Published in final edited form as:

J Periodontol. 2013 March ; 84(3): 389–395. doi:10.1902/jop.2012.120004.

Stimulation of Periodontal Ligament Stem Cells by Dentin Matrix Protein 1 Activates Mitogen-Activated Protein Kinase and Osteoblast Differentiation

Sangeetha Chandrasekaran*, Amsaveni Ramachandran†, Asha Eapen†, and Anne George†

*Department of Periodontology, University of Illinois at Chicago, Chicago, IL

†Brodie Tooth Development Genetics and Regenerative Medicine Research Laboratory, University of Illinois at Chicago

Abstract

Background—Periodontitis can ultimately result in tooth loss. Many natural and synthetic materials have been tried to achieve periodontal regeneration, but the results remain variable and unpredictable. We hypothesized that exogenous treatment with dentin matrix protein 1 (DMP1) activates specific genes and results in phenotypic and functional changes in human periodontal ligament stem cells (hPDLSCs).

Methods—hPDLSCs were isolated from extracted teeth and cultured in the presence or absence of DMP1. Quantitative polymerase chain reactions were performed to analyze the expression of several genes involved in periodontal regeneration. hPDLSCs were also processed for immunocytochemical and Western blot analysis using phosphorylated extracellular signal-regulated kinase (pERK) and ERK antibodies. Alkaline phosphatase and von Kossa staining were performed to characterize the differentiation of hPDLSCs into osteoblasts. Field emission scanning electron microscopic analysis of the treated and control cell cultures were also performed.

Results—Treatment with DMP1 resulted in the upregulation of genes, such as matrix metalloproteinase-2, alkaline phosphatase, and transforming growth factor β 1. Activation of ERK mitogen-activated protein kinase signaling pathway and translocation of pERK from the cytoplasm to the nucleus was observed. Overall, DMP1-treated cells showed increased expression of alkaline phosphatase, increased matrix, and mineralized nodule formation when compared with untreated controls.

Conclusion—DMP1 can orchestrate a coordinated expression of genes and phenotypic changes in hPDLSCs by activation of the ERK signaling pathway, which may provide a valuable strategy for tissue engineering approaches in periodontal regeneration.

Keywords

Bone; bone regeneration; guided tissue regeneration; periodontal; mitogen-activated protein kinases

---

Periodontitis and the resultant inflammatory process affect the integrity of the attachment to teeth and can ultimately result in tooth loss. A major challenge in periodontology has been

---

Correspondence: Dr. Anne George, Brodie Tooth Development Genetics and Regenerative Medicine Research Laboratory, University of Illinois at Chicago, Chicago, IL 60612. Fax: 312/996-6044; anneg@uic.edu..

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

to develop strategies to predictably regenerate periodontal tissues that are lost as a result of the inflammatory process.

The periodontal ligament (PDL) has stem cells that have the ability to regenerate lost periodontal tissues.¹ During PDL formation, the inner layer of the dental follicle cells penetrate disintegrated Hertwig epithelial root sheath and contact with root dentin to form periodontium.² Several root conditioning agents, such as EDTA, are still being used to expose dentin proteins to positively influence the wound healing in the periodontium.³ A previous study⁴ has demonstrated that dentin proteins might function as regulatory signals for various mesenchymal and inflammatory cells to enhance healing potential of periodontal tissues. One such non-collagenous protein present in the dentin is dentin matrix protein 1 (DMP1). DMP1 has been shown to have pivotal effects in osteogenesis, odontogenesis, and phosphate homeostasis.^{5–7}

This study looked at the biologic effects of DMP1 on human periodontal ligament stem cells (hPDLSCs) in vitro and assessed the advantages of using DMP1 as a biologic mediator to enhance the regenerative potential of the periodontium.

To the best of our knowledge, this is the first study looking into the effect of a dentin matrix protein, namely DMP1, on periodontal tissues.

MATERIALS AND METHODS

Expression and Purification of DMP1

The recombinant DMP1 (rDMP1) protein was expressed in *Escherichia coli* as described previously.⁸

Cell Culture

Briefly, PDL was isolated from extracted teeth and pooled as single-cell suspensions. Stem cells were identified by single colony selection and magnetic activated cell sorting as published previously.¹ hPDLSCs were cultured in a minimum essential medium supplemented with 10% fetal bovine serum, 1% penicillin—streptomycin, 10 mM L-ascorbic acid, and 2 mM glutamine. Cells were seeded in six-well plates, grown to 70% confluency, and then treated with rDMP1 (250 ng/mL) for 24 hours. Cells only up to passage 4 were used for all experiments.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total ribonucleic acid was isolated after 24 hours of rDMP1 treatment using the protocol of the manufacturer.[‡] Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously.⁹ Expression of bone alkaline phosphatase (ALP), matrix metalloproteinase 2 (MMP2), transforming growth factor β 1 (TGF β 1), and GAPDH transcripts was analyzed during its linear phase. The primer sequences for the above genes are listed in Table 1. Experiments were performed in triplicate.

Immunofluorescence

hPDLSCs were seeded on glass coverslips grown overnight at 37 °C. The following day, the media were changed to serum-free media. The test plate was treated for 45 minutes with 250 ng/mL rDMP1. Untreated cells were used as controls. The cells were then fixed with 4% paraformaldehyde for 2 hours. Fixed cells were rinsed thrice with 1× phosphate-buffered

[‡]Trizol reagent, Invitrogen, Carlsbad, CA.

saline (PBS) and permeabilized with a 0.05% non-ionic surfactant.[§] The cells were washed, and 5% bovine serum albumin was used for blocking for 1.5 hours. This was followed by incubation with primary antibodies for phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2)^{||} at concentrations of 1:100 overnight at 4 °C. The cells were subsequently washed three times with 1× PBS and incubated with fluorescein-conjugated goat antirabbit immunoglobulin G (IgG)[¶] at 1:100 concentration, and nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). The images were examined using confocal microscopy. Experiments were performed in triplicate.

Western Blot Analyses

Total protein was extracted from hPDLSCs using reagent.[#] A total of 20 µg protein was resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. The proteins were transferred overnight at 4 °C onto a nitrocellulose membrane at 70 V in a trans-blot electrophoretic transfer cell. After the transfer, the membranes were incubated with blocking buffer containing 5% non-fat dry milk for 5 hours. Membranes were then incubated with primary antibodies against the proteins under investigation (pERK1/2 at 1:1,000 dilution; ERK1/2 at 1:1,000 dilution; tubulin at 1:1,000 dilution) overnight at room temperature and then washed with 1× PBS with a 0.1% non-ionic surfactant^{**} three times for 10 minutes each. The secondary antibodies antirabbit IgG for pERK and ERK and antimouse IgG for tubulin were used, and the membranes were incubated for 5 hours. After washing three times with 1× PBS, the blot was developed using ALP conjugate substrate kit.

ALP Staining

hPDLSCs were plated in 100-mm plates. Cells were grown to 70% confluency and then treated with rDMP1 for 24 hours, and untreated cells served as controls. The media were changed once every 2 days for a period of 27 days. The cells were then washed with 3× PBS and fixed with cold methanol for 15 minutes as per the instructions of the manufacturer.^{††} The stained cultures were analyzed for ALP-positive area using a digitized image analysis system.^{‡‡} Experiments were performed in triplicate.

von Kossa Staining

hPDLSCs were plated in 100-mm plates. Cells were grown to 70% confluency and then treated with rDMP1 for 24 hours, and untreated cells served as controls. The media were changed once every 2 days for a period of 27 days. The cells were fixed in methanol, washed twice with PBS, and subsequently stained with 5% silver nitrate solution. Experiments were performed in triplicate.

Field Emission Scanning Electron Microscopy

hPDLSCs were plated in 100-mm plates. Cells were grown under mineralization conditions to 70% confluency and then treated with rDMP1 for 24 hours, and untreated cells served as controls. The media were changed once every 2 days for a period of 27 days. They were then washed twice with PBS and fixed in 1.5% glutaraldehyde in 0.15 M sodium cacodylate buffer for 24 hours at 4 °C. The samples were dehydrated by passing through a series of graded ethanol solutions for 10 minutes each. The samples were finally dehydrated by

[§]Triton X-100, Fisher Scientific, Hanover Park, IL.

^{||}pERK1/2 antibody, Santa Cruz Biotechnology, Santa Cruz, CA.

[¶]Goat antirabbit IgG, Sigma-Aldrich, St. Louis, MO.

[#]M-PER Mammalian Protein Extraction Reagent, Pierce, Rockford, IL.

^{**}Tween 20, Fisher Scientific.

^{††}Alkaline phosphatase assay, Bio-Rad, Hercules, CA.

^{‡‡}NIH ImageJ, National Institutes of Health, Bethesda, MD.

immersing them in a solution of hexamethyldisilazane for 10 minutes, followed by air drying. Specimens were sealed with paraffin to avoid dust accumulation. The surface was sputter coated with a thick layer of platinum/palladium.^{§§} The coated samples were examined with a field emission scanning electron microscope at an acceleration voltage of 20 kV using the in-lens secondary electron detector.

RESULTS

Genes Regulated by rDMP1 Stimulation in hPDLSCs

RT-PCR results showed an upregulation of MMP2, ALP, and TGF β 1 in rDMP1-treated cells compared with untreated controls. RT-PCR results also showed downregulation of Twist1 in rDMP1-treated cells. They were found to be statistically significant as assessed by *t* test and *P* value <0.05 (Fig. 1).

Nuclear Translocation of pERK in rDMP1-Treated hPDLSCs

Immunofluorescence analysis of control hPDLSCs reveals predominant localization of pERK in the cytoplasm (Figs. 2A through 2C). However, rDMP1 treatment results in the translocation of pERK into the nucleus (Figs. 2D through 2G).

Increased Phosphorylation of ERK in rDMP1-Treated hPDLSCs

Proteins from untreated controls and DMP1-treated cells were subjected to Western blot analysis and probed for pERK. DMP1-treated samples showed an increased amount of pERK corresponding to two bands on the Western blot of 42 and 44 kDa (Fig. 3). Samples were also probed for total ERK.

Increased ALP Staining and Mineralized Nodule Formation in rDMP1-Treated hPDLSCs

rDMP1-treated cells showed increased ALP staining compared with untreated controls (Fig. 4). Also, increased extracellular matrix (ECM) calcification as depicted by phosphate deposits were seen in rDMP1-treated cells compared with controls, as confirmed with von Kossa staining (Fig. 5).

Abundant and Organized Matrix Formation With rDMP1 Treatment

Morphologic analysis with field emission scanning electron microscopy (FE-SEM) demonstrated that hPDLSCs with rDMP1 treatment grown for 27 days produced an abundant, organized ECM compared with control hPDLSCs with a very diffuse and un-organized matrix (Fig. 6).

DISCUSSION

It is estimated that $\approx$ 50% of the 45- to 65-year-old adult population has bone loss around the teeth as a result of periodontal disease.¹⁰ Periodontal regeneration is a complex process involving reformation of hard and soft tissues around a periodontally affected tooth. Outcomes from therapeutic approaches attempting to regenerate the periodontium have been variable and inconsistent. Regeneration is primarily dependent on recruitment of progenitor cells that can differentiate into specialized cells, followed by their proliferation and synthesis of the specialized components of periodontium.¹¹

DMP1 is a member of a family of non-collagenous proteins in dentin termed the SIBLINGs (small integrin-binding ligand, *N*-linked) glycoprotein family.¹² DMP1 has a large number

^{§§}208HR high-resolution sputter coater, Cressington Scientific Instruments, Watford, U.K.

of acidic domains, suggesting a regulatory role in mineralized tissue formation.⁵ Potential responsive elements for transcriptional factors essential for bone- and tooth-specific regulation, such as AP1, JunB, Runx2, Msx1/2, Tef/Lef, C/EBP, and YY1, are present in the DMP1 promoter sequences.¹³ DMP1 also promotes cell attachment through RGD motif in a cell- and tissue-specific manner.¹⁴ When DMP1 is added to exposed dental pulp, it acts as a morphogen and promoter of differentiation of undifferentiated ectomesenchymal cells in pulp toward odontoblastic lineage.⁶ Deletion of DMP1 gene leads to defects in odontogenesis and mineralization.⁷ The phenotype includes a partial failure of maturation of predentin–dentin, enlarged pulp chambers, and increased width of predentin zone with a reduced dentin wall, hypomineralization, a three-fold reduction in dentin appositional rate, and abnormalities in the dentinal tubule system. The DMP1 null mice also have hypophosphatemia, a novel role for DMP1 in regulation of phosphate homeostasis.¹⁵ Phosphate homeostasis has also been shown to be important for cementoblast function, such as matrix formation and mineralization.

The effects of DMP1 on PDLSCs are examined in vitro in the present study. Gene expression analysis revealed that rDMP1 treatment of hPDLSCs resulted in upregulation of MMP2. MMPs are a family of zinc-dependent endopeptidases responsible for the turnover of matrix components and are activated in inflammation, neoplasms, wound healing, angiogenesis, and matrix remodeling.¹⁶ Turnover of the ECM consisting of synthesis, degradation, and replacement is a fundamental process of homeostasis. MMP2 may accordingly be a major enzyme involved in remodeling of the ECM in periodontal tissue.

ALP (orthophosphoric-monoester phosphohydrolase) is a calcium- and phosphate-binding protein. This enzyme is abundant in developing teeth and periodontal tissue.¹⁷ ALP activity is important for bone formation and as a phenotypic marker of osteoblast cells.¹⁸ A positive correlation has been reported between ALP activity and thickness of cementum.¹⁹ In fact, in patients suffering from hypophosphatasia, it has been observed that cementum thickness is affected based on the severity of disease.²⁰ In mice lacking ALP activity, there was a severe inhibition in the formation of the acellular cementum, and as a result the PDL was not firmly anchored by Sharpey fibers.²¹ ALP is also upregulated in regenerated human periodontal cells obtained from membranes and the regenerated tissue underlying the membrane.²² Increased ALP expression in hPDLSCs after rDMP1 treatment observed in this study may therefore be significant for both alveolar bone and cementum formation.

Gene expression analysis revealed that rDMP1 treatment of hPDLSCs resulted in upregulation of TGFβ1. TGFβ1 is a polypeptide growth factor important in repair and regeneration of tissues.²³ Studies have demonstrated that TGFβ1 is a potent inhibitor of epithelial cell proliferation but enhances the proliferation of mesenchymal cells, such as fibroblasts and osteoblasts.^{24,25} TGFβ1 accelerates all stages of wound healing by stimulating granulation tissue formation, angiogenesis, and collagen production.^{26,27}

Twist is a basic helix-loop-helix protein that has been demonstrated to play an important role in cell-type determination, differentiation, and early osteogenesis.²⁸ Twist inhibited osteoblastic differentiation by inhibiting Runx2 function.²⁹ It was demonstrated that decreased levels of Twist shifts PDL cells to preosteoblasts, thereby acting as a negative regulator of osteogenesis.³⁰ We observed similar decreased expression of Twist in hPDLSCs treated with rDMP1.

Our results also demonstrated an increased amount of pERK in rDMP1-treated cells compared with untreated controls. ERK1/2 is the prototypical mitogen-activated protein (MAP) kinase pathway that is activated by mitogenic differentiation stimuli and cytokines. Activation of the ERK pathway is the key signal in integrating environmental stimuli into

specific cell responses. Binding of ligands to their specific cell-surface receptors activates guanosine triphosphatase Ras, which recruits the MAP kinase kinase Raf to the membrane for activation by phosphorylation. Activated Raf isoforms phosphorylate MAP kinase kinase1/2, which in turn phosphorylates and activates effector MAP kinase ERK1/2. Activated ERK1/2 phosphorylates a vast array of substrates located in all cellular compartments.^{31,32} Sustained activation of ERK1/2 is necessary for cell proliferation.^{33,34} The ERK pathway, in addition to upregulating proliferation-associated genes, has also been shown to lead to downregulation of antiproliferative genes.³⁵

In our study, we demonstrate that rDMP1 activated ERK and translocated it to the nucleus. This is crucial for activating cell response because cytosolic retention of ERK1/2 has been shown to inhibit survival and proliferative genes in the nucleus. ERK1/2 translocation to the nucleus is essential for G₁-to-S phase progression.³⁶ The rapid and persistent nuclear transfer of ERK1/2 is crucial for mediating the growth response.³⁷ During translocation to the nucleus, activated ERK1/2 phosphorylates the ternary complex factors Elk-1, Sap-1a, and TIF-1A and thereby regulates cellular activities, such as gene expression, mitosis, embryogenesis, cell differentiation, movement, metabolism, and apoptosis.

In this study, we also demonstrate that rDMP1-treated hPDLSCs showed increased ALP staining and greater amount of mineralized deposits compared with untreated controls. The FE-SEM pictures of rDMP1-treated cells demonstrated abundant, organized ECM.

CONCLUSIONS

DMP1 can orchestrate expression of genes and phenotypic changes in hPDLSCs by activation of ERK signaling pathway, which may provide a valuable strategy for tissue engineering approaches in periodontal regeneration. The use of periodontal stem cells is very attractive because we exploit the natural healing capacity of the periodontium for regeneration. This potential can further be enhanced using rDMP1. In this study, early makers of osteoblast differentiation are analyzed. Future studies must be performed to analyze gene expression that occurs later in the osteoblast differentiation cascade. Future animal and human studies must also evaluate whether rDMP1 will alone be sufficient to attract hPDLSCs to the periodontal defect site. Delivery methods, wound management, outcomes of treatment compared with conventional treatments, and maintenance of regenerated tissues must all be addressed.

Acknowledgments

This study was supported by National Institutes of Health Grant DE 11657. hPDLSCs were a kind gift from Dr. Songtao Shi (Associate Professor, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA).

REFERENCES

1. Mrozik K, Gronthos S, Shi S, Bartold PM. A method to isolate, purify, and characterize human periodontal ligament stem cells. *Methods Mol Biol.* 2010; 666:269–284. [PubMed: 20717790]
2. Berkovitz, BKB.; Holland, GR.; Moxham, BJ. *A Colour Atlas and Textbook of Oral Anatomy, Histology, and Embryology.* Wolfe; London: 1992.
3. Egelberg J. Regeneration and repair of periodontal tissues. *J Periodontal Res.* 1987; 22:233–242. [PubMed: 2955107]
4. Ma Z, Li S, Song Y, et al. The biological effect of dentin noncollagenous proteins (DNCPs) on the human periodontal ligament stem cells (HPDLSCs) in vitro and in vivo. *Tissue Eng Part A.* 2008; 14:2059–2068. [PubMed: 18939934]

5. Narayanan K, Ramachandran A, Hao J, George A. Transcriptional regulation of dentin matrix protein 1 (DMP1) by AP-1 (c-fos/c-jun) factors. *Connect Tissue Res.* 2002; 43:365–371. [PubMed: 12489182]
6. Narayanan K, Ramachandran A, Hao J, et al. Dual functional roles of dentin matrix protein 1. Implications in biomineralization and gene transcription by activation of intracellular Ca^{2+} store. *J Biol Chem.* 2003; 278:17500–17508. [PubMed: 12615915]
7. Ye L, MacDougall M, Zhang S, et al. Deletion of dentin matrix protein-1 leads to a partial failure of maturation of predentin into dentin, hypomineralization, and expanded cavities of pulp and root canal during postnatal tooth development. *J Biol Chem.* 2004; 279:19141–19148. [PubMed: 14966118]
8. Srinivasan R, Chen B, Gorski JP, George A. Recombinant expression and characterization of dentin matrix protein 1. *Connect Tissue Res.* 1999; 40:251–258. [PubMed: 10757113]
9. Eapen A, Sundivakkam P, Song Y, et al. Calcium-mediated stress kinase activation by DMP1 promotes osteoblast differentiation. *J Biol Chem.* 2010; 285:36339–36351. [PubMed: 20841352]
10. Oliver RC, Brown LJ, Le H. Periodontal diseases in the United States population. *J Periodontol.* 1998; 69:269–278. [PubMed: 9526927]
11. Bartold PM, McCulloch CA, Narayanan AS, Pitaru S. Tissue engineering: A new paradigm for periodontal regeneration based on molecular and cell biology. *Periodontol 2000.* 2000; 24:253–269. [PubMed: 11276871]
12. George A, Sabsay B, Simonian PA, Veis A. Characterization of a novel dentin matrix acidic phosphoprotein. Implications for induction of biomineralization. *J Biol Chem.* 1993; 268:12624–12630. [PubMed: 8509401]
13. Qin C, Brunn JC, Cook RG, et al. Evidence for the proteolytic processing of dentin matrix protein 1. Identification and characterization of processed fragments and cleavage sites. *J Biol Chem.* 2003; 278:34700–34708. [PubMed: 12813042]
14. Narayanan K, Gajjaraman S, Ramachandran A, Hao J, George A. Dentin matrix protein 1 regulates dentin sialo-phosphoprotein gene transcription during early odontoblast differentiation. *J Biol Chem.* 2006; 281:19064–19071. [PubMed: 16679514]
15. Ye L, Mishina Y, Chen D, et al. Dmp1-deficient mice display severe defects in cartilage formation responsible for a chondrodysplasia-like phenotype. *J Biol Chem.* 2005; 280:6197–6203. [PubMed: 15590631]
16. Stygar D, Wang H, Vladic YS, Ekman G, Eriksson H, Sahlin L. Increased level of matrix metalloproteinases 2 and 9 in the ripening process of the human cervix. *Biol Reprod.* 2002; 67:889–894. [PubMed: 12193399]
17. Groeneveld MC, Everts V, Beertsen W. A quantitative enzyme histochemical analysis of the distribution of alkaline phosphatase activity in the periodontal ligament of the rat incisor. *J Dent Res.* 1993; 72:1344–1350. [PubMed: 8360383]
18. Schmidt-Gayk H, Roth HJ, Becker S, Reichel H, Boneth HG, Knuth UA. Noninvasive parameters of bone metabolism. *Curr Opin Nephrol Hypertens.* 1995; 4:334–338. [PubMed: 7552099]
19. Groeneveld MC, Everts V, Beertsen W. Alkaline phosphatase activity in the periodontal ligament and gingiva of the rat molar: Its relation to cementum formation. *J Dent Res.* 1995; 74:1374–1381. [PubMed: 7560388]
20. Bruckner RJ, Rickles NH, Porter DR. Hypophosphatasia with premature shedding of teeth and aplasia of cementum. *Oral Surg Oral Med Oral Pathol.* 1962; 15:1351–1369. [PubMed: 14016153]
21. Beertsen W, VandenBos T, Everts V. Root development in mice lacking functional tissue non-specific alkaline phosphatase gene: Inhibition of acellular cementum formation. *J Dent Res.* 1999; 78:1221–1229. [PubMed: 10371245]
22. Kuru L, Griffiths GS, Petrie A, Olsen I. Alkaline phosphatase activity is upregulated in regenerating human periodontal cells. *J Periodontol Res.* 1999; 34:123–127. [PubMed: 10207841]
23. Terranova VP, Odziemiec C, Tweden KS, Spadone DP. Repopulation of dentin surfaces by periodontal ligament cells and endothelial cells. Effect of basic fibroblast growth factor. *J Periodontol.* 1989; 60:293–301. [PubMed: 2550605]

24. Graves DT, Cochran DL. Biologically active mediators: Platelet-derived growth factor, monocyte chemoattractant protein-1, and transforming growth factor-beta. *Curr Opin Dent.* 1991; 1:809–815. [PubMed: 1807487]
25. Royce LS, Baum BJ. Physiologic levels of salivary epidermal growth factor stimulate migration of an oral epithelial cell line. *Biochim Biophys Acta.* 1991; 1092:401–403. [PubMed: 2049404]
26. Lynch SE, Williams RC, Polson AM, et al. A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration. *J Clin Periodontol.* 1989; 16:545–548. [PubMed: 2778088]
27. Dennison DK, Vallone DR, Pinero GJ, Rittman B, Caffesse RG. Differential effect of TGF-beta 1 and PDGF on proliferation of periodontal ligament cells and gingival fibroblasts. *J Periodontol.* 1994; 65:641–648. [PubMed: 7608839]
28. Lee MS, Lowe GN, Strong DD, Wergedal JE, Glackin CA. TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. *J Cell Biochem.* 1999; 75:566–577. [PubMed: 10572240]
29. Bialek P, Kern B, Yang X, et al. A twist code determines the onset of osteoblast differentiation. *Dev Cell.* 2004; 6:423–435. [PubMed: 15030764]
30. Komaki M, Karakida T, Abe M, et al. Twist negatively regulates osteoblastic differentiation in human periodontal ligament cells. *J Cell Biochem.* 2007; 100:303–314. [PubMed: 16888803]
31. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res.* 1998; 74:49–139. [PubMed: 9561267]
32. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocr Rev.* 2001; 22:153–183. [PubMed: 11294822]
33. Nakamura KD, Martinez R, Weber MJ. Tyrosine phosphorylation of specific proteins after mitogen stimulation of chicken embryo fibroblasts. *Mol Cell Biol.* 1983; 3:380–390. [PubMed: 6601755]
34. Cooper JA, Sefton BM, Hunter T. Diverse mitogenic agents induce the phosphorylation of two related 42,000-dalton proteins on tyrosine in quiescent chick cells. *Mol Cell Biol.* 1984; 4:30–37. [PubMed: 6199661]
35. Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S, Nishida E. Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. *Curr Biol.* 2006; 16:1171–1182. [PubMed: 16782007]
36. Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J.* 1999; 18:664–674. [PubMed: 9927426]
37. Lenormand P, Sardet C, Pagès G, L'Allemain G, Brunet A, Pouyssegur J. Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J Cell Biol.* 1993; 122:1079–1088. [PubMed: 8394845]

Figure 1.

RT-PCR results showing effect of rDMPI on gene expression compared with control (untreated hPDLSCs). **A)** Upregulation of MMP2. **B)** Upregulation of ALP. **C)** Upregulation of TGFb1. **D)** Downregulation of Twist1.

*Results were found to be statistically significant, $P < 0.05$.

Figure 2.

DMP1 stimulates nuclear translocation of pERK. Confocal images showing nuclear and cytoplasmic staining of pERK in unstimulated (**A through C**) and rDMP1-stimulated hPDLSCs (**D through F**). The arrows in A point to very low nuclear pERK signal compared with the cytoplasm, whereas in the rDMP1-stimulated hPDLSCs, predominant nuclear localization of pERK is seen, as shown by the arrows in D. Scale bar = 10 μm. **G**) Proposed model showing nuclear translocation of pERK after DMP1 stimulation resulting in genotypic changes in hPDLSCs. P = phosphorylated.

Figure 3.

Increased expression of pERK with rDMP1 stimulation. Western blot analysis of control and rDMP1-treated hPDLSCs with pERK and ERK antibodies. Increased expression of pERK and similar expression of ERK are observed in rDMP1-treated hPDLSCs.

Figure 4. Increased ALP staining of hPDLSC cultures with rDMP1 treatment. Increased ALP staining seen in rDMP1-treated hPDLSCs (**B**) compared with untreated controls (**A**). N = 3. Scale bar = 50 μ m.

Figure 5.

Effect of rDMP1 on mineralized nodule formation. von Kossa staining shows that secreted calcified ECM (black deposits indicated by the arrows) are greater in rDMP1-treated hPDLSCs (**B**) compared with untreated controls (**A**). N = 3. Scale bar = 50 μm.

Figure 6.

Abundant and organized ECM with rDMP1 stimulation. hPDLSCs were grown on coverslips for 27 days, and the topography was analyzed using FE-SEM. Diffuse, amorphous matrix without any apparent organization is seen in control cells (A) compared with well-organized and homogeneous ECM (indicated by arrows) seen after rDMP1 stimulation of hPDLSCs (B). N = 3.

Table 1

Primer Sequences for RT-PCR

Sequence No.	Gene of Interest	Forward Primer	Reverse Primer
1	ALP	GGACCATTCCACGTCTTCAC	CCTTGTAGCCAGGCCCATG
2	MMP2	CGACCGCGACAAGAAGTA	GCACACCACATCTTCCGTCA
3	TGF β 1	GCCCTGGACACCAACTATTGC	GCTGCACTTGCAGGAGCGCAC
4	GAPDH	ACCACAGTCCATGCCATCACTG	GCCTGCTTCACCACCTTCTTGA
5	Twist	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG