Cellular responses and expression profiling of human bone marrow stromal cells stimulated with enamel matrix proteins *in vitro*

Z. C. Song*, R. Shu* and X. L. Zhang†

*Department of Periodontology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China and †Shanghai Key Laboratory of Stomatology, Shanghai, China

Received 16 February 2009; revision accepted 19 May 2009

Abstract

Objectives: The aim of this study was to investigate biological effects and gene expression profiles of enamel matrix proteins (EMPs), on human bone marrow stromal cells (HBMSCs), for preliminary understanding of mechanisms involved in promoting periodontal regeneration by EMPs.

Materials and methods: EMPs were extracted using the acetic acid method, and HBMSCs from human bone marrow aspirates were cultured. Attachment levels, level of cells morphologically attenuated, cell proliferation, alkaline phosphatase (ALP) activity and staining of HBMSCs were measured in the absence and in the presence of EMPs. Microarray analysis was performed to detect gene profiles of HBMSCs by treatment with 200 µg/ml EMPs, for 5 days. Four differential genes were selected for validation of the microarray data using real-time PCR.

Results: EMPs promoted proliferation and ALP activity of HBMSCs in a time- and dose-dependent manner, and at a concentration of 200 µg/ml significantly enhanced proliferation and ALP expression. However, there were no significant changes between EMP-treated groups and the control group in cell attachment and cell process attenuation levels. Twenty-seven genes were differentially expressed by HBMSCs in the presence of EMPs. Expressions of 18 genes were upregulated and expressions of nine genes were found to be downregulated. There was good consistency between data obtained from the validation group and microarray results.

Conclusions: EMPs promoted cell proliferation and differentiation and gene expression profiles of HBMSCs were affected. This may help elucidation of mechanisms involved in promoting regeneration of periodontal tissues by EMPs.

Introduction

The fundamental goal of periodontal treatment is regeneration of periodontal tissues and formation of new periodontium. Numerous pre-clinical and clinical studies have explored various methods of promoting periodontal regeneration with procedures such as placement of bone grafts or bone substitutes, root surface conditioning and use of organic or synthetic barrier membranes in guided tissue regeneration (1). Periodontal tissue engineering of autologous bone marrow stromal cells (BMSCs) for transplantation may be a feasible future alternative for patients with periodontal defects. BMSCs are pluripotent cells with the ability to differentiate into a number of phenotypes and they have been identified as a major source of osteogenic cells (2,3). Human BMSCs (HBMSCs) can be obtained easily and repeatedly from a patient by bone marrow aspiration; reimplantation of the cells cultured with autologous serum into the source patient would not cause an immune reaction nor raise ethical concerns. Thus, transplantation of HBMSCs into periodontal tissue defects would seem to be an effective strategy to accelerate periodontal tissue regeneration (4,5).

Enamel matrix proteins secreted by Hertwig's epithelial root sheath play an important role in cementogenesis and in development of the periodontal attachment apparatus (6–8). The major constituents of enamel matrix proteins are amelogenins, which comprise more than 90% of enamel matrix proteins (EMPs). They are hydrophobic proteins and share high degrees of homology across different species (9,10). Due to their important role in development of the periodontium, EMPs have been applied

Correspondence: R. Shu, PhD, Professor, Department of Periodontology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, No. 639 ZhiZaoJu Road, Shanghai 200011, China. Tel.: +86 21 23271699 5510; Fax: +86 21 63135412; E-mail: shurong123@hotmail.com

successfully in pre-clinical and clinical studies for regeneration of periodontal tissues (11); studies have shown that EMPs successfully promote cementum and alveolar bone formation (12,13). Recent results have indicated that EMPs are able to induce regenerative processes in periodontal tissues in a way similar to that of embryogenic development. In addition, enamel matrix derivative (EMD) has been introduced to enhance periodontal regenerative healing, that is, regeneration of the cementum, periodontal ligament and bone (14,15).

To understand the mechanisms underlying development and regeneration of periodontal tissues, several investigators, through *in vitro* studies, have attempted to clarify the mode of action of EMPs. These undertakings have focused on specific cell functions associated with the regenerative response, including cell recruitment, proliferation, and differentiation into mature periodontal ligament (PDL) cells and osteoblasts (16–19). In our previous study, we showed that porcine BMSCs could be differentiated into cementoblasts under EMP induction *in vivo* (20). In other investigations, BMSCs from rat and dog were selected to investigate effects of EMPs (11,21). However previously, there have not been any reports on HBMSCs stimulated with EMPs. Here, the biological effect of EMPs on HBMSCs and gene expression profiles of the cells were investigated, using cDNA microarray analysis. Hopefully, it will help elucidate mechanisms involved in promoting periodontal regeneration under the influence of EMPs.

Methods

Preparation of porcine enamel matrix proteins

Porcine EMPs were extracted as described previously (20). Briefly, dental germs were dissected freshly from the jaws of 6-month-old pigs. The EMPs were then extracted from the unmineralized enamel matrix using acetic acid, then were lyophilized to obtain EMP powder. Using sodium dodecyl sulphate–polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining, the protein showed typical molecular weight distribution of amelogenins. The total lyophilized powder was then dissolved in 5 mM acetic acid at a concentration of 4 mg/ml, sterilized by filtration and stored at -80°C .

Culture of human BMSCs

HBMSCs were isolated and expanded by modification of methods reported previously (22). Bone marrow aspirates were obtained from healthy adult donors after obtaining informed consent and under the protocol approved by our Institutional Review Board. Bone marrow aspirates were

suspended in Dulbecco's modified Eagle's medium (DMEM, high glucose) with 500 U/ml heparin. After centrifugation at 160g for 10 min, cells were then suspended in DMEM supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells obtained, including erythrocytes, were seeded in tissue culture dishes and incubated under standard conditions (5% CO_2 , 37°C , saturation humidity). Culture medium was changed every second day thereafter. Non-adherent and haematopoietic cells were removed at each medium change. When cells were approaching confluence, passages were performed. Third-passage HBMSCs were used for the following experiments.

Flow cytometric analysis

Passage 3 (P3) HMSCs were used for flow cytometric analyses. Confluent cultures were harvested in 0.125% trypsin and pelleted at 1000 rpm for 5 min. Cells were resuspended in fluorescence-activated cell sorting (FACS) buffer [PBS + 0.1% (w/v) sodium azide + 1% (w/v) bovine serum albumin] and incubated with primary conjugated antibodies [CD34-phycoerythrin (PE), CD44-PE, CD45-CY, CD105-PE, CD90-fluorescein isothiocyanate (FITC)] (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at room temperature. Cells were then washed and fixed with 1% (w/v) paraformaldehyde in PBS prior to analysis and were analysed using a flow cytometer (BD FACScalibur; BD Biosciences). Data were interpreted using FCSEXPRESS software (BD Biosciences, San Jose, CA, USA).

Attachment assay

The attachment assay was performed as employed by Lyngstadaas (23). In essence, 24-well culture plates were coated with EMPs overnight at various concentrations (50, 100, 200 and 300 $\mu\text{g/ml}$). To assess cell attachment level during the first 7 h after seeding, 5×10^4 cells were cultured on EMP-coated surfaces for 1, 3, 5 or 7 h before the cultures were vigorously washed in PBS to remove all non-attached cells. The washing solution was centrifuged, and numbers of non-attached cells were analysed. Attached cells were then removed from surfaces by trypsinization and were counted in a similar fashion as that for the controls. Uncoated dishes were used as negative control.

Spreading, attenuation assay

A cell spreading assay was performed using the methods of Palioto *et al.* (24) with some modification. 24-well culture plates were coated with EMPs at various concentrations (50, 100, 200 and 300 $\mu\text{g/ml}$). BMSCs cultured in DMEM without EMPs served as control. Cells were

suspended in DMEM with 10% FBS at a concentration of 5×10^4 cells/ml, and 1 ml of this was plated on to each well. After incubation for 1, 3, 5 and 7 h respectively, non-adherent cells were removed by three washes in PBS. Attached cells were fixed in 70% alcohol. Photographic images of attached BMSCs were recorded using an inverted microscope. Areas of attenuated fixed cells were measured. Numbers of attached cells and morphologically spread out cells were established in wells in five random light micrograph fields, at magnification of $\times 100$. The cell spreading assay was performed by analysing those micrographs taken at predetermined sites of each well. Cell attenuation levels were calculated by counting spread out cells per 100 cells.

Proliferation assay

Proliferation of BMSCs was investigated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, which stains proliferating cells (25). EMPs were added to 96-well culture plates at concentrations of 50, 100, 200 and 300 $\mu\text{g/ml}$ and left overnight for attachment, and wells without EMPs served as controls. First, 100 μl of DMEM with 10% FBS was added to each well. Then third passage HBMSCs were suspended in DMEM with 10% FBS at a concentration of 5×10^3 cells/100 μl , and 100 μl of this was plated on to each well of 96-well plates. Cells were seeded in triplicate wells for each treatment. Observation of cell proliferation lasted for a period of 8 days. At the end of each incubation period, 20 μl MTT (0.5 mg/ml; AMERSCO, Cleveland, OH, American) was added and cells were incubated for 4 h under standard cell culture conditions (37 °C, 100% humidity, 95% air and 5% CO_2). Then the liquid was removed, followed by addition of 200 μl DMSO to each well. Absorbance was measured using an ELISA reader at 490 nm.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity assay was performed by modification of methods reported previously (26). For this experiment, mineralized induction medium was used; this was complete DMEM supplemented with 10% FBS, ascorbic acid (50 mg/l; Sigma, St. Louis, MO, USA), 10 mM beta glycerophosphate disodium salt (Sigma) and 10^{-8} M dexamethasone (Sigma). EMPs were added to 96-well culture plates at concentrations of 50, 100, 200 and 300 $\mu\text{g/ml}$ and left overnight. Wells without EMPs served as controls. Cells were seeded in triplicate wells for each treatment. ALP activity was evaluated for each group at days 1, 3, 5 and 7 respectively. The cell layer was washed three times in PBS and extracted using 0.2% Triton X-100

for 30 min at 37 °C, followed by overnight storage at 4 °C. ALP activity was determined using a kinetic spectrophotometric enzyme assay with p-nitrophenol phosphate as a substrate. Absorbance was measured using an ELISA reader at 405 nm.

ALP staining

HBMSCs were treated with EMPs at a concentration of 200 $\mu\text{g/ml}$. BMSCs without EMPs served as control. ALP staining of BMSCs was performed 5 days after EMP treatment. Cells were fixed for 10 min at 4 °C and incubated with a mixture of naphthol AS-MX phosphate, *N,N*-dimethylformamide, and fast blue BB salt (ALP kit; Renbao, Shanghai, China). Mean intensity of positive stain was evaluated using an image analysis program (IPP; Olympus, Tokyo, Japan).

RNA extraction and probe labelling

Third-passage HBMSCs were used for EMP stimulation at a concentration of 200 $\mu\text{g/ml}$, for 5 days during which EMPs significantly promoted BMSC proliferation as recorded by the proliferation assay. Total RNA was isolated from HBMSCs using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was quantified by measuring absorbance using Gene Quant Pro. Fluorescent cDNA probes were prepared through reverse transcription of isolated mRNAs and then purified according to the methods of Schena *et al.* (27). RNA samples from untreated cells were labelled with Cy3-dUTP and those from EMP-treated cells with Cy5-dUTP. The two colour probes were then mixed and diluted to 500 μl with TE, and concentrated using Microcon YM-30 filter (Millipore, Bedford, MA, USA) to 10 μL . Samples were then dried.

Microarray analysis

The microarray (4096 chip; United Gene Holdings Ltd., Shanghai, China) was pre-hybridized with hybridization solution [$5\times$ SSC (0.75 mol/L NaCl and 0.075 mol/l sodium citrate), 0.4% SDS and 50% formamide] containing 0.5 mg/ml denatured salmon sperm DNA at 42 °C for 6 h. Fluorescent probe mixtures were denatured at 95 °C for 5 min, and denatured probe mixtures were applied to the pre-hybridized chip under a coverslip. Chips were hybridized in a homemade chamber at 42 °C for 16 h and hybridized chips were then washed at 60 °C for 10 min each, in solutions of $2\times$ SSC, 0.2% SDS, $0.1\times$ SSC, 0.2% SDS, and $0.1\times$ SSC, and then dried at room temperature.

Chips were scanned using a ScanArray 3000 (GSI Lumonics, Bellerica, MA, USA) at two wavelengths, to

detect emission from both Cy3 and Cy5. Acquired images were analysed using ImaGene 3.0 software (BioDiscovery, Los Angeles, CA, USA). Intensities of each spot at each wavelength represent quantity of Cy3-dUTP and Cy5-dUTP, respectively, hybridized to each spot. Ratios of Cy5 to Cy3 were computed for each location on each microarray. Overall intensities were normalized with a correction coefficient obtained using ratios of 40 house-keeping genes. To minimize artefacts arising from low expression values, only genes with raw intensity values for both Cy3 and Cy5 of >800 counts were chosen for differential analysis. Genes were identified as differentially expressed if absolute value of the natural logarithm of the ratios was >0.69.

Differential gene analysis was performed between controls and EMP-treated HBMSCs, using a threshold of 2-fold or greater of hybridization intensity. Threshold value of 2 and above was considered as upregulation, whereas ratio of 0.5 and below indicated downregulation in gene expression.

Real-time PCR

For validation of microarray data, real-time PCR was performed to access relative expression of genes of interest. The same total RNA pool used for hybridization was converted to cDNA and amplified by polymerase chain reaction (RT-PCR) (Takara). RT-PCR was performed in a model rotor-gene-3000A (Corbett Research, Sydney, Australia). Cycle conditions were as follows: after initial 2-min hold at 95 °C, 45 cycles denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s. Data were expressed as n-fold differences in target gene expression relating to both endogenous control gene expression and calibrator, which were determined using the $2^{-\Delta\Delta CT}$ method. Designs of PCR primers and reaction conditions are shown in Table 1.

Table 1. Primer sequences of target genes and amplified product by PCR

Target gene	Primer sequence	Amplified product (bp)
Serpin b2	Forward 5'-CCAGAGAACAACCAGATTGA-3' Reverse 5'-TCTGGATCTGCTGCATGAAC-3'	282
NRG1	Forward 5'-GCGTGAGCAGGACGGTGATA-3' Reverse 5'-GCCTCGCTCCTTCTTCTTGC-3'	403
EED	Forward 5'-CCTCTTAATCCAACGGACCT-3' Reverse 5'-TGCTCAGCTTCTGCTTCTTG-3'	388
SDC1	Forward 5'-GAGAGGAATCCGGCAGTAGA-3' Reverse 5'-GAGCCATCTTGATCTTCAGG-3'	284

Statistical analysis

Data are presented as mean $\pm$ SEM, and all data were subjected to one-way analysis of variance (ANOVA). *P*-values less than 0.05 were considered significant.

Results

Morphology and flow cytometry analysis of HBMSCs

For the first 3 days after seeding, cells appeared as a mixture of BMSCs, haematopoietic cells and others, but by the seventh day, by removing non-adherent and haematopoietic cells, the remaining cell layer consisted of densely packed spindle-shaped cells; some of these developed clustered colonies. Twelve to 14 days after seeding, cultures showed a monolayer of elongated cells. As shown in Fig. 1, subcultured HBMSCs showed typical fibroblast-like cells with long, thin cytoplasmic arms at each extremity, which occurred as radiate arrangements.

To investigate surface epitopes of the cells, third-passage HBMSCs were used for detection of cell-surface markers from three different individual donors, using flow cytometric analysis. They were negative for expression of haematopoietic stem cell marker CD34 or common leukocyte marker CD45 (Fig. 2). Negative expression of haematopoietic molecules in this study indicates that there was no contamination by haematopoietic cells in the bone marrow mesenchymal stem cell separation. A panel of various cell-surface molecules related to cell adhesion (CD44), migration and multipotency (CD90 and CD105) was used for cell characterization; the HBMSCs were positive for all of these (Fig. 2), which indicated characteristics of mesenchymal/stromal stem cells. Figure 2h is

Figure 1. Isolation of HBMSCs. Passage 3 HBMSCs were cultured for 3 days after seeding (reverse phase contrast microscope, $\times 100$).

Figure 2. Flow cytometric analysis of cell-surface antigen expression. Histograms representing fluorescence intensity of cell-surface molecules on HBMSCs. (a) and (e) negative control of fluorescent dye. (b) haematopoietic stem cell marker CD34. (c) cell adhesion molecule CD44. (d) stem cell marker CD105. (f) stem cell marker CD90. (g) common leucocyte marker CD45. It was noted that HBMSCs did not express CD45 or CD34, whereas they expressed CD44, CD90 and CD105. (h) summary of surface marker expression in HBMSCs. Data represent mean of three different experiments.

the summary of surface marker expression of HBMSCs from the three different individuals.

Effect of EMPs on attachment of HBMSCs

The experiments showed that attachment levels during the first hour after seeding were very low. As culture time progressed, attachment increased rapidly; five hours later, attachment was up to 90%. However, EMPs failed to exhibit any improvement in attachment, and results of EMP-coated surfaces did not differ significantly from the control ($P > 0.05$) (Fig. 3).

Figure 3. Effect of EMPs at different concentrations on attachment of HBMSCs. EMPs failed to exhibit any improvement in attachment rates ($P > 0.05$).

Effect of EMPs on spreading out of HBMSCs

By the first hour after seeding, most of the cells were round and had no cytoplasmic arms when viewed using an inverted microscope; this indicated that the cells had not spread out. However, 3 h later, some cells had attenuated and had cytoplasmic processes; after 5 h, spreading out of cells increased and round cells, were rare. After 7 h almost all the cells displayed the attenuated phenotype, with long extensions of cytoplasmic processes and numerous filopodia. At the end of each incubation period, however, there was no significant difference between treatment groups and the control group ($P > 0.05$) (Fig. 4). This indicated that influence of EMPs did not contribute to the spread out shape of the cells.

Effect of EMPs on proliferation of HBMSCs

As shown in Fig. 5, no significant difference was noticed between EMP-treated groups and the control group on days 1 and 2. From day 3, 200 μg/ml concentration caused a higher level of cell proliferation compared to the control group ($P < 0.05$). This increase was persistent up to the eighth day. Cell proliferation increased with extended incubation time and reached plateaux around the fifth day. From day 4 on, there was a significant difference between the 100 μg/ml concentration-treatment group

Figure 4. Effect of EMPs at different concentrations on level of morphological spread of HBMSCs. At the end of each incubation period, there were no significant differences between treatment groups and control group ($P > 0.05$).

Figure 5. Effect of EMPs at different concentrations on proliferation of HBMSCs. Starting from day 3, 200 µg/ml concentration had higher levels of proliferation compared to the control group ($P < 0.05$); this increase persisted until the eighth day. Cell proliferation increased with extended incubation time. From day 4, there was significant difference between the 100 µg/ml concentration group and the control group ($P < 0.05$). On day 7, the 300 µg/ml concentration resulted in marked increase in cell numbers. EMPs at concentration of 50 µg/ml failed to provide greater proliferation as compared to the control group ($P > 0.05$).

and the control group ($P < 0.05$). On day 7, 300 µg/ml concentration-treatment resulted in a marked increase in cell viability. EMPs at concentration of 50 µg/ml failed to demonstrate greater cell proliferation compared to the control group ($P > 0.05$) in this study. 200 µg/ml EMP concentration significantly enhanced proliferation of HBMSCs, and thus was taken as the optimal concentration for the following experiments.

Effect of EMPs on ALP activity of HBMSCs

Given that ALP expression is an important phenotype of osteoblasts, it was used as a marker to gauge effects of

Figure 6. Effect of EMPs at different concentrations on ALP activity of HBMSCs cultured in mineralized induction medium. From day 3, EMPs significantly enhanced ALP activity in HBMSCs ($P < 0.05$), 200 µg/ml concentration group was the highest on day 5. EMP treatment group versus control group, * $P < 0.05$, ** $P < 0.01$.

EMP on differentiation of HBMSCs. There was no significant difference between EMP-treated groups and the control group on day 1 ($P > 0.05$), but from day 3, treatment with EMPs significantly enhanced ALP activity ($P < 0.05$). On day 5, there was marked significance of ALP activity at EMP concentration of 100 and 200 µg/ml ($P < 0.01$), and ALP activity at concentration of 200 µg/ml was the highest among those groups (Fig. 6).

Effect of EMPs on ALP staining of HBMSCs

To assess the phenotype of EMP-treated HBMSCs, their ALP expression was also determined by EMPs concentration of 200 µg/ml for 5 days. Our result showed that the area of the ALP-positive staining in the EMP group was larger than that of the control group (Fig. 7a,b). Results of image analysis showed that there was significant difference between EMP treatment group (0.25 ± 0.02) and the control group (0.21 ± 0.03) ($P < 0.05$) (Fig. 7c).

Gene expression of HBMSCs regulated by EMPs

To identify gene expression profiles regulated by EMPs, HBMSCs were stimulated by EMPs at a concentration of 200 µg/ml, for 5 days. cDNA microarray analysis was first performed and scatter plots of relative gene expression levels between control and EMP-treated cells showed a highly concordant distribution pattern. Direct comparisons were made between EMP-treated and untreated BMSC expression profiles, based on colour-coded designation for each hybridized dot. Among 4096 target genes, 27 were differentially expressed by HBMSCs between controls and EMP-treated cells. Nine genes were found to be downregulated as shown in Table 2, whereas 18 genes were upregulated as shown in Table 3. These genes were related to cell proliferation or protein synthesis, such as

Figure 7. Alkaline phosphatase staining in HBMSCs treated with EMPs for 5 days. (a) control group, (b) EMPs group (original magnification $\times 25$), (c) quantitative expression of ALP in the presence or absence of EMPs. Result of image analysis showed that there was significant difference between EMP treatment group and the control group ($P < 0.05$).

neuregulin 1 (*NRG1*), and *Homo sapiens* syndecan 1 (*SDC1*).

Verification of microarray results using real-time PCR

To confirm the microarray data, four genes (*SDC1*, *NRG1*, *EED* and *Serpinb2*) were selected with various levels of differential expression, which were related to cell adhesion

and proliferation, for analysis by real-time PCR (Fig. 8). Relative amounts of *SDC1* mRNA to GAPDH was 3.25-fold higher in the EMP-treated group than in the control group. The relative amount of *NRG1* mRNA was 1.91-fold higher in EMP-treated cells than in the control group. These results were concordant with their 4.05- and 2.522-fold differential expressions in microarray results, respectively. Relative ratio of *EED* to GAPDH in EMP-treated cells was 2.57-fold higher than in the control group, whereas transcript level of *Serpinb2* was 0.32-fold lower in EMP-treated cells than in the control group. These results were comparable to the 2-fold differential expression for both the genes observed by microarray analysis.

Discussion

EMPs have been widely used to promote regeneration of periodontal tissues (28–30). In our previous study, induction effect of EMPs on differentiation of porcine BMSCs into cementoblasts suggested that EMPs may effectively improve the periodontium-forming ability in BMSC-based periodontal tissue engineering (20). However, studies on effects of EMPs on HBMSCs are rarely reported. In this study, the biological effect of EMPs on HBMSCs, and gene expression profiles were investigated, to understand the preliminary mechanisms involved in promoting periodontal regeneration by EMPs.

In tissue engineering, cell adhesion and cell spreading out on extracellular matrix substrata are critical for cell motility, growth and organization of tissues (31,32). As cellular attachment and migration are essential events for regeneration of periodontal tissues, it was necessary to

Table 2. Genes downregulated in human BMSCs after EMPs treatment

Genbank ID	Gene name	Ratio (mean $\pm$ SEM)*	Function
AK125026	<i>Highly similar to Sodium- and chloride-dependent betaine transporter</i>	0.376 $\pm$ 0.02	Chemokine activity, signal transduction, immune response
BQ189472	<i>Homo sapiens cocaine- and amphetamine-regulated transcript</i>	0.391 $\pm$ 0.01	Signal transduction, neuro-peptide signaling pathway, cell–cell signalling
AK091034	<i>Homo sapiens osteoclast stimulating factors 1</i>	0.442 $\pm$ 0.09	Ossification, protein binding, signal transduction
NM_014325	<i>Homo sapiens coronin, actin binding protein</i>	0.458 $\pm$ 0.01	Cell–cell signaling and transporter protein
AK126930	<i>Homo sapiens heparan sulphate (glucosamine) 3-o-sulphotransferase 5</i>	0.471 $\pm$ 0.04	Protein binding, protein amino acid sulphation
AK128094	<i>RAS p21 protein activator 3</i>	0.473 $\pm$ 0.1	GTPase activator activity, metal ion binding, intracellular signaling cascade
AL080215	<i>Homo sapiens tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)</i>	0.474 $\pm$ 0.02	Protease inhibitor activity, blood coagulation
BX647782	<i>Homo sapiens nuclear transport factor 2</i>	0.489 $\pm$ 0.04	Protein binding, protein transport
BC012609	<i>Homo sapiens serpin peptidase inhibitor</i>	0.497 $\pm$ 0.06	Serine-type endopeptidase inhibitor activity, plasminogen activator activity, anti-apoptosis

*Numbers indicate ratio of Cy5/Cy3, i.e. the change in expression, and are the mean $\pm$ SEM of two independent hybridizations. Ratio of 0.5 and below indicate downregulation in gene expression.

Table 3. Genes upregulated in human BMSCs after EMPs treatment

Genbank ID	Gene name	Ratio (mean $\pm$ SEM)*	Function
L03427	<i>Human sapiens basophilin 1</i>	2.067 $\pm$ 0.15	Transcription factor activity, positive regulation of cell proliferation development
NM_006122	<i>Homo sapiens mannosidase, alpha</i>	2.078 $\pm$ 0.11	Alpha-mannosidase activity, carbohydrate metabolism
BC033897	<i>Homo sapiens chromosome 3 open reading frame 19</i>	2.101 $\pm$ 0.37	Signal transduction
AK096367	<i>Homo sapiens cDNA FLJ39048 fis</i>	2.125 $\pm$ 0.09	Unknown
BC013326	<i>Homo sapiens cDNA FLJ39048 fis</i>	2.135 $\pm$ 0.255	Cell cycle, regulation of cell shape, apoptosis
NM_152991	<i>Homo sapiens embryonic ectoderm development (EED)</i>	2.236 $\pm$ 0.17	Transcriptional repress or activity
BC063127	<i>Homo sapiens pregnancy-specific beta-1-glycoprotein 4, transcript variant 1,</i>	2.318 $\pm$ 0.43	Cell signaling and transporter protein, defence response
CR749478	<i>Homo sapiens phenylalanine-tRNA synthetase 2 (mitochondrial)</i>	2.368 $\pm$ 0.23	Protein biosynthesis, tRNA binding
X53586	<i>Human mRNA for integrin alpha 6</i>	2.405 $\pm$ 0.37	Cell-substrate junction assembly, cell-matrix adhesion, receptor activity, calcium ion binding, signalling pathway
BX648892	<i>Homo sapiens male sterility domain containing 2</i>	2.415 $\pm$ 0.5	Cellular metabolism
NM_013994	<i>Homo sapiens discoidin domain receptor family, member 1</i>	2.440 $\pm$ 0.33	Cell adhesion, transmembrane receptor protein tyrosine kinase signalling pathway
NM_013957	<i>Homo sapiens neuregulin 1 (NRG1)</i>	2.522 $\pm$ 0.47	Embryonic development, negative regulation of transcription, cell differentiation
BQ067599	<i>AGENCOURT_6758954</i>	2.559 $\pm$ 0.35	Unknown
AB007932	<i>Homo sapiens plexin A2</i>	2.652 $\pm$ 0.27	Development, receptor activity
AK095158	<i>RNA binding motif protein 4B</i>	2.969 $\pm$ 0.45	Nucleotide binding, mRNA processing
BC020652	<i>Homo sapiens pregnancy-specific beta-1-glycoprotein 6</i>	3.361 $\pm$ 0.58	Defence response, pregnancy
M23575	<i>Human pregnancy-specific beta-1 glycoprotein</i>	3.538 $\pm$ 0.64	Pregnancy, defence response
NM_001006946	<i>Homo sapiens syndecan 1 (SDC1)</i>	4.052 $\pm$ 0.95	Cytoskeletal protein binding

*Numbers indicate ratio of Cy5/Cy3, i.e. the change in expression, and are the mean $\pm$ SEM of two independent hybridizations. Ratio of 2 and above was considered as upregulation.

Figure 8. Confirmation of differentially expressed genes observed in microarray results. Four genes (a: *SDC1*, b: *NRG1*, c: *EED* and d: *Serpinb2*) selected from array results were analysed using real-time PCR. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Data are presented as mean $\pm$ SEM (** P < 0.01, n = 3).

explore the effects of EMPs on attachment, attenuation and proliferation of HBMSCs. EMPs can act as a matrix for cells at a periodontal regenerative site. Aggregates

formed by EMPs at the physiological pH and temperature are responsible for creating a positive environment for cells to proliferate and differentiate, as required for regen-

eration of the periodontium, then EMPs may influence cell functions. This experiment showed that EMPs could promote proliferation ability of HBMSCs in a time- and dose-dependent manner, with no effect on their attachment or morphological spreading. However, effects of EMPs on attachment and spreading of cells show controversial results. Lyngstadaas (23) found that attachment level of PDL cells during the first hours after seeding in sarstedt dishes is nearly five times more efficient when the surface of the culture dish is coated with EMD, but Gestrelus (33) has indicated that EMD had no significant effect on migration nor attachment and attenuation of PDL cells. van der Pauw (16) noted that human periodontal ligament fibroblasts attached and spread out within 24 h, whereas human gingival fibroblasts barely attached and spread out on EMP-coated substrata. Our studies have indicated that EMPs did not increase attachment nor spreading out levels of HBMSCs. The conflicting results probably resulted from different kinds of cells or EMPs used by the various investigators.

Cell proliferative effects may be important in the early phase for enhancement of osteogenic cells in a wound space and is a prerequisite for bone formation at later stages. In this sense, cell proliferation contributes to wound healing. Keila (11) found that EMD at a concentration of 25 µg/ml increased BMSC numbers in rats and Gestrelus (33) indicated that EMD enhanced proliferation of PDL cells. In our study, EMPs increased cell viability in a time- and dose-dependent manner. Our results demonstrated that HBMSCs treated with 200 µg/ml EMPs showed higher cell viability than the control group, which indicated that EMPs promoted cell proliferation significantly. These differences may be related to different cell types adopted in the actual applications, which provides some evidence for the mechanism used by EMPs in promoting the periodontal tissue regeneration. EMPs *in vitro* can form protein aggregates, thereby providing a unique environment for cell-matrix interaction. Under these conditions, effects of EMPs on various kinds of cells were different. On the other hand, some studies have shown that EMPs enhance cellular activities involved in different aspects of tissue regeneration for different cell types. Further studies are expected to determine exact mechanisms exerted by EMPs that promote proliferation of HBMSCs *in vitro*.

Characteristically, cells of osteogenic lineage express high levels of osteoblastic markers such as ALP activity (15). To study the effects of EMPs on differentiation of HBMSCs, their ALP activity was investigated and the ALP staining was performed. Our results verified high mineralization potential of HBMSCs. The experiment showed clear enhancement in mineralization by EMP-treatment. These measurements are indicative of induction of osteogenic differentiation parameters.

With the advent of the cDNA microarray technique, it is now possible to explore expression profiles and analyse thousands of genes simultaneously (34,35). In the previous studies, Brett and Parkar used selective cDNA arrays to elucidate some of the changes in gene expression in PDL cells exposed to EMD (19,34). They partly explain apparent efficacy of EMD application for periodontal regeneration. However, gene expression profiles of HBMSCs treated with EMPs were still unclear. In the present study, gene expression profile of EMP-treated HBMSCs was explored in this manner and 4096 sequences, including full-length and partial complementary DNAs (cDNAs), representing novel, known and control genes whose identity was provided by United Gene Holdings (36). This cDNA microarray is comprised of a wide range of functional genes involved in transcription, cell-cycle regulation, apoptosis, and expression of cell surface receptors and signal transduction mediators. Most of the genes regulated by EMPs in our data are functionally related to cell proliferation or signal mediators, providing new insight into the molecular mechanisms of EMP-mediated periodontal regeneration. Furthermore, four genes were selected for real-time PCR analysis – three upregulated genes (*EED*, *NRG1* and *SDC1*) and one downregulated gene (*Serpinb2*) – to validate the microarray conclusions. Results showed that expression levels of all four selected genes obtained from real-time PCR were consistent with cDNA microarray data.

Syndecans are a family of cell-surface transmembrane heparan-sulphate proteoglycans (37). They regulate cell behaviour through interactions with various effectors including heparin-binding growth factors and insoluble matrix components (38). They interact with growth factors, matrix components and other extracellular proteins, and are thought to be involved in processes such as cell proliferation, differentiation and adhesion (39). Syndecan-1 belongs to the family which is composed of four closely related proteins (syndecan-1, -2, -3 and -4) coded by four different genes. Our results showed that EMPs significantly upregulated expression level of *Syndecan1 1* (*SDC1*) in HBMSCs. It may be concluded that one of the mechanisms that promote proliferation of HBMSCs by EMPs may be involved in interaction between EMPs and a Syndecan.

Neuregulin 1 was originally identified as a 44-kDa glycoprotein that interacts with the NEU/ERBB2 receptor tyrosine kinase, to increase its phosphorylation on tyrosine residues. Through interaction with ERBB receptors, NRG1 induces proliferation and differentiation of epithelial, neuronal, glial and other cell types. NRG1, a widely expressed growth and differentiation factor that is structurally related to EGF, is currently the best candidate for providing the signal that activates synapse-specific

transcription (40,41). In our study, NRG1 was also upregulated, which may induce proliferation of HBMSCs in the presence of EMPs.

Embryonic ectoderm development gene (*EED*) encodes a member of the Polycomb-group (PcG) family. PcG family members form multimeric protein complexes, which are involved in maintaining the transcriptional repressive state of genes over successive cell generations. This protein mediates repression of gene activity through histone deacetylation and may act as a specific regulator of integrin function. However, integrins may mediate attachment of BMSCs to matrix proteins. As integrin-mediated attachment occurs, transmembrane receptors begin to cluster and then initiate signalling cascades that ultimately regulate events such as morphological cell spreading, migration, proliferation and differentiation (42). In this study, it was found that EMPs enhanced proliferation of HBMSCs and had no effect on their attachment nor spreading. This phenomenon may be explained by upregulation of *EED* and consequent inhibition of integrin.

Serpin peptidase inhibitor (*Serpinb2*) is a member of subgroup serpinb of serpin superfamily. Serpins inhibit serine proteinases by an irreversible suicide substrate mechanism when the interaction proceeds down the inhibitory arm of a branched pathway. *Serpinb2* may also be defined as PAI-2 (plasminogen activator inhibitor type-2). PAI-2 has been shown to have a number of intracellular roles: it can alter gene expression, and influence the level of cell proliferation and differentiation in a manner independent of urokinase inhibition. PAI-2 has also been reported to inhibit keratinocyte proliferation and to play a role in keratinocyte differentiation (43). Our results also demonstrated that downregulation of *Serpinb2* may play a role in proliferation of HBMSCs induced by EMPs. As described above, genes including *SDC1*, *NRG1*, *EED* and *Serpinb2* are related to cell adhesion and proliferation, and may contribute to the proliferation of HBMSCs by EMP treatment for periodontal regeneration, yet further investigation into the detailed mechanisms exerted by EMPs on HBMSCs is needed.

In summary, EMPs promoted proliferation ability of human BMSCs, but had no effect on their attachment or morphological spreading out. The results of cDNA microarray analysis demonstrated that EMPs are able to alter gene expression profiles of human BMSCs and have provided some new insights into molecular mechanism of EMP-mediated periodontal regeneration.

Acknowledgements

This research was supported by the National Nature Science Foundation of China (Project No.30672315,

30801292) and Shanghai Leading Academic Discipline Project (Project No. S30206).

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