). To simulate the pressured periodontium in OTM, a modified "weight" method was used. Briefly, a cover glass and a bottle of granules were placed on the PDLtm to produce a compression of 25 g/cm², which has been proved to be the optimal force level for this model¹⁸. In the compression+KN-93 group, 0.01 mM KN-93 (Sigma) was added to the media to suppress the CAMK II pathway.

Real-time PCR

Three, six and twelve hours after applying interventions, total RNA (n=5) was extracted by dissolving the PDLtm using TRIZOL reagent (Invitrogen, Carlsbad, USA). The quality and integrity of extracted RNA samples were validated before their use. Real-time PCR was performed with a SYBR Green reaction Kit (Roche Diagnostics, China) in a LightCycler according to the manufacturer's instruction to investigate the mRNA expression of RANKL, OPG, and Nuclear factor of activated T-cells (NFAT) C2. GAPDH served as the internal control. The sequences of relevant primers were shown in Figure 1.

Western blotting

Twelve hours after force application, the total proteins (n=5) were collected using total protein extraction kit (Keygen Biotech, China). The 15 μL prepared samples (40 μg of protein) per lane were separated by SDS-PAGE and then transferred to the polyvinylidene difluoride (PVDF) membrane. After that, the PVDF membranes were probed with antibodies to RANKL (1:1000, Santa-Cruz, USA), OPG (1:1000, Santa-Cruz, USA) GAPDH (1:1000, Beyotime, China) overnight at 4°C. Subsequently, the membranes were immersed in secondary antibody (1:5000, Beyotime, China) for

Gene	GeneBank	Primer sequence (5'-3')	
		Reverse	Forward
RANKL	NM_033012	GACAGACTCACTTTATGGGAACC	CAACATATCGTTGGATCACAGCA
OPG	NM_002546	CCACTCCAAATCCAGGAGGG	GTGTGCGAATGCAAGGAAGG
NFATC2	NM_173091	CCAGAGAGACTAGCAAGGGG	GAGCCGAATGCACATAAGGTC
GAPDH	NM_002046	GTAGAGGCAGGGATGATGTTCT	CTTTGGTATCGTGGAAGGACTC

Figure 1- Primer used in real-time PCR analysis

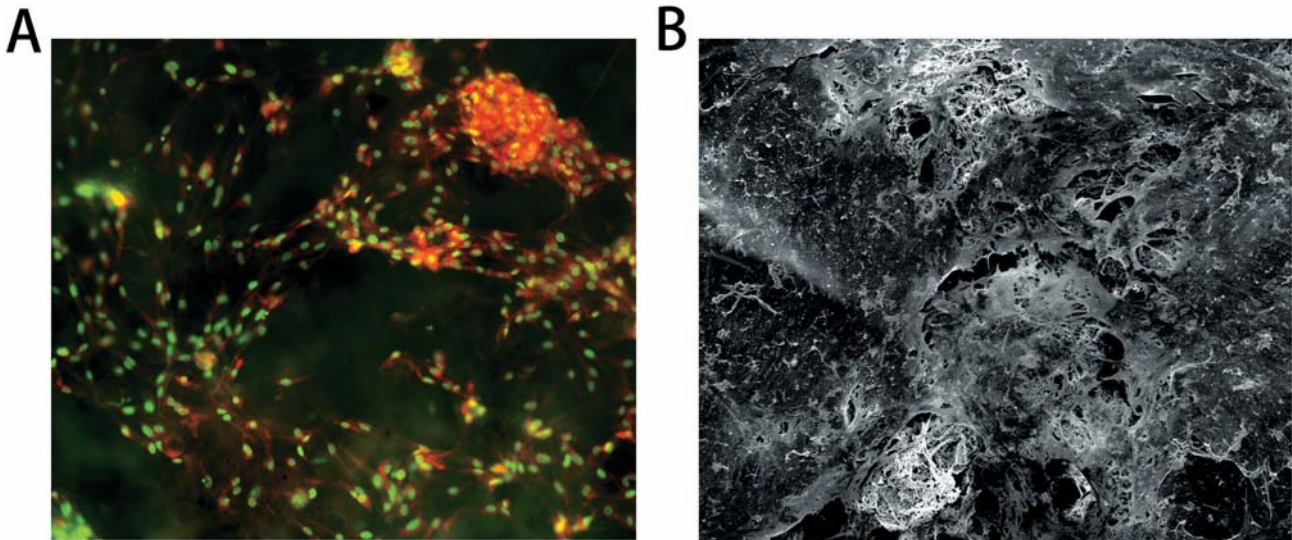


Figure 2- The microscopic observations of PDLtm. (A) Spindle PDLcs grew densely with nuclei in green-yellow and cytoplasm in orange-red, 200x; (B) The PDLcs, secreted extracellular matrix (SEM) and PLGA scaffolds were integrated, 300x

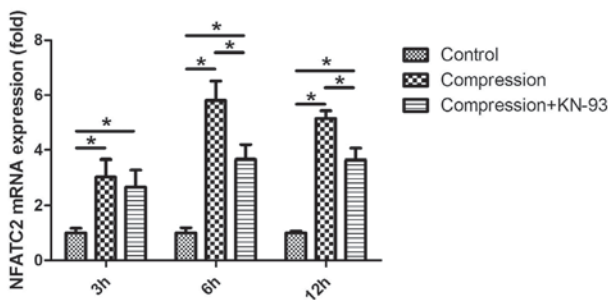


Figure 3- The time-course expression of NFATC2 in loaded PDLcs at mRNA level. Compression enhanced the NFATC2 expression throughout the experiment, while its expression was inhibited by KN-93 after 6 and 12 h. * $p < 0.05$

1 h. The immunoreactive proteins were visualized by a chemiluminescence kit (Millipore). The band intensities were evaluated using Quantity One software (Bio-Rad, Hercules, USA).

Statistical analysis

All data was expressed as mean \pm SD. The comparison among 3 groups was conducted by one-way analysis of variance (ANOVA) followed by LSD *post hoc* test using SPSS software of version 13.0. Differences with $p < 0.05$ were set as significant.

RESULTS

The 3-D cultured PDLcs

By acridine orange staining, the growth of PDLcs was observed under microscope (Figure

2A). In addition, the secreted extracellular matrix, the PDLcs and the scaffolds were observed to be interconnected under SEM (Figure 2B). More characterization of this PDLtm has been previously shown¹⁸.

The NFATC2 expression

Under static compression, the NFATC2 expression was significantly up-regulated, which peaked in 6 h (Figure 3). KN-93 partially suppressed the compression-induced up-regulation of NFATC2 in 6 and 12 h.

The OPG/RANKL expression

The real-time PCR analysis revealed the significant up-regulation of RANKL expression under static compression, which peaked in 6 h (Figure 4A). In contrast, the OPG expression was significantly down-regulated after 3 h, while up-regulated in 12 h (Figure 4B). The OPG/RANKL ratio was reduced in 3 and 6 h while enhanced in 12 h (Figure 4C).

Notably, after 12 h, the up-regulation of RANKL, OPG and OPG/RANKL ratio was partially suppressed by KN-93, suggesting that the CAMK II pathway took part in the up-regulation of RANKL and OPG in PDLcs induced by long-term stimulation of static compression (Figure 4).

Moreover, the western blotting method showed results similar to the PCR assay, indicating that after 12 h of static compression there was an increased protein expression of RANKL and OPG, which was partially suppressed by KN-93 (Figure 5).

