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Expression of *OPG*, *RANKL*, and *RUNX2* in rabbit periodontium under orthodontic force

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ABSTRACT. This study aims to investigate the expression changes of *RANKL*, *RUNX2*, and *OPG* in rabbit periodontal tissues under orthodontic force and explore its effect on the remodeling of periodontal tissues. A total of 16 specific pathogen-free rabbits were used in this study. The maxillary appliance was worn on the right (experimental) side, and the appliance-free left side was used as the control. Rabbits were sacrificed after 3, 5, 7, and 14 days of treatment. Changes in the expression levels of *OPG*, *RANKL*, and *RUNX2* in the periodontium were detected using real-time PCR and western blotting methods. The *OPG* expression levels decreased after 3 to 14 days of treatment, while the expression levels of *RANKL* and *RUNX2* increased after 3 to 14 days. The *OPG* expression levels decreased while those of *RANKL* and *RUNX2* increased during orthodontic tooth movement,

which suggested that they play a role in the osteogenesis process and the reconstruction of periodontal tissue.

Key words: Orthodontic force; Periodontium; *OPG*; *RANKL*; *RUNX2*; Western blotting

INTRODUCTION

Fault micromaxillary deformity refers to irregular teeth, upper and lower dental arch dislocation, abnormal jaw position, or size and other deformities caused by genetic or environmental factors during growth and development. It is a common disease that affects the facial appearance and function, and it causes dental caries, periodontal disease, and temporomandibular joint disease (Indira et al., 2014; Al-Moraissi et al., 2015). Orthodontic treatment is the main treatment method for fault micromaxillary deformity, and it can improve the occluding relationship in order to solve problems associated with aesthetics and function. The orthodontic force acting on the periodontal tissue can activate osteoblasts and osteoclasts, and this leads to the absorption and deposition of the alveolar bone, which are accompanied by cell growth and collagen renewal (Wise and King, 2008; Wise, 2009).

The molecular mechanisms of orthodontic tooth movement are not clear. Studies demonstrated that cementoblasts that were stimulated by mechanical signals affected cementoblast functions (Brezniak and Wasserstein, 2002). Osteoprotegerin (*OPG*) and receptor activator of nuclear factor-κB ligand (*RANKL*) were thought to be widely involved in the growth of bone cells (Rody et al., 2001; Wagner and Fahrleitner-Pammer, 2010). However, whether mechanical stimulation affected the expression of *OPG*, *RANKL,* and runt-related transcription factor-2 (*RUNX2*) remained unclear. Therefore, we explored this question in the current study using an animal model.

MATERIAL AND METHODS

Experimental animals

A total of 16 specific pathogen-free (SPF) rabbits (weighing 1.0 to 2.5 kg) were obtained from the Animal Experimental Center of Vital River Laboratories (Beijing, China). The rabbits were pre-fed for seven days, and they had free access to food and water while adapting to the environment. Food and water were regularly changed, and good room ventilation and natural lighting were present in housing area. The rabbits were anesthetized with 2% pentobarbital sodium (2 mL/kg) via the ear vein. The maxillary appliance (0.78 N force) was worn on the right (experimental) side, and the appliance-free left side was used as a control. Rabbits were sacrificed after 3, 5, 7, and 14 days of treatment.

Housing and procedures involving experimental animals were in accordance with the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Care of Experimental Animals Committee of our hospital.

Real-time PCR

Total RNA was extracted from periodontal tissue using SunShineBio™ Total RNA

Extraction Reagent (SunShineBio Ltd., Hnagzhou, China) according to the manufacturer protocol. Total RNA was reverse transcribed and qRT-PCR was conducted using SYBR Green Supermix (Bio-Rad-172-5264, USA). The PCR primers are listed in Table 1. The amplification conditions were as follows: 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. For relative quantification, we calculated the n-fold differential expression using the $2^{-\Delta\Delta Ct}$ method (Ct denotes the threshold PCR amplification cycle at which product is first detected by fluorescence). This method compares the amount of target gene amplification, which is normalized to the *GAPDH* endogenous reference.

Table 1. Primers used in real-time PCR analyses.

Gene	GenBank accession No.	Primer pair (5'-3')	Length of product (bp)
OPG	NM_002546.3	F: AACCCCAGAGCGAAATAC R: AAGAATGCCTCCTCACAC	205
RANKL	NM_057149.1	F: GGTTCCCATAAAGTGAGTCTGT R: TTAAAAGCCCCAAAGTATG	101
RUNX2	XM_008262992.1	F: GACTGTGGTTACCGTCATGGC R: ACTTGGTTTTTCATAACAGCGGA	172
GAPDH	NM002046	F: CCTCAAGATTGTCAGCAAT R: ACCACAGTCCATGCCATCAC	141

OPG = osteoprotegerin; *RANKL* = receptor activator of nuclear factor kappa B ligand; *RUNX2* = runt-related transcription factor-2; *GAPDH* = glyceraldehyde phosphate dehydrogenase; F = forward; R = reverse.

