Cell Prolif. 2007, **40**, 381–396

A study of enamel matrix proteins on differentiation of porcine bone marrow stromal cells into cementoblasts

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Received 16 *December* 2006*; revision accepted* 22 *January* 2007

Abstract. *Objective*: To further explore the role of enamel matrix proteins (EMPs) in periodontal regeneration, we have used porcine bone marrow-derived stromal cells (BMSCs) to observe whether the EMPs could have an effect on their differentiation into cementoblasts. *Materials and methods*: In this study, EMPs were extracted from porcine tooth germs by the use of acetic acid. BMSCs obtained from porcine iliac marrow aspiration were inoculated onto the surface of autologous root slices treated with or without EMPs. Following 7-day co-culture, all the BMSC-seeded root slices, with their respective non-cell-inoculated control specimens, were pocketed with expanded polytetrafluoroethylene membrane and were transplanted subcutaneously into 11 nude mice. The animals were sacrificed after 3 and 8 weeks, and the new specimens were processed for haematoxylin and eosin staining. *Results*: Histological analysis demonstrated new cellular cementum-like tissue formed along EMP-treated root slices. *Conclusion*: Our work has indicated for the first time, differentiation of BMSCs into cementoblasts using an EMP-based protocol.

INTRODUCTION

Regeneration of periodontal tissues destroyed or lost after periodontitis is a major objective in periodontal therapy. Due to the critical role played by cementum in maintaining periodontal structure, and regeneration of connective tissue attachment to the root surface, there has been a considerable interest in cementum development. Cementum is a calcified connective tissue that covers the tooth root surface, it regulates interaction between the periodontal ligament and tooth root (Bosshardt & Selvig 1997; Cho & Garant 2000). Cementogenesis is initiated after root dentin formation, and is regulated by interaction between Hertwig's epithelial root sheath (HERS) and dental follicle mesenchymal cells (Bosshardt & Schroeder 1996). Thus, a derivative of enamel matrix proteins (EMPs) extracted from porcine tooth buds, here is introduced as a biological mediator to achieve better periodontal regeneration. The rationale for use of this protein-based

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product is the assumption that EMPs, synthesized and secreted by cells of the Hertwig's epithelial root sheath, are active during embryogenesis of cementum, the periodontal ligament and supporting bone (Hammarström 1997).

Although recent clinical studies have reported some success in the promotion of both cementum and alveolar bone formation with EMP application, results have been unpredictable and unreliable. As early studies failed to detect the presence of growth factors in enamel matrix derivative (EMD) preparations (Gestrelius *et al*. 1997), it is postulated that EMD acts as a matrix enhancement factor, creating a positive environment for cell (osteoblast and cementoblast) proliferation, differentiation and matrix synthesis. The effect of EMD on matrix synthesis has been investigated by application of cultured periodontal fibroblasts (Haase & Bartold 2001), but there still exists controversy concerning effects on cell differentiation. Some studies have indicated that EMD is an osteoconductive agent (Schwartz *et al*. 2000) rather than an osteoinductive one. However, recent *in vitro* studies have suggested that EMD may have the ability to induce osteochondral progenitor cells to differentiate into osteoblasts and/or chondroblasts (Ohyama *et al*. 2002). Furthermore, until now, no information has been reported with regard to the particular effect of EMPs on osteoblastic or cementoblastic differentiation without disturbance of the periodontal environment, in any *in vivo* model system. This is of vital importance in the study of mechanisms of EMP influence.

A better understanding of the function of cells, their origin and factors influencing their capacity for tissue formation can be attained by re-transplantation of cells from these tissues (Lang *et al*. 1995). Bone marrow-derived stromal cells (BMSC) have been shown to have the potential to develop into mature cells that form liver, fat, cartilage, bone, epithelium, neuronal and muscle tissue, which depends on the conditions under which they are cultured (Tuan *et al*. 2003; Vaananen 2005). Extensive renewing potential in combination with their developmental plasticity has given rise to a tremendous interest in the prospective use of mesenchymal stem cells to regenerate damaged tissues. Here thus, cultured porcine bone marrow stromal cells, after induction in appropriate media, were co-implanted with root slices *in vivo*. The purpose was to discover whether EMPs would have an effect on cell differentiation into cementoblasts, in an effort to further explore the role of EMPs in periodontal regeneration.

MATERIALS AND METHODS

Extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of porcine enamel matrix proteins

According to the methods of Moe *et al*. (1984) and Shu Rong *et al*. (1999), enamel matrix proteins were prepared from secretory-stage enamel obtained from non-erupted, developing molars of approximately 6-month-old pigs, which was followed by demineralization using 0.5 M/l acetic acid and 20% trichloroacetic acid before the lyophilization.

Extracted enamel matrix lyophilized powder was analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Proteins were dissolved in SDS sample buffer (160 mM Tris-HCl, pH 6.8, 2% SDS, 26% glycerol, 0.1% bromophenol blue) at 5 mg/ml and was heated for 5 min at 100 °C. Then, 20 μl of each sample was loaded onto a 5% stacking gel and was separated on 15% resolving gels at a constant voltage of 100 V for 4 h at 20 °C. The gels were then fixed and stained for 0.5 h in 0.25% Coomassie blue stain in 10% acetic acid and were distained in de-ionized water overnight. Analysis by means of SDS-PAGE showed predominating bands at 20.13 and 5 kDa, which was typical for amelogenin (Brookes

et al. 1995; Maycock *et al*. 2002). The lyophilized powder was dissolved in 5 mM of acetic acid at a concentration of 4 mg/ml, was sterilized by filtration and was stored at –80 °C.

