Local osteoprotegerin gene transfer inhibits relapse of orthodontic tooth movement

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Introduction: In orthodontic treatment, teeth can relapse after tooth movement without retention. The aim of this study was to evaluate the inhibition effects of local osteoprotegerin (OPG) gene transfer on orthodontic relapse. **Methods:** Eighteen male Wistar rats were divided into 3 groups. The maxillary right first molars of all animals were subjected to orthodontic force and moved mesially. Three weeks later, the force was removed, and the teeth relapsed. During the 2-week relapse period, the 3 groups of rats received local OPG gene transfer (experimental group), mock vector transfer (mock group), and no injections (control group). Tooth movement and relapse were measured by using palatal superimpositions of 3-dimensional digital models. Histomorphometric analysis was used to quantify osteoclasts, and microcomputed tomography analysis was done to quantify the alveolar bone and the tibia. **Results:** Relapse was significantly inhibited and the number of osteoclasts was reduced in the experimental group. On the other hand, bone mineral density and bone volume fraction of alveolar bone were significantly increased. Bone mineral density and bone volume fraction of the tibia showed no significant difference between the groups. **Conclusions:** Local OPG gene transfer to periodontal tissues could inhibit relapse after orthodontic tooth movement, through the inhibition of osteoclastogenesis. (Am J Orthod Dentofacial Orthop 2012;141:30-40)

A challenging problem in orthodontics is tooth relapse. After orthodontic tooth movement, a retainer is needed to maintain teeth in their corrected positions until the treatment result becomes more stable. Without a phase of retention, there is a tendency for teeth to return toward their initial positions. The reasons behind relapse are unclear, although several factors have been suggested, including the recoil of gingival and periodontal tissues, the surrounding soft tissues, growth of skeletal bases, and other dental factors.[1,2] In this study, we focused on the periodontal supporting tissues, especially the remodeling of surrounding alveolar bone.

During active orthodontic tooth movement, stresses and strains are thought to be built up and stored in the periodontal and transseptal fiber system.[3] After removal of the orthodontic appliance, these stresses are released, and the teeth begin to relapse to their original positions.[4-6] However, even though the periodontal fiber is primarily responsible for the generation of forces on moved teeth, osteoclastic resorption and osteoblastic formation of surrounding alveolar bone are necessary for relapse to occur.[7,8] It has been reported that relapse pressure persisted until the alveolar bone resorption was completed,[4] and relapse of moved teeth can be prevented by properly manipulating alveolar bone remodeling after orthodontic tooth movement.[7-10] Administration of bisphosphonate has been reported to inhibit bone resorptive functions of osteoclasts and reduce relapse.[8] Simvastatin can prevent relapse through inhibition of the bone-resorbing activity of osteoclasts and by stimulating bone formation.[7] Bone morphogenetic proteins have been used to prevent relapse, and the results showed that they can promote new bone and cementum formation.[10] These data suggest that relapse could be inhibited by manipulating alveolar bone remodeling.

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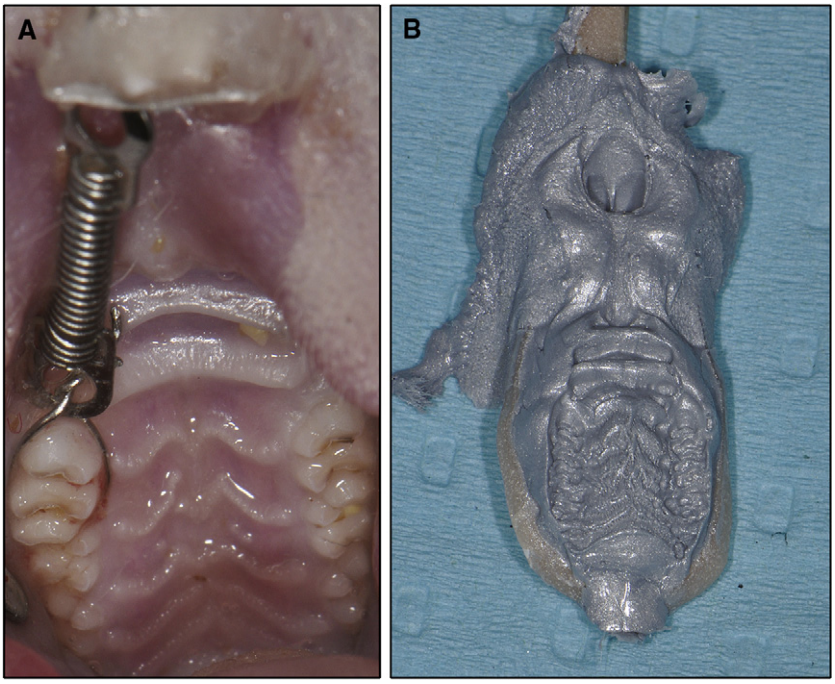

Fig 1. A, Intraoral photograph of the orthodontic appliance; **B,** silicone impression of the maxillary dentition of a rat.

The axis consisting of receptor activator of nuclear factor kappa B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) has recently been shown to play an important role in orthodontic bone remodeling.[11-13] RANKL, a member of the tumor necrosis factor superfamily, is produced by osteoblast lineage cells, periodontal ligament cells, and T lymphocytes. RANKL binds to its receptor RANK, which is located on the surface of osteoclasts and osteoclast precursors. RANK-RANKL interactions lead to preosteoclast recruitment, fusion into multinucleated osteoclasts, osteoclast activation, and osteoclast survival. Each of these responses can be fully inhibited by OPG, which is a soluble decoy receptor against RANKL and produced by osteoblasts and other cells.

An increase in exogenous OPG expression can lead to the indirect inhibition of bone resorption and loss. This inhibition has been observed in many bone destruction-related diseases including rheumatoid arthritis,[14] primary osteoprosis,[11] postmenopausal bone loss,[15] periodontal disease,[16] and others. It has also been shown that exogenous OPG has an inhibitory effect on orthodontic tooth movement.[17,18] When the fusion protein OPG-Fc was locally delivered, inhibition of osteoclastogenesis and active orthodontic tooth movement was observed.[17] Previously, we had reported that local OPG gene transfer to periodontal tissues significantly diminished, whereas RANKL gene transfer significantly enhanced, orthodontic tooth movement.[18,19] OPG expression in periodontal tissues was observed to increase significantly after local OPG gene transfer; this was followed by RANKL-mediated osteoclastogenesis inhibition. As a result, the experimental orthodontic tooth movement in rats was inhibited without eliciting any systemic effects.[18] Simvastatin can inhibit relapse by enhancing the expression of OPG and reducing the expression of RANKL.[7] These data suggested the possibility of manipulating bone remodeling with local OPG gene transfer to inhibit post-orthodontic relapse.

In this study, we hypothesized that local OPG gene transfer to periodontal tissues would inhibit the relapse after orthodontic tooth movement. The specific objectives of this study were to assess the magnitude of relapse after orthodontic tooth movement in rats with or without local OPG gene transfer, and to determine the effects of local OPG gene transfer on the bone mineral density (BMD) and the bone volume fraction (BVF) of the alveolar bone (local effects) and the tibia (systemic effects) through microcomputed tomography analysis.

MATERIAL AND METHODS

Eighteen male Wistar rats (age, 6 weeks; approximate weight, 180-190 g) were used in this study. Six-week-old

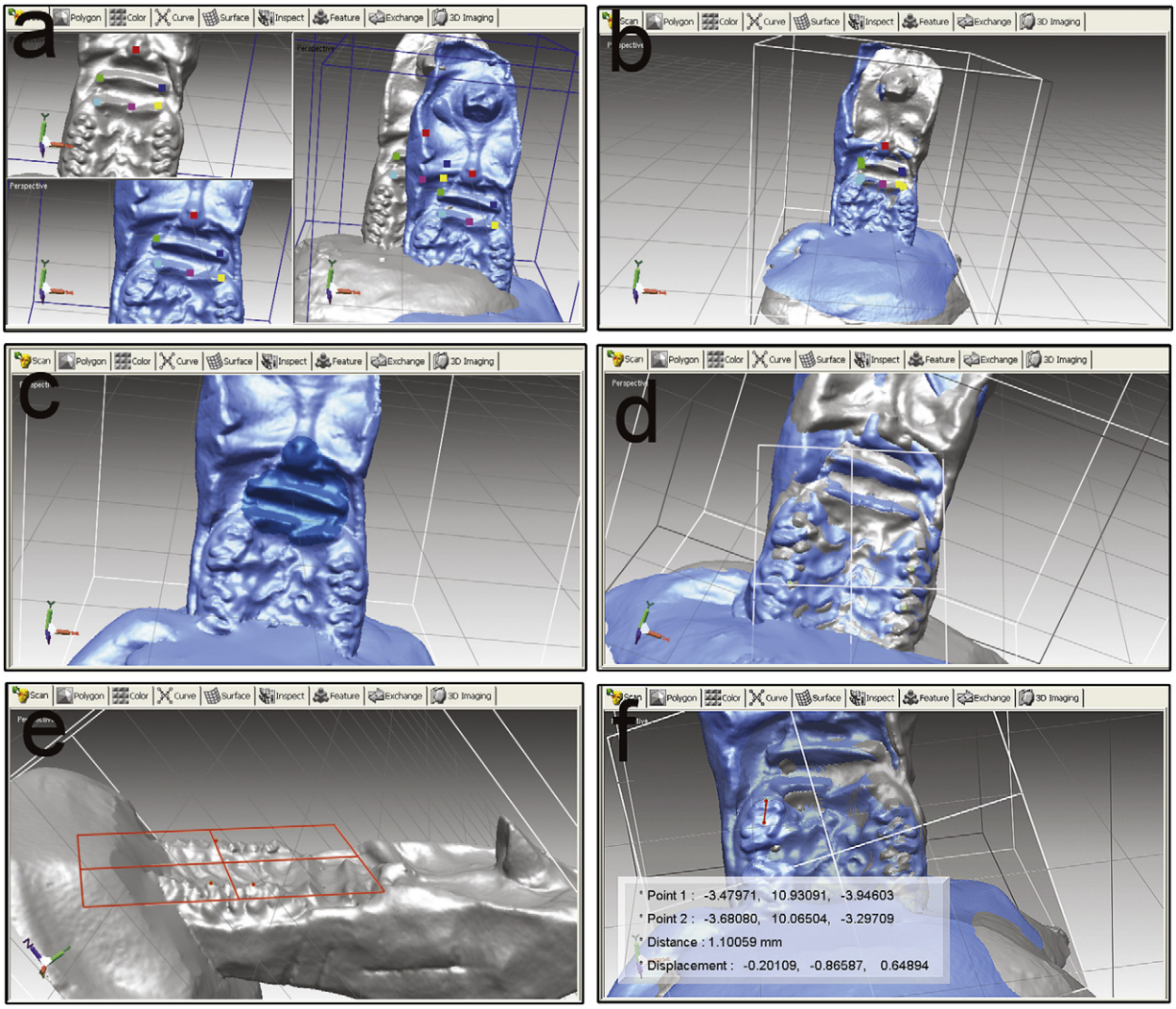

Fig 2. The distance of tooth movement was measured by using palatal superimposition of digital models, with the Rapidform 2006 software. *Gray model,* Pretreatment; *blue model*, posttreatment. **A,** Image of initial registration made by selecting 6 stable points on the palate; **B,** initial registration image of pretreatment and posttreatment models; **C,** fine tuning of superimposition using regional registration; **D,** regional registration image of pretreatment and posttreatment models; **E,** occlusal plane on which the distance of tooth movement was measured (the occlusal plane was an imaginary plane passing through 3 points: the mesiobuccal cusp tips of the maxillary right second molar, left first molar, and left second molar); **F,** measurement of tooth movement: *red points*, midpoints of the mesial marginal ridge of the maxillary right first molars on the pretreatment and posttreatment models. The distance was measured on the occlusal plane.

rats were considered as adolescent, since male rats reach sexual maturity at 8 weeks of age.[20] All rats were housed under normal conditions with a 12-hour circadian cycle and fed a standard rat-chow diet and water ad libitum. The experimental procedures were approved by the Ethics Committee of Peking University Health Science Center, Beijing, China.

The 18 rats were randomly divided into 3 equal groups: experimental, mock, and control groups. The maxillary right first molars of all animals were subjected to orthodontic force and moved mesially by a closed-coil nickel-titanium spring (Sentalloy, Tomy, Tokyo, Japan). The spring was ligated to the maxillary right first molar and incisor, exerting a force of approximately 40 g

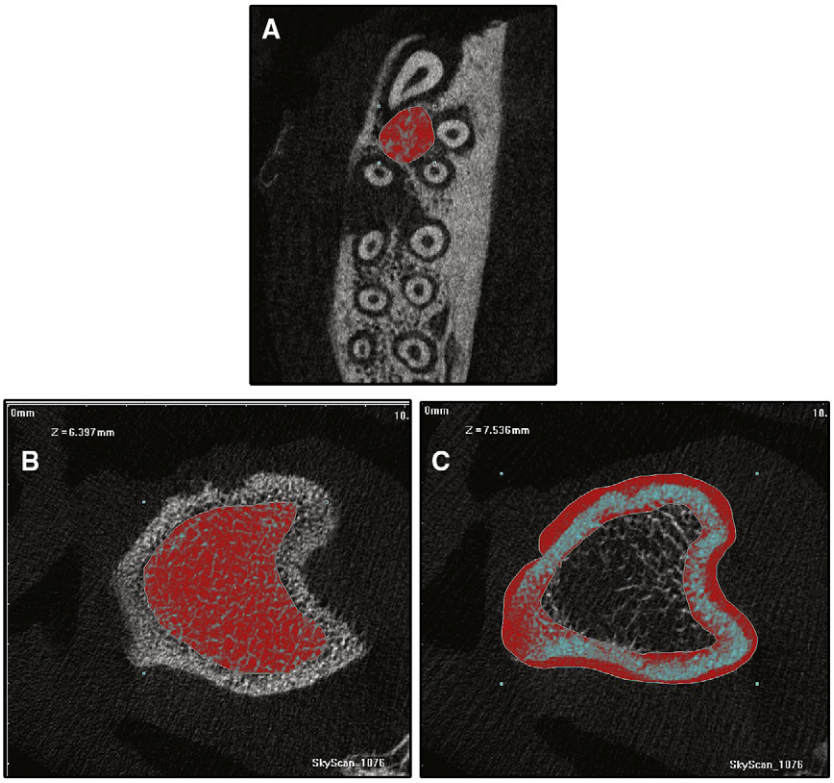

Fig 3. Microcomputed tomography images of the maxilla and the tibia. BMD and BVF were measured in 100 slices of these images by using the SkyScan system's software. This figure shows 3 slices of microcomputed tomography images: **A,** axial slice of the maxilla (the *red area* was selected to measure the BMD and BVF of the alveolar bone); **B,** axial slice of the tibia (the *red area* was selected to measure the BVF and BMD of cancellous bone in the tibia); **C,** axial slice of tibia (the *red area* was selected to measure the BMD of cortical bone in the tibia).

(Fig 1, *A*). The activation force of the spring was evaluated with a force gauge, and the spring could provide a force of 40 ± 2 g at 3.2 mm of activation. Three weeks later, the springs were removed, and the teeth were left without retainers. This allowed relapse of the mesialized molars to occur by returning toward their distal positions. At the time of spring removal, the 3 groups of rats received different treatments: local OPG gene transfer (experimental group), mock vector transfer (mock group), and no injections (control group). The duration of relapse was 2 weeks.

For in-vivo gene transfer, we used an inactivated hemagglutinating virus of Japan (HVJ) envelope vector (GenomONE, Ishiara-sangyo kaisha, Osaka, Japan) and an OPG expression plasmid. The cloning of the mouse OPG gene and details of the OPG expression plasmid (pcDNA3.1(+)-mOPG) have been described previously, and functional in-vitro protein expression has been confirmed.[10] The rats of the experimental group were administered the HVJ envelope vector, which contained the pcDNA-mOPG plasmid, on the initial day of relapse. Under anesthesia, 5 μL of vector solution were injected into the palatal mucosa adjacent to the distal surface of the maxillary right first molar, by using 26s-gauge microneedles (Hamilton, Reno, Nev). All injections were volumetrically equivalent and were administered twice weekly. After 2 weeks, the animals were killed.

The distances of tooth movement and relapse were measured by palatal superimposition of 3-dimensional (3D) digital models. Silicone (Affinis precious light body, Coltene Whaledent, Altstatten, Switzerland) impressions were taken of the rats' maxillary dentitions at 3 times: baseline (pretreatment impression), the last day of the 3-week tooth movement (posttreatment impression), and the last day of the 2-week relapse (relapse impression; Fig 1, *B*). After the fabrication of precise stone models, 3D scanning of the maxillary dental casts was performed by using a 3D spot laser scanner (LPX-1200, Roland, Shizuoka, Japan). The validity and precision of the scanner were 0.05 mm. The scanned data

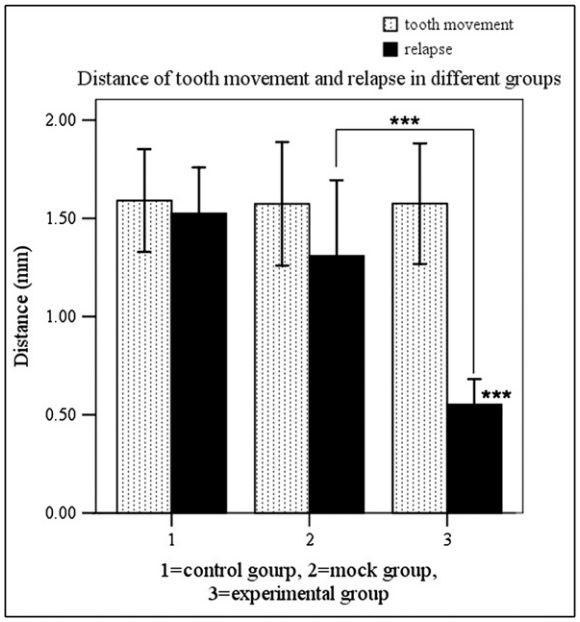

Fig 4. The distances of tooth movement and relapse in the 3 groups. The average amounts of tooth movement and relapse in each group from 6 rats are shown. ***P <0.001. There were no significant differences of tooth movement among the 3 groups. Relapse was significantly inhibited in the experimental group by local OPG gene transfer.

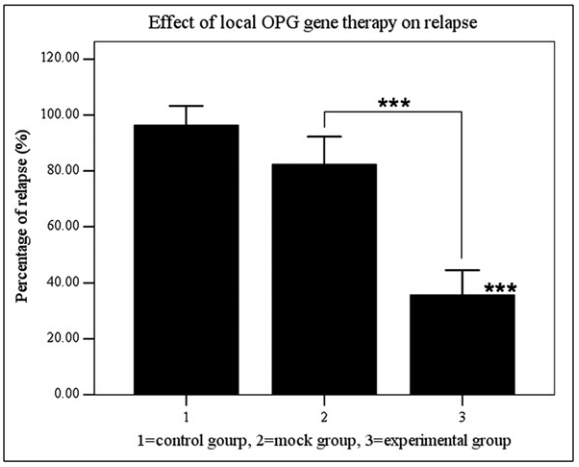

Fig 5. The percentages of relapse in the groups. The percentage of relapse is distance of relapse/distance of tooth movement. The percentages of relapse values from 6 rats in each group are shown. ***P <0.001. Local OPG gene transfer significantly inhibited the percentage of relapse compared with the control and mock groups. There were no significant differences between these groups.

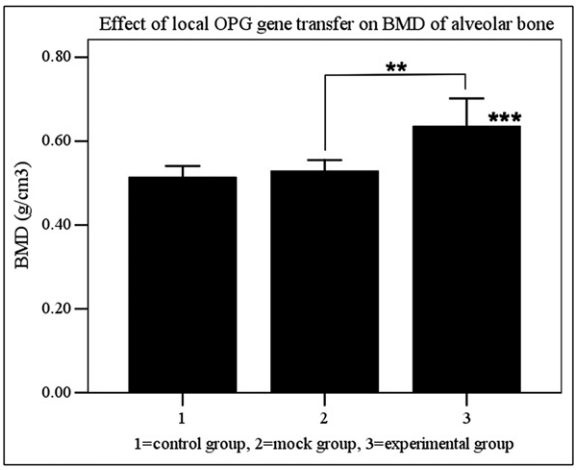

Fig 6. BMD of the alveolar bones in the groups. The average BMD values from 6 rats in each group are shown. **P <0.01; ***P <0.001. Local OPG gene transfer significantly increased the BMD of alveolar bone in the experimental group.

were used to reconstruct and analyze the 3D images; this was performed by using reverse engineering software (Rapidform 2006; Inus Technology, Seoul, Korea). After reconstruction and analysis, the pretreatment, posttreatment, and relapse models were superimposed on the area of the palatal rugae (Fig 2, *A-D*). After superimposition, the amounts of tooth movement and relapse were calculated by using the software Rapidform 2006. First, the occlusal plane was chosen as the reference plane (Fig 2, *E*). Then the midpoints of the mesial marginal ridge of the maxillary right first molars were marked on the pretreatment, posttreatment, and relapse models. The distances between these points were measured on the reference plane (Fig 2, *F*). All measurements were repeated 3 times by 2 investigators (N.Z. and W.L.).

To evaluate the effect of 5-week growth on the stability of the rats' palatal surfaces, we performed the following preliminary experiment. Three rats received no orthodontic forces. For each rat, the maxilla models at baseline and subsequent growth after 5 weeks were scanned and superimposed on the area of the palatal rugae. The results showed that the 3D positions of landmarks on the palatal surface were stable.

Microcomputed tomography analysis was performed to quantify alveolar bone in the proximity of the first molar roots. It was also used to measure the BMD and the BVF of the tibia (Fig 3). Density and volume fraction are important parameters in describing the status of bone metabolism.[11,13,14] BVF is the fraction of solid bone volume to total volume, which is measured to evaluate the trabecular microstructure. The accuracy of BVF and BMD measured by microcomputed tomography has been confirmed in previous studies.[21]

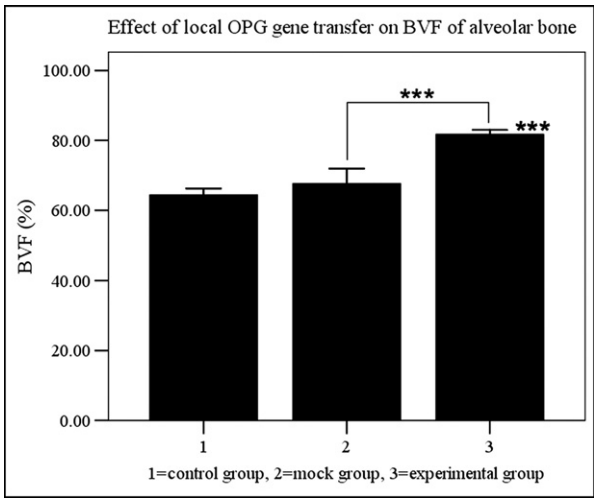

Fig 7. BVF of the alveolar bones in the groups. ***P <0.001. BVF, a fraction of bone volume/total volume. Local OPG gene transfer significantly increased the BVF of alveolar bone in the experimental group.

In this experiment, the head and left tibia of each study animal were scanned, under general anesthesia, with an x-ray microtomography system (model 1076; SkyScan, Kontich, Belgium). The BMD and BVF of the alveolar bone and the tibia were analyzed by using the system's software. The image voxel sizes of alveolar bone and tibia were 9.488 and 18.97 μm, respectively. All measurements were repeated 3 times by 2 investigators.

BMD and BVF of alveolar bone were measured in the first molar furcation area. The furcation area was chosen since it provides reproducible morphologic landmarks.[17]

The animals were killed under pentobarbital anesthesia 2 weeks after spring removal. After final impressions and scanning with microcomputed tomography, block biopsies of the maxillae were harvested, immediately fixed, and stored in 10% neutral-buffered formalin solution for 24 hours at 4°C. The maxillae, including the molars, were dissected and decalcified by using 10% ethylene diamine tetra-acetic acid for 4 weeks at 4°C before being embedded in paraffin. Horizontal specimens were obtained from the root of the maxillary first molar. The sections were stained with hematoxylin and eosin for descriptive histology. The OPG immunohistochemical staining was performed to identify the OPG gene transfection. A minimum of 6 randomly selected slides per animal were deparaffinized and incubated overnight with the anti-OPG antibody (1:200 dilution; sc-8468; Santa Cruz Biotechnology, Santa Cruz, Calif) at 4°C. The anti-OPG antibody can recognize both mouse and rat OPG. The slides were stained with the 2-step plus poly-HRP anti-goat IgG detection system (ZSGB-Bio, Beijing, China), followed by color development with diaminobenzidine. The intensity of immunohistochemistry was measured with the software Image-Pro Plus (version 6.0; Media Cybernetics, Bethesda, Md). Tartrate-resistant acid phosphatase (TRAP) staining of the sections at the bifurcation level was performed with a leukocyte acid-phosphatase kit (387A-1KT; Sigma-Aldrich, St Louis, Mo). Counting was performed of TRAP-positive multinucleated cells that formed resorption lacunae on the alveolar bone surface adjacent to the distopalatal root of the maxillary right first molar.

Table. BMD and BVF of the tibia

	Control group	*Mock group*	*Experimental group*
BMD of cortical bone (g/cm^3)	0.96 ± 0.06	0.90 ± 0.03	0.96 ± 0.07
BMD of cancellous bone (g/cm^3)	0.26 ± 0.07	0.22 ± 0.02	0.24 ± 0.04
BVF (%)	21.47 ± 7.92	20.37 ± 1.77	19.35 ± 3.99

The values from 6 rats in each group are shown. BVF, a fraction of bone volume/total volume (BV/TV). There were no significant differences among the 3 groups.

Statistical analysis

All data were expressed as means ± standard deviations. Statistical significance was calculated by 1-way analysis of variance (ANOVA) and the least-significant difference test. Differences with a P value less than 0.05 were considered significant.

RESULTS

The orthodontic appliance and force only slightly affected the animals's weights during the first 3 days. There were no significant differences in weight gain among the groups. The local injections did not affect the rats' growth during the 2-week relapse. Twice-weekly local OPG gene transfer seemed to cause no appreciable macroscopic changes, such as edema, redness, or erosion at the local injection site.

Local OPG gene transfer resulted in substantial relapse of the first molar tooth movement when compared with the animals in the mock and control groups (Figs 4 and 5). The distances of tooth movement and the relapse of the first molars in the 3 groups are shown in Figure 4. After 3 weeks of force application, tooth movement of the first molars was about 1.57 to 1.59 mm, and there were no significant differences between the groups. After 2 weeks of relapse, the distance of relapse in the experimental group (0.55 ± 0.13 mm) was reduced significantly (P <0.001) when compared with the control

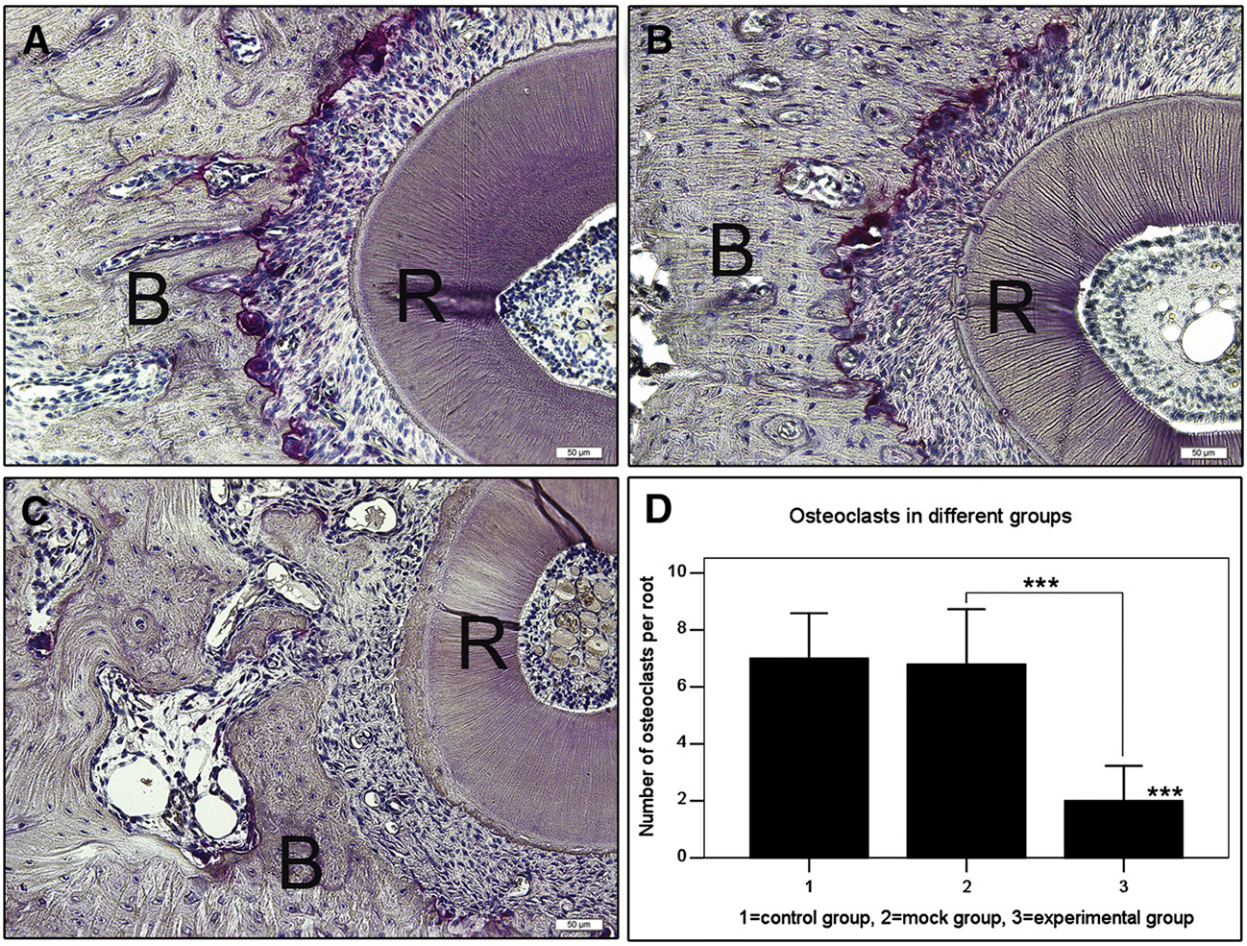

Fig 8. Osteoclasts in the groups: TRAP staining of **A,** control group; **B,** mock group; and **C,** experimental group. Representative photographs from 6 samples in each group are shown. Bar = 50 μm. *R*, Root; *B*, bone. **D,** Number of osteoclasts in the groups. TRAP-positive multinucleated cells that formed resorption lacunae were counted around the distopalatal roots of the maxillary right first molars. The numbers of osteoclasts in the experimental group were significantly fewer than in the control and mock groups. ***P <0.001.

(1.53 ± 0.23 mm) and mock (1.31 ± 0.39 mm) groups. The percentage of relapse (distance of relapse/distance of tooth movement) in the experimental group (35.7% ± 8.9%) was significantly (P <0.001) less than in the mock group (82.3% ± 10.0%) and the control group (96.3% ± 7.0%). No statistical significance was observed between the control and mock groups. These results suggest that RANKL-dependent osteoclast regulation plays a role not only in orthodontic tooth movement, but also in relapse.[17,18]

The results of microcomputed tomography analysis showed that the BMD and the BVF of alveolar bone were significantly increased by local OPG gene transfer in the experimental group compared with the other groups (BMD, P <0.01; BVF, P <0.001) (Figs 6 and 7). Local OPG gene transfer did not affect the bone remodeling of the tibia, since there were no significant differences in the average BMD and BVF values of the tibia among the groups (Table). These results suggest that local OPG gene transfer into periodontal tissue could inhibit bone resorption or increase bone formation in alveolar bone without any effects on systemic bone metabolism.

The analysis of TRAP staining showed that the number of osteoclasts was reduced by local OPG gene transfer in periodontal tissues (Fig 8). The experimental group showed significantly fewer osteoclasts than did the control (P <0.001) and mock (P <0.001) groups (Fig 8).

OPG transfection had been previously validated by in-vitro analysis.[18] OPG protein production was

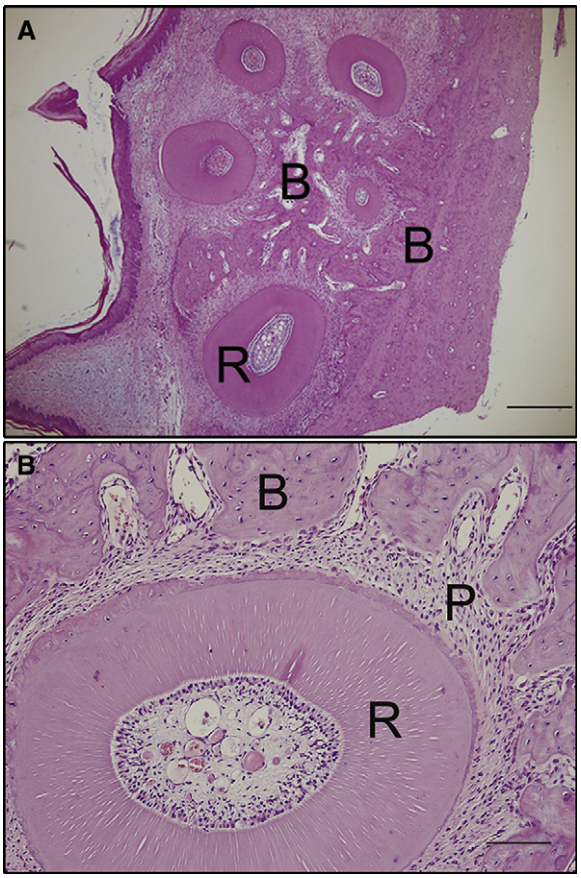

Fig 9. Hematoxylin and eosin staining of the mesial root of the maxillary right first molar. Representative photographs from 6 samples are shown: **A,** scale bar = 500 μm; **B,** scale bar = 100 μm. There were no severe inflammations in the periodontal tissue. *R*, Root; *P*, periodontal ligament; *B*, bone.

confirmed by western blot analysis, and the functional activity of OPG was tested by examination of bone resorption.[18]

The sections were stained with hematoxylin and eosin to assess whether repeated local gene transfer could induce inflammation. Section analysis showed no severe inflammations, such as lymphocytic infiltration, in the periodontal tissues when repeated local OPG gene transfers were performed (Fig 9).

The immunohistochemical analysis of OPG showed that, when the HVJ envelope vector containing pcDNA-mOPG was injected into the experimental group, OPG protein expression was facilitated locally in the peridontium (Fig 10).

DISCUSSION

In this study, we used an experimental tooth movement system with a fixed orthodontic appliance. The orthodontic force generated by this device was 40 g, which constantly generated effective tooth movement of the first molars. Analysis of the effect of this force in rats showed that it did not significantly affect local periodontal health or body weight. In addition, there were no significant differences in the average amounts of tooth movement among the groups. These results suggest that the magnitude and duration of the applied force in this study were appropriate for observing the extent of relapse in each group.

The HVJ envelope vector gene delivery system was used to deliver OPG gene transfer to the periodontal tissues without local inflammation or systemic effects. The HVJ envelope vector is a nonviral gene transfer system and has several advantages over other gene transfer systems.[22-24] The low immunogenicity of the HVJ envelope vector is superior to highly immunogenic vectors such as an adenoviral vector, and it is beneficial for long-term gene expression and safety.[22] The HVJ envelope vector has been reported to have high in-vivo gene transfer efficiency.[22,23] Furthermore, this transfer system is safe, and administration can be repeated without causing undesirable side effects, such as inflammation.[24] In this study, the repeated administration of vector solution, with or without the OPG gene, did not affect the BMD and the BVF of the tibia. These results suggest that local OPG gene transfer did not affect the systemic bone remodeling. Gene transfer was administered twice weekly because of the attenuation time of our local gene transfer system. Previously, we had demonstrated that twice-weekly administration of the HVJ envelope vector, containing pc-DNA mOPG, could maintain a locally effective concentration and prolonged protein expression.[18]

In this study, the distances of tooth movement and relapse were measured by palatal superimposition of the 3D digital models. This method has previously been used to analyze tooth movement in clinical studies.[25-29] The accuracy and reliability of palatal superimposition of 3D digital models have been confirmed.[25,26] In animal research, there are no studies focusing on the stability of the palatal rugae. Therefore, we examined the palatal surface by 3D analysis and found it stable throughout the experimental period.

Previous reports directly measured the distance of tooth movement with a digital caliper while the animals were under anesthesia[30] or on a stone model.[7] The amount of tooth movement was calculated as the distance between the first and second molars. However, the distance between the first and second molars might not be the actual distance moved by the first molar. Since we were unsure whether the second molar was stable, there might have been a mesial-moving tendency for

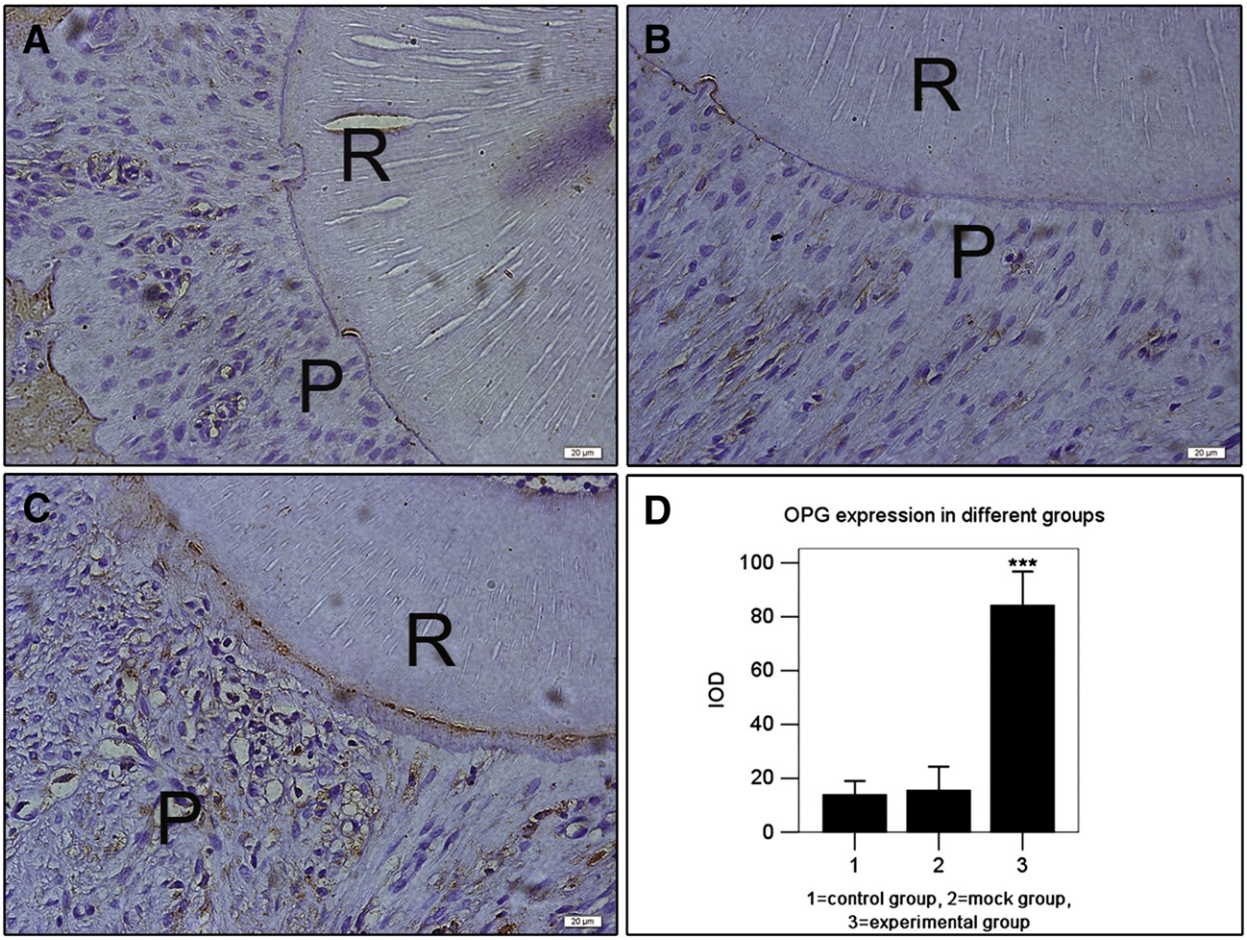

Fig 10. Immunohistochemical analysis of OPG: **A,** control group; **B,** mock group; **C,** experimental group. Representative photographs from 6 samples in each group are shown. Bar = 100 μm. *R*, Root; *P*, periodontal ligament. **D,** The intensity of immunohistochemistry. *IOD*, Integrated optical density. The intensity of immunohistochemistry was measured on samples from 6 photographs in each group. ***P <0.001.

the second molar when the first molar moved mesially. To overcome this problem, palatal superimposition of the 3D digital models was used to measure the distances of tooth movement and relapse. Therefore, this prevented the influence of second molar movement on measurement and enhanced the accuracy.

In this study, local OPG gene transfer was used as a biologic method to prevent or inhibit relapse after orthodontic treatment. In the experimental group, the number of osteoclasts was significantly fewer, whereas the BMD and the BVF of alveolar bone significantly increased, when compared with the control and mock groups. The measurement results of tooth movement and relapse showed that molar movement was successfully inhibited by local OPG gene transfer. A possible mechanism is that, by inhibiting osteoclastogenesis and coordinating bone remodeling, tooth relapse can be inhibited.

BMD and BVF are commonly used to evaluate the status of bone metabolism. BMD is a strong predictor of bone strength, and BVF is an important parameter in describing the trabecular microstructure.[21,31,32] Microcomputed tomography analysis is a nondestructive examination, with a demonstrated ability to measure BMD and BVF with high degrees of accuracy and precision.[21,31,32]

Our immunohistochemical results showed that local OPG gene transfer could induce local OPG expression in periodontal tissues. Exogenous OPG expression affected local bone remodeling, and osteoclastogenesis was significantly inhibited. Furthermore, the BMD and the BVF of the alveolar bones in the rats' first molar furcation areas were significantly increased. When the tibia

was analyzed, no significant differences in BMD and BVF were observed. These data suggest that the overexpressed OPG inhibited osteoclastogenesis only in the alveolar bone and not in the tibia, which was far from the injection site.

Biologic retention methods to overcome relapse have recently come under study. Local or systemic administration of bisphosphonate and systemic administration of simvastatin were shown to decrease the extent of initial relapse in experimentally moved rat molars.[7-9,33] However, since bisphosphonate and simvastatin are rapidly distributed by blood circulation, only daily systemic administration was required. Using a different approach, Hassan et al[10] injected dried bone matrix containing bone morphogenetic proteins into the periodontal tissues of experimental sheep, and then the relapse of incisor movement was inhibited. However, this was a pilot study, so the number of animals was small, and further studies are required for confirmation of this biologic mechanism.

In this study, we indicated that local OPG gene transfer might be a biologic retention method. However, the biologic mechanism behind it is still unclear. Further studies are required to evaluate the role of the RANK/RANKL/OPG axis on relapse.

CONCLUSIONS

OPG gene transfer to periodontal tissues increased the BMD and the BVF of alveolar bone and inhibited the relapse of tooth movement. The OPG gene transfer did not elicit any detectable systemic effects. Therefore, local OPG gene transfer might be a useful tool for preventing orthodontic relapse.

The SkyScan 1076 x-ray microtomography system was provided by the School of Biological Science and Medical Engineering, Beijing University of Aeronautics and Astronautics. The 3D spot laser scanner LPX-1200 and Rapidform 2006 software were provided by the 3D Laboratory of Orthodontic Department at Peking University, School and Hospital of Stomatology. We thank Hongti Jia, Yaqiong Jin, Zhe Zhong, and Gui Chen for their kind support.

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