

## Original article

# Effects of cell-mediated osteoprotegerin gene transfer and mesenchymal stem cell applications on orthodontically induced root resorption of rat teeth

Nisa Gul Amuk<sup>1</sup>, Gökmen Kurt<sup>2</sup>, Yusuf Baran<sup>3,4</sup>, Volkan Seyrantepe<sup>3</sup>, Melis Kartal Yandim<sup>3,5</sup>, Aysun Adan<sup>3,4</sup>, Secil Akyildiz Demir<sup>3</sup>, Yagmur Kiraz<sup>3,4</sup> and Mehmet Fatih Sonmez<sup>6</sup>

<sup>1</sup>Department of Orthodontics, Faculty of Dentistry, Erciyes University, Kayseri, <sup>2</sup>Department of Orthodontics, Faculty of Dentistry, Istanbul Yeni Yüzyıl University, <sup>3</sup>Department of Molecular Biology and Genetics, Faculty of Science, Izmir Institute of Technology, <sup>4</sup>Department of Molecular Biology and Genetics, Faculty of Life and Natural Sciences, Abdullah Gul University, Kayseri, <sup>5</sup>Department of Medical biology, Faculty of Medicine, Izmir University of Economics, and <sup>6</sup>Department of Histology and Embryology, Faculty of Medicine, Erciyes University, Kayseri, Turkey

Correspondence to: Nisa Gul Amuk, Department of Orthodontics, Faculty of Dentistry, Erciyes University, 38039 Melikgazi, Kayseri, Turkey. E-mail: [nisa.gul86@hotmail.com](mailto:nisa.gul86@hotmail.com)

## Summary

**Aim:** The aim of this study is to evaluate and compare therapeutic effects of mesenchymal stem cell (MSCs) and osteoprotegerin (OPG) gene transfer applications on inhibition and/or repair of orthodontically induced inflammatory root resorption (OIIRR).

**Materials and methods:** Thirty Wistar rats were divided into four groups as untreated group (negative control), treated with orthodontic appliance group (positive control), MSCs injection group, and OPG transfected MSCs [gene therapy (GT) group]. About 100g of orthodontic force was applied to upper first molar teeth of rats for 14 days. MSCs and transfected MSC injections were performed at 1st, 6th, and 11th days to the MSC and GT group rats. At the end of experiment, upper first molar teeth were prepared for genetical, scanning electron microscopy (SEM), fluorescent microscopy, and haematoxylin eosin-tartrate resistant acid phosphatase staining histological analyses. Number of total cells, number of osteoclastic cells, number of resorption lacunae, resorption area ratio, SEM resorption ratio, OPG, RANKL, Cox-2 gene expression levels at the periodontal ligament (PDL) were calculated. Paired *t*-test, Kruskal–Wallis, and chi-square tests were performed.

**Results:** Transferred MSCs showed marked fluorescence in PDL. The results revealed that number of osteoclastic cells, resorption lacunae, resorption area ratio, RANKL, and Cox-2 were reduced after single MSC injections significantly ( $P < 0.05$ ). GT group showed the lowest number of osteoclastic cells ( $P < 0.01$ ), number of resorption lacunae, resorption area ratio, and highest OPG expression ( $P < 0.001$ ).

**Conclusions:** Taken together all these results, MSCs and GT showed marked inhibition and/or repair effects on OIIRR during orthodontic treatment on rats.

## Introduction

Orthodontic tooth movement is achieved by remodelling of the periodontal ligament (PDL) and alveolar bone. At the pressure side of PDL, bone resorption was performed by osteoclasts and at the tension side new bone formation was achieved by osteoblasts (1). Root resorption process has similarities with bone resorption in many aspects. The most important biological markers that are accepted as determinants of bone resorption process are osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) which also plays a role with root resorption process (2). Cementoclasts that are responsible from cement resorption are similar to osteoclasts in morphology, activity, function, and features (3). Both cell types can be evaluated clearly by tartrate-resistant acid phosphatase (TRAP) staining (4).

One of the main function of OPG/RANK/RANKL is to regulate resorptive cell differentiation (2). RANKL is very important about multinuclear and mononuclear phagocytic cell maturation and OPG is known as osteoclast inhibitor. OPG also activates alkaline phosphatase expression which has critical importance at early differentiation phase of osteoblasts and promotes differentiation of preosteoblasts into mature osteoblasts (5, 6). Another important cytokine of root resorption is cyclooxygenase-2 (Cox-2), and this cytokine also participates in inflammatory process of orthodontic tooth movement as well as of orthodontically induced inflammatory root resorption (OIIRR). In addition, this arachidonic acid derivate regulates pain formation (7).

Expression of RANKL is induced at the pressure side during tooth movement for osteoclastic activation (8) while OPG expression rises at the tension side (9). During the remodelling of hard tissues, root surfaces also were affected from resorptive activity on alveolar bone via RANKL and osteoclasts (2). On the other hand, overexpression of OPG inhibits RANKL and osteoclastic activity which plays an important role in root resorption.

Regeneration and repair effects of stem cells have not been investigated as a treatment method of OIIRR in the literature. These cells might present therapeutic results because of their anti-inflammatory effects (10) and proliferation–differentiation capacity to the needed repair cells in related region. If prolonged OPG expression of these excellent cells can be achieved, they may also avoid osteoclast, cementoclast differentiation and may stimulate osteoblastic maturation through enhanced alkaline phosphatase expression and increased matrix mineralization (6).