The animal model

Three male healthy crossbreed piglets (aged 3 months; each weighing 20–25 kg) and 11 nude Balb/c mice (aged 5 weeks; each weighing 20 g) were used for this experiment. The study was performed in accordance with the regulations and approval of the Institutional Animal Care and Use, Committee of the Shanghai Ninth People's Hospital and in conformity with standards of the Association for Assessment and Accreditation of Laboratory Animal Care. They were housed together for 1 week to become acclimatized to the environment and diet. Throughout the experiment, the pigs were monitored for their general appearance, activity, excretion and weight.

Isolation and expansion of porcine bone marrow stromal cells

Porcine bone marrow stromal cells (pBMSCs) were harvested and were isolated according to a modification of the procedure of Pittenger *et al*. (1999) and Jaiswal *et al*. (1997). Under general anaesthesia, bone marrow was aspirated from the pig ilium using a 16-gauge bone marrow aspiration needle. Four millilitres of bone marrow aspirate was collected into a syringe containing 2000 units of heparin. Bone marrow samples were washed with Dulbecco's modified Eagle's medium (DMEM) (high glucose, Gibco, Grand Island, NY, USA) and were centrifuged at 900 ***g***. Cell pellets were re-suspended in DMEM and were loaded onto 70% Percoll (Sigma, St. Louis, MO, USA) gradients. These gradients were centrifuged at 1100 ***g*** for 30 min, and the pBMSC-enriched density fraction (the upper part) was collected. Cells were re-suspended in complete DMEM supplemented with 10% foetal bovine serum (Hyclon, Logan, UT, USA) and 2% antibiotics (penicillin and streptomycin, Gibco BRL), and were plated at 1.0×10^6 cells/cm^2 into 55-cm^2 cell culture dishes (Corning, New York, USA). Cells were incubated under standard cell culture conditions. Media were changed after 5 days and every 3 days thereafter. Non-adherent and haematopoietic cells were removed at each medium change. When cultures became near-confluent the cells were detached with 0.125% trypsin (Sigma) containing 0.02% ethylenediaminetetraacetic acid (EDTA) for 3 min at 37 °C and were re-plated at 2×10^4 cells/cm^2 in fresh dishes. In this experiment, pBMSCs were cultured in complete DMEM medium until being used.

For the following experiment, BMSCs were cultured in general growth medium or in mineralized induction medium. The general growth medium, GG medium, was complete DMEM supplemented with 10% foetal bovine serum and ascorbic acid (50 mg/l, Sigma). The mineralized induction medium, MI medium, was GG medium additionally supplemented with 10 mM beta glycerophosphate disodium salt (Sigma) and 10^{-8} mM dexamethasone (Sigma).

The animal study

Here, autogenous root slices were used to load the pBMSCs following application of EMPs and were transplanted subcutaneously into the nude mice.

Root slice preparation

The pigs were anaesthetized with pentobarbital sodium intraveneously and teeth, including the incisors and premolars, were extracted. Meanwhile, two excised blocks of porcine alveolar bone with teeth anchored in were prepared for light microscopic observation to examine the normal periodontal tissue of the pig. Extracted roots were treated with careful scaling and root planning in order to remove fibres and the cementum. Then, roots were cut with a diamond blade in longitudinal section. In this way, two symmetrical pieces were obtained and internal pulpal tissue was cleared. Pieces were conditioned with 24% EDTA (Biora AB, Malmo, Sweden) for

Table 1. Distribution of root slices groups and specimens per group

Groups	EMPs treatment (#)	Medium	BMSCs inoculation (*)	Number
E1	–	GG	+	9
E2	+	GG	+	9
E3	–	MI	+	10
E4	+	MI	+	8
C1	–	GG	–	1
C2	+	GG	–	1
C3	–	MI	–	1
C4	+	MI	–	1

E1, E2, E3 and E4 groups refer to the experimental groups 1, 2, 3 and 4; and C1, C2, C3 and C4 the respective control groups 1, 2, 3 and 4;
#column: '–' means 'with no EMPs treatment' and '+' 'with EMPs treatment';
*column: '–' means 'with no BMSCs inoculation' and '+' 'with BMSCs inoculation'.

2 min and then were washed with a copious quantity of physiological solution, followed by sterilization under ultraviolet light for 4 h.

EMP treatment and BMSC inoculation

Root slices were placed into 48-well plates (Corning) with one chip in each well, scaled side upwards. After EMP treatment, pBMSC inoculation and application of different media, root slices were divided into four experimental groups and four control groups as illustrated in Table 1. EMP solution at concentration of 200 μg/ml was applied to the samples for 3 h at 37 °C, then, second passage pBMSCs, suspended in GG medium or MI medium were plated onto the 48-well plates at 160 000 cells/well.

Root slice transplantation into the nude mice

After 7 days of co-culture with change of medium twice, all root slices were rinsed with phosphate-buffered saline (PH7.4) and were wrapped with sterilized, expanded polytetrafluoroethylene (ePTFE, Shanghai Plastics Research Institute, China) and were transplanted subcutaneously into the nude mice (under general anaesthesia). After insertion of the ePTFE membrane-wrapped root slices beneath the dorsal subcutaneous skin, incisions through the skin were sutured and an antibiotic was administered orally to the animals to reduce risk of infection and inflammation. Each mouse received transplantation of four specimens of the same medium culture but different EMP treatment.

