Int J Clin Exp Pathol 2015;8(9):11895-11900
www.ijcep.com /ISSN:1936-2625/IJCEP0013744

Original Article

Expression and function of osteogenic genes runt-related transcription factor 2 and osterix in orthodontic tooth movement in rats

Jinyou Han[1,2], Hong He[1]

[1]*Department of Orthodontics, School & Hospital of Stomatology, Wuhan University, Hubei Province, Wuhan 430079, China;* [2]*Liaocheng People's Hospital, Shandong Province, China*

Received July 31, 2015; Accepted August 28, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Objective: To investigate the expression and function of osteogenic genes osterix (OSX) and runt-related transcription factor 2 (RUNX2) in the rat periodontal tissues under orthodontic force for the remodeling of the periodontal tissues. Methods: 24 Wistar rats were randomly divided into 4 groups of orthodontic tooth movements for 1, 3, and 7 days (experimental groups) and control group (without orthodontic force). The expression of RUNX2 and OSX in the periodontal tissues was analyzed using real time PCR for mRNA and Western blot analysis for protein. The data were also analyzed for involvement of the two genes in signal pathways using bioinformatics tools. Results: The mRNA levels of RUNX2 and OSX increased in the periodontal tissues after subjected to the orthodontic force for 1 to 7 days, with the highest level occurring at day 7. The relative expression levels of RUNX2 and OSX mRNA were 1.85±0.12, 304±0.06 and 4.16±0.068, and 1.52±0.09, 1.83±0.03 and 2.56±0.06 at day 1, 3 and 7, respectively. The results of Western blot analysis were consistent with the mRNA results. Conclusion: In orthodontic tooth movement, the expression of RUNX2 and OSX was upregulated as a result of external stimulation, suggesting that the two genes is involved in periodontal tissue remodeling and plays an important role in periodontal tissue remodeling.

Keywords: Orthodontic force, periodontal tissue, osteogenic gene

Introduction

Orthodontic treatment applies orthodontic force to the tooth through a variety of appliances to treat wrong jaw deformity and can improve occlusion to address the esthetic and functional problems. However, its longer treatment period has limited its clinical application. Therefore, effective control of tooth movement and shortening of the treatment cycle are important clinical issues to be addressed for better treatment [1, 2].

Orthodontic tooth movement is a complex biological and mechanical process involving a variety of molecules. Orthodontic force exerted on the periodontal tissue can activate osteoblast and osteoclast, resulting in their resorption and deposition in the alveolar bone, and stimulate the growth of cells and regeneration of collagen [3]. More specifically, the cells can be induced by the mechanical force to degenerate collagen on the pressed side and enhance bone absorption. Under the force, cells in the extended pericementum begin to proliferate and bone tissue begins to deposit, resulting in bone remodeling and tooth movement. Orthodontic tooth movement depends on the rebuilding of alveolar bone. The formation of osteoclasts is the prerequisite of alveolar bone remodeling. Under pressure stimulation, osteoclast generates osteoclastic reaction. It has been shown that the tooth movement can be effectively regulated by controlling the differentiation and function of osteoclast [4]. Therefore, a better understanding of molecular mechanism underlying the growth and differentiation of osteoclast is important for elucidating the molecular mechanism for orthodontic tooth movement.

Many studies have investigated how mechanical stimuli are transformed into biochemical signals to activate signaling pathways to regulate the synthesis of relevant proteins [5, 6]. Early work shows that the function of cementoblast may be altered when stimulated by me-

Table 1. Primer used in this study

Gene	GenBank Accession NO.	Primer sequence (5'-3')	Length of expected product (bp)
OSX	NM_130458.3	For: GGAAAGGAGGCACAAAGAAGC Rev: CCCCTTAGGCACTAGGAGC	165
RUNX2	XM_008262992.1	For: GACTGTGGTTACCGTCATGGC Rev: ACTTGGTTTTTCATAACAGCGGA	172
GAPDH	NM002046	For: CCTCAAGATTGTCAGCAAT Rev: ACCACAGTCCATGCCATCAC	141

