# Regulation of osteoprotegerin and receptor activator of nuclear factor-k B ligand in human dental follicle cells

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**Purpose**: To determine the regulation of the expression of osteoprotegerin and receptor activator of nuclear factor-kappa B ligand in human dental follicle cells by colony-stimulating factor-1, parathyroid hormone-related protein and bone morphogenetic protein-2.

**Materials and Methods**: Primary human dental follicle cells were cultured. The fifth passaged human dental follicle cells were separately cocultured with colony-stimulating factor-1 (25 ng/mL) parathyroid hormone-related protein (10 ng/mL) and bone morphogenetic protein-2 (100 ng/mL) for 0.5, 1, 3, 6, 12, and 18 hours. Using enzyme-linked immunosorbent assays and reverse transcription polymerase chain, the expression of osteoprotegerin and receptor activator of nuclear factor-kappa B ligand were examined.

**Results**: The results of enzyme-linked immunosorbent assays and reverse transcription polymerase chain revealed that colony-stimulating factor-1 and parathyroid hormone-related protein decrease the secretion of osteoprotegerin significantly after incubation for 1 hour ($p<0.01$). Conversely, the expression of receptor activator of nuclear factor-kappa B ligand increased significantly after incubation with colony-stimulating factor-1 and parathyroid hormone-related protein separately for 3 hours ($p<0.01$). While bone morphogenetic protein-2 regulates them in the contrary.

**Conclusion**: At defined concentration, colony-stimulating factor-1 and parathyroid hormone-related protein decrease osteoprotegerin secretion of human dental follicle cells and increase the expression of receptor activator of nuclear factor-kappa B ligand. Thus, a reduction in secretion of the osteoprotegerin protein and an increase in the expression of receptor activator of nuclear factor-kappa B ligand at defined times may promote the osteoclastogenesis needed for eruption, but enhancement of osteoprotegerin and decrease of receptor activator of nuclear factor-kappa B ligand may promote alveolar bone formation. **(Int Chin J Dent 2006; 6: 97-104.)**

**Key Words**: colony-stimulating factor-1, dental follicle, osteoprotegerin, parathyroid hormone, tooth eruption.

## Introduction

Regulation of alveolar bone resorption is a critical event in tooth eruption because an eruption pathway must be formed within the alveolar bone in order for the tooth to escape its bony crypt. The cellular events that culminate in this resorption are recruitments of mononuclear cells (osteoclast precursors) to the dental follicle of the unerupted tooth followed by the fusion of these cells to form the osteoclasts needed for resorption. Osteoprotegerin (OPG) is constitutively expressed in the dental follicle of the first mandibular molar of the rat, but its expression is markedly reduced at day 3 postnatally.[1] Day 3 is the time of maximal influx of mononuclear cells into the follicle and the time of maximal number of osteoclasts seen on the alveolar bone surrounding the first molar.[2,3] Thus, we have postulated that this reduction in OPG expression enables osteoclasts to form and resorb the alveolar bone such that an eruption pathway is formed.[1]

But inhibition of OPG expression alone is not sufficient for osteoclastogenesis to occur, i.e., a positive stimulus is required. The key molecule for this would be receptor activator of nuclear factor-kappa B ligand (RANKL), a protein that is a member of the tumor necrosis factor (TNF) ligand family, which induces osteoclast formation and activation from osteoclast precursor cells. Yao et al.[4,5] have proved that in vivo RANKL is expressed in rat dental follicle at days 1-9 postnatally by laser capture microdissection. Besides, colony-stimulating factor-1 (CSF-1) is expressed in rat dental follicle at earlier stage and has the ability of accelerating tooth eruption. Parathyroid hormone-related protein (PTHrP) is expressed in the stellate reticulum adjacent to the dental follicle in mice, rat molars[6] and the enamel epithelium of the tooth germs of rat,[7] mice,[8]

and humans.[9] The proximity of the stellate reticulum to the dental follicle make it act as paracrine control. But, bone morphogenetic protein-2 (BMP-2) as a positive regulator can stimulate alveolar bone formation.

