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REGULAR ARTICLE

Establishment of immortalized dental follicle cells for generating periodontal ligament in vivo

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Abstract The dental follicle is a mesenchymal tissue that surrounds the developing tooth germ. During tooth root formation, periodontal components, viz., cementum, periodontal ligament (PDL), and alveolar bone, are created by dental follicle progenitors. Here, we report the presence of PDL progenitors in mouse dental follicle (MDF) cells. MDF cells were obtained from mouse incisor tooth germs and immortalized by the expression of a mutant human papilloma virus type 16 *E6* gene lacking the PDZ-domain-binding motif. MDF cells expressing the mutant *E6* gene ($MDF^{E6\text{-}EGFP}$ cells) had an extended life span, beyond 150 population doublings (PD). In contrast, normal MDF cells failed to proliferate beyond 10 PD. $MDF^{E6\text{-}EGFP}$ cells expressed tendon/ligament phenotype-related genes such as *Scleraxis* (*Scx*), *growth and differentiation factor-5*, *EphA4*, *Six-1*, and *type I collagen*. In addition, the expression of *periostin* was observed. To elucidate the differentiation capacity of $MDF^{E6\text{-}EGFP}$ cells in vivo, the cells were transplanted into severe combined immunodeficiency mice. At 4 weeks, $MDF^{E6\text{-}EGFP}$ cell transplants had the capacity to generate a PDL-like tissue that expressed *periostin*, *Scx*, and *type XII collagen* and the fibrillar assembly of type I collagen. Our findings suggest that $MDF^{E6\text{-}EGFP}$ cells can act as PDL progenitors, and that these cells may be a useful research tool for studying PDL formation and for developing regeneration therapies.

Keywords Dental follicle · Progenitor · Development · Immortalization · Periodontal ligament · Mouse (ICR)

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Introduction

The periodontal ligament (PDL), which surrounds the tooth root, absorbs occlusal forces and functions as a sense organ (Ten Cate 1994). In periodontitis, a chronic inflammatory disease, the PDL is irreversibly damaged. Despite a number of novel approaches, no one has yet succeeded in reliably forming PDL (D'Errico et al. 1999).

Hence, there is considerable interest in the developmental mechanisms of PDL.

The PDL originates from dental follicle cells formed during the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from cranial neural crest cells (Chai et al. 2000). Progenitors in the dental follicle are thought to contribute to the formation of all periodontal tissues, namely cementum, PDL, and alveolar bone (Bosshardt and Schroeder 1996). After the formation of tooth root dentin, cementoblast progenitors in the dental follicle migrate onto the tooth root surface and differentiate into cementoblasts (Bosshardt and Schroeder 1996). Almost simultaneously, PDL progenitors within the dental follicle cells differentiate into PDL cells. Finally, both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus. Although a specific marker for PDL is not available, tendon/ligament phenotype-related genes are thought to be involved in the differentiation of PDL progenitors. Growth and differentiation factors (GDFs)-5, 6, and 7 are members of the bone morphogenetic protein family that regulate tendon/ligament formation (Wolfman et al. 1997) and have been shown to be expressed by both dental follicle and PDL cells (Morotome et al. 1998; Nakamura et al. 2003; Sena et al. 2003). Screlaxis (*Scx*), a basic helix-loop-helix transcription factor that serves as a tendon progenitor marker gene, has also been found to be expressed by PDL stem cells (Brent et al. 2003; Seo et al. 2004). Non-collagenous extracellular matrix has been shown to be involved in the formation of PDL (Matias et al. 2003). *Periostin* is a marker for preosteoblasts but is also found in the periosteum and PDL (Horiuchi et al. 1999). During tooth germ development, *periostin* is initially expressed in the dental follicle cells and is then restricted to postnatal PDL cells during tooth root formation (Kruzynska-Frejtag et al. 2004). *Periostin-/-* mice develop a periodontal-disease-like phenotype within 3 months of birth, suggesting that this protein plays a critical role in maintenance of the PDL (Rios et al. 2005). These findings suggest that both tendon/ligament phenotype-related genes and extracellular matrices, which are highly expressed in PDL, are involved in PDL formation and maintenance. However, details of the mechanisms involved in PDL formation are yet to be clarified, because of the scarcity of PDL progenitor culture systems.

Recently, we have demonstrated the presence of cementoblast progenitors in bovine dental follicle cells (Handa et al. 2002). A cementoblast progenitor cell line, designated as BCPb8, has the capacity to form PDL- and cementum-like tissue when transplanted into severe combine immunodeficiency (SCID) mice (Saito et al. 2005). Although BCPb8 is a clonal cell line useful for the study of dental follicle progenitors, the use of these cells derived from a bovine species is restricted, because the cDNA database is inadequate, and because antibodies are limited. Thus, little is known about the biological properties of dental follicle progenitors or the mechanisms that regulate their differentiation. Investigations directed at addressing these key questions are essential if we are to understand the developmental mechanisms of PDL. In this study, we have attempted to establish immortalized mouse dental follicle (MDF) cells for the study of progenitors in dental follicle cells.

Materials and methods

Tissue culture

MDF cells were isolated from the dental follicle tissue of the incisor tooth germs of 1-day postnatal (P1) ICR mice. Briefly, mouse dental follicle tissue was mechanically stripped from the lingual posterior region of mouse incisor and placed onto a 24 multi-well plate. The tissues were then incubated with α-minimum essential medium (α-MEM: Sigma, St. Louis, USA) containing 10% fetal bovine serum (FBS; BioWhittaker, Maryland, USA), 50 μg/ml ascorbic acid, 100 U/ml streptomycin and penicillin, in a humidified atmosphere of 5% CO_2 at 37°C. When the cells reached approximately 80% confluence, they were passaged with 0.25% trypsin/1 mM EDTA and maintained as MDF cells. These cells were plated into six wells at a density of 3×10^4 cells/ml, and the medium was changed every 2 days.

Infection of retrovirus constructs and establishment of $MDF^{E6\text{-}EGFP}$ cells

The construction of pCLXSN-$16E6^{\Delta146\text{-}151}$ and the production of LXSN-$16E6^{\Delta146\text{-}151}$ retrovirus have been described previously (Kyo et al. 2003). An aliquot of 1 ml producer cell culture fluid was added to MDF cells (passage 1) in the presence of polybrene (8 μg/ml), and the cells were subsequently selected in the presence of G418 (100 μg/ml). Transduced cells were maintained in the medium described above. Following infection with LXSN-$16E6^{\Delta146\text{-}151}$, MDF cells were transduced with EGFP lentivirus under the control of a CMV promoter to obtain stably expressed EGFP ($MDF^{E6\text{-}EGFP}$).

Osteogenic differentiation

Cells were plated into six wells at a density of 3×10^4 cells/ml and cultured in the medium described above supplemented with 100 nM dexamethasone, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate. The culture medium was

replaced every 2 days, and the cells were maintained for 3 weeks.

Alkaline phosphatase activity and alizarin red staining

To evaluate alkaline phosphatase (ALP) activity, $MDF^{E6\text{-}EGFP}$ cells were fixed with 4% paraformaldehyde for 20 min at 21°C. After being washed with PBS, the cells were incubated for 20 min in a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% N-N dimethyl formamide (Sigma), 2 mM $MgCl_2$, 0.6 mg/ml Fast Blue BB salt (Sigma) in 0.1 M TRIS-HCl (pH 8.5) at room temperature. Calcium accumulation was detected by staining preparations with 2% alizarin red S (pH 6.4) (Sigma). MC3T3E1 (purchased from RIKEN BioResource Center, Japan) and NIH3T3 were used as controls.

Probes for in situ hybridization

The cDNA of mouse *osteopontin* open reading frame (885 bp), mouse *Scx* 3′ untranslated region (UTR; 291 bp), or mouse *type XII collagen* (500 bp) region was amplified by reverse transcription/polymerase chain reaction (RT-PCR) by using the following primers: *osteopontin*, 5′-ATGAGATTGGCAGTGATTTG-3′ and 5′-GTTGACCT CAGAAGATGAAC-3′; *Scx*, 5′-AAGAGGT GATGCCAC TAGTG-3′and 5′-TATACAAAATTTCCA GACTTTAT ATTATCAT-3′; *type XII collagen*, 5′-TCCCCATCAAA GAACAGACC-3′ and 5′-TGACTGCTGGATGA CAAAGG-3′. The amplicons for *osteopontin* and *Scx* were subsequently cloned into the pCRII vector (Invitrogen, Carlsbad, Calif., USA), and those for *type XII collagen* were cloned into pCR 4-TOPO (Invitrogen). A 623-bp mouse *periostin* cDNA fragment was isolated by RT-PCR with partial T7 and T3 promoter-containing primers (below, in italics) at the 5′ end and the 3′ end, respectively: T7: 5′-*CACTATAGGGC*GGCTGAA GATGGTTCCTCTC-3′, and T3: 5′-*CACTAAAGGGC* CATGTGGCTGTGTAAGG CATTC-3′. These cDNA fragments were further amplified by using the following adaptor primers to install full T7 and T3 promoter sequences into the fragments: 5′-GTAATAC GACTCAC TATAGGGC-3′ for T7, and 5′-AAT TAACCCTCAC TAAAGG-3′ for T3.

In situ hybridization

To generate antisense and sense digoxygenin-labeled riboprobes, linearized *osteopontin*, *Scx*, and *type XII collagen* plasmids and *periostin* cDNA fragments (see previous section) were transcribed by T7, T3, or Sp6 RNA polymerase as described elsewhere (Wilkinson 1995). Heads of P1 C57BL/6 mice were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), and 10-μm-thick sagittal sections were cut. Mandibles of P35 mice were fixed in 4% paraformaldehyde at 4°C overnight, decalcified in 12.5% EDTA containing 2.5% paraformaldehyde for 6 weeks, and then embedded in OCT compound. In situ hybridization was carried out on these sections as previously described with some modification (Iseki et al. 1999). Polyvinyl alcohol was used as buffer during the color reaction.

RNA preparation and RT-PCR

Total RNA was isolated from cells by using ISOGEN (Nippon Gene, Tokyo, Japan) as described previously (Handa et al. 2002). cDNAs were synthesized from 1 μg total RNA in a 20-μl reaction volume containing 10× reaction buffer, 1 mM dNTP mixture, 1 U/μl RNase inhibitor, 0.25 U/μl reverse transcriptase (M-MLV reverse transcriptase; Invitrogen), and 0.125 μM random 9-mers (Takara, Tokyo, Japan). Amplification was performed in a PCR Thermal Cycler SP (Takara, Tokyo, Japan) for 25 cycles with the following reaction profile: 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s. Synthesized cDNA served as a template for subsequent PCR amplification with mouse-specific primers: *bone sialoprotein* (*BSP*): sense 5′-AGGGAACTGACCAGTGTTGG-3′, antisense 5′-TCGTTGCCTGTTTGTTCGTA-3′; *osteocalcin* (*OC*): sense 5′-CATGAGGACCCTCTCTCTGC-3′, antisense 5′-GCCGGAGTCTGTTCACTACC-3′; *osteopontin*: sense 5′-TGCACCCAGATCCTATGACC-3′, antisense 5′-TGTGGTCATGGCTTTCATTG-3′; *periostin*: sense 5′-TTGAAGGTGTCCCGSCTTC-3′, antisense 5′-TGAT TCGTTCTTCCCGAGTC-3′; *Scx*: sense 5′-CTTTCTTC CACAAGCGGTCGT-3′, antisense 5′-TGTCACGGTCTT TGCTCAAC-3′; and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*): sense 5′-TGTTCCTACCCCCAA TTGTGT-3′, antisense 5′-AGGAGACAACCTGGTCC TCA-3′. Specific primers for *six-1*, *EphA4*, *GDF-5*, *collagen I* (*Coll*), and *osterix* have been described previously (Salingcarnboriboon et al. 2003).

In vivo differentiation assay

The differentiation potential of $MDF^{E6\text{-}EGFP}$ cells was assessed by transplantation of the cells into SCID mice as described previously (Handa et al. 2002). Briefly, cells were inoculated subcutaneously into 5-week-old male CB-17 scid/scid (SCID) mice (Nihoncrea, Tokyo, Japan) after incubation of 1.5×10^6 cells in a mixture of 40 mg hydroxyapatite powder (Osferion, Olympus, Tokyo, Japan) and fibrin clot (mixture of mouse fibrinogen and thrombin: both from Sigma). Transplantation analysis was carried out three times, and three transplants were prepared per group. The mice were sacrificed after 4 weeks and subjected to

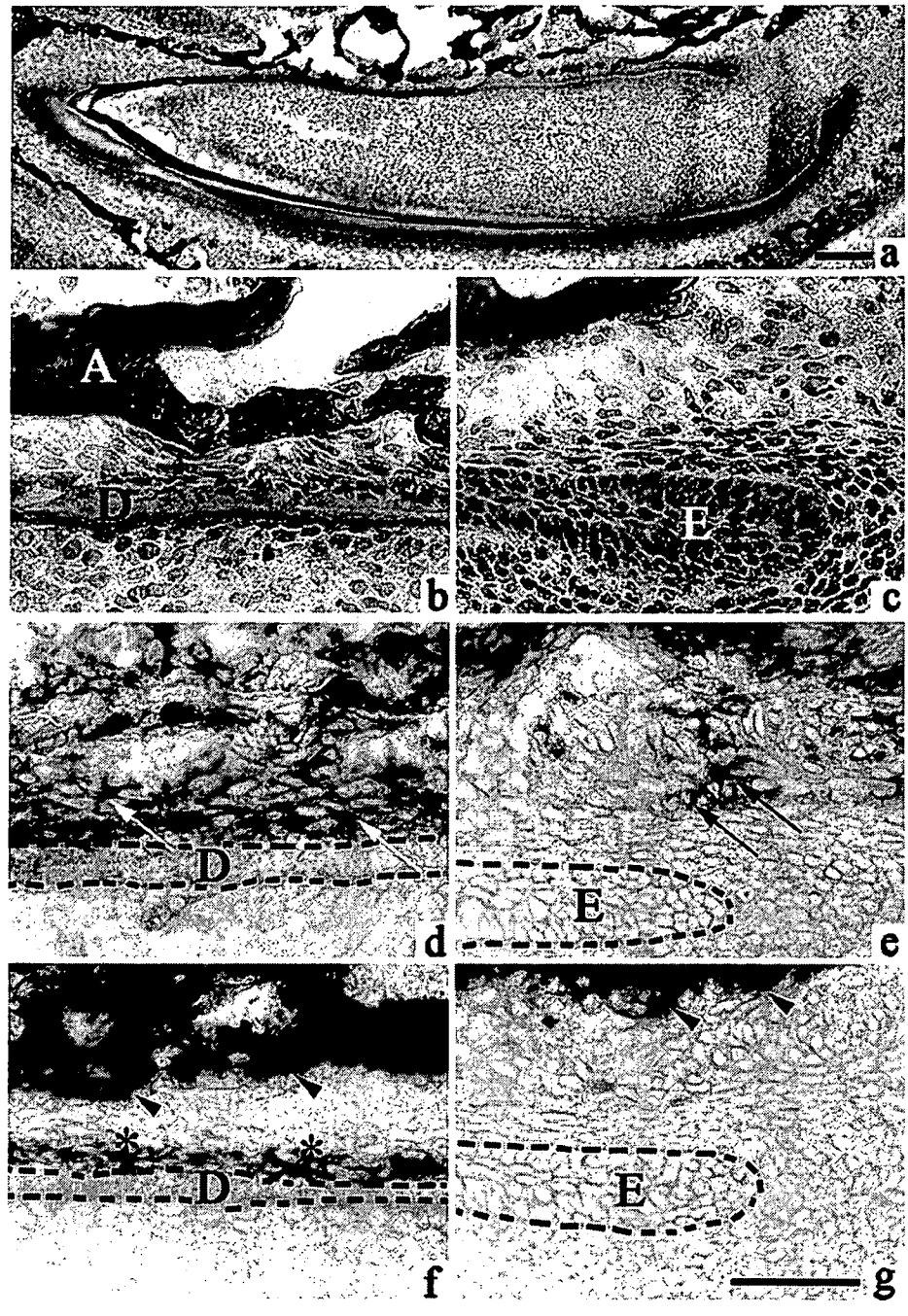

Fig. 1 In situ hybridization for *periostin* and *osteopontin* mRNA in newborn mice (*A* alveolar bone, *D* dentin, *E* dental epithelium). Sagittal sections of the incisor in the lower jaw of P1 mouse (**a**) were examined by hematoxylin and eosin staining (**a–c**) or in situ hybridization for *periostin* (**d, e**) or *osteopontin* (**f, g**). Higher magnifications of the anterior (**b, d, f**) and posterior (**c, e, g**) regions of the lingual side of the incisor are shown. Intense expression of *periostin* is seen in the developing PDL in the anterior region (**d**, *arrows*), with little expression in the posterior region (**e**, *arrows*). *Osteopontin* is expressed strongly throughout the alveolar bone (**f, g**, *arrowheads*) and in the differentiated cementoblasts in the anterior region (**f**, *asterisks*). No expression of *osteopontin* is observed in the dental follicle tissue in the posterior region (**g**). *Bars* 100 μm

histochemical analysis with immunohistochemical staining or in situ hybridization as described below. NIH3T3 cells were used for comparison.

Histochemical analysis

The transplants were fixed in 4% paraformaldehyde for 1 day, decalcified with 12.5% EDTA containing 2.5% paraformaldehyde for 3 days, and then embedded in OCT compound for the production of frozen sections. Subsequently, 30 serial sections of 5 μm in thickness were cut per implant and analyzed histochemically. Morphology was examined by hematoxylin and eosin staining. Observation by fluorescence microscopy (Axio imager, Carl Zeiss, Germany) was performed to distinguish between the cells of donor origin and host tissue. Expression of mRNA for *periosotin* and *Scx* was examined by in situ hybridization as described above. For immunohistocemical analysis, the

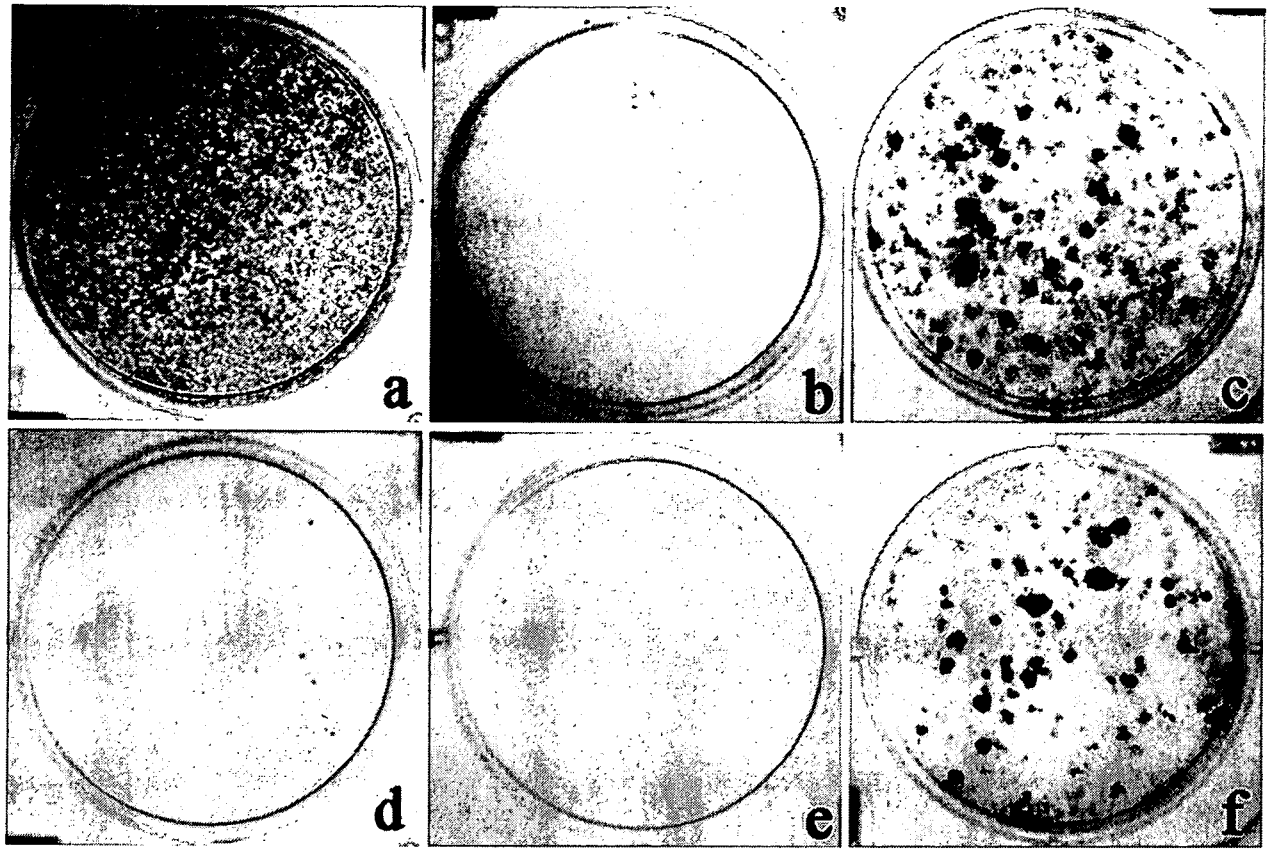

Fig. 2 ALP activity and mineralization potential of MDF$^{E6\text{-}EGFP}$ cells in vitro. MDF$^{E6\text{-}EGFP}$ cells were grown for 21 days in osteogenic differentiation medium in order to analyze ALP activity (**a–c**) and mineralization potential (**d–f**). High ALP activity by MDF$^{E6\text{-}EGFP}$ cells and MC3T3E1 (**a**, **c**) cells was apparent, whereas no activity was observed for NIH3T3 cells (**b**). Alizarin red staining showed no mineralized nodule formation for MDF$^{E6\text{-}EGFP}$ cells and NIH3T3 cells (**d**, **e**), whereas mineralized deposits were readily apparent for MC3T3E1 cells (**f**)

sections were blocked with 1% bovine serum albumin and probed with goat anti-type I collagen polyclonal antibody (Southern Biotech, Birmingham, Ala., USA) for 1 h. Sections were then probed with donkey anti-goat Alexa 555 (Invitrogen). After several washes, fluorescence in the sections was observed by fluorescence microscopy.

Results

Localization of periostin mRNA in the incisor tooth

Sequential developmental process of the dental follicle was observed in a sagittal section (in an anterior-posterior direction) of the P1 mice incisor (Fig. 1a). Immature cells were located posteriorly (Fig. 1c,e,g), and differentiation progressed toward the anterior region (Fig. 1b,d,f). To locate immature dental follicle cells, we first investigated the expression pattern of a PDL marker gene, *periostin*, and a cementoblast/osteoblast marker, *osteopontin*, in the lower incisor tooth germ of P1 mice. In a section stained with hematoxylin and eosin, dentin was seen in the anterior region (Fig. 1b), and invaginating dental epithelium was observed lingually in the posterior region (Fig. 1c). Intense expression of *periostin* was observed in the dental follicle cells close to the dentin layer in the anterior region (Fig. 1d, arrows). In contrast, only patchy expression of *periostin* was observed in the dental follicle cells in the posterior region (Fig. 1e, arrows). *Osteopontin* was expressed intensely throughout the alveolar bone (Fig. 1f,g, arrowheads). In accordance with the *periostin* expression pattern, *osteopontin* was expressed in the cementoblast adjacent to the dentin-forming layer in the anterior region (Fig. 1f, asterisks), but not in the posterior region (Fig. 1g). These data confirmed that the dental follicle cells in the posterior region were in an immature stage, whereas those in the anterior region were differentiated. We thus dissected dental follicle cells from the posterior region in order to establish a dental follicle progenitor cell culture system.

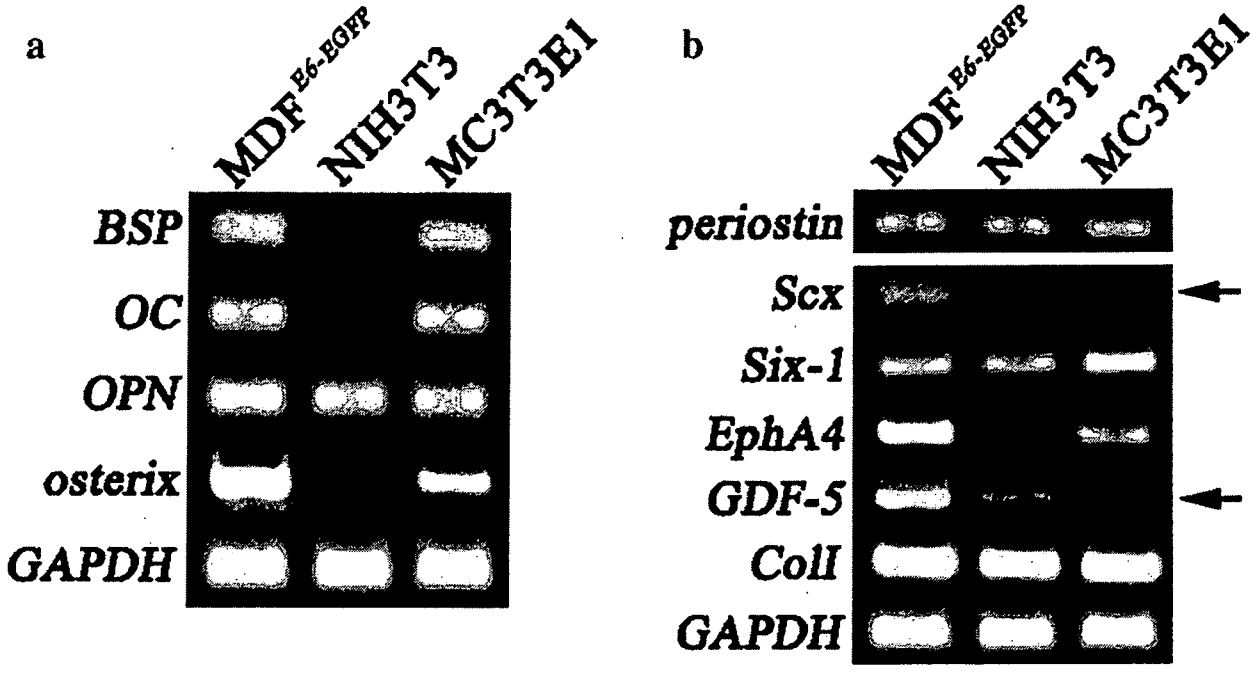

Fig. 3 RT-PCR analysis of $MDF^{E6\text{-}EGFP}$ cells. Expression of osteoblastic phenotype-related genes such as *bone sialoprotein* (*BSP*), *osteocalcin* (*OC*), *osteopontin* (*OPN*), and *osterix* (**a**), and PDL and tendon/ligament phenotype-related genes, such as *periostin*, *scleraxis* (*Scx*), *Six-1*, *EphA4*, *growth and differentiation factor 5* (*GDF5*), and *type I collagen* (*ColI*; **b**) were examined by RT-PCR analysis. Cells were cultured with osteoblastic differentiation (**a**) or normal medium (**b**), and total RNA was extracted (*arrows* strong expression of *Scx* and *GDF-5* in $MDF^{E6\text{-}EGFP}$ compared with NIH3T3 cells or MC3T3E1 cells)

Characterization of $MDF^{E6\text{-}EGFP}$ cells

MDF cells were isolated from the posterior region of the incisor tooth germ, and their life span was extended by using a retrovirus-expressing human papillomavirus type 16 (HPV16) *E6* gene lacking the PDZ-domain-binding motif ($E6^{\Delta 146\text{-}151}$), together with a lentivirus expressing *EGFP* for fluorescence detection. After viral infection, the expression of $E6^{\Delta 146\text{-}151}$ was confirmed by RT-PCR analysis (data not shown). $MDF^{E6\text{-}EGFP}$ cells maintained their original morphology and cell proliferation activity, even when the cells were cultured beyond population doubling (PD) 150, indicating that they had overcome replicative senescence. In contrast, normal MDF cells were only able to propagate until PD 10 (data not shown). We thus used $MDF^{E6\text{-}EGFP}$ cells for further analysis.

To assess the osteogenic potential of $MDF^{E6\text{-}EGFP}$ cells, the cells were treated with osteogenic differentiation medium supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone for 21 days. ALP activity was observed in $MDF^{E6\text{-}EGFP}$ cells (Fig. 2a) and in osteoblast-like MC3T3E1 cells (Fig. 2c). Mineralized nodule formation by the $MDF^{E6\text{-}EGFP}$ cells was not observed (Fig. 2d), whereas MC3T3E1 cells were able to deposit mineralized nodules (Fig. 2f). Neither ALP activity nor mineral deposition was observed in the fibroblastic cells (NIH3T3 cells; Fig. 2b,e). RT-PCR analysis was performed to characterize the $MDF^{E6\text{-}EGFP}$ cells by using primers for osteoblast phenotype-related genes *BSP*, *OC*, and *osterix*. $MDF^{E6\text{-}EGFP}$ cells expressed all of these osteoblast marker genes, with a similar expression pattern to that of MC3T3E1 cells. In contrast, no expression of osteoblast phenotype-related genes was observed in NIH3T3 cells, except for *BSP*. From these data, we considered that osteoblastic populations were present in $MDF^{E6\text{-}EGFP}$ cells (Fig. 3a).

To characterize the PDL-forming properties of $MDF^{E6\text{-}EGFP}$ cells, gene expression of *periostin* and tendon/ligament phenotype-related genes encoding *Scx*, *EphA4*, *Six-1*, *GDF-5*, and *ColI* were examined by RT-PCR analysis. $MDF^{E6\text{-}EGFP}$ cells expressed all of these genes suggesting that they possessed PDL and tendon cell properties (Fig. 3b). Stronger expression of *Scx* and *GDF-5* was observed in $MDF^{E6\text{-}EGFP}$ cells than in NIH3T3 cells or MC3T3E1 cells (Fig. 3b, arrows). Expression patterns of *Scx* and *periostin* mRNA were examined in postnatal (P35) mouse molar PDL (Fig. 4a) by in situ hybridization. Intense expression of *periostin* was observed throughout the PDL (Fig. 4b, arrows), and *Scx* was also expressed in the PDL (Fig. 4c, arrowheads).

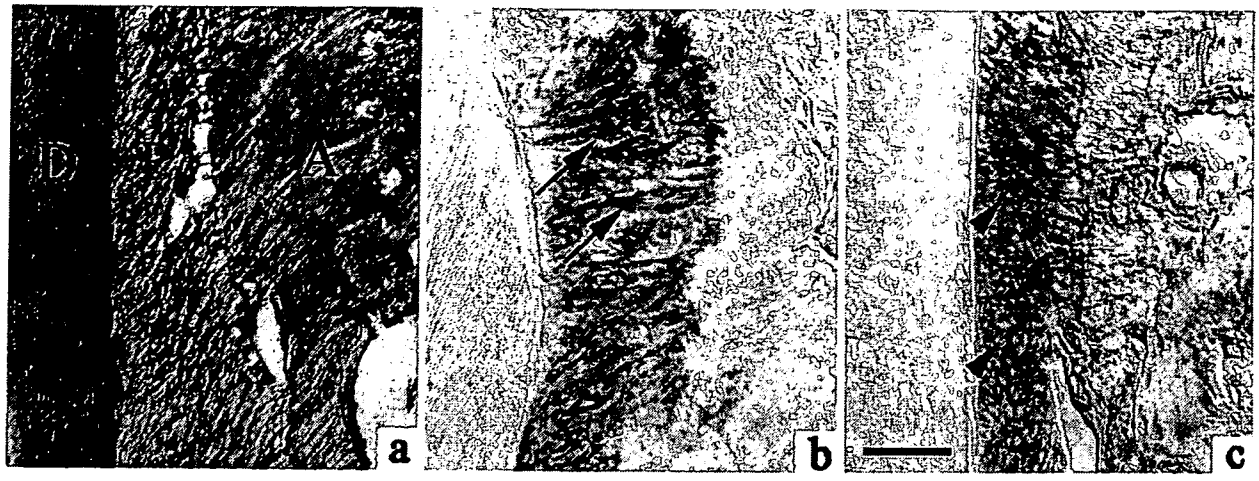

Fig. 4 In situ hybridization for *Scx* and *periostin* mRNA in postnatal PDL (*A* alveolar bone, *D* dentin, *P* PDL). Frontal sections of the molar tooth of P35 mouse (**a**) were examined by hematoxylin and eosin staining (**a**) or in situ hybridization with *periostin* (**b**) or *Scx* (**c**). Intense expression of *periostin* was seen within PDL (**b**, *arrows*). Expression of *Scx* can be seen throughout the PDL (**c**, *arrowheads*). *Bars* 100 μm

In vivo characterization of MDF$^{E6\text{-}EGFP}$ cells

To investigate the differentiation potential of MDF$^{E6\text{-}EGFP}$ cells, the cells were implanted into SCID mice. After 4 weeks, MDFE6-EGFP cell transplants formed PDL-like fibrous tissue (Fig. 5a-a) including scattered bone-like tissue formation (Fig. 5a-b). The PDL-like tissues resembled the structure of PDL with sheet-like cells (Fig. 5a-a, a-d, arrows). Cells within the PDL- and bone-like tissues were strongly positive for EGFP (Fig. 5a-d, a-e). In contrast, no such tissues were formed in the transplants without mouse cells (HAP transplants; Fig. 5a-f). Immunohistochemical staining revealed dense type I collagen fibril assembly in the PDL-like tissue (Fig. 5b-a), whereas type I collagen fibril assembly was not evident in the NIH3T3 cell or HAP transplants (Fig. 5b-b, b-c). To validate the capacity of MDF$^{E6\text{-}EGFP}$ cells to differentiate into PDL in vivo, the expression of *periostin* and *Scx* was examined in the transplants by in situ hybridization. Expression of *type XII collagen* has been shown in the PDL during tooth root formation; therefore, we also examined the expression of this gene in the transplants (MacNeil et al. 1998). As expected, the expression of *periostin*, *Scx*, and *type XII collagen* was observed in MDF$^{E6\text{-}EGFP}$ cells (Fig. 6a,d,g, arrows), whereas these genes were not expressed in the NIH3T3 cell transplants (Fig. 6b,e,h) or HAP transplants (Fig. 6c,f,i). All the transplants except HAP transplants were EGFP-positive, indicating that cells within the MDF$^{E6\text{-}EGFP}$ and NIH3T3 transplants had originated from the donor (Fig. 6j,k,l).

Discussion

We have immortalized dental follicle cells from cells isolated from mice incisor tooth germs. These cells express *Scx*, *GDF-5*, *EphA4*, *Six-1*, and *Coll*, which are expressed in a developing tendon (Bonnin et al. 2005; Brent et al. 2003; Luukko et al. 2005; Settle et al. 2003). Our findings also suggest that MDF$^{E6\text{-}EGFP}$ cells act as PDL progenitors since they form a PDL-like structure that expresses *periostin*, *Scx*, and *type XII collagen* and that is capable of producing dense collagen fibril assembly in vivo.

In the present study, MDF$^{E6\text{-}EGFP}$ cells have been isolated from the undifferentiated dental follicle region of the incisor in which there is minimal expression of *periostin* and no expression of *osteopontin*. Our findings indicate that MDF$^{E6\text{-}EGFP}$ cells act as PDL progenitors, as shown by their capacity to generate PDL-like tissue in vivo. MDF$^{E6\text{-}EGFP}$ cells are similar to tendon progenitors with respect to their expression of *Scx* and *GDF-5*, a marker for tendon/ligament. Since PDL is morphologically similar to the tendon/ligament in vivo, dental follicle cells may have a similar phenotype as tendon progenitors. The expression of *Scx* by mouse PDL has been seen in the present study, suggesting that the PDL has some characteristics similar to tendon. Salingcamboriboon and coworkers (2003) have reported that a tendon-derived cell line isolated from mouse Achilles tissue shows expression of *Scx*, and that these cells are able to form tendon-like tissue when they are implanted into a mouse tendon-defect model. MDF$^{E6\text{-}EGFP}$ cells show a similar phenotype to these cells, and the transplants form PDL-like tissues comprising cells

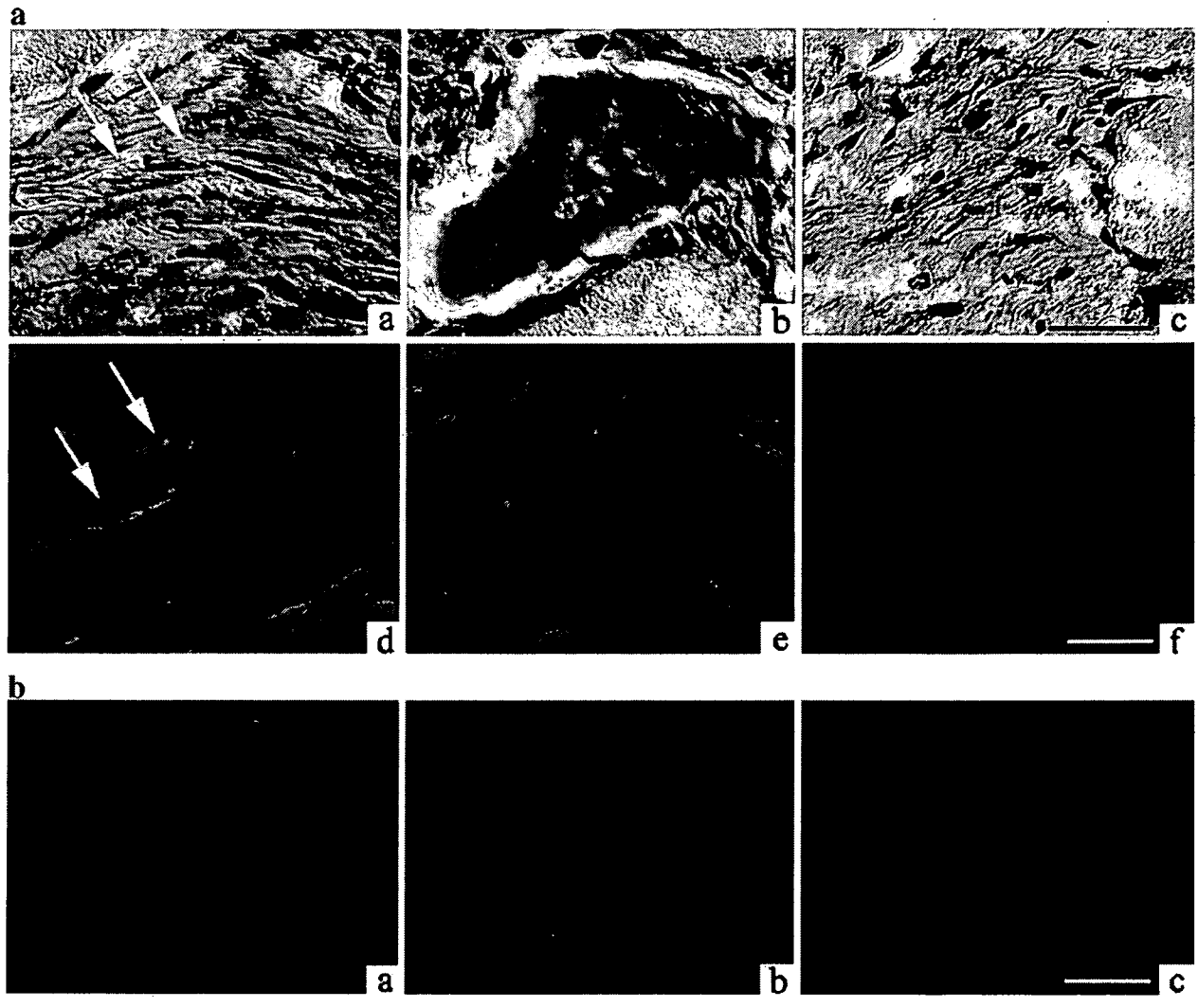

Fig. 5 Differentiation potential of MDF$^{E6\text{-}EGFP}$ cells in vivo. **a** Representative sections of MDF$^{E6\text{-}EGFP}$ cell transplants (**a-a**, **a-b**, **a-d**, and **a-e**) and transplants without mouse cells (HAP transplants; **a-c**, **a-f**). The transplant was stained with hematoxylin and eosin (**a–a** to **a-c**) or viewed for EGFP fluorescence (**a-d** to **a-f**). PDL-like fibrous tissues (**a-a**, **a-d**, *arrows*) and bone-like tissues (**a-e**) are visible in the MDF$^{E6\text{-}EGFP}$ transplants. Cells within the MDF$^{E6\text{-}EGFP}$ transplants are positive for EGFP (**a-d**, **a-e**). **b** Immunohistochemical staining with anti-type I collagen polyclonal antibody in the MDF$^{E6\text{-}EGFP}$ transplants (**b-a**), NIH3T3 cell transplants (**b-b**), or HAP transplants (**b-c**) are shown. Dense type I collagen fibril assemblies are seen in the MDF$^{E6\text{-}EGFP}$ transplants. *Bars* 50 μm

with sheet-like extensions surrounding a densely packed, collagen fibril assembly. Our data are also coincident with previous findings that human PDL cells are able to form PDL-like tissue upon implantation into immunodeficient mice for 4 weeks, suggesting that the differentiation potential of MDF$^{E6\text{-}EGFP}$ is comparable with these cells (Grzesik et al. 2000; Seo et al. 2004). In the present study, the PDL differentiation of MDF$^{E6\text{-}EGFP}$ cells has been assessed by the expression of *periostin*, *Scx*, and *type XII collagen* (Bohme et al. 1995; Karimbux and Nishimura 1995). The expression of *periostin* is observed in both alveolar bone cells and dental follicle cells on P1. However, previous findings and our data have shown that the expression of *periostin* is limited to the adult periodontal ligament, indicating that it could be used as a marker for differentiated PDL (Kruzynska-Frejtag et al. 2004). In the case of MDF$^{E6\text{-}EGFP}$ transplants, they form PDL-like tissue expressing *periostin*, *Scx*, and *type XII collagen*, thus indicating that the tissue is almost identical to PDL. From these findings, we suggest that the MDF$^{E6\text{-}EGFP}$ cells have PDL progenitors able to differentiate into PDL in vivo. Although the role of the tendon/ligament related genes in PDL development is not clear, these data strongly support our hypothesis that MDF$^{E6\text{-}EGFP}$ cells possess PDL progenitors that resemble tendon progenitors.

Progenitors for cementoblasts, PDL cells, and osteoblasts are generally believed to be present in dental follicle cells (Ten Cate 1994), and we have previously shown that

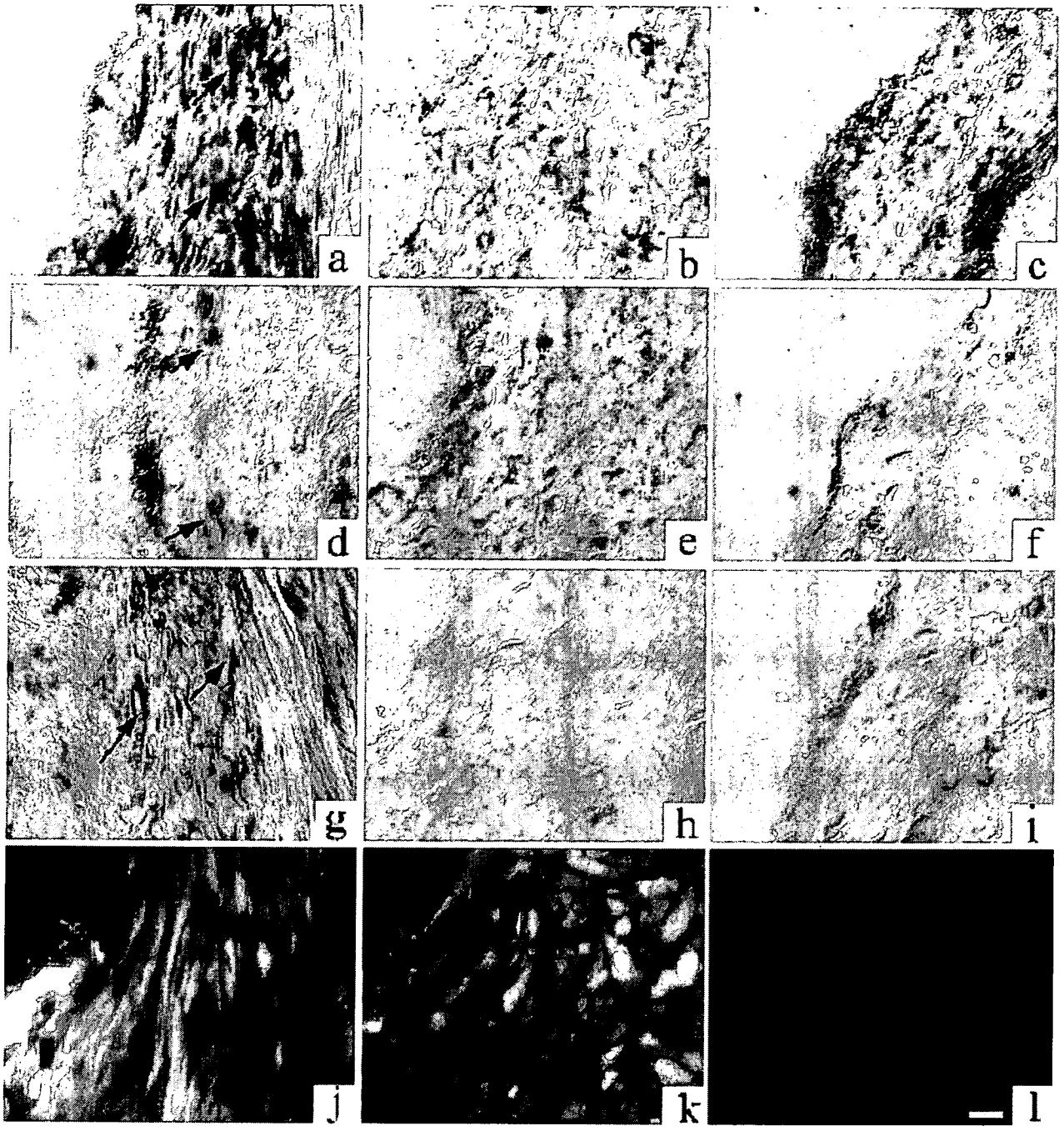

Fig. 6 In situ hybridization for *periostin*, *Scx*, and *type XII collagen* mRNA in $MDF^{E6\text{-}EGFP}$ cell transplants. Representative sections of $MDF^{E6\text{-}EGFP}$ cell transplants (**a**, **d**, **g**, **j**), NIH3T3 cell transplants (**b**, **e**, **h**, **k**), and transplants without mouse cells (HAP transplants; **c**, **f**, **i**, **l**) were analyzed by in situ hybridization for *periostin* (**a–c**), *Scx* (**d–f**), or *type XII collagen* (**g–i**), or viewed for EGFP fluorescence (**j–l**). Expression of *periostin*, *Scx*, or *type XII collagen* is seen in the $MDF^{E6\text{-}EGFP}$ transplants (**a**, **d**, **g**, *arrows*). Cells within the $MDF^{E6\text{-}EGFP}$ and NIH3T3 transplants are positive for EGFP (**j**, **k**). *Bar* 100 μm

cementoblast progenitors can be obtained from bovine dental follicle tissues (Handa et al. 2002). Cementoblast differentiation does not occur in $MDF^{E6\text{-}EGFP}$ cell transplants. This discrepancy may be explained by the isolation technique used for $MDF^{E6\text{-}EGFP}$ cells. The cells grown from mouse dental follicle tissues have been employed in this study, whereas in a previous study, we isolated bovine dental follicle cells by bacterial collagenase digestion; this

suggests that isolation of cementoblast progenitors requires enzyme digestion (Handa et al. 2002). We have also found that MDF$^{E6\text{-}EGFP}$ cells can act as osteoblast progenitors, since they form bone-like tissue in vivo. Although MDF$^{E6\text{-}EGFP}$ cells express an array of osteoblast marker genes, they cannot form mineralized nodules in vitro, as described previously for bovine dental follicle cells (Handa et al. 2002). This may be because the difference in time required for the calcification of MDF^{E6EFGP}. The timing and extent of mineralization varies substantially depending on the origin of the cells or experimental conditions. Mineralization of MDF^{E6EFGP} may require a longer period compared with that for MC3T3E1. Recently, Yoshizawa et al. (2004) have reported that PDL cells do not have the ability to form mineralized nodules, and Msx-2 plays a central role in suppressing matrix mineralization in these cells. Our findings suggest that PDL progenitors present in MDF$^{E6\text{-}EGFP}$ cells are similar to those in PDL, and that the cells preventing or delaying the mineralization of osteoblast progenitors are also present in MDF$^{E6\text{-}EGFP}$ cells. Based on these findings, future studies are necessary to elucidate the mechanism by which MDF$^{E6\text{-}EGFP}$ cells differentiate into osteoblastic cells. From our present findings, we suggest that PDL progenitors and osteoblast progenitors co-exist in MDF$^{E6\text{-}EGFP}$ cells. In addition, the slight expression of BSP observed in NIH3T3 cells might be attributable to the effect of the osteogenic differentiated medium.

Normal MDF cells proliferate in culture for a finite number of PD because of cellular senescence. Therefore, attempts have been made to establish immortalized MDF cells in order to analyze their differentiation potential. A deficiency in p53 is sufficient for the establishment of mouse clonal cell lines from various tissues (Hanazono et al. 1997). HPV16E6 has been shown to abrogate the function of p53 and has the ability to immortalize various cell types (Fehrmann and Laimins 2003; Kiyono et al. 1998). However, it also has other biological functions that depend on its C-terminal PDZ-domain-binding motif, such as cell transformation and skin hyperplasia in transgenic mice (Nguyen et al. 2003). For instance, mice expressing HPV-16 E6 in their epidermis develop epithelial hyperplasia and squamous carcinomas (Song et al. 1999). However, transgenic mice expressing HPV-16 E6 lacking the PDZ-binding motif fail to display epithelial hyperplasia but retain the ability to inactivate p53 (Nguyen et al. 2003). In the present study, we have used a mutant version of E6 that lacks the C-terminal PDZ-domain binding-motif to extend the life span of MDF cells and have succeeded in immortalizing MDF cells without affecting their differentiation potential. In addition, we have confirmed that no tumor formation occurs in MDF$^{E6\text{-}EGFP}$ cell transplants (data not shown). This suggests that the immortalization system used in this study might be useful for the immortalization of MDF cells.

In summary, we have established an immortalized mouse dental follicle cell culture system that possesses PDL progenitors. MDF$^{E6\text{-}EGFP}$ cells might provide new insights into the mechanisms of PDL formation, including those pertaining to PDL cell differentiation. They may also be a powerful tool in the development of therapeutic strategies for the treatment of periodontitis.

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Establishment of gene expression profiling database from human periodontal ligament.

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ABSTRACT

Development of periodontal ligament (PDL) occurred as a series of events that requires temporal and spatial expression of numerous genes regulated differentiation of dental follicle (DF) cells. Nevertheless the genes required for PDL development have not been identified yet. To solve this problem, we used in silico approach with a human PDL database of expressed sequence tags (ESTs) named "periome" database. ESTs were obtained by partially sequencing of 5' end of 10,000 cDNA clones from human PDL cDNA library, and identified 4,384 unique EST clusters. 617 EST clusters appearing more than 3 times were collected, and classified by functional annotation to produce periome database. From periome database, we screened DF specific EST clusters by *in situ* hybridization. The result showed that *SPARC like 1, Nidogen 1, Spondin 1* specifically expressed in DF. From these findings, periome database was successfully established, and this database provides a transcriptome resource for analyzing gene clusters involved in PDL development.

Keyword: Periodontal ligament t / ESTs / cDNA library / Expression profiling

INTRODUCTION

Periodontium is a tooth supporting tissue composed of periodontal ligament (PDL), cementum, and alveolar bone. Among these, PDL plays an central role for stress- breaking capability towards occlusal force, but also acts as the sensory organ that stimulates the central nerve system(1). In periodontitis, a chronic inflammatory disease, the PDL is irreversibly damaged. Despite a number of novel approaches, it has not yet been possible to reliably form PDL (2). Hence, there is considerable interest in the developmental mechanisms of PDL.

PDL cell is originated from the dental follicle (DF) cells that derived from neural crest derived the ectomesenchymal cells(3). After the formation of tooth root dentin, progenitor presented in DF cells migrated onto the tooth root surface to be differentiated into cementoblast. Almost at a simultaneous period, PDL progenitors within DF cells lead to differentiate into PDL cells for inserting fibers into cementum matrix, known as sharpy's fiber. Fiber insertion also occurred along the lining alveolar bone osteoblast(4). Finally both bone-derived and PDL cells derived fiber are coalescing in the PDL to form the intermediate plexus, like tendinous tissue. Each of these stages during transition of dental follicle cells to PDL cells is characterized by specific temporal and spatial gene expression patterns. It is important to clarify the cascade of these genes regulating PDL development. However most of these developmental processes are not fully understood and genes involved are only partly known.

Expressed sequence tags (ESTs), short single-pass sequence reads of randomly selected clones from cDNA library, constitute a valuable source to identify the specific genes in tissue of interest(5). Consequently, attempts are made to establish expression profiling of PDL by EST sequencing. Recently EST profile has been created from 1,752 cDNA sequences of human PDL cDNA library, and *PLAP-1* (periodontal ligament associated protein-1) / *Asporin*, a novel extracellular matrix (ECM) that involved PDL formation was identified from the database. Thus, the PDL EST dataset provide useful information for investigating the genes related to PDL formation. However, the dataset were comparatively small information due to the ESTs was analyzed before the human genome draft is completed.

In the present study we described the expression profiling obtained through evaluation of 4,384 ESTs generated from human PDL cDNA library. Since ECM involved in the organization of tissue specificity(6), we have focused on ESTs that classified as ECM for the screening of genes involved in the PDL development.

MATERIALS AND METHODS

Construction of human PDL EST library.

After signed informed consent was obtained, third molar tooth were harvested with approval (approval number 18) of Kanagawa dental college hospital. Total RNA is extracted by

ISOGEN (NIPPON GENE) from PDL tissue isolated from extracted teeth. Then, mRNA was extracted from total RNA with MACS (Mitenyi Biotec). From provided mRNA, we synthesized cDNA following Superscript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen) with some modifications. The resulting cDNAs were size- fractionated through as agarose gel electrophoresis, and cDNA fragments longer than 1kbp were extracted from the gel. The cDNA fragments then were inserted into pBluescriptSK (-) (Stratagene). The ligated DNA was electroporated into DH10B- competent cells by using E-coli Pulser (Bio-Rad). Plasmid DNA was purified after the culture of transformed bacteria for 16 hours.

DNA Sequencing and sequence data analysis.

The plasmids were amplified using TempliPhi DNA amplification kit (Amasham), and M13 primer. The 5' end of cDNA clones sequenced. Then, a total of about 10,000 cDNA sequences from human PDL library were analyzed. We obtained the ESTs information using the BLAST network client to access BLAST at the NCBI.

Probes for *in situ* hybridization.

The mouse homologue of the candidate ECM gene clusters were searched from homologene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=homologene) respectively. The cRNA probes were generated from the mouse homologue genes that were amplified by PCR using mouse specific primer: *SPARC like 1* (5'- AAT GAA CTG GAC CAG CAT CC -3' and 5'- AAA CGC AGA TGC ACA GAG TG -3'), *Nidogen 1* (5'- ACG TCA TGG GAA TCT TCA GC -3' and 5'- TGC AAA CCG AAC TTC TGA TG-3') and *Spondin-1* (5'- AGA CGG TCT ACT GGG CAC TG -3' and 5'- TGC AAA AGG ATG TGG TGG TA -3') Mouse fragments were cloned into pCR-4 TOPO vector (Invitrogen). The plasmids were linearized by *Not* I (antisense) and *Spe* I (sense). To generate antisense and sense transcripts, digoxygenin - labeled riboprobes were prepared as described previously(7) using T7 or T3 RNA polymerase.

In situ hybridization analysis on sections.

For the expression analysis on sections, the C57BL mouse head at embryonic (E) 13 days, E15, E17 and postnatal (P) 1day were immediately frozen after embedded in OCT compound (Sakura Finetechnical Co.) and 10 μm frontal sections were prepared. *In situ* hybridization was performed as described with some modifications(8). BCIP (Boeringer Mannheim) was used for immunodetection of digoxygenin.

RESULTS

Sequence and analysis of ESTs.

In order to analyze the expression profile in human PDL cDNA library, a total of 11,520 clones were randomly selected, and sequenced from 5' ends. Among the total of 11,520 clones, sequenced clones were 9,600 (83.3%), in addition there were 255 no insert clones, and 1,665 clones were poor quality. The sequenced clones were annotated by BLAST at NCBI.

A total of 9,600 clones from human PDL library were sequenced. Of these analyses these were grouped into 4,384 clusters. In the detail of the expression frequency, 20% clusters were distributed the expression frequency of more than 3 times. Out of these 617 clusters, 481 (78%) were known function clusters. Interestingly, the 101 (16%) were unknown function clusters, and 35 (6%) were unknown transcripts.

Functional annotation of the known function clusters.

617 kinds of clusters which classified more than frequency 3 times, were collected and classified into 12 categories according to their appropriate function(9). Among the known function clusters, 481(78%) were known function clusters shown by Genbank. The remaining 101(16%) were unknown function clusters, and 35(6%) were unknown transcripts. 107(17%) clusters were secreting molecules including 39(6%) extracellular matrix, 44(7%) plasma membrane, 12(2%) proteases and protease inhibitors, 12 (2%) signaling molecules.

Expression patterning of the extracelluar matrix genes.

The ECM has been shown to play an important role in the cellular differentiation and the maintenance of the organs. (6). Thus, we screened the ESTs that specifically expressed in DF from ECM category by *in situ* hybridization. As a result, *SPARC like 1, Nidigen1, Spondin 1* found to express intensely in the DF cells. Expression pattern of these three genes during tooth morphogenesis showed that they initiate to express in DF cells of tooth germ at E15 cap stage, and became intensely expressed at P1 late bell stage.

DISCUSSION

In this study, periome database has successfully established in silico analysis revealed that periome database reflected to human PDL phenotype according to EST expression frequency within ECM category. Among these, *SPARC like 1, Nidogen 1, Spondin 1* intensely expressed during DF development, suggesting that they could serve as a marker for DF cells.

The gene expression profile was analyzed by three different parameters, frequency of gene expression, functional annotation and specific temporal and spatical gene expression patterns. Based on the top 20 ESTs within ECM list, highly expression of *Col I, Col III, SPARC, Periostin* and *PLAP-1 / Asporin* were observed. These genes have an important role for PDL biology. *Col I* and *Col III* are the most abundant gene in PDL(10). *SPARC* is observed strongly in the PDL(11). *Periostin* is also abundant ECM from PDL(12). *PLAP-1 / Asporin* belongs to the small leucine-rich repeat proteoglycan family, and it has been shown that it was highly expressed in the PDL(9). Periome database was similar to that of previously reported human PDL EST(9). From these findings periome database reflects human PDL phenotype. Interestingly 16% of genes are unknown function genes. This may be the reason why

developmental mechanisms of PDL have been unclear for long time.

In situ hybridization screening showed that *SPARC like 1, Nidogen 1, Spondin 1* intensely expressed in DF. *SPARC like 1* is down-regulated in many cancers and is a negative regulator of cell growth and proliferation(13). *Nidigen 1* is a basement membrane component, homozygous mutants often displayed seizure - like symptoms and loss of muscle control in the hind legs(14). *Spondin 1* is a secreted signaling molecule implicated in neuronal development and repair, binds to the conserved central extracellular domain of APP and inhibits -secretase cleavage of APP(15).

As described above, no report was found about relationship between these genes and PDL formation. On the other hand, they expressed in dermal papilla which require epithelial-mesenchymal interaction for development. These finding suggested that epithelial- mesenchymal interaction could involve in the formation of PDL (16).

In summary, we have established the periome database that provides a transcriptome resource for analyzing gene clusters involved in PDL development, and found specific ECM clusters such as *spondin-1* and *tenascin-N* that could serve as a marker for DF or PDL, respectively. Periome database has various information of human PDL, and novel molecules responsible for human PDL formation may be contained this database.

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Establishment of dental follicle cells culture system that generating periodontal ligament in vivo

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ABSTRACT

The dental follicle is a mesechymal tissue that surrounds the developing tooth germ. During tooth root formation, periodontal components (cementum, periodontal ligament [PDL] and alveolar bone) are created by dental follicle progenitors. Here, we report the presence of PDL progenitors in mice dental follicle (MDF) cells. MDF cells were obtained from mice incisor tooth germs, and immortalized by expression of a mutant human papilloma virus type 16 *E6* gene lacking the PDZ domain-binding motif. MDF cells expressing mutant *E6* gene ($MDF^{E6\text{-}EGFP}$ cells) had an extended life span, beyond 150 population doublings (PD). In contrast, normal MDF cells failed to proliferate beyond 10 PD. $MDF^{E6\text{-}EGFP}$ cells expressed tendon/ligament phenotype-related genes such as *Scleraxis (Scx)*, *growth and differentiation factor (GDF)-5*, *EphA4*, *Six-1* and *type I collagen*. In addition, expression of PDL-specific gene, such as *periostin*, was observed. To elucidate the differentiation capacity of $MDF^{E6\text{-}EGFP}$ cells *in vivo*, cells were transplanted into severe combined immunodeficiency (SCID) mice. At 4 weeks, $MDF^{E6\text{-}EGFP}$ cell transplants had the capacity to generate a PDL-like tissue with strong expression of *periostin*, and fibrillar assembly of type I collagen. Our findings suggest that PDL progenitors are present in $MDF^{E6\text{-}EGFP}$cells, and these cells may provide useful information for the formation of PDL for research purposes and for the development of regeneration therapies.

Keyword: Dental follicle / Regeneration / periodontal ligament Progenitor / Immortalization / Differentiation

INTRODUCTION

The periodontal ligament (PDL) surrounds the tooth root, and acts to absorb occlusal forces and function as a sense organ (1). In periodontitis, a chronic inflammatory disease, the PDL is irreversibly damaged. Despite a number of novel approaches, it has not yet been possible to predictably form PDL (2). For this reason, there is considerable interest in the developmental mechanisms of PDL.

The PDL originates from dental follicle cells formed at the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from cranial neural crest cells (3). Progenitors present in the dental follicle are thought to contribute to the formation of all periodontal tissues, that is cementum, PDL and osteoblasts (4). After the formation of tooth root dentin, cementoblast progenitor in the dental follicle migrate onto the tooth root surface and differentiate into cementoblasts (4). Almost simultaneously, PDL progenitors within the dental follicle cells differentiate into PDL cells. Finally both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus. Since PDL is similar to tendon with respect to its dense collagen fiber structure, it has been suggested that tendon/ligament phenotype-related genes are involved in the differentiation of PDL progenitors. Growth and differentiation factors (GDFs)-5, 6, and 7 are members of the bone morphogenetic proteins that regulate tendon/ligament formation (5), and have been shown to be expressed in both dental follicle and PDL cells (6). *Screlaxis* (*Scx*), a basic helix-loop-helix (bHLH) transcription factor that serves as a tendon progenitor marker gene has also been found to be expressed in the PDL stem cells (7). Periostin is a marker for preosteoblasts, but it is also found in the periosteum and PDL (8). During tooth germ development, periostin is initially expressed in the dental follicle cells and is then deposited in postnatal PDL cells during tooth root formation(9). Altenatively periostin -/- mice develop periodontal disease-like phenotype in 3 month after birth, suggesting that critical role for maintenance of PDL (Rios, et al. 2005). These findings suggested that both tendon/ligament phenotype-related genes and PDL-specific extracellular matrices are involved in PDL formation and maintenance. However, details of the mechanisms involved PDL formation have yet to be clarified, due to the lack of periodontal ligament progenitor culture system.

Recently, we have shown the presence of cementoblast progenitors in bovine dental follicle cells (10). Cementoblast progenitor cell line designated BCPb8 has the capacity of forming PDL and cementum-like tissue when transplanted into severe combine immunodeficiency (SCID) mice(11). Although BCPb8 is a useful clonal cell line for the study of dental follicle progenitors, there are limitations

in using these cells derived from a bovine species since there is an inadequate cDNA database and antibodies are limited. To overcome these difficulties with BCPb8, we attempted to establish immortalized mice dental follicle (MDF) cells for the study of progenitors in the dental follicle.

MATERIALS AND METHODS

In situ hybridization

To generate antisense and sense digoxygenin-labelled riboprobes, linearized *osteopontin* and *Scx* plasmids and *periostin* cDNA fragments were transcribed by T7, T3 or Sp6 RNA polymerase as described by Wilkinson (Wilkinson 1995). Heads of 1-day postnatal C57BL/6 mice were embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), sagittal sections were cut at 10 μm. Mandible of 35-day postnatal were fixed in 4% paraformaldehyde at 4°C overnight, and decalcified in 12.5% EDTA containing 2.5% paraformaldehyde for 6 wks, and then embedded in OCT compound. In situ hybridization was carried out on these sections as previously described with some modification (Iseki, et al. 1999). Polyvinyl alcohol was used for buffer during color reaction buffer.

Tissue Culture

The MDF cells were isolated from mice dental follicle tissue of 1-day old (=P1) the incisor tooth germs. Briefly, mice dental follicle tissue was mechanically stripped from the lingual posterior region of mice incisor, and placed onto a 24 multi-well plate. The tissues were then incubated with α-minimum essential medium (α-MEM: Sigma, St.Louis, USA) containing 10% fetal bovine serum (FBS; BioWhittaker, Maryland, USA), 50 μg/ml of ascorbic acid, 100 units/ml of streptomycin and penicillin, in a humidified atmosphere of 5% CO_2 at 37°C. When the cells reached approximately 80% confluence, they were passaged with 0.25% trypsin/1 mM EDTA and maintained as MDF cells. These cells were plated into 6 wells at a density of 3×10^4 cells/ml, and the medium was changed every 2 days.

Infection of retrovirus constructs and establishment of $MDF^{E6\text{-}EGFP}$ cells

Construction of pCLXSN-16E6$^{\Delta 146\text{-}151}$ and production of LXSN-16E6$^{\Delta 146\text{-}151}$ retrovirus have been described previously (Kyo, et al. 2003). One milliliter of producer cell culture fluid was added to MDF (passage 1) in the presence of polybrene (8 μg/ml), and subsequently selected in the presence of G418 (100 μg/ml). Transduced cells were maintained in the medium described above. Following infection with LXSN-16E6$^{\Delta 146\text{-}151}$, MDF cells were transduced with EGFP lentivirus under the control of a CMV promoter for obtaining stably expressed EGFP ($MDF^{E6\text{-}EGFP}$).

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) as described previously (10). cDNAs were synthesized from 1 μg of total RNA in a 20 μl reaction containing 10x reaction buffer, 1 mM dNTP mixture, 1 U/μl RNase inhibitor, 0.25 U/μl reverse transcriptase (M-MLV reverse transcriptase, Invitorgen Corporation, Carlsbad, CA, USA) and 0.125 μM random 9-mers (Takara, Tokyo, Japan). Amplification was performed in a PCR Thermal Cycler SP (Takara, Tokyo, Japan) for 25 cycles using the following reaction profile ; 94°C for 1 min, 60°C for 30 sec and 72°C for 30 sec. Synthesized cDNA served as a template for subsequent PCR amplification, and specific primer used for *periostin, Scx, six-1, EphA4, GDF-5, collagen I (ColI)* and *glyceraldehydes-3-phosphate dehydrogenase (GAPDH).*

In vivo differentiation assay

The differentiation potential of $MDF^{E6\text{-}EGFP}$ cells was assessed by transplantation of the cells into SCID mice as described previously (10). Briefly, cells were inoculated subcutaneously into 5 weeks-old male CB-17 scid/scid (SCID) mice (Nihoncrea, Tokyo, Japan) after incubating 1.5×10^6 cells in a mixture of 40 mg of hydroxyapatite powder (Osferion, Olympus, Tokyo, Japan) and fibrin clot (mixture of mouse fibrinogen and thrombin: both from Sigma, St. Louis, MO, USA). Mice were sacrificed after 4 weeks, and implants analyzed histochemically. NIH3T3 cells were used for comparison.

Histochemical analysis

The transplants were fixed in 4% paraformaldehyde for 1 day, decalcified with 12.5% EDTA containing 2.5% paraformaldehyde for 3 days and then embedded in OCT compound to make frozen section. Subsequently, 5 μm of section were cut, and stained with hematoxylin and eosin. Fluorescence was observed by fluorescence microscopy (Axio imager, Carl Zeiss, Germany). Expression of *periostin* mRNA was examined by in situ hybridization as described above. For immunohistocemical analysis, the sections were blocked with 1% bovine serum albumin, and probed with goat anti-type I collagen polyclonal antibody (SouthernBiotech, Birmingham, AL, USA) for 1 hour. Then sections were probed with donkey anti-goat alexa 555 (Invitrogen Corp., Carlsbad, CA, USA). After washing, fluorescence was observed by fluorescence microscopy.

RESULT AND DISCUSSION

Localization of periostin mRNA in the incisor tooth

Sequential developmental process of the dental follicle was observed in a sagittal section of P1 mice incisor. Immature cells were located posteriorly and differentiation was progressed towards anterior. To obtain immature dental follicle cells, we first investigated the expression pattern of a PDL-specific gene, *periostin*, and a cementoblast/osteoblast marker, *osteopontin*, in the lower incisor tooth germ of P1 mice. Intense expression of *periostin* was observed in the dental follicle cells close to the dentin layer in the anterior region. In contrast, only patchy expression of *periostin* was observed in the dental follicle cells in the posterior region. *Osteopontin* was expressed intensely throughout the alveolar bone. In accordance with the *periostin* expression pattern, *osteopontin* was expressed in the cementoblast next to the dentin-forming layer in the anterior region, but not in the posterior region. These data confirmed that the dental follicle cells in the posterior region were at an immature stage, while those in the anterior region were differentiated. We thus dissected dental follicle cells in the posterior region for establishing a dental follicle progenitor cell culture system.

Immortarization of $MDF^{E6\text{-}EGFP}$ cells

MDF cells were isolated from the posterior region of the incisor tooth germ, and their life span was extended using a retrovirus expressing human papillomavirus type 16 (HPV16) *E6* gene deleted with PDZ domain-binding motif ($E6^{\Delta 146\text{-}151}$), and a lentivirus expressing *EGFP* for fluorescence detection. After viral infection, expression of $E6^{\Delta 146\text{-}151}$ was confirmed by RT-PCR analysis. $MDF^{E6\text{-}EGFP}$ cells maintained their original morphology and cell proliferation activity, even when the cells were cultured beyond population doublings (PD) 150. In contrast, normal MDF cells were only able to propagate until PD 10. We thus used $MDF^{E6\text{-}EGFP}$ cells for further analysis.

Characterization of $MDF^{E6\text{-}EGFP}$ cells

RT-PCR analysis was performed to characterize the PDL forming properties of $MDF^{E6\text{-}EGFP}$ cells. $MDF^{E6\text{-}EGFP}$ cells expressed *periostin* and tendon/ligament phenotype-related genes encoding *Scx, EphA4, Six-1, GDF-5* and *ColI* , suggesting that they possessed PDL and tendon cell properties. Especially, stronger expression of *Scx* and *GDF-5* were observed in $MDF^{E6\text{-}EGFP}$ cells than with NIH3T3 cells or MC3T3E1 cells. This finding suggested that $MDF^{E6\text{-}EGFP}$ cells were similar to tendon progenitors. Since PDL is morphologically similar to the tendon/ligament *in vivo*, dental follicle cells may have a similar phenotype to tendon cells. Among these genes, we examined the expression pattern of *Scx* in the PDL(P35 mouse molar) by in situ hybridization. Intense expression of *periostin* was observed throughout the PDL, and *Scx* was also expressed in the PDL, suggesting that the PDL shares some characters with the tendon. *Scx* has been revealed as a maker not only for a somitc compartment in the embryo that give rise to tendinous tissue, but also for the cells of adult tendon and ligament tissue (7) (Schweitzer, et al. 2001). Salingcarnboriboon et al reported that a tendon-derived cell line isolated from mouse achilles tissue showed expression of *Scx*, and that these cells were able to form tendon-like tissue when they were implanted into a mouse tendon defect model (Salingcarnboriboon, et al. 2003). Expression of *Scx* has also been observed in PDL stem cells which can form a ligament-like structure upon implantation (Seo, et al. 2004). Although the role of the *Scx* in periodontal ligament development is not known, these data strongly support our hypothesis that $MDF^{E6\text{-}EGFP}$ cells possess PDL progenitors that resemble tendon progenitors.

To investigate the differentiation potential of $MDF^{E6\text{-}EGFP}$ cells, they were implanted into SCID mice. After 4 weeks, $MDF^{E6\text{-}EGFP}$ cell transplants formed PDL-like fibrous tissue. The PDL-like tissues resembled a PDL structure with sheet-like cells. Immunohistochemical staining revealed that dense type I collagen fibers were deposited in the PDL-like tissue. To validate the capacity of $MDF^{E6\text{-}EGFP}$ cells to differentiate into PDL *in vivo*, expression of *periostin* was examined in the transplants by in situ hybridization. As expected, intense expression of *periostin* was observed in $MDF^{E6\text{-}EGFP}$ cells, indicating that PDL-like tissue formed by $MDF^{E6\text{-}EGFP}$ cells were indeed PDL(9)

In summary, we established an immortalized mice dental follicle cell culture system that possessed PDL progenitors. $MDF^{E6\text{-}EGFP}$ cells could provide a new insight into the mechanisms of PDL formation, including those pertaining to PDL cell differentiation. They also provide a powerful tool for the development of therapeutic strategies for the treatment of periodontitis.

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Regulation of PLAP-1 Expression in Periodontal Ligament Cells

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RESEARCH REPORTS
Biological

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Regulation of PLAP-1 Expression in Periodontal Ligament Cells

ABSTRACT

Periodontal-ligament-associated protein-1 (*PLAP-1*) is preferentially expressed in the periodontal ligament (PDL) and encodes a novel small leucine-rich repeat proteoglycan protein. *PLAP-1* expression was induced during the course of cytodifferentiation of PDL cells into mineralized-tissue-forming cells *in vitro*, suggesting the possible involvement of PLAP-1 in the mineralization process of PDL cells. In this study, we hypothesized that PLAP-1 expression is regulated by mineralization-related cytokines in PDL cells. *PLAP-1* expression was clearly down-regulated when the cytodifferentiation of PDL cells was reversibly inhibited by fibroblast growth factor-2 (FGF-2). In contrast, bone morphogenetic protein-2 (BMP-2) enhanced *PLAP-1* expression. Up-regulation of *PLAP-1* expression by BMP-2 was confirmed at the protein level when PDL cells were immunostained with anti-PLAP-1 polyclonal antibody. These results revealed the cytokine-mediated regulatory mechanisms of PLAP-1 expression and suggested that PLAP-1 expression may be associated with the process of cytodifferentiation of PDL cells.

KEY WORDS: PLAP-1, FGF-2, BMP-2, periodontal ligament cells, mineralization.

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INTRODUCTION

The periodontal ligament (PDL) is a connective tissue interposed between the roots of teeth and the inner wall of the alveolar bone socket. Its fibers form a meshwork that stretches out between the cementum and the bone and is firmly anchored by Sharpey's fibers. PDL is rich in extracellular matrix (ECM). The ECM provides important functions within the PDL in maintaining structural integrity and regulation of cellular activity and function. The principal elements of ECM in PDL may be considered as a collagenous fibrous network providing structural support embedded in and interacting with a non-collagenous matrix consisting of proteoglycan and various glycoproteins (Waddington and Embery, 2001).

We recently reported the gene expression profile describing quantitative aspects of the genes active in the human PDL and identified a novel gene, *PLAP-1* (*periodontal-ligament-associated protein-1*), which is frequently and predominantly expressed in the PDL tissue (Yamada *et al.*, 2001). Other groups have discovered an identical gene (Henry *et al.*, 2001; Lorenzo *et al.*, 2001). They named this gene *Asporin*, due to its unique aspartic stretch at the N terminus of the translated open reading frame. The *PLAP-1/Asporin* gene encoded a novel SLRP (small leucine-rich repeat proteoglycan) protein, which resembled Decorin and Biglycan. Interestingly, expression of the *PLAP-1* gene was enhanced during the course of the cytodifferentiation of the PDL cells into mineralized-tissue-forming cells (Yamada *et al.*, 2001). This suggests the possible involvement of PLAP-1 in the process of mineralized matrix formation in PDL tissue. PLAP-1 has no glycosaminoglycan attachment site in its predicted amino acid sequence (Yamada *et al.*, 2001), implying that PLAP-1 is not a proteoglycan and may function differently in PDL tissue compared with other SLRP proteins such as Decorin and Biglycan in the PDL.

In this study, we hypothesized that PLAP-1 expression is regulated by the mineralization-related cytokines in human PDL cells.

MATERIALS & METHODS

All experiments were performed according to institutionally approved guidelines, and informed consent was obtained from the patients (Osaka University IRB/Ethical Committee approval #1488).

Cell Culture

Human PDL cells were isolated in accordance with the method described previously (Takayama *et al.*, 1997). The cells were cultured in α-MEM supplemented with 10% FCS, 50 units/mL penicillin G, and 50 μg/mL streptomycin (standard medium) at 37°C in 5% CO_2.

Stimulation of Human PDL Cells with Cytokines

Human PDL cells were cultured in standard medium. The next day, the medium was replaced with FCS-free α-MEM. After serum deprivation for 48 hrs, the cells were stimulated with FGF-2 (Kaken Pharmaceutical, Tokyo, Japan) (100 ng/mL), platelet-derived growth factor BB (PDGF-BB) (SIGMA, St. Louis,