Subculture affects the phenotypic expression of human PDL cells and their response to FGF-2 and BMP-7 in vitro

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My parents, sisters and brother for all the sacrifices

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Zusammenfassung

Obwohl humane Parodontalligamentzellen (PDL) mehrere osteoblastäre Merkmale aufweisen, ist ihr Phänotyp noch immer nicht vollständig charakterisiert. Es bleibt eine ungeklärte Frage, ob sie ein ausdifferenziertes Zellstadium oder eine Zwischenstufe der zellulären Differenzierung darstellen und womöglich in ihrer phänotypischen Expression durch bestimmte Stimuli in eine eher fibroblastäre oder osteoblastäre Richtung beeinflusst werden können.

Zur weiteren Erforschung der PDL-Zellcharakteristika wurden humane PDL Zellen der ersten und vierten Passage kultiviert und jeweils für fünf Tage mit FGF-2 bzw. BMP-7 stimuliert. Anschließend wurde die Zellzahl mit Hilfe eines Zellzählers bestimmt. Der Einfluss auf die Differenzierung der Zellen wurde anhand des biochemischen Nachweises der alkalische-Phosphatase-Aktivität in Zelllysaten sowie mit Hilfe der Bestimmung des Osteokalzingehaltes des konditionierten Mediums bestimmt. Zur Beleuchtung der Frage, ob sich PDL-Zellen bei einer solchen Stimulation eher wie Fibroblasten oder wie Osteoblasten verhalten, dienten dermale Fibroblasten und Osteoblasten-ähnlichen Zellen (MG63) als Referenzzelllinien.

Nach Ablauf der experimentellen Phase war die Zellzahl der Zellen der ersten Passage höher als die der vierten was auf ein großes Proliferationsvermögen dieser Zellen hinweist. Die Behandlung mit FGF-2 erhöhte die Zellzahl in beiden Passagen wobei ALP und Osteocalcin Produktion unverändert blieben. Im Gegensatz dazu führte eine BMP-7 Exposition zu einer Reduzierung der Zellzahl in beiden Passagen, während die ALP und Osteocalcin Produktion erhöht wurde.

In dermalen Fibroblasten blieben die Differenzierungsparameter unverändert. MG63 Zellen verhielten sich ähnlich wie PDL Zellen.

Diese Ergebnisse deuten darauf hin, dass 1) die Subkultivierung einen Einfluss auf die phänotypische Expression von humanen PDL Zellen hat, und 2) dass der Phänotyp dieser Zellen durch Wachstumsfaktoren modifiziert werden kann.

Introduction

The periodontal ligament (PDL) is a specialized connective tissue interposed between the roots of teeth and the inner wall of the alveolar bone socket (Fig. 1). Its fibers form a meshwork that stretches out between the cementum and the bone. This meshwork provides both anchorage and cushioning of mechanical stresses which teeth are subjected to (Waddington and Embery 2001, Lecik et al. 2001).

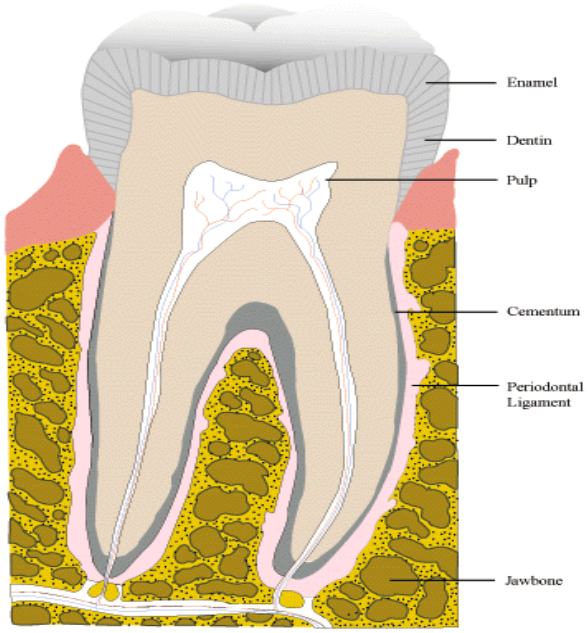

Fig. 1: the periodontal ligament binds the roots of the tooth to the alveolar bone providing anchorage.

Identifying the biological properties of the cells residing within the periodontal ligament will help to understand the role these cells play in the various functions of the periodontal ligament, and will improve the success of clinical procedures such as orthodontic tooth movement (Basdra and Komposch 1997) and aid the therapeutical periodontal regeneration processes (Takayama et al. 1997).

Within the mixed population of PDL cells, PDL fibroblasts represent the majority of cells. The exact phenotypic characteristics of PDL fibroblasts are still not 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 which potentially can be directed toward a fibroblastic, cementoblastic or osteoblastic phenotype by certain growth factors.

PDL fibroblasts exhibit several osteoblastic traits such as osteoblastic marker gene expression and enhanced alkaline phosphatase specific activity in response to 1,25-$(OH)_2D_3$ (Basdra and Komposch 1997, Chen et al. 2002, Jackson et al. 2006, Wang et al. 2007). Recently it has been demonstrated that PDL fibroblasts respond to parathyroid hormone in an osteoblast-like manner, too (Lossdörfer et. al. 2005, 2006 a, b, c). Thus, PDL cells are believed to be involved in the regulatory events in the course of periodontal repair processes following inflammatory periodontal disease or orthodontic tooth movement.

In order to benefit from those properties and direct the differentiation process, several biological stimuli have been used. Among these factors, bone morphogenetic protein (BMP)-7, which is one of at least 15 structurally and functionally related BMPs that belong to the TGF-β superfamily of polypeptides, which was used to induce new bone formation *in vivo* and *in vitro*, as well as cementogenesis for bridging of periodontal bone defects (Ripamonti et al. 1996, Ripamonti and Reddi 1997, Franceschi et al. 2000, Krebsbach et al. 2000, Nakashima and Reddi 2003).

Fibroblast growth factor (FGF)-2, also known as basic FGF, which is a member of the FGF family, currently comprised of 19 related mitogenic proteins, stimulates the proliferation of all cells of mesodermal origin and many cells of neuroectodermal, ectodermal and endodermal origin (Fering et al. 1994, Coulier et al. 1997). Studies have also shown that FGF-2 induces the proliferation of bone marrow stromal cells (Hankemeier et al. 2005), gingival fibroblasts (Takayama et al. 2002), and PDL cells, while inhibiting PDL cell differentiation as evidenced by a reduction of alkaline phosphatase specific activity and mineralization in response to this growth factor (Okamoto et al. 1997, Murakami et al. 1999).

Previous studies showed that the cellular response to biological stimuli depends on cellular developmental age. Some *in vitro* studies employed fourth to eighth passage cells in their

experimental set up (Hou et al. 2007, Wescott et al. 2007). Others used different stages of confluence as a model of cellular maturation where increased alkaline phosphatase activity and osteocalcin production were associated with the confluent stage indicating a more mature phenotype as compared to pre-confluent cells (Chen et al. 2002, Jackson et al. 2006, Wang et al. 2007). Recently this was also demonstrated in PDL cells (Lossdörfer et. al. 2005, 2006 a, b, c)

However, it is unknown whether cells of different passages would respond differently to the same stimuli and whether those results could be compared to those involving different stages of confluence. Therefore, it was the aim of the present investigation to help identifying the traits of PDL cells by exposing them to BMP-7 and FGF-2 and to determine the role of the cell passage for the cellular response to such stimulation.

Based on these considerations, we hypothesized that BMP-7 and FGF-2 would alter the PDL cells characteristics in that FGF-2 would enhance proliferation of the cells and minimally affect their differentiation, whereas we speculated that BMP-7 would inhibit proliferation but stimulate the differentiation of those cells towards a more osteoblastic phenotype. In addition, it was hypothesized that those effects would be dependent on cell passage as a model for cellular developmental age.

Materials and Methods

PDL-Cell Culture

Human periodontal ligament cells were obtained from premolar teeth of six healthy human donors aged between 12 and 14 years. The teeth showed no clinical signs of periodontitis and they had been extracted for orthodontic reasons, with informed parental consent and following a protocol approved by the ethics committee of the University of Bonn.

Periodontal tissue was scraped from the middle third of the roots in order to avoid any contamination of the culture with gingival fibroblasts or pulp cells (Fig. 2).

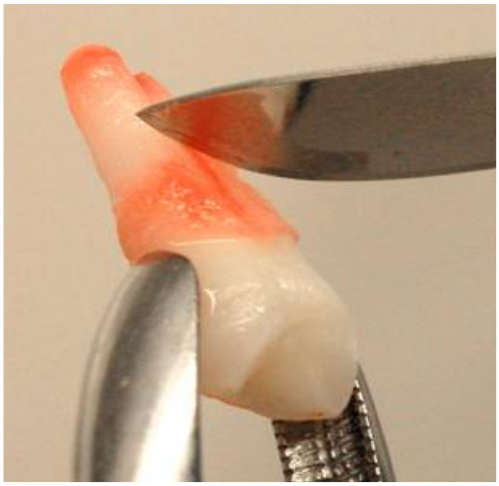

Fig. 2: Obtaining the PDL cells by scraping the middle third of the tooth with a scalpel.

Cells that grew from those explants were then cultured in DMEM containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin; Biochrom AG, Berlin, Germany) at 37º C in an atmosphere of 100% humidity and 5% CO_2. Medium was changed every 48 hours (Fig. 3).

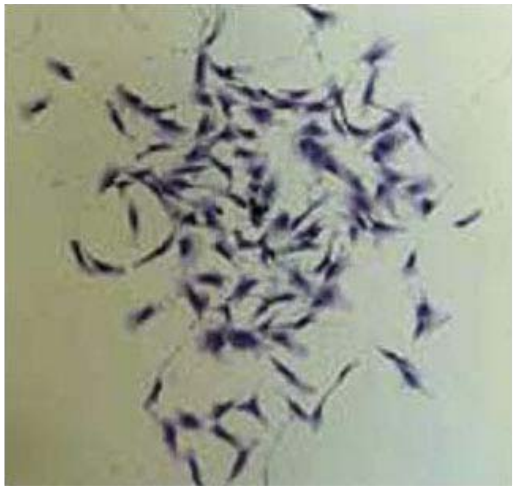

Fig. 3: Cells in culture as seen under the microscope (Magnification x50).

At confluence, cells were detached from the culture flask using 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA and transferred to 24-well-plates where they were subcultured at a density of 10000 cells/well (n=6)

FGF-2 and BMP-7 Administration

First and fourth passage cells were cultured for 5 days in the presence of either 50ng/ml FGF-2 or 50ng/ml BMP-7 (R&D Systems GmbH, Wiesbaden, Germany).

FGF-2 vehicle was prepared using sterile phosphate-buffered saline containing 1µg/mL sodium heparin and 0.1% bovine serum albumin. BMP-7 vehicle on the other hand was prepared using 4mM HCL containing 0.1% bovine serum albumin. Vehicle-treated cultures for each treatment group served as controls. The media were changed after 24h and thereafter every 48h until the end of the experiment.

Cell Culture of Dermal Fibroblasts and MG63-Osteoblast-like-Cells

Commercially available human dermal fibroblasts of the 13th passage (1BR.3.G; European Collection of Cell Cultures) and 4th passage human MG63-osteoblast-like cells (European Collection of Cell Cultures) were used as reference cell lines for the characterization of the periodontal ligament cells, as well as for the comparison of periodontal ligament cell

response to FGF-2 and BMP-7 stimulation. The cells were cultured under same conditions as the PDL-cells and were treated with FGF-2 and BMP-7 accordingly.

Effect of the Growth Factors on Proliferation

After an experimental period of 5 days, the supernatant was collected for later osteocalcin analysis and the cells were released from the culture surface by trypsinization for 5 min at 37°C. This reaction was stopped by the addition of DMEM containing 10% FBS. The cell suspension was then 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, Hilgen, Germany). Cells harvested in this manner exhibited >95% viability based on Trypan-blue exclusion.

Effect of the Growth Factors on Differentiation

Alkaline-phosphatase-specific activity was measured in lysates of isolated cells, as described previously (Bretaudiere and Spillman, 1984). The alkaline phosphatase assay kit uses pNPP (p-Nitrophenyl phosphate) as a colorimetric substrate for alkaline phosphatase-based ELISA secondary antibody detection systems. The procedure included pipetting 100µl of each sample into 96-well plates. 50µl of buffer solution (AMP-pNPP-MgCl2) were added to each well and the plates were incubated at 37ºC until a change in color was noted in the majority of wells taking into account that the total incubation does not exceed 3 hours. The reaction was then stopped by adding 100µl of NaOH and the plates were read using an absorbance mircoplate reader detecting the absorbance at 405nm. The samples were then compared to the standards prepared in the same assay. The levels of osteocalcin in the conditioned media were assayed with the use of a commercially available enzyme-linked immunoassay kit (IBL GmbH, Hamburg; Immundiagnostik AG, Bensheim; Germany) where 25µl of standards, controls and samples were pipetted in 96-well-microplates. 125µl of anti-osteocalcin antibody were added to each well. The plates were then incubated at room temperature for 2 hours. Afterwards, all wells were washed three times using 300µl/well of the washing solution provided with the kit. Thereafter, 150µl/well enzyme conjugate were added and the plates were incubated at room temperature for 1 hour. 150µl substrate

solution was added to each well and the plates were left at room temperature for another 40 minutes. 50µl/well stop solution was then added and the plates were read within 15 minutes using an absorbance mircoplate reader detecting the absorbance at 405nm.

Statistical Analysis

For any given experiment the mean ± SEM of 6 independent cultures was calculated. Variance and statistical significance of data were analyzed by Bonferroni's modification of Student's t-test. P-values < 0.05 were considered to be significant. Each set of experiments was repeated twice and analyzed separately. Both sets of experiments had comparable results; hence the results of only one of the two sets are presented.

Results

Alkaline phosphatase and Osteocalcin were detectable in both first and fourth passage cells. When first and fourth passage PDL cells were cultured for five days in the absence of FGF-2 or BMP-7, cell number and alkaline phosphatase specific activity were higher in first passage cells, whereas osteocalcin production was higher in fourth passge cells (Fig. 4, Fig. 5, Fig. 6).

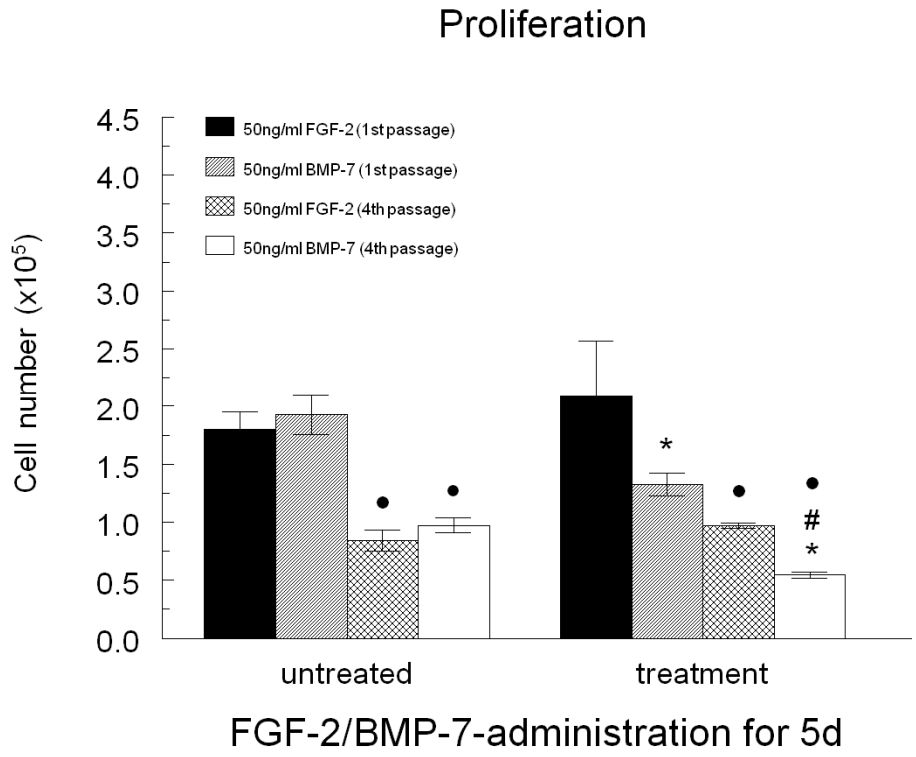

Fig. 4: Proliferation of PDL cells of the first and fourth passage with and without treatment with FGF-2 and BMP-7. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, 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; ●$p < 0.05$, particular group vs. same treatment regime in first passage cells. BMP-7, bone morphogenetic protein-7; FGF-2, fibroblast growth factor-2.

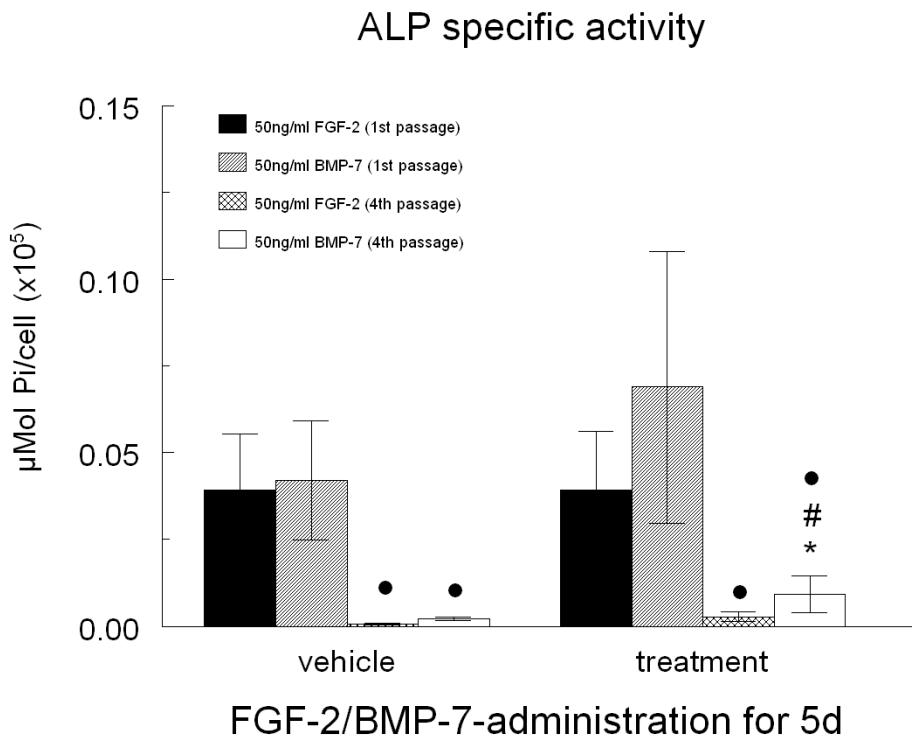

Fig. 5: ALP specific activity of PDL cells of the first and fourth passage with and without treatment with FGF-2 and BMP-7. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *$p < 0.05$, 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; ●$p < 0.05$, particular group vs. same treatment regime in first passage cells. BMP-7, bone morphogenetic protein-7; FGF-2, fibroblast growth factor-2.

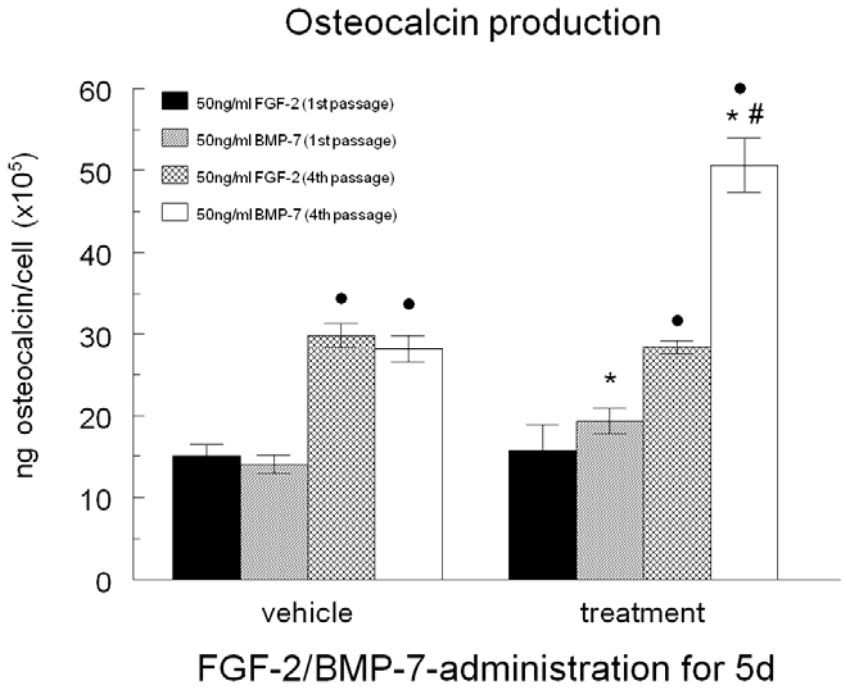

Fig. 6: Osteocalcin production of PDL cells of the first and fourth passage with and without treatment with FGF-2 and BMP-7. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± standard error of the mean for six independent cultures. *p < 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; ●p < 0.05, particular group vs. same treatment regime in first passage cells. BMP-7, bone morphogenetic protein-7; FGF-2, fibroblast growth factor-2.

Exposing PDL cells to FGF-2 led to an increase in cell number. This effect displayed just a trend without statistical significance and was more prominent in cells of the 1st passage than in those of the 4th one (Fig. 4).

Similar effects were observed in the dermal and osteoblast reference cell lines where FGF-2 evoked a statistically significant increase in cell number in both dermal fibroblasts, with an increase of almost 131%, and in MG63 cells with an average increase of 46% (Fig. 7, Fig. 8).

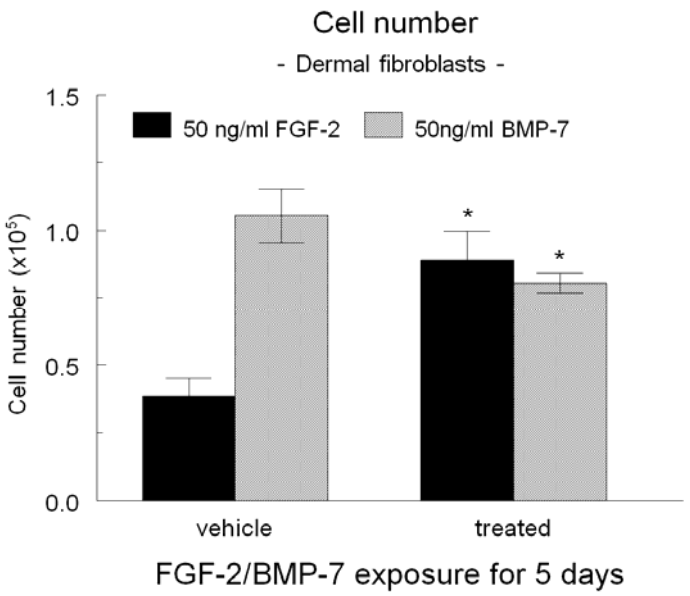

Fig. 7: Effect of FGF-2 and BMP-7 administration for five days on the proliferation of DF cells. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for 6 independent cultures. *P<0.05, particular group vs. same group after 5 days in culture; #P<0.05, particular group vs. same group of the preceding culture period.

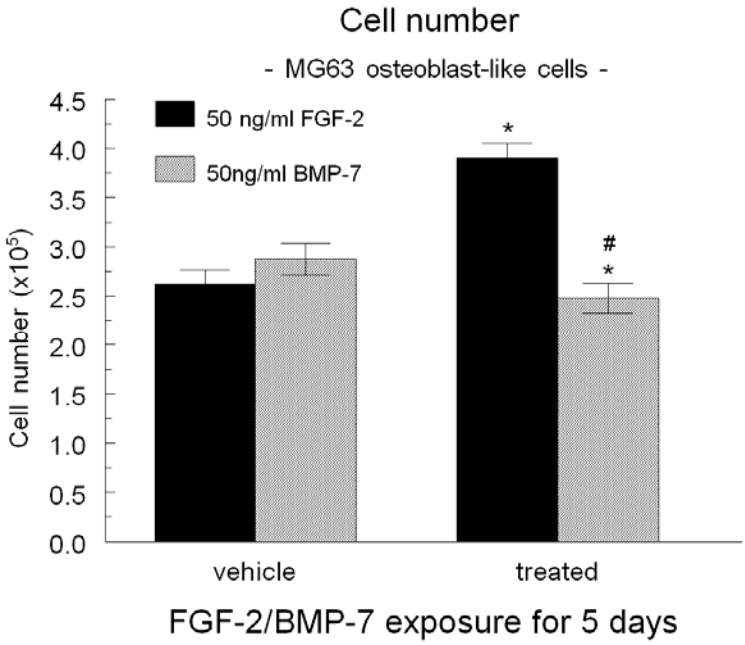

Fig. 8: Effect of FGF-2 and BMP-7 administration for five days on the proliferation of MG63 cells. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for 6 independent cultures. *P<0.05, particular group vs. same group after 5 days in culture; #P<0.05, particular group vs. same group of the preceding culture period.

The opposite was observed after treating PDL cells with BMP-7 where a statistically significant reduction in cell number resulted. This reduction was seen in both passages but was more evident in 4th passage cells with an average decrease of 43% (Fig. 4).
A statistically significant reduction in cell number was also seen in dermal fibroblasts and MG63 cells with an average of 23% and 10.3%, respectively (Fig. 7, Fig. 8).

Regarding the differentiation parameters, PDL cells of the 1st passage showed no difference in the ALP activity following treatment with FGF-2. A slight, statistically nonsignificant increase was seen in cells of the 4th passage and a similar trend was seen in the DF cells (Fig. 5, Fig. 9). In contrast, a statistically significant increase which averaged about 110% was seen in the ALP activity in MG63 cells (Fig. 10).

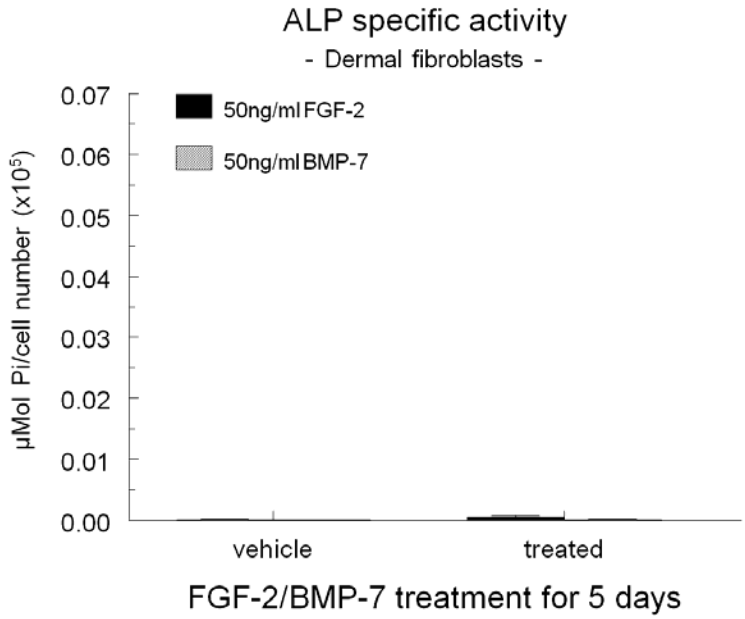

Fig. 9: Effect of FGF-2 and BMP-7 administration for five days on the ALP specific activity of DF cells. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for 6 independent cultures. *P<0.05, particular group vs. same group after 5 days in culture; #P<0.05, particular group vs. same group of the preceding culture period.

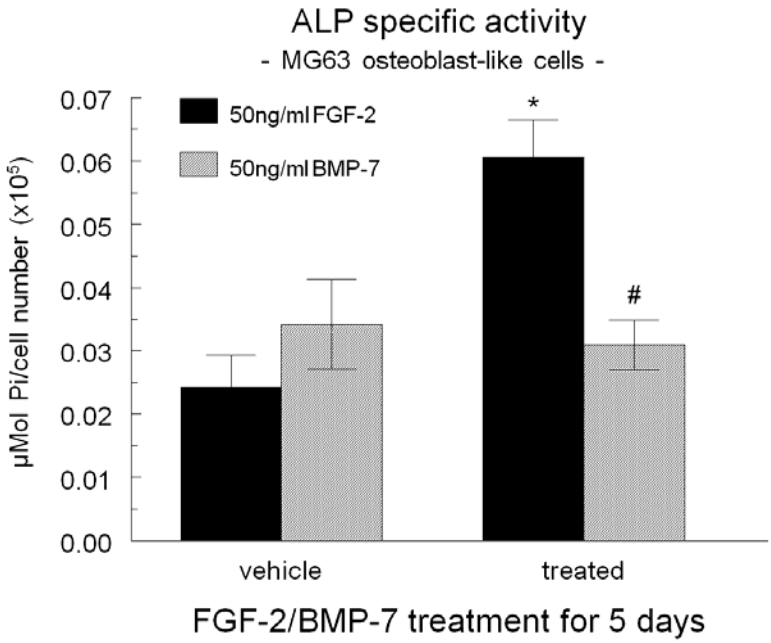

Fig. 10: Effect of FGF-2 and BMP-7 administration for five days on the ALP specific activity of MG63 cells. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for 6 independent cultures. *P<0.05, particular group vs. same group after 5 days in culture; #P<0.05, particular group vs. same group of the preceding culture period.

Unlike FGF-2, an increase in ALP activity of PDL cells was triggered by BMP-7 regardless of the passage whereas a statistically significant more pronounced effect was detected in cells of the 4th passage where the ALP activity increased by about 300% (Fig. 5).

An opposite effect was observed in MG63 cells where a reduction of 21% in treated cells in comparison to the controls was noticed (Fig. 10). In dermal fibroblasts basal expression of ALP specific activity was very low and remained unaffected by BMP-7 (Fig. 9)

The unchanged ALP activity of PDL cells of the 1st passage after being treated with FGF-2 was accompanied by a slight increase in osteocalcin production. In contrast, cells of the 4th passage showed a slight decrease (Fig. 6).

In this aspect, PDL cells of the 4th passage were comparable to MG63 cells which also showed a statistically nonsignificant reduction in osteocalcin production after exposure to FGF-2 (Fig. 11).

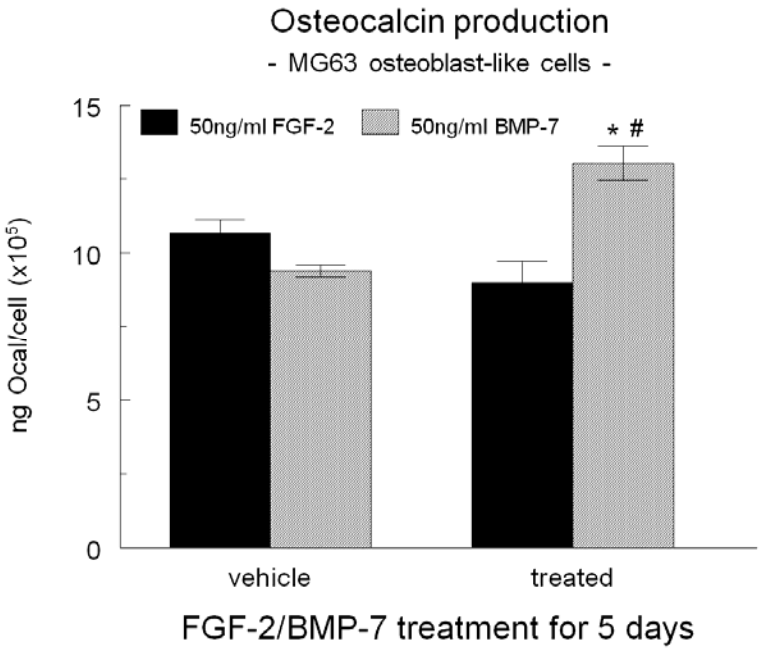

Fig. 11: Effect of FGF-2 and BMP-7 administration for five days on the osteocalcin production of MG63 cells. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for 6 independent cultures. *P<0.05, particular group vs. same group after 5 days in culture; #P<0.05, particular group vs. same group of the preceding culture period

DF cells exhibited a very low production of osteocalcin that was hardly influenced by the administration of FGF-2 (Fig. 9).

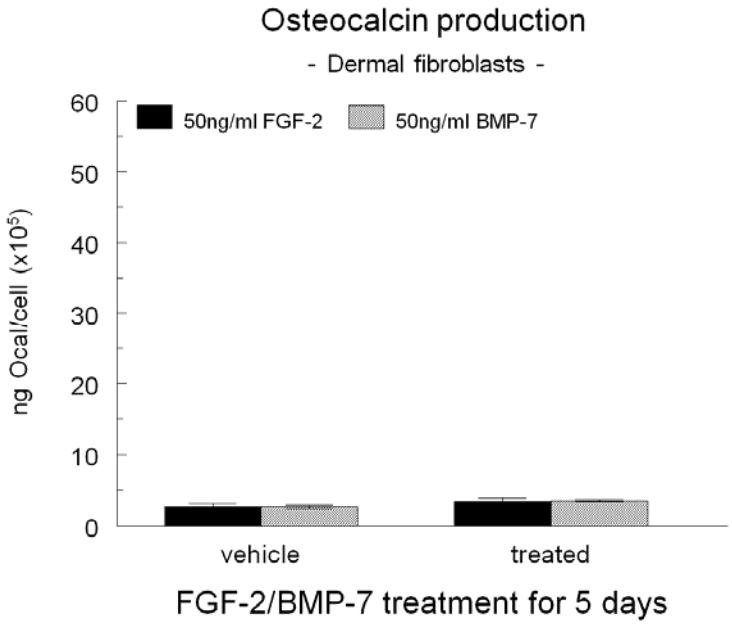

Fig. 12: Effect of FGF-2 and BMP-7 administration for five days on the osteocalcin production of DF cells. Data were acquired from one of two separate experiments, both yielding comparable results. Each value is the mean ± SEM for 6 independent cultures. *P<0.05, particular group vs. same group after 5 days in culture; #P<0.05, particular group vs. same group of the preceding culture period

Treating PDL cells with BMP-7 resulted in a statistically significant increase in osteocalcin production in both 1st and 4th passage cells with an average increase of 37% and 80% respectively (Fig. 6). A similar effect was noticed after treating MG63 cells with BMP-7 (Fig. 11). In dermal fibroblasts basal expression of osteocalcin was very low and remained unaffected by BMP-7 (Fig. 12).

Discussion

In this study, the effect of a FGF-2 and BMP-7-simulation on the phenotypic expression of human PDL cells with special attention directed to the role of cellular developmental age was examined. The cellular response was compared to a dermal and to an osteoblastic reference cell line.

Proliferation was measured using a cell counter (Moelab, Hilgen, Germany) allowing for determination of cell number as well as the differentiation parameters within the same experiment. The cell number was used as an indicator of proliferation in our experiment. The ratio of proliferation versus apoptosis was not further investigated. Changes in differentiation on the other hand were assessed using two parameters, osteocalcin and alkaline phosphatase content in the conditioned medium.

Alkaline phosphatase (ALP) is one of the most widely recognized biochemical markers for early osteoblast differentiation (Sabokbar et al. 1994). The osteocalcin immunoassay quantitatively measures intact osteocalcin in collected medium. Osteocalcin studies in developing embryos and bone cell models have validated its high specificity as a marker for the mature osteoblastic phenotype. As osteocalcin is expressed by osteoblasts, it is often used as a biochemical marker for the bone formation process (Gomez et al. 1994, Delmas 1995).
The ELISA allows for a quantitative assessment. Since data was expressed as a function of cell number it was assured that changes actually result from changes in the expression by single cells and not just from an increase in cell number.

The characteristics of PDL cells and their response to different stimuli were compared to a dermal and to an osteoblastic reference cell line in order to verify whether the phenotype resembles more that of dermal fibroblasts or osteoblasts and whether it could be altered towards fibroblasts or osteoblasts after treatment with FGF-2/BMP-7.

Different models of cellular developmental age have been used previously. Some studies have used cells at different stages of confluence as a model for cellular maturation (Lossdörfer et al. 2005, Lossdörfer et al. 2006a, 2006b, 2006c). In our study, cell passage was used. However, it is not clear whether the PDL cells of a higher passage actually display a more mature phenotype than those of a lower passage and whether the results of such an experimental setup can be compared to those obtained from experiments with different states of confluence. Lossdörfer and co-workers (2006) have reported increased alkaline phosphatase specific activity and osteocalcin production in confluent PDL cells compared with lower levels in pre-confluent cultures indicating a more mature phenotype of confluent cells. It is unclear whether these distinct differences hold true for different cell passages as well.

In this study, the proliferative activity of PDL cells decreased with cellular developmental age. This decrease was accompanied by an increase of osteocalcin production indicating the differentiation towards a more mature osteoblast phenotype. These findings are coincident with results obtained from earlier experiments done in our laboratory where PDL cells that were cultured for up to 3 weeks showed an increase in differentiation and a decrease in mitogenic activity with time (Lossdörfer et al. 2008). An early gene expression for alkaline phosphatase and a later Osteocalcin expression was also observed in bone marrow stromal cells (Huang 2004) and differentiating rat osteoblasts that were cultured for up to four weeks (Owen 1990).

Proliferation and alkaline phosphatase specific activity were stronger in first passage cells as compared to fourth passage cells. Apparently, cells of lower passages exhibit a stronger proliferative potential than those of higher cell passages. This conclusion is supported by data comparing the expression of molecules associated with tissue mineralization in freshly isolated PDL tissue and PDL cells in culture where fresh PDL cells expressed larger quantities of periostin, osteopontin and alkaline phosphatase activity (Lallier and Spencer 2007). An early gene expression for alkaline phosphatase and later osteocalcin expression was observed in bone marrow stromal cells (Huang et al. 2004) and differentiating rat

osteoblasts (Owen et al. 1990, Thomas et al. 2001) that were cultured for up to four weeks. Sawa and coworkers observed a reduction in ALP activity in aged human osteogenic PDL fibroblasts (Sawa et al. 2004) whereas an increase of ALP activity with time was reported in another study (Basdra and Komposch 1997). This conflicting data could be explained by the fact that Sawa and coworkers have used human fibroblasts from donors aging between 15 to 51 years.

Regarding the effect of growth factors on PDL cell proliferation and differentiation, FGF- 2 increased the proliferative activity without changing alkaline phosphatase activity in both first and fourth passage cells. A similar enhancement of proliferation after treating PDL cells with FGF-2 was also reported in other studies (Zhang et al. 2001, Ling and Li 2004). Yamada and coworkers (2006) have confirmed that the addition of FGF-2 to a long-term human PDL cell culture resulted in a down-regulation of ALP activity. An increase of proliferation and a down regulation of ALP activity were also reported by Takayama and coworkers (Takayama et al. 1997). Ge and Yang (2001) have reported an increase of proliferation with an inhibition of cytodifferentiation of PDL cells after FGF-2 treatment.
On the other hand, the osteocalcin content increased significantly after the administration of FGF-2. This is in line with results reported by Tirashima and coworkers (2008) who investigated the effect of FGFs on the periodontal ligament in a mouse model and found that FGF-2 induces a unique expression of another marker of differentiation namely osteopontin, which increased with the progression of PDL cell differentiation. Similar results were observed when using exogenous FGF-5 (Lallier and Spencer 2007).
In vitro studies showing an inhibitory effect of FGF-2 on PDL cell maturation seem to be contradictory to the *in vivo* trials demonstrating that topical application of exogenous FGF-2 enhanced periodontal regeneration in experimental bony defects (Murakami et al. 1999). Given the pro-mitogenic effect of FGF-2 opposing its inhibitory actions on differentiation, these data might be interpreted in terms of a role for FGF-2 in maintaining a pool of PDL cells in a multipotent, naive state that might later on be directed to differentiate by other factors in the local microenvironment. In fact, a high density of immature cells was

recognized histologically after the application of FGF-2 to bone defects in beagle dogs (Murakami et al. 1999).

Administrating FGF-2 to dermal fibroblasts led to a significant increase of their proliferation. The same was noticed in a study done by Root and Shipley (1991).
Dermal fibroblasts displayed a very low level of alkaline phosphatase activity that was hardly influenced by treatment with FGF-2. Similar results were shown by Whyte and co-workers (1987). The osteocalcin production of dermal fibroblasts was also minimal and was not affected by treatment with FGF-2 in our study. No information in the literature could be found on the expression of osteocalcin by dermal fibroblast and the effect of FGF-2 on this parameter.

In MG63 cells, FGF-2 induced an increase of proliferative activity. This was also reported in other studies (Kim et al. 2007, Takechi 2008). Alkaline phosphatase specific activity increased significantly. A similar effect was reported in another study in which the addition of FGF-8 to mouse bone marrow cultures effectively increased initial cell proliferation as well as subsequent osteoblast-specific alkaline phosphatase production (Valta et al. 2006)

BMP-7 suppressed the proliferation but induced PDL cells to assume a more differentiated phenotype in our experiments. This effect was more pronounced in fourth passage cells. Lallier and Spencer (2007) also reported an enhanced alkaline phosphatase activity following BMP-7 exposure of PDL cells. Besides BMP-7, other BMPs were shown to promote osteoblastic differentiation of human PDL cells while inhibiting their proliferation (Markopoulou 2003). They also induced an increase in the expression of osteopontin, alkaline phosphatase (Xu et al. 2004), and osteocalcin (Yamada et al. 2006) committing them to differentiate towards the osteo-cementoblastic phenotype (Kobayashi et al. 1999, Zhao et al. 2002).

Adding BMP-7 to dermal fibroblasts significantly suppressed their proliferation in contrast to another study in which the role of BMP-2 in normal skin development and fetal wound

healing in fetal mammals was investigated (Stelnicki 1998). In that study, BMP-2 induced massive dermal and epidermal growth when compared with controls. This difference could have resulted from the use of BMP-2 in fetal mammals as it was the case in the study of Stelnicki. Support for the physiological relevance of the in vitro findings was provided by the demonstration of an involvement of BMP-7 with the stimulation of periodontal regeneration in animal models (Taba et al. 2005).

In conclusion, according to the present investigation, PDL cells displayed greater similarity to osteoblasts than to dermal fibroblasts, possibly reflecting their common origin (the neural crest). Both FGF-2 and BMP-7 demonstrate their potential in periodontal regeneration by a different mode of action on PDL cells with FGF-2 maintaining a pool of multipotent cells that might be committed to osteoblastic differentiation by BMP-7 and other local factors.

In summary the present study adds to a better characterization of the PDL cell phenotype and points out the critical role of cell passage in this respect. The data provide a theoretical basis which is crucial for the development of promising strategies to influence the differentiation process of PDL cells and aid periodontal repair processes following periodontal disease or orthodontic tooth movement.

Summary

Although periodontal ligament cells (PDL) 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 which potentially can be directed towards a fibroblastic or osteoblastic phenotype. To further explore the PDL cells' characteristics, first and fourth passage human PDL cells were used. Cell number was determined using a cell counter. Alkaline-phosphatase-specific activity was measured in lysates of isolated cells and the levels of osteocalcin in the conditioned media were assayed with the use of a commercially available enzyme-linked immunoassay kit. Furthermore, the cellular response to FGF-2 and BMP-7 was examined in first and fourth passage cells. Dermal fibroblasts and osteoblast-like cells (MG63) served as reference cell lines.

Proliferation decreased over time and was highest in first passage cells. The expression of differentiation parameters increased with culture time and was higher in first passage cells than in the fourth one. FGF-2 administration enhanced cell numbers in both passages whereas ALP and osteocalcin production remained unchanged. In contrast, BMP-7 exposure of PDL cells resulted in a reduction of cell number in both passages while ALP and osteocalcin production were enhanced. In dermal fibroblasts, differentiation parameters did not respond to both stimuli. MG63 cells behaved similarly to PDL cells. These results indicate that subcultivation affects the phenotypic expression of human PDL cells with respect to characteristics that these cells share with osteoblasts. Furthermore, the PDL cell phenotype can be altered by fibroblastic and osteoblastic growth factors.

Literaturverzeichnis

Basdra EK, Komposch G. Osteoblast-like properties of human periodontal ligament cells: an in vitro analysis. Eur J Orthod 1997; 19: 615-621

Bretaudiere JP, Spillman T. Alkaline phosphatases. In: Bergmeyer HU (ed) Methods of Enzymatic Analysis. Weinheim, Germany: Verlag Chemica, 1984: 75-92.

Chen FP, Lee N, Wang KC, Soong YK, Huang KE. Effect of estrogen and 1alpha,25(OH)2-vitamin D3 on the activity and growth of human primary osteoblast-like cells in vitro. Fertil Steril 2002; 77: 1038-1043

Coulier F, Pontarotti P, Roubin R, Hartung H, Goldfarb M, Birnbaum D. Of worms and men: an evolutionary perspective on the fibroblast growth factor (FGF) and FGF receptor families. J Mol Evol 1997; 44: 43-56

Delmas PD. Biochemical markers of bone turnover. Acta Orthop Scand Suppl 1995; 266: 176-182

Franceschi RT, Wang D, Krebsbach PH, Rutherford RB. Gene therapy for bone formation: in vitro and in vivo osteogenic activity of an adenovirus expressing BMP7. J Cell Biochem 2000; 78: 476-486

Hankemeier S, Keus M, Zeichen J, Jagodzinski M, Barkhausen T, Bosch U. Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engineering of tendons and ligaments. Tissue Eng 2005; 11: 41-49

Hou LT, Li TI, Liu CM, Liu BY, Liu CL, Mi HW. Modulation of osteogenic potential by recombinant human bone morphogenic protein-2 in human periodontal ligament cells: effect of serum, culture medium, and osteoinductive medium. J Periodontal Res 2007; 42: 244-252

Huang CY, Reuben PM, D'Ippolito G, Schiller PC, Cheung HS. Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. Anat Rec A Discov Mol Cell Evol Biol 2004; 278: 428-436

Jackson RA, Nurcombe V, Cool SM. Coordinated fibroblast growth factor and heparan sulfate regulation of osteogenesis. Gene 2006; 379: 79-91

Kim JB, Leucht P, Luppen CA, Park YJ, Beggs HE, Damsky CH. Reconciling the roles of FAK in osteoblast differentiation, osteoclast remodeling, and bone regeneration. Bone 2007; 41: 39-51

Kobayashi M, Takiguchi T, Suzuki R, Yamaguchi A, Deguchi K, Shionome M. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic differentiation in cells isolated from human periodontal ligament. J Dent Res 1999; 78: 1624-1633

Krebsbach PH, Gu K, Franceschi RT, Rutherford RB. Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone in vivo. Hum Gene Ther 2000; 11: 1201-1210

Lallier TE, Spencer A. Use of microarrays to find novel regulators of periodontal ligament fibroblast differentiation. Cell Tissue Res 2007; 327: 93-109

Lekic P, Rojas J, Birek C, Tenenbaum H, McCulloch CA. Phenotypic comparison of periodontal ligament cells in vivo and in vitro. J Periodontal Res 2001; 36: 71-79

Ling JQ, Li JP. Insulin-like growth factor-II and basic fibroblast growth factor affect periodontal ligament cells expressing osteoprotegerin in vitro. Hua Xi Kou Qiang Yi Xue Za Zhi 2004; 22: 366-369

Lossdorfer S, Gotz W, Jager A. PTH(1-34) affects osteoprotegerin production in human PDL cells in vitro. J Dent Res 2005; 84: 634-638

Lossdorfer S, Gotz W, Jager A. Parathyroid hormone modifies human periodontal ligament cell proliferation and survival in vitro. J Periodontal Res 2006a; 41: 519-526

Lossdorfer S, Gotz W, Rath-Deschner B, Jager A. Parathyroid hormone(1-34) mediates proliferative and apoptotic signaling in human periodontal ligament cells in vitro via protein kinase C-dependent and protein kinase A-dependent pathways. Cell Tissue Res 2006b; 325: 469-479

Lossdorfer S, Stier S, Gotz W, Jager A. Maturation-state dependent response of human periodontal ligament cells to an intermittent parathyroid hormone exposure in vitro. J Periodontal Res 2006c; 41: 62-72

Lossdorfer S, Fiekens D, Salik MI, Gotz W, Jager A. 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. J Periodontal Res 2008; 43: 563-569

Markopoulou CE, Vrotsos IA, Vavouraki HN, Dereka XE, Mantzavinos ZS. Human periodontal ligament cell responses to recombinant human bone morphogenetic protein-2 with and without bone allografts. J Periodontol 2003; 74: 982-989

Murakami S, Takayama S, Ikezawa K, Shimabukuro Y, Kitamura M, Nozaki T. Regeneration of periodontal tissues by basic fibroblast growth factor. J Periodontal Res 1999; 34: 425-430

Nakashima M, Reddi AH. The application of bone morphogenetic proteins to dental tissue engineering. Nat Biotechnol 2003; 21: 1025-1032

Okamoto T, Yatsuzuka N, Tanaka Y, Kan M, Yamanaka T, Sakamoto A. Growth and differentiation of periodontal ligament-derived cells in serum-free defined culture. In Vitro Cell Dev Biol Anim 1997; 33: 302-309

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 1990; 143: 420-430

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

Ripamonti U, Reddi AH. Tissue engineering, morphogenesis, and regeneration of the periodontal tissues by bone morphogenetic proteins. Crit Rev Oral Biol Med 1997; 8: 154-163

Root LL, Shipley GD. Human dermal fibroblasts express multiple bFGF and aFGF proteins. In Vitro Cell Dev Biol 1991; 27A: 815-822

Sabokbar A, Millett PJ, Myer B, Rushton N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. Bone Miner 1994; 27: 57-67

Sawa Y, Yamaoka Y, Kuroshima S, Yoshida S. Reduction of alkaline phosphatase activity in aged human osteogenic periodontal ligament fibroblasts exhibiting short telomeres. Cell Tissue Res 2004; 315: 331-337

Stelnicki EJ, Longaker MT, Holmes D, Vanderwall K, Harrison MR, Largman C. Bone morphogenetic protein-2 induces scar formation and skin maturation in the second trimester fetus. Plast Reconstr Surg 1998; 101: 12-19

Taba M, Jr., Jin Q, Sugai JV, Giannobile WV. Current concepts in periodontal bioengineering. Orthod Craniofac Res 2005; 8: 292-302

Takayama S, Murakami S, Miki Y, Ikezawa K, Tasaka S, Terashima A. Effects of basic fibroblast growth factor on human periodontal ligament cells. J Periodontal Res 1997; 32: 667-675

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

Takechi M, Tatehara S, Satomura K, Fujisawa K, Nagayama M. Effect of FGF-2 and melatonin on implant bone healing: a histomorphometric study. J Mater Sci Mater Med 2008; 19: 2949-2952

Terashima Y, Shimabukuro Y, Terashima H, Ozasa M, Terakura M, Ikezawa K. Fibroblast growth factor-2 regulates expression of osteopontin in periodontal ligament cells. J Cell Physiol 2008; 216: 640-650

Thomas GP, Baker SU, Eisman JA, Gardiner EM. Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts. J Endocrinol 2001; 170: 451-460

Valta MP, Hentunen T, Qu Q, Valve EM, Harjula A, Seppanen JA. Regulation of osteoblast differentiation: a novel function for fibroblast growth factor 8. Endocrinology 2006; 147: 2171-2182

Waddington RJ, Embery G. Proteoglycans and orthodontic tooth movement. J Orthod 2001; 28: 281-290

Wang IE, Shan J, Choi R, Oh S, Kepler CK, Chen FH. Role of osteoblast-fibroblast interactions in the formation of the ligament-to-bone interface. J Orthop Res 2007; 25: 1609-1620

Wescott DC, Pinkerton MN, Gaffey BJ, Beggs KT, Milne TJ, Meikle MC. Osteogenic gene expression by human periodontal ligament cells under cyclic tension. J Dent Res 2007; 86: 1212-1216

Whyte MP, Rettinger SD, Vrabel LA. Infantile hypophosphatasia: enzymatic defect explored with alkaline phosphatase-deficient skin fibroblasts in culture. Calcif Tissue Int 1987; 40: 244-252

Xu WP, Shiba H, Mizuno N, Uchida Y, Mouri Y, Kawaguchi H. 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

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

Zhang SJ, Liang YH, Yang PS. Effects of basic fibroblast growth factor on growth of human periodontal ligament cells. Shanghai Kou Qiang Yi Xue 2001; 10: 135-137

Zhao M, Xiao G, Berry JE, Franceschi RT, Reddi A, Somerman MJ. Bone morphogenetic protein 2 induces dental follicle cells to differentiate toward a cementoblast/osteoblast phenotype. J Bone Miner Res 2002; 17: 1441-1451

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