OSX: Transcription factor Sp7; RUNX2: Runt-related transcription factor-2; GAPDH, glyceraldehyde phosphate dehydrogenase; For, forward; Rev, reverse.

chanical signal [7], and a number of growth factors have been shown to be involved in the orthodontic tooth movement, such as insulin-like growth factor, transforming growth factor, fibroblast growth factor, bone morphogenetic protein [8, 9]. Runt-related transcription factor-2 (RUNX2) is a multifunctional transcriptional factor and expressed in the whole process of osteoblast cell differentiation [10]. Osterix (OSX) is zinc finger transcriptional factor and also indispensable regulator for the growth and differentiation of osteoblast and development of bone tissue [11]. However, little is known about their expression in periodontal tissues during orthodontic tooth movement.

In this study, we used animal models to investigate the expression of the two genes in periodontal tissues and analyzed their involvements in signaling pathways to elucidate their possible functions in orthodontic tooth movement.

Materials and methods

Animal model and orthodontic treatment

Male Wistar rats (SPF, weighing 250±50 g), purchased from Silaike Experimental Animal Co., Ltd. (Shanghai, China), were to use for the study. The rats were fed with standard pellet feed in animal cages (5 per cage) with free access to drinking water and feed during the experiments. After 7 days of adaptive feeding, the rats were randomly divided to establish orthodontic tooth movement model as described [12]. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/kg), and shallow concaves of about 0.5-1.0 mm deep were cut on the axial plane angle in the first molar of left upper jaw and on the side of labial surface of the incisors in left and right upper jaws using fine emery bur using a low speed dental turbine. The shallow concaves were used to fasten the orthodontic stainless steel ligature wire of 0.25 mm in diameter. One end of the wire was tied to the neck of the first molar and the other end was attached to nickel titanium orthodontic spring (IMD, Inc, Shanghai, China) to provide orthodontic force of 40 g. In experimental groups, the orthodontic force was maintained for 1, 3 and 7 days, while in control, the same orthodontic appliance was used without orthodontic force. Six rats were used in each treatment. The experimental protocols were approved by the Ethics Committee on Laboratory Animal Use, Liaocheng People's Hospital.

Periodontal tissue

Rats were sacrificed by cervical dislocation, and tissues surrounding the first molar were taken, washed in pre-chilled PBS buffer and snap-frozen in liquid nitrogen.

Real-time PCR

Total RNA was extracted from the frozen samples using a RNA extract kit (Qiagen, USA) according to the manufacturer's instructions and reversely transcripted into cDNA using a reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions and quantified using real-time PCR using gene-specific primers (**Table 1**) and the 2(-Delta Delta C(T)) method [13]. Glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as internal reference for the quantification. All determinations were repeated three times. The RT-PCR reaction contained 5 μL SsoAdvanced SYBR Green Super mix (Bio-Rad, USA), 0.3-0.45 μL each of primers (300-450 nM), 100 ng cDNA template and nuclease-free water to fill up to a total reaction volume of 10 μL, and performed according to the manufacturer's protocols (Invitrogen, USA).