Given the above, whether the expressions of OPG and RANKL are regulated by CSF-1 and PTHrP in dental follicle cells in order to contribute to form the major burst of osteoclasts and if BMP-2 can participate the alveolar bone growth via OPG/RANKL pathway? The present study was designed to answer these questions, at the same time to determine whether some regulations of OPG and RANKL by rat dental follicle also happen in human dental follicle cells in vitro.

## Materials and Methods

**Culture of human dental follicle cells**

Human dental follicles from third mandibular molars were surgically removed from adolescents who need for orthodontics treatment after informed content, then trypsinized and cultured as previously described.[10] The human dental follicle cells (HDFCs) were cultured in 250 mL T-flasks with 25 mL Dulbecco's modified Eagle medium (DMEM, HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 1 mmol/L sodium pyruvate, in an incubator at 37˚C in an atmosphere of 5% $CO_2$. HDFCs of passage 5 were grown in 250 mL T-flasks until confluence. Cells at these passages still express the characteristics of dental follicle cells and are not transformed or differentiated into another cell type. Prior to treatments, cells were serum-deprived DMEM containing 0.2% fatty-acid-free and growth-factor-depleted bovine serum albumin for 12 hours.

**Incubation experiments**

Human dental follicle cells of the 5 passage were grown in T-25 tissue-culture flasks until they reached confluence. The cells were then placed in 5 mL of serum-free Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 0.3% bovine serum albumin and incubated for 3 hours prior to the incubation experiments. Then, PTHrP, CSF-1, or BMP-2 was added in each group. For CSF-1, the concentration was 25 ng/mL of medium; for PTHrP, the concentration was 10 ng/mL and the incubation time both was 0-12 hours. The BMP-2 of 100 ng/mL was added to the medium, and the incubation time was 0-18 hours. The concentrations of CSF-1 and PTHrP used are those that can increase the cell proliferation. The time selected for incubation with CSF-1 and PTHrP covers the time when the expression of OPG mRNA has started to be reduced by treatment with either CSF-1 or PTHrP. Controls were incubated in serum-free DMEM containing BSA only.

**Enzyme-linked immunosorbent assay**

In order to quantify the amount of OPG secreted after incubating the dental follicle cells with PTHrP, CSF-1, or BMP-2, immunoassay (R&D Systems, Minneapolis, MN, USA) was employed using a monoclonal antibody for human OPG that had been precoated onto microplates. Each sample of 50 μL from test and control was mixed with 50 μL of assay buffer, pipetted into separate enzyme-linked immunosorbent assay (ELISA) well, and incubated for 2 hours at 37˚C and then overnight at 4˚C. After washing with wash buffer, 100 μL of enzyme linked goat polyclonal antibody for human OPG (R&D Systems) was added to the wells. Following three washes to remove any unbound antibody-enzyme reagent, a tetramethyl benzidine (TMB) substrate solution of 100 μL was added to the well. The absorbance was read at 450 nm with the correction wavelength set at 570 nm using a microplate reader model 550 (Bio-Rad, Hercules, CA, USA). The intensity of the blue color measured was in proportion to the amount of human OPG bound. All experiments were repeated three times.

