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# Subculture affects the phenotypic expression of human periodontal ligament cells and their response to fibroblast growth factor-2 and bone morphogenetic protein-7 *in vitro*

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*Background and Objective:* Although periodontal ligament cells display several osteoblastic traits, their phenotypic expression is still not well established. It remains a matter of debate whether they resemble a terminally differentiated cell type or an intermediate maturation state that potentially can be directed towards a fibroblastic or an osteoblastic phenotype.

*Material and Methods:* To explore the characteristics of periodontal ligament cells in greater detail, fourth-passage, sixth-passage and eighth-passage human periodontal ligament cells were cultured for up to 3 wk. Ki-67, alkaline phosphatase, osteocalcin, osteoprotegerin and receptor activator of nuclear factor-κB ligand (RANKL) mRNA expression was quantified by real-time polymerase chain reaction. Furthermore, the cellular response to fibroblast growth factor-2 and bone morphogenetic protein-7 was examined in first-passage and fourth-passage cells. Dermal fibroblasts (1BR.3.G) and osteoblast-like cells (MG63) served as reference cell lines.

*Results:* Proliferation decreased over time and was highest in fourth-passage cells. The expression of differentiation parameters, osteoprotegerin and RANKL increased with culture time and was higher in fourth-passage cells than in cells of later passages. The RANKL/osteoprotegerin ratio increased steadily until day 21. Administration of fibroblast growth factor-2 enhanced cell numbers in both passages, whereas alkaline phosphatase and osteocalcin production remained unchanged. By contrast, exposure of periodontal ligament cells to bone morphogenetic protein-7 resulted in a reduction of cell number in the first and fourth passages, whereas the production of alkaline phosphatase and osteocalcin was enhanced. In dermal fibroblasts, differentiation parameters did not respond to both stimuli. MG63 cells behaved similarly to periodontal ligament cells.

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*Conclusion:* These results indicate that subculture affects the phenotypic expression of human periodontal ligament cells with respect to the characteristics that these cells share with osteoblasts. Furthermore, the periodontal ligament cell phenotype can be altered by fibroblastic and osteoblastic growth factors.

Human periodontal ligament cells have been attributed several osteoblastic traits such as osteoblastic marker gene expression (1) and enhanced alkaline phosphatase expression in response to 1,25-dihydroxyvitamin$(OH)_2D_3$ (2). Furthermore, changes in proliferation, apoptosis, differentiation and local factor production following exposure to parathyroid hormone have been described (3–6). Despite these findings, the exact phenotype of periodontal ligament cells has still not been well established. It remains a matter of debate whether these cells resemble a terminally differentiated cell type of fibroblastic nature or whether they represent an intermediate maturation state that potentially can be directed towards a fibroblastic, cementoblastic or osteoblastic phenotype by certain growth factors. Amongst others, bone morphogenetic proteins (7–10) and fibroblast growth factors (11–13) have been demonstrated to contribute to the regulation of the differentiation of mesenchymal cells along osteoblastic lineages and of mature periodontal tissues, including the periodontal ligament and the cementum. Bone morphogenetic proteins, and specifically bone morphogenetic protein-7, have been associated with mineralized tissue formation (14) and, within the periodontium, with the induction of cementogenesis (15). Fibroblast growth factors were shown to induce proliferation of bone marrow stromal cells (16), gingival fibroblasts (17) and periodontal ligament cells while inhibiting periodontal ligament cell differentiation (18,19). Thus, periodontal ligament cells, as well as bone morphogenetic proteins and fibroblast growth factors, were suggested to be involved with the regulatory events in the course of periodontal repair processes following inflammatory periodontal disease or orthodontic tooth movement.

Therefore, it is necessary to obtain a better understanding of the phenotypic characteristics of these cells in order to develop purposeful strategies to influence their reparative capacities.

Many *in vitro* studies employed fourth-passage to eighth-passage cells in their experimental set up (20,21). In other experiments, cells at different stages of confluence were used as a model for cellular maturation (3–6). However, it is not clear whether eighth-passage periodontal ligament cells actually display a more mature phenotype than fourth-passage cells and whether the results of such an experimental set up can be compared with those acquired from experiments with different states of confluence. Previously, we reported increased alkaline phosphatase specific activity and osteocalcin production in confluent periodontal ligament cells compared with the levels found in preconfluent cultures, indicating a more mature phenotype of confluent cells (4). It is unclear whether these distinct differences also hold true for different cell passages.

Therefore, the rationale for the present investigation was to characterize in greater detail the phenotypic periodontal ligament cell maturation in long-term culture. Special attention was directed to the role of cell passage. Understanding the effects of cell passage on maturation of the periodontal ligament cell phenotype might enable us to compare the expression of various parameters for proliferation, differentiation and the regulation of hard tissue remodeling by periodontal ligament cells with data obtained from osteoblasts that has already been published in the literature.

Based on the osteoblastic characteristics of periodontal ligament cells described above, we hypothesized that periodontal ligament cells would display decreased mitogenic activity with culture age but enhanced expression of osteoblastic differentiation parameters as an indicator of cellular maturation. Along these lines, we assumed that periodontal ligament cells would express an increased ratio of receptor activator of nuclear factor κB ligand (RANKL) over osteoprotegerin with an increasing length of culture period, supporting the idea that periodontal ligament cells take on an osteoblastic rather than a fibroblastic phenotype with culture age. We further speculated that these changes would vary with cell passage.

Furthermore, it was hypothesized that fibroblast growth factor-2 and bone morphogenetic protein-7 would alter the phenotypic expression of periodontal ligament cells, with a stronger effect of both factors in less mature cells. We assumed that fibroblast growth factor-2 would induce enhanced proliferation of periodontal ligament cells with only marginal effects on differentiation, whereas bone morphogenetic protein-7 would inhibit proliferation but promote differentiation of periodontal ligament cells towards a more osteoblastic phenotype.

## Material and methods

### Cell culture

Human periodontal ligament cells were scraped from the middle third of the roots of premolars of six different human donors, aged between 12 and 14 years, who showed no clinical signs of periodontitis. The teeth had been extracted for orthodontic reasons, after informed parental consent had been obtained and following an approved protocol of the ethics committee of the University of Bonn. Cells were cultured in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum and 0.5% antibiotics, at 37°C in an atmosphere of 100% humidity and 5% $CO_2$. Fourth-passage, sixth-passage and eighth-passage cells were plated in 24-well plates ($n = 6$) at a seeding density

of 10,000 cells/well and then cultured for 3, 6, 9, 12, 15, 18 and 21 d.

Commercially available human dermal fibroblasts (1BR.3.G; European collection of cell cultures Taufkirchen, Germany) and human MG63 osteoblast-like cells (European collection of cell cultures) were cultured according to the manufacturer's instructions and served as reference cell lines to characterize the periodontal ligament cell response to fibroblast growth factor-2 and bone morphogenetic protein-7.

### RNA isolation and reverse transcription

At harvest, total RNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Afterwards, 1 μg of RNA was reverse transcribed with 200 ng of the respective glyceraldehyde-3-phosphate dehydrogenase, Ki-67, alkaline phosphatase specific activity, osteocalcin, osteoprotegerin or RANKL antisense primer in a 15 μL volume using the Amersham-Pharmacia-Biotech RT kit (Amersham Biosciences, Piscataway, NJ, USA). The primers used to amplify human cDNA were purchased from Invitrogen (Karlsruhe, Germany).

### Real-time polymerase chain reaction

Optimal oligonucleotide primers were purchased from Qiagen. Real-time polymerase chain reaction (PCR) was performed on a light-cycler (Roche, Mannheim, Germany) using the software LIGHT CYCLER SOFTWARE 3.5.3. PCR amplification reactions were carried out in a total volume of 20 μL in PCR master mix containing 10 μL of SYBR® Green (Qiagen GmbH, Hilden, Germany), 2 μL of 10× QuantiTect primer assay (Qiagen GmbH, Hilden, Germany) and 2 μL of the reverse transcription product filled up to 20 μL with RNase-free $H_2O$. The amplifications were performed in duplicate for each sample and the optimal annealing temperature for all primers was 55°C for 40 cycles. To normalize the content of cDNA samples, the comparative threshold (Ct) cycle method, consisting of the normalization of the number of target gene copies vs. the number of copies of the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase, was used. The Ct is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold baseline when amplification of the PCR product is first detected. For comparative analysis of gene expression, data were obtained by using the $\Delta$Ct method derived from a mathematical elaboration described previously. Through this method, a single sample, represented in our experiments by cells without any treatment, was designed as a calibrator and used for comparison of gene expression level of any unknown samples.

### Fibroblast growth factor-2 and bone morphogenetic protein-7 administration

To examine the response of periodontal ligament cells to agents that stimulate either a fibroblastic or an osteoblastic phenotype, first-passage and fourth-passage periodontal ligament cells were exposed to either 50 ng/mL of fibroblast growth factor-2 or 50 ng/mL of bone morphogenetic protein-7 (R&D Systems, Wiesbaden, Germany) for 5 d continuously. Both factors were added to serum-containing medium. For each experimental group, vehicle-treated cultures served as controls. Upon cell harvest, cell number, alkaline phosphatase-specific activity and osteocalcin expression were determined.

### Fibroblast growth factor-2 and bone morphogenetic protein-7 effect on cell number and differentiation parameters

Cells were released from the culture surface by trypsinization. Thereafter, the cell suspension was centrifuged and the cell pellet was resuspended in 0.9% NaCl. Finally, the cell number was determined by the use of a cell counter (Moelab, Hilden, Germany). Cells harvested in this manner exhibited > 95% viability based on Trypan blue exclusion.

Alkaline phosphatase-specific activity was measured in the lysates of isolated cells, as described previously (22). The level of osteocalcin in the conditioned media was assayed using a commercially available enzyme-linked immunoassay kit (Immundiagnostik AG, Bensheim, Germany).

### Statistical analysis

For any given experiment, each data point represents the mean ± standard error of the mean of six independent cultures. Variance and statistical significance of the data were analyzed using Bonferroni's modification of the Student's *t*-test; *p*-values of $< 0.05$ were considered to be significant. Each set of experiments was repeated twice and analyzed separately, and both sets of experiments yielded comparable results. Only one set of results from the two sets of experiments are presented.

## Results

Fourth-passage cells expressed significantly more Ki67 mRNA on day 3 than higher cell passages. These basal levels decreased in a time-dependent manner (Fig. 1). With respect to differentiation parameters, both alkaline phosphatase and osteocalcin mRNA expression increased with increasing culture period, with the highest expression levels observed after 18 and 21 d in fourth-passage cells (Fig. 2A,B). Osteoprotegerin mRNA expression increased significantly over time and was higher in eighth-passage cells than in sixth-passage and fourth-passage cells (Fig. 3A). This time-dependent elevation of mRNA in culture was also observed for RANKL, but the highest expression levels were observed in fourth-passage cells (Fig. 3B). The RANKL/osteoprotegerin ratio increased with culture period, and fourth-passage periodontal ligament cells displayed the highest ratio.

When first-passage and fourth-passage periodontal ligament cells were cultured for 5 d in the absence of fibroblast growth factor-2 or bone morphogenetic protein-7, cell number and alkaline phosphatase specific activity were higher in first-passage

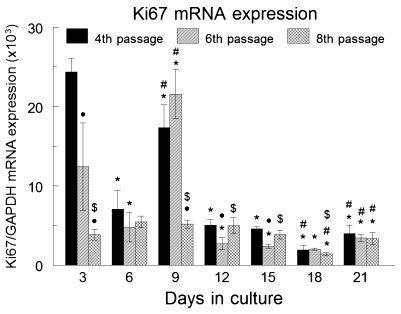


*Fig. 1.* Cell passage-dependent decrease of the proliferative activity of human periodontal ligament cells during 3 wk of culture, as determined by quantification of Ki-67 mRNA expression by means of real-time polymerase chain reaction. Fourth-passage, sixth-passage and eighth-passage cells were cultured for up to 3 wk. Ki-67 mRNA expression was determined every 3 d and normalized to the endogenous reference gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, a particular group vs. the same group after 3 d in culture; $p < 0.05$, a particular group vs. the same group of the preceding culture period; •$p < 0.05$, a particular group vs. fourth-passage cells after the same culture period; and \$$p < 0.05$, a particular group vs. sixth-passage cells after the same culture period.

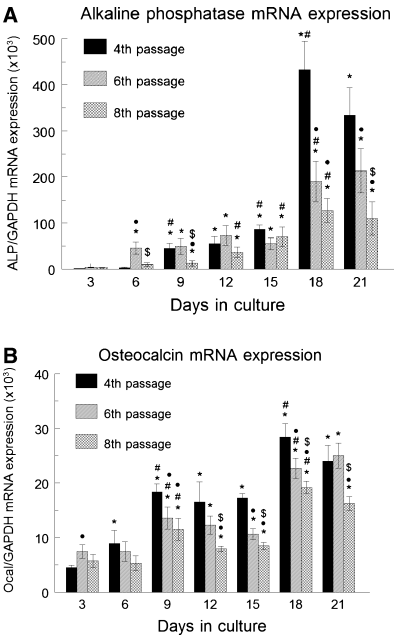


*Fig. 2.* Impact of culture period and cell passage on the expression of differentiation parameters by human periodontal ligament cells, as determined by alkaline phosphatase (A) and osteocalcin (B) mRNA expression. Fourth-passage, sixth-passage and eighth-passage cells were cultured for up to 3 wk. Alkaline phosphatase and osteocalcin mRNA expression was determined every 3 d and normalized to the endogenous reference gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, a particular group vs. the same group after 3 d in culture; $p < 0.05$, a particular group vs. the same group in the preceding culture period; •$p < 0.05$, a particular group vs. fourth-passage cells after the same culture period; \$$p < 0.05$, a particular group vs. sixth-passage cells after the same culture period. ALP, alkaline phosphatase; Ocal, osteocalcin.

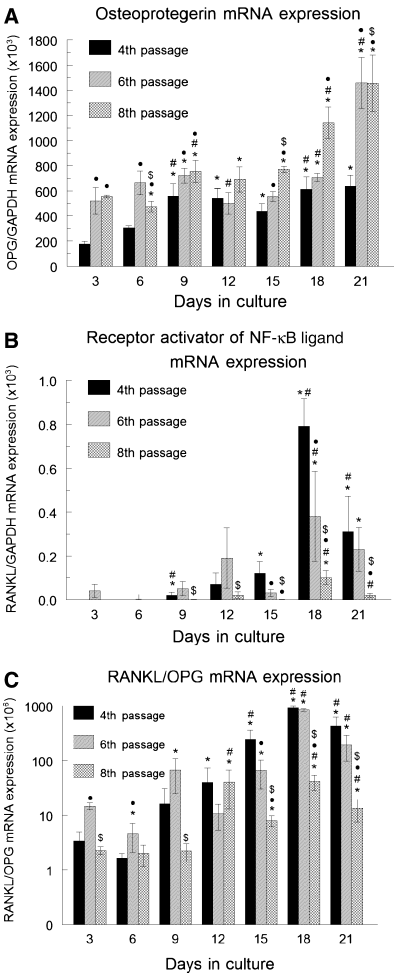


*Fig. 3.* Cell passage-dependent local factors that regulate hard tissue remodeling during 3 wk of culture of human periodontal ligament cells. mRNA expression of osteoprotegerin (A) and receptor activator of nuclear factor κB ligand (B) was determined by means of real-time polymerase chain reaction every 3 d in fourth-passage, sixth-passage and eighth-passage cells and normalized to the endogenous reference gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH). (C) Development of the RANKL/osteoprotegerin ratio over the culture period. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, a particular group vs. the same group after 3 d in culture; $p < 0.05$, a particular group vs. the same group in the preceding culture period; •$p < 0.05$, a particular group vs. fourth-passage cells after the same culture period; \$$p < 0.05$, a particular group vs. sixth-passage cells after the same culture period. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand.

cells. By contrast, osteocalcin protein synthesis was highest in fourth-passage cells. The presence of fibroblast growth factor-2 in the culture medium slightly enhanced cell numbers in both passages, but this was just a trend and not statistically significant (Fig. 4A). As for the differentiation parameters, fibroblast growth factor-2 did not significantly alter alkaline phosphatase specific activity and osteocalcin production (Fig. 4B,C). By contrast, the exposure of periodontal ligament cells to bone morphogenetic protein-7 resulted in a reduction of cell number, both in first-passage and fourth-passage cells (Fig. 4A). Alkaline phosphatase specific activity was enhanced by bone morphogenetic protein-7 in both first and fourth passages (Fig. 4B). Similar results were obtained for osteocalcin production in response to bone morphogenetic protein-7, with a greater effect observed on fourth-passage cells (Fig. 4C).

In the dermal fibroblast reference cell line, similar observations were made for the fibroblast growth factor-2 and bone morphogenetic protein-7 effect on cell number. The cell number was enhanced in response to fibroblast growth factor-7, whereas bone morphogenetic protein-7 induced a reduction in cell number. Basal levels of

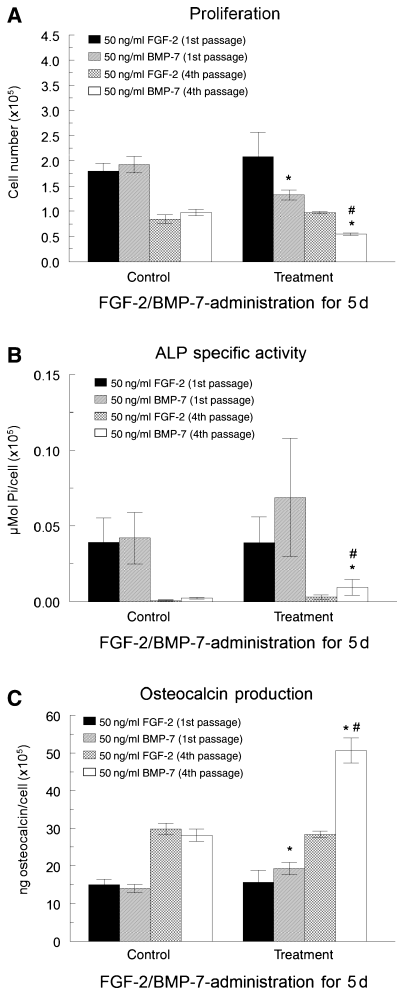


*Fig. 4.* Effect of fibroblast growth factor-2 and bone morphogenetic protein-7 administration for 5 d on the proliferation (A), alkaline phosphatase specific activity (B) and osteocalcin production (C) of first-passage and fourth-passage human periodontal ligament cells. First-passage and fourth-passage cells were cultured in the presence of either 50 ng/mL of fibroblast growth factor-2 or 50 ng/mL of bone morphogenetic protein-7 for 5 d. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. $*p < 0.05$, experimental group vs. vehicle-treated control within the same passage; $p < 0.05$, experimental group vs. fibroblast growth factor-2-treated group within the same cell passage. BMP-7, bone morphogenetic protein-7; FGF-2, fibroblast growth factor-2.

alkaline phosphatase specific activity and osteocalcin production were very low in dermal fibroblasts and unaltered by both stimuli (data not shown). In MG63 osteoblast-like cells, fibroblast growth factor-2 enhanced cell number and alkaline phosphatase specific activity. Bone morphogenetic protein-7 had an inhibitory effect on these parameters. Osteocalcin production was slightly reduced by fibroblast growth factor-2 but significantly enhanced following the administration of bone morphogenetic protein-7 (data not shown).

## Discussion

The present study examined cell-passage-dependent phenotypic characteristics of human periodontal ligament cells during a 3-wk culture period and compared their response to fibroblast growth factor-2 and bone morphogenetic protein-7 with the responses of a dermal reference cell line and an osteoblast reference cell line.

Periodontal ligament cells displayed a culture time-dependent decrease of proliferative activity that was paralleled by an increase in alkaline phosphatase specific activity and osteocalcin production, indicating a shift from an initial increase in number of cells towards initialization of differentiation towards a mature osteoblast phenotype. These findings parallel data obtained from bone marrow stromal cells (23) and differentiating osteoblasts (24,25).

Proliferation and expression of osteoblast-specific genes were stronger in fourth-passage cells than in higher cell passages. Apparently, cells of lower passages exhibit a higher osteogenic potential than those of higher passages. This conclusion is supported by the detection of significantly larger quantities of alkaline phosphatase and other molecules associated with tissue mineralization in freshly isolated periodontal ligament tissue compared with periodontal ligament cells in culture (26,27) and the accumulation of more total DNA in primary cultures of rat osteoblasts than in subcultured cells (28). These findings imply phenotypic alterations of cultured cells and an important role of cell passage in the phenotypic expression of the cells. This consideration has to be kept in mind when interpreting data obtained from experiments with periodontal ligament cells of different cell passages. These cells do not necessarily represent the same stage of phenotypic maturation and therefore might respond differently to mechanical or hormonal stimuli.

Besides alkaline phosphatase and osteocalcin, periodontal ligament cells expressed osteoprotegerin and RANKL mRNA. Both factors were demonstrated to be present in periodontal tissues (29) and were found to be expressed by osteoblasts as key molecules in the regulation of osteoclast differentiation and activity (30,31). Time course assays of RANKL and osteoprotegerin gene expression in osteoblast cell lines showed reduced RANKL mRNA, whereas osteoprotegerin mRNA was increased in undifferentiated cell conditions after 3 wk in culture (32,33). By contrast, Huang *et al.* (23) reported an increase of the RANKL/osteoprotegerin ratio with culture time in human marrow stromal cells, suggesting that the osteoclast induction potential of osteoblasts increases with maturation. From these conflicting data it becomes obvious that species source and cell origin are critical. The basal expression of RANKL was very low in our experiments but its enhancement over time dominated the concurrent increase in osteoprotegerin expression, resulting in an increase of the RANKL/osteoprotegerin ratio with time. These findings further substantiate the osteoblastic nature of periodontal ligament cells and their potential role in the regulation of periodontal remodeling processes.

When the effect of growth factors on periodontal ligament cells was examined, fibroblast growth factor-2 enhanced the proliferative activity, whereas the differentiation parameters remained unchanged in both cell passages examined. Similar observations were made for exogenous fibroblast growth factor-5 administration (26). The inhibitory effect of fibroblast growth factor-2 on periodontal ligament cell maturation *in vitro* might appear contradictory to the *in vivo* phenomenon that topical application of exogenous fibroblast growth factor-2

enhanced periodontal regeneration in experimental bony defects (19). Given the promitogenic effect of fibroblast growth factor-2 opposing its inhibitory actions on differentiation, these data suggest a role for fibroblast growth factor-2 in maintaining a pool of periodontal ligament cells in a multipotent, naive state that might later on be committed to osteoblastic differentiation by other factors in the local microenvironment. This interpretation correlates with a high density of immature cells after the application of fibroblast growth factor-2 to bone defects in beagle dogs (19).

Bone morphogenetic protein-7 suppressed proliferation but induced periodontal ligament cells to assume a more differentiated phenotype in our experiments. This effect was more pronounced in fourth-passage cells. These data are in line with those reported by Lallier and coworkers for bone morphogenetic protein-7 (26) and other bone morphogenetic proteins (34–36). The involvement of bone morphogenetic protein-7 with the stimulation of periodontal regeneration in animal models (37) supports the physiological relevance of the *in vitro* findings.

In conclusion, the results of previous *in vitro* studies on periodontal ligament cells cultured under different conditions and at different cell maturation stages often make comparisons and interpretations relevant to real tissue difficult. The present study provides data that help characterize the periodontal ligament cell phenotype in greater detail and points out the critical role of cell passage in this respect. The data provide a theoretical basis that is crucial in the development of promising strategies to aid periodontal repair processes.

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## References

1. Chou AM, Sae-Lim V, Lim T *et al.* Culturing and characterization of human periodontal ligament fibroblasts - a preliminary study. *Mater Sci Eng* 2002;**20:** 77–83.
2. Basdra EK, Komposch G. Osteoblast-like properties of human periodontal ligament cells: an in vitro analysis. *Eur J Orthod* 1997;**19:**615–621.
3. Lossdörfer S, Götz W, Jäger A. PTH(1-34) affects OPG production in human PDL cells *in vitro*. *J Dent Res* 2005; **84:**634–638.
4. Lossdörfer S, Stier S, Götz W, Jäger A. Maturation-state dependent response of human periodontal ligament cells to an intermittent parathyroid hormone exposure *in vitro*. *J Periodontal Res* 2006;**41:** 62–72.
5. Lossdörfer S, Götz W, Jäger A. Parathyroid hormone modifies human periodontal ligament cell proliferation and survival *in vitro*. *J Periodontal Res* 2006;**41:**519–526.
6. Lossdörfer S, Götz W, Jäger A. PTH (1-34) mediates proliferative and apoptotic signaling in human PDL cells via PKC- and PKA-dependent pathways. *Cell Tissue Res* 2006;**325:**469–479.
7. Sekiya I, Colter DC, Prockop DJ. BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. *Biochem Biophys Res Commun* 2001;**284:** 411–418.
8. Diefenderfer DL, Osyczka AM, Garino JP, Leboy PS Regulation of BMP-induced transcription in cultured human bone marrow stromal cells. *J Bone Joint Surg Am* 2003; **85(A)** (suppl 3)**:** 19–28.
9. Chang SC, Chuang H, Chen YR, *et al.* Cranial repair using BMP-2 gene engineered bone marrow stromal cells. *J Surg Res* 2004;**119:**85–91.
10. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ. Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. *Cell Tissue Res* 2005;**320:**269–276.
11. Batouli S, Miura M, Brahim J *et al.* Comparison of stem-cell-mediated osteogenesis and dentinogenesis. *J Dent Res* 2003;**82:**976–981.
12. Bianchi G, Banfi A, Mastrogiacomo M *et al.* Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res* 2003;**287:**98–105.
13. Chaudhary LR, Hofmeister AM, Hruska KA. Differential growth factor control of bone formation through osteoprogenitor differentiation. *Bone* 2004;**34:** 402–411.
14. Wan M, Cao X. BMP signaling in skeletal development. *Biochem Biophys Res Commun* 2005;**328:**651–657.
15. Ripamonti U, Heliotis M, Rueger DC, Sampath TK. Induction of cementogenesis by recombinant human osteogenic protein-1 (hop-1/bmp-7) in the baboon (*Papio ursinus*). *Arch Oral Biol* 1996; **41:**121–126.
16. Hankemeier S, Keus M, Zeichen J *et al.* Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engeneering of tendons and ligaments. *Tissue Eng* 2005;**11:**41–49.
17. Takayama S, Yoshida J, Hirano H, Okada H, Murakami S. Effects of basic fibroblast growth factor on human gingival epithelial cells. *J Periodontol* 2002; **73:**1467–1473.
18. Okamoto T, Yatsuzuka N, Tanaka Y *et al.* Growth and differentiation of periodontal ligament-derived cells in serum-free defined culture. *In Vitro Cell Dev Biol Anim* 1997;**33:**302–309.
19. Murakami S, Takayama S, Ikezawa K *et al.* Regeneration of periodontal tissues by basic fibroblast growth factor. *J Periodontal Res* 1999;**34:**425–430.
20. Hou L, Li T, Liu C, Liu B, Mi H. Modulation of osteogenic potential by recombinant human bone morphogenetic protein-2 in human periodontal ligament cells: effect of serum, culture medium, and osteoinductive medium. *J Periodont Res* 2007;**42:**244–252.
21. Wescott D, Pinkerton M, Gaffey B, Beggs T, Milne T, Meikle M. Osteogenic gene expression by human periodontal ligament cells under cyclic tension. *J Dent Res* 2007;**86:**1212–1216.
22. Bretaudiere JP, Spillman T. Alkaline phosphatases. In: Bergmeyer HU, ed. *Methods of Enzymatic Analysis*. Weinheim, Germany: Verlag Chemica, 1984:75–92.
23. Huang J, Sakata T, Pfleger L *et al.* PTH differentially regulates expression of RANKL and OPG. *J Bone Miner Res* 2004;**19:**235–244.
24. Owen T, Aronow M, Shalhoub V *et al.* Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in the expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* 1990;**143:**420–430.
25. Thomas G, Baker S, Eisman J, Gardiner E. Changing RANKL/OPG mRNA expression in differentiating primary murine osteoblasts. *J Endocrinol* 2001; **170:**451–460.
26. Lallier T, Spencer A. Use of microarrays to find novel regulators of periodontal

ligament fibroblast differentiation. *Cell Tissue Res* 2007;**327:**93–109.

27. Nohutcu R, McCauley L, Shigeyama Y, Somerman M. Expression of mineral-associated protein by human periodontal ligament cells: in vitro vs. ex vivo. *J Periodontal Res* 1996;**31:**369–372.
28. Aronow M, Gerstenfeld L, Owen T, Tassanari M, Stein G, Lian J. Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvarial cells. *J Cell Physiol* 1990; **143:**213–221.
29. Kanzaki H, Chiba M, Shimizi Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res* 2001;**80:**887–891.
30. Simonet WS, Lacey DL, Dunstman CR *et al*. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;**89:**309–319.
31. Lacey DL, Timms E, Tan HL *et al*. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;**93:**165–176.
32. Gori F, Hofbauer L, Dunstan C, Spelsberg T, Khosla S, Riggs L. Expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. *Endocrinology* 2000;**141:**4768–4776.
33. Deyama Y, Takeyama S, Koshikawa M *et al*. Osteoblast maturation suppresses osteoclastogenesis in co-culture with bone marrow cells. *Biochem Biophys Res Commun* 2000;**274:**249–254.
34. Xu W, Shiba H, Mizuno N *et al*. Effect of bone morphogenetic proteins-4, -5 and -6 on DNA synthesis and expression of bone-related proteins in cultured human periodontal ligament cells. *Cell Biol Int* 2004;**28:**675–682.
35. Yamada S, Ozawa Y, Tomoeda M, Matoba R, Matsubara K, Murakami S. Regulation of PLAP-1 expression in periodontal ligament cells. *J Dent Res* 2006;**85:**447–451.
36. Zhao M, Xiao G, Berry J, Franceschi R, Reddi A, Somerman M. Bone morphogenetic protein 2 induces dental follicle cells to differentiate toward a cementoblast/osteoblast phenotype. *J Bone Miner Res* 2002;**17:**1441–1451.
37. Taba M, Jin Q, Sugai J, Giannobile W. Current concepts in periodontal bioengineering. *Orthod Craniofac Res* 2005;**8:** 292–302.