Sample harvest and specimen processing

At the third week and eighth week after surgery, 2× and 9× mice, respectively, were anaesthetized by ether inhalation. Transplanted specimens were excised, including the surrounding skin and subcutaneous tissue. Specimens were fixed in freshly prepared 10% formalin for 1 day. Some specimens were then de-mineralized in 14% EDTA, were dehydrated with increasing concentrations of ethanol and were embedded in paraffin wax. Longitudinal 5-μm serial sections were cut in the mesio-distal plane and four sections of each specimen were stained with haematoxylin and eosin. Other specimens, which were undecalcified, were infiltrated with and embedded in methyl-methacrylate. These were allowed to polymerize for 3–5 days at room temperature. Twenty

Table 2. The wound healing and number distribution of the specimens following the root slice transplantation

Wound healing	E1	E2	E3	E4	C1	C2	C3	C4
Membrane exposure	1	2	1					
Abscess formation		1						
3-week specimens	1	1	1	2				
8-week specimens	6	6	7	6	1	1	1	1

micrometre sections were taken by saw microtome (Leica SP 1600, Leica Microsystems, Nussloch, Germany) in the mesio-distal plane and were stained with a Stevenel's Blue – picric acid stain.

RESULTS

Healing of wounds

Healing of the dorsal skin wounds was uneven; there were three specimens with little membrane exposure although no inflammation was found around the wound. The rest were found to be normal with no membrane exposure after transplantation; one specimen showed abscess formation. Wound healing and distribution of the specimens are shown in Table 2.

Histological observations

Normal periodontium of the pig

Normal porcine cementum is different from that of humans, in which acellular cementum is the main component. In the pig, there was cell-rich cementum with approximately the same depth of colour as stained dentine, with cube-shaped cementoblasts aligned along the cementum. Alveolar bone was characterized by its bone marrow content. Periodontal ligaments aligned densely with fibres, inserted into the cementum and alveolar bone was clearly detected (Fig. 1).

Three weeks post-transplantation

E1, E2 and E3 groups consisted of only one specimen in each, which exhibited similar histological results. There was old cellular cementum left on some root surfaces. Between the membrane and the pre-dentine aspect, no new tissue was seen to have formed, which demonstrated that the membrane could, during the first 3 weeks after transplantation, bar cells of the nude mice from entering the membrane. Fibrous tissue had formed along root surfaces with spaces splitting new tissue and root surfaces (Fig. 2). In group E4, one specimen revealed cellular cementum-like tissue formation along the root surface with fibrous tissue adjacent or inserted into it. New fibrous tissue contained large numbers of cells. In some areas, cementum-like tissue was attached to root surfaces without split spaces (Fig. 3).

Eight weeks post-transplantation

Six specimens were obtained from group E1, all of which illustrated the same structure along the root surface with fibrous tissue formation (Fig. 4). In group E2, four of the six specimens showed tissue formation, similar to that in group E1, only one showing cellular cementum-like tissue formation along the root surface. Embedded cells in hard tissue were few in number; there

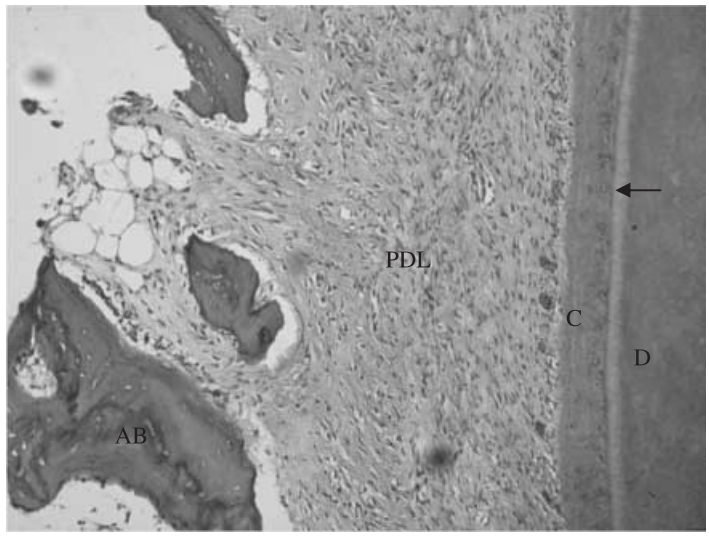

Figure 1. Histological observation of porcine normal periodontium. Dentino-cemental junction stained light red and fibrillar area across clearly seen (black arrow). Haematoxylin and eosin stain, ×100. AB, alveolar bone; PDL, periodontal ligament; C, cementum; D, dentin.

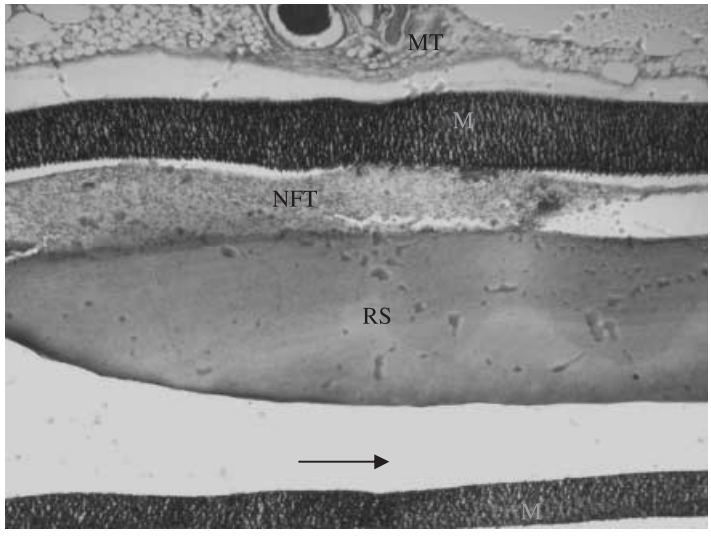

Figure 2. Three-week post-transplantation. New cell-rich fibrous tissue formed between ePTFE membrane and the outside of root slices. Black arrow, no tissue found along obverse side root slices (haematoxylin and eosin stain, ×40). M, ePTFE membrane; NFT, new fibrous tissue; RS, root slices; MT, mouse tissue.

were cube-shaped cells along the new cementum-like tissue and in some places, fibres were found inserted into it (Fig. 5). A further specimen displayed a small area of bone-like tissue formation with considerable amounts of new fibrous tissues. In group E3, four of the seven specimens showed bone-like tissue formed along the root surface without adjacent connective

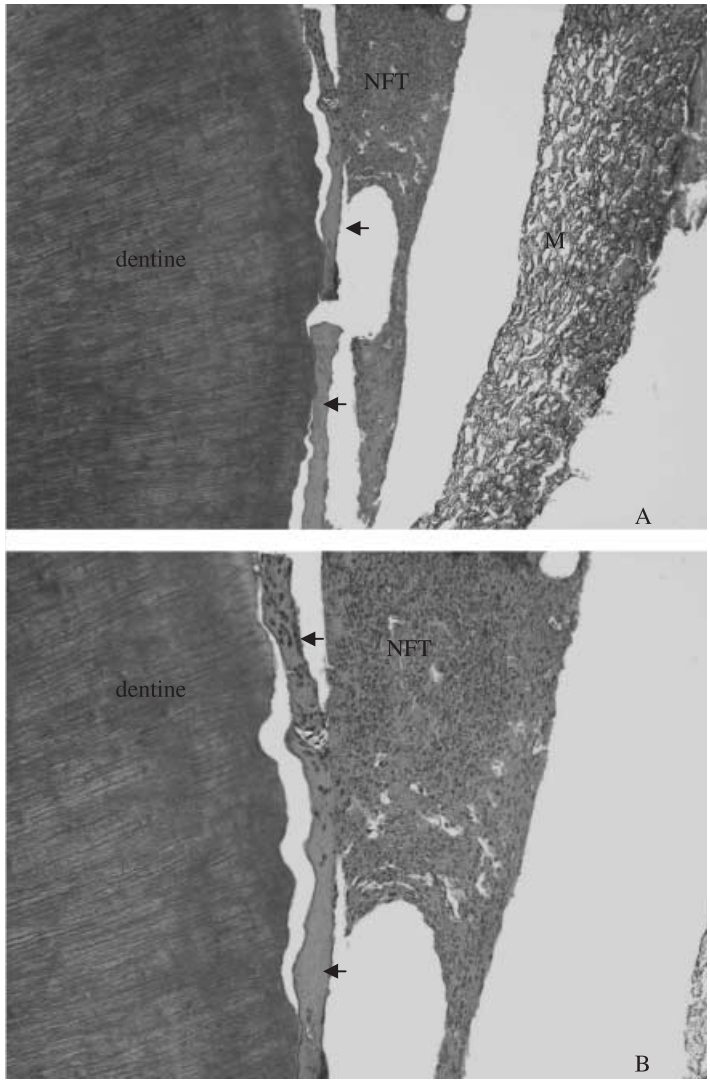

Figure 3. Three-week post-transplantation, Group E4 specimen. New cellular cementum-like tissue (arrow heads) formed along the root slice with cell-rich fibrous tissue adjacent. Haematoxylin and eosin stain (A) ×100; (B) ×200. NFT, new fibrous tissue.

tissue. Embedded cells were much more numerous than in those of group E2 (Fig. 6). Interestingly, two of the specimens illustrated bone marrow-like structures, which were comparable to that of the alveolar bone (Fig. 7). In group E4 (Fig. 8), four of the six specimens showed similar cellular cementum-like tissue formed along the root surface as described for those of group E2, which was comparable to that of normal pig cellular cementum.

In control groups, both sides had formation of fibrous tissue, with a broad space between the root surface and new tissue; no hard tissue formation was observed in these (Fig. 9). Table 3 indicates the ratio distribution of newly formed tissue characteristics to numbers of specimens acquired after transplantation into the nude mice, including third-week and eighth-week specimens.

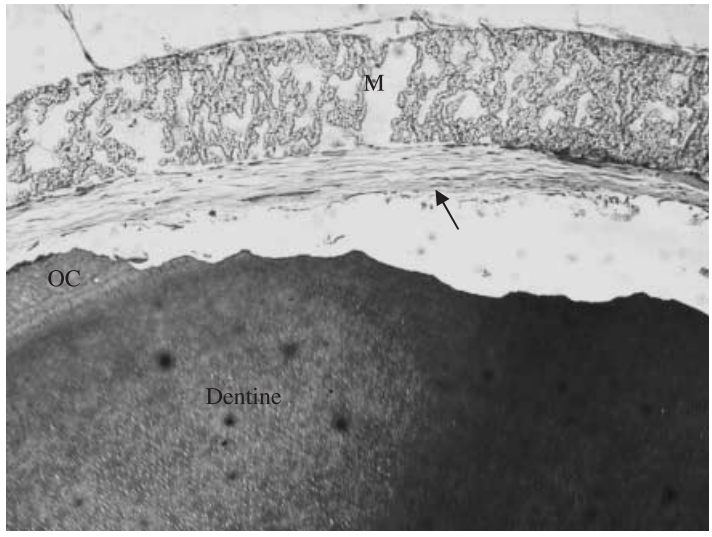

Figure 4. Eight-week post-transplantation, Group E1. Fibrous tissue formed along the root slice (black arrows), no new calcified tissue formed. Haematoxylin and eosin stain, ×100. OC, old cementum; M, membrane.

DISCUSSION

The *in vivo* animal study

Although the effects of EMPs on differentiation of BMSCs have been investigated *in vitro* (Du *et al*. 2004; Keila *et al*. 2004), capacity of the differentiated cell population for tissue formation was yet to be determined. A better understanding of a cell type's function, its origin and factors influencing its capacity for tissue formation can be acquired by a re-transplantation of the cells from their tissues of origin. Thus, in our study, cultured pBMSCs, induced by different media, were co-implanted with the root slice carrier, *in vivo*. Results show that different structures are formed by different treatments.

When pBMSCs were seeded on the EMP-treated root slices and were co-cultured with GG medium, one specimen alone showed cellular cementum-like tissue formation. This had connective tissue adjacent to, or embedded in, the newly formed hard tissue (resembling normal pig cellular cementum), with cubic cells aligned on its surface. When pBMSCs, seeded on the EMP-treated slices, had been co-cultured with inductive media then transplanted, however, many more specimens displayed cementum-like tissue formation. These findings suggest that pBMSCs, cultured with EMP supplement, promoted differentiation of the cells into cementoblasts and that the combination of EMPs and the inductive mineralized media may have resulted in more predictable cementoblast differentiation.

In contrast, when the root was not treated with EMPs yet co-cultured with an MI medium, very many more specimens were likely to demonstrate bone-like tissue formation, which could be confirmed by two specimens showing bone marrow formation in the new calcified tissue. This suggested that the inductive medium could induce pBMSC differentiation into osteoblasts; this is in line with findings of a previous study in which BMSCs cultured in a mineralized inductive medium differentiated into osteoblasts and formed bone-like tissue *in vivo* (Jaquiery *et al*. 2005; Kim *et al*. 2005).

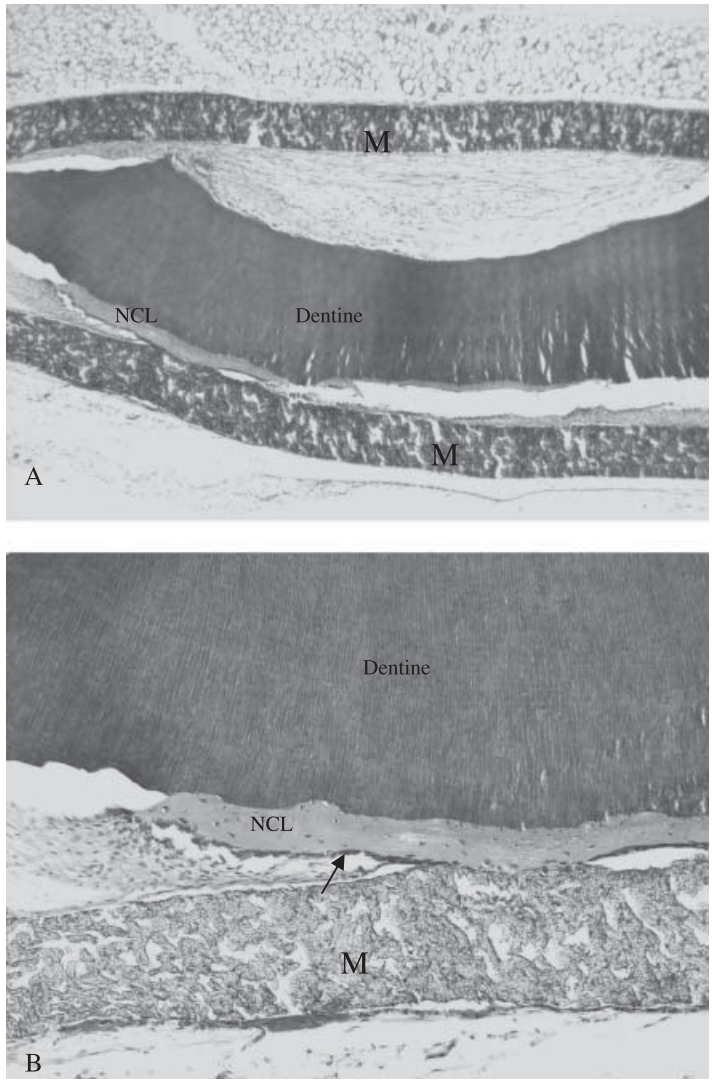

Figure 5. Eight-week post-transplantation, Group E2 specimen. New cellular cementum-like tissue formed along root slice with fibrous tissue adjacent. Cube-like cells (black arrows), similar to cementoblasts, aligned on the surface of the newly formed calcified tissue. NCL, new cementum-like tissue; M, ePTFE membrane. (A, B) Haematoxylin and eosin stain, ×40, ×100, respectively.