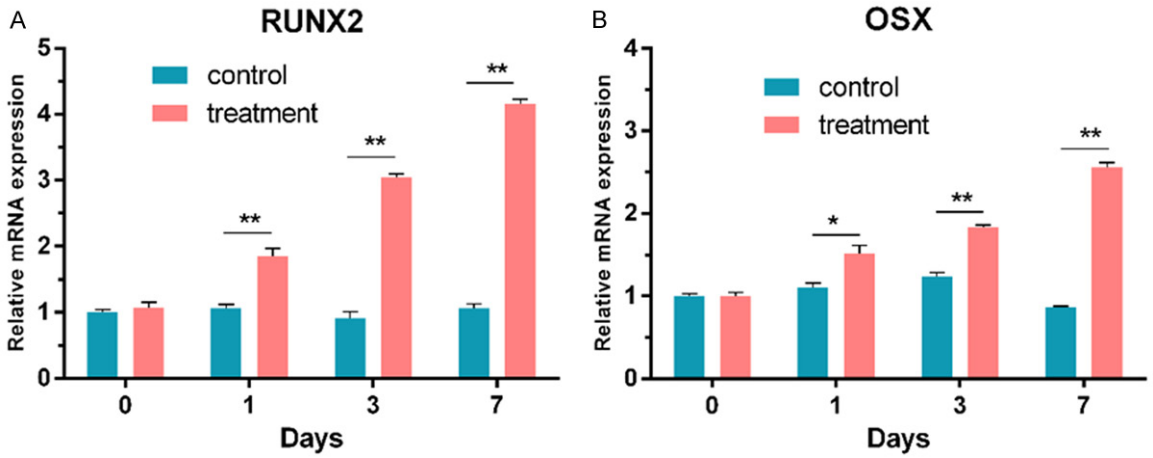

Figure 1. Relative mRNA levels of the RUNX2 (A) and OSX (B) genes determined by RT-PCR. Control, rats not applied with orthodontic force; treatment, rats applied with orthodontic force.

Western blot analysis

Total protein was extracted using a lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA) supplemented with a protease inhibitor cocktail kit and a phosphatase inhibitor cocktail kit (Hoffman-La Roche Ltd., Basel, Switzerland). The protein extracts were loaded, size-fractionated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). After blocking, the membranes were incubated with the primary monoclonal antibodies against RUNX2 and OSX (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and rabbit anti-β-actin (Proteintech Group Inc., Chicago, IL, USA) at 4°C overnight. Protein expression was determined using horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, followed by detection using enhanced chemiluminescence (EMD Millipore). Band intensity was visualized using a JS-1035 image analysis scanning system (Shanghai Peiqing Science & Technology, Co., Ltd., Shanghai, China), and quantitatively analyzed using Quantity one v4.62.

Bioinformatics analysis

Online analysis tools STRING9.05 (http://string-db.org) and KEGG pathway database (http://www.genome.jp) were used to identify the signaling pathways that Runx2 and OSX participate and proteins they interact with.

Statistical analysis

Experimental data were analyzed using SPSS16.0 statistical software for statistical analysis. The measurement data were expressed mean ± SD. Means between the two groups were compared using Student-t test and value was considered as significant or highly significant if $P < 0.05$, or < 0.01.

Results

OSX and RUNX2 expression at mRNA level

Expression of the OSX and RUNX2 genes were measured using RT-PCR in the periodontal tissues after different days of orthodontic movement treatment. The results showed that compared with rats applied no orthodontic force (control), the expression of the two genes were up-regulated at all sampling times (day 1, 3 and 7) and increased with increasing treatment time with the highest levels occurring on day 7 (**Figure 1**). One other hand, the expression of the two genes did not change in the control group over the experimental period ($P > 0.05$) (**Figure 1**). As a result, the difference in the expression levels between the experimental and control groups increased over the treatment period (**Figure 1**). The relative quantities of RUNX2 and OSX mRNA in the experiment groups were 1.85±0.12, 3.04±0.06 and 4.16±0.068, and 1.52±0.09, 1.83±0.03 and 2.56±0.06, at day 1, 3 and 7 following the orthodontic treatment, respectively.

