Journal of Clinical Periodontology
Official Journal of The European Federation of Periodontology
Founded by the British, Dutch, French, German, Scandinavian, and Swiss Societies of Periodontology

J Clin Periodontol 2008; 35 (Suppl. 8): 87–105 doi: 10.1111/j.1600-051X.2008.01264.x

Biological mediators and periodontal regeneration: a review of enamel matrix proteins at the cellular and molecular levels

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Bosshardt DD. Biological mediators and periodontal regeneration: a review of enamel matrix proteins at the cellular and molecular levels. *J Clin Periodontol 2008; 35 (Suppl. 8): 87–105. doi: 10.1111/j.1600-051X.2008.01264.x*.

Abstract

Background: Despite a large body of clinical and histological data demonstrating beneficial effects of enamel matrix proteins (EMPs) for regenerative periodontal therapy, it is less clear how the available biological data can explain the mechanisms underlying the supportive effects of EMPs.

Objective: To analyse all available biological data of EMPs at the cellular and molecular levels that are relevant in the context of periodontal wound healing and tissue formation.

Methods: A stringent systematic approach was applied using the key words ''enamel matrix proteins'' OR ''enamel matrix derivative'' OR ''emdogain'' OR ''amelogenin''. The literature search was performed separately for epithelial cells, gingival fibroblasts, periodontal ligament cells, cementoblasts, osteogenic/chondrogenic/bone marrow cells, wound healing, and bacteria.

Results: A total of 103 papers met the inclusion criteria. EMPs affect many different cell types. Overall, the available data show that EMPs have effects on: (1) cell attachment, spreading, and chemotaxis; (2) cell proliferation and survival; (3) expression of transcription factors; (4) expression of growth factors, cytokines, extracellular matrix constituents, and other macromolecules; and (5) expression of molecules involved in the regulation of bone remodelling.

Conclusion: All together, the data analysis provides strong evidence for EMPs to support wound healing and new periodontal tissue formation.

Key words: cell biology; enamel matrix derivative; enamel matrix proteins; periodontal regeneration

Accepted for publication 20 May 2008.

Regeneration of the periodontium is a major goal in the treatment of teeth affected by periodontitis. The peculiarities, biological problems, and technical complications associated with periodontal wound healing and tissue regeneration have been reviewed extensively (Schroeder 1992, Pitaru et al. 1994, MacNeil & Somerman 1999, Wikesjö & Selvig 1999, Grzesik & Narayanan 2002, Wang et al. 2005). There are several techniques used alone or in combination considered to achieve periodontal regeneration, including root surface modification, bone grafts or substitutes, guided tissue regeneration, and biological mediators. The latter technique comprises (1) extracellular matrix proteins and cell attachment factors; (2) mediators of cell metabolism and activity; and (3) growth and differentiation factors.

Growth factors are molecules that regulate cell proliferation, cell activity, chemotaxis, and/or cell differentiation. Insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), epidermal growth factor (EGF), platelet-derived growth factors (PDGFs), vascular endothelial growth factor (VEGF), parathyroid hormone (PTH), transforming growth factor-β (TGF-β), and bone morphogenetic proteins (BMPs) are among the

Conflict of interest and source of funding statment

There is no conflict of interests.
This review was supported by the Clinical Research Foundation (CRF) for the Promotion of Oral Health, University of Berne, Switzerland.
The 6th European Workshop on Periodontology was supported by an unrestricted educational grant from Straumann AG.

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growth factors that have been tested in animal experiments. The efficacy of exogenous growth factors to regenerate the periodontium has been reviewed exhaustively both for clinical and for pre-clinical applications (Caffesse & Quinones 1993, Ripamonti & Reddi 1997, Cochran & Wozney 1999, King & Cochran 2002, Giannobile & Somerman 2003, Nakashima & Reddi 2003, Shimono et al. 2003, Dereka et al. 2006, Ripamonti & Renton 2006). The most promising growth factors appear to be the BMPs, members of the TGF-β superfamily. In particular, BMP-2 and BMP-7 (osteogenic protein 1; OP-1) have been widely used in animal experiments. Despite the fact that very heterogeneous pre-clinical studies were performed (i.e., different species, very different defect designs, different growth factor doses, single or combined use with other growth factors, different vehicles), most authors concluded that the evaluated growth factors achieved successful periodontal regeneration and it is just a matter of time until their therapeutic application. However, despite a long history of pre-clinical evaluation with promising results, the routine use of growth factors as therapeutic agents for periodontal regeneration is not reality yet. Why is this so? Even the most promising growth factors, the BMPs, are not yet approved for periodontal applications. In contrast, recombinant human BMP-2 (rhBMP-2) and rhBMP-7 are currently in clinical use in the orthopaedic field for problematic cases; non-union, open tibial fractures, and spinal fusions are the three conditions for which there is clinical approval in the United States and Europe. It has to be clearly understood that these BMPs are only used when all other treatment options have failed. Many circumstances must be considered and many problems are associated with growth factor-based periodontal regeneration, including:

(1) *Structural and functional complexity of the periodontium*

The fact that more than one tissue must be reconstructed, namely alveolar bone, periodontal ligament, root cementum, and gingiva, makes it much more difficult to find both the right combination and the doses of growth factors.

(2) *Why are high doses required in humans?*

Regarding BMPs, very high doses (much higher doses than in animal experiments) must be applied to be effective in humans. A decreasing responsiveness with increasing patient age may be one of the reasons for this. Furthermore, a high dose may be required to compensate partially for the rapid clearance of the BMPs.

(3) *What about the carrier and release system?*

To overcome the rapid clearance of growth factors, a carrier system must be found that stores and releases the growth factors over a longer period of time so that their resident time is prolonged. Although many carrier systems have been tested, none of them appears to be ideal. Furthermore, because probably more than one growth factor is needed for periodontal regeneration, different release kinetics may be desirable to adjust for the different growth rates of the periodontal tissues.

(4) *Is protein therapy the right approach at all?*

Because of these shortcomings, a major question is whether or not protein-based tissue regeneration is the right way to go at all. Advances in gene transfer technology provide an opportunity to deliver complementary DNAs that can encode growth factors. This strategy, which is currently tested under experimental conditions, achieves a sustained local presence of the growth/differentiation factor with minimal exposure of non-target sites. Whether or not gene transfer technology will find its way into routine treatment of periodontitis, which is not a life-threatening disease, is debatable.

(5) *What about the costs?*

While high developmental and therapeutic costs appear justified for severe skeletal conditions such as non-unions, open fractures, spinal fusion, and large bone defects for example in the mandible, the same cannot necessarily be said for relatively small and non-life-threatening periodontal defects where preventive and maintenance measures are still mandatory and therapeutic alternatives exist.

Thus, although there is a vast amount of data available on the functions of BMPs and other growth factors for embryonic development, tissue formation, and tissue repair, the translation of these findings into a clinical application with the aim to regenerate periodontal tissues appears to be very difficult. The opposite is true for another group of molecules, the enamel matrix proteins (EMPs). For more than 10 years, EMPs are in clinical use, nowadays in more than 50 countries on five continents, to treat intrabony periodontal defects, although the mechanism of action is still regarded as obscure. How is this possible? There are many clinical studies that have shown the beneficial effects of Emdogain for the treatment of periodontitis, and many reviews of clinical and histological studies document its beneficial effects (Kalpidis & Ruben 2002, Venezia et al. 2004, Esposito et al. 2005, Sculean et al. 2007). However, in the context of wound healing and periodontal regeneration, a pertinent question must be addressed: What functions other than traditionally associated with EMPs do these proteins have? Therefore, the aim of this review is to analyse these functions of EMPs at the cellular and molecular levels.

The Pre-Therapeutic Era of EMPs and Tooth Developmental Studies

Traditionally, EMPs are associated with amelogenesis. Ameloblasts synthesize and secrete a number of EMPs, including amelogenins, ameloblastin (also called amelin or sheathlin), amelotin, tuftelin, and enamelin (Bartlett et al. 2006, Margolis et al. 2006). Amelogenins self-aggregate into supramolecular aggregates, so-called nanospheres, and play a crucial role in regulating the initiation and growth of hydroxyapatite crystals during the formation of enamel. However, in the context of wound healing and tissue regeneration, it is important to know whether EMPs have functions that go beyond enamel biomineralization. EMPs are also considered to be involved in cell differentiation processes occurring during the epithelial–mesenchymal interactions of crown development (Bègue-Kirn et al. 1998, Fong et al. 1998, Nanci et al. 1998, MacDougall et al. 2000, Oida et al. 2002, Papagerakis et al. 2003, Le et al. 2007). Of particular interest are observations suggesting that specific amelogenin splice products may function as potential epithelial–mesenchymal signalling molecules during tooth development (Veis et al. 2000, Tompkins & Veis 2002, Veis 2003, Tompkins et al. 2005).