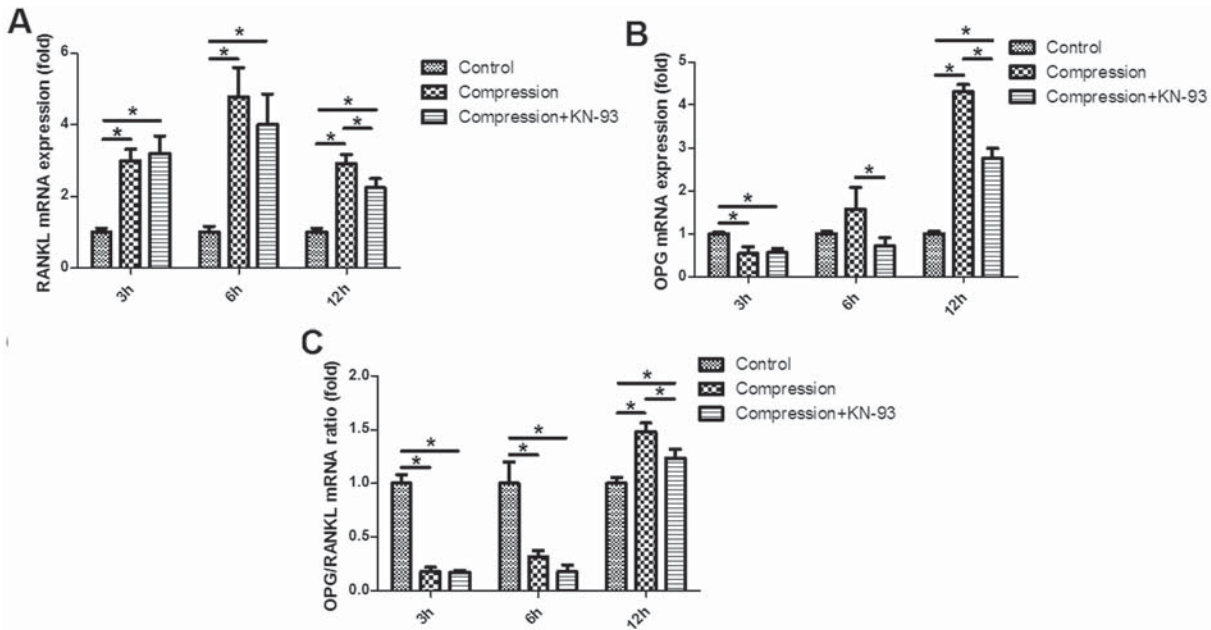


Figure 4- The time-course expression of osteoprotegerin (OPG) and RANKL in loaded PDLCs at mRNA level. (A) RANKL; (B) OPG; (C) OPG/RANKL ratio. * $p < 0.05$

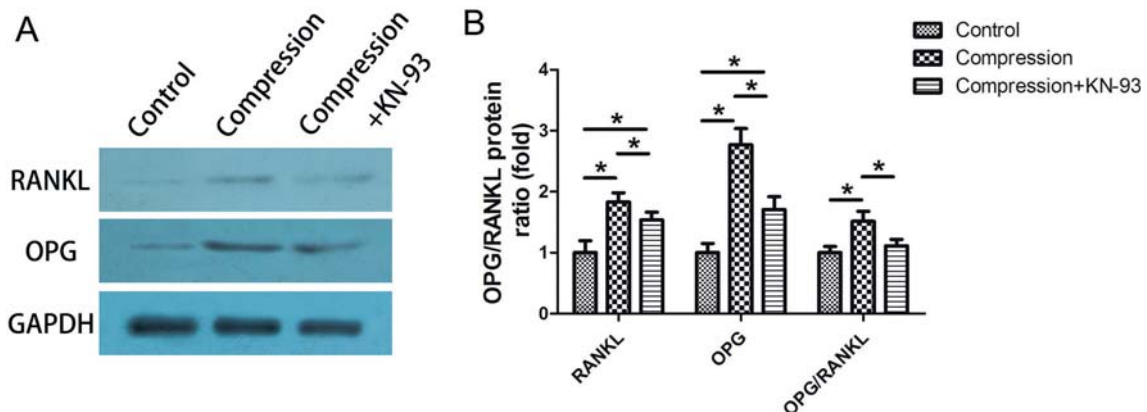


Figure 5- (A) The protein expression of osteoprotegerin (OPG) and RANKL in the loaded PDLCs after 12 h. The static compression significantly elevated the OPG and RANKL expression in PDLCs, while the enhancement was suppressed by KN-93; (B) The fold change of the expression of OPG, RANKL and OPG/RANKL ratio. * $p < 0.05$

