A cell line with characteristics of the periodontal ligament fibroblasts is negatively regulated for mineralization and Runx2/Cbfa1/Osf2 activity, part of which can be overcome by bone morphogenetic protein-2

Yoshinori Saito[1,2,*], Tatsuya Yoshizawa[1,*], Fumio Takizawa[1,2], Mika Ikegame[1,3], Osamu Ishibashi[1], Kazuhiro Okuda[2], Kohji Hara[2], Kotaro Ishibashi[4], Masuo Obinata[5] and Hiroyuki Kawashima[1,‡]

Divisions of [1]Cell Biology and Molecular Pharmacology, [2]Periodontology and [3]Anatomy and Cell Biology of the Hard Tissue, Niigata University Graduate School of Medical and Dental Sciences, 5274 2-Bancho, Gakkocho-dori, Niigata-city, Niigata 951-8514, Japan
[4]Daiichi Pharmaceutical Co. Ltd., 1-16-13 Kita-kasai, Edogawa-ku, Tokyo 134-8630, Japan,
[5]Department of Cell Biology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8575, Japan

*These authors contributed equally to this study
‡Author for correspondence (e-mail: kawashim@dent.niigata-u.ac.jp)

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Summary

The periodontal ligament (PDL) is a connective tissue located between the cementum of teeth and the alveolar bone of the mandibula. It plays an integral role in the maintenance and regeneration of periodontal tissue. The cells responsible for maintaining this tissue are thought to be fibroblasts, which can be either multipotent or composed of heterogenous cell populations. However, as no established cell lines from the PDL are available, it is difficult to assess what type of cell promotes all of these functions. As a first step to circumvent this problem, we have cloned and characterized cell lines from the PDL from mice harboring a temperature-sensitive SV 40 large T-antigen gene. RT-PCR and in situ hybridization studies demonstrated that a cell line, designated PDL-L2, mimics the gene expression of the PDL in vivo: it expresses genes such as alkaline phosphatase, type I collagen, periostin, runt-related transcription factor-2 (Runx2) and EGF receptor, but does not express genes such as bone sialoprotein and osteocalcin. Unlike osteoblastic cells and a mixed cell population from the PDL, PDL-L2 cells do not produce mineralized nodules in the minearlization medium. When PDL-L2 cells were incubated in the presence of recombinant human bone morphogenetic protein-2 alkaline phosphatase activity increased and mineralized nodules were eventually produced, although the extent of mineralization is much less than that in osteoblastic MC3T3-E1 cells. Furthermore, PDL-L2 cells appeared to have a regulatory mechanism by which the function of Runx2 is normally suppressed.

Key words: Periodontal ligament, Cell line, Runx2/Cbfa1/Osf-2, Mineralization, BMP-2

Introduction

The periodontal ligament (PDL) is a soft connective tissue interposed between the roots of teeth and the inner wall of the alveolar socket. This tissue is characterized by rapid turnover and a high remodeling capacity, which give it adaptability, maintaining a constant width despite being exposed to rapidly changing physical forces such as mastication, speech and orthodontic tooth movement (Beertsen, 1975; Berkovitz and Shore, 1995). The PDL also has a remarkable capacity for renewal and repair, playing a pivotal role in periodontal regeneration (Berkovitz and Shore, 1995; Beertsen et al., 1997). Since fibroblasts are predominant in the PDL (Berkovitz and Shore, 1995; Beertsen et al., 1997) and are capable of producing and digesting matrix components, fibroblastic cells have been assumed to be responsible for self-renewal of the PDL and regeneration of periodontal tissue. Indeed, experiments dealing with periodontal wound healing have suggested that PDL fibroblast populations may play an integral role in PDL regeneration (Melcher et al., 1986; McCulloch et al., 1987; Magnusson et al., 1990; Beertsen et al., 1997). Since these experiments were carried out with mixed cell populations of PDL tissues and/or cells of endosteal spaces, however, it is difficult to determine the cellular phenotypes that are required for regeneration.

Fibroblastic cells of the PDL have also been suggested to be a source of osteoblasts for continued remodeling of alveolar bone of the mandibula (Roberts et al., 1982). Therefore, PDL fibroblastic cells have been suggested to be multipotent cells (McCulloch and Bordin, 1991) or composed of heterogenous cell populations that have the capacity to differentiate into either osteoblasts or cementoblasts depending on the microenvironment (Gould et al., 1980; Roberts et al., 1982; McCulloch and Melcher, 1983). Indeed, recent studies

demonstrated that cells isolated from the PDL have osteoblast-like properties, such as alkaline phosphatase (ALPase) activity (Yamashita et al., 1987), being responsive to PTH (Nojima et al., 1990) and producing bone sialoprotein in response to 1,25-dihydroxyvitamin D_3 (Nojima et al., 1990). In addition, primary rat PDL cells in culture formed mineralized nodules in vitro, although the mineralized nodules appeared to be different from those produced by osteoblasts (Cho et al., 1992). A similar result was observed with human PDL cells (Arceo et al., 1991). However, since it has been shown that there are subsets of fibroblastic cells in the PDL (Roberts and Chamberlain, 1978; Limeback et al., 1982; Rose et al., 1987), it is not clear whether these osteoblastic phenotypes and functions were due to one type of cell or to a combination of cells in the PDL.