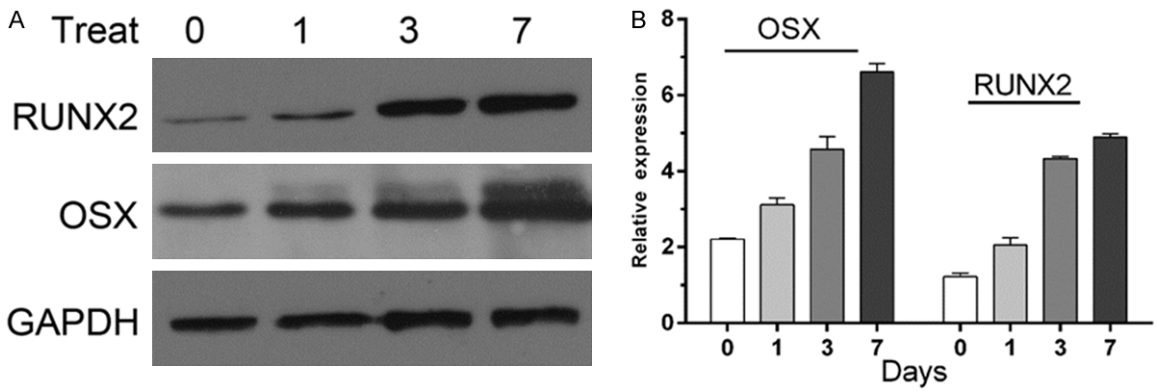

Figure 2. Expression of RUNX2 and OSX at protein level. A. Western blot; B. Gray value analysis based on trace tracking using software Quantity one v4.62.

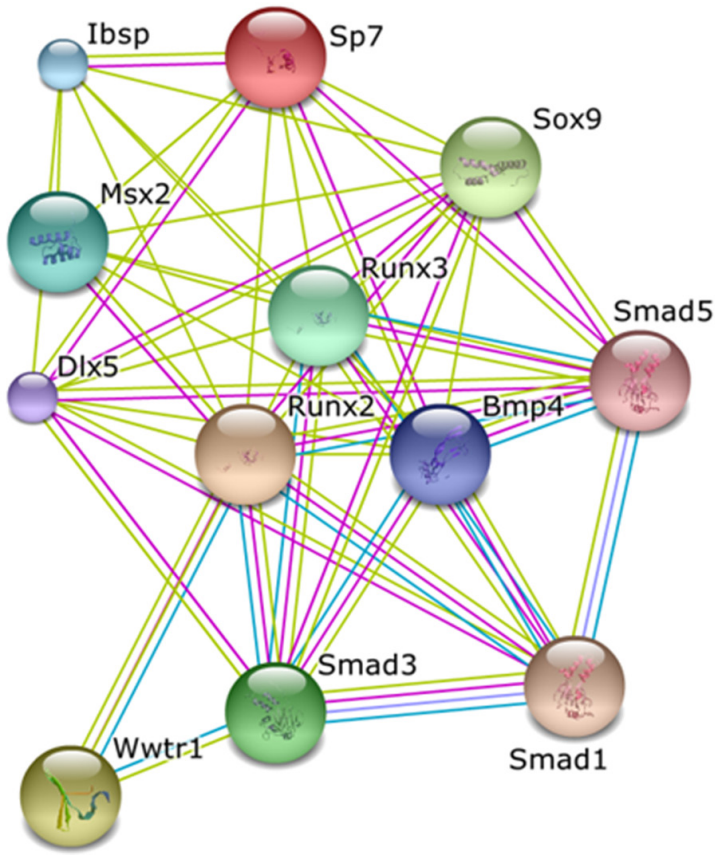

Figure 3. Interaction of RUNX2 and OSX with other proteins.

OSX and RUNX2 expression at protein level

We then analyzed the expression of the two genes at protein level in the periodontal tissue using Western blot analysis. The blot and quantitative analysis results showed that the expression of the two genes at protein level increased with increasing treatment times and reached the highest levels at day 7 (**Figure 2A** and **2B**).

Bioinformatics analysis

Search with STRING9.05 was made for Runx2 and OSX. The results showed that a number of proteins are interacting with Runx2 and OSX as network signal molecules, including WW domain containing transcription regulator 1(Wwtr1); SRY box containing gene 9 (Sox9); Smad family member 3 (Smad3); runt related transcription factor 3 (RUNX3); homeobox msh-like 2 (Msx2) (**Figure 3**). Among them Sox9, Msx2 and Run3 are related to the formation of osteoblasts. KEGG Pathway database showed that RUNX2 and OSX are involved in a number of pathways that regulate the differentiation of osteoblast.

Discussion

Mechanical force in orthodontic tooth movement has been shown to bring a number of bone cell biological activities through signal transduction, resulting in alveolar bone resorption and deposition, and causing the remodeling of periodontal tissues [1, 2, 14]. This process requires the participation of osteoclasts and cementoblast, the latter can not only participate in the repair of root through the synthesis of new cementum, but also regulate osteoclast differentiation and absorption by expressing regulatory proteins [3, 15-19].

Runx2 is osteoblast specific transcriptional factor that activates and initiates the differentiation of bone marrow mesenchymal cells into osteoblasts and regulate the maturation of osteoblasts; it also regulates the expression osteoprotegerin [20, 21]. OSX is a zinc finger transcriptional factor and an important regulatory factor in the growth and differentiation of osteoblasts and the development of bone tissue. In this study, we used orthodontic tooth movement (OTM) animal model [22] to study the expression of the two genes for their role in periodontal tissue rebuilding process.

The experimental results showed that the orthodontic force upregulated the expression of Runx2 and OSX at mRNA and protein level over the 7 day experiment period compared with the control group; and the upregulation increased with increase in experimental time and reached the highest at the end of study. On other hand, there was no change in RUNX2 and OSX expression in rats not subjected to the orthodontic force treatment. These findings suggest that RUNX2 and OSX may be involved in the early response of bone cells to mechanical signal.

Fibroblasts are the pluripotent cells in periodontal tissue, and can differentiate into osteoblast like cells by expressing proteins that confer osteoblast phenotype and functions under external mechanical forces. In this process, osteoblast specific transcriptional factor plays a central role [23]. Biologically, the upregulation of RUNX2 and OSX expression by the mechanical stimulation may activate and initiate the differentiation of the bone marrow mesenchymal stem cells into bone cells and regulate the maturation of bone cells. Furthermore, RUNX2 may regulate the expression of osteocalcin to promote the maturation of osteoblast. Thus, RUNX2 is likely playing key roles in the regulation of bone cell differentiation.

Analysis of interaction of RUNX2 with other proteins in signaling pathways showed that they interact directly or indirectly with a number of molecules involved in bone remodeling, such as Run3, Sox9 and Msx2, forming a signaling network (**Figure 3**). In the osteoblast differentiation and bone formation, RUNX2 as a marker for osteoblast and transcriptional factor with the runt domain can bind to the core regions in a number of enhancers and promoters. For example, RUNX2 is shown to interact with Sox9 to play an important role in cartilage differentiation [24]. Msx2 also plays regulatory role in the osteoblast differentiation. For example, Shirakabe et al showed that it acts directly with RUNX2 in an immunoprecipitation assay [25]. It would be our next topic to elucidate how RUNX2 and OSX participate in the regulation of the complex networks.

Acknowledgements

This study was supported by Shandong Province Natural Science Fund (ZR2014HL052) and Shandong Province Medical Science and Technology Development Plan Projects (2014 ws-0048).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hong He, Department of Orthodontics, School & Hospital of Stomatology, Wuhan University, 237 Luoyu Road, Hongshan District, Wuhan 430079, Hubei Province, China. Tel: 86-27-87686265; E-mail: 1755850704@qq.com

References

[1] Li S, Zhou J, Ren C. Adult orthodontic technique: development and challenge. West China Journal of Stomatology 2013; 31: 549-551.

[2] Wang X, Wu C, Li Y, Wang R. Research progress on relationship between malocclusion as well as orthodontic treatment and psychosocial behavior. China Medical Herald 2014; 16: 162-165.

[3] Thirunavukkarasu K, Halladay DL, Miles RR, Yang X, Galvin RJ, Chandrasekhar S, Martin TJ, Onyia JE. The osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. J Biol Chem 2000; 275: 25163-25172.

[4] Caetano-Lopes J, Canhao H, Fonseca JE. Osteoblasts and bone formation. Acta Reumatol Port 2007; 32: 103-110.

[5] Wise GE, King GJ. Mechanisms of tooth eruption and orthodontic tooth movement. J Dent Res 2008; 87: 414-434.

[6] Wise GE. Cellular and molecular basis of tooth eruption. Orthod Craniofac Res 2009; 12: 67-73.

[7] Brezniak N, Wasserstein A. Orthodontically induced inflammatory root resorption. Part I: The basic science aspects. Angle Orthod 2002; 72: 175-179.

[8] Xi J, Wang Y, Zhang P, He L, Nan X, Yue W, Pei X. Human fetal liver stromal cells that overexpress bFGF support growth and maintenance of human embryonic stem cells. PLoS One 2010; 30: e14457.
[9] Desai N, Rambhia P, Gishto A. Human embryonic stem cell cultivation: historical perspective and evolution of xeno-free culture systems. Reprod Biol Endocrinol 2015; 13: 9.
[10] Wang W. Role of Runx1 and its co-transcription factor CBF b in osteoblast differentiation and bone development. Acta Zhejiang University 2014; 55: 125-140.
[11] Farshdousti Hagh M, Noruzinia M, Mortazavi Y, Soleimani M, Kaviani S, Abroun S, Dehghani Fard A, Mahmoodinia M. Different Methylation Patterns of RUNX2, OSX, DLX5 and BSP in Osteoblastic Differentiation of Mesenchymal Stem Cells. Cell J 2015; 17: 71-82.
[12] Chen J, Chen WC, Gu M, Wang T. Expression of Runx2 in periodontal ligment during orthodontic tooth movement. Stomatology 2010; 30: 286-290.
[13] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.
[14] Rody WJ Jr, King GJ, Gu G. Osteoclast recruitment to sites of compression in orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2001; 120: 477-489.
[15] Iliadi A, Kloukos D, Gkantidis N, Katsaros C, Pandis N. Failure of fixed orthodontic retainers: A systematic review. J Dent 2015; 43: 876-896.
[16] Hendesi H, Barbe MF, Safadi FF, Monroy MA, Popoff SN. Integrin mediated adhesion of osteoblasts to connective tissue growth factor (CTGF/CCN2) induces cytoskeleton reorganization and cell differentiation. PLoS One 2015; 10: e0115325.
[17] Boabaid F, Berry JE, Koh AJ, Somerman MJ, McCcauley LK. The role of parathyroid hormone-related protein in the regulation of osteoclastogenesis by cementoblasts. J Periodontol 2004; 75: 1247-1254.
[18] Marchesan JT, Gerow EA, Schaff R, Taut AD, Shin SY, Sugai J, Brand D, Burberry A, Jorns J, Lundy SK, Nuñez G, Fox DA, Giannobile WV. Porphyromonas gingivalis oral infection exacerbates the development and severity of collagen-induced arthritis. Arthritis Res Ther 2013; 15: R186.
[19] Yang X, Wang Y, Han X, Shu R, Chen T, Zeng H, Xu X, Huang L, Ren A, Song J, Cao L, Bai D. Effects of TGF-β1 on OPG/RANKL expression of cementoblasts and osteoblasts are similar without stress but different with mechanical compressive stress. ScientificWorldJournal 2015; 2015: 718180.
[20] Nagatake T, Fukuyama S, Sato S, Okura H, Tachibana M, Taniuchi I, Ito K, Shimojou M, Matsumoto N, Suzuki H, Kunisawa J, Kiyono H. Central Role of Core Binding Factor β2 in Mucosa-Associated Lymphoid Tissue Organogenesis in Mouse. PLoS One 2015; 10: e0127460.
[21] Baek JE, Choi JY, Kim JE. Skeletal analysis and differential gene expression in Runx2/Osterix double heterozygous embryos. Biochem Biophys Res Commun 2014; 451: 442-448.
[22] Krishnan S, Pandian S, Kumar SA. Effect of bisphosphonates on orthodontic tooth movement-an update. J Clin Diagn Res 2015; 9: ZE01-05.
[23] Patil AK, Shetty AS, Setty S, Thakur S. Understanding the advances in biology of orthodontic tooth movement for improved ortho-perio interdisciplinary approach. J Indian Soc Periodontol 2013; 17: 309-318.
[24] Wang J, Lü D, Mao D, Long M. Mechanomics: an emerging field between biology and biomechanics. Protein Cell 2014; 5: 518-531.
[25] Shirakabe K, Terasawa K, Miyama K, Shibuya H, Nishida E. Regulation of the activity of the transcription factor Runx2 by two homeobox proteins, Msx2 and Dlx5. Genes Cells 2001; 6: 851-856.