

Lentivirus-mediated TNF- α gene silencing and overexpression of osteoprotegerin inhibit titanium particle-induced inflammatory response and osteoclastogenesis *in vitro*

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Abstract. Macrophages and osteoclasts release proinflammatory factors and promote osteoclastogenesis following the phagocytosis of wear particles. During this pathological process, receptor of nuclear factor κ B ligand (RANKL) and tumor necrosis factor (TNF)- α are critical factors contributing to resorption and the inflammatory response. The present study aimed to construct recombination lentivirus vectors carrying TNF- α small interfering (si)RNA and osteoprotegerin (OPG) cDNA, and to examine the effects of Lenti-siTNF α -OPG on the wear particle-induced inflammatory response and osteoclastogenesis in a titanium (Ti) particle-induced-inflammatory response cell model. Lenti-siTNF α -OPG vectors were constructed and transfected into RAW264.7 and MC3T3-E1 cells, respectively, prior to particle stimulation. The protein levels of TNF- α , OPG and RANKL were evaluated using western blot analysis and enzyme-linked immunosorbent assays, and the mRNA expression levels of the inflammatory factors, TNF- α , interleukin (IL)-1 β and IL-6, as well as OPG and RANKL, were measured using reverse transcription-quantitative polymerase chain reaction analysis. The activity of alkaline phosphatase (ALP) was examined using an ALP kit. In the presence of the Lenti-siTNF α -OPG vector, the mRNA expression levels of the inflammatory factors and RANKL were downregulated, as were the protein levels of TNF- α . The mRNA expression and protein levels of OPG were upregulated, and ALP activity was increased. These

findings suggested that Lenti-siTNF α -OPG transfection inhibited the wear particle-induced inflammatory response and osteoclastogenesis, which warrants further investigation for the prevention and/or treatment of wear particle-induced osteolysis.

Introduction

Total hip replacement improves quality of life, in terms of its reduction in pain and improved function (1). However, peri-prosthetic osteolysis is a major complication following total hip replacement (2,3). Histopathological studies have shown the infiltration of macrophages, osteoblasts, osteoclasts and fibroblasts into peri-prosthetic tissues (4) and the interstitial membrane (5,6). Each of these cells are involved in the web of interactions, which govern periprosthetic bone loss (1).

The receptor of nuclear factor κ B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) axis is at the core of the biological response of osteolysis (7), and RANKL activates nuclear factor (NF) κ B, which results in osteoclastogenesis. The RANKL/OPG ratio is a crucial indicator of bone mass and skeletal integrity (8). OPG is a decoy receptor for RANKL; it is secreted by osteoblasts and regulates osteoclast activity by providing an alternative binding site for RANKL, thus inhibiting the interaction between RANKL and RANK (9).

TNF- α is involved in the development of the osteolytic response (10) and controls the release of other proinflammatory factors, including interleukin (IL)-1 β and IL-6 (11,12). It is also reported that TNF- α inhibits osteoblast differentiation and promotes osteoblast apoptosis (13,14). In addition, it has been suggested that TNF- α may act dependently (15) or independently of RANKL (10) to induce osteolysis.

TNF- α small interfering (si)RNA has been demonstrated to be effective in inhibiting wear particle-induced osteolysis, however certain evidence had shown that the most sensitive osteolytic response of bone to TNF- α is through the activation of existing osteoclasts (12). Therefore, osteoclast precursors may retain the ability to differentiate into osteoclasts through interaction with RANKL, whose decoy is OPG. OPG protein has been confirmed to have the ability to prevent periprosthetic osteolysis, however, due to the short-half life of the biological

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agent and the chronic nature of the particle-associated peri-prosthetic osteolysis, it is difficult to utilize conventional therapeutic methods to administer a sufficient quantity of OPG protein to osteolytic sites around the loosening prosthesis (8).

In previous years, several studies have shown that gene therapy offers a more efficient, localized, long-term option (16), compared with drugs, and have the ability prevent or treat periprosthetic osteolysis. Therefore, the present study aimed to construct a lentivirus-mediated siRNA targeting TNF- α in RAW264.7 cells and, at the same time, induce the overexpression of OPG in MC3T3-E1 cells, to determine whether the recombinant lentivirus, Lenti-siTNF α -OPG, has the ability to inhibit titanium (Ti) particle-induced osteolysis.

Materials and methods

Particle preparation. Commercial pure Ti particles (diameter range, 1–10 μ m) with a purity of 93% were obtained from Johnson Matthey Pharma Services (Ward Hill, MA, USA). The particles were incubated in 75% ethanol for 48 h for sterilization and to remove endotoxin, which was followed by washing three times in sterile phosphate-buffered saline (PBS; Wuhan Boster Biological Technology, Ltd., Wuhan, China). The levels of endotoxin in the particle solutions was measured using a Limulus Amebocyte Lysate assay (Xiamen Houshiji, Ltd., Xiamen, China), and the results showed that the endotoxin level was <0.2 EU/ml. The particles were then suspended in sterile PBS at 0.1 mg/ml and stored at 4°C until use.

Lentiviral vector construction and recombinant lentivirus production. The siRNA target sequences (CCCAAAGGGATGAGAAGTT) were designed and cloned into a GV118 lentivirus vector (GeneChem Co., Ltd., Shanghai, China) by restriction endonuclease *HpaI* and *XhoI* double digestion (GeneChem Co., Ltd.) and T4 DNA ligase ligation, to construct a pLenti-PU6-siTNF- α -PU6iEGFP backbone. Based on the mouse OPG gene sequences, PCR primers were designed (GeneChem Co., Ltd.) to clone the full length of OPG cDNA, which were cloned into the pLenti-PU6-siTNF- α -PU6iEGFP backbone using *AgeI* and *BamHI* sites to construct the Lenti-siTNF α -OPG vector. Following construction, the recombinant lentivirus vector and pPACK Packaging Plasmid mix (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were cotransfected into 293T cells (GeneChem Co., Ltd.). Three short hairpin RNAs (shRNAs) were selected based on the sequences of the mouse TNF- α gene (GenBank: NM_013693; listed in Table I), and a scrambled shRNA served as a negative control. Preliminary experiments indicated that shRNA2 (sense, 5'-CCAACGGCATGGATCTCAA-3') downregulated TNF- α mRNA more markedly than the other tested shRNAs (data not shown). The pGag/Pol, pRev, pVSV-G, and recombinant lentivirus were packaged into plasmid vectors, and the recombinant lentivirus was amplified by transforming 293T cells with the packaging plasmids using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). pLenti-PU6-siTNF- α -PU6iEGFP was harvested after 48 h. Full-length OPG cDNA was cloned into the pLenti-PU6-siTNF- α -PU6iEGFP using *AgeI* and *BamHI* sites to construct Lenti-siTNF α -OPG. The plasmids were then amplified by transfection into 293T cells and purified with

three rounds of density gradient centrifugation with CsCl. At 48 h post-transfection, the lentiviruses were harvested and centrifuged at 4,000 x g for 10 min at 4°C to remove cell debris. Condensation was performed by filtration of the supernatant into a filtrate collection tube through a filter cup, followed by centrifugation at 4,000 x g for 13 min. The filter cup was removed and a sample collection cup was inserted into the filtrate collection tube, this was then centrifuged at 1,000 x g for 2 min. Ultimately, a concentrated lentivirus solution was obtained, with a final titer of 1.5x10⁹ TU/l.

Cell culture. RAW264.7 mouse macrophage/monocyte cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in α -minimum essential medium (α -MEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37.6°C under 5% CO₂ and 95% humidity. MC3T3-E1 (American Type Culture Collection) murine osteoblast-like cells were maintained in the same media and conditions.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity of the RAW264.7 and MC3T3-E1 cells transfected with Lenti-siTNF α -OPG was examined using the MTT assay. The cells (5x10³ cells/well) were cultured in 96-well tissue culture plates for 24 h and incubated with 0.5 mg/ml MTT at 37°C for 4 h. Following the removal of the supernatant, the insoluble formazan crystals were dissolved in 200 μ l dimethyl sulfoxide, and the absorbance was measured using a Synergy HT microtiter plate reader (BioTek Instruments, Inc., Winooski, Vermont, USA) at a wavelength of 570 nm.

Collection of conditioned media (CM). The RAW264.7 cells were plated in 24-well plates at a density of 1.0x10⁵ cells in complete α -MEM. Following 24 h attachment at 37°C, the cells were washed with PBS and stabilized in serum-free Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences) for 1 h at 37°C. Subsequently, the cells were subjected to Ti particles (0.1 mg/ml) with or without Lenti-siTNF α -OPG (5.0x10⁶/ml). Control groups were treated with equal volumes of PBS and transfection was conducted by adding 5.0x10⁶/ml Lenti-siTNF-OPG to each well with 5 μ g/ml polybrene and 5 μ g/ml Enhanced Infection Solution for 72 h. Multiplicity of infection (MOI) was determined by observation of the decrease in TNF- α expression and overexpression of OPG. Following 24 h of incubation at 37°C, the cells in the control CM group (Cont CM), CM with Ti particles group (Ti CM) and CM with Ti particles and Lenti-siTNF α -OPG group (Ti-lenti CM) were collected, centrifuged at 1,000 x g for 5 min to remove any cell debris and stored at -20°C until use.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA in the RAW264.7 and MC3T3-E1 cells following treatment was extracted in 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized from the total RNA. qPCR was used to detect the mRNA expression levels of TNF- α , OPG and RANKL. The sequences of the PCR

Table I. Primers for reverse transcription-quantitative polymerase chain reaction analysis.

Target	Forward primer (5'→3')	Reverse primer (3'→5')
TNF- α	TCCTCACCCACACCGTCAG	GCTGAGTTGGTCCCCCTTC
OPG	GATCCTGGACAGCTTCACAA	AAACAGCCCAGTGTCCATGC
RANKL	AGATTTGCAGGACTCGACTC	CCCACAATGTGTTGCAGTTC
IL-1 β	TTCTCGCAGCAGCACATC	CAGCAGGTTATCATCATCATCC
IL-6	TCCATCCAGTTGCCTTCTTG	TTTCTCATTTCCACGATTTCCC
GAPDH	TGGTGAAGGTCGGTGTGAAC	GCTCCTGGAAGATGGTGTATGG

TNF- α , tumor necrosis factor- α ; OPG, osteoprotegerin; RANKL, receptor of nuclear factor κ B ligand, IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

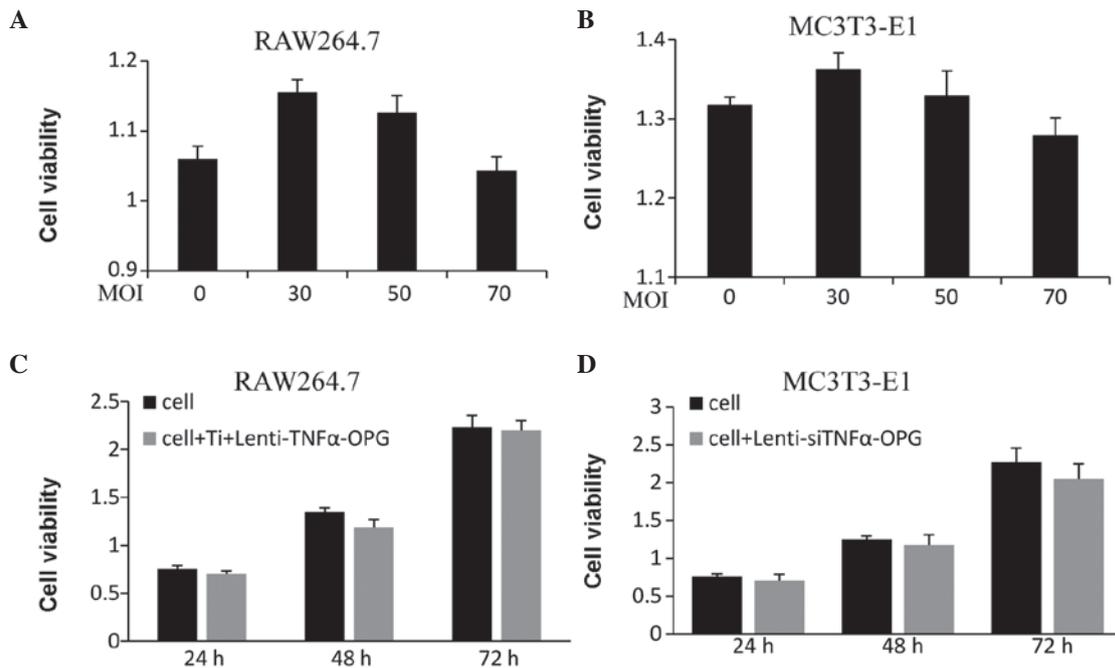


Figure 1. Effects of Lenti-siTNF α -OPG on cell viability, determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (A and B) RAW264.7 and MC3T3-E1 cells were transfected with different MOIs of Lenti-siTNF α -OPG for 48 h. (C) RAW264.7 were treated with Ti and 50 MOI RAW264.7 for 24, 48 and 72 h. (D) MC3T3-E1 cells were transfected with 50 MOI Lenti-siTNF α -OPG for 24, 48 and 72 h. Data are presented as the mean \pm standard deviation. Ti, titanium; TNF- α , tumor necrosis factor- α ; OPG, osteoprotegerin; si, small interfering; MOI, multiplicity of infection.

primers are listed in Table I. Total RNA was extracted from the MC3T3-E1 and RAW264.7 co-cultures using 1 ml TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA purity was determined using the 260/280 nm absorbance ratio (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). First-strand cDNA was synthesized with 2 mg total RNA (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA), and one-tenth of the total cDNA was used for each PCR mixture containing 5 μ l Express SYBR Green (Takara Bio, Inc., Otsu, Japan) and 5 μ l PCR Supermix (Fermentas; Thermo Fisher Scientific, Inc.). The PCR primers (0.5 μ l upstream and downstream, respectively) used to amplify TNF- α , OPG, IL-1 β , IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table II. The reaction mixture (20 μ l) was subjected to a 45-cycle amplification in a DNA Thermal Cycler (PerkinElmer, Inc., Waltham, MA, USA) at 95°C for

15 sec and 95°C for 5 sec, followed by 60°C for 30 sec. Relative mRNA expression levels of the selected genes (TNF- α , OPG and RANKL) were normalized to GAPDH and quantified using the $\Delta\Delta$ Cq method.

Enzyme-linked immunosorbent assay (ELISA). The RAW264.7 cells were incubated with/without Ti particles in the presence or absence of Lenti-siTNF α -OPG for 24 h, and the cell supernatants were harvested and centrifuged to remove the cell particles, as described above. Aliquots were stored at -20°C for TNF- α measurement. A mouse TNF- α ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA) was used for quantitative measurement, according to the manufacturer's protocol.

Western blot analysis. The cells were lysed in radioimmuno-precipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) with protease inhibitors (Beyotime Institute

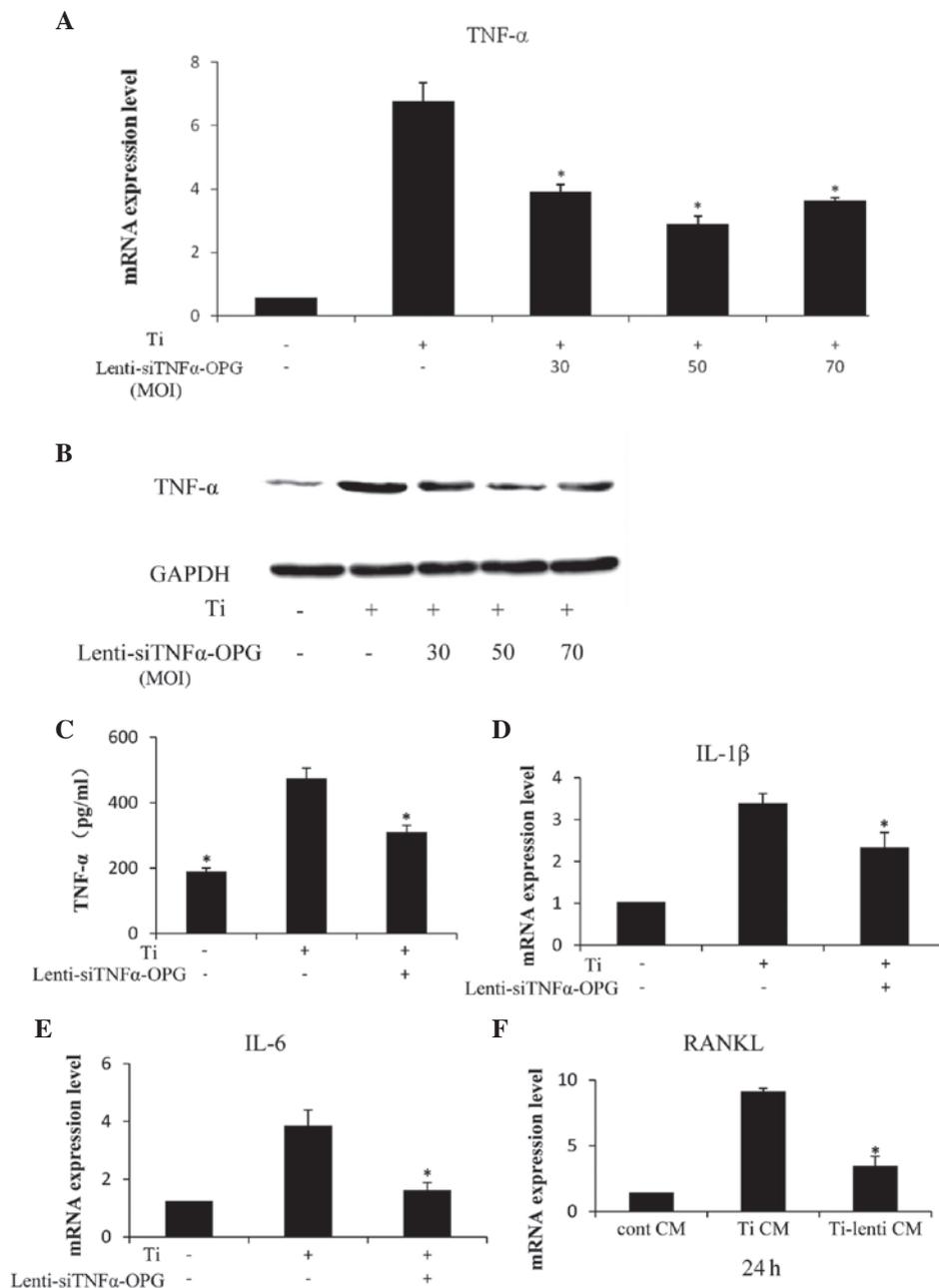


Figure 2. mRNA and protein expression levels of proinflammatory cytokines in RAW264.7 cells, and the mRNA expression of RANKL in MC3T3-E1 cells. (A) RAW264.7 cells were treated with/without Ti particles in the presence or absence of Lenti-siTNF α -OPG for 48 h. mRNA expression levels of TNF- α were significantly decreased at 50 MOI. (B) Protein levels of TNF- α were examined using western blotting after 48 h. (C) Protein levels of TNF- α were assessed using an enzyme-linked immunosorbent assay following exposure to Ti particles, or Ti particles and 50 MOI Lenti-siTNF α -OPG for 24 h. (D and E) mRNA expression levels of IL-6 and IL-1 β were significantly downregulated by Lenti-siTNF α -OPG at 50 MOI. (F) mRNA expression levels of RANKL were significantly decreased in the MC3T3-E1 cells in the Ti-lenti CM group, compared with the Ti CM group. Data are presented as the mean \pm standard deviation. * P <0.05 vs. Ti CM. Ti, titanium; TNF- α , tumor necrosis factor- α ; IL, interleukin; OPG, osteoprotegerin; RANKL, receptor of nuclear factor κ B ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection; cont, control; CM, conditioned media; si, small interfering.

of Biotechnology). Subsequently, the protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). The total protein (22 μ g) was electrophoresed via 10% SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes (PVDF; EMD Millipore, Billerica, MA, USA) and blocked in TBS for 1 h. The PVDF membranes were incubated overnight at 4°C with rabbit anti-mouse monoclonal TNF- α (1:300; Abcam, Cambridge, MA; cat. no. ab11564) and rabbit anti-mouse polyclonal OPG (1:200) antibodies

(Abcam; cat. no. ab9986). GAPDH served as a protein loading control. Following incubation with the primary antibody, the membranes were washed twice with TBST for 10 min and then washed with TBS for 10 min. Subsequently, the blots were incubated with goat anti-rabbit monoclonal secondary antibody (1:500; cat. no. 7074P2; Cell Signaling Technology, Inc., Shanghai, China) at 20°C for 2 h. Following incubation with the secondary antibody, the membranes were washed twice in TBST for 10 min and then washed with TBS for 10 min. Following incubation, the proteins were detected by enhanced

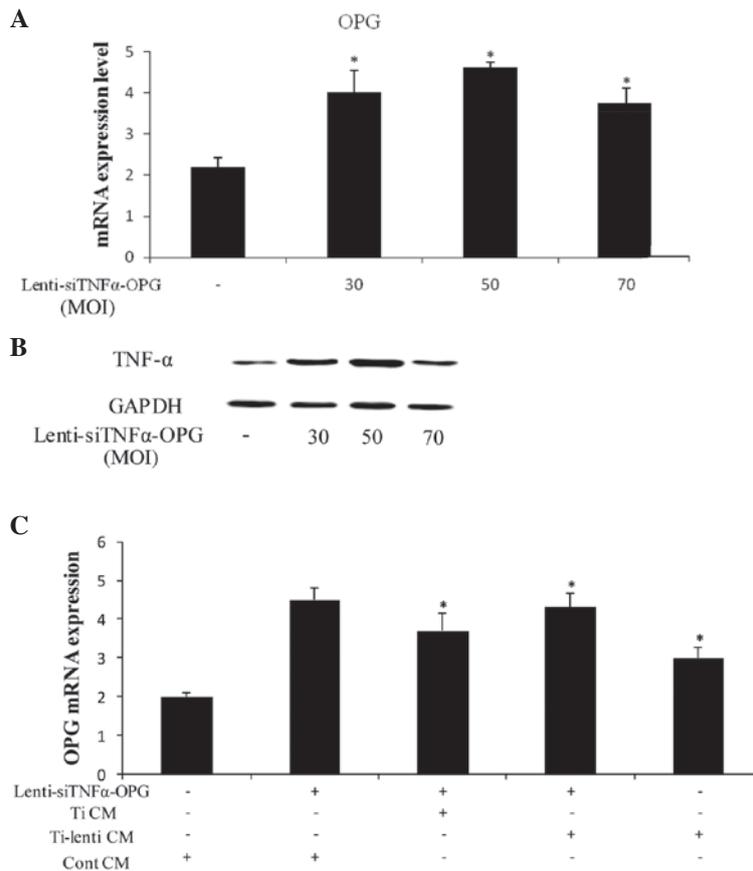


Figure 3. Expression levels of OPG in MC3T3-E1 cells treated with Lenti-siTNF α -OPG and/or different CM. (A) RAW264.7 were transfected with Lenti-siTNF α -OPG at different MOIs for 48 h. The mRNA expression of OPG was significantly increased at 50 MOI. *P<0.05 vs. 0 Lenti-siTNF α -OPG. (B) Protein levels of OPG were determined using western blotting after 48 h. (C) Expression levels of OPG were significantly increased in the MC3T3-E1 cells in the Lenti-siTNF α -OPG and Ti-lenti CM-treated group, compared with the Lenti-siTNF α -OPG and Ti CM-treated and Ti-lenti-treated groups. Data are presented as the mean \pm standard deviation. *P<0.05 vs. Cont CM + Lenti-siTNF α -OPG. Ti, titanium; TNF- α , tumor necrosis factor- α ; IL, interleukin; OPG, osteoprotegerin; RANKL, receptor of nuclear factor κ B ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection; Cont, control; CM, conditioned media; si, small interfering.

chemiluminescence with BeyoECL Plus (Beyotime Institute of Biotechnology) and scanned using Quantity One analysis software, version 4.6 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ALP activity assay. ALP activity was measured using a QuantiChrom™ Alkaline Phosphatase Assay kit (BioAssay Systems, Hayward, CA, USA). In brief, the culture medium was removed, and following being washed with PBS, the cells were lysed in 0.5 ml 0.2% Triton X-100 in distilled water with agitation for 20 min at room temperature. The samples were then incubated with a mixture of assay buffer (pH 10.5), 5 mM Mg acetate (final) and 10 mM pNPP liquid substrate at room temperature for 10 min. The optical density at 405 nm was determined (t=0), and this was measured again after 4 min (t=4 min) on a plate reader (Multiskan Plus; Thermo Fisher Scientific, Inc.). The quantity of released nitrophenolate was calculated photometrically, according to the manufacturer's protocol.

Statistical analysis. Data from three independent experiments were analyzed and are presented as the mean \pm standard deviation. Differences between groups were analyzed using one-way analysis of variance. P<0.05 was considered to indicate a

statistically significant difference. All statistical analyses were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Variation in the MOI of Lenti-siTNF α -OPG transfection has no effect on cell viability. The results of the MTT assay revealed no significant differences among the RAW264.7 and MC3T3-E1 cells transfected with different MOIs (30, 50 and 70 MOI) of Lenti-siTNF α -OPG for 48 h (Fig. 1A and B). As the mRNA expression levels of TNF- α and OPG were significantly downregulated at 50 MOI, the present study analyzed the viability of RAW264.7 cells treated with Ti particles and 50 MOI Lenti-siTNF α -OPG at 24, 48 and 72 h, which showed no significant difference, compared with the untransfected cells (Fig. 1C). Similarly, no significant difference was observed between the viability of the MC3T3-E1 cells transfected with 50 MOI Lenti-siTNF α -OPG (Fig. 1D) at each time point, compared with the untransfected cells.

Lenti-siTNF α -OPG inhibits the expression of cytokines in RAW264.7 cells. Compared with 30 and 70 MOI, the mRNA expression of TNF- α in the RAW264.7 cells transfected with

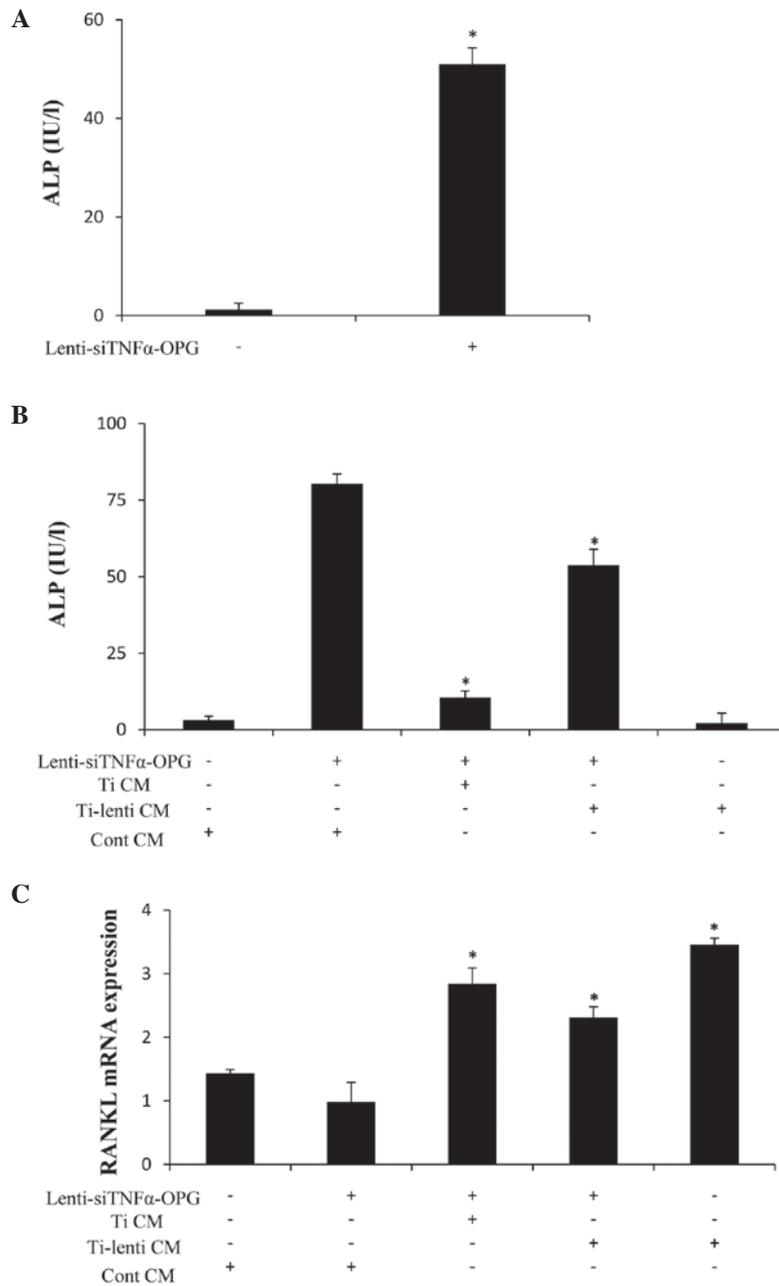


Figure 4. ALP activity and mRNA expression levels of RANKL in MC3T3-E1 cells. (A) MC3T3-E1 cells transfected with 50 MOI Lenti-siTNF α -OPG for 48 h. ALP activity was significantly higher, compared with the control. *P<0.05 vs. cont. (B) ALP activity was significantly increased in the MC3T3-E1 cells of the Lenti-siTNF α -OPG and Ti-lenti CM-treated group, compared with the Lenti-siTNF α -OPG and Ti CM and Ti-lenti-treated groups. *P<0.05 vs. cont CM + Lenti-siTNF α -OPG. (C) mRNA expression levels of RANKL were significantly decreased in the MC3T3-E1 cells of the Lenti-siTNF α -OPG and Ti-lenti CM-treated group, compared with the Lenti-siTNF α -OPG and Ti CM and Ti-lenti-treated groups. *P<0.05 vs. cont CM + Lenti-siTNF α -OPG. Data are presented as the mean \pm standard deviation. Ti, titanium; TNF- α , tumor necrosis factor- α ; IL, interleukin; OPG, osteoprotegerin; RANKL, receptor of nuclear factor κ B ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection; cont, control; CM, conditioned media; si, small interfering.

50 MOI Lenti-siTNF α -OPG was lowest at 48 h (Fig. 2A). The protein expression of TNF- α , determined using western blot analysis, revealed similar results (Fig. 2B). These results confirmed that 50 MOI Lenti-siTNF α -OPG significantly reduced the expression levels of TNF- α in the RAW264.7 cells treated with 0.1 mg/ml Ti particles. Following 24 h incubation with a combination of Ti particles and 50 MOI Lenti-siTNF α -OPG, the protein expression of TNF- α was inhibited in the RAW264.7 cells, compared with the cells in the Ti CM group, as determined using ELISA

analysis (Fig. 2C). To evaluate the particle-induced inflammatory response, the present study examined the expression levels of proinflammatory cytokines, including IL-1 β and IL-6. It was found that the downregulation of TNF α by siTNF α resulted in decreases in the mRNA expression levels of IL-1 β (Fig. 2D) and IL-6 (Fig. 2E), compared with the Ti CM group, which indicated that TNF- α may control the mRNA expression levels of IL-6 and IL-1 β mRNA. It was also observed that the mRNA expression of RANKL in the MC3T3-E1 cells decreased markedly when cultured

in lenti-Ti CM, compared with the Ti CM group, which indicated that TNF- α may have an effect on the expression of RANKL (Fig. 2F).

Lenti-siTNF α -OPG upregulates the expression of OPG in MC3T3-E1 cells. To assess the mRNA expression levels of OPG, the MC3T3-E1 cells treated with different MOIs (30, 50 and 70) were examined using RT-qPCR. The results showed that the expression level of OPG was highest at an MOI of 50 at 48 h (Fig. 3A). At 48 h post-transfection with the different MOIs of Lenti-siTNF α -OPG, overexpression of OPG protein was demonstrated in the MC3T3-E1 cells using western blot analysis (Fig. 3B). As expected, the MC3T3-E1 cells transfected with 50 MOI Lenti-siTNF α -OPG exhibited higher protein expression levels of OPG, compared with those transfected with 30 and 70 MOI. The present study also examined the protein expression levels of OPG in the OPG-overexpressing MC3T3-E1 cells cultured in Cont CM, Ti CM and Ti-lenti CM using ELISA. The Lenti-siTNF α -OPG and Ti-lenti CM-treated group exhibited the highest protein expression of OPG, compared with the Ti CM and Ti-lenti CM-treated groups (Fig. 3C), suggesting that the overexpression of OPG is more marked when the expression of TNF- α is decreased.

Lenti-siTNF α -OPG promotes osteoblast differentiation and inhibits osteoclastogenesis in transfected MC3T3-E1 cells. ALP is a marker of matrix maturation and, during differentiation from mesenchymal cells to mature osteoblasts, ALP begins to be expressed in osteoprogenitors and is expressed at high levels in mature osteoblasts (17). Using an ALP kit, the present study assessed the activity of ALP in the MC3T3-E1 cells transfected with or without Lenti-siTNF α -OPG for 48 h. The results revealed that ALP activity in the MC3T3-E1 cells increased following transfection with Lenti-siTNF α -OPG (Fig. 4A). It was also found that ALP activity was significantly higher in the OPG-overexpressing MC3T3-E1 cells when treated with the different CM (Fig. 4B). These data indicated that Lenti-siTNF α -OPG transfection alleviated Ti particle-induced osteolysis by promoting osteoblast differentiation.

RANKL has a high level of involvement in osteoclastogenesis due to its binding to the receptor activator of RANK. In the present study, RT-qPCR analysis revealed differences in the mRNA expression levels of RANKL in the OPG-overexpressing MC3T3-E1 cells cultured in Cont CM, Ti CM and Ti-lenti CM (Fig. 4C). The Lenti-siTNF α -OPG and Ti-lenti CM-treated group revealed the lowest mRNA expression level of RANKL, compared with the Ti CM and Ti-lenti CM-treated groups. These results indicated that the Lenti-siTNF α -OPG may have suppressed Ti particle-induced osteoclastogenesis.

Discussion

At present, there is no satisfactory treatment option for periprosthetic osteolysis, with the exception of revision, which requires complicated and expensive surgery, and is frequently associated with considerable patient morbidity, and even mortality rates (18). There has been a focus on nonsurgical methods to prevent aseptic loosening (19), however, there

remains no satisfactory method for the prevention of aseptic loosening of joint prostheses. To the best of our knowledge, the present study is the first study to address the effects of the combination of TNF- α siRNA and overexpression of OPG by construction of a recombinant lentivirus.

RANKL is essential for the promotion of osteoclastogenesis. It binds to its signaling receptor, RANK, on the membranes of macrophages and osteoclast precursors, thereby providing signals required for their survival, maturation and activation (20). Mature osteoclasts secrete large quantities of inflammatory factors, including TNF- α , IL-1 β and IL-6, which in turn activate osteoclasts and prompt an inflammatory response (10). Among these inflammatory factors, TNF- α acts as a link between inflammatory processes and osteoclastogenesis. It is involved in the osteolytic response predominantly by two mechanisms: An indirect mechanism, in which TNF- α enhances the inflammatory response by promoting the expression levels of RANKL, IL-6 and IL-1 β (21) and a direct mechanism, in which TNF- α synergizes with RANKL to enhance osteoclast formation of bone erosions (22). In the present study, Lenti-siTNF α -OPG transfection inhibited osteolysis by the two mechanisms. It decreased the level of TNF- α secreted by osteoclasts and subsequently suppressed the expression levels of IL-1 β and IL-6 stimulated by the Ti particles. It also decreased the expression of TNF- α , which may have led to the reduction in the expression of RANKL, controlling osteoclast maturation and function (23). As shown in the data of the present study, Lenti-siTNF α -OPG transfection effectively reduced the expression of RANKL in the MC3T3-E1 cells by interfering with the levels of the proinflammatory cytokines secreted from the RAW264.7 cells induced by Ti particles. In addition, TNF- α and RANKL support osteoclast survival, therefore, downregulation in the expression of TNF- α reduces the numbers of osteoclasts (24). Therefore, Lenti-siTNF α -OPG transfection may prevent the inflammatory response, which is important in periprosthetic osteolysis.

ALP is a vital early marker of matrix maturation, which is expressed in preosteoblasts during osteoblast differentiation and exhibits upregulated expression levels in mature osteoblasts and downregulated expression levels in osteocytes (25). In the present study, Lenti-siTNF α -OPG transfection promoted osteoblast differentiation, accompanied by the expression of ALP. Furthermore, the transfection of Lenti-siTNF α -OPG resulted in suppression of the expression of RANKL and the inhibition of osteoclastogenesis (26). Compared with preosteoblasts, the ratio of RANKL to OPG is markedly higher in mature osteoblasts (17). In addition, in the presence of TNF- α , the level of apoptosis in mature osteoblasts is higher than in preosteoblasts (27). Therefore, Lenti-siTNF α -OPG transfection may induce osteoblast maturation and inhibit osteoclast differentiation.

OPG has been identified as a negative regulator of the RANKL/RANK/OPG axis (28). Overexpression of OPG upregulates the OPG/RANKL ratio, inhibiting the interaction of RANKL and RANK, leading to suppression of the inflammatory response and osteoclastogenesis. The results of the present study clearly revealed that Lenti-siTNF α -OPG transfection increase the expression of OPG in the MC3T3-E1 cells. In addition, comparison of the mRNA expression levels of RANKL

among the MC3T3-E1 cells of the Lenti-siTNF α -OPG and Ti-lenti CM-treated, Lenti-siTNF α -OPG and Ti CM-treated and Ti-lenti CM-treated groups, the Lenti-siTNF α -OPG and Ti-lenti CM-treated MC3T3-E1 cells exhibited the lowest mRNA expression level of RANKL. This indicated that the combination of the decreased expression of TNF- α in RAW264.7 cells with increased the expression of OPG in MC3T3-E1 cells downregulated the mRNA expression levels of RANKL more effectively. As RANKL is critical for osteoclastogenesis, it was hypothesized that Lenti-siTNF α -OPG transfection may be more effective in inhibiting osteoclastogenesis, compared with siRNA that target only the downregulation of TNF- α or upregulation of OPG.

Gene therapy is an attractive option for treatment of osteolysis. Major problems in the approaches to treat localized chronic inflammatory/osteolytic disorders, including aseptic loosening, include the lack of adequate suppressive agents and effective specific therapeutic delivery systems (18). Although the conventional system of administering biological drugs, including bisphosphonates, relies on vascular perfusion to the local sites of loosening, viral vector mediated gene therapy provides a novel means of delivering therapeutic genes to the site of disease to express gene products in a persistent and localized manner (18). In addition, investigations have been performed on TNF- α siRNA or OPG cDNA gene delivery (29,30), and they have been demonstrated to be more potent, efficacious and cost-effective in inhibiting wear particle-induced inflammatory response and osteoclastogenesis. As a promising vector of siRNA, the lentivirus compares favorably with other transgenic methods for transducing genes *in vivo*. Transfection with a lentivirus is more efficient than other methods due to its stable expression of siRNA in mammalian cells (31). Overall, Lenti-siTNF α -OPG appears to be an effective mechanism to prevent Ti particle-induced osteolysis.

For investigations aim to examine the effect of Lenti-siTNF α -OPG on periprosthetic osteolysis *in vitro*, and to fully understand the molecular mechanisms underlying the therapeutic effects, as well as safety concerns.

In conclusion, the present study demonstrated that the inhibition of TNF- α and overexpression of OPG by recombinant lentivirus transfection effectively alleviated the Ti particle-induced inflammatory response and osteoclastogenesis *in vitro*, and indicated that Lenti-siTNF α -OPG may be a potential therapeutic method for the prevention of Ti particle-induced osteolysis.

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