To circumvent these problems, it is necessary to establish a cell line possessing the exact nature of PDL fibroblastic cells. Therefore, we have tried to establish immortalized cell lines from transgenic mice harboring the temperature-sensitive (ts) Simian Virus 40 large tumor antigen (SV 40 large T-antigen) gene (Yanai et al., 1991). Here, we report that one of the established cell lines is indistinguishable from the fibroblastic cells of PDL in terms of gene expression. Furthermore, it produces mineralized nodules in the presence of recombinant human bone morphogenetic protein (rhBMP)-2.

Materials and Methods

Tissues for histochemistry and in situ hybridization

C57BL/6J mice, 7, 21 and 35 days old, were purchased from Japan Clea (Tokyo, Japan) and perfused transcardially with 4% paraformaldehyde under ether anesthesia. Mandibles were removed and fixed with 4% paraformaldehyde in diethyl pyrocarbonate (DEPC)-treated PBS for a further 3 hours. The tissues were washed with DEPC-treated PBS and then decalcified with 10% EDTA (pH 7.2) at 4°C for 1 week. Specimens were dehydrated with increasing concentrations of ethanol (70%, 80%, 90%, 95% and 100%) and embedded in paraffin. The specimens were then deparaffinized with xylene, and 6 μm sections were cut. The sections were subjected to in situ hybridization. For histochemistry, frozen sections were used. Mice were treated under the principles and procedures outlined in the NIH Guide for the Care and Use of laboratory Animals.

Histochemistry

Frozen sections were treated with 0.1 M Tris-maleate buffer (pH 7.4) containing 50 mM $MgSO_4$ at 37°C for 1 hour, and ALPase activity was detected according to the azo-dye method of Burstone (Burstone, 1962).

Probe preparation

Digoxigenin UTP-labeled sense and anti-sense single-stranded RNA probes were prepared with a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. For *type I collagen (Col. I)* (Ac. No. X06753), *osteocalcin (OCN)* (Ac. No. X4142) and *bone sialoprotein (BSP)* (Ac. No. L20232), each corresponding cDNA fragment, 202, 470 and 1048 bp, respectively, was obtained by RT-PCR and subcloned into *pBluescript SK(–)* (Stratagene, La Jolla, CA). For periostin, 2.5 kb fragment of mouse cDNA (Xiao et al., 1998) was used in a similar manner. After transcription, 40 units of RNase-free DNase (Roche Diagnostics) was added to the reaction and incubated for an additional 10 minutes at 37°C. Transcription products were recovered by ethanol precipitation with 25 μg of RNase-free glycogen (Roche Diagnostics) as a carrier, and precipitates were washed once in 70% ethanol, air-dried and resuspended in 50 μl of diethyl pyrocarbonate-treated water. For runt-related transcription factor-2 (*Runx2)/core binding factor a1 (Cbfa1)/osteoblast-specific transcription factor-2 (Osf2)*, a 0.3 kb fragment of exon I of mouse cDNA (Horiuchi et al., 1999) was subcloned into *pBluescript KS(–)* (Stratagene). The fragment of *Runx2/Osf2* is known to detect the isoform expressed specifically in osteoblasts and chondrocytes. The subcloned fragments were confirmed to be identical to the respective cDNA sequence using the ABI prism™ 377 DNA Sequencing System (PE Co., Foster City, CA).