Recent investigations have indicated that mesenchymal stem cells (bone marrow-derived stromal cells), are pluripotent and can be differentiated into mature cells that form liver, fat, cartilage, bone, epithelium, neuronal and muscle tissues under appropriate culture conditions. Dexamethasone, for example, plays an important role in the differentiation of the multi-potential stem cells into various cells of the same lineage (Morsczeck *et al*. 2005) and can induce osteogenic differentiation (Pittenger *et al*. 1999; Nuttelman *et al*. 2006). In addition, it can act with other growth factors and induce BMSCs to differentiate into yet more phenotypes, for example, under conditions of high glucose DMEM medium, insulin-like growth factor, transforming growth

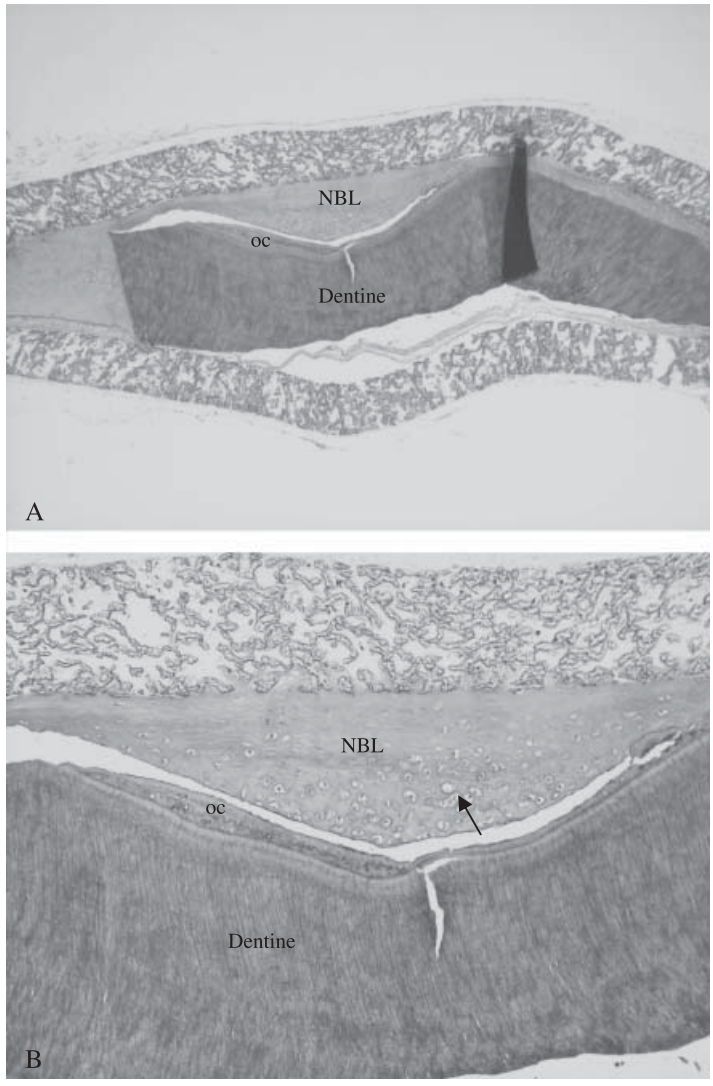

Figure 6. Eight-week post-transplantation, Group E3 specimens. The formed tissue resembled bone with more embedded cells (black arrow). NBL, newly formed bone-like tissue. OC, old cementum; (A, B) Haematoxylin and eosin stain, ×40, ×100, respectively.

factor and dexamethasone, bone marrow-derived stromal cells have been shown to be able to differentiate into chondrocytes (Pittenger *et al*. 1999; Sekiya *et al*. 2001). For BMSC adipogenesis, medium consisting of complete culture medium supplemented with 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 50 μM indomethacin has been successful (Sekiya *et al*. 2004). Thus, the concept of EMPs role in development of cementum formation is valid; interaction of the proteins with dexamethasone to promote BMSC differentiation into cementoblasts seems likely.

A number of experiments *in vivo* have demonstrated that EMPs stimulate regeneration of periodontal tissue, including alveolar bone (Araujo & Lindhe 1998; Sculean *et al*. 1998, 2000).