**RNA extraction and RT-PCR**

Total RNA was extracted from the cultured HDFCs cells with Trizol. The pellet of total RNA was washed briefly with 75% ethanol, resuspended in 20 μL DEPC-treated water, and stored at -80˚C. The concentration of the total RNA was determined by measuring the optical density at 260 nm with the use of a spectrophotometer. And the OD 260/280 was determined to be greater than 1.6. For each sample, reverse transcription was performed using 5 μg RNA extracted from a sample, 200-U M-MuLV reverse-transcriptase (Fermentas Inc., Hanover, MD, USA), 5,000 ng oligo dT, 20 μmol dNTP, and 20 U RNase inhibitor in 20 μL RT reaction volume. The reaction was incubated at 37˚C for 1 hour followed by a 10-minute incubation at 70˚C. To amplify the cDNA with PCR, the primers were designed based on the published human RANKL, OPG, and β-actin mRNA sequences. Specifically, the RANKL primer pair was: 5'-GGC TCA TGG TTA GAT CTG GC-3' (forward) and 5'-TGA CCA ATA CTT GGT GCT TCC-3' (reverse), which yields a 351 bp amplicom. The OPG primer pair was 5'-TCA AGC AGG AGT GCA ATC G-3' (forward) and 5'-AGA ATG CCT CCT CAC ACA GG-3 (reverse), which yields an amplicom of 342 bp. For the β-actin primer pair, the forward primer was 5'-CCG GAT CCA TCT CTT GCT CGA AGT CCA-3' and the reverse primer was 5'-AGA GGC ATA CAG GGA CAA CAC A-3', which yields a 326 bp amplicom. A total of 40 cycles of PCR was performed by mixing 3 μL cDNA with dNTP, primers, PCR buffer and Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) to 25 μL in each reaction with the amplification protocol of 94˚C for 30 s (denatureation), 58.5˚C for 2 s (annealing), and 74˚C for 30 s (extention). For loading controls, identical amplification procedures were done with β-actin primers. The PCR product (20 μL of each reaction) was separated by electrophoresis in a 1.5% agarose I gel (Amresco, Solon, OH, USA) and visualized with ethidium bromide staining, then photographed under UV light. The densitometric evaluation of data was normalized to β-actin, and the ratios are graphs under each PCR profile.