DISCUSSION

Orthodontic tooth movement (OTM) has been generally featured with bone remodeling, a fundamental biological process consisting of bone resorption and bone formation²³. Particularly, the initiated bone resorption at compression sites results in tooth movement, and the following bone formation surrounding roots strengthen the new position of the teeth²⁹. Numerous studies focused on the osteoclastogenesis at the pressure side and sketched the contour of underlying mechanisms^{12,13}, while the subsequent bone formation was less studied.

The CAMK family contains a series of proteins playing critical roles in bone modeling and remodeling. Afamin enhances osteoclastogenesis

by decreasing intracellular cAMP levels via the CAMK pathways¹⁴. The role of the CAMK II pathway in the parathyroid hormone-related protein (PTHrP)-regulating osteoclast inhibitory action has been recently identified³². Though KN-93 was observed to exert effects on other pathways⁸, it has been applied as a classic CAMK II pathway inhibitor in many studies^{25,27}. Recently, by using KN-93, the enhancement of OPG in osteoblast under mechanical stimuli was suppressed¹¹. However, limited information concerning its effect on OTM is available till now.

The microarray screening for gene expression profiles in our previous study has revealed the potential role of the CAMK II pathway in the mechanoreponse of PDLCs¹⁷. In the present study, PDLCs were embedded in 3-D PLGA scaffolds and

cultured under static compressive force. The ratio of OPG/RANKL expression was significantly down-regulated 3 and 6 h after loading, indicating a potential role of osteoclastogenesis induction^{24,31}. On the other hand, the OPG expression declined in 3 h, while started to elevate in 6 h and was significantly up-regulated after 12 h. As an important anti-osteoclastic and pro-osteogenic factor, the marked up-regulation of OPG after 12h indicates suppression for bone resorption¹⁵. However, this elevation was greatly impeded by KN-93, a specific CAMK II pathway inhibitor, suggesting that CAMK II pathway takes part in the OPG up-regulation induced by long-term static compression stimulation. Notably, the CAMK II pathway has also been reported to regulate the mechano-induced OPG enhancement in osteoblast¹¹, which is to a great extent similar to PDL fibroblast².

The RANKL–RANK–OPG axis mediates osteoclast formation through activation of RANK on the osteoclast precursors by RANKL²⁸. Although the constitutional expression of OPG in PDLCs is much higher than RANKL²⁶, numerous studies demonstrated the comparatively slight up-regulation of RANKL in PDLCs induced by compressive force could promote osteoclast formation and the following tooth movement^{13,18}. Therefore, the large ratio of total OPG vs. RANKL seems meaningless accounting for osteoclastic induction. A reasonable explanation has been given that it could be due to the tight cell–cell contact between PDLCs and osteoclast precursors, which could create a favorable micro-environment for RANKL–RANK binding, thereby preventing the interaction of OPG with RANKL⁵.

On the other hand, at the sites away from the cell–cell contact area, the expression of OPG could play an important role. In contrast to the study reporting reduced or unchanged OPG expression in PDLCs under compression²⁰, in the present study the OPG expression was significantly up-regulated after long-term mechanical stimuli, consistent with our previous data¹⁸. The delayed but marked increase of OPG could account for the subsequent bone formation at pressure periodontium, which prevents from alveolar bone loss and strengthens tooth again after movement. In this sense, targeting the CAMK II pathway might potentially benefit the OTM periodontium by stimulating OPG expression.

In the present study, we observed that the expression of NFATC2 in PDLCs was enhanced by compressive force while partially inhibited by KN-93 treatment (Figure 3). NFATC2 is a transcription factor which plays an undisputable role in the mechanoresponse of bone tissue³⁰. As downstream factors of Wnt-Ca²⁺ pathways, both NFATC2

and CAMK II are involved in the Wnt-Ca²⁺ bone formation regulated by pathways⁷. Interestingly, Wnt-Ca²⁺ pathways were up-regulated when PDLCs were treated with compressive force in our previous study¹⁷. Therefore, the enhancement of OPG in PDLCs under compressive force might be regulated via Wnt-Ca²⁺ pathways, which should be further identified in future.

Last but not the least, it is interesting to compare the masticatory force and orthodontic force, both of which are transmitted to alveolar bone through PDL, while it results in opposite effects on alveolar bone metabolism. Obviously, the present theory of “compression-PDL-osteoclastogenesis” process cannot interpret this contradictory phenomenon. Based on our results, it is reasonable to speculate that the CAMK II pathways may be much more sensitive to masticatory force (intermittent force) than orthodontic force (static force), which should be further identified.

CONCLUSION

The OPG expression was significantly up-regulated in PDLCs after long-term static compression stimulation, which is at least partially regulated by the CAMK II pathway. These results have enriched the present understanding to molecular mechanisms in bone remodeling modulation in OTM.

ACKNOWLEDGEMENT

This study was supported by NSFC - National Natural Science Foundation of China (11372202 and 31201087), and by Sichuan University Fund for Outstanding Young Scholars (2082604164243).

REFERENCES

- 1- Alhashimi N, Frithiof L, Brudvik P, Bakhiet M. Orthodontic tooth movement and de novo synthesis of proinflammatory cytokines. *Am J Orthod Dentofacial Orthop.* 2001;119(3):307-12.
- 2- Alves LB, Mariguela VC, Grisi MF, Souza SL, Novaes Junior AB, Taba Junior M, et al. Expression of osteoblastic phenotype in periodontal ligament fibroblasts cultured in three-dimensional collagen gel. *J Appl Oral Sci.* 2015;23(2):206-14.
- 3- Ang ES, Zhang P, Steer JH, Tan JW, Yip K, Zheng MH, et al. Calcium/calmodulin-dependent kinase activity is required for efficient induction of osteoclast differentiation and bone resorption by receptor activator of nuclear factor kappa B ligand (RANKL). *J Cell Physiol.* 2007;212(3):787-95.
- 4- Araújo AS, Fernandes AB, Maciel JV, Netto JN, Bolognese AM. New methodology for evaluating osteoclastic activity induced by orthodontic load. *J Appl Oral Sci.* 2015;23(1):19-25.
- 5- Bloemen V, Schoenmaker T, de Vries TJ, Everts V. Direct cell-cell contact between periodontal ligament fibroblasts and osteoclast precursors synergistically increases the expression of genes related to osteoclastogenesis. *J Cell Physiol.* 2010;222(3):565-73.
- 6- Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys.* 2008;473(2):139-46.

- 7- Chen Y, Alman BA. Wnt pathway, an essential role in bone regeneration. *J Cell Biochem.* 2009;106(3):353-62.
- 8- Gao L, Blair LA, Marshall J. CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. *Biochem Biophys Res Commun.* 2006;345(4):1606-10.
- 9- Hoeflich KP, Ikura M. Calmodulin in action: diversity in target recognition and activation mechanisms. *Cell.* 2002;108(6):739-42.
- 10- Huiskes R, Ruimerman R, van Lenthe GH, Janssen JD. Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. *Nature.* 2000;405(6787):704-6.
- 11- Kaneuji T, Ariyoshi W, Okinaga T, Toshinaga A, Takahashi T, Nishihara T. Mechanisms involved in regulation of osteoclastic differentiation by mechanical stress-loaded osteoblasts. *Biochem Biophys Res Commun.* 2011;408(1):103-9.
- 12- Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res.* 2001;80(3):887-91.
- 13- Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. *J Bone Miner Res.* 2002;17(2):210-20.
- 14- Kim BJ, Lee YS, Lee SY, Park SY, Dieplinger H, Yea K, et al. Afamin stimulates osteoclastogenesis and bone resorption via G-coupled receptor and Ca²⁺/calmodulin-dependent protein kinase (CaMK) pathways. *J Endocrinol Invest.* 2013;36(10):876-82.
- 15- Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature.* 1999;402(6759):304-9.
- 16- Lee YH, Nahm DS, Jung YK, Choi JY, Kim SG, Cho M, et al. Differential gene expression of periodontal ligament cells after loading of static compressive force. *J Periodontol.* 2007;78(3):446-52.
- 17- Li Y, Li M, Tan L, Huang S, Zhao L, Tang T, et al. Analysis of time-course gene expression profiles of a periodontal ligament tissue model under compression. *Arch Oral Biol.* 2013;58(5):511-22.
- 18- Li Y, Zheng W, Liu JS, Wang J, Yang P, Li ML, et al. Expression of osteoclastogenesis inducers in a tissue model of periodontal ligament under compression. *J Dent Res.* 2011;90(1):115-20.
- 19- Liu L, Igarashi K, Kanzaki H, Chiba M, Shinoda H, Mitani H. Clodronate inhibits PGE(2) production in compressed periodontal ligament cells. *J Dent Res.* 2006;85(8):757-60.
- 20- Nishijima Y, Yamaguchi M, Kojima T, Aihara N, Nakajima R, Kasai K. Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement and effect of compression force on releases from periodontal ligament cells *in vitro*. *Orthod Craniofac Res.* 2006;9(2):63-70.
- 21- Park-Min KH, Ji JD, Antoniv T, Reid AC, Silver RB, Humphrey MB, et al. IL-10 suppresses calcium-mediated costimulation of receptor activator NF-kappa B signaling during human osteoclast differentiation by inhibiting TREM-2 expression. *J Immunol.* 2009;183(4):2444-55.
- 22- Ren Y, Maltha JC, Kuijpers-Jagtman AM. Optimum force magnitude for orthodontic tooth movement: a systematic literature review. *Angle Orthod.* 2003;73(1):86-92.
- 23- Roberts WE, Goodwin WC Jr, Heiner SR. Cellular response to orthodontic force. *Dent Clin North Am.* 1981;25(1):3-17.
- 24- Sağlam M, Köseoğlu S, Hatipoğlu M, Esen HH, Köksal E. Effect of sumac extract on serum oxidative status, RANKL/OPG system and alveolar bone loss in experimental periodontitis in rats. *J Appl Oral Sci.* 2015;23(1):33-41.
- 25- Saito A, Miyajima K, Akatsuka J, Kondo H, Mashiko T, Kiuchi T, et al. CaMKIIbeta-mediated LIM-kinase activation plays a crucial role in BDNF-induced neuritogenesis. *Genes Cells.* 2013;18(7):533-43.
- 26- Sokos D, Everts V, de Vries TJ. Role of periodontal ligament fibroblasts in osteoclastogenesis: a review. *J Periodontal Res.* 2015;50(2):152-9.
- 27- Souza CF, Carneiro AB, Silveira AB, Laranja GA, Silva-Neto MA, Costa SC, et al. Heme-induced *Trypanosoma cruzi* proliferation is mediated by CaM kinase II. *Biochem Biophys Res Commun.* 2009;390(3):541-6.
- 28- Tanaka S. Signaling axis in osteoclast biology and therapeutic targeting in the RANKL/RANK/OPG system. *Am J Nephrol.* 2007;27(5):466-78.
- 29- Wise GE, King GJ. Mechanisms of tooth eruption and orthodontic tooth movement. *J Dent Res.* 2008;87(5):414-34.
- 30- Wu Y, Yang Y, Yang P, Gu Y, Zhao Z, Tan L, et al. The osteogenic differentiation of PDLSCs is mediated through MEK/ERK and p38 MAPK signalling under hypoxia. *Arch Oral Biol.* 2013;58(10):1357-68.
- 31- Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A.* 1998;95(7):3597-602.
- 32- Zheng F, Liang H, Liu R, Quan JX, Li XX, Dai CL, et al. Parathyroid hormone-related protein regulates osteoclast inhibitory lectin expression via multiple signaling pathways in osteoblast-like cells. *Endocrine.* 2009;35(1):47-56.