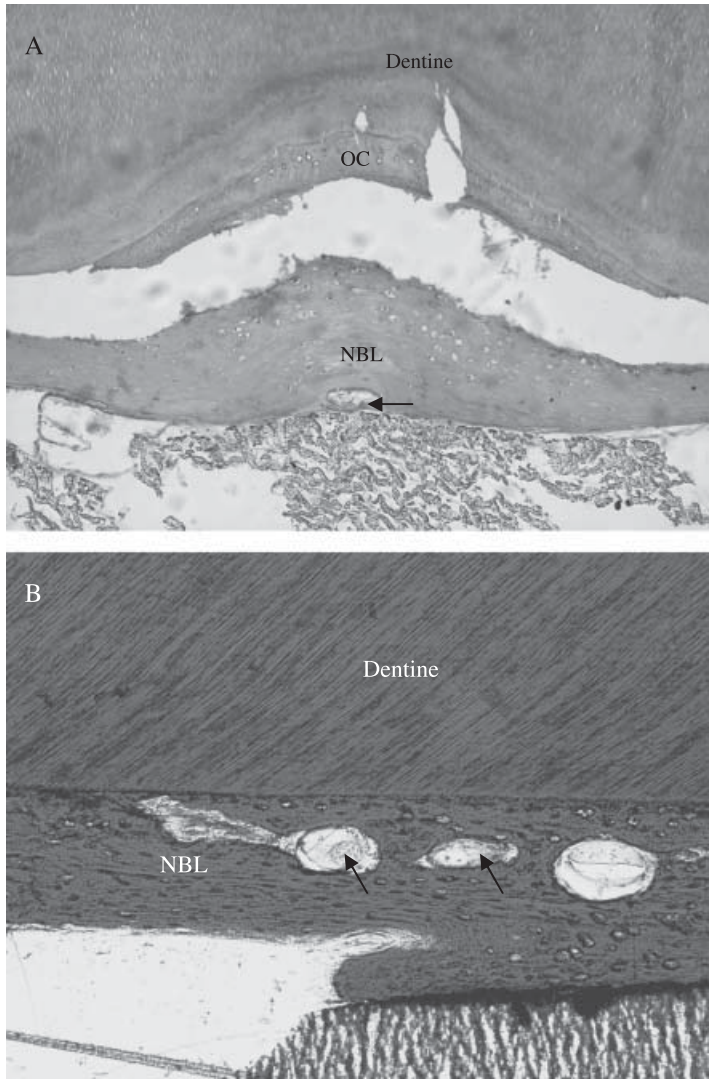

Figure 7. Eight-week post-transplantation, Group E3 specimens. Formed tissue resembled bone with bone marrow-like structures (black arrows). (A) Haematoxylin and eosin stain, ×100; (B) Stevenel blue stain for the hard tissue slices, × 100. OC, old cementum; NBL, new bone-like tissue.

The rationale for investigating EMPs for periodontal regeneration is based on the assumption that enamel matrix proteins, synthesized and secreted by cells of the Hertwig epithelial root sheath, trigger differentiation of dental follicle cells into cementoblasts (Hammarström 1997), representing a case of 'biomimicry' (Gestrelius *et al*. 2000). Moreover, it has been suggested that EMPs, particularly amelogenins, specifically induce formation of acellular extrinsic fibrillar cementum (Hammarström *et al*. 1997). However, in our study, there were differences between normal cementum of the experimental animal (pig) and that of normal humans. Pig cementum is highly cellular (different from that of humans) and EMPs induced newly formed cementum

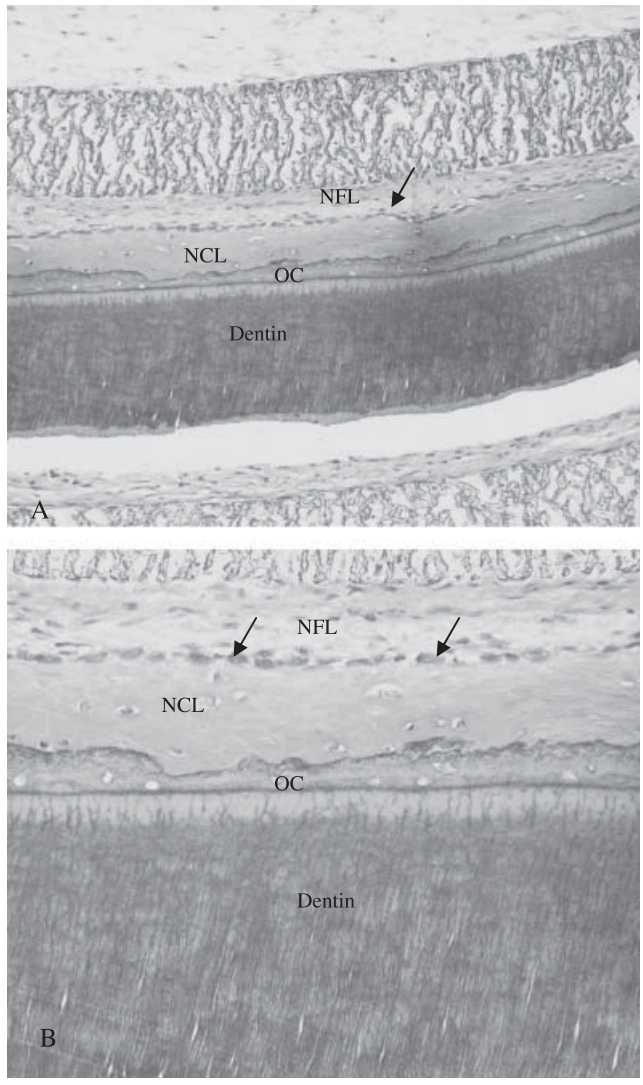

Figure 8. Eight-week post-transplantation, Group E4 specimens. New cellular cementum-like tissue formed on old cememtum with fibrous tissue adjacent. Cube-shaped cells (arrow head), similar to cementoblasts, aligned on the surface of the newly formed calcified tissue. NCL, new cementum-like tissue; OC, old cementum; NFT, newly formed fibrous tissue. (A, B) Haematoxylin and eosin stain, ×100, ×200, respectively.

comparable with old cementum of the pig. Even in humans, several studies have demonstrated cellular cementum formed under induction of the EMPs (Bosshardt *et al*. 2005; Majzoub *et al*. 2005), although the exact mechanism of the EMP activity is still unclear. It has been reported that non-commercial fractionated enamel extracts from developing porcine teeth are found to contain low levels of bone morphogenic protein (Iwata *et al*. 2002) and prospective studies should perhaps focus further on exploration of the growth factor-associated role of EMPs in modulating gene expression.