Western blotting method

Total proteins were extracted from periodontal tissue and analyzed with SDS-PAGE electrophoresis. Periodontal tissues were homogenized using RIPA buffer (10 µg/mL) and a protease inhibitor. Total proteins were isolated, and their concentrations were measured using the bicinchoninic acid (BCA) method. Proteins were then electrotransferred to the PVDF membrane, and the membrane containing the proteins was used for immunoblotting with required antibodies. Proteins were blocked with 5% non-fat milk in TBST buffer for 2 h, and they were then incubated with the primary antibodies (Santa Cruz Biotechnology; sc-8468, sc-9073, and sc-390715) at 4°C overnight. Proteins were subsequently incubated with secondary antibodies that were conjugated with horseradish peroxidase for 1 h at room temperature. *GAPDH* was used as an internal control. The images were analyzed using the Quantity One (version 4.62) software.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS, version 18.0) program. The data are reported as means ± SD, and differences between experimental groups were analyzed using Student *t*-tests. $P < 0.05$ was considered to be significant.

RESULTS

Real-time PCR results

Real-time PCR results are shown in Figure 1. The results showed that the *OPG* expression

levels were 0.83 ± 0.06, 0.62 ± 0.04, 0.40 ± 0.06, and 0.36 ± 0.07 after treatment for 3, 5, 7, and 14 days, respectively, and they decreased significantly compared to the control group ($P < 0.05$). The *RANKL* expression levels were 1.52 ± 0.09, 1.41 ± 0.03, 1.38 ± 0.06, and 1.26 ± 0.07 after treatment for 3, 5, 7, and 14 days, respectively, and they increased significantly compared to the control group ($P < 0.05$). The *RUNX2* expression levels were 2.21 ± 0.09, 1.96 ± 0.03, 1.82 ± 0.06, and 1.88 ± 0.07 after treatment for 3, 5, 7, and 14 days, respectively, and they increased significantly compared to the control group ($P < 0.05$).

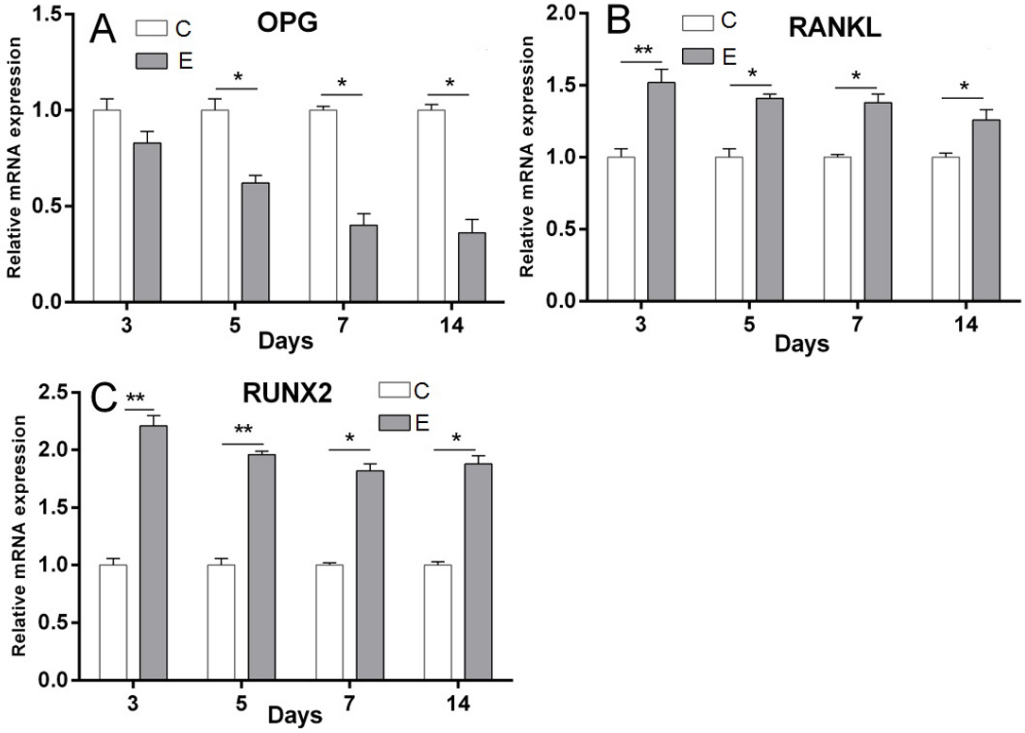

Figure 1. Real-time PCR results. **A.** Relative expression level of *OPG*. **B.** Relative expression level of *RANKL*. **C.** Relative expression level of *RUNX2*; *P < 0.05; **P < 0.01. C = control; E = experimental group.

