

# Enamel Factors Regulate Expression of Genes Associated With Cementoblasts

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**Background:** In order to design predictable periodontal regenerative therapies, it is important to understand the responsiveness of cells within the local environment to factors considered attractive candidates. The aim of this study was to determine the effect of an enamel matrix derivative (EMD) on cementoblast behavior in vitro and in vivo.

**Methods:** Osteocalcin (OC) promoter SV40 transgenic mice were used to obtain cementoblasts. For comparison, pre-osteoblasts from these mice, as well as another murine pre-osteoblast cell line, MC3T3-E1 cells, were used. Cells exposed to EMD were evaluated for changes in: 1) proliferation over an 8-day period by cell counting; 2) gene expression using Northern blot analysis; and 3) biomineralization by von Kossa stain, in vitro and by preparing histological samples from implants retrieved from immunodeficient (SCID) mice, where cementoblasts were treated with EMD prior to implantation.

**Results:** EMD promoted proliferation of all cell types. EMD down-regulated osteocalcin transcripts in cementoblasts and MC3T3-E1 cells and up-regulated osteopontin gene expression markedly in MC3T3-E1 cells and slightly in cementoblasts at day 8. In vitro, EMD decreased cementoblast-mediated biomineralization. In contrast, mineralization was noted in implants retrieved from SCID mice, where cells were pretreated with EMD.

**Conclusion:** These results indicate that EMD can influence activities of cementoblasts and osteoblasts, and thus may be able to regulate cell activities at a periodontal regenerative site. *J Periodontol 2000;71:1829-1839.*

## KEY WORDS

**Protein, enamel matrix; osteoblasts; dental cementum; osteoclasts; osteopontin; sialoglycoprotein.**

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Periodontal diseases are marked by destruction of periodontal support; i.e., periodontal ligament (PDL), cementum, and bone, with subsequent tooth loss if left untreated. The recognition that periodontal regeneration can be achieved (formation of new bone, new cementum, and supportive PDL) has resulted in attempts to understand the cellular and molecular mechanisms and factors regulating formation of these tissues during development and regeneration.<sup>1</sup> This enhanced knowledge also has resulted in increased efforts at the clinical level to develop improved regenerative therapies based on sound biological principles.<sup>2</sup>

As one approach to understanding the mechanisms and factors involved in regenerating periodontal tissues, researchers have mapped the temporal and spatial expression of specific factors during development of these tissues. While the mechanisms, cells, and factors involved in regenerating periodontal tissues do not exactly follow those associated with development, investigations focused at developmental stages have provided important information as to potential factors that may prove beneficial during regeneration of periodontal tissues. In this regard, one area that has received attention, the use of enamel factors to promote periodontal regeneration, is based on the knowledge that epithelial-mesenchymal interactions are required for formation of several tissues. For example, appropriate interactions be-

tween neural crest cells and epithelial cells result in formation of enamel and dentin.<sup>3,4</sup> It is less clear whether epithelial-mesenchymal signaling molecules are involved in cementum formation and in development of a functional periodontal ligament. Several molecules associated with the epithelial root sheath have been suggested to have a role in controlling root development and these include laminin,<sup>5</sup> amelin (ameloblastin, sheathlin),<sup>6-8</sup> and other enamel and enamel-like products.<sup>9-12</sup>

Importantly, while it is not established whether epithelial-mesenchymal interactions are required for cementum formation, enamel-like molecules such as a porcine enamel matrix derivative<sup>¶</sup> (EMD) have been reported to promote proliferation, migration, adhesion, and differentiation of cells associated with healing periodontal tissues *in vivo*.<sup>13</sup> With regard to cell differentiation, this would be analogous to the known ability of ameloblasts-odontoblasts to secrete factors required for the development of dentin and enamel; i.e., epithelial-mesenchymal interactions.

Data from several studies suggest that EMD, where the principal protein is amelogenin, can augment cell activities both *in vitro* and *in vivo*. Hammarstrom and colleagues, using monkey models, reported enhanced regeneration of periodontal tissues; i.e., bone, cementum, and PDL with the use EMD versus vehicle.<sup>14,15</sup> Further studies in humans have reported promising results as measured histologically and also based on clinical attachment levels and subtraction radiography.<sup>16,17</sup> *In vitro* studies indicate that PDL cells exposed to EMD exhibit enhanced protein production, cell proliferation, and ability to promote mineral nodule formation.<sup>18</sup>

These findings suggest strongly that EMD has a positive effect on cells within a healing periodontal site; however, the mechanism by which EMD promotes cell activity, as well as the selectivity of EMD for specific cell types, remains unknown. As a next step to address these issues, we determined whether EMD had an effect on gene expression, cell proliferation, and mineral nodule formation in cementoblasts and also preosteoblasts *in vitro*. Since previous studies, based on temporal and spatial localization of enamel-like proteins during root development, suggested that they promote cementoblast activity, it was important to determine whether EMD had a direct effect on cementoblast behavior. In addition, OCT-1 cells, an immortalized preosteoblast calvaria cell line from OC-TAg transgenic mice and a murine osteoprogenitor cell line, MC3T3-E1 cells, were used.

As presented below, the results indicated that EMD enhances proliferation of all cells types studied and also alters gene expression, but in different fashions for each cell type. Further, EMD modifies mineral nodule formation both *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Cell Culture

Methods used to generate immortalized cementoblasts have been previously described in detail.<sup>19</sup> Briefly, osteocalcin promoter-driven SV-40 TAg transgenic mice (OC-TAg) (provided by Dr. Jolene Windle) were used to obtain cementoblasts exclusive from the surrounding periodontal ligament (PDL) fibroblasts.<sup>20</sup> These cells are termed osteocalcin promoter cementoblasts (OC-CM cells). The rationale here is that only those cells expressing osteocalcin, root surface cells/cementoblasts, and not PDL fibroblasts, would be immortalized and therefore survive *in vitro*. In previous studies we demonstrated that *in situ* osteocalcin is expressed by root surface cells, but not by cells within the periodontium.<sup>21</sup> Mice at day 41 of development (day 0, vaginal plug date) were used to obtain cells. Selection of this time point was based on previous studies,<sup>21</sup> where we showed that at day 41 of development in mice, root cementum formation is well established and cells along the root surface, cementoblasts, but not PDL cells express transcripts for bone sialoprotein (BSP) and osteocalcin (OCN), markers associated with cells involved in promoting mineralized tissues. This genotype is maintained when cells are cultured. All procedures involving mice were performed in compliance with regulations administered by the University of Michigan Unit for Lab Animal Medicine.

To obtain root surface cells, mandibular first molars were removed using a dissecting microscope to separate PDL from surrounding alveolar bone and cells released using collagenase/trypsin cocktail.<sup>19,21,22</sup> Cells were maintained in Dulbecco's modified Eagle's medium (DMEM),<sup>#</sup> supplemented with 10% fetal bovine serum (FBS) containing 100 U/ml of penicillin<sup>#</sup> and 100 µg/ml of streptomycin<sup>#</sup> in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. A clonal cell line, OC-CM30 established from the parent OC-CM cell line, was used. This cell population exhibits strong transcripts for both BSP and OCN. Two murine osteoblast cells lines also were used for these studies as comparisons.

OCT-1 cells, (a gift from Dr. D. Chen) are a preosteoblast cell line generated from calvaria of OC-TAg mice.<sup>20</sup> These cells were selected to control for possible variations in response of cells obtained from OC-TAg mice regardless of the tissue from which the cells were generated. These cells require BMP 2 in order to differentiate along the osteoblast pathway. For studies here, cells were maintained in culture using the same media as for OC-CM cells and BMP was not included. The other cell line selected, MC3T3-E1, is osteoprogenitor cells derived from new born calvaria that are known to differentiate along the osteoblast pathway in

¶ Emdogain, Biora AB, Malmo, Sweden.

# Gibco, BRL, Gaithersburg, MD.

the presence of ascorbic acid.<sup>23</sup> These cells were selected since they provide an excellent model for determining the ability of factors to regulate osteoblast differentiation.<sup>23-25</sup> Upon exposure to ascorbic acid they express mRNA for OCN and BSP, both markers of active osteoblasts. These cells were maintained in  $\alpha$ -modified Eagle's Medium<sup>#</sup> supplemented with 10% FBS and antibiotics as above and used between passages 10 to 15.

### Cell Proliferation

For proliferation assays, cells were plated in 24-well dishes, at 5,000 cells/ml in triplicate wells and cultured in media described above, in the presence of ascorbic acid. After allowing cells to adhere overnight, media were changed to 2% FBS containing EMD (5 to 100  $\mu$ /ml) with media changed on days 2 and 5.

Cells were harvested on days 2, 5, and 7 and cell number determined by Coulter counter. Appropriate controls included cells cultured in media without EMD, containing 10% FBS, a positive control, or 2% FBS, a negative control, where in initial studies we showed that cells can survive over a 10-day period in 2% FBS, but exhibit minimal cell growth. Experiments were performed on 3 separate occasions.

### Northern Blot Analysis

For analysis of gene expression, cells were plated in 100 mm dishes at an initial density of 50,000 cells per dish. After a 24-hour attachment period, media were changed to media with or without ascorbic acid, with or without 100  $\mu$ g/ml EMD, and with 5% FBS for OC-CM and OCT-1 cells and 2% FBS for MC3T3-E1 cells. The reason for using 5% FBS for OC-CM cells was to parallel the mineralization assays. Early on in these studies we noted that OC-CM cells cultured in 2% FBS expressed low levels of BSP and exhibited limited ability to promote mineral nodule formation. Thus for consistency, while comparable results on gene expression were noted with 2% FBS, results using 5% FBS are shown. RNA was isolated at days 3, 5, and 8 for OC-CM cells, at days 3 and 8 for OCT-1 cells and day 8 for MC3T3-E1 cells, using the procedure of Xie and Rothblum.<sup>26</sup> Briefly, for Northern blot analyses, total RNA was isolated using a guanidinium isothiocyanate procedure and quantified by spectrophotometer. Total RNA (10 to 20  $\mu$ g) was denatured, fractionated, on a 6% formaldehyde, 1.2% agarose gel, transferred to a nylon membrane\*\* and cross-linked by UV irradiation.††

Blots were hybridized with random-primed <sup>32</sup>P radiolabeled probes‡‡ and were exposed to film at -70 °C with intensifying screens for 24 to 72 hours.

Probes used for Northern blots were BSP = M-BSP consists of 1 kb of mouse cDNA in PCR II<sup>27</sup> (a gift from Drs. M. Young and L. Fisher, NIDCR/NIH, Bethesda, Maryland); OPN = MCP-3 consists of 1 kb

of mouse OPN cDNA in CR II<sup>28</sup> (a gift from Drs. M. Young and L. Fisher); OCN = 400 bp of mouse OCN cDNA originally cloned into pSP65 cloning vector was transferred to Bluescript Sk<sup>29</sup> (obtained from Dr. J. Wozney, Genetic Institute, Cambridge, Massachusetts). Ethidium bromide stained gels, 18S, were used to determine relative RNA loading. To normalize for differences in loading, data were quantitated by obtaining relative density of specific RNAs and 18S RNA and expressing data as a ratio of specific RNA to 18S RNA. Experiments were performed at least 2 times.

### Mineralization Assay

**In vitro.** The in vitro mineralization assay was performed on cementoblasts and OCT-1 cells as reported previously.<sup>19,25,30</sup> Cells were plated at  $1.0 \times 10^4$  cells/cm<sup>2</sup> in 24-well plates. Upon confluence, designated day 0, media were removed and cells were incubated in DMEM containing 5% FBS and supplemented with 50  $\mu$ g/ml ascorbic acid and 10mM  $\beta$ -glycerol phosphate,  $\pm$  EMD (5, 50, or 100  $\mu$ g/ml). Samples were processed on day 8 and von Kossa assay performed to detect mineral nodule formation. Experiments were performed 3 times.

**Ex vivo.** The procedures described below were approved by the University of Michigan Committee and the Unit for Laboratory Animal Medicine (ULAM), and were compliant with State and Federal laws as well as the guiding principles of use and care of animals. To avoid host rejection of cell transplants, immunocompromised mice (SCID mice)<sup>§§</sup> were employed to serve as subcutaneous transplant recipients. Previous studies have shown that this animal model provides an excellent environment for supporting formation of mineralized tissues by several implanted cell types, including human bone marrow stromal cells,<sup>31</sup> mouse osteoblastic cells (MC3T3-E1 cells),<sup>32</sup> and human cementum-derived cells.<sup>33</sup> Transplant vehicles were prepared by soaking 3  $\times$  3 mm squares of a mixture of hydroxyapatite and collagen<sup>|||</sup> in DMEM containing 10% FBS for 30 to 45 minutes, followed by compression between filter paper to remove air. Cells treated for 24 or 72 hours with 50  $\mu$ g/ml of EMD in vitro were trypsinized, pelleted, and resuspended to approximately  $2.0 \times 10^6$  cells/per ml. The sponges alone served as negative controls and untreated cells served as positive controls. The compressed sponges were allowed to absorb the cell suspension by capillary action and held at 37°C until implantation. SCID mice were anesthetized using methoxyflurane.<sup>¶¶</sup> Midsagittal incisions were made and

\*\* Darakib-UV, Stratagene, Inc., La Jolla, CA.

†† Stratalinker, Stratagene, Inc.

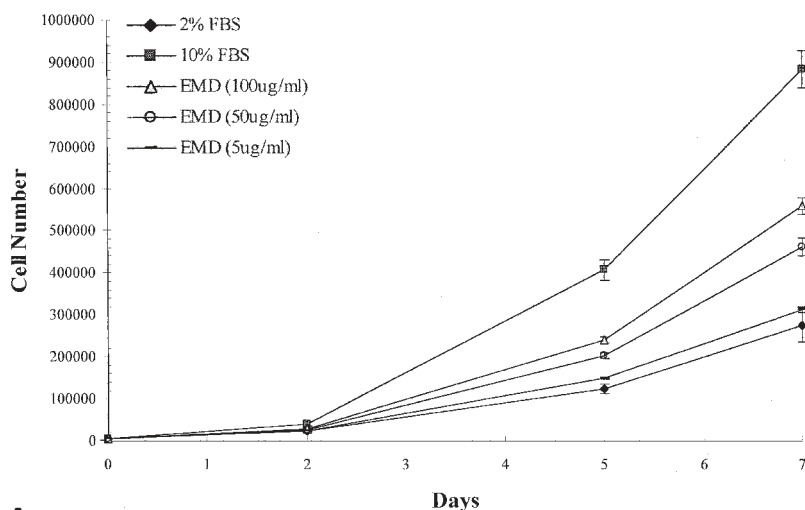
‡‡ Rediprime, Amersham-Pharmacia, Arlington Heights, IL.

§§ C.B-17 SCID, Taconic, Germantown, NY.

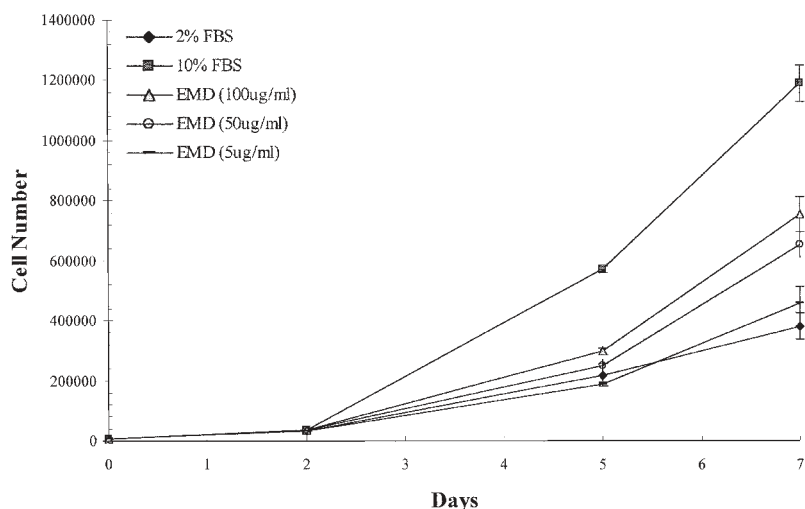
||| Zimmer Corp., Warsaw, IN.

¶¶ Mallinckrodt Veterinary, Mundelein, IL.

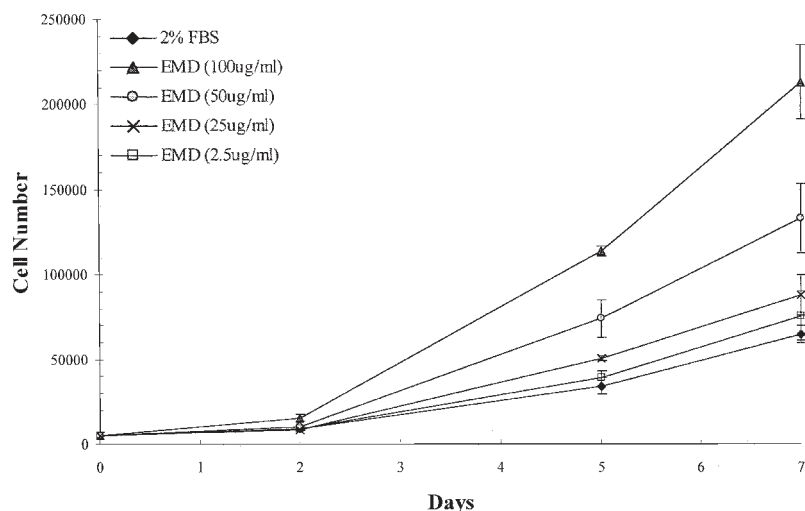
**CEMENTOBLASTS (OC-CM)**



**A**  
**OCT-1**



**B**  
**MC3T3-E1**



**C**

implants inserted into the surgical pockets (4 in 2 animals with 2 on each side) and the sites stapled closed. Eight SCID mice were used. Implants were removed 6 weeks following surgery, fixed using 10% neutral buffered formalin, and embedded in paraffin. Five-micron sections were prepared and stained with hematoxylin and eosin.

**Statistical Analysis**

For statistical analysis, one way analysis of variance (ANOVA) and Tukey Kramer multiple comparison tests were performed.

**RESULTS**

**Cell Proliferation**

As shown in Figure 1 and Table 1, EMD promoted proliferation of all cell types assayed. A dose-response effect was noted, where by day 5, cells treated with 50 or 100 µg/ml EMD exhibited enhanced cell proliferation when compared to 2% FBS control.

**Northern Blot Analysis**

Results for cells treated with EMD are summarized in Table 2, where data are expressed as a ratio of specific RNA to 18S RNA and detailed below, with Northern blots presented in Figures 2 and 3.

**OC-CM Cells**

**Day 3 (Fig. 2A).** Cells exposed to ascorbic acid in the absence of EMD had greater levels of OPN mRNA when compared with non-ascorbic acid treated cells. At day 3, cementoblasts exposed to EMD, -AA, showed a slight increased expression for OPN mRNA when compared to 5% FBS control. In the presence of AA this effect was not apparent and in fact a slight decrease in OPN mRNA was noted. BSP and OCN mRNAs were not detected at this time.

**Day 5 (Fig. 2B).** At day 5, BSP and OCN mRNA expression was apparent in control cells, where AA enhanced expression when compared with cells not exposed to AA. Cells exposed to EMD in the absence of AA exhibited a marked

**Figure 1.** Effect of EMD on cell proliferation. **A.** OC-CM 30: cementoblasts from OC-TAg mice. **B.** OCT-1: preosteoblasts from OC-TAg mice. **C.** MC3T3-E1: preosteoblasts from mouse calvaria. Cells were cultured in media with 2% FBS, 50 µg/ml ascorbic acid, ±EMD. For OCT-1 and OC-CM cells, a 10% FBS control was used. Cell number was determined by Coulter counter on days 2, 5, and 7. Statistical analysis is shown in Table 1.

**Table 1.**  
**Statistical Analysis for Figure 1**

Cementoblasts (OC-CM)	Day 5 (2% FBS + EMD)			Day 7 (2% FBS + EMD)				
	5 ug	50 ug	100 ug	5 ug	50 ug	100 ug		
2%FBS	n.s.	‡	‡	n.s.	†	‡		
+EMD (5ug)		*	‡		*	‡		
+EMD (50ug)			n.s.			n.s.		
Preosteoblasts (OCT-1)	Day 5 (2% FBS + EMD)			Day 5 (2% FBS + EMD)				
	5 ug	50 ug	100 ug	5 ug	50 ug	100 ug		
2%FBS	n.s.	n.s.	‡	n.s.	‡	‡		
+EMD (5ug)		*	‡		‡	‡		
+EMD (50ug)			n.s.			n.s.		
Preosteoblasts (MC3T3-E1)	Day 5 (2% FBS + EMD)				Day 7 (2% FBS + EMD)			
	2.5 ug	25 ug	50 ug	100 ug	2.5 ug	25 ug	50 ug	100 ug
2%FBS	n.s.	*	‡	‡	n.s.	n.s.	†	‡
+EMD (2.5ug)		n.s.	‡	‡		n.s.	†	‡
+EMD (25ug)			†	‡			*	‡
+EMD (50ug)				‡				‡

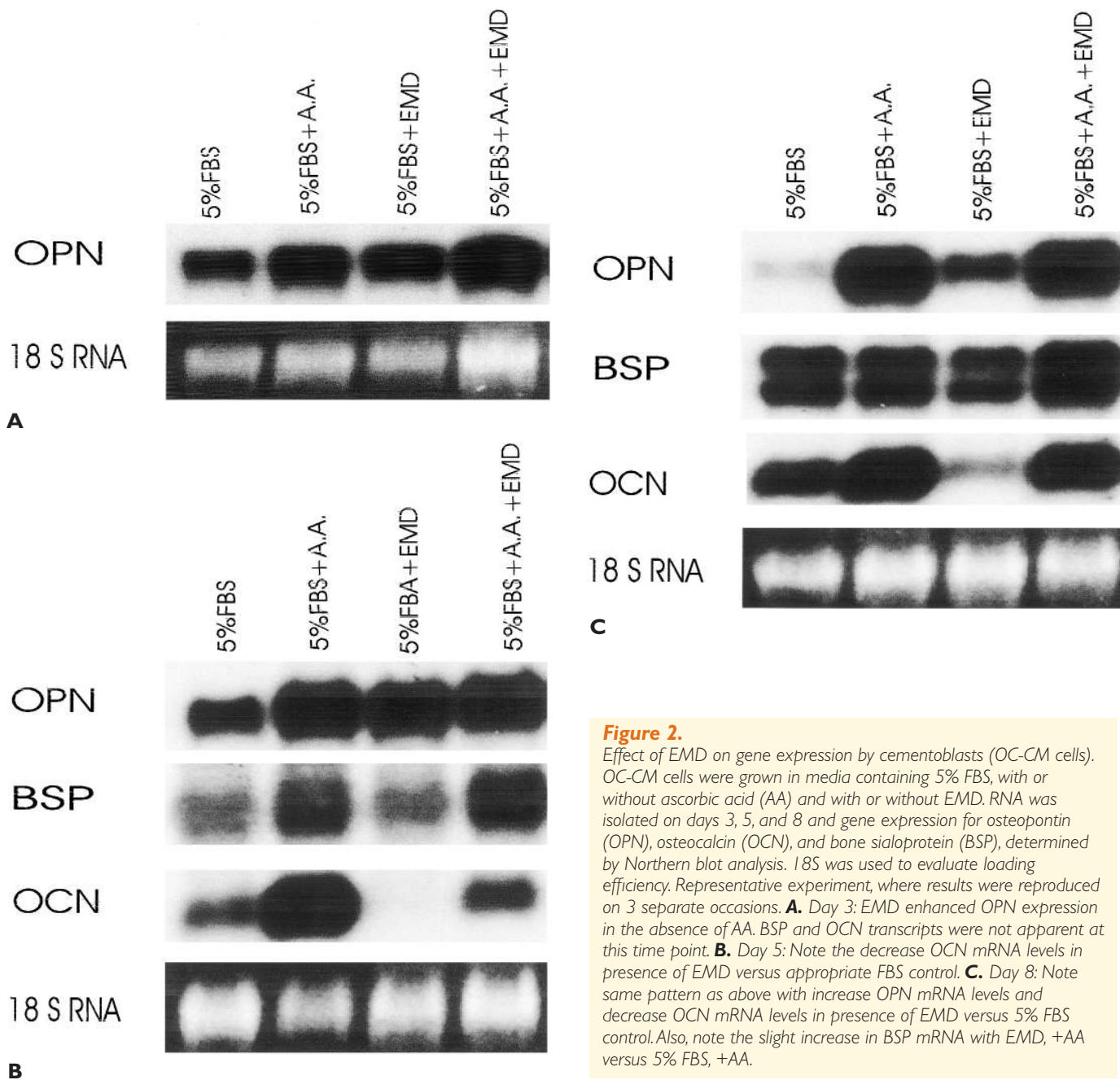
n.s. = not significant; \*  $P < 0.05$ ; †  $P < 0.01$ ; ‡  $P < 0.001$ .

**Table 2.**  
**Effect of EMD on Gene Expression\***

mRNA	OC-CM						OCT-1		MC3T3-E1	
	Day 3		Day 5		Day 8		Day 8		Day 8	
	-A.A.	+A.A.	-A.A.	+A.A.	-A.A.	+A.A.	-A.A.	+A.A.	-A.A.	+A.A.
OPN										
-EMD	1.7	2.7	0.9	2.5	0.3	0.8	3.0	5.0	0.3	0.8
+EMD	2.3	1.8	1.7	2.3	1.7	2.2	2.8	4.4	2.0	2.0
BSP										
-EMD	N.E.†		1.0	2.5	2.5	1.8	N.E.		0.9	2.5
+EMD			1.0	1.7	1.8	2.4			1.5	2.0
OCN										
-EMD	N.E.		0.6	2.8	1.5	1.8	N.E.		0.0	1.5
+EMD			0.0	0.7	0.3	1.8			0.0	0.0

\* Summary of Northern blot analysis shown in Figures 2 and 3. Data expressed as specific RNA/18S RNA ratio. Representative results from one experiment are shown, where experiments were repeated twice for OCT-1 cells and three times for MC3T3-E1 and OC-CM cells with comparable results.

† Not expressed.

**Figure 2.**

Effect of EMD on gene expression by cementoblasts (OC-CM cells). OC-CM cells were grown in media containing 5% FBS, with or without ascorbic acid (AA) and with or without EMD. RNA was isolated on days 3, 5, and 8 and gene expression for osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP), determined by Northern blot analysis. 18S was used to evaluate loading efficiency. Representative experiment, where results were reproduced on 3 separate occasions. **A.** Day 3: EMD enhanced OPN expression in the absence of AA. BSP and OCN transcripts were not apparent at this time point. **B.** Day 5: Note the decrease OCN mRNA levels in presence of EMD versus appropriate FBS control. **C.** Day 8: Note same pattern as above with increase OPN mRNA levels and decrease OCN mRNA levels in presence of EMD versus 5% FBS control. Also, note the slight increase in BSP mRNA with EMD, +AA versus 5% FBS, +AA.

down regulation of OCN mRNA when compared with 5% FBS control. Further, while OCN transcripts were noted in cells treated with EMD, + AA, expression remained lower when compared to 5% FBS, AA control. An increased expression of OPN mRNA was apparent in cells treated with EMD in the absence of AA, when compared to 5% FBS control, similar to that observed at day 3.

**Day 8 (Fig. 2C).** At day 8, the ability of EMD to enhance OPN expression and inhibit OCN expression persisted for cells exposed to EMD, without AA. In addition, a modest increase in BSP mRNA level was noted in cells exposed to EMD in the presence of AA

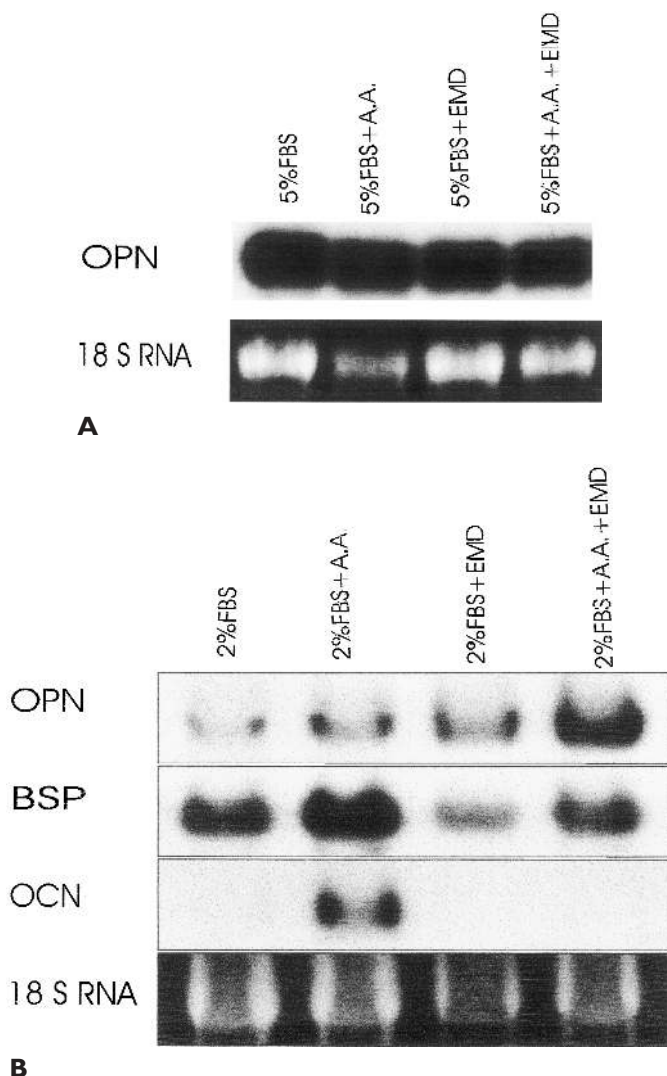
when compared with 5% FBS, AA control and this response was reproducible.

#### OCT-1 Cells (Fig. 3A)

At all time points, cells with or without AA and/or EMD did not express transcripts for BSP or OCN (data not shown). At day 8 no significant differences were noted in OPN transcripts between EMD treated versus untreated cells.

#### MC3T3-E1 Cells (Fig. 3B)

Northern blot analysis was done at day 8 only. As seen in Figure 3B, EMD blocked AA mediated induction of OCN



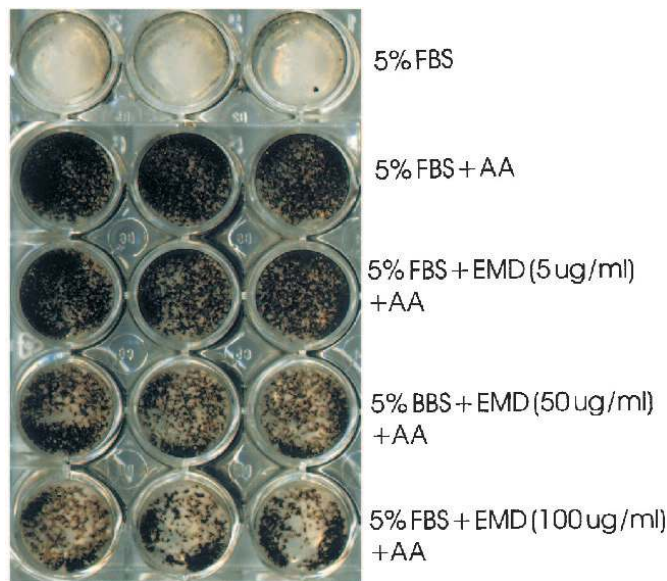
**Figure 3.**

Effect of EMD on gene expression in osteoblast cell lines. **A.** Effect of EMD on gene expression by OCT-1 cells (OC-Tag preosteoblasts). OCT-1 cells were grown in media containing 5% FBS,  $\pm$ AA,  $\pm$ EMD. RNA was isolated on day 8 and gene expression for OPN, OCN, and BSP determined by Northern blot analysis. 18S was used to evaluate loading efficiency. Under conditions used here, neither BSP mRNA nor OCN mRNA were detected (data not shown). EMD had no significant effect on OPN expression at this time point. **B.** Effect of EMD on gene expression by MC3T3-E1 cells (mouse preosteoblasts). MC3T3-E1 cells were grown in media containing 5% FBS,  $\pm$ EMD,  $\pm$ AA. RNA was isolated on day 8 and gene expression for OPN, BSP, and OCN determined by Northern blot analysis. 18S was used to evaluate loading efficiency. Note that EMD decreased gene expression for OCN, but increased OPN mRNA levels when compared with respective controls.

gene expression, while OPN transcripts were increased in EMD treated cells compared to untreated cells.

#### Mineralization: (Figs. 4 and 5)

**In vitro (Fig. 4).** For these studies only OC-CM and OCT-1 cells were examined. As shown in Figure 4,



**Figure 4.**

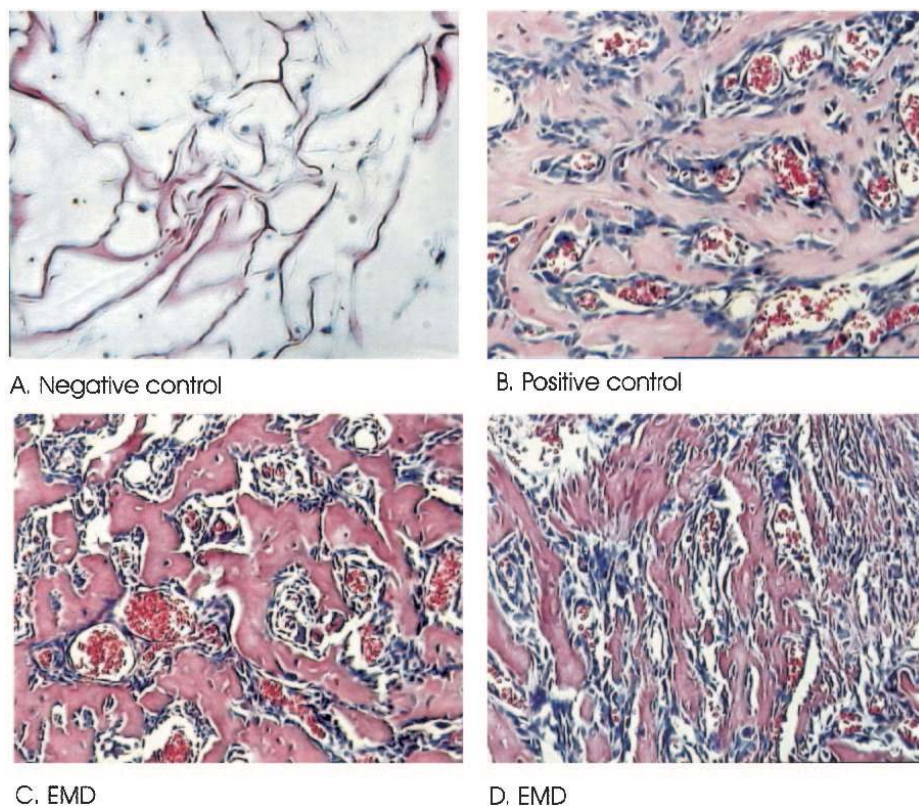
*In vitro* mineralization. Effect of pretreatment of cementoblasts with EMD on biomineralization *in vitro*. For these studies, cells were cultured in media containing 5% FBS, 50  $\mu$ g/ml AA, 10mM  $\beta$ -glycerophosphate and EMD at 5, 50, or 100  $\mu$ g/ml. On day 8, von Kossa stain was used to determine extent of mineral nodule formation. EMD inhibited OC-CM mediated mineral nodule formation, in a dose-dependent fashion, where at 5  $\mu$ g/ml EMD had no inhibitory effect, but 50  $\mu$ g/ml EMD and 100  $\mu$ g/ml EMD had an inhibitory effect on mineral formation.

EMD inhibited OC-CM mediated mineralization *in vitro* in a dose-response fashion. At 5  $\mu$ g/ml of EMD mineral nodule formation was comparable to control cells treated with AA plus  $\beta$ -glycerophosphate, however, at 50  $\mu$ g/ml and at 100  $\mu$ g/ml mineral nodule formation was decreased but not blocked. OCT-1 cells did not promote mineral formation under any of the conditions examined (data not shown).

**In vivo (Fig. 5).** When OC-CM cells were first treated with EMD and then transplanted into SCID mice, they were able to promote mineral nodule formation (Fig. 5C and D). Interestingly, when compared with sections obtained from cells treated with 2% or 10% serum without EMD prior to transplantation (Fig. 5B), approximately 50% of the sections, obtained from cells treated with EMD for 24 or 72 hours prior to transplantation, exhibited areas with a greater cell number to mineral tissue ratio (Fig. 5D). The pattern of cell-mineral formation shown in Figure 5D was never observed in sections obtained from cells treated with 2% or 10% FBS.

#### DISCUSSION

The results presented here indicate that EMD has the ability to promote a variety of activities in mesenchymal derived cells. Specifically, these studies focused on the interaction of cementoblasts with EMD, but 2 osteoblast cell lines also were examined as a com-



**Figure 5.**

*In vivo* mineralization. Effect of pretreatment of cementoblasts with EMD on biomineralization, *in vivo*. For these studies cells were pretreated with 50  $\mu\text{g/ml}$  EMD for 24 or 72 hours and then seeded on sponges and transplanted subcutaneously in SCID mice. Grafts were retrieved 6 weeks later, fixed and 5  $\mu$  sections, prepared, and H&E stained. **A.** Graft only control. **B.** OC-CM cells, in 10% FBS. Note mineral formation. **C.** OC-CM cells pretreated with EMD for 24 hours prior to transplantation. Note mineral formation. **D.** OC-CM cells pretreated with EMD for 24 hours prior to transplantation. Note mineral formation; however, when compared with B or C, an increase in cell to mineral ratio was noted. This finding was observed in approximately 50% of the tissues obtained from cells pretreated with EMD for 24 hours (shown here), as well as 72 hours (data not shown), but not in cells pretreated with 10% FBS (Fig. 5B) or 2% FBS (data not shown).

parison to cementoblasts. Importantly, and as reported previously for human PDL cells,<sup>18</sup> EMD promoted proliferation of all 3 cell lines. In addition, EMD altered gene expression of all cell types, but the pattern of change was different for each cell type. EMD inhibited cementoblast-mediated mineral nodule formation *in vitro*, while mineral formation was observed in transplants obtained from SCID mice, when cells were pretreated with EMD *in vitro* prior to transplantation.

Based on these findings, as well as past studies, it is clear that EMD promotes cell proliferation, and thus has properties similar to growth factors such as platelet-derived growth factor (PDGF). Previously, we explored the possibility that EMD may contain growth factors known to mediate proliferation of PDL cells; e.g., PDGF and IGF and others, and based on the immunoassays used, these molecules were not identified in EMD.<sup>18</sup> At this point, we do not know the mechanism by which EMD promotes cell proliferation,

but the possibility that amelogenin itself or other as yet to be identified molecules present in EMD work through growth factor associated receptors such as receptor tyrosine kinases and serine-threonine receptor kinases needs to be explored.<sup>34,35</sup> Regardless of the mechanism, factors that promote proliferation of cells within a wound healing would be advantageous for providing a critical mass of cells required for synthesizing the necessary regenerative matrix.

Beyond the effect of EMD on cell proliferation, evidence from the *in vitro* models suggest that EMD may alter gene expression when used for regenerative therapies. The most dramatic effect of EMD was on OCN gene expression. EMD decreased OCN expression in both the cementoblast cell line and the preosteoblast cell line, MC3T3-E1 cells. The ability of an agent that promotes proliferation to decrease expression of genes associated with differentiation is not surprising. For example, evidence from several *in vitro* studies indicate that the strong mitogen, PDGF, promotes osteoblast proliferation, but decreases expression of osteoblast-associated genes, *in vitro*.<sup>36-39</sup>

Cementoblasts exposed to EMD, +AA for 8 days, exhibited a modest increase in BSP gene expression when compared with 5% FBS, +AA control. In contrast, EMD did not alter BSP

transcripts in 8-day cultured MC3T3-E1 cells. The reason for this dissimilarity in response to EMD is most likely related to differences in stage of cell maturation versus differences in response of osteoblasts versus cementoblasts to EMD. We also determined the effect of EMD on OPN gene expression, where OPN gene and product are found in high concentrations in bones and teeth.<sup>40</sup> By day 8, both MC3T3-E1 cells and cementoblasts, when exposed to EMD, exhibited an increase in OPN mRNA levels.

While these are *in vitro* results, these data suggest that EMD can alter genes associated with cementoblast and osteoblast maturation. In examining the genes that were decreased by EMD, it is interesting to note that the most dramatic effect was on OCN mRNA levels. Osteocalcin, based on time of expression, appears to play a role in early phases of mineralization, but in addition, may also regulate extent of crystal growth.<sup>41-43</sup> Similarly, OPN also appears to regulate extent of crystal growth and



in addition promotes migration and attachment of both osteoblasts and osteoclasts to sites of bone remodeling.<sup>40,44,45</sup> However, unlike EMD's inhibitory effect on OCN transcripts, EMD promoted expression of OPN mRNA, *in vitro*. Thus, the ability to regulate crystal growth, and hence overgrowth of mineralized tissues, may be a function of EMD. This function may be coupled with EMD's ability to maintain or slightly enhance BSP transcripts. BSP is a mineral-associated protein that promotes attachment of both osteoblasts and osteoclasts and, in cell-free systems, acts as a nucleator of hydroxyapatite crystal formation.<sup>41,46</sup>

Conceivably, EMD could act by first promoting cell proliferation, thereby providing a sufficient cell population to synthesize the appropriate extracellular matrix. Having the critical cell mass, EMD may then act by regulating the level of mineralization versus soft connective tissue formation (i.e., PDL formation).

To determine the effects of EMD on cementoblast-mediated mineralization, *in vitro* and *in vivo* assays were used. In the *in vitro* model, EMD inhibited cementoblast-mediated mineral nodule formation in a dose-response fashion. This is in contrast to previous studies by Gestrelus et al.,<sup>18</sup> where EMD was shown to promote PDL-mediated mineral formation. There are several possibilities for these discrepancies, including differences in cell types and experimental design between these studies. In the studies here, cementoblasts were cultured in the presence of AA,  $\beta$ -glycerophosphate  $\pm$  EMD. In contrast, in the previous studies with PDL cells dexamethasone was included in the medium. Further, cementoblasts promote mineral nodule formation within 8 days, while for PDL cells, 21 days is required. Therefore, *in vitro* it is not surprising that such differences in EMD's effects on mineralization were noted. While EMD decreased OC-CM mediated-mineral formation *in vitro*, when cells were first treated with EMD and then implanted subcutaneously in SCID mice, cells pretreated with EMD retained the ability to promote mineral formation. Conceivably, EMD *in vivo* may provide the environment necessary to promote regeneration by enhancing cell proliferation. While more studies are needed to confirm this, increased cellularity noted in some sections obtained from implants where cells were pretreated with EMD versus sera treated control cells (Fig. 5B versus 4D), supports this concept.

With regard to the reported effects of EMD on mineralization, it is interesting to review the effects of amelogenin on hydroxyapatite formation, where there exists evidence that both amelogenin and enamel play a role in initiation of mineralization,<sup>47-51</sup> while others indicate that amelogenin has no specific crystal-modulating properties.<sup>52</sup> Possible explanations for differences noted include assay systems and purity, species and nature of the amelogenin used; i.e., in the first 2 cases

bovine and porcine amelogenins were used, while in the latter situation a recombinant mouse amelogenin was used.<sup>51-53</sup> Furthermore, amelogenin is processed rapidly during amelogenesis, where studies in pigs have shown that ameloblasts secrete a 25KDa nascent protein into the newly formed enamel which is processed into smaller fragments (23K, 20K, 13K) during maturation.<sup>54</sup> The exact role of these individual fragments remains unknown, but may explain, in part, differences in activities of amelogenin reported by different laboratories. The EMD used for the studies here is of porcine origin. However, whether amelogenin was the factor responsible for inhibition of mineralization needs further investigation.

Based on the results summarized here and on data from clinical studies using EMD, it is clear that EMD can have a positive effect on periodontal tissues. However, clinical results are often not predictable. While it is possible to imagine that EMD would be well suited for slow-delivery therapy where first phase release would bring the critical mass of cells to the healing site and, in the second phase, EMD and/or other factors in combination would act to regulate expression of genes and their products. Continued studies targeted at defining the factor(s) in EMD responsible for promoting cell proliferation and altering gene expression; determining the signaling pathways triggered by EMD; determining whether EMD influences expression of metalloproteinases; and designing appropriate delivery systems for EMD, as well as other factors, will enable us to design more predictable clinical therapies based on sound biological principles.

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