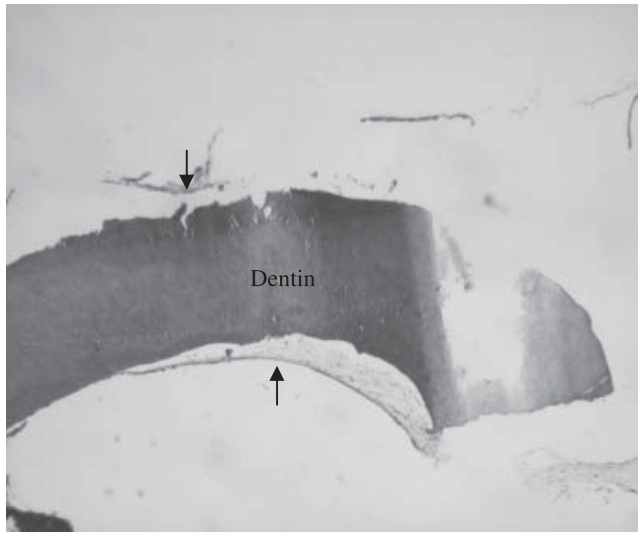

Figure 9. Histological observation of the control group: fibrous tissue formed along root slices Group E4 specimens (arrow head). Haematoxylin and eosin, ×40.

Table 3. The ratio distribution of the newly formed tissue characteristics and the number of the specimens acquired after the transplantation into the nude mice

Group	Cementum-like tissue	Bone-like tissue	Fibre-like tissue
E1	0/7	0/7	7/7
E2	1/7	1/7	5/7
E3	0/8	4/8	4/8
E4	4/8	0/8	4/8

In our hands, we have found it difficult to demonstrate unequivocally the newly formed tissue in the absence of a specific marker. Here, EMP groups and EMPs plus dexamethasone groups demonstrated that the newly formed calcified tissue was adjacent to fibrous tissue formation, which was comparable to normal cementum alignment in the pig, and which was morphologically distinct from tissue formed under induction of the mineralized inductive medium.

Animal model study establishment

In our study, we used root slices as a carrier for BMSCs, to mimic clinical cell transplantation. Periodontal ligament cells can be co-cultured with root slices to simulate the natural environment, and subsequently formation of periodontal-like tissue is found *in vitro* (Preisig & Schroeder 1988). Cells originating from different periodontal tissues can be co-cultured on extracted roots and be re-implanted. The cultured cells retain the capacity to form differentiated periodontal tissues after re-implantation, depending on the cells' origin type (Lang *et al*. 1995). Severe combined immunodeficiency mice are incapable of rejecting xenograft transplants because of their immune dysfunction due to genetic defects (Bosma *et al*. 1983; Malynn *et al*. 1988); thus, they represent a suitable animal model for studying cementogenesis (Grzesik *et al*. 1998; D'Errico

et al. 2000). In these, a cementum-like matrix has found to have been deposited, *in vivo*. Previous studies have reported that human cementoblasts (obtained from cellular cementum) upon implantation into severe combined immunodeficiency mice form cementum-like tissues, and that cells are phenotypically distinct from osteoblasts (Grzesik *et al*. 1998; Grzesik *et al*. 2000). In our study, we have used membranes, in which to pocket transplanted root slices, to reduce potential effects of the nude mouse immunological environment on BMSCs. The membrane's main component is polytetrafluoroethylene that has its pore size in the region of 50 μm. The results demonstrated that no structure had formed along the 'no-cell inoculation' side for 3 weeks after surgery, which indicated that the membrane was impervious to cells of nude mice for the initial 3 weeks. By this time-point, the BMSCs had proliferated and had developed into mature cells. Thus, the model was valid for application in our studies of cell differentiation activity and of mechanisms for EMP acctivity, *in vivo*, without disturbance of periodontal-related factors.

In our control groups, there was no mineralized tissue formed on root dentine, indicating that any mineralized tissue formed was by the bone marrow-derived stromal cells. Thus, our study has shown that BMSCs can be induced to seed cells in periodontal tissue engineering. We can conclude that EMPs can promote BMSC differentiation into cementobasts, and that they can also promote regeneration of periodontal tissues via differentiation pathways. It follows that EMPs can be used to induce the BMSC differentiation *in vitro* to be transplanted clinically into periodontal defects to regenerate lost periodontal tissue.

ACKNOWLEDGEMENTS

This work was completed in the Shanghai Key Laboratory of Stomatology. We would like to thank Xinquan Jiang and Xiuli Zhang for their excellent technical support. This work was supported by a generous grant from the National High Technology Research and Development Program of China (863 Program, 2002AA205011) and the Shanghai Education Development Foundation of China (2002-02).

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