These goals may be achieved by cell-mediated gene therapy (GT) applications. Although decrease in amount of orthodontic tooth movement was reported as a result of OPG transfer via non-viral vectors (11) and as a form of fusion proteins (12) at previous studies which investigated effects of OPG on tooth movement, transfer of OPG gene in mesenchymal stem cell (MSC) to the PDL area for reducing and repairing root resorption has not been reported in the literature.

The aim of this experimental study was to evaluate and to compare the effects of MSC and cell-mediated OPG transfer GT applications on inhibition and repair of OIIRR.

## Materials and methods

### Animals

A total of 30 healthy, 12-week-old Wistar rats were divided randomly into four groups as untreated group (negative control), treated with orthodontic appliance group (positive control), MSCs injection group, and OPG transfected MSCs (GT) group. Animal selection,

management, and experiment protocols were approved by Erciyes University, Regional Animal Research Ethics Committee (approval code: 11/136). Cell isolation, gene preparation, and transfection steps were performed at Izmir Institute of Technology, Department of Molecular Biology and Genetics.

### Orthodontic appliance design

The rats were anaesthetized with an intraperitoneal injection of 1.0 mg/kg ketamin hydrochloride (Gedeon Richter Ltd, Budapest, Hungary) and 0.5 mg/kg xylazine (Rompoun Bayer, Leverkusen, Germany) combination. A special oral retractor was used for providing appropriate mouth opening, head position, and soft tissue retraction. Orthodontic tooth movement achieved by modified technique described by Brudvik and Rygh (13). The force of coil springs was adjusted to 100 g.

### Isolation and cultivation of MSCs

The bone marrow aspirates obtained from tibia and femoral parts of the compact rat bone were used as a source of MSCs (14). The bones were sterilized with 70% ethanol and then washed with 1 × PBS at least two times. Then, the epiphyses were removed by using sterile scissors. A syringe needle was placed into the bone marrow cavity and the marrow was completely flushed out with PBS. The resulting bone marrow suspension was centrifuged at 1000 rpm for 10 minutes. The pellet was suspended and cultured in *Dulbecco's modified Eagle's medium* (DMEM) (Invitrogen, Paisley, UK) containing 15% foetal bovine serum (FBS) and 1% penicillin-streptomycin (37°C and 5% CO<sub>2</sub> incubator). Cultured bone marrow mesenchymal stem cells (BM-MSCs) were observed under the microscope in order to control their expansion and morphology. When 80–90% confluency is obtained, cells were trypsinized using Trypsin/EDTA solution and cells were seeded onto fresh plates (15).

### Characterization of BM-MSCs

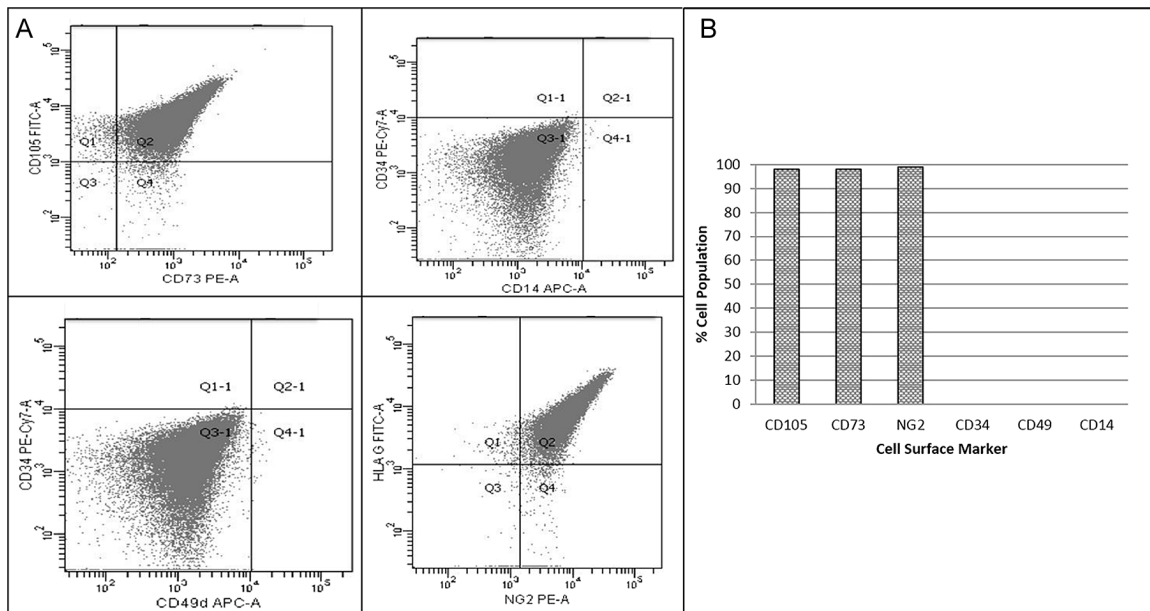
We incubated the cells with antibodies against CD45 PE, CD73 PE, CD105 FITC, and NG2 PE antibodies. A total of 2 × 10<sup>4</sup> cells/sample at a flow rate of approximately 200 cell events/s were recorded to obtain Fluorescence histograms. Coulter Epics XL-MCL was used during the experiments and the data were analyzed by using EXPO 32 ADC software (Beckman Coulter, Inc., Miami, Florida, USA). Flow cytometry analysis revealed that there were significant expressions of CD105, CD73, and NG2 that are specific to MSC antigens while there was no detection of CD45 that is specific to hematopoietic marker antigen (Figure 1). These results showed that these cells are MSCs.

### Transfection of MSCs by pEGFP-N2 (green fluorescent protein) and pEGFP-N2-OPG plasmids

Total RNA was extracted from the mouse osteoblastic cells isolated from callus tissue on alveolar and tibial bony defects of rats and then reverse transcribed.

OPG gene was enhanced using specific PCR primers including cDNA plus restriction site, primer XhoI site + OPG upstream 5'-AAACTCGAGTGAGGTTTCCCAGGACCAC-3' and -downstream + with KpnI-Hind III site (5'-AGGTACCAAGCTTTAA GCAGCTTATTTTACGG-3') using PCR amplification.

Obtained OPG cDNA was inserted to the TA cloning vector [TA Cloning® Kit (Invitrogen-K2000-01)]. TA vector and green fluorescent protein (GFP) containing PEGFP-N2 united for obtaining OPG–GFP fusion protein for GT group. Presence of OPG gene in the TA vector and pEGFP-N2-OPG vector was verified with EcorI



**Figure 1.** Characterization of the mesenchymal stem cell population from rat bone marrow. (A) Representative flow cytometry dot plots showing the principal mesenchymal stem cell markers. (B) Representative histogram plots of the flow cytometry analysis revealed that there were significant expressions of CD105, CD73, and NG2 specific to MSC antigens.

and Hind III restriction reactions. For single MSCs application pEGFP-N2 vector was used without OPG gene carrying TA vector. With GFP gene production both MSCs and transfected MSCs could be visualized *in vivo* by fluorescent microscopy. After combining, pEGFP-N2-OPG vector was enhanced. GT group stem cells were transfected with pEGFP-N2-OPG vector and MSCs group stem cells was transfected with pEGFP-N2 vector only by using Metafectene Pro Transfection Reagent (Biontex Laboratories GmbH, Germany). About 48–72 hours after the transfection, the transfected cells were visualized under the fluorescent microscope (Figure 2).

### Transfer of MSCs to the rats

For transfer of MSCs to the PDL of upper first molar of experiment group rats, injections was performed under general anaesthesia at the 1st, 6th, and 11th days of experiment. For each tooth of MSCs and GT groups,  $25 \times 10^3$  cells were prepared and injected from mesio-vestibular and mesio-palatinal corners of upper first molar teeth. Attention had been paid not to create traumatic application.

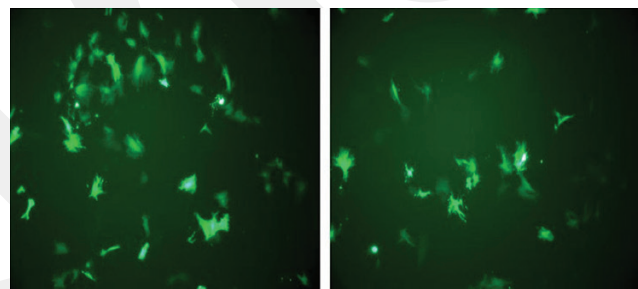
### Fluorescent microscopy

At the end of 14th experimental day, experiment and control group rats were euthanized with overdose of anaesthetic (200 mg/kg sodium-pentobarbital pentol, Abbot, ABD).

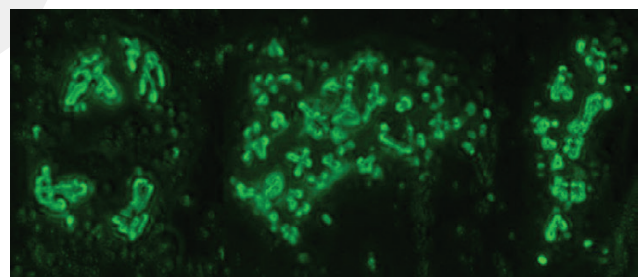
To investigate the presence of the transferred GFP containing MSCs at the GT and MSCs groups, PDL tissue was prepared for fluorescent microscope examination. Smear from PDL tissue was spread on slides and micrographs were evaluated in terms of GFP shining (Figure 3).

### Scanning electron microscopy

Totally 18 extracted teeth were evaluated in order to investigate the resorptive changes on the molar root surface. Scanning electron microscope (SEM) (SEM; Leo 440; M/s Leo Electron Microscopy, Cambridge, UK) examinations were performed on the mesial surfaces of the mesio-buccal root of the maxillary first molars and



**Figure 2.** Fluorescent microscopy images of transfected mesenchymal stem cell with pEGFP-N2-OPG and pEGFP-N2 vector, prior to injection showing stem cells and expressing of green fluorescent protein. Magnification:  $\times 40$ .



