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ORIGINAL ARTICLE

Effect of coating Straumann® Bone Ceramic with Emdogain on mesenchymal stromal cell hard tissue formation

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Abstract Periodontal tissue engineering requires a suitable biocompatible scaffold, cells with regenerative capacity, and instructional molecules. In this study, we investigated the capacity of Straumann® Bone Ceramic coated with Straumann® Emdogain, a clinical preparation of enamel matrix protein (EMP), to aid in hard tissue formation by post-natal mesenchymal stromal cells (MSCs) including bone marrow stromal cells (BMSCs) and periodontal ligament fibroblasts (PDLFs). MSCs were isolated and ex vivo-expanded from human bone marrow and periodontal ligament and, in culture, allowed to attach to Bone Ceramic in the presence or absence of Emdogain. Gene expression of bone-related proteins was investigated by real time RT-PCR for 72 h, and ectopic bone formation was assessed histologically in subcutaneous implants of Bone Ceramic containing MSCs with or without Emdogain in NOD/SCID mice. Alkaline phosphatase activity was also assessed in vitro, in the presence or absence of Emdogain. Collagen-I mRNA was up-regulated in both MSC populations over the 72-h time course with Emdogain. Expression of BMP-2 and the osteogenic transcription factor Cbfa-1 showed early stimulation in both MSC types after 24 h. In contrast, expression of BMP-4 was consistently down-regulated in both MSC types with Emdogain. Up-regulation of osteopontin and periostin mRNA was restricted to BMSCs, while higher levels of bone sialoprotein-II were observed in PDLFs with Emdogain. Furthermore, alkaline phosphatase activity levels were reduced in both BMSCs and PDLFs in the presence of Emdogain. Very little evidence was found for ectopic bone formation following subcutaneous implantation of MSCs with Emdogain-coated or -uncoated Bone Ceramic in NOD/SCID mice. The early up-regulation of several important bone-related genes suggests that Emdogain may have a significant stimulatory effect in the commitment of mesenchymal cells to osteogenic differentiation in vitro. While Emdogain inhibited AP activity and appeared not to induce ectopic bone formation, longer-term studies are required to determine whether it promotes the final stages of osteoblast formation and mineralization at gene and protein levels. While used in clinical applications, whether Emdogain and other commercial preparations of EMPs truly possess the capacity to induce the regeneration of bone or other components of the periodontium remains to be established.

Keywords Straumann® Emdogain · Straumann® Bone Ceramic · Bone marrow stromal/stem cells · Periodontal ligament stem cells

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Introduction

With improving understanding of the molecular processes associated with tissue repair and regeneration, polypeptide

growth factors applied to root surfaces have been used to facilitate periodontal regeneration [1, 2]. To date, these have included epidermal growth factor, fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor, tumor-derived growth factor, and bone morphogenetic proteins [1, 3, 4]. Combinations of growth factors, such as those present in platelet-rich plasma preparations, may be useful in promoting periodontal regeneration [5, 6]. However, the current literature concerning the clinical outcomes using such combinations is still scant. Although not a growth factor per se, the enamel matrix derivative Emdogain has shown considerable promise for the regeneration of periodontal defects when applied to the root surface during periodontal surgery [7–9].

Key factors in attaining successful periodontal regeneration are the correct recruitment of cells to the site and the production of a suitable extracellular matrix consistent with the periodontal tissues. Tissue engineering, aimed at developing techniques for the fabrication of new tissues to replace damaged or diseased tissues, is based on principles of cell biology, developmental biology, and biomaterials [10, 11]. Recent advances in growth factor biology and biodegradable polymers have set the stage for successful tissue engineering of cartilage, bone, and other tissues, of which the periodontium could be considered a prime candidate for such procedures. Studies to date have shown that periodontal ligament cells can be transplanted into periodontal defects and result in tissue regeneration with no adverse immunologic or inflammatory consequences [12–14]. The immunomodulatory effects of mesenchymal stromal cells (MSCs) have been reported recently and could account for these favorable regenerative outcomes [15, 16]. Thus, a tissue engineering strategy for periodontal regeneration that exploits the regenerative capacity of MSCs residing within the periodontium is an attractive thesis [17–19]. By using such an approach, the need for recruitment of cells to the site is negated, and the predictability of the outcome may be enhanced.

It now seems likely that a combination of several regenerative techniques may offer the most likely chance of success for periodontal regeneration. Through a combination of transplanted biomaterials containing appropriately selected and primed cells, together with an appropriate mix of regulatory factors and extracellular matrix components to allow growth and specialization of the cells, new therapies such as tissue engineering are emerging to be of significant clinical potential [11]. Therefore, the purpose of the present study was to investigate the capacity of a commercially available, fully synthetic particulate HA-TCP, Straumann® Bone Ceramic, coated with Straumann® Emdogain, to aid in hard tissue formation by MSC populations including bone marrow stromal cells (BMSCs) and periodontal ligament fibroblasts (PDLFs).

Methods

Cell culture

Human BMSCs were isolated and cultured according to previous reports [20]. Human PDLFs were isolated from pre-molars extracted for orthodontic purposes as described previously [17]. Primary cultures were maintained in alpha modification Minimum Essential Medium Eagle (α-MEM; Sigma-Aldrich Inc., St. Louis, MO) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine (Sigma-Aldrich Inc.), 100 μM L-ascorbate-2-phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1 mM sodium pyruvate (Sigma-Aldrich Inc.), 50 U/ml penicillin G (JRH Biosciences, Inc., Lenexa, KS), and 50 μg/ml streptomycin (JRH Biosciences, Inc.) in a humidified atmosphere (37°C, 5% CO_2). For ex vivo expansion, adherent cells at 70% confluence were rinsed once with Hanks' Balanced Salt Solution (HBSS; Sigma-Aldrich Inc.) and liberated by enzymatic digestion using 3 ml 0.05% Trypsin/EDTA solution per 75 cm^2 tissue culture flask for 5 min at 37°C. Detached cells were washed in HBSS with 5% FCS and then re-plated in α-MEM with supplements.

Flow cytometry

To characterize the immunophenotype of BMSCs and PDLFs, ex vivo-expanded cells were trypsinized from tissue flasks as described above, washed twice in HBSS supplemented with 5% FCS, and incubated in blocking buffer (HBSS supplemented with 20 mM Hepes), 1% normal human AB serum, 1% bovine serum albumin (BSA; Cohn fraction V, Sigma-Aldrich Inc.), and 5% FCS for 30 min on ice. Aliquots of 2×10^5 BMSCs or PDLFs were incubated with primary murine monoclonal antibodies specific to MSC-associated markers (CD44 [clone H9H11, IgG_1; kindly provided by Prof. A.C.W. Zannettino, Division of Haematology, Institute of Medical and Veterinary Science, Adelaide, Australia], CD73 [IgG_1; BD Biosciences, San Jose, CA], CD105 [IgG_1; Becton Dickinson], CD146 [Clone CC9, IgG_{2a}] [21], and STRO-4 [HSP90β, IgG_1] [22]), hematopoietic markers (CD14 [IgG_2; Beckman Coulter Inc., Brea, CA], CD34 [IgG_1; Beckman Coulter

Inc.] and CD45 [IgG_1; Beckman Coulter Inc.]) or isotype-matched controls (IgG_1 [clone 1B5] and IgG_{2a} [clone 1D4.5] kindly provided by Prof. L.K. Ashman, University of Newcastle, Newcastle, Australia) at 20 μg/ml for 1 h on ice. Cells were then washed twice in 1 ml of HBSS with 5% FCS and incubated with secondary detection reagent; goat anti-mouse IgG-FITC conjugated antibody (1:50, Southern Biotechnology, Birmingham, AL) for 45 min on ice. The cells were then washed in 1 ml of HBSS with 5% FCS and fixed in 500 μl of FACS Fix solution. Analysis was performed on a fluorescence-activated cell sorter fitted with a 250 MW argon laser (Beckman Coulter Cytomics FC500), using CXP Cytometry List Mode Data Acquisition and Analysis Software version 2.2 (Beckman Coulter, Miami, FL). Positivity for each antibody was defined as the level of fluorescence greater than 99% of what was observed with isotype-matched control, non-binding control antibodies were used.

Differentiation assays

Osteogenic differentiation and mineralization was induced as previously described [23, 24]. Briefly, BMSCs and PDLFs at passage 4 were seeded at $8\times10^3/cm^2$ in 24-well plates and cultured in α-MEM supplemented with 5% FCS, 100 μM L-ascorbate-2-phospahte, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin, dexamethasone (10^{-7} M; Mayne Pharma, Mulgrave, VIC, Australia), and 1.8 mM inorganic phosphate (KH_2PO_4, BDH Chemicals, Poole, UK) for 28 days with media changed twice weekly. Mineral deposit formation was visualized by Alizarin Red (Alizarin Red S; Sigma-Aldrich) staining. Adipogenesis was induced as previously described [24, 25]. Briefly, BMSCs and PDLFs at passage 4 were seeded at $8\times10^3/cm^2$ in 24-well plates and cultured for 28 days in α-MEM with supplements (as described above) in the presence of 0.5 μM hydrocortisone (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich) and 0.5 mM IBMX (3-isobutyl-1-methyl-xanthine; Sigma-Aldrich) with media changed twice weekly. Formation of lipid-laden fat cells was demonstrated by Oil Red O (MP Biomedicals, Solon, OH) staining.

Real-time reverse transcription-PCR

Bone Ceramic (40 μg; 400–700 μm; purchased from Straumann AB, Switzerland; Lot Numbers: 1036; F1203, F5860, F6846; F68226, G9806, G9811) was pre-washed in α-MEM while rotating for 1 h at 37°C (2 ml α-MEM per 40 μg lot of Bone Ceramic) and then added to each well of six 6-well culture plates. To half the wells, 0.1 ml Emdogain alginate (purchased from Straumann AB, Switzerland; Lot Numbers 1134, E8235, F6826, F3752, J8226) was added and gently mixed to coat the Bone Ceramic (3 mg/ml Emdogain alginate final). To each well, $1.0–2.5\times10^5$ ex vivo-expanded BMSCs or PDLFs were then added in 1 ml α-MEM culture medium (described above) to achieve 80% confluency after 24, 48, and 72 h. At 24, 48, and 72 h, triplicate wells of BMSCs or PDLFs seeded into Emdogain-coated or -uncoated Bone Ceramic were rinsed three times in phosphate-buffered saline and RNA extracted using the TRIzol extraction method (Invitrogen Life Technologies, CA) according to the manufacturer's recommendations. Isolated RNA was then subjected to reverse transcription using Oligo dT primer and Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Real-time reverse transcription-PCR was performed using RT^2 SYBR Green/ROX qPCR Master Mix (SuperArray Bioscience, Frederick, MD) and Rotor-Gene 6000 Real Time Thermal Cycler (Corbett Research, Sydney, Australia) (50°C/2 min; 95°C/15 min [95°C/15 s; 60°C/26 s; 72°C/10 s—40 cycles]; 72°C/3 min). Bone- and periodontal ligament-related primer sets used in this study include:

β-actin (157 bp)	5′-GAT CAT TGC TCC TCC TGA GC-3′
NM_001101	5′-GTC ATA GTC CGC CTA GAA GCA T-3′
Alkaline phosphatase (134 bp)	5′-CGT GGC TAA GAA TGT CAT CAT GTT-3′
NM _000478	5′-AGG GGA ACT TGT CCA TCT CC-3′
Bone morphogenetic protein-2 (200bp)	5′-TCA AGC CAA ACA CAA ACA GC-3′
NM_001200	5′-ACG TCT GAA CAA TGG CAT GA-3′
Bone morphogenetic protein-4 (188 bp)	5′-CTT TAC CGG CTT CAG TCT GG-3′
NM_001202	5′-GGG ATG CTG CTG AGG TTA AA-3′
Bone sialoprotein-II (123 bp)	5′-ATG GCC TGT GCT TTC TCA ATG-3′
NM_004967	5′-AGG ATA AAA GTA GGC ATG CTT G-3′
CBFA-1 (137 bp)	5′-GTG GAC GAG GCA AGA GTT TCA-3′
NM_001024630	5′-CAT CAA GCT TCT GTC TGT GCC-3′
Collagen-1 (225 bp)	5′-AGG GTC CCA ACG AGA TCG AGA TCC G-3′
NM_000088	5′-TAC AGG AAG CAG ACA GGG CCA ACG TCG-3′
Osteocalcin (257 bp)	5′-ATG AGA GCC CTC ACA CTC CTC G-3′
NM_199173	5′-GTC AGC CAA CTC GTC ACA GTC C-3′
Osteopontin (92 bp)	5′-GCA GAC CTG ACA TCC AGT ACC-3′
NM_001040060	5′-GAT GGC CTT GTA TGC ACC ATT C-3′
Periostin (239 bp)	5′-GAT GGA GTG CCT GTG GAA AT-3′
NM_006475	5′-AAC TTC CTC ACG GGT GTG TC-3
PLAP-1 (176 bp)	5′-TCGAAAATGGGAGTCTTGCT-3′
NM_017680	5′-CTTTGGCACTGTTGGACAGA-3′

Amplified products were further analyzed by electrophoresis on a 1.5% (*w*/*v*) agarose gel and visualized by ethidium bromide staining. β-actin expression was used as

an internal control of RNA integrity and efficiency of the reverse transcription process.

Alkaline phosphatase activity assay

BMSCs and PDLFs each derived from two different donors were plated at 8,000 cells/well in a 96-well plate and maintained in either α-MEM culture medium (described above) or osteogenic inductive media (α-MEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 μM L-ascorbate-2-phosphate, dexamethasone 10^{-7} M and 3 mM inorganic phosphate). After 24 h, Emdogain was added to triplicate wells at 0.75, 1.5, and 3 mg/ml following dilution in α-MEM culture medium or osteogenic inductive media. α-MEM culture medium and osteogenic inductive media without Emdogain was used as a control, and all media were changed twice weekly. After 7 and 14 days, cells were rinsed three times in PBS and then lysed for 10 min in 100 mM Tris base with 1% Triton-X100. Alkaline phosphatase activity (AP activity) was determined in the lysate by measuring the release of *p*-nitrophenol using SIGMAFAST™ *p*-nitrophenyl phosphate (Sigma) as a substrate after 45 min at 37°C. Absorbance was measured at 405 nm using a Microplate Reader (EL808, BioTek Instruments, Inc., Winooski, VT).

Subcutaneous implantation into immunocompromised (NOD/SCID) mice and recovery

Ex vivo-expanded BMSCs or PDLFs ($2–4\times10^6$) were added and mixed with Emdogain-coated or -uncoated pre-washed Bone Ceramic particles (40 μg per lot) for 2 h while rotating at 37°C in α-MEM for cell attachment. Duplicate test and control samples were then loaded into gamma-irradiated needle-pricked gelatin capsules (size 5; Pfizer Australia, West Ryde, NSW, Australia) and subcutaneously implanted into the dorsal surface of 6-week old NOD/SCID mice as previously described [17, 21]. Negative controls consisted of Emdogain-coated and-uncoated Bone Ceramic without MSCs. Positive controls consisted of MSCs attached to Zimmer hydroxyapatite/tricalcium phosphate (HA/TCP; 40 μg per lot, Warsaw, IN) (Table 1). Procedures were performed in accordance to specifications of an approved animal protocol with institutional animal ethics approval (Institute of Medical and Veterinary Science, Animal Ethics Committee # 33/05). Two non-identical implants were placed per mouse. The transplants were recovered at 6–8 weeks post-transplantation, fixed with 4% formalin for 2 days, decalcified for 10 days in buffered 10% EDTA (pH 8.0), and then embedded in paraffin. Sections were then deparaffinized and stained with hematoxylin and eosin (H&E) for assessment of hard tissue (bone/cementum) formation.

Table 1 Experimental groups of subcutaneous transplants in NOD/SCID mice

Group number	Transplant
1	Straumann® Bone Ceramic alone
2	Straumann® Bone Ceramic + Emdogain
3	Straumann® Bone Ceramic + PDLF
4	Straumann® Bone Ceramic + BMSCs
5	Straumann® Bone Ceramic + Emdogain + PDLF
6	Straumann® Bone Ceramic + Emdogain + BMSC
7	Zimmer HA/TCP + PDLF
8	Zimmer HA/TCP + BMSC

Statistical analysis

Statistical analysis was performed using the paired Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Results

Characterization of BMSCs and PDLFs

Fluorescence-activated cell sorting (FACS) analysis was performed to characterize the phenotype of BMSCs and PDLFs following ex vivo expansion. Both populations demonstrated a similar expression profile of MSC-related markers CD44, CD73, CD105, CD146, and STRO-4 (HSP-90β) and negativity for hematopoietic markers CD14, CD34, and CD45 as previously described [15, 22, 26, 27]. The ability of BMSCs and PDLFs to undergo osteogenic and adipogenic differentiation was also assessed following 4 weeks of culture in inductive media (Figs. 1 and 2). BMSCs and PDLFs exhibited the capacity to form both alizarin red-positive mineralized nodules and oil red O-positive lipid clusters similar to previous findings [17, 24].

Genes up-regulated in BMSCs and PDLFs co-cultured with Bone Ceramic and Emdogain

Collagen-I gene expression was up-regulated with Emdogain at all time-points in PDLFs and after 48 and 72 h in BMSCs (Fig. 3a, b). Bone morphogenetic protein-2 (BMP-2) mRNA expression was up-regulated more than tenfold in both BMSCs and PDLFs in the presence of Emdogain after 24 h. While the up-regulation in BMSCs was sustained at later time-points, albeit at much lower levels, the up-regulation in PDLFs was diminished after 72 h (Fig. 3c, d). Similarly, up-regulated expression of mRNA for the osteogenic transcription factor core binding

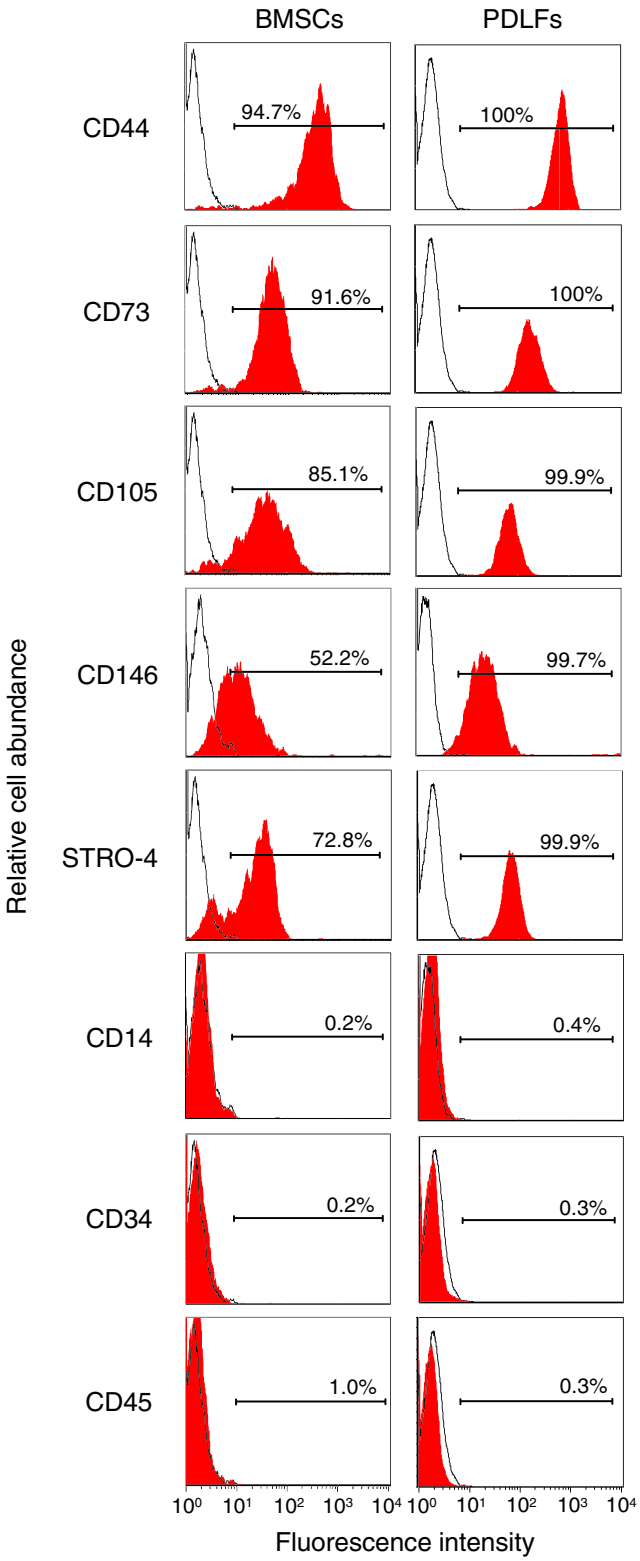

Fig. 1 Flow-cytometric expression of MSC-like markers CD44, CD73, CD105, CD146, and STRO-4 and hematopoietic markers CD14, CD34, and CD45 in BMSCs and PDLFs (*solid histogram*). Corresponding isotype controls IgG_1 and IgG_{2a} are shown (*lined histogram*)

factor alpha-1 (Cbfa-1) was noted in both BMSCs and PDLFs after 24 h with Emdogain. However, sustained up-regulation after 72 h was only observed in BMSCs (Fig. 3g, h). Bone Sialoprotein-II (BSP-II) expression was consistently up-regulated in PDLFs co-cultured with Emdogain at each time point. In contrast, BSP-II expression was higher in BMSCs with Emdogain after 24 h but was down-regulated at later time-points (Fig. 3i, j). Expression levels of osteopontin (OPN) mRNA were consistently higher in BMSCs treated with Emdogain over

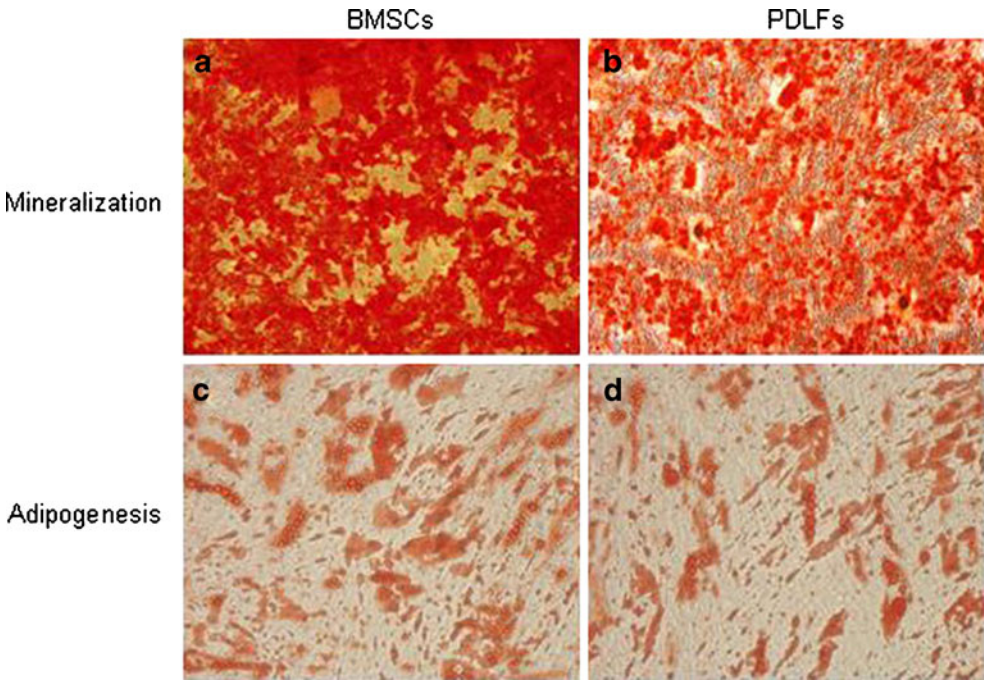

Fig. 2 Differentiation capacity of BMSCs and PDLFs after 4 weeks in osteogenic (**a** and **b**) and adipogenic (**c** and **d**) induction media. Representative images of alizarin red-stained mineralized nodules (×4 magnification) and oil red O-stained lipid globules are shown (×40 magnification)

the time course, while levels in PDLFs were higher after 72 h (Fig. 3k, l). Osteocalcin (OCN) expression was elevated in PDLFs with Emdogain after 48 and 72 h (Fig. 3n). Despite initial down-regulation in PDLFs, alkaline phosphatase expression steadily increased over the time course and was significantly up-regulated after 72 h with Emdogain (Fig. 3p). Expression of periostin mRNA was also found to be higher in BMSCs co-cultured with Emdogain at each time point. Notably, levels of periostin were strongly up-regulated at each time point in BMSCs compared to PDLFs with or without Emdogain (Fig. 3q, r). A late up-regulation in PLAP-1 mRNA expression was observed in PDLFs with Emdogain treatment (Fig. 3t).

Genes down-regulated in BMSCs and PDLFs co-cultured with Bone Ceramic and Emdogain

Expression of bone morphogenetic protein-4 (BMP-4) mRNA was consistently down-regulated in both BMSCs and PDLFs co-cultured with Emdogain after 24, 48, and 72 h (Fig. 3e, f). Expression of alkaline phosphatase mRNA was lower in BMSCs at the 24 and 72 h time-points with Emdogain (Fig. 3o). Alkaline phosphatase mRNA was similarly down-regulated in PDLFs after 24 h; however, levels increased over the remaining time-points as previously mentioned (Fig. 3p). In contrast to increased OCN mRNA in PDLFs, expression levels were consistently down-regulated in BMSCs with Emdogain (Fig. 3m, n). Periodontal ligament associated protein-1 (PLAP-1) mRNA expression was also significantly lower in BMSCs after 24 and 72 h with Emdogain (Fig. 3s). Despite initial up-regulation, Cbfa-1 mRNA in PDLFs steadily decreased over the time course in the presence of Emdogain and was significantly down-regulated after 72 h (Fig. 3h).

Genes unchanged in BMSCs and PDLFs co-cultured with Bone Ceramic and Emdogain

Despite up-regulation after 48 h, no significant trend was observed in periostin mRNA expression over the time course following treatment of PDLFs with Emdogain (Fig. 3r).

Alkaline phosphatase activity of BMSCs and PDLFs co-cultured with Emdogain

The presence of Emdogain inhibited AP activity in BMSCs at days 7 and 14 in mineralization medium. Compared to control AP activity levels without Emdogain (100% activity), treatment of BMSCs with 0.75, 1.5, and 3 mg/ml Emdogain showed a reduction in AP activity to 13% at day 7 and 20% at day 14 in mineralization medium. Highest inhibition levels of AP activity were achieved at 3 mg/ml Emdogain (Fig. 4a, b). Similarly, Emdogain also inhibited the AP activity of PDLFs at days 7 and 14 in mineralization medium, although the extent of inhibition varied between donors (Fig. 4c, d). AP activity levels were reduced to 2% at day 7 and 3% at day 14 in donor one and 44% at days 7 and 14 in donor two, of AP activity levels without Emdogain. Highest inhibition of AP activity in PDLFs was observed at 1.5 and 3 mg/ml Emdogain. These

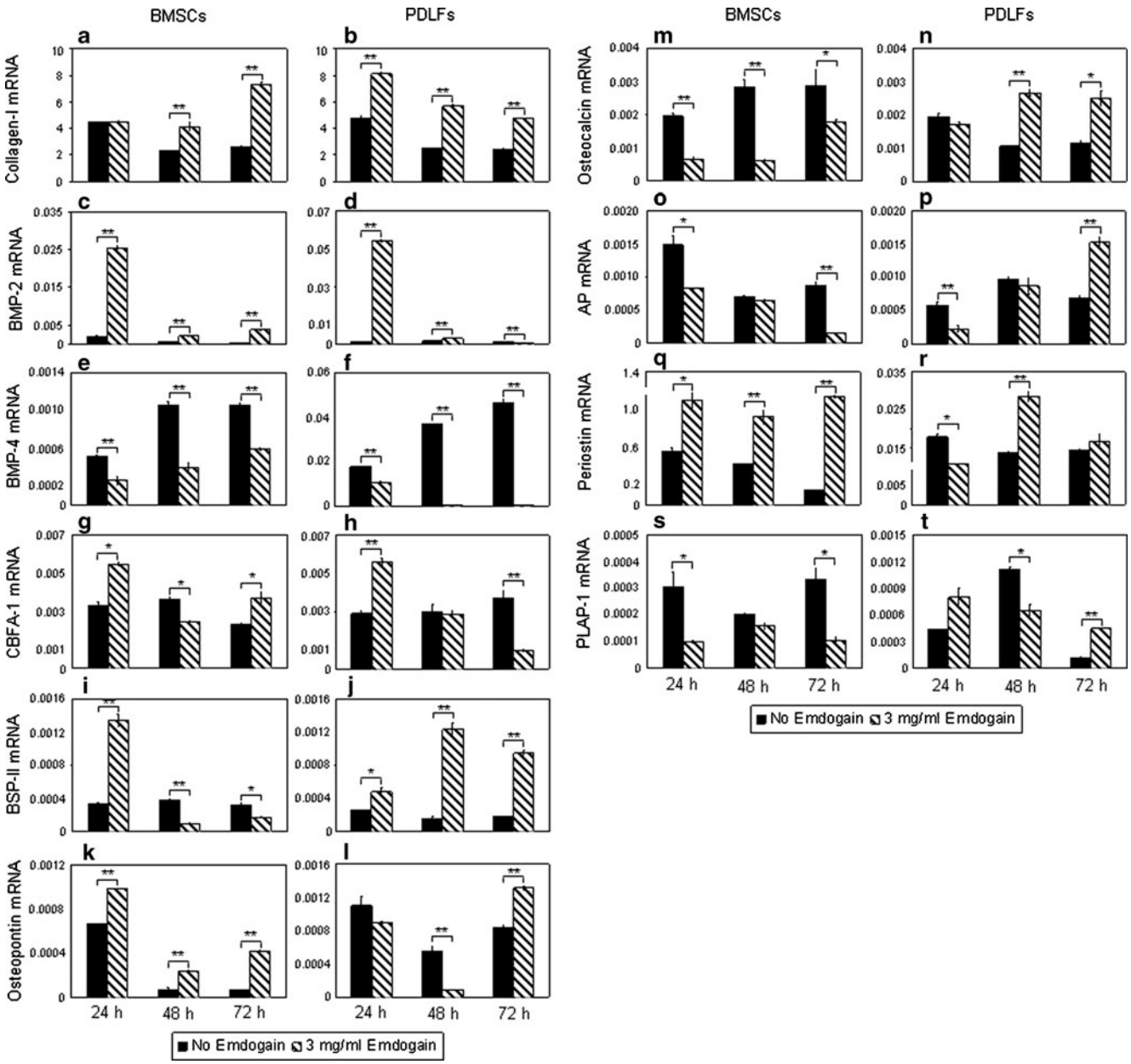

Fig. 3 Effect of Emdogain on mRNA expression levels in BMSCs and PDLFs co-cultured with Bone Ceramic after 24, 48, and 72 h. Collagen-I (**a** and **b**), bone morphogenetic protein-2 (BMP-2) (**c** and **d**), bone morphogenetic protein-4 (BMP-4) (**e** and **f**), core binding factor alpha-1 (Cbfa-1) (**g** and **h**), bone sialoprotein-II (BSP-II) (**i** and **j**), osteopontin(**k** and **l**), osteocalcin (**m** and **n**), alkaline phosphatase (AP) (**o** and **p**), periostin(**q** and **r**) and periodontal ligament associated protein-1 (PLAP-1) (**s** and **t**). Expression levels are normalized to β-actin. Data is presented as the mean±SEM of three experimental replicates representative of one BMSC and one PDLF donor with statistical significance of $*P<0.05$ or $**P<0.01$

results represent a down-regulation of alkaline phosphatase activity with Straumann® Emdogain in equivalent mesenchymal cell populations derived from four different donors. Similar inhibition of AP activity was also observed in both BMSCs and PDLFs using 3 mg/ml Emdogain in standard α-MEM culture medium (data not shown).

Ectopic bone formation in NOD/SCID mice following subcutaneous implantation of BMSCs and PDLFs attached to Bone Ceramic with and without Emdogain

Subcutaneous implants containing BMSCs and PDLFs attached to Bone Ceramic particles with and without Emdogain were well tolerated by NOD/SCID mice.

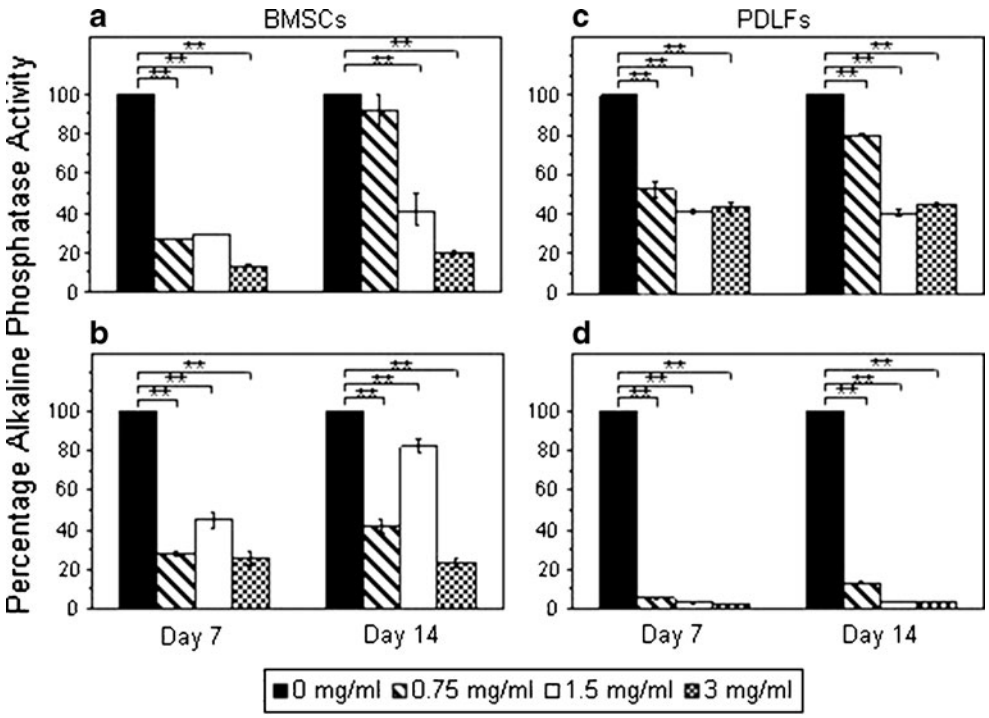

Fig. 4 Percentage inhibition of alkaline phosphatase activity in BMSCs and PDLFs co-cultured with Emdogain after 7 and 14 days in mineralization medium relative to 0 mg/ml Emdogain (100% activity), measured as absorbance at 540 nm. **a** BMSC donor 1, **b** BMSC donor 2, **c** PDLF donor 1, and **d** PDLF donor 2. Data is presented as the mean±SEM of three experimental replicates with statistical significance of $*P<0.05$ or $**P<0.01$

Histological analysis of each implant demonstrates that the majority of Bone Ceramic particles were lost during tissue processing. Areas which contained Bone Ceramic appeared as empty spaces; however, some residual Bone Ceramic is evident.

Recovered implants of Bone Ceramic alone demonstrated a significant amount of fibrous tissue and some vascularization. Although the collagen was quite dense in some fibrous areas, no mineralization was observed (Fig. 5a). Furthermore, no obvious difference was observed in the histology between implants containing Bone Ceramic with Emdogain (Fig. 5b) compared to Bone Ceramic alone. Following implantation of PDLFs or BMSCs attached to Bone Ceramic particles only, small pockets of acellular dense collagen were observed amongst predominantly fibrous tissue possibly suggesting early stages of mineralized tissue formation (Fig. 5c, d). Vascularization was also observed in each mouse. Following implantation of PDLFs attached to Bone Ceramic with Emdogain, pockets of acellular dense collagen were observed similarly to equivalent implants without Emdogain (Fig. 5e). An interesting observation was the number of multi-nucleated cells, possibly osteoclasts, lining the spaces where Bone Ceramic was originally located. Small pockets of acellular dense collagen were also consistently observed in mice implanted with BMSCs attached to Bone Ceramic particles with Emdogain (Fig. 5f). Many multi-nucleated cells lining the Bone Ceramic spaces were also observed but less than within implants containing PDLFs attached to Bone Ceramic particles with Emdogain. Overall, no obvious ectopic bone formation was observed in any implants containing Bone Ceramic with or without Emdogain. Furthermore, no significant differences were observed in early stage bone formation by PDLFs and BMSCs attached to Bone Ceramic with or without Emdogain. In contrast, a large amount of bone/cementum-like tissue and PDL-like fibrous tissue was observed within implants containing PDLFs and Zimmer HA/TCP (Fig. 5g). Similar quantities of bone were observed within implants containing BMSCs and Zimmer HA/TCP (Fig. 5h).

Discussion

Enamel matrix proteins (EMPs) secreted by Hertwig's epithelial root sheath during root development play an important role in cementogenesis and the development of periodontal attachment tissues during root development [28]. Clinical preparations of EMPs, such as Emdogain, are thought to promote regeneration of periodontal tissues, albeit not in a completely predictable or consistent manner [7–9]. However, the mechanisms and important factors within Emdogain thought to mediate tissue regeneration are yet to be elucidated [29]. Previous studies have shown that EMPs have a stimulatory effect on PDL fibroblast proliferation, thus increasing the progenitor pool available for differentiation [30, 31]. In addition, EMPs have been demonstrated to significantly affect the gene expression of numerous tran-

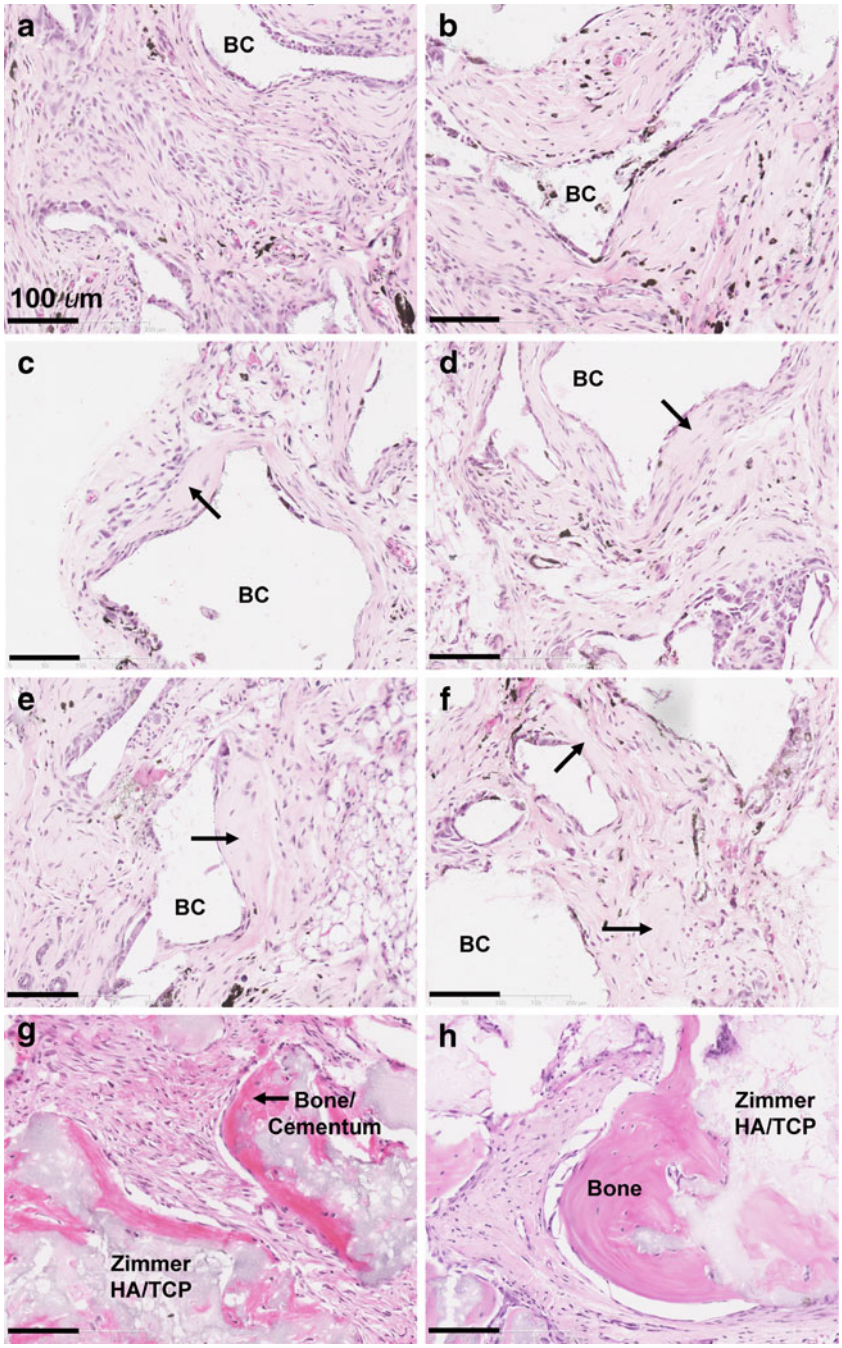

Fig. 5 Histological appearance of ectopic bone formation following subcutaneous implantation of Bone Ceramic coated with or without Emdogain and seeded with or without PDLFs or BMSCs in NOD/SCID mice. **a** Bone Ceramic alone, **b** Bone Ceramic with Emdogain, **c** Bone Ceramic and PDLFs, **d** Bone Ceramic and BMSCs, **e** Bone Ceramic with Emdogain and PDLFs, and **f** Bone Ceramic with Emdogain and BMSCs. Positive controls of Zimmer HA/TCP seeded with (**g**) PDLFs and (**h**) BMSCs. Areas of acellular dense collagen (*arrows*) and ectopic bone are shown. Bone Cermaic (BC). ×20 magnification

scription factors, growth factors, cytokines, and extracellular matrix molecules [32–34].

In this study, the effect of coating Bone Ceramic with Emdogain on hard tissue formation by BMSCs and PDLFs was investigated. Based on their capacity to generate structures at ectopic sites in immunocompromised mice resembling the tissues from which they were originally derived in [17, 24, 35], MSCs have provided a promising alternative to conventional therapies for regeneration of periodontal tissues. Increasingly, studies are investigating the capacity of PDLFs and BMSCs to regenerate periodontal tissues using large-animal models with surgically created periodontal defects representing periodontal disease [36, 37].

We investigated the effect of Emdogain on early expression of a variety of genes for molecules associated with mineralized tissue formation in both BMSCs and PDLFs for up to 72 h. While both populations are phenotypically similar in vitro [17], we observed similarities and differences in their expression patterns following co-culture with Emdogain most likely reflecting the heterogeneity of these populations given their different tissue regeneration capacity in vivo. Whether differences in

the regulation of some bone-associated proteins with Emdogain compared to previous studies using equivalent cell populations is, at least in part, attributable to the presence of Bone Ceramic in our co-culture experiments remains to be established.

Bone morphogenetic proteins (BMPs) play an important role in skeletal development and bone repair [38] by inducing osteogenic differentiation of mesenchymal progenitor cells [39, 40]. After 24 h in both cell types, we found that Emdogain significantly increased expression of BMP-2 and also the BMP-2-inducible osteogenic transcription factor Cbfa-1/Runx2 thought to regulate gene expression in developing osteoblasts [41]. Consistent with previous findings, we postulate that diminished Cbfa-1 expression in BMSCs and PDLFs demonstrated at subsequent time-points is mediated by down-regulated BMP-2 expression in the presence of Emdogain [42]. In addition, expression levels of extracellular matrix proteins collagen-I and non-collagenous Bone Sialoprotein-II mRNA, precursors to the onset of mineralization, were consistently stimulated by Emdogain in PDLFs and at various time-points in BMSCs. Collectively, these findings suggest that Emdogain may enhance the early stages of osteoblastic differentiation in mesechymal cells.

In addition to early down-regulation of AP mRNA expression, Emdogain markedly decreased the activity of AP protein in both cell types after days 7 and 14. Notably, these finding were consistent with previous reports for BMSCs but contradictory for equivalent PDL populations [43, 44]. Given that AP expression in functional osteoblasts plays an important role in hydroxyapatite crystal deposition during mineralization processes, we postulate that despite enhancing initial osteoblastic differentiation, Emdogain may not possess the capacity to stimulate the differentiation of osteoprogenitor cells into mineral-secreting osteoblasts. Interestingly, previous studies have demonstrated that EMP stimulation is cell-stage specific and does not stimulate the differentiation of pre-osteoblastic cells [45]. Expression of another early marker of osteogenic differentiation, osteopontin, was also enhanced by Emdogain in both cell types at various time-points similarly to calvarial cells in previous studies [46]. Emdogain also enhanced mRNA expression of the mature osteoblast marker osteocalcin in PDLFs but consistently inhibited expression in BMSCs. Further investigations are required to determine the significance of osteopontin and osteocalcin up-regulation with Emdogain in these early stages of cultures given previous studies have demonstrated that peak expression of these genes occurs by days 16–20 during extracellular matrix formation and maturation in vitro [47]. Similarly, the consistent inhibition of BMP-4, a molecule thought to promote both osteo- and chrondro-genesis in both cell types with Emdogain, requires further studies.

We also investigated the effect of Emdogain on the expression of PDL-associated markers PLAP-1 and periostin. PLAP-1, a small leucine-rich proteoglycan predominantly expressed in human PDL is thought to negatively regulate the mineralization of PDL cells by regulating BMP-2 activity [48]. With this in mind, we speculate that the up-regulated PLAP-1 mRNA levels in PDLSCs after 72 h and down-regulated expression in BMSCs may, in part, relate to the pattern of BMP-2 mRNA expression observed in the presence of Emdogain. Periostin is an important regulator of fibrosis and development of tooth structure, and is highly expressed in collagen-rich fibrous connective tissues including the periosteum and PDL [49]. While no obvious trend was observed in PDLFs cultured with Emdogain over the time course, periostin expression was consistently higher in BMSCs. Periostin expression has also been associated with progression of bone marrow fibrosis [50], suggesting BMSCs may have an increased propensity for collagen deposition in the presence of Emdogain.

While Emdogain has been reported to possess osteopromotive effects on bone healing during repair of bone defects [51, 52], other studies have indicated that Emdogain is not truly osteogenic in terms of its ability to induce bone production when implanted into ectopic sites [53, 54]. Interestingly, Emdogain has been reported to inhibit the ectopic osteogenic capacity of dentin [53]. In the present study, while areas of acellular dense collagen, indicative of early mineral formation, were observed, no obvious ectopic bone was observed by PDLFs or BMSCs attached to Bone Ceramic in vivo. Furthermore, no bone formation was observed following Emdogain-coating of Bone Ceramic with PDLFs or BMSCs. No bone formation was observed with Bone Ceramic alone or when coated with Emdogain. While not the principal focus of this study, it is important to note that HA-TCP preparations can differ in their osteoconductive capacity. Although the uncoated Straumann® Bone Ceramic material was not osteoconductive, another HA/TCP preparation, Zimmer, demonstrated an osteoconductive capacity similar to previously described [17]. Similar findings of heterogeneity amongst HA-TCP preparations have been reported [55, 56].

In conclusion, Emdogain treatment of BMSCs and PDLFs resulted in the up-regulation of several bone-related genes, namely BMP-2 and Cbfa1, after 24 h, suggesting that it may have a significant stimulatory effect in the commitment of mesenchymal cells to osteogenic differentiation in vitro. While Emdogain inhibited AP activity and appeared not to induce ectopic bone formation, longer-term studies are required to determine whether it promotes the final stages of osteoblast formation and mineralization at gene and protein levels. While Emdogain is used in clinical

applications, whether it truly possesses the capacity to induce the regeneration of bone or other components of the periodontium, remains to be established.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Giannobile WV (1996) Periodontal tissue engineering by growth factors. Bone 19:23S–37S
2. Dereka XE, Markopoulou CE, Vrotsos IA (2006) Role of growth factors on periodontal repair. Growth Factors 24:260–267
3. Ripamonti U, Reddi AH (1997) Tissue engineering, morphogenesis, and regeneration of the periodontal tissues by bone morphogenetic proteins. Crit Rev Oral Biol Med 8:154–163
4. Taba M Jr, Jin Q, Sugai JV, Giannobile WV (2005) Current concepts in periodontal bioengineering. Orthod Craniofac Res 8:292–302
5. Carlson NE, Roach RB Jr (2002) Platelet-rich plasma: clinical applications in dentistry. J Am Dent Assoc 133:1383–1386
6. Pradeep AR, Pai S, Garg G, Devi P, Shetty SK (2009) A randomized clinical trial of autologous platelet-rich plasma in the treatment of mandibular degree II furcation defects. J Clin Periodontol 36:581–588
7. Heijl L, Heden G, Svardstrom G, Ostgren A (1997) Enamel matrix derivative (EMDOGAIN) in the treatment of intrabony periodontal defects. J Clin Periodontol 24:705–714
8. Hirooka H (1998) The biologic concept for the use of enamel matrix protein: true periodontal regeneration. Quintessence Int 29:621–630
9. Sculean A, Nikolidakis D, Schwarz F (2008) Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials—biological foundation and preclinical evidence: a systematic review. J Clin Periodontol 35:106–116
10. Bartold PM, McCulloch CA, Narayanan AS, Pitaru S (2000) Tissue engineering: a new paradigm for periodontal regeneration based on molecular and cell biology. Periodontol 24:253–269
11. Bartold PM, Xiao Y, Lyngstaadas SP, Paine ML, Snead ML (2006) Principles and applications of cell delivery systems for periodontal regeneration. Periodontol 2000(41):123–135
12. van Dijk LJ, Schakenraad JM, van der Voort HM, Herkstroter FM, Busscher HJ (1991) Cell-seeding of periodontal ligament fibroblasts. A novel technique to create new attachment. A pilot study. J Clin Periodontol 18:196–199
13. Lang H, Schuler N, Nolden R (1998) Attachment formation following replantation of cultured cells into periodontal defects—a study in minipigs. J Dent Res 77:393–405
14. Malekzadeh R, Hollinger JO, Buck D, Adams DF, McAllister BS (1998) Isolation of human osteoblast-like cells and in vitro amplification for tissue engineering. J Periodontol 69:1256–1262
15. Wada N, Menicanin D, Shi S, Bartold PM, Gronthos S (2009) Immunomodulatory properties of human periodontal ligament stem cells. J Cell Physiol 219:667–676
16. Wada N, Bartold PM, Gronthos S (2011) Human foreskin fibroblasts exert immunomodulatory properties by a different mechanism to bone marrow mesenchymal stem cells. Stem Cells Dev 20:647–659
17. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang C, Shi S (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364:149–155
18. Bartold PM, Shi S, Gronthos S (2006) Stem cells and periodontal regeneration. Periodontol 40:164–172
19. Ivanovski S, Gronthos S, Shi S, Bartold PM (2006) Stem cells in the periodontal ligament. Oral Dis 12:358–363
20. Gronthos S, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino AC (2007) A novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. Stem Cells Dev 16:953–963
21. Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18:696–704
22. Gronthos S, McCarty R, Mrozik K, Fitter S, Paton S, Menicanin D, Itescu S, Bartold PM, Xian C, Zannettino ACW (2009) Heat shock protein-90 beta is expressed at the surface of multipotential mesenchymal precursor cells: generation of a novel monoclonal antibody, STRO-4, with specificity for mesenchymal precursor cells from human and ovine tissues. Stem Cells Dev 18:1253–1262
23. Gronthos S, Graves SE, Ohta S, Simmons PJ (1994) The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood 84:4164–4173
24. Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortesidis A, Simmons PJ (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 116:1827–1835
25. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
26. Gronthos S, Mrozik K, Shi S, Bartold PM (2006) Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. Calcif Tissue Int 79:310–317
27. Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S (2006) Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS ONE 1:e79
28. Hammarstrom L (1997) Enamel matrix, cementum development and regeneration. J Clin Periodontol 24:658–668
29. Zilm PS, Bartold PM (2011) Proteomic identification of proteinase inhibitors in the porcine enamel matrix derivative, EMD((R)). J Periodontal Res 46:111–117
30. Gestrelius S, Andersson C, Lidstrom D, Hammarstrom L, Somerman M (1997) In vitro studies on periodontal ligament cells and enamel matrix derivative. J Clin Periodontol 24:685–692
31. Rodrigues TL, Marchesan JT, Coletta RD, Novaes AB Jr, Grisi MFM, Souza SLS, Taba M Jr, Palioti DB (2007) Effects of enamel matrix derivative and transforming growth factor-beta1 on human periodontal ligament fibroblasts. J Clin Periodontol 34:514–522
32. Brett PM, Parkar M, Olsen I, Tonetti M (2002) Expression profiling of periodontal ligament cells stimulated with enamel matrix proteins in vitro: a model for tissue regeneration. J Dent Res 81:776–783
33. Parkar MH, Tonetti M (2004) Gene expression profiles of periodontal ligament cells treated with enamel matrix proteins in vitro: analysis using cDNA arrays. J Periodontol 75:1539–1546
34. Barkana I, Alexopoulou E, Ziv S, Jacob-Hirsch J, Amariglio N, Pitaru S, Vardimon AD, Nemcovsky CE (2007) Gene profile in periodontal ligament cells and clones with enamel matrix proteins derivative. J Clin Periodontol 34:599–609
35. Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, Robey PG (1997) Single-colony derived strains of

human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 12:1335–1347
36. Kawaguchi H, Hirachi A, Hasegawa N, Iwata T, Hamaguchi H, Shiba H, Takata T, Kato Y, Kurihara H (2004) Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. J Periodontol 75:1281–1287
37. Liu Y, Zheng Y, Ding G, Fang D, Zhang C, Bartold PM, Gronthos S, Shi S, Wang S (2008) Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine. Stem Cells 26:1065–1073
38. Rosen V (2009) BMP2 signaling in bone development and repair. Cytokine Growth Factor Rev 20:475–480
39. Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V (1992) Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology 130:1318–1324
40. Wang EA, Israel DI, Kelly S, Luxenberg DP (1993) Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors 9:57–71
41. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754
42. Takayama T, Suzuki N, Narukawa M, Tokunaga T, Otsuka K, Ito K (2005) Enamel matrix derivative stimulates core binding factor alpha1/Runt-related transcription factor-2 expression via activation of Smad1 in C2C12 cells. J Periodontol 76:244–249
43. Guida L, Annunziata M, Carinci F, Di Feo A, Passaro I, Oliva A (2007) In vitro biologic response of human bone marrow stromal cells to enamel matrix derivative. J Periodontol 78:2190–2196
44. Lossdorfer S, Sun M, Gotz W, Dard M, Jager A (2007) Enamel matrix derivative promotes human periodontal ligament cell differentiation and osteoprotegerin production in vitro. J Dent Res 86:980–985
45. Schwartz Z, Carnes DL Jr, Pulliam R, Lohmann CH, Sylvia VL, Liu Y, Dean DD, Cochran DL, Boyan BD (2000) Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. J Periodontol 71:1287–1296
46. Hama H, Azuma H, Seto H, Kido J, Nagata T (2008) Inhibitory effect of enamel matrix derivative on osteoblastic differentiation of rat calvaria cells in culture. J Periodontal Res 43:179–185
47. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990) Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430
48. Tomoeda M, Yamada S, Shirai H, Ozawa Y, Yanagita M, Murakami S (2008) PLAP-1/asporin inhibits activation of BMP receptor via its leucine-rich repeat motif. Biochem Biophys Res Commun 371:191–196
49. Horiuchi K, Amizuka N, Takeshita S, Takamatsu H, Katsuura M, Ozawa H, Toyama Y, Bonewald LF, Kudo A (1999) Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. J Bone Miner Res 14:1239–1249
50. Oku E, Kanaji T, Takata Y, Oshima K, Seki R, Morishige S, Imamura R, Ohtsubo K, Hashiguchi M, Osaki K, Yakushiji K, Yoshimoto K, Ogata H, Hamada H, Izuhara K, Sata M, Okamura T (2008) Periostin and bone marrow fibrosis. Int J Hematol 88:57–63
51. Kawana F, Sawae Y, Sahara T, Tanaka S, Debari K, Shimizu M, Sasaki T (2001) Porcine enamel matrix derivative enhances trabecular bone regeneration during wound healing of injured rat femur. Anat Rec 264:438–446
52. Sawae Y, Sahara T, Kawana F, Sasaki T (2002) Effects of enamel matrix derivative on mineralized tissue formation during bone wound healing in rat parietal bone defects. J Electron Microsc 51:413–423
53. Koike Y, Murakami S, Matsuzaka K, Inoue T (2005) The effect of Emdogain on ectopic bone formation in tubes of rat demineralized dentin matrix. J Periodontal Res 40:385–394
54. Donos N, Kostopoulos L, Tonetti M, Karring T, Lang NP (2006) The effect of enamel matrix proteins and deproteinized bovine bone mineral on heterotopic bone formation. Clin Oral Implants Res 17:434–438
55. Fellah BH, Gauthier O, Weiss P, Chappard D, Layrolle P (2008) Osteogenicity of biphasic calcium phosphate ceramics and bone autograft in a goat model. Biomaterials 29:1177–1188
56. Eid K, Zelicof S, Perona BP, Sledge CB, Glowacki J (2001) Tissue reactions to particles of bone-substitute materials in intraosseous and heterotopic sites in rats: discrimination of osteoinduction, osteocompatibility, and inflammation. J Orthop Res 19:962–969