## Results

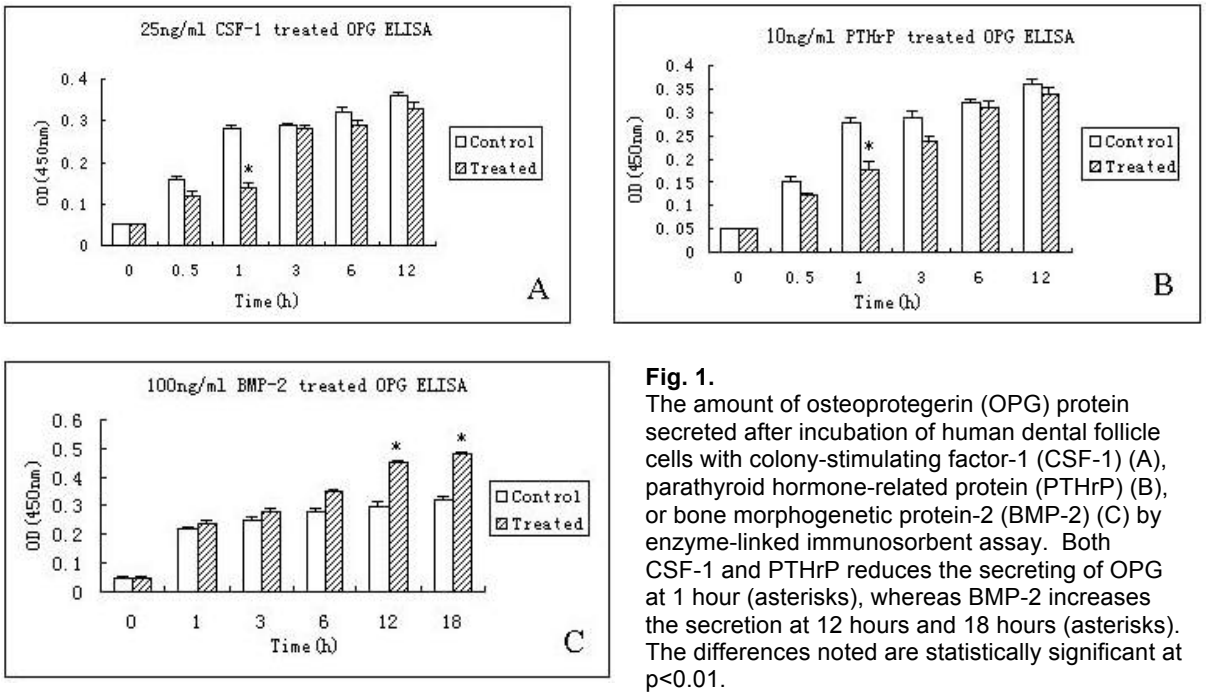


**Fig. 1.**
The amount of osteoprotegerin (OPG) protein secreted after incubation of human dental follicle cells with colony-stimulating factor-1 (CSF-1) (A), parathyroid hormone-related protein (PTHrP) (B), or bone morphogenetic protein-2 (BMP-2) (C) by enzyme-linked immunosorbent assay. Both CSF-1 and PTHrP reduces the secreting of OPG at 1 hour (asterisks), whereas BMP-2 increases the secretion at 12 hours and 18 hours (asterisks). The differences noted are statistically significant at $p<0.01$.

As seen in Fig. 1A, treatment of the cells with CSF-1 resulted in a significant decrease of OPG secretion after

incubation with 25 ng/mL CSF-1 for 1 hour. Using a t-test, this reduction was significant at $p<0.001$. At later time-points of 3 and 6 hours, no reduction in OPG secretion was observed. Treatment with 10 ng/mL PTHrP showed similar results, with a significant reduction in secretion at 1 hour, $p<0.01$ (Fig. 1B). Conversely, BMP-2, at a concentration of 100 ng/mL, significantly increased ($p<0.01$) the secretion of OPG by the dental follicle cells at 12 and 18 hours (Fig. 1C).

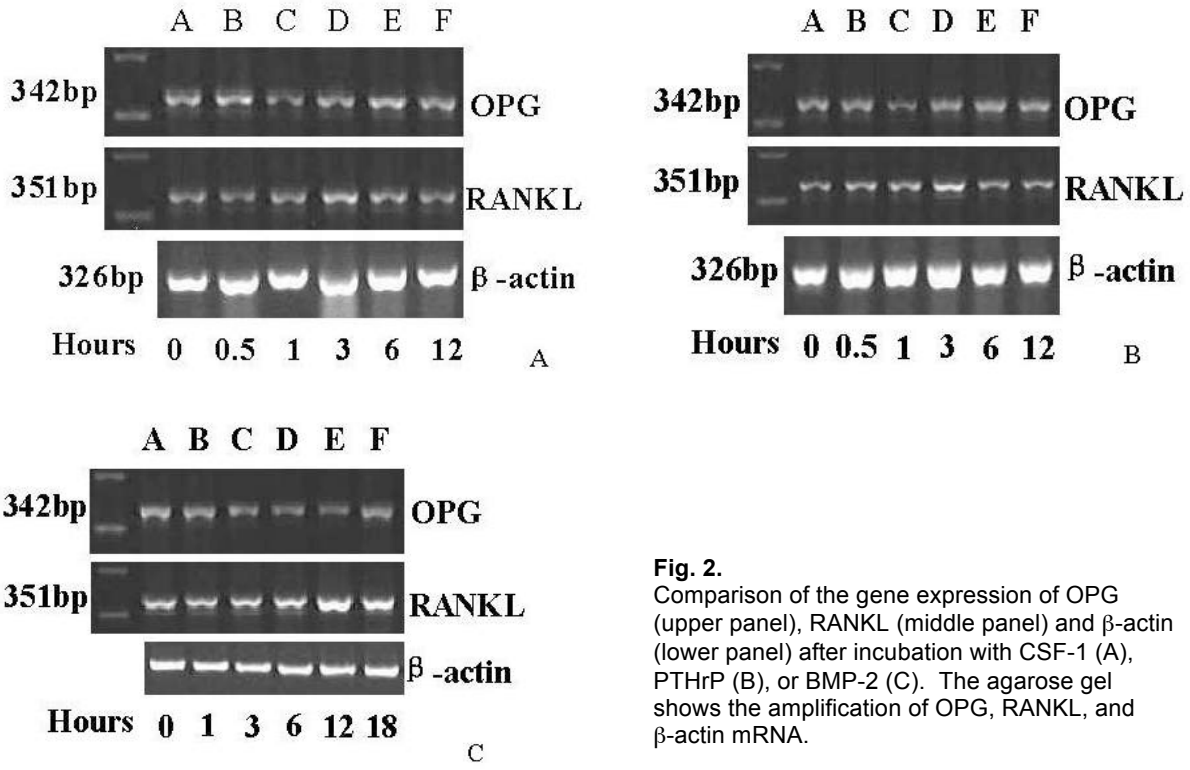


**Fig. 2.**
Comparison of the gene expression of OPG (upper panel), RANKL (middle panel) and β-actin (lower panel) after incubation with CSF-1 (A), PTHrP (B), or BMP-2 (C). The agarose gel shows the amplification of OPG, RANKL, and β-actin mRNA.

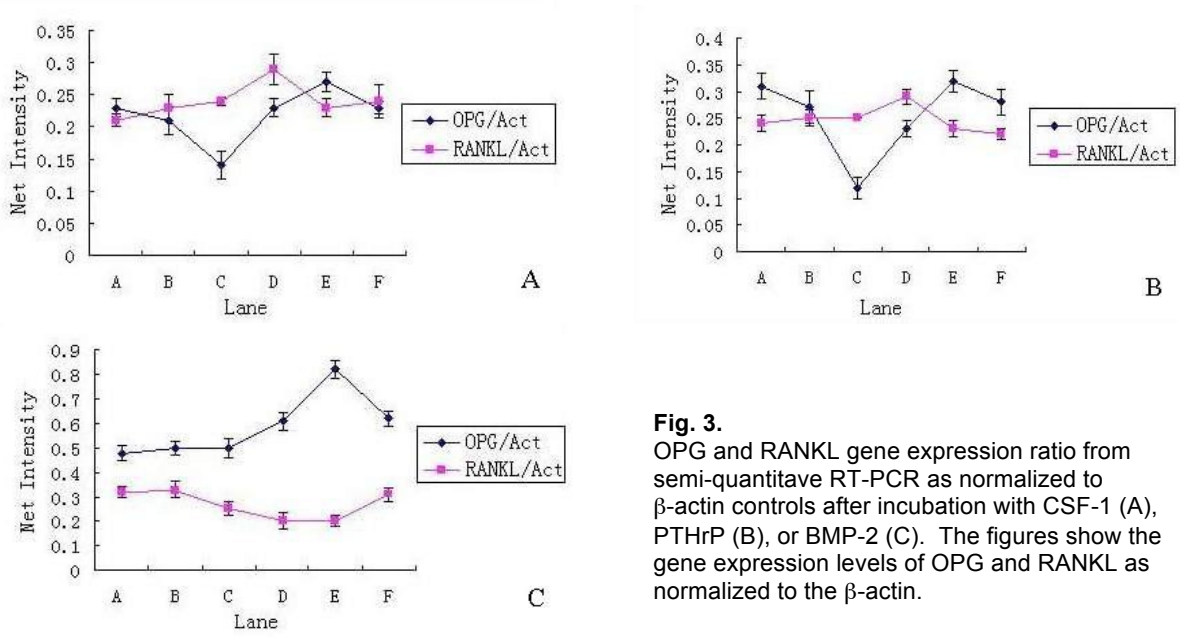


**Fig. 3.**
OPG and RANKL gene expression ratio from semi-quantitave RT-PCR as normalized to β-actin controls after incubation with CSF-1 (A), PTHrP (B), or BMP-2 (C). The figures show the gene expression levels of OPG and RANKL as normalized to the β-actin.

The OPG and RANKL gene is expressed in vitro in the human dental follicle cells. OPG expression is inhibited when the cells are incubated in either 25 ng/mL CSF-1 or 10 ng/mL PTHrP, OPG gene began to reduce after 0.5 hour of coculture, with maximal inhibition seen after 1 hour (Figs. 2A and 3A). Similar results are seen

when incubating with 10 ng/mL PTHrP (Figs. 2B and 3B). But when incubated in 100 ng/mL BMP-2, the expression of OPG gene is enhanced. At 3 hours the gene expression began increase, with the maximal enhancement at 12 hours (Figs. 2C and 3C). Incubation of the human dental follicle cells with 25 ng/mL CSF-1 or 10 ng/mL PTHrP enhanced RANKL expression. The expression began at 0.5 hour, with the maximal enhancement at 3 hours (Figs. 2A, 2B, 3A, and 3B). But when incubated in 100 ng/mL BMP-2, RANKL gene is inhibited. At 3 hours the gene began increase, with the maximal enhancement at 12 hours (Figs. 2C and 3C).

## Discussion

The osteoprotegerin (OPG) is a member of the tumor necrosis factor (TNF) superfamily, and it inhibits osteoclastogenesis by binding to receptor activator of nuclear factor-k B ligand (RANKL) to prevent cell-to-cell signaling between osteoclast precursors and the cells producing RANKL.[11-13] Tooth eruption requires the presence of the dental follicle (DF) around the unerupted tooth.[14,15] The follicle regulates the cellular events of tooth eruption, including recruitment of mononuclear cells to the follicle and the subsequent osteoclastogenesis needed for alveolar bone resorption.[16] At the molecular level, the major burst of osteoclastogenesis seen on day 3 in the rat first mandibular molar appears to occur as the result of a decrease in OPG expression in the DF on that day.[1] At this time, CSF-1 is maximally expressed in the follicle,[17] and CSF-1 acts to down-regulate OPG gene expression in vivo.[18] It is postulated that this reduction in OPG expression enables osteoclastogenesis and the formation of eruption pathway .

In addition to the above, a positive stimulus, namely RANKL, is needed to initiate osteoclastogenesis. RANKL is present in the DF in vivo.[4] The presence of RANKL in DF would enable the OPG that is expressed in the follicle to regulate osteoclastogenesis, as we have previously hypothesized.[1,19] In essence, the reduction of OPG expression at day 3 in the follicle of the first mandibular molar of the rat (day 5 in the mouse)[1] would enable more RANKL to be available to promote osteoclastogenesis. This may not be the only mechanism by which RANKL availability is regulated, however. It is possible that the gene expression of RANKL also is enhanced at these times. The present study shows that RANKL gene expression is significantly up-regulated on postnatal days 9 and 11 compared with the earlier postnatal days.[5] In view of the fact that the major burst of osteoclastogenesis for eruption of the first mandibular molar occurs on day 3,[1] the absence of up-regulation of RANKL at this time point emphasizes the importance of down-regulation of OPG on day 3. A steady-state output of RANKL, coupled with the decrease in OPG, would increase the RANKL/OPG ratio to favor osteoclastogenesis at that time.

In vitro, we have been shown that two probable eruption molecules, CSF-1 and PTHrP, appear to reduce OPG expression.[1] Given that CSF-1 and PTHrP are localized in the dental follicle[17] and adjacent satellite reticulum, respectively,[6] either one or both molecules could signal to inhibit OPG expression. In particular, CSF-1 is maximally transcribed and translated in the dental follicle of the first mandibular molar of the rat at day 3 postnatally,[17] the time of reduction in OPG expression and the time of maximal osteoclast formation.[2,3] Evidence suggests that PTHrP is essential for tooth eruption.[6,7,20] The rescued PTHrP-knockout mice can survive but the eruption pathway fails to form, and developing tooth germs of neonatal homozygous PTHrP-knockout mice are compressed or penetrated by surrounding alveolar bone via impaired osteoclast formation and activation.[21] Moreover, organ culture of tooth germs of homozygous PTHrP-knockout mice showed a significant decrease in the number of TRAP-positive multinucleated osteoclastic cells after culture

whereas the number was increased in wild-type mice.[22] All this evidence suggests that PTHrP, expressed by enamel organ, is indispensable to creation of the eruption pathway and to induction of osteoclasts around the erupting teeth.

In this study, the effects of PTHrP and CSF-1 on OPG gene expression are improved in the human dental follicle cells. Although the tissue sample at variable phases of human tooth eruption in vivo is not available, we postulate that the decrease of OPG in human dental follicle can also contribute to osteoclastogenesis and eruption. We found both reduce OPG expression, less effective at certain times. Let us consider first the response of PTHrP on OPG expression and secretion: there is a slight decrease of OPG at 15 minutes before it drops to the lowest at 1 hour when incubated in PTHrP (Figs. 2B, 3B, and 4B). Again, in another time-course study there is a slight initial increase in OPG at 5 minutes before it declines to a low at 30 minutes and 1 hour.[1] Regarding CSF-1, it inhibits OPG expression at 50 ng/mL but less so at 100 ng/mL.[1] But, in this study, culturing with above 50 ng/mL CSF-1 decreases the proliferation of HDFCS, so we select 25 ng/mL for time-course study. Both the expression and secretion decrease maximally at 1 hour although there is a slight increase expression at 6 hours (Figs. 2A, 3A, and 4A). It is similar to the previous study.[23]

The present study shows that RANKL gene expression is stable for the earlier postnatal days but significantly up-regulated on postnatal days 9 and 11.[5] The up-regulation of RANKL gene expression on days 9-11 may be moderated by TNF-α because TNF-α is maximally expressed on day 9 in the follicle.[24] Others have also shown that TNF-α up-regulates RANKL expression.[25,26] In this study, RANKL gene expression is up-regulated by PTHrP in vitro (Figs. 3B and 4B). We have known PTHrP is present in the satellite reticulum adjacent to the DF, but the chronology of its expression is not clear during tooth eruption. It is postulated that PTHrP participates the osteoclastogenesis on days 9-11. We also found CSF-1 can also up-regulate the RANKL gene expression in this study (Figs. 3A and 4A). However, in vivo RANKL expression is not up-regulated early postnatally, in contrast to its up-regulation on days 9-11.[5] This suggests that the effect of these molecules on RANKL expression in the follicle in vivo may be inhibited either by a blockage of the signal transduction pathway for up-regulation of RANKL in the cells or by some intrinsic refractoriness of the dental follicle to the molecular stimulus during these early postnatal days.

In contrast to the two molecules above that down-regulate OPG expression and up-regulate RANKL expression in the HDFCS, BMP-2 acts the other way. This would be expected that BMP-2, act as stimulator of bone formation,[27] can up-regulate OPG expression in osteoblast lineage cells,[28] and enhance OPG protein secretion in the DF.[22] These effects of BMP-2 on the human dental follicle cells make it a prime candidate for stimulating alveolar bone formation at the base of the tooth during eruption. Given that both CSF-1 and BMP-2 were expressed in the dental follicle and PTHrP expressed in adjacent satellite reticulum, and that they have contrasting effects on OPG and RANKL gene expression, how can both the burst of osteoclastogenesis and alveolar bone formation be stimulated? It is likely that the explanation is based on the location within the follicle as to which gene is expressed (i.e., the coronal half of the follicle above the crown might primarily express CSF-1 for the osteoclastogenesis and bone resorption to occur in the coronal region of the bony crypt, whereas the BMP-2 would be primarily expressed in the basal half of the follicle to promote bone formation in the basal region of the bony crypt). Ultrastructural analysis of the alveolar bony crypts of the third and fourth permanent mandibular molars of the dog demonstrate that the topographic features of the crypts indicate bone resorption in the coronal half and bone formation in the basal half.[29] Thus, further study needs to determine

whether spatial differences exist in the expression of CSF-1 and BMP-2 in the HDFCSs.

In summary, the reduction of OPG expression and enhancement of RANKL expression are related to the burst of osteoclast numbers in rat and mouse dental follicles. From the results of above study, we postulate that CSF-1 and/or PTHrP secretion participates the regulation of OPG and RANKL gene expression in the dental follicle, then enable mononuclear cells in the follicle to form the osteoclasts needed to resorb alveolar bone and to form an eruption pathway. Alternatively, BMP contributes to the alveolar bone formation. In human conditions and syndromes, impacted teeth and delayed tooth eruption are often seen.[30-33] However, the precise mechanisms of these abnormalities are not clear, and it is quite important to clarify the mechanisms of tooth eruption and make clear these points for the patients suffering these problems.

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