**Figure 3.** Fluorescent microscopy images of periodontal ligament (PDL) smear samples of cell transfer group teeth. Expressing of green fluorescent protein of transfected stem cells revealed the presence of transfected stem cells in PDL tissue of cell transfer group teeth. Magnification:  $\times 10$ .

measurement were processed with ImageJ (1.47v; Java1.6.0, Research Services Branch, USA). Healthy cement shows smooth surface characteristics (4) while marked resorption lacunae have views of dentin tubules through the craters (Figure 4A) (16). The repair layer of resorption lacunae were recognized with smooth cement surface coverage onto the dentin tubule orifices in crater or with

shallowing of resorption craters compared with near lacunae borders (4). General resorption crater borders were observed and signed for each image at  $\times 100$  magnification. The surface structure of root was examined in terms of repair layers and the borders of resorption areas were rearranged by leaving the repaired areas out of SEM resorption measurement area (Figure 4B). Resorption areas were proportioned to entire frontal root surface and then SEM resorption area ratio was calculated.

### Histologic preparation and evaluation

Twenty upper first molar teeth and surrounding maxilla were totally dissected and transferred to the histology laboratory in 10% formaldehyde. After histological procedures,  $5\ \mu\text{m}$  parasagittal sections from the longest length of the mesiobuccal root of first molar were cut and stained with haematoxylin-eosin (H&E) and TRAP. Photomicrographs were taken digitally with  $\times 4$ ,  $\times 10$ , and  $\times 20$  objective lens with a microscope and digital camera system (Olympus CX41/DP25; Olympus Corp., Tokyo, Japan).

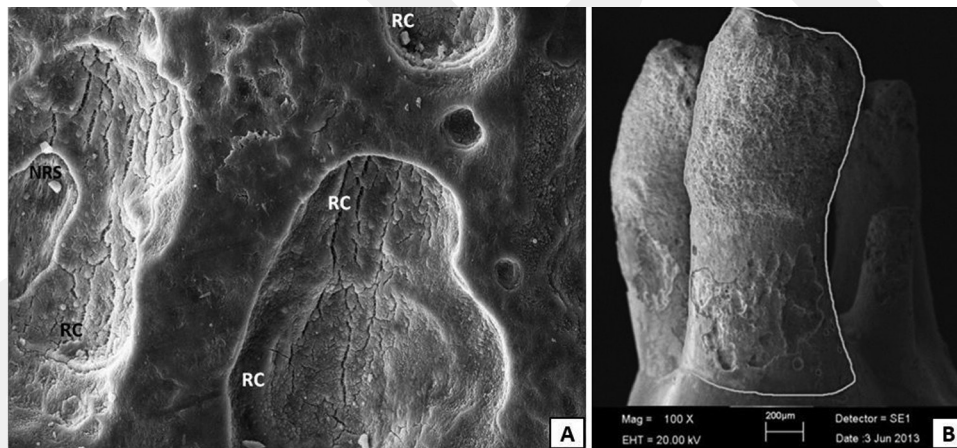
To calculate the percentage of root resorption on H&E staining slides, grids were placed onto the images using Adobe Photoshop CS3 version 10.0 and resorption area ratio results were determined by dividing the number of lacunae grids to the number of entire root surface excluding pulp area (Figure 5A). For determining total cell count on the H&E slides greater sized grids were used (Figure 5B). One apiece grid was selected from apical, middle, and cervical PDL field for each sample. The average of the number of cells in

the selected grids is calculated. Total number of lacunae were also counted and scored in terms of their depth qualifications. Resorption lacunae qualification scores have a range between 1 and 4; 1 indicates resorptions in the cemental region; 2, resorptions progressed to the cement-dentin border; 3, resorptions deepened into the dentin up to cement thickness; 4, resorptions deepened into the dentin more than cement thickness.

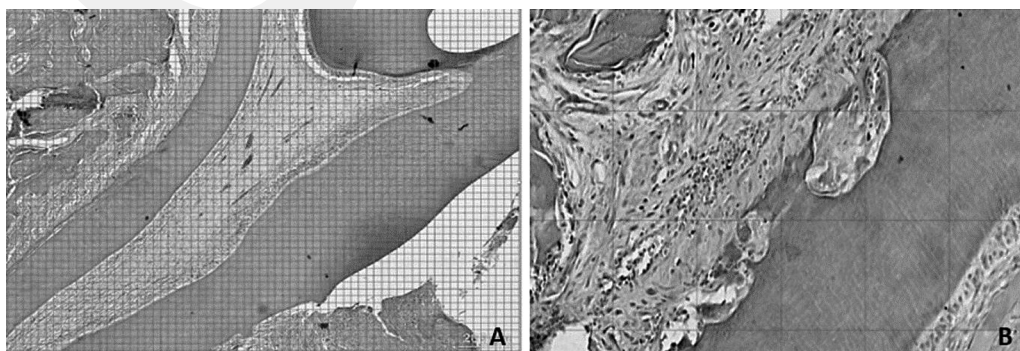
For osteoclast–cementoclast cell investigation, TRAP staining slides were evaluated in same way with H&E slides. Grids were superimpositioned and TRAP (+) cell counting performed from three horizontal levels (apical, middle, and cervical) of PDL close to teeth, middle PDL and PDL close to bone. The values for five consecutive sections were averaged for each specimen and used to calculate the mean and standard deviation for each experimental group.

### Determining expression levels of OPG, RANKL, and Cox-2

Upper first molars extracted from alveolar socket carefully. The extracted teeth containing PDL tissue on their roots were placed in mRNA fixation solution. RNA was isolated from samples by QIAamp RNA Blood Mini Kit (QIAGEN—52304). cDNA was synthesized from RNA isolates with First Strand cDNA Synthesis Kit (Thermo Scientific—K1612). Obtained material was analyzed with real-time polymerase chain reaction (PCR) for OPG, RANKL, and Cox-2 (for details on PCR primer and probe sequence, see Supplementary Table 1).



**Figure 4.** Resorption and repair image on SEM analysis. (A) RC (*repair cement*): repair of resorption lacunae. NRS (*naked resorption surface*): naked unrepaired dentin surface on the centre of lacunae, because repair progresses from perimeter to the deepest centre of lacunae. (B) Resorption area ratio evaluation on SEM images.



**Figure 5.** Hematoxylin and Eosin (H&E) staining of histological specimens from upper first molar teeth of rats. (A) Resorption area ratio and (B) number of total cells calculation using grids.

## Statistical analysis

Statistical Package for Social Sciences, 20.0 program ((SPSS for Windows; SPSS Inc, Chicago, Illinois, USA) was used for all statistical analysis. Initial and final body weight of rats was compared with paired sample *t*-test. Kruskal–Wallis analysis was used for inter-group comparison of number of total cells, number of osteoclastic cells, number of resorption lacunae, resorption area ratio, SEM resorption area ratio, OPG, RANKL, and Cox-2 mRNA gene expression levels. Dunn's method was preferred for multiple comparisons of parameters. Comparison of resorption lacunae scores between groups was performed with chi-square test. When the *P* value was less than 0.05, the statistical test was determined as significant. Linear regression analyses and correlation evaluations were performed for OPG versus number of osteoclasts, number of resorption lacunae, resorption area ratio variables and RANKL versus number of osteoclasts, number of resorption lacunae, resorption area ratio.

## Results

Statistically significant weight loss was observed only at the positive control group animals until end of the experiment. One animal died from MSCs group because of natural reasons (for details on body weight changes between T0 and T1, see Supplementary Table 2). Staining with H&E indicated that total cell number was significantly greater at the GT and MSCs groups ( $P < 0.001$ ) while TRAP staining results revealed reduction of osteoclast number at GT ( $P < 0.05$ ) and MSCs ( $P < 0.05$ ) groups compared with positive

control group (Table 1). Accordingly, GT and MSCs treated group samples showed significantly lower resorption area ratio at SEM ( $P < 0.05$ ) and histological examinations ( $P < 0.05$ ) and resorption lacunae number ( $P < 0.05$ ). However, osteoclast number and resorption lacunae area results of GT group were significantly lower than MSCs at the same time ( $P < 0.05$ ) (Table 2). Resorption lacunae scores were not different between groups significantly ( $P = 0.141$ ). The highest OPG mRNA expression was found in GT ( $P < 0.001$ ) group and MSC group values follows GT with significantly higher values than control groups ( $P < 0.05$ ). RANKL ( $P < 0.001$ ) and Cox-2 ( $P < 0.05$ ) expression results showed similar levels at MSC and GT groups and lower than positive control significantly.

Regression and correlation analyses showed a negative relationship between OPG and osteoclast number, resorption lacunae number and resorption area ratio (Figure 6), while positive relationship was found between RANKL and resorption lacunae forming and osteoclast number (Figure 7).

## Discussion

Prevention and repair of root resorption have clinical importance because of maintaining long-term vitality of teeth and stability of the orthodontic treatment. Our study aimed to present therapeutic results of two advanced cellular and genetical approaches on OIRR.

MSCs are often preferred cell groups due to their differentiation capacity to many other cell types, leading and accelerating feature in

**Table 1.** Comparison of variables between groups.

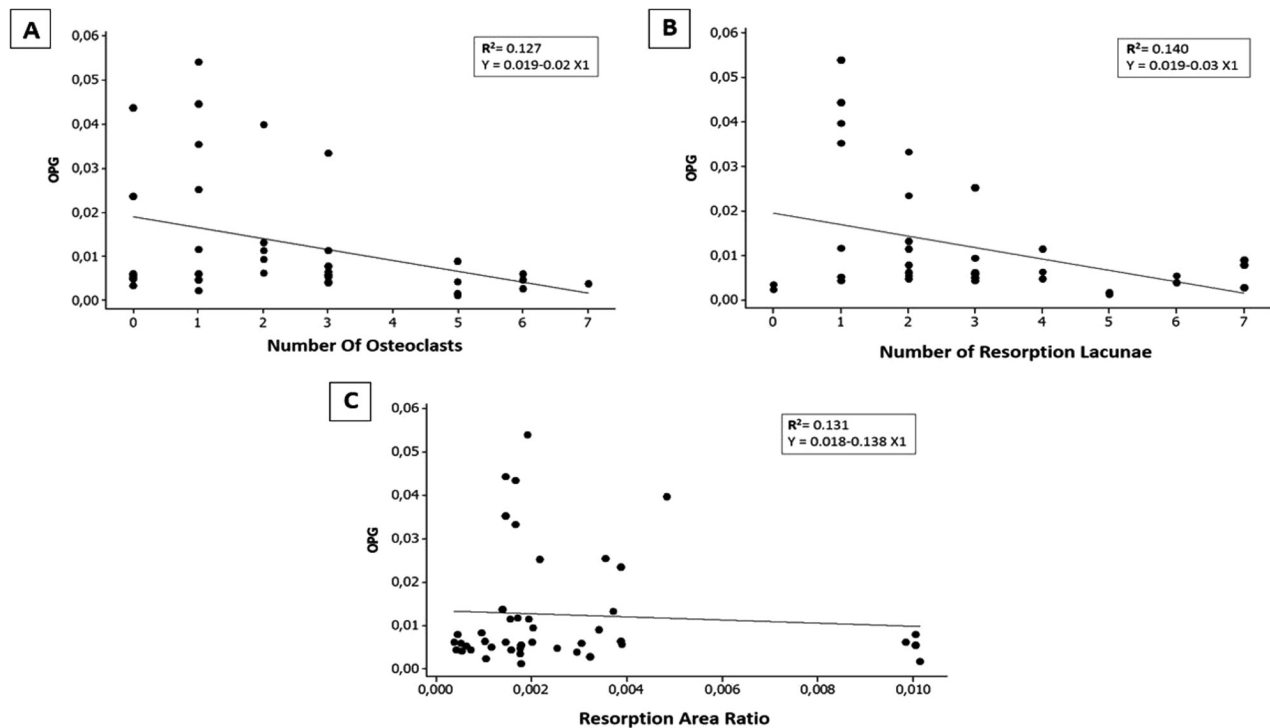
Group	GT			MSCs			PC			NC			<i>P</i>
	Median	%25	%75	Median	%25	%75	Median	%25	%75	Median	%25	%75	
NTC	68.5 <sup>a</sup>	64.50	80.5	73.5 <sup>a</sup>	71.5	76.5	43.5 <sup>b</sup>	41.5	48.0	36.0 <sup>b</sup>	30.5	40.0	<0.001
RAR	0.01 <sup>b</sup>	0.008	0.013	0.03 <sup>ab</sup>	0.02	0.03	0.1 <sup>a</sup>	0.09	0.12	0.01 <sup>b</sup>	0.00	0.001	<0.001
NRL	1.00 <sup>b</sup>	1.00	1.75	2.00 <sup>ab</sup>	2.00	2.50	5.50 <sup>a</sup>	5.25	6.50	0.50 <sup>b</sup>	0.00	1.00	<0.001
NOsC	1.00 <sup>b</sup>	0.75	1.50	2.50 <sup>ab</sup>	1.50	3.00	5.50 <sup>a</sup>	4.00	6.00	0.00 <sup>b</sup>	0.00	0.50	<0.01
Score	2.00	1.50	2.50	—	—	—	3.50	2.50	4.00	—	—	—	0.141
SRAR	0.01 <sup>b</sup>	0.02	0.04	0.05 <sup>ab</sup>	0.03	0.06	0.16 <sup>a</sup>	0.14	0.18	0.00 <sup>b</sup>	0.00	0.01	<0.001
OPG	0.03 <sup>a</sup>	0.013	0.047	0.009 <sup>ab</sup>	0.005	0.012	0.005 <sup>b</sup>	0.004	0.006	0.004 <sup>b</sup>	0.003	0.005	<0.001
RANKL	0.002 <sup>ab</sup>	0.001	0.003	0.003 <sup>ab</sup>	0.002	0.003	0.006 <sup>a</sup>	0.004	0.009	0.001 <sup>b</sup>	0.0008	0.0016	<0.001
Cox-2	0.004 <sup>ab</sup>	0.003	0.007	0.005 <sup>ab</sup>	0.003	0.014	0.009 <sup>a</sup>	0.004	0.015	0.001 <sup>b</sup>	0.0008	0.0019	<0.05

There is statistically significant difference at  $P < 0.05$ . Groups with different letters are significantly different from each other. GT, gene therapy; MSCs, mesenchymal stem cells; PC, positive control; NC, negative control; NTC, number of total cell; RAR, resorption area ratio; NRL, number of resorption lacunae; NOsC, number of osteoclastic cell; SRAR, SEM resorption area ratio.

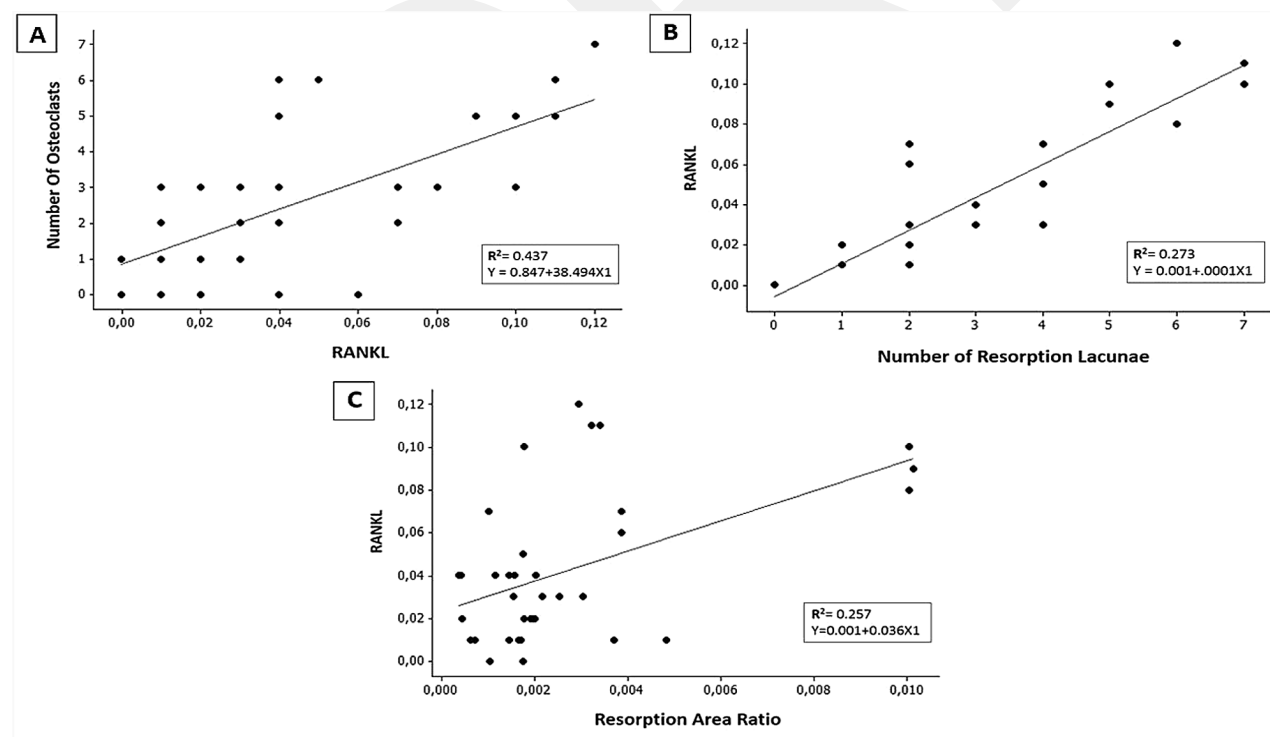
**Table 2.** Multiple comparison and statistical significance.

Multiple comparison	<i>P</i> value					
	GT-MSCs	GT-PC	GT-NC	MSCs-PC	MSCs-NC	PC-NC
NTC	NS	<0.05	<0.05	<0.05	<0.05	NS
RAR	<0.05	<0.05	NS	<0.05	<0.05	<0.05
NRL	<0.05	<0.05	NS	<0.05	<0.05	<0.05
NOsC	<0.05	<0.05	NS	<0.05	<0.05	<0.05
Score	—	—	—	—	—	—
SRAR	<0.05	<0.05	NS	<0.05	<0.05	<0.05
OPG	<0.05	<0.05	<0.05	<0.05	<0.05	NS
RANKL	NS	<0.05	<0.05	<0.05	<0.05	<0.05
Cox-2	NS	<0.05	<0.05	<0.05	<0.05	<0.05

There is statistically significant difference at  $P < 0.05$ . GT, gene therapy; MSCs, mesenchymal stem cells; PC, positive control; NC, negative control; NTC, number of total cell; RAR, resorption area ratio; NRL, number of resorption lacunae; NOsC, number of osteoclastic cell; SRAR, SEM resorption area ratio.



**Figure 6.** Scatter plot with fitted regression line showing negative relationship between OPG and osteoclast number, resorption lacunae number and resorption area ratio. (A) OPG versus number of osteoclasts ( $r = -0.357$ ,  $P = 0.015$ ). (B) OPG versus number of resorption lacunae ( $r = -0.374$ ,  $P = 0.012$ ). (C) OPG versus resorption area ratio ( $r = -0.363$ ,  $P = 0.015$ ).



**Figure 7.** Scatter plot with fitted regression line showing positive relationship between RANKL and osteoclast number, resorption lacunae number and resorption area ratio. (A) RANKL versus number of osteoclasts ( $r = 0.661$ ,  $P < 0.001$ ). RANKL versus number of resorption lacunae ( $r = 0.523$ ,  $P = 0.001$ ). (C) RANKL versus resorption area ratio ( $r = 0.507$ ,  $P = 0.001$ ).

terms of regeneration and repair process (17). Yang *et al.* (18) transferred BM-MSCs to the experimental periodontal defects of rats by labelling with GFP. They reported increase in new bone, cement and

PDL tissue regeneration. We also preferred these cells for due to their new cement formation ability, easy availability, and similarity with PDL stem cells (19).

Stem cell application to the experimental periodontal defects generally requires a scaffold or vehicle for retention of cells in the cavity. A new non-invasive and non-traumatic cell delivery method was developed for this study with dental injection to PDL. No inflammatory response or bleeding was observed during or after cell injections.

Recently, MSCs sources were increased which offers stem cells have similar regenerative characteristics with BM-MSCs (20). Although BM-MSCs were used for this study, differentiation of MSCs from PDL, dental pulp, dental follicle, gingival tissue into osteogenic, dental tissue cells were also demonstrated (20–23). PDL, dental follicles, or pulpal tissues of collected or cryopreserved extracted teeth might be available MSCs sources for patients whose orthodontic treatment plan involves premolar or wisdom teeth extractions (24). For non-extraction treatments gingival tissues also serve as MSCs sources (23). Further studies may investigate efficacy of MSCs from dental tissues on inhibition of OIIRR because of their convenience of isolation in orthodontic patients.

Prolonged and over-expression of OPG in the resorption areas was achieved by cell-mediated GT in the current study. MSCs did not only serve as a mediator for related gene expression by autocrine and paracrine mechanism, but also they became a part of target tissue by differentiating, rapidly proliferating, and taking an active role in the repair process (25). Similar with our study, Edward *et al.* (26) also used MSCs for regeneration and gene transfer at the cranial defects and reported significantly increase in new bone formation.

MC3T3-E1 osteoblastic cell lines have been used previously for obtaining OPG gene material (11). However obtaining this cell line, keeping alive and usable during and after transfer was suspicious and costly. A new cell source was defined for extraction of total RNA that belongs to osteoblasts. In this way, genetic material at desired quantity and quality was obtained successfully, less costly, and faster. After transfection, transfer of MSCs was achieved by newly described cell transfer method to the PDL. Infiltrative and intraligamentary injections were used for the delivery of MSCs and OPG carrier MSCs to the intact PDL. Kanzaki *et al.* (11, 27) also transferred genes to the PDL by injections successfully, but differently from us, they used vector-mediated gene transfer instead of cells. Presence of GFP labelled MSCs in PDL were proven both with rise in total cell count in PDL and GFP shining in fluorescent microscope images, indicating successful cell transfer. OPG over-expression by transfected MSCs was evaluated with PCR analysis on PDL tissue samples and marked rise of OPG mRNA expression was observed.

MSCs applications also created a reduction in osteoclastic activity by itself. This situation can be explained with anti-inflammatory effects of stem cells (28). The other evidence about the suppression of inflammation effect of MSCs was decline in Cox-2 mRNA expression which is responsible for the synthesis of prostaglandins from the pro-inflammatory cytokines. At the same time decrease in Cox-2 mRNA expression in MSCs and GT group compared with positive control can be interpreted as relief on orthodontically induced pain. In addition, rise in OPG mRNA expression and decline in RANKL mRNA expression was observed as a result of MSCs transfer. Beside these anti-inflammatory and reparative effects of MSCs, with GT, prolonged and over-expression of OPG inhibited RANK–RANKL linkage and the highest decrease in osteoclast–cementoclast number was found at GT group samples. Most of studies reported successful determination of multinuclear bone cells with TRAP staining (13). In this study, multinuclear cells in PDL could be observed clearly and consistent with Kanzaki *et al.*

(11) study that they reported reduction on number of osteoclasts as a result of OPG gene transfer.

Decreased number of resorption lacuna with MSCs transfer to the PDL can be explained with proliferation and differentiation of MSCs to reparative cells like cementoblasts and cementoblast-like cells after the metabolic regeneration request and to create an anti-inflammatory activity. Moreover, the lowest resorption was found in OPG gene transfer group related with inhibition of RANKL–RANK link and osteoclast differentiation in addition to reparative effects of MSCs. Beside the findings of present study indicated inhibition and/or repair of root resorption with OPG transfer, findings of Kanzaki and Dunn (11, 12) revealed that rising in OPG levels in PDL decelerates tooth movement as a result of reduction on the alveolar bone resorption. Likewise anti-inflammatory effects of MSCs application also may decrease amount of tooth movement in same manner. Although decelerating of tooth movement seems as a serious disadvantage of over-expression of OPG and anti-inflammatory effects of MSCs, the orthodontic management of some cases who have tendency to root resorption or some situations of severe root resorption during orthodontic treatment may necessitate the preference of OIIRR inhibition rather than accelerated tooth movement. When severe root resorption was detected during orthodontic treatment, the main purpose of treatment may change into long-term viability of the dentition. In such cases, clinicians should content with decelerated tooth movement for achieving inhibition and repair of root resorption. In these aspects, treatment approaches for effective elimination and repair of root resorption should be improved for distinctive indications although they cause reduction on velocity of tooth movement. Moreover, decrease in the amount of tooth movement because of rising of OPG level in PDL were reported as dose-dependent (12). Further studies are required to determine the optimum dosage of OPG transfer for both resorption inhibition and sufficient tooth movement.

This study has presented a viable method and basis for further investigations on advanced cellular and genetical approaches for inhibition and repair of OIIRR. In case of these advanced approaches are improved for clinical practice, they would provide benefits in the progressive resorption cases that have already root resorption before orthodontic treatment, high root resorption risk due to identified predisposing factors, inflammatory tendency or genetically potential to root resorption and in adult patients which can be more prone to root resorption taking into account the slowdown of tooth movement velocity.

## Conclusions

1. Transfer of MSCs to the intact PDL tissue was successful. Desired amount of cells and continuity of OPG mRNA expression was provided by transmission of transfected MSCs.
2. MSCs transfer to the PDL of teeth under orthodontic force reduced number and area of resorption lacunae, number of osteoclasts and Cox-2 expression. Therefore, decreased root resorption lacunae were found. Single MSCs and transfected MSCs transfer exhibited therapeutic effects by reducing root resorption and/or evoking a reparative effect.
3. GT showed the most successful results about preventing and/or repairing of root resorption induced by orthodontic tooth movement.
4. Positive correlation between RANKL and number of osteoclastic cells, number of lacunae and resorption area ratio; negative correlation between OPG and number of osteoclastic cells, number of lacunae and resorption area ratio proves the important role of OPG and RANKL on root resorption.

## Supplementary material

Supplementary material is available at *European Journal of Orthodontics* online.

## Conflict of interest

The authors declare that they have no conflict of interest.

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