In situ hybridization

Deparaffinized sections were mounted on silane-coated slides (Dako Japan Co., Ltd., Kyoto, Japan), washed in PBS, treated with 0.2 N HCl for 10 minutes and incubated with 0.5 μg/ml of proteinase K (Roche Diagnostics) in 10 mM Tris-HCl (pH 8.0) at 37°C for 30 minutes. The sections were then refixed with 4% paraformaldehyde in PBS at room temperature for 30 minutes and further treated with 0.25% acetic anhydride in 0.1 M triethanolamine for acetylation. The acetylated sections were hybridized with probes at a final concentration of 1.0 μg/ml in a hybridization mixture containing 50% deionized formamide, 2% blocking agent (Roche Diagnostics), 5×SSC (1×SSC=0.15 M NaCl, pH 7.5, 15 mM sodium citrate, 0.02% SDS (sodium dodecyl sulfate), 0.1% N-lauroylsarcosine, 200 μg/ml yeast tRNA and 100 μg/ml salmon sperm DNA at 55°C for 18 hours, then washed twice with 0.1×SSC at 60°C for 30 minutes. This was followed by RNase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) treatment at 37°C for 30 minutes. The sections were further washed twice with 2×SSC at 60°C for 15 minutes and then twice with 0.1×SSC at 60°C for 15 minutes. Specific transcripts were detected with ALPase-conjugated anti-digoxigenin antibody according to the manufacturer's protocol (DIG Detection Kit, Roche Diagnostics). The sections were counterstained with nuclear fast red or methyl green.

Cloning of cell lines

Molars were aseptically removed from 5-week-old transgenic mice harboring the temperature-sensitive SV40 large T-antigen gene under ether anesthesia, and the PDL tissues were scraped off from the root surface of the mandibula. Tissue explants were placed in 24-well tissue culture plates (Falcon, Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) containing Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies Inc., Rockville, MD) supplemented with 10% FBS (Gibco BRL), penicillin G (100 IU/ml) (Gibco BRL) and streptomycin (100 μg/ml) (Gibco BRL). The explants were covered with a coverslip (NUNC, Nalge Nunc International, Roskilde, Denmark) and incubated at 33°C and 5% CO_2 in air. The outgrown cells from explants were cloned according to the limited dilution method after 10 passages. Nineteen clonal cell lines were obtained and one of them (designated as PDL-L2) was chosen for further characterization by ALPase activity, mineralization assay and reverse transcriptase (RT)-PCR. A mixture of uncloned cell population was also used as PDL cells for the control. After the PDL-L2 was established, the cell line has maintained its phenotype over 40 passages.

Cell culture

In addition to the PDL-L2 and PDL cells, four other cell lines were used in the study. They are murine mesenchymal pluripotent cell line, C3H10T1/2 (Human Science Research Resources Bank, Osaka, Japan), murine osteoblastic cell line, MC3T3-E1 (Riken Gene Bank, Wako, Japan), murine fibroblastic cell line, NIH3T3 (Cell Resource Center for Biomedical Research Institute of Development, Aging and

Cancer, Tohoku University, Sendai, Japan) and rat osteoblastic cell line ROS 17/2.8 (kindly provided from Yamanouchi Pharmaceutical Co. Ltd., Tsukuba, Japan). C3H10T1/2, MC3T3-E1, NIH3T3, and ROS 17/2.8 cells were cultured at 37°C in basal MEM with 10% heat-inactivated FBS, αMEM with 10%FBS, DMEM with 10% FBS and DMEM/F12 with 10% FBS, respectively.

Semi-quantitative RT-PCR

Cells were plated at 5×10^4 cells/35 mm dish (Falcon) containing the medium described above and cultured for the indicated number of days at 33°C for PDL-L2 and PDL cells, and at 37°C for MC3T3-E1 and NIH3T3 cells. Total RNA of each cell line was extracted according to the acid guanidinium isothiocyanate-phenol-chloroform method. Then, the first-strand cDNA was synthesized using random primers (nine-mers) (Takara Shuzo, Osaka, Japan) and Superscript II (Gibco BRL) as a reverse transcriptase. For PCR, aliquots of synthesized cDNA were added to PCR mixtures containing 3′ and 5′ primers (0.2 μM each), dNTP mixture (0.2 mM each) (Gibco BRL) and Taq polymerase (0.05 unit/μl) (Gibco BRL). Cycling conditions were 94°C/30 seconds, 53°C/45 seconds, and 72°C/30 seconds for 20 cycles for *Col. I*, 94°C/30 seconds, 55°C/45 seconds, 72°C/40 seconds for 19 cycles for *periostin*, 94°C/30 seconds, 58°C/45 seconds, 72°C/40 seconds for 24 cycles for *BSP*, 94°C/30 seconds, 62°C/45 seconds, 72°C/40 seconds for 21 cycles for *OCN*, 94°C/30 seconds, 62°C/45 seconds, 72°C/30 seconds for 33 cycles for *Runx2/Cbfa1/Osf2*, 94°C/20 seconds, 54°C/30 seconds, 72°C/30 seconds for *epidermal growth factor receptor* (*EGFR*) for 32 cycles and 94°C/30 seconds, 55°C/45 seconds, 72°C/30 seconds for 12 cycles for *Glyceraldehyde-3-phosphate dehydrogenase* gene (*GAPDH*), respectively. Primers for PCR were as follows:

5′-ACC ATC Tgg CAT CTC ATg gC-3′ and 5′-gCA ACA CAA TTg CAC CTgAgg-3′ for *type I collagen* gene, 5′-ATC CCC ATg ACT gTC TAT Ag-3′ and 5′-CAA ATA AgT gAC CAT CgC CA-3′ for *periostin*, 5′-AAC AAT CCg TgC CAC TCA-3′ and 5′-ggA ggg ggC TTC ACT gAT-3′ for *BSP*, 5′-TgC gCT CTg TCT CTC TgA CC-3′ and 5′-CTg TgA CAT CCA TAC TTg Cag g-3′ for *osteocalcin* gene, 5′-gAg ggC ACA AgT TCT ATC Tgg A-3′ and 5′-ggT ggT CCg CgA TgA TCT C-3′ for *Runx2/Osf2*, 5′-Aag gAT gTg AAg TgT gg-3′ and 5′-ACT TTC TCA CCT TCT gg-3′ for *EGFR*, and 5′-AAg ATg gTg AAg gTC ggT gT-3′ and 5′-gCA Tgg ACT gTg gTC Atg Ag-3′ for *GAPDH*.

PCR products were fractionated on a 1% agarose gel, transferred to positively charged nylon membranes, and cross-linked by ultraviolet light. Membranes were hybridized with DIG-labeled (Roche Diagnostics) DNA probes and detected with CDP-Star substrate (NEW ENGLAND BioLabs. Inc., Beverly, MA) according to manufacture's standard protocols.

Western blot analysis

To discover whether Runx2/Cbfa1/Osf2 is produced in the PDL cells, western blot analysis was carried out. PDL-L2 and MC3T3-E1 cells were grown in 10 cm diameter tissue culture plates, and nuclear extracts of cells at confluence were prepared using the Nuclear Extract Kit (ACTIVE MOTIF, Carlsbad, CA). The nuclear extract of each plate was separated by 8% SDS-PAGE and then subjected to immunoblot analysis with a 1:8,000 dilution of anti-Runx2/Osf2 antibody (generated against the N-terminal peptide sequence of Osf2) (kindly provided by Dr Karsenty of Baylor College of Medicine) and horseradish-peroxidase-conjugated anti-rabbit IgG antibody (1:250,000) followed by ECL+Plus detection (Amersham Biosciences, Tokyo, Japan).

Alkaline phosphatase activity and mineralization assay

To assess alkaline phosphatase (ALPase) activity, cells were incubated with normal medium (α-MEM containing 5% FBS) or differentiation medium (normal medium supplemented with 1 μM dexamethazone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid). Cells were cultured for 3 days and for an additional 3 days in the presence or absence of 250 ng/ml rhBMP-2 (kindly provided by Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan). The cultured cells were fixed with 2% formaldehyde for 15 minutes and subjected to ALPase staining. For the mineralization study, cells at confluence were cultured for 3 days in the differentiation medium in the presence or absence of 250 ng/ml rhBMP-2 and cultured for an additional 25 days. The incubation medium was changed every 3 days without further addition of rhBMP-2. Cells were then fixed with 2% formaldehyde for 15 minutes and subjected to Alizarin red-S staining.

Alizarin red-S (AR-S) staining

At the end of each experiment, the cultures were rinsed with PBS and fixed for 15 minutes at 4°C with 2% paraformaldehyde in PBS. Fixed cultures were rinsed with PBS and Nanopure water, and stained with 40 mM AR-S (Wako Pure Chemical Industries, Ltd.) (pH 4.2, 1 ml/35 mm dish) at room temperature with gentle rotation. Cultures were then washed five times with water followed by a 15 minutes rinse with PBS with gentle rotation to reduce nonspecific AR-S stain. Stained cultures were photographed and then destained by incubating in 10% (w/v) cetylpyridinium chloride (CPC) (Wako Pure Chemical Industries, Ltd) in 10 mM sodium phosphate buffer (pH 7.0) for 1 hour at room temperature. Aliquots of these AR-S extracts were diluted 10-fold in the 10% CPC solution, and the AR-S concentration of each sample was determined by absorbance at 562 nm for quantification (Stanford et al., 1995).

Expression and reporter plasmid

For the mammalian expression plasmid encoding *Runx2/Cbfa1/Osf2*, the RT-PCR-amplified N-terminal portion of *Osf2* (ATG-*Nae*I 0.26 kb) was fused to *PEBP2αA* (*Nae*I-*Xba*I 3 kb) from *pBKS2αA* (kindly provided by T. Komori), and this was cloned into the *pcDNA3* