

Osteoprotegerin Promotes Cementoblastic Activity of Murine Cementoblast Cell Line in vitro

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Objective: To investigate the effect of osteoprotegerin (OPG) on the cementoblastic activity of a clonal population of immortalised murine cementoblasts (OCCM-30) in vitro.

Methods: OCCM-30 cells were transiently transfected with the mouse OPG using the Avalanche transfection reagent. The ectopic expression of OPG was confirmed by enzyme-linked immunosorbent assay and quantitative polymerase chain reaction. The cell counting Kit-8 assay was used to investigate the effect of OPG on cell proliferation. The expression levels of cementoblastic-related mRNA and protein in the transfected OCCM-30 cells were detected using real-time PCR, Western blotting and immunohistochemical staining.

Results: Satisfactory transfection efficiency was observed 48 h after transfection. The results of the cell proliferation assay indicated that the expansion rate of the OPG transfection group was greater than that of the control group at both 72 h and 96 h. The mRNA levels of osterix (*Osx*), protein kinase B (*Akt1*), cementum attachment protein (*CAP*) and osteopontin (*Opn*) were significantly upregulated ($P < 0.05$) in the OPG group. Protein levels of OPN, bone sialoprotein II (*BSP II*), osteocalcin (*OC*) and *CAP*, which are responsible for osteogenetic and cementoblastic activity, were significantly increased in the OPG-overexpressing group.

Conclusion: Overexpression of OPG in OCCM-30 cells promotes cementoblastic activity.

Key words: cementoblastic, OCCM-30, osteoprotegerin (OPG), root repair, root resorption
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External root resorption (ERR) is a very common complication during orthodontic treatment. Studies have shown that 93% of all adult orthodontic patients experienced different degrees of root resorption¹. Root resorption terminates when root repair initiates, during

the retention phase of orthodontic treatment. Cementoblasts distributed adjacent to the cementum in the periodontal ligament begin to express proteins associated with mineralization, such as OC and BSP II, and secrete matrix to form calcified nodules and new bone², in order to repair bone resorption pits. Many proteins and transcription factors are involved in the repair process³.

OPG, a secretory glycoprotein discovered by Simonet et al in 1997⁴, is a member of the tumor necrosis factor receptor superfamily. Many related studies have been performed on the mechanism of promotion of alveolar bone formation by OPG-transfected osteoblasts^{5,6}. Cementoblasts and osteoblasts have similar progenitors, namely, mesenchymal stem cells and periodontal ligament stem cells. Consequently, they react similarly to signalling factors and both have the ability to mineralise tissue. In our previous experiments in rats, we found that overexpression of OPG can significantly improve the repair of root absorption during the maintenance phase of orthodontic treatment⁷. Similar results were reported by other researchers⁸. We hypothesised that OPG influences the molecular biology of cementoblastic biomarkers, thus promoting the repair of root

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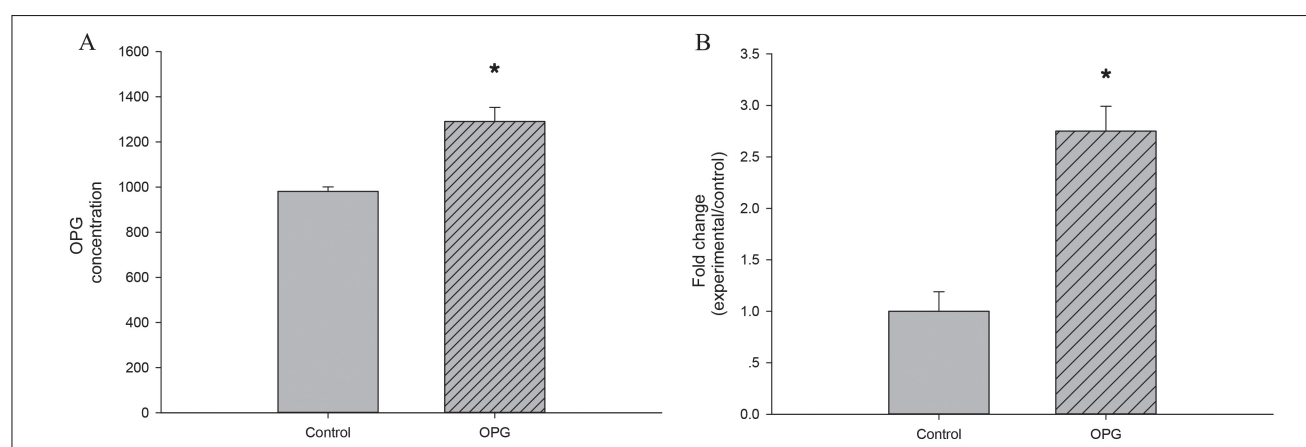


Fig 1 Ectopic expression of OPG. **A)** The concentration of secreted OPG in the OPG-transfected group was 1291.00 ± 62.21 pg/ml, which is significantly higher than the control group ($P < 0.05$). **B)** qPCR demonstrated that OPG transfection promotes OPG transcription in OCCM-30 cells ($P < 0.05$).

absorption. The OCCM-30 cell line, a clonal population of immortalised murine cementoblasts, was originally isolated by D'Errico in 2000⁹, and is now widely used in cementoblast research. In our study, we used a vector carrying the mouse-OPG gene (denoted the pcDNA-mOPG vector) to transfect OCCM-30 cells, in order to evaluate the expression of protein and mRNA associated with the repair of cementoblasts.

Materials and methods

Cell culture

OCCM-30 cells were kindly provided by Professor Somerman (Washington, USA)¹⁰. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, New York, USA) containing 10% fetal bovine serum (FBS; Equitech, Texas, USA), supplemented with 100 U/mL penicillin (Gibco) and 100 ug/mL streptomycin (Gibco); at 37°C in a humidified atmosphere, which consisted of 5% CO₂ in air.

Cell transfection

The pcDNA3.1 (+)-mOPG vector was described in a previous study¹¹. Plasmids were transfected into OCCM-30 cells using the Avalanche Transfection Reagent (EZ Biosystems, Maryland, USA), according to the manufacturer's instructions. After reaching 50% to 60% confluence in a 100 mm dish, cells were transferred into 10 ml DMEM, containing 10% FBS. The transfection complex mixture was formed by mixing 12 µg DNA with 24 µl transfection reagent. After incubation for 15 min at room temperature, the mixture was added to the cells. After

24 h at 37°C in a humidified atmosphere of 5% CO₂ in air, the medium was replaced with fresh DMEM and 10% FBS. Cells were harvested 48 h after transfection.

CCK-8 assay

The Cell Counting Kit-8 (CCK-8, DOJINDO, Kumamon, Japan) was used for the cell proliferation assay according to the manufacturer's instructions. Briefly, cells were seeded onto 96-well plates at a cell density of 1,000/well, 16 h after transfection. Cell viability was analysed at 24, 48, 72, 96 and 120 h after transfection. The CCK-8 working solution was added to the wells and incubated at 37°C for 4 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-rad, California, USA).

Real-time quantitative polymerase chain reaction (qPCR)

In our study, the primers were either previously used in other studies or designed using the NCBI database (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and successfully validated before use in experiments. Cells were harvested and total mRNA was isolated using the RNA Extraction Kit (CWBI, Beijing, China), according to the manufacturer's instructions. Two-step real-time PCR was performed and analysed on an Applied Biosystems 7500 thermal cycler (Bio-rad), using Power SYBR Green (Invitrogen, California, USA), according to the manufacturer's instructions. Briefly, 1.5 µg DNA, 10 µl SYBR Green and 2 µl (10 pmol) of forward and reverse primers were mixed with DNase-free water to create a 20 µl reaction mixture. Thermal cycling con-

sisted of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s (denaturation step), 60°C for 45 s (combined annealing) and 72°C for 45 s (extension step), followed by the final extension of 72°C for 5 min. The primer sequences were as follows: OPG forward, 5'-AGGCTCATGGTTGGATGTGG-3' and reverse 5'-GCATTGATGGTGAGGTGTGC-3'; CAP forward, 5'-TCTGACGACTCTGCTTCACG-3' and reverse, 5'-TTCAGGGCATGTGTGATGCT-3'; Akt1 forward, 5'-ATGAACGACGTAGCCATTGTG-3' and reverse, 5'-TTGTAGCCAATAAAGGTGCCAT-3'; Ogn forward, 5'-GTAGGGACGATTGGAGTGA-3' and reverse, 5'-GCCAAGAAGACATCCCTGAA-3'; OC forward, 5'-AGCAGCTTGCCCCAGACCTA-3' and reverse, 5'-TAGCGCCGGAGTCTGTTCCTACTAC-3'; Osx forward, 5'-CTGCCTGACTCCTTGGGACC-3' and reverse, 5'-GGGGACTGGAGCCATAGTGA-3'.

Western blotting

Cells were lysed and protein was extracted using Trizol (Thermo, Massachusetts, USA). The protein content in the supernatant was determined using the BCA protein assay (Thermo). Ten micrograms of lysate was subjected to denatured 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane, according to conventional protocol. The membrane was incubated with OPN, OC, BSP II, CAP and actin primary antibodies (Santa Cruz Biotechnology, Texas, USA). Bands were visualised using chemiluminescence (Cyanagen, Bologna, Italy) and the results were analysed using a Bio-Rad chemiDoc (Bio-Rad) and Image J software.

Immunohistochemical (IHC) staining

Cells were fixed with 4% paraformaldehyde after endogenous peroxidase was quenched by 3% hydrogen peroxide. CAP and OC antibodies (Abcam, Massachusetts, USA) were added to the slides, following the instructions supplied with the Histostain-Plus Kit (4A Biotech, Beijing, China). Slides were incubated for 1 h at room temperature before 3,3'-diaminobenzidine (DAB) staining was performed.

Enzyme-linked immunosorbent Assay (ELISA)

A mouse OPG ELISA kit (4abio) was used to detect the expression of OPG in the supernatant. After centrifugation (1000 × g, 4°C, 20 min), the supernatant was added to the plates. The plates were incubated with an OPG antibody, conjugated with HRP and stained with tetra-

methylbenzidine (TMB). The optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad).

Statistical analysis

The RT-PCR and ELISA results were subjected to statistical analysis using the Student's t-test. $P \leq 0.05$ was considered to be statistically significant.

Results

OPG expression

The level of OPG protein in supernatant collected after 48 h was evaluated using ELISA. The concentration of secreted OPG was 1291.00 ± 62.21 pg/ml in the OPG-transfected group, compared to 981.67 ± 19.76 pg/ml in the control group (Fig 1A). The protein expression level was significantly different between the two groups ($P < 0.05$). Real time quantitative polymerase chain reaction (qPCR) analysis indicated that OPG gene expression was increased in the OPG-transfected group, compared with control cells ($P < 0.05$, Fig 1B). These ELISA and qPCR results confirm the successful transfection of the OPG vector.

Cell proliferation

Both the OPG-transfected group and the control group exhibited low growth rates from 24 h to 72 h, with no

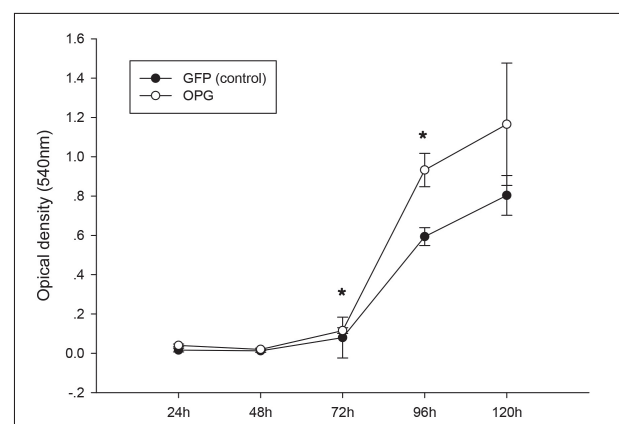


Fig 2 OCCM-30 cell proliferation after transfection of OPG. Both the OPG-transfected group and the control group showed a low growth rate from 24 h to 72 h. The OPG-transfected group began growing rapidly at 72 h, reaching an expansion rate, double that of the control group at 96 h. The growth rate started to decline after 96 h in the OPG-transfected group; however, it was still higher than in the control group at 120 h, although it was not statistically different ($P < 0.05$).

significant difference between the groups. The OPG-transfected group began growing rapidly at 72 h, reaching double the proliferation rate of the control group at 96 h. After 96 h, the growth rate began to slow, but still remained higher than the control group at 120 h, although the difference was not statistically significant ($P < 0.05$, Fig 2).

Expression of adhesion molecules

Western blotting and immunofluorescence assays confirmed the overexpression of OPN, CAP and BSP II protein in the OPG-transfected group (Figs 3A to 3C). These results were confirmed by quantification of mRNA levels. RT-PCR analysis indicated that Opn gene expression was increased in the OPG-transfected group, and that the level of CAP mRNA in these cells was almost double that of the control group (Fig 3B).

Expression of mineralisation-related molecules

Expression of the Osx, Akt1 and Oc genes were 5.5, 3.5 and 2.0 times higher, respectively, in the OPG-transfected group relative to the control group (Fig 4B). Western blot analysis revealed that the OC and BSP II protein levels in the cytoplasm were increased, compared with the control group (Figs 4A to 4C).

Discussion

Cementum is very similar to bone, both in terms of the source of development and composition. The two substances respond similarly to signalling factors¹². Overexpression of OPG promotes pre-osteoblast differentiation in mature osteoblasts⁵ and promotes osteogenesis in mice⁶. We hypothesise that because OPG promotes osteoblastic activity, it may also influence the cementoblastic activity of OCCM-30 cells. In our previous study, we demonstrated that the overexpression of OPG in rats can significantly improve the repair of root absorption during the retention phase of orthodontic treatment⁷. Li Xiaoyan⁸ found that, compared to the natural repair control group, the quantity of OPG overexpression in the ultrasound repair group was obviously higher and the cementum repair on the surface of the roots was also enhanced in rats. We infer that OPG promotes the repair of root absorption by influencing the cementoblastic activity of OCCM-30 cells.

We observed that the transfection of OCCM-30 cells with OPG promotes proliferation. Previous reports on the effect of OPG overexpression on cell proliferation are inconsistent. Corey et al and Chanda et al

showed that OPG overexpression did not affect cell proliferation^{13,14}. Yu et al demonstrated that OPG-overexpressing cells grew more slowly than the control group⁵. OPG acts as an antagonist of RANK by neutralising RANKL. Che et al showed that RANKL, by binding to its receptor RANK, inhibits cell proliferation in osteoblast cells *in vitro* via MALAT1 upregulation¹⁵. By preventing RANKL-RANK interaction, OPG was able to promote cell proliferation when overexpressed. These inconsistent results may be a consequence of the different experimental conditions, procedures and cells used. Further research is required in order to clarify the influence of OPG overexpression on cell proliferation.

The repair of root resorption begins when odontoclasts disappear from the resorption lacunae. Next, repair cells, such as cementoblasts, migrate to the resorption lacunae and begin to differentiate. Then, new cementum is deposited at the surface of resorption lacunae by cementoblastic cells^{2,16}, with insertion of the periodontal ligament into the cementum and the alveolar bone. During this process, many cytokines play an important role in regulating the adhesion of cementoblasts and the mineralisation of cementum.

OPG transfection increased cementoblastic activity by increasing the adhesion of OCCM-30 cells to the root surface. Western blot analysis showed that the expression of OPN and BSP II in the cytoplasm increased in OPG-transfected OCCM-30 cells. Both BSP II and OPN contain the most effective and extensive adhesion domain arginine-glycine-aspartic acid (RGD)¹⁷. Cells are anchored to synthetic surfaces by the binding of RGD to specific integrin receptors¹⁸. The expression of BSP II and OPN was upregulated after transfection of OPG into OCCM-30 cells, making more RGD domains available for reacting with integrin receptors, enabling more OCCM-30 cells to anchor to the root surface. BSP II and OPN play a major role as anchoring molecules, promoting migration and cell adhesion *in vitro*¹⁹⁻²¹. RT-PCR analysis indicated that Cap mRNA expression was almost two times higher in the OPG-transfected group, relative to the control. By determining the phenotypic expression of periodontal ligament cell populations, BarKana et al demonstrated that CAP is capable of attracting putative cementoblastic cells to the root surface *in vitro*²². These data suggest that OPG overexpression promotes the attachment of OCCM-30 cells to the root surface, as a consequence of increased expression of adhesion molecules, such as BSP II, OPN and CAP.

Transfection of OCCM-30 cells with OPG increased cementoblastic activity, by influencing the relative expression level of mineralization-related biomarkers, such as BSP II, OC, AKT1, OSX and OPN. BSP,

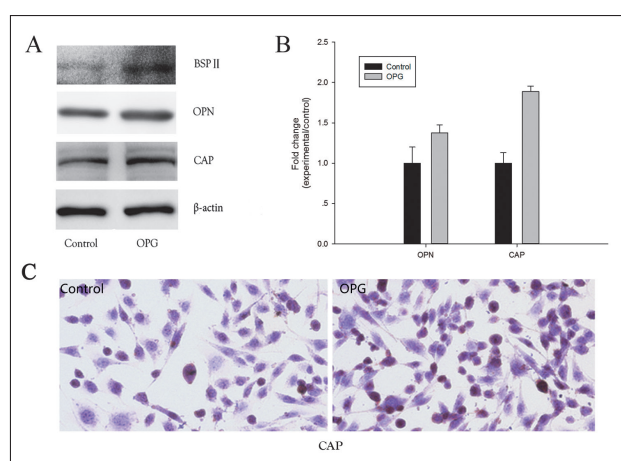


Fig 3 OPG transfection promoted the expression of adhesion molecules. **A)** Western blot analysis confirmed the increased expression of BSP II, OPN and CAP protein in the OPG-transfected group. **B)** RT-PCR revealed that OPG overexpression promotes *Opn* and *Cap* transcription in OCCM-30 cells ($P < 0.05$). **C)** Immunofluorescence analysis confirmed the overexpression of CAP protein in the OPG-transfected group.

a multifunctional extracellular matrix protein, highly expressed in mineralised tissues, is able to bind type I collagen. Hydroxyapatite (HA) crystals are deposited onto a scaffold made by type I collagen. By binding to type I collagen, BSP II is capable of nucleating HA with an increasing potency²³ and increasing calcium incorporation and nodule formation²⁴. Similar results were reported by other researchers *in vitro*²⁵. These data are also consistent with *in vivo* results. Foster et al confirmed a critical role for BSP in the process of cementogenesis, by demonstrating that *Bsp*(-/-) mouse molars lacked functional acellular cementum *in vivo*¹². After transfection with OPG, OCCM-30 cells upregulated BSP II, which increased HA nucleation and nodule formation. OPG overexpression, therefore, promotes cementoblastic activity at the beginning of mineralization.

RT-PCR analysis indicated that *Oc* gene expression was two times higher in the OPG-transfected group. This result was confirmed by Western blot analysis of OC protein expression. OC, which has the ability to bind calcium ions, is currently considered to be the most specific osteoblast marker, expressed at the latest stage of differentiation; undetectable in pre-osteoblasts and abundantly expressed only in postmitotic osteoblasts²⁶. OPG overexpression increased the cementum mineralization of binding calcium ions, at the final stage, by upregulating OC expression.

The phosphoinositide-dependent serine-threonine protein kinase Akt plays a key role in the signalling

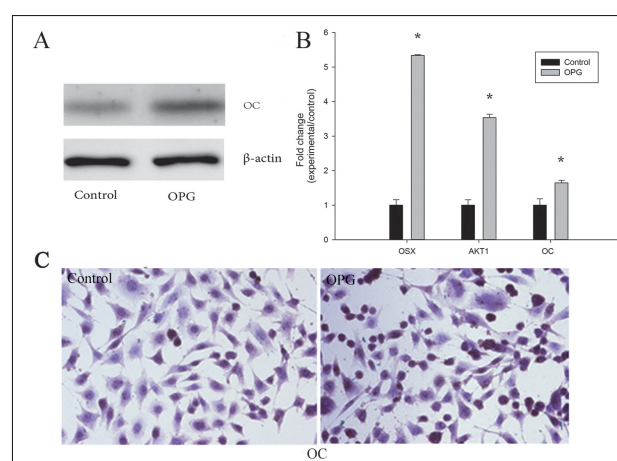


Fig 4 OPG transfection promoted the expression of mineralization-related molecules. **A)** Western blot analysis confirmed the upregulation of OC protein expression in the OPG-transfected group. **B)** RT-PCR demonstrated that OPG overexpression promotes *Osx*, *Akt1* and *Oc* transcription in OCCM-30 cells ($P < 0.05$). **C)** Immunofluorescence analysis confirmed the overexpression of OC protein in the OPG-transfected group.

of potent bone anabolic factors. Akt1 phosphorylates FoxO3a to prevent it from translocating into the nucleus, which leads to suppression of osteoblast apoptosis²⁷. Consistent with the idea that cementoblasts and osteoblasts react similarly to signalling factors, the overexpression of Akt1 mRNA increased the anabolism of cementum, thus resulting in increased cementoblastic activity in OCCM-30 cells.

The upregulated expression of OPN in OPG-transfected cells was confirmed by Western blotting and immunofluorescence assays. From the results of earlier studies, OPN was thought to be an inhibitor of mineralization^{28,29}; however, more researchers began to believe that OPN plays an important role in the regulation of biomineralization, as well as in the modulation of HA formation³⁰⁻³³. *Osx* is a transcription factor that was first found in osteoblasts. Homozygous mutation of *Osx* in mice results in a complete lack of osteoblast differentiation and bone formation. The associated inhibition of the Wnt pathway reveals a potential novel feedback control mechanism involved in bone formation³⁴. The increased expression of *Osx* mRNA in OPG-transfected cells indicates that OPG promotes the regulation of mineralization of OCCM-30 cells. Through the upregulation of OPN and *Osx*, OPG overexpression increases the efficiency of cementoblastic activity, by regulating the location, size and shape of crystal growth³¹.

In conclusion, our results indicate that OPG transfection promotes the cementoblastic activity of OCCM-30

cells. This is the first report to verify that OPG can promote the proliferation and cementoblastic activity of OCCM-30 cells. As a result, OPG may improve the repair of root absorption during the maintenance phase of orthodontic treatment. However, the molecular mechanisms of how OPG promotes cementoblastic activity, and the interactions amongst related molecular pathways need to be explored further. It is also important that *these in vitro* studies are validated *in vivo* through animal experiments.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contributions

- Dr Yan Liu and Jiu Xiang Lin for the design and supervision of the experiments and the manuscript revision.
- Dr Ying Ying Zhang and Dr Hua Xiang Zhao for performing the experiments, for interpretation of the results and for preparing the manuscript.
- Dr Zhi Bin Chen for assistance in the experiments.

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