Western blotting results

Western blotting results are shown in Figure 2. The results were similar to the RT-PCR results. The results indicated that the *OPG* expression levels decreased and those of *RANKL* and *RUNX2* increased significantly compared to the control group after treatment ($P < 0.05$).

DISCUSSION

Fault micromaxillary deformity is caused by genetic or environmental factors during growth and development. It not only affects facial appearance and function, but it also causes dental caries and periodontal disease (Rody et al., 2001; Indira et al., 2014).

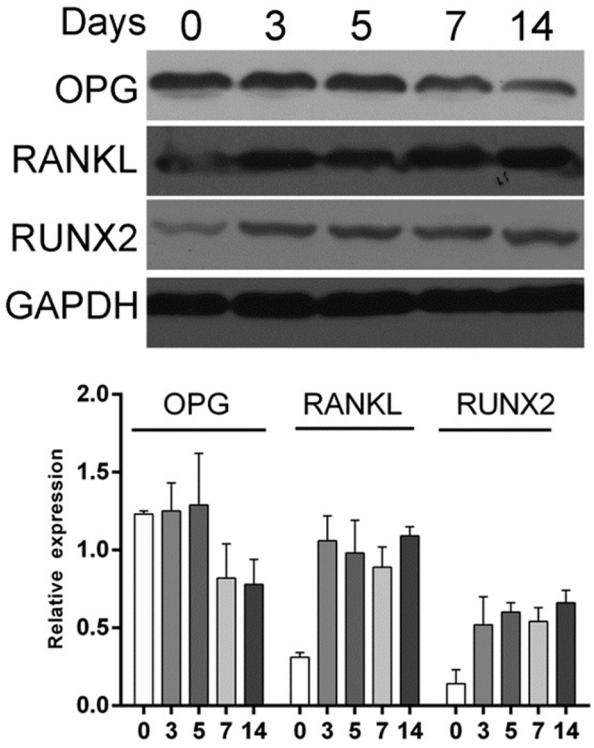

Figure 2. Western blotting results.

Orthodontic treatment can improve these symptoms. Mechanical force applied to periodontal tissues induced a cellular response, and it resulted in the degradation of collagen and bone tissue absorption on the stressed side. Periodontal ligament cells on the stressed side grew, and bone tissue precipitation occurred, causing bone remodeling and tooth movement (Iliadi et al., 2015; Krishnan et al., 2015).

Orthodontic treatment caused an inflammatory reaction in periodontal tissues under the action of the external force, and orthodontic force was transferred to the periodontal membrane, which induced periodontal tissue remodeling (Thirunavukkarasu et al., 2000; Hendesi et al., 2015). This process required osteoclasts and cementoblasts. Cementoblasts participated in the root repair process and in the regulation of the differentiation and absorption functions of osteoclasts, and they also affected root absorption (Boabaid et al., 2004; Marchesan et al., 2013; Yang et al., 2015). In recent years, the *OPG* and *RANKL* system was found to be an important signal pathway in the differentiation of osteoclasts, which was widely involved in bone cell growth. The RANKL receptor is located on the cell membrane of osteoclasts. OPG belongs to the superfamily of TNF receptors, and its main function is to inhibit the differentiation of osteoclasts and to induce apoptosis. However, OPG is a false receptor of RANKL. Osteoblasts expressed *RANKL*, which can promote the differentiation of bone cells and bone resorption after the gene product combines with RANK. OPG secreted by osteoblasts can competitively combine with RANKL to inhibit the binding between RANK and RANKL (Boyce and Xing, 2007; Tokuyama and Tanaka, 2010; Shimamura et

al., 2014). In this study, we found that *OPG* expression was downregulated by orthodontic force, while *RANKL* expression was upregulated. Therefore, the expression ratio of *OPG* and *RANKL* can reflect the early response of cementum cells to mechanical signals. The fibroblast group is a cell group in the periodontal tissues with differentiation potential, and the group can successfully differentiate osteoblasts based on the expression of the phenotype and functional osteoblast proteins that are subjected to mechanical forces. Moreover, osteoblast-specific transcription factors play a central regulatory role in the process. RUNX2 is a specific transcription factor that can activate bone marrow mesenchymal cells for differentiation into osteoblasts. Furthermore, it can regulate the maturation of osteoblasts and *OPG* expression, and it also plays a regulatory role in bone formation and bone resorption (Baek et al., 2014; Nagatake et al., 2015). In this study we found that mechanical stimulation can upregulate the expression of *RUNX2*, which may be caused by decreased *OPG* expression.

Conflicts of interest

The authors declare no conflict of interest.

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