Far less is known about these cell–cell and cell–matrix interactions when the tooth root is developing. However, ultrastructural findings indicative of synthetic activity in the cells of the Hertwig's epithelial root sheath (HERS) (Owens 1978, 1980, Slavkin et al. 1988, Bosshardt & Nanci 2004) suggest that these cells secrete molecules into the

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extracellular milieu. A great debate started concerning the nature of these molecules. While some studies showed EMP expression on the developing root surface (Slavkin et al. 1989, Fong et al. 1996, Hammarström 1997, Thomas et al. 1997, Bosshardt & Nanci 1998, 2000, 2004, Fong & Hammarström 2000), others have claimed that HERS' cells produce cementum proteins (Bosshardt & Nanci 1997, 1998, 2000, Bosshardt et al. 1998) (for reviews, see Slavkin 1976, Bosshardt & Schroeder 1996, Bosshardt 2005). Based on the findings of the occasional expression of EMPs along the forming root, the idea developed that EMPs play a pivotal role in the differentiation of progenitor cells into cementoblasts that specifically produce acellular extrinsic fibre cementum (Hammarström 1997, Hammarström et al. 1997). It should be pointed out here that experimental data confirming a cause–effect relationship between EMPs and cementoblast differentiation and that EMPs specifically induce the formation of acellular extrinsic fibre cementum are lacking. This does, of course, not exclude the possibility of a causal relationship. That EMPs and cementogenesis are somehow linked is shown by studies in which an increased number of cementicles has been observed in amelogenin knockout mice (Hatakeyama et al. 2003). However, it has to be clearly understood that there is cementum formation, even if the amelogenin gene is knocked out. The effect of the gene deletion is related to the resorption process and not the cementum deposition (see later). However, of great interest in the context of hard tissue regeneration is that there is evidence that demineralized enamel (Urist 1971) and amelogenin combined with plaster of Paris, used as a carrier (Wang 1993), possess bone-inductive activity.

The Era of Therapeutic Use of EMPs

EMPs are available as a therapeutic agent under the brand name Emdogain® since 1997. Emdogain® consists of an enamel matrix derivative (EMD), water, and a carrier, propylene glycol alginate (PGA). Clinically, Emdogain® is used for periodontal regeneration of teeth affected by periodontitis, root coverage procedures, and tooth replantation. Experimentally, it has also been used for dentin repair, tooth movement, anticancer treatment evaluation, and skin wound healing. The most interesting findings regarding the effects of EMPs appeared in the literature after EMPs were launched for the dental market.

Search strategy: Because this review will not analyse clinical data, the criteria for a systematic review cannot be applied. However, in order to gather all available biological data relevant for periodontal wound healing and regeneration, a systematic approach was applied. Furthermore, an attempt was made to carry out a structured listing of the findings. The literature search was performed in Medline. Because Emdogain consists of EMD (a mixture of EMPs, mainly amelogenins), the following key words were used: ''enamel matrix proteins'' OR ''enamel matrix derivative'' OR ''emdogain''. Because in some studies specific amelogenin isoforms or recombinant forms of amelogenin were used, an additional search was undertaken using the key word ''amelogenin''. In view of summarizing the effects of EMPs on wound healing and periodontal regeneration, the Medline literature search was performed on the effects of EMPs on (see Table 1):

(1) Epithelial cells
(2) Gingival fibroblasts
(3) Periodontal ligament (PDL) fibroblasts
(4) Cementoblasts
(5) Osteogenic and bone marrow cells
(6) Wound healing
(7) Bacteria

Inclusion criteria

- Articles written in English
- In vitro and in vivo studies

Exclusion criteria

- Articles written in languages other than English
- Clinical or radiographic studies
- Periodontal regeneration studies with descriptive histology, histomorphometry, immunohistochemistry, or in situ hybridization
- In vivo and in vitro tooth developmental studies (with ameloblasts)

Presentation of data: In each section, there are subsections, where the data are listed in chronologic order. This may lengthen some parts of the text where data could be grouped. However, it was the explicit aim to avoid presentation of data in a condensed form in order to leave room for additional detailed information, such as dose- and time-dependency, and comments on the methodology used for each individual study. However, because of the

Table 1. Search strategy

Effects on Epithelial Cells
epithel*
AND
(enamel matrix proteins **OR** enamel matrix derivative **OR** emdogain)
epithel*
AND
amelogenin
Effects on Gingival Fibroblasts
gingiva*
AND
(enamel matrix proteins **OR** enamel matrix derivative **OR** emdogain)
gingiva*
AND
amelogenin
Effects on Periodontal Ligament Fibroblasts
(periodontal ligament **OR** dental follicle)
AND
(enamel matrix proteins **OR** enamel matrix derivative **OR** emdogain)
(periodontal ligament **OR** dental follicle)
AND
amelogenin
Effects on Cementoblasts
(cement*)
AND
(enamel matrix proteins **OR** enamel matrix derivative **OR** emdogain)
(cement*)
AND
amelogenin
Effects on Cells of the Osteoblast and Chondrocyte Lineages
(osteo* **OR** bone **OR** bone marrow **OR** chondro*)
AND
(enamel matrix proteins **OR** enamel matrix derivative **OR** emdogain)
(osteo* **OR** bone **OR** bone marrow **OR** chondro*)
AND
amelogenin
Effects on Wound Healing
(wound healing **OR** angiogenesis **OR** vascul*)
AND
(enamel matrix proteins **OR** enamel matrix derivative **OR** emdogain)
(wound healing **OR** angiogenesis **OR** vascul*)
AND
amelogenin
Effects on Bacteria
(bacteria **OR** microorganisms)
AND
(enamel matrix proteins **OR** enamel matrix derivative **OR** emdogain)
(bacteria **OR** microorganisms)
AND
amelogenin

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complexity of the subject (i.e., many combinations of the various forms of EMPs, EMD, many cell types, and many parameters studied) it was decided to include two tables that summarize the results (Tables 2 and 3). In addition, these tables may represent an opportunity for researchers to fill some gaps of knowledge by creating appropriate studies.

Effects of EMPs on epithelial cells

While the search strategy (enamel matrix proteins OR enamel matrix derivative OR emdogain) AND (epithel*) retrieved 57 papers, (amelogenin AND epithel*) retrieved 127 papers. From these 184 papers, 6 met the inclusion/exlusion criteria.

DNA synthesis, cell proliferation, and cell viability

Gestrelius et al. (1997) showed that when rat tongue epithelial cells were exposed to 100 μg EMD/ml culture medium, a slow cell proliferation was observed. However, this effect was not statistically significant when compared with the negative control (2% foetal bovine serum). Kawase et al. (2000) examined the effects of EMD on the proliferation of oral epithelial cells (SCC25, a carcinoma-derived cell line). Their results showed that EMD, in a dose-dependent manner, inhibited cell division and concomitantly arrested cell cycle at the G1 phase. However, no apoptosis was observed. The authors concluded that EMD acts as a cytostatic rather than a cytotoxic agent on epithelial cells. Lyngstadaas et al. (2001) showed that EMD has a growth-inhibitory effect on epithelial (HeLa) cells. Kawase et al. (2002) showed that EMD reduced, in a dose-dependent manner, DNA synthesis. The evidence suggests that the suppression of epithelial cell growth may be mediated by TGF-β. However, this principle may not be applicable to another special type of epithelial cells, the epithelial cell rests of Malassez (ERM). Rincon et al. (2005) showed that DNA synthesis by the ERM was significantly increased after EMD stimulation. The ERM represent a special group of cells, known to respond to inflammatory mediators by at least cell proliferation, and may be involved in periodontal regeneration.

Cell migration, attachment/adhesion, and spreading

Kawase et al. (2001) showed that 50 μg EMD/ml culture medium promoted adhesion of epithelial cells (SCC25) and stimulated cytoskeletal actin polymerization. Rincon et al. (2005) provided evidence of increased attachment to EMD-coated tissue culture wells, compared with the untreated (negative) controls.

Expression of growth factors, cytokines, and extracellular matrix proteins

Human epithelial cells (HeLa) growing in the presence of EMD showed a rapid and strong secretion of PDGF-AB when compared with controls (Lyngstadaas et al. 2001). The ERM showed a dose-dependent and significant increase in OPN mRNA band intensities following exposure to EMD (Rincon et al. 2005).

Levels of cyclic adenosine monophosphate (cAMP)

Human epithelial cells (HeLa) exposed to EMD exhibited highly increased intracellular levels of cAMP when compared with controls (Lyngstadaas et al. 2001). However, in another study (Kawase et al. 2001), EMD failed to stimulate cAMP production in a human epithelial cell line (SCC25).

Effects of EMPs on gingival fibroblasts

While the search strategy (enamel matrix proteins OR enamel matrix derivative OR emdogain) AND (gingiva*) retrieved 137 papers, (amelogenin AND gingiva*) retrieved 8 papers. From these 145 papers, 9 papers were found that met the inclusion/exclusion criteria. Two additional papers were identified, while reading the selected papers from other categories. Thus, a total of 11 papers are included.

DNA synthesis, cell proliferation, and cell viability

Attached human gingival fibroblasts, when exposed to EMD, showed a significantly higher incorporation of ^{3}H-thymidine than the corresponding human PDL fibroblasts (Van der Pauw et al. 2000). Kawase et al. (2000) showed that EMD substantially stimulated the proliferation of human gingival fibroblasts in a dose-dependent manner over 3 days. EMD dose-dependently stimulated DNA synthesis in human gingival fibroblastic cells (Kawase et al. 2002). Rincon et al. (2003) showed a significant increase in ^{3}H-thymidine incorporation into DNA of human gingival fibroblasts when compared with the controls (0.2% FCS). EMD increased dose-dependently the number of rat gingival fibroblasts up to two-fold when compared with negative controls (Keila et al. 2004). Porcine gingival fibroblasts revealed an increase in ^{3}H-thymidine incorporation into DNA after stimulation with EMD, whereas the effect on porcine PDL fibroblasts was more pronounced (Rincon et al. 2005). EMD increased both ^{3}H-thymidine incorporation into DNA and cell proliferation in primary human gingival fibroblasts, whereby the effects were dependent on the presence of serum growth factors (Zeldich et al. 2007a). In another study, Zeldich et al. (2007b) showed that EMD protects human gingival fibroblasts from tumour necrosis factor (TNF)-induced apoptosis.

Cell migration, attachment/adhesion, and spreading

Compared with human PDL fibroblasts, human gingival fibroblasts attached and spread much less and slower (Van der Pauw et al. 2000). In another study by the same group, the authors concluded that integrins are involved in the interaction of gingival fibroblasts with EMD (Van der Pauw et al. 2002). EMD produced a significant increase in cell attachment of porcine gingival fibroblasts (Rincon et al. 2005). Cell attachment was more pronounced in gingival fibroblasts than in porcine PDL fibroblasts.

Expression of growth factors, cytokines, and extracellular matrix constituents

EMD significantly stimulated the release of TGF-β1 by human gingival fibroblasts (Van der Pauw et al. 2000). Haase & Bartold (2001) showed that EMD stimulation significantly affected mRNA expression of the matrix proteoglycans versican, biglycan, and decorin in human gingival fibroblasts. EMD also stimulated hyaluronan synthesis in human gingival fibroblasts, and human gingival fibroblasts appeared to be more responsive to EMD than human PDL fibroblasts. EMD increased the

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Table 2. Effects of EMD on various cell types in vitro

Process/ molecules	Epithelial cells	Gingival fibroblasts	PDL or follicle cells	Cemento-blasts	Osteoblasts	Pre-osteoblasts	BMSC or mesenchymal progenitors	Chondrogenic cells	Endothelial cells
Cell attachment, cell spreading, chemotaxis	+,+	+,+	−,+,+,+,−,+,−		+,+				+,+
Cell proliferation	−,−,−,−,+	+,+,+,+,+,+,+	+,+,+,+,+,+,−+,+,−,+,+,−,+,−,+,+,+	+	+,−+,−,+,+,+,+,−,+,−,+	+,+,+	−,+,−,+	+,+	−,+,+
Growth factors, cytokines									
TGF-β1		+	+,+,+,+,+		+,+,+			−	
BMP-2			+						
BMP-7			+						
PDGF-AB	+		+						
CTGF					+				
FGF-2					+				
IGF-1			+,+,+		−	+			
VEGF			+						
TNF-α									
IL-6			+		+				
IL-8									
PGE_2								+	
COX2			+		+				
Total protein synthesis, extracellular matrix molecules									
Total protein		+	+,−,+						
Collagen type I			−		+,+,+	+	−,−		
Collagen type II								+	
Collagen type X							+	+	
Proteoglycans	+	+	+					+	
OPN	+	+	+,+,+	+	+,+	+			
BSP		−	+,+,−,+	+	+,+	+			
OC			−,+,+	−	−,+,+,+	−,+	−,+,−		
CAP			+						
CP-23			+						
MMPs					+,−				+
Mineralization									
In vitro mineralized nodule formation		−	+,−,+,−+,+		+,+,+,−		+,−	+	
ALP		+,−	+,−,+,−,+,+		+,+,−,+,+,+,−	+	−,+,+,−	−,+	
Bone remodeling									
RANKL			−		−				
OPG			−,+		+	+			
Intracellular signaling molecules, transcription factors									
Cbfa1/Runx2			−			+	+,+		
Osx									
Sox9							+	+	
Zfp60								+	
AJ18								+	
cAMP	+,−	−	+						

+, positive effect; −, negative or no measurable effect; empty cells, not determined or not applicable; BMSC, bone marrow stromal cells.
Every single + or − indicates one single study or single cell line, if more than one cell line was used in the same study.

Table 3. Effects of various EMP forms/formulations on various cell types in vitro and in vivo

Process molecules	Epithelial cells	Gingival fibroblasts	PDL or follicle cells	Cementoblasts	Osteblasts	Preosteoblasts	BMSC or stem cells	In vivo studies
Cell attachment, cell spreading, chemotaxis			+(5,6)		+(13)	+(16)		
Cell proliferation			−(1,2),+(3,4,5,6)	−(9,10,11)			−(12)	
Growth factors, cytokines								
No information available								
Extracellular matrix molecules								
Collagen type I			−(5,6)			+(16)		
OPN				+(10,11)				+(14,15)
BSP			+(5)	−(9,10)			+(12,20)	+(14,15)
OC			+(5,6)	−(9,10,11)	+(17)	+(16)	+(12)	
FN					−(19)			
Mineralization								
In vitro mineralized nodule formation			+(7)	−(9,10,11)			+(12,20)	
ALP			+(7,8)		−(7),+(18)	+(16)	+(12)	
Bone remodeling								
RANKL				−(5,11)	−(19)			
OPG				+(11)				
M-CSF					−(19)			
Intracellular signaling molecules, transcription factors								
Cbfa1/Runx2				−(11)				
Osx							1(20)	
Sox9								+(14,15)
RP59								+(14,15)

+, positive effect; −, negative or no measurable effect; empty cells, not determined or not applicable; BMSC, bone marrow stromal cells.

Numbers in brackets indicate the specific type of EMP molecule tested and the corresponding reference.

(1) EMPs from freshly extracted porcine teeth, separation into 4 fractions (Nagano et al. 2006).
(2) Amelogenin (not further specified or recombinant amelogenin, rpAmel)) (Chong et al. 2006).
(3) Recombinant mouse amelogenin (Zeichner-David et al. 2006).
(4) Recombinant mouse ameloblastin (Zeichner-David et al. 2006).
(5) LRAP (leucine-rich amelogenin peptide) (Hatakeyama et al. 2006).
(6) P172 (a porcine homolog of mouse M180) (Hatakeyama et al. 2006).
(7) Fraction No. 3 from EMPs from freshly extracted porcine teeth (Nagano et al. 2006).
(8) 17 kDa sheath protein from fraction No. 1 from freshly extracted porcine teeth (Fukae et al. 2006).
(9) rp(H)M180 (full length murine amelogenin protein) (Viswanathan et al. 2003).
(10) TRAP (tyrosine-rich amelogenin peptide) (Swanson et al. 2006).
(11) LRAP (leucine-rich amelogenin peptide) (Boabaid et al. 2004).
(12) Ostoinductive fraction (OFE), containing mainly 20-, 23-, and 20 kDa and perhaps additional low-molecular mass amelogenins, derived from EMPs from freshly extracted porcine teeth (Iwata et al. 2002).
(13) Recombinant porcine amelogenin (rP172) (Hoang et al. 2002).
(14) A+4, low-molecular-weight (~ 5 kDa) amelogenin isoform (Lacerda-Pinheiro et al. 2006).
(15) A −4, low-molecular-weight (~ 5 kDa) amelogenin isoform (Lacerda-Pinheiro et al. 2006).
(16) Recombinant murine amelogenin (rM179) (Du et al. 2005).
(17) rp(H)M180 (full length murine amelogenin protein) (Svensson et al. 2006).
(18) Fraction No. 2 from EMPs from freshly extracted porcine teeth (Nagano et al. 2006).
(19) Recombinant mouse amelogenin (rAMEL) (Nishiguchi et al. 2007).
(20) LRAP (leucine-rich amelogenin peptide) (Warotayanont et al. 2008).

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amount of extracellular matrix and protein content in a dose-dependent manner compared with controls (Keila et al. 2004). EMD stimulated mRNA expression of osteopontin (OPN), whereas the gene expression of bone sialoprotein (BSP) was not affected (Rincon et al. 2005).

Levels of cyclic adenosine monophosphate (cAMP)

EMD failed to stimulate cAMP production in human gingival fibroblast cell line (Kawase et al. 2001).

Mineralization and alkaline phosphatase (ALP) activity

EMD significantly stimulated ALP activity in human gingival fibroblasts, although the levels were much lower as compared with those of human PDL fibroblasts (Van der Pauw et al. 2000). Keila et al. (2004) showed negligible ALP activity and absence of in vitro mineralization in rat gingival fibroblasts cultured in the presence of EMD.

Effects of EMPs on PDL fibroblasts

While the search strategy (enamel matrix proteins OR enamel matrix derivative OR emdogain) AND (periodontal ligament OR dental follicle) retrieved 114 papers, (amelogenin AND (periodontal ligament OR dental follicle)) retrieved 28 papers. From these 142 papers, 31 met the inclusion/exclusion criteria. Three additional papers were found, while reading the selected papers from other categories. Thus, a total of 34 papers were included in the analysis of EMPs' effects on PDL fibroblasts.

DNA synthesis, cell proliferation, and cell viability

A marked enhancement of cell proliferation was observed in human PDL fibroblasts exposed to EMD (Gestrelius et al. 1997). Kawase et al. (2000) showed that EMD substantially stimulated the proliferation of human PDL fibroblasts in a dose-dependent manner over 3 days. Attached human PDL fibroblasts showed a significantly lower incorporation of ^{3}H-thymidine uptake than the corresponding human gingival fibroblasts (Van der Pauw et al. 2000). PDL cell density and DNA synthesis were significantly increased when EMD was present in cultures (Lyngstadaas et al. 2001). EMD had a significant proliferative effect on virus-transformed murine dental follicle cells (Hakki et al. 2001). Brett et al. (2002) did not notice a difference in DNA synthesis between EMD-treated human PDL fibroblasts and controls. However, Matsuda et al. (2002) showed that EMD significantly enhanced DNA synthesis of a clone of PDL fibroblastic cells (OM 3-8) and Okubo et al. (2003) found that EMD stimulated in a dose- and time-dependent manner the cell growth of human PDL fibroblasts. Using human PDL fibroblasts, a trend was noted over time for EMD to enhance cell proliferation when compared with a positive control (FBS) (Davenport et al. 2003). However, the authors did not compare their test data with a negative control, as is done in most other studies. Data derived from a cell viability assay performed in the same study suggested that EMD had the tendency to decrease cell viability in a time- and dose-dependent manner. Rincon et al. (2003) showed a significant increase in ^{3}H-thymidine incorporation into DNA of human PDL fibroblasts when compared with the controls (0.2% FCS). Cattaneo et al. (2003) measured a significant increase in cell number of human PDL fibroblasts exposed to EMD. Addition of EMD to cultures of rat PDL cells (a mixture of fibroblasts and epithelial cells) decreased the total DNA content (Inoue et al. 2004). Palioto et al. (2004) showed a significant time- and dose-dependent increase in cell proliferation of EMD-stimulated human PDL fibroblasts. In a study by Nagano et al. (2004), EMD did not show any growth stimulation of human PDL fibroblasts. Porcine PDL fibroblasts exhibited increased ^{3}H-thymidine incorporation into DNA after stimulation with EMD (Rincon et al. 2005). The effect on porcine gingival fibroblasts was less pronounced. Instead of using commercially available EMD, Nagano et al. (2006) processed and examined EMPs from freshly extracted teeth from young pigs. They separated the EMPs into four fractions. At concentrations of 50 μg/ml, none of these fractions showed any stimulatory effect on human PDL cell proliferation.

Ashkenazi & Shaked (2006) evaluated the in vitro clonogenic capacity of human PDL fibroblasts cultured in the presence or absence of EMD. The presence of EMD decreased the percentage of cells with the ability of giving rise to colonies with 75%–100% confluence. The authors concluded that this was probably due to an increased cell differentiation effect of EMD. Using human PDL fibroblasts, Chong et al. (2006) demonstrated greater ALP activity for one cell line over another one. These two cell lines were used for a cell proliferation assay with or without EMD, other molecular factors, or combinations thereof. ALP-positive cells demonstrated no significant effects by EMD or amelogenin. However, a statistically significant cell growth over negative control media was seen when EMD was combined with PDGF-BB or amelogenin was combined with PDGF-BB. In contrast, ALP-negative cells showed no significant increase in cell numbers with each of the molecular factors tested alone. Only the combination of EMD and PDFG-BB significantly increased cell numbers. Pischon et al. (2006), using an organoid culture system, showed that EMD caused a significant increase in BrdU incorporation in human PDL fibroblasts.

Zeichner-David et al. (2006) determined the effect of purified recombinant mouse amelogenin and ameloblastin on cell proliferation of immortomouse-derived PDL fibroblasts. Both recombinant EMPs had a statistically significant positive effect on cell proliferation. Using LRAP (leucine-rich amelogenin peptide) and P172 (a porcine homologue of mouse M180), two amelogenin isoforms, Hatakeyama et al. (2006) showed that either amelogenin peptide dose-dependently increased cell proliferation of a mixture of PDL cells and cementoblasts. However, in amelogenin-knockout mice, the increase in cell proliferation was less pronounced than in the wild-type mice. Rodrigues et al. (2007) showed a significant increase in cell proliferation when human PDL fibroblasts were exposed to EMD. Comparing the proliferative response of human PDL fibroblasts with EMD and other devices used for periodontal regeneration, Kasaj et al. (2007) observed enhanced cell proliferation under the influence of EMD. Short-term exposure of human PDL cells to EMD resulted in a reduction of cell number compared with the negative control, whereas long-term exposure resulted in a significant increase in cell number at the highest EMD concentration used (Lossdörfer et al. 2007).

Cell migration, attachment/adhesion, and spreading

EMD had no significant effect on migration, attachment, and spreading of

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human PDL fibroblasts (Gestrelius et al. 1997). Compared with human gingival fibroblasts, human PDL fibroblasts attached and spread much better and faster (Van der Pauw et al. 2000). PDL cell attachment rate was significantly increased when EMD was present in cultures (Lyngstadaas et al. 2001). When human PDL fibroblasts were seeded on EMD-coated culture wells, cell attachment was significantly increased compared with controls (carrier alone) (Suzuki et al. 2001). Cell attachment assays led the authors to conclude that the cell attachment was mediated by interaction between a BSP-like molecule and the cell surface receptor integrin $\alpha v\beta 3$. Likewise, Van der Pauw et al. (2002) concluded that integrins are involved in the interaction of PDL fibroblasts with EMD. Cell migration and adhesion of human PDL fibroblasts was not affected by EMD in a study by Palioto et al. (2004). EMD produced a significant increase in cell attachment of porcine gingival fibroblasts only at the highest concentration, and cell attachment was much more stimulated in gingival fibroblasts than in porcine PDL fibroblasts (Rincon et al. 2005). Zeichner-David et al. (2006) determined the effect of purified recombinant mouse amelogenin and ameloblastin on cell adhesion of immortomouse-derived PDL fibroblasts. Both recombinant EMPs had a statistically significant positive effect on cell adhesion. In contrast, EMD had a statistically significant inhibitory effect on cell adhesion of human PDL fibroblasts (Rodrigues et al. 2007).

Expression of growth factors, cytokines, extracellular matrix constituents, and transcription factors

The synthesis of total protein was enhanced by human PDL fibroblasts exposed to EMD (Gestrelius et al. 1997). EMD significantly stimulated the release of TGF-β1 by human PDL fibroblasts (Van der Pauw et al. 2000). PDL cell metabolism was significantly increased when EMD was present in cultures, and there was increased autocrine production of TGF-β1, interleukin 6 (IL-6), and PDGF-AB when compared with controls (Lyngstadaas et al. 2001). Human PDL fibroblasts responded to EMD by a significantly increased and dose-dependent proteoglycan synthesis, and mRNA expression for versican and biglycan increased, whereas that for decorin decreased (Haase & Bartold 2001). Furthermore, EMD significantly increased the synthesis of hyaluronan, and gingival fibroblasts appeared to be more responsive to EMD than PDL fibroblasts. EMD increased both BSP and OPN mRNA expression, whereas osteocalcin (OC) mRNA expression was decreased (Hakki et al. 2001). Brett et al. (2002) observed no significant difference in protein synthesis of human PDL fibroblasts between EMD-treated cells and controls. However, RNA synthesis in these cells was elevated in the presence of EMD compared with controls. Hybridization of the cDNA prepared from this RNA to gene array filters showed that 121 genes, most of which had not been associated previously with periodontal regeneration, were differentially expressed. EMD stimulated the gene and protein expression of IGF-1 and TGF-β1 (Okubo et al. 2003). Synthesis of type I collagen was not affected by EMD in human PDL cells (Palioto et al. 2004). An up-regulation of mRNA expression of ALP, BSP, OPN, and OC was observed in PDL fibroblasts exposed to EMD (Nagano et al. 2004). EMD up-regulated the mRNA expression of IGF-1 and TGF-β1 in an established cell line cloned from PDL cells (OM 3-8) (Inaba et al. 2004).

Parkar & Tonetti (2004) suggested in a gene array study that in human PDL cells, EMD down-regulated the expression of genes involved in early inflammatory events of wound healing, whereas genes encoding growth and repair-promoting molecules were up-regulated. At the highest concentration of EMD, porcine PDL fibroblasts showed a significant increase of OPN mRNA intensity (Rincon et al. 2005). Although BSP mRNA expression of EMD-stimulated cells was also observed, the levels of expression were not above the negative control level. Takayanagi et al. (2006) examined the effects of EMD on bone-related mRNA expression in human PDL cells in vitro. Their results showed a significant increase in cyclooxygenase 2 (COX2) mRNA levels in cells exposed to EMD. No effects were noted on mRNA levels for core binding factor α1 (Cbfa1). Receptor activator of nuclear factor kappa B ligand (RANKL) mRNA levels were significantly decreased, whereas osteoprotegerin (OPG) mRNA levels showed minimal effects with EMD treatment. Of interest is, however, that the RANKL/OPG ratio showed a 40%–55% reduction with higher concentrations of EMD. Yuan et al (2006) showed that using EMD at concentrations of 50 and 100 μg/ml TGF-β1 levels in the culture medium were significantly higher than those without addition of EMD. Furthermore, these authors suggested that the IgG from patients undergoing regenerative periodontal therapy with EMD did not significantly neutralize the increase in TGF-β1 synthesis induced by EMD in human PDL fibroblasts. In an organoid culture system, Pischon et al. (2006) showed that in human PDL fibroblasts, EMD did not affect ^{3}H-proline incorporation.

Zeichner-David et al. (2006) determined the effect of purified recombinant mouse amelogenin and ameloblastin on bone-related gene expression of immortomouse-derived PDL fibroblasts. Both recombinant EMPs modulated BMP expression, down-regulated the expression of type I collagen, and induced the de novo expression of OC. Recombinant mouse amelogenin also induced the expression of BSP. Using DNA microarray analysis, Barkana et al. (2007) showed that EMD up-regulated expression of genes related to nucleic acid metabolism, protein metabolism, and signal transduction in a subpopulation of PDL cells that form mineralized tissue. In a subpopulation of PDL cells exhibiting a fibroblastic phenotype, up-regulated genes were related to nucleic acid metabolism, signal transduction, and cell adhesion. Evaluating the response of human dental follicle cells to EMD in vitro, Kémoun et al. (2007) noted increased expression of BMP-2, BMP-7, BSP, cementum attachment protein (CAP) and cementum protein-23 (CP-23), two putative cementum markers. EMD significantly stimulated total protein synthesis by human PDL fibroblasts (Rodrigues et al. 2007). EMD exposure to human PDL fibroblasts resulted in significantly enhanced OC and OPG protein levels (Lossdörfer et al. 2007).

Levels of cAMP

PDL fibroblasts exposed to EMD showed increased intracellular levels of cAMP signalling (Lyngstadaas et al. 2001).

Mineralization and alkaline phosphatase (ALP) activity

EMD significantly promoted in vitro mineral nodule formation of human

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PDL fibroblasts (Gestrelius et al. 1997), and significantly stimulated ALP activity (Van der Pauw et al. 2000). In contrast, Hakki et al. (2001) noticed that EMD blocked cell-mediated mineralization in vitro. Likewise, Cattaneo et al. (2003) observed a lack of ALP activity in human PDL fibroblasts grown on EMD-treated culture dishes. In a study by Nagano et al. (2004), mRNA expression for ALP was increased and ALP activity dose-dependently increased in human PDL fibroblasts exposed to EMD. Furthermore, in vitro biomineralization was also enhanced. Fraction No. 3 of processed EMPs from young pig teeth induced ALP activity and in vitro mineralized nodule formation in human PDL cells (Nagano et al. 2006). Under the culture conditions of a study by Pischon et al. (2006), human PDL fibroblasts exposed to EMD neither showed altered ALP activity nor was calcium accumulation affected. Based on histological findings of one-wall defects created in beagle dogs and treated with various fractions of EMPs or EMD as a positive control, Fukae et al. (2006) determined fraction 1 as containing EMPs showing activity to regenerate cementum. Fraction 1 was further fractionated and contained mostly 13-, 15-, and 17-kDa sheath proteins, which were then examined for ALP activity of human PDL fibroblasts. The 17-kDa sheath protein and one corresponding synthetic peptide were the only molecules that enhanced the ALP activity. Kémoun et al. (2007) showed that long-term stimulation of human dental follicle cells with EMD significantly increased ALP activity and mineralized nodule formation. EMD significantly increased ALP activity and increased in vitro mineralized nodule formation in human PDL fibroblasts (Rodrigues et al. 2007). EMD exposure to human PDL fibroblasts resulted in significantly enhanced ALP activity (Lossdörfer et al. 2007).

Effects of EMPs on cementogenic cells

While the search strategy (enamel matrix proteins OR enamel matrix derivative OR emdogain) AND (cement*) retrieved 118 papers, (amelogenin AND cement*) retrieved 11 papers. From these 129 papers, 4 met the inclusion/exclusion criteria. The search strategy retrieved 5 papers. One paper used a mixture of cementoblastic and PDL cells and was therefore moved to the PDL group. The remaining 4 papers are included. While only one paper was examining the effects of EMD, the other 3 papers used specific amelogenin forms.

DNA synthesis, cell proliferation, and cell viability

Immortalized murine cementoblasts exposed to EMD showed significantly enhanced cell proliferation (Tokiyasu et al. 2000, Viswanathan et al. 2003), whereas exposure to a full-length murine amelogenin protein (rp(H)M180) (Viswanathan et al. 2003) or the N-terminal, proteolytically cleaved, tyrosine-rich amelogenin peptide (TRAP) (Swanson et al. 2006) showed no difference from untreated controls. Using another alternatively spliced amelogenin product, a leucine-rich amelogenin peptide (LRAP), Boabaid et al. (2004) did not observe an effect on cell proliferation of immortalized murine cementoblasts up to 6 day, with a decrease in cell growth observed at the highest dose by 9 days.

Expression of growth factors, cytokines, and extracellular matrix constituents

EMD down-regulated OC and slightly up-regulated OPN mRNA expression, whereas gene expression for BSP was modestly increased only towards the end of the cultivation period (Tokiyasu et al. 2000). The lowest dose of a full-length murine amelogenin protein (rp(H)M180) showed slightly enhanced BSP and OC gene expression, whereas at the highest dose, a dramatic decrease in both BSP and OC expression was observed (Viswanathan et al. 2003). However, both BSP and OC transcripts recovered with increasing time. LRAP down-regulated OC and up-regulated OPN gene expression in a dose- and time-dependent manner (Boabaid et al. 2004). Transcripts for OPG were increased in LRAP-treated cells, but RANKL and Cbfa1 mRNA levels were not affected. Gene expression of OC, OPN, and BSP in TRAP-treated immortalized murine cementoblasts showed down-regulation, up-regulation, and no significant change, respectively (Swanson et al. 2006).

Mineralization and alkaline phosphatase (ALP) activity

In vitro mineralized nodule formation was not blocked but dose-dependently decreased in immortalized cementoblasts exposed to EMD (Tokiyasu et al. 2000), a full-length murine amelogenin protein (rp(H)M180) (Viswanathan et al. 2003), LRAP (Boabaid et al. 2004), or TRAP (Swanson et al. 2006).

Effects of EMPs on osteogenic and bone marrow cells and myoblasts

By far the highest number of papers could be retrieved from Medline for this category. The search strategy (enamel matrix proteins OR enamel matrix derivative OR emdogain OR amelogenin) AND (osteo* OR bone OR bone marrow OR chondro*) retrieved 442 papers; only 56 met the inclusion/exclusion criteria. Not all papers used EMD as the test substance. Particularly for this category, it may be advantageous to subdivide the findings into an ''EMD'' and a ''non-EMD'' group.

Experiments using EMPs other than EMD as the test substance

The bone matrix is known to contain BMPs, highly potent growth/differentiation factors that induce the differentiation of progenitor cells into osteoblasts. However, the dentin matrix appears to have a higher osteoinductive capacity (for reviews, see Veis 2003, Bosshardt 2005). This could mean that the dentin matrix has a heightened content of BMPs, or, alternatively, that dentin contains additional chondrogenic/osteogenic factors. Nebgen et al. (1999) identified the active chondro-/osteogenic fraction of proteins extracted from bovine dentin as a small splice product of the amelogenin gene. Thereafter, Veis et al. (2000) identified two specific cDNAs from a rat incisor tooth odontoblast pulp cDNA library and produced the corresponding recombinant proteins, $r[A+4]$ and $r[A-4]$. *In vitro* and *in vivo*, both recombinant amelogenin polypeptides showed chondrogenic and osteogenic activities, respectively. As mentioned earlier, these findings are in line with those obtained with enamel matrix and amelogenin by Urist (1971) and Wang (1993), respectively.

Iwata et al. (2002) fractionated enamel matrix extracts from developing porcine teeth and found an osteoinductive fraction (OFE) containing mainly 20-, 23-, and 20 kDa proteins. The OFE enhanced ALP activity and in vitro

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mineralized nodule formation, and up-regulated OC, BSP, and ALP mRNA expression in ST2 cells, a mouse bone marrow stromal cell line. Cell proliferation, however, was not affected at the concentrations selected (i.e., 0 to 10 μg/ml). The methodology used in this study could not exclude the possibility that the OFE may contain additional low-molecular-mass amelogenins. Hoang et al. (2002) demonstrated that recombinant porcine amelogenin (rP172) promotes adhesion of MG63 cells, a human osteosarcoma cell line. Recombinant murine amelogenin (rM179) incorporated into a biomimetic apatite layer caused a significant increase in the mRNA expression of type I collagen, ALP and OC, as well as enhanced cell attachment and spreading in human embryonic palatal mesenchymal pre-osteoblasts (HEPM 1486) (Du et al. 2005). Commercially available primary human osteoblasts (NHOst cell system) exposed to rp(H)M180, a recombinant murine histidine-tagged amelogenin, or EMD showed an approximately twofold increase of secreted OC, compared with untreated controls (Svensson et al. 2006). The addition of leucine-rich amelogenin peptide (LRAP) to co-cultures of cementoblasts/PDL cells and mouse bone marrow cells significantly reduced RANKL expression and the number of cells positive for tartrate-resistant alkaline phosphatase (Hatakeyama et al. 2006). Exposure of osteoblast-like cells (ST2), a mouse bone marrow stromal cell line, to fraction No. 3 of EMPs extracted from porcine permanent molars reduced ALP activity, whereas fraction No. 2 caused an increase in ALP activity (Nagano et al. 2006). Further analysis led the authors to conclude that porcine enamel protein fractions contain TGF-β1.

In a study by Lacerda-Pinheiro et al. (2006), agarose beads with or without either $A+4$ or $A-4$, two low-molecular-weight amelogenin isoforms, were implanted in the cheek mucosa of mice. Agarose beads alone or with either amelogenin isoform induced the recruitment of CD45-positive cells. When the beads were coated with either amelogenin isoform, RP59, Sox9, BSP and OPN, markers of osteo-/chondrogenic lineages, were expressed. Only $A-4$ had the capacity to induce BSP protein expression. Using co-cultures of mouse calvarial osteoblasts and bone marrow cells, Nishiguchi et al. (2007) showed that recombinant mouse amelogenin (rAMEL) is a negative regulator of osteoclastogenesis via down-regulation of RANKL, macrophage colony stimulating factor (M-CSF), and fibronectin expression in osteoblasts. In another study, porcine bone marrow-derived stromal cells were inoculated onto the surface of tooth root disks with or without EMPs extracted from porcine tooth germs (Song et al. 2007). After a 7-day culturing period, the root disks were transplanted subcutaneously into nude mice, and histology was performed 3 and 8 weeks later. The authors concluded that a new cellular cementum-like tissue formed along EMP-treated root surfaces. It is important to point out that there are difficulties to differentiate between bone and cellular cementum (Bosshardt 2005). Warotayanont et al. (2008) provided data supporting the function of leucine-rich amelogenin peptide (LRAP) as a signalling molecule that enhances osteoblastic cell differentiation in mouse embryonic stem cells. The authors demonstrated an increase of as much as 4000-fold for BSP expression, 5-fold for osterix(Osx) expression, and 6-fold for calcium accumulation in LRAP-treated cultures, and concluded that LRAP's role as an osteoinductive molecule is equal to or more potent than BMP-2 during osteogenic differentiation in their in vitro model.

Experiments using EMD as the test substance

Cell attachment, motility, proliferation, and viability. Gurpinar et al. (2003) showed that EMD failed to enhance proliferation of stromal osteoblastic cells obtained from the bone marrow of young adult male rats. In contrast, treatment with EMD of human osteoblastic cells (SaM-1) from one patient (Mizutani et al. 2003) and of rat femoral bone marrow stroma (Keila et al. 2004), and MC3T3-E1 cells, a mouse pre-osteoblastic cell line (He et al. 2004a, b, Jiang et al. 2006), significantly stimulated cell proliferation. Studying the effects of EMD on SaOs2 cells, a human osteoblastic cell line, in vitro in the presence of titanium disks, no proliferative effect was observed over the controls (Schwarz et al. 2004). However, cell viability at higher EMD concentrations was higher than in the controls. In another study, osteoblasts isolated from mouse calvaria were used (Hägewald et al. 2004). While EMD increased BrdU incorporation in monolayers, ^{3}H-proline incorporation was not affected by EMD exposure in 3-dimensional organoid cultures. However, when alveolar bone cells were used, no effect of EMD on cell proliferation was observed, but the bone cells showed the greatest attachment response to EMD (Rincon et al. 2005). In contrast, Galli et al. (2006) showed that growth of human mandibular osteoblasts from one patient was significantly increased by EMD. In a heterogeneous cell population from rat bone marrow, EMD had no significant effect on cell proliferation, ALP activity, and mRNA expression of type I collagen, OC, and ALP (Van den Dolder et al. 2006). Using an organoid culture system with human primary osteoblasts, Pischon et al. (2006) observed a significant increase in cell proliferation. Klein et al. (2007) observed that EMD promotes motility of different osteoblastic cell lines better than the control groups, whereas the proliferation rates depended on the cell type. In another study where a chondrogenic cell line, ATDC5, was used, EMD markedly increased cell proliferation (Narukawa et al. 2007a). Guida et al. (2007) showed that EMD stimulated proliferation of human bone marrow stromal cells in a dose-dependent manner. EMD treatment of human osteoblastic cells (Saos-2) increased cell proliferation (Heng et al. 2007).

Expression of molecules, cell signalling, cell differentiation, and ectopic bone formation. Using an ectopic model, Boyan et al. (2000) could not show that EMD is osteoinductive. There results, however, showed that EMD is osteopromotive. The same group showed in vitro that EMD has differential effects on cells of the osteogenic lineage (Schwartz et al. 2000). Using ALP and OC as differentiation markers, they showed that EMD affects early states of cell maturation by stimulating proliferation, but enhances differentiation, as cells mature. Furthermore, EMD increased the cell number of normal human osteoblasts and stimulated TGF-β1 release into the culture medium.

EMD promoted proliferation of pre-osteoblasts (cell lines OCT-1 and MC3T3-E1), down-regulated OC, and up-regulated OPN gene expression in MC3T3-E1 cells (Tokiyasu et al. 2000). Another group showed that EMD in primary osteoblasts enhances

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gene expression of type I collagen, IL-6, and prostaglandin G/H synthase 2 (PGHS-2), but not of OC and IGF-1 (Jiang et al. 2001a), and prolongs proliferation of primary osteoblasts (Jiang et al. 2001b). Ohyama et al. (2002) used a typical, commercially available pluripotent mesenchymal cell line (C2C12) to evaluate the differentiation pathway under the influence of EMD. They showed that EMD induced high ALP activity and a marked increase in the mRNA expression of ALP, OC, and type X collagen. These results were interpreted as clear evidence that EMD directs the differentiation pathway of C2C12 cells into the osteoblast and/or the chondroblast lineage. The purpose of another study was to test the response of chondrocytes in the endochondral pathway at two stages of cell maturation (Dean et al. 2002). The less mature chondrocytes responded to EMD with an increase in cell proliferation and PGE_2 synthesis, and a decrease in differentiation as measured by ALP activity. However, EMD had no measurable effect on collagen synthesis, proteoglycan sulphation, and TGF-β1 production. The more mature chondrocytes also responded to EMD with enhanced proliferation, but ALP activity was not affected and the increase in PGE_2 production was only modest. The authors' conclusion was that the differential effects of EMD are related to the cell maturation state. Yoneda et al. (2003) evaluated the response of osteoblastic cells (ST2 cells and KUSA/A1 cells). They showed that EMD did not stimulate cell growth in ST2 cells, whereas in KUSA/A1 cell proliferation was enhanced. EMD enhanced ALP activity in both cell lines. In KUSA/A1 cells, EMD enhanced in vitro mineralized nodule formation, mRNA expression of type I collagen, OPN, OC, and TGF-β1, and the synthesis of matrix metalloproteinases (MMPs). The conclusion was that the effects of EMD depend on the cell type and that the overall effect of EMD on osteoblastic cells is stimulatory rather than inhibitory.

Treatment of human osteoblastic cells (SaM-1) from one patient with EMD enhanced mRNA expression of COX2 and both protein and mRNA expression of FGF-2, but decreased mRNA expression of ALP and MMP-1 (Mizutani et al. 2003). EMD significantly enhanced in vitro mineralized nodule formation and ALP activity in rat femoral bone marrow stromal cells at concentrations of 25 μg/ml (Keila et al. 2004). MC3T3-E1 cells, a mouse pre-osteoblastic cell line, responded to EMD with a significantly enhanced ALP activity, and an up-regulation of mRNA expression of type I collagen, BSP, OC, OPG, and IGF-1, whereas the expression of Cbfa1 mRNA levels was elevated after longer culturing periods only (He et al. 2004a). Shimizu et al. (2004) observed an increase in BSP mRNA levels in an osteoblastic cell line (ROS 17/2.8 cells) exposed to EMD and concluded that EMD may mediate BSP expression at the gene transcriptional level. He et al. (2005) showed that TNFα-induced apoptosis of MC3T3-E1 cells, a mouse osteoblastic cell line, is inhibited by EMD. In C2C12 cells, a subclone of mouse C2 myoblasts, EMD substantially increased both protein and mRNA expression of Cbfa1/Runx2 (Takayama et al. 2005). Using demineralized or undemineralized dentin matrix with or without EMD implanted into rectus abdominis muscles in rats, Koike et al. (2005) demonstrated that EMD does not have the ability to induce hard tissue formation in this model. Otsuka et al. (2005) showed that fraction numbers 21–25 of EMD induced the formation of osteoclasts in mouse marrow cultures. The presence of RANKL in mouse primary osteoblastic cells stimulated with EMD or its purified fractions was also demonstrated, and OPG completely inhibited osteoclast formation.

Injection of EMD into the back of rats induced cartilage-like tissue formation (Kim et al. 2005). However, it must be said that these findings are solely based on histological observations without the use of any histochemical or immunohistochemical techniques. In an organoid culture system, EMD enhanced ALP activity, calcium accumulation, and in vitro mineralized nodule formation of osteoblasts isolated from mouse calvaria (Hägewald et al. 2004). In porcine alveolar bone cells, the expression of OPN and BSP mRNA was significantly enhanced (Rincon et al. 2005). EMD caused an increase in ALP activity and an increase in mRNA expression of type I collagen and OC in commercially available human osteoblasts (NHOst cell system) (Reseland et al. 2006). A stimulatory effect on osteoclasts was also observed, and EMD appeared to be taken up by the osteoblastic cells. Using the DNA microarray technique, several up- and down-regulated genes were identified in an osteoblast-like cell line (MG-63) when exposed to EMD (Carinci et al. 2006). The differentially expressed genes covered functional activities such as signal transduction, transcription, translation, cell cycle regulation, cell proliferation, apoptosis, immune system, vesicular transport and lysosome activity, as well as cytoskeleton, cell adhesion, and extracellular matrix production. When human mandibular osteoblasts from one patient were exposed to EMD, a significant increase in the production of OPG and OC was observed, whereas RANKL production was decreased (Galli et al. 2006). Furthermore, ALP activity was enhanced and mineralized nodules were larger and more numerous. Itoh et al. (2006) used mouse bone marrow cells and mouse monocytic RAW 264.7 cells (ATCC TIB 71) to test the effect of EMD fractions. Purified EMD fractions (fraction numbers 21–25, EMD peak 2) were found to enhance the formation and function of RAW 264.7 cells induced by RANKL. The authors concluded that EMD supports the formation of osteoclasts through interaction with RANKL.

In an organoid culture system with human primary osteoblasts, Pischon et al. (2006) showed that ALP activity and calcium accumulation were not affected by EMD. C3H10T1/cell line, a typical pluripotent undifferentiated mesenchymal cell line, exposed to EMD substantially increased mRNA levels of osteogenesis- and chondrogenesis-related transcription factors, as well as Cbfa1/Runx2 and Sox9 protein expression (Narukawa et al. 2007b). In a subsequent paper by the same group, EMD markedly increased ALP activity, and induced the formation of Alcian blue-positive cartilage matrix and mineralized nodules in a chondrogenic cell line (ATDC5) (Narukawa et al. 2007a). Gene expression encoding for typical cartilage proteins (type II and type X collagen, aggrecan) and chondrogenesis-related transcription factors (Sox9, Zfp60, and AJ18) was also markedly increased in the presence of EMD. Using human bone marrow stromal cells, Guida et al. (2007) showed that EMD down-regulated type I collagen synthesis and ALP activity, whereas the decrease in OC synthesis was not statistically significant. In vitro mineralization was reduced in EMD-treated cells. Using an in vitro metal implant model, Dacy et al. (2007) showed that EMD up-regulated the release of TGF-β1 from primary rat osteoblasts

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into the culture medium. When human osteoblastic cells (Saos-2) were treated with EMD, a dose-dependent increase in the expression of connective tissue growth factor (CTGF), a mediator of TGF-β, was observed (Heng et al. 2007). EMD-induced CTGF expression and in vitro mineralization were significantly reduced in the presence of TGF-β inhibitor and treatment with anti-CTGF antibody, respectively.

Healing of bone defects. Using a bone wound-healing model in rat femurs Kawana et al. (2001) and in rat parietal bone (Sawae et al. 2002), the authors noted a significantly higher bone volume fraction of newly formed bone trabeculae 7 days after injury in the EMD group compared with the PGA control. Interestingly, marked differences in the immunostaining intensity of BSP and the lysosomal cysteine proteinase, cathepsin K, and the formation of a ruffled border in multinucleated cells were noted between these two studies, suggesting that the effects of EMD also depend on the local osseous environment. EMD applied to rat skull defects revealed significantly more bone formation 2 weeks post-injury (Yoneda et al. 2003). Donos et al. (2004) showed that EMD alone did not completely fill critical-size calvarial defects in rats. Heterotopic bone formation in the rat mandibular ramus under a dome-shaped capsule was also not altered by the addition of EMD (Donos et al. 2005). Analysing the bone healing 4 and 8 weeks after defect creation in the tibia of rabbits, Cornelini et al. (2004) noted no histological differences between the EMD group and the unfilled (empty) control.

Healing of bone defects around implants. Placing titanium implants in the corticotrabecular area of the femur in rats, Shimizu-Ishiura et al. (2002) observed that EMD treatment produced a significantly greater trabecular bone area around the implants when compared with the negative control (carrier PGA alone) at both 14 and 30 days postimplantation. In contrast, healing periods of 6 weeks did not demonstrate any beneficial effects of EMD treatment on bone formation around titanium implants placed in femurs and tibia of rats (Franke-Stenport & Johansson 2003). Craig et al. (2006) reported that transplantation of porcine PDL cells with EMD before insertion of metal implants into the mandible of minipigs led to good bone-to-implant contact, whereas omitting the EMD resulted in good bone-to-implant contact with strands of epithelial cells in the implant–connective tissue interface.

Effects of EMPs on wound healing

Using the search strategy, 111 studies were retrieved from Medline and 8 met the inclusion/exclusion criteria. While reading the papers from all the other categories, it became evident that another 6 studies could be assigned to this category. Thus, a total of 14 studies were analysed. In most of these studies, an in vitro wound-healing model was applied. Wounding in vitro is usually performed by creating an incision across the surface of the well (scratching and scraping off of cells using a rubber policeman) covered with a subconfluent cell monolayer.

DNA synthesis, cell migration, cell proliferation, and cell viability

Exposure of PDL cells, gingival fibroblasts, and MG-63, an osteosarcoma cell line, to EMD resulted in enhanced wound-fill rates (Hoang et al. 2000). At early time points, the effect was statistically greater for PDL cells than for both gingival fibroblasts and MG-63 cells. Accelerated wound filling stimulation by EMD over negative controls was also observed in another study where human PDL cells were used (Rodrigues et al. 2007). Rincon et al. (2003), using human PDL cells and gingival fibroblasts, showed that the in vitro wound-fill process is achieved by a combination of cell proliferation and cell migration. The most rapid wound closure was observed when cells were cultured in 10% FCS or at a concentration of 20 μg/ml EMD, which promoted cell proliferation. Using human umbilical vein endothelial cell (HUVEC) cultures, Yuan et al. (2003) observed no significant difference between the negative control and EMD groups in their proliferation assay. However, compared with the controls, EMD treatment did exhibit a significantly greater and dose-dependent chemotactic effect on these cells. Furthermore, their in vitro angiogenesis assay revealed new blood vessel outgrowth in the EMD groups, but not in the negative control group. Histology of subcutaneously implanted collagen membranes soaked with EMD in mice showed significantly more endothelial cells than the controls. Using the relaxed dermal equivalent (DE) in vitro model for early wound contraction, Grayson et al. (2006) evaluated the effects of EMD *versus* a recombinant porcine amelogenin (rP172) on human skin fibroblasts. Both EMD and rP172 significantly increased contraction and fibroblast numbers. Because this effect was significantly associated with elevated levels of TGF-β1 levels in conditioned medium, the authors concluded that the observed effects were at least partially due to increased endogenous production of TGF-β1.

Using the in vitro wound-fill model, Chong et al. (2006) showed that EMD but not recombinant amelogenin protein (rpAmel) significantly enhanced PDL cell migration at the wound edge, whereas at the centre of the wound, neither EMD nor the recombinant amelogenin had a significant effect. In addition, EMD+PDGF-BB had additive effects on ALP-negative PDL cells at the wound edge, whereas the combination of EMD and PDGF-BB additively increased wound-fill for both ALP-positive and ALP-negative PDL cells. In another study, both LRAP and P172, two amelogenin isoforms, dose-dependently increased cementoblast/PDL cell migration after in vitro wounding (Hatakeyama et al. 2006). In another in vitro model, the effects of EMD on human microvascular endothelial cells (HMVECs) were investigated (Schlueter et al. 2007). EMD at low concentrations resulted in significant stimulation of HMVEC proliferation, and HMVEC chemotaxis when PDL cells were present. All doses tested increased angiogenesis. HMVECs in combination with EMD stimulated a 750% increase in PDL cell migration compared with controls. ELISA determined an almost 400% increase in VEGF concentration by ALP-positive PDL cells and a significant increase in TGF-β production in both ALP-positive and ALP-negative PDL cells in EMD-stimulated conditioned media. Narani et al. (2007) showed that EMD proteins bind to wound extracellular matrix proteins and that this interaction tended to favour fibroblast adhesion over epithelial cells.

Immune/inflammatory cells

Petinaki et al. (1998) showed that EMD produced a slightly enhanced proliferation of lymphocytes, restricted

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to the CD25 (IL-2 receptor) fraction of the CD4-positive T-lymphocytes, and a concomitant decrease of CD19-positive B-lymphocytes. Furthermore, the authors noted that EMD was not cytotoxic.

Expression of mediators involved in inflammation and wound healing

Brett et al. (2002) showed that 121 genes, most of which had not been associated previously with periodontal regeneration, were differentially expressed in human PDL cells exposed to EMD. Among these were genes that are involved in wound healing and remodelling of the extracellular matrix. Mirastschijski et al. (2004) exposed adult human dermal fibroblasts to EMD and observed a significant increase of secreted VEGF. EMD also significantly increased release of MMP-2 from the fibroblasts and from human microvascular endothelial cells. In vivo, EMD increased the amount of granulation tissue and significantly accelerated the completion of epithelialization by 3 days, as determined in full-thickness, circular 2-cm skin wounds in white rabbits. Parkar & Tonetti (2004) demonstrated that in human PDL cells EMD down-regulates the expression of genes involved in early inflammatory events of wound healing, whereas genes encoding growth and repair-promoting molecules were up-regulated. Myhre et al. (2006) challenged whole blood from humans by lipopolysaccharide or peptidoglycan and incubated it with different concentrations of EMD or a cAMP analogue. Their results showed attenuated release of TNF-α and IL-8, while the release of IL-10 was unchanged. They concluded that EMD limits the release of pro-inflammatory cytokines induced by lipopolysaccharide or peptidoglycan in human blood.

Levels of cyclic adenosine monophosphate (cAMP)

In a study by Myhre et al. (2006), a four-fold increase in the cAMP levels was found in peripheral blood mononuclear cell lysates exposed to EMD.

Effects of EMPs on bacteria

While the search strategy (enamel matrix proteins OR enamel matrix derivative OR emdogain) AND (bacteria OR microorganisms) retrieved 15 papers [amelogenin AND (bacteria OR microorganisms)], retrieved 19 papers. From these 34 papers, 5 met the inclusion/exclusion criteria. In all papers, EMD was used as the test substance. In one paper, amelogenin was additionally used to evaluate its effects on bacteria. Examinations were performed in vitro, ex vivo, and in vitro.

The first paper that appeared on this topic was by Sculean et al. (2001). They evaluated the effects of EMD on ex vivo dental plaque vitality. Plaque samples from 24 patients with chronic periodontitis were covered with various solutions for 2 min, followed by vitality measurements. When EMD was used, 54% of the bacteria remained vital. However, when EMD+PGA (= Emdogain) was used, only 21.4% of the bacteria remained vital. When PGA, the carrier, was used alone, the vitality of the bacteria declined to only 19.6%. NaCl, used as a negative control, and chlorhexidine as a positive control showed 76.8% and 32.3% vitality, respectively. These results suggest that Emdogain(EMD+PGA) has an antibacterial effect and that PGA contributed most to this activity.

The aim of a study of Spahr et al. (2002) was to evaluate the effect of EMD on the in vitro growth of gram-negative periodontal pathogens like *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, and *Prevotella intermedia*. Their results revealed a marked inhibitory effect of EMD+PGA on the growth of these gram-negative bacteria. Interestingly, PGA alone had the same inhibitory effect.

Arweiler et al. (2002) examined the antibacterial efficacy of EMD on established supragingival plaque in periodontally healthy dental students. Biofilm vitality was 86.7%, 70.4%, 67.5%, and 56.2% after application of NaCl, EMD+PGA, PGA alone, and chlorhexidine, respectively. This study shows that EMD+PGA, PGA alone, and chlorhexidine possess significantly high antimicrobial properties when compared with a standard NaCl solution. As in the study of Sculean et al. (2001), the PGA alone appeared to contribute primarily to the antibacterial properties.

Newman et al. (2003) studied the in vitro effects of EMD on *P. gingivalis* and showed that EMD+PGA, and PGA alone had antimicrobial effects. An amelogenin fraction of EMD did not show an antibacterial effect on *P. gingivalis*, but stimulated the growth of this bacterium. Thus, the authors clearly and correctly concluded that the antimicrobial effects could be attributed to the vehicle PGA. Walter et al. (2006) basically came to the the same conclusion.

Summary and conclusions

EMPs have attracted considerable attention since their launch as medical devices. There is a vast amount of biological information available on functions of EMPs that go beyond both the regulation of enamel mineral crystal growth and the original idea of a function in cementoblast differentiation, which was actually the basis for commercialization. It is now evident that EMPs affect many different cell types and that not all the results are consistent. It is also clear that the results cannot always be consistent. There are several reasons for this, including the use of (1) different types of EMPs; (2) different concentrations of EMPs; (3) different observation periods; (4) different cell types; (5) different states of cell differentiation; (6) different experimental in vitro systems or conditions; and (7) different local in vivo environments. Nevertheless, there is a large body of information available that provides a biological rationale for the use of EMPs for periodontal regeneration. Overall, the available data suggest the following:

(1) *Cell attachment, spreading, and chemotaxis*

In most studies, EMPs caused an increase in cell attachment of epithelial cells, gingival fibroblasts, and PDL fibroblasts. Regarding differences in the rate and extent of cell attachment between gingival and PDL fibroblasts, inconsistent observations were made. A promotion of adhesion of osteogenic cells also does occur, but appears to be dependent on the cell differentiation/maturation state. Cell–matrix adhesion appears to be mediated, at least in part, by integrins. EMD also has a chemotactic effect on endothelial cells.

(2) *Cell proliferation and survival*

Most information is available on the effects of EMPs on cell proliferation. EMPs favour cell proliferation of PDL fibroblasts over gingival fibroblasts and over epithelial cells. Epithelial cells appear to respond the least to EMPs by cell proliferation. However, the effect of

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EMPs on epithelial cells appears to be cytostatic, but not cytotoxic. The influence of EMPs on cell proliferation of osteogenic cells including various progenitors appears to decrease with increasing cell differentiation/maturation state. Accelerated wound-fill rates in vitro using PDL fibroblasts, gingival fibroblasts, and osteoblast-like cells appear to be due to enhanced cell migration and proliferation. EMPs stimulate the outgrowth of new blood vessels and increase the number of endothelial cells. EMD, and, in particular, its vehicle PGA, have antibacterial properties. The antibacterial effect of PGA has been known for many years (Olitzky 1965).

(3) *Expression of transcription factors*

EMPs increase the expression of transcription factors (Osx, Cbfa1/Runx2, Sox9, Zfp60, AJ18) that are related to chondroblast and osteoblast/cementoblast differentiation.

(4) *Expression of growth factors, cytokines, extracellular matrix constituents, and other macromolecules*

EMPs cause a stimulation of total protein synthesis and synthesis of specific extracellular matrix molecules (glycoproteins and proteoglycans). Overall, EMPs down-regulate the expression of genes involved in early inflammatory events of wound healing and up-regulate the expression of genes encoding growth and repair-promoting molecules. The type of molecule affected by EMP-treatment appears to depend on the cell type and differentiation/maturation state. Among the up-regulated molecules are TGF-β1, BMP-2, BMP-7, PDFG-AB, VEGF, CTGF, FGF-2, IGF-1, TNF-α, IL-6, IL-8, PGE_2, OPN, collagen types II and X, MMP-2, and ALP. In particular, BSP, OC, and type I collagen showed inconsistent results.

(5) *Expression of molecules involved in the regulation of bone remodelling*

Normal bone remodelling depends on a delicate balance between bone formation and resorption. Bone resorption is regulated by a system constituting RANK and its ligand RANKL, which are members of the tumour necrosis factor ligand and receptor families, and OPG. RANKL is expressed by bone marrow stromal cells, osteoblasts, and certain fibroblasts, whereas RANK is expressed by osteoclast precursors and mature osteoclasts. The binding of RANK to RANKL induces osteoclast differentiation and activity, and regulates their survival. OPG, which is produced by bone marrow stromal cells, osteoblasts, and certain fibroblasts, however, is a soluble decoy receptor for RANKL that competes for this binding. Thus, OPG is a natural inhibitor of osteoclast differentiation and activation. Any interference with this system can shift the balance between bone apposition and resorption. The expression of M-CSF plays an essential role in this regulatory system. Interestingly, EMPs have an influence on this system by modulating the expression of OPG and RANKL. While a few studies suggest an up-regulation of RANKL, most studies show a down-regulation of RANKL and an up-regulation of OPG. This suggests that EMPs modulate the RANK-RANKL-OPG system most likely towards bone apposition. Of interest in this context is the observation that amelogenin knockout mice show increased hard tissue resorption (Hatakeyama et al. 2003). Furthermore, it has also to be taken into consideration that some of the growth factors and cytokines that are up-regulated by EMPs directly up-regulate OPG and down-regulate RANKL production. Thus, EMPs appear to be indirectly involved in the regulation of bone remodelling.

Issue that remain to be resolved

There are certainly many issues that require much more clarification. However, one aspect appears to be very pertinent. A number of studies suggest that EMD contains TGF-β members or both BMP-like and TGF-β-like molecules, whereas other studies suggest that certain cell types exposed to EMPs up-regulate the expression of TGF-β members (BMP-2, BMP-7, TGF-β1) (Kawase et al. 2001, 2002, Suzuki et al. 2001, Iwata et al. 2002, Matsuda et al. 2002, Boabaid et al. 2004, Shimizu et al. 2004, 2005, Suzuki et al. 2005, Takayama et al. 2005, Fukae et al. 2006, Grayson et al. 2006, Nagano et al. 2006, Yuan et al. 2006, Kémoun et al. 2007). While there is good evidence that part of the cell-stimulatory effects of EMPs can be attributed to endogenous production of TGF-β members, the issue of the (exogenous) presence of TGF-β superfamily members needs further clarification. There is the possibility that EMD contains true TGF-β members. Alternatively, it may also be possible that some EMP molecules behave like members of the TGF-β superfamily. However, it appears unlikely that there is a coincidental amino acid sequence homology. Sire et al. (2006) provided evidence for an evolutionary link between EMPs and SPARC (secreted protein, acid, and rich in cysteine), also known as osteonectin, a major bone-related protein. Furthermore, Spahr et al. (2006) showed gene and protein expression of ameloblastin during craniofacial bone formation in rats, and Haze et al. (2007) demonstrated amelogenin expression in long bone cells, in cartilage cells, and in bone marrow progenitor cells. Thus, it is very likely that EMPs stimulate a variety of different cell types both directly and indirectly and one of the signalling pathways is related to the TGF-β superfamily.

Perspectives for EMPs as therapeutic agents

It is interesting to note that Xelma (Mölnlycke, Gothenburg, Sweden), a second amelogenin-based product, is available on the medical market since 2006. Xelma, like Emdogain, consists of EMPs (mainly amelogenins), PGA, and water, and is used for hard-to-heal skin wounds, underlying the scientific evidence for both EMPs and PGA to support and enhance wound healing in general. The much-longed-for third product, which specifically enhances bone formation in fields other than periodontology such as orthopaedics and implantology, is still not available, despite a vast amount of data suggesting a causal link between specific EMP variants and chondro-/osteogenesis. There is strong evidence supporting the existence of an EMP-based osteogenic factor, likely a small amelogenin peptide in the order of 5 kDa (A-4, LRAP, rH58). Recent reports (Mumulidu et al. 2007) indicate that the search is still on. It may perhaps just be a question of time until such a product will appear on the medical market. However, the mode of application and release may require some modifications. As we all know from the BMPs and other growth/differentiation factors, this appears to be the greatest challenge, particularly so for the amelogenins, which are very hydrophobic.

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Clinical Relevance

Scientific rationale for the study: Although there is a wealth of information available on the functions of growth/differentiation factors in embryonic development, tissue formation, and tissue repair, the translation of this knowledge into a clinical application with the aim to regenerate periodontal tissues appears to be very difficult. The opposite is true for EMPs: for more than 10 years, they are widely used to treat intrabony periodontal defects, although the mechanism of action is regarded as obscure.

Principal findings: Information from 103 papers, mostly in vitro studies, demonstrates at the cellular and molecular levels that EMPs support wound healing and periodontal tissue formation.

Practical implications: There is a large body of information available that provides a biological rationale for the use of EMPs for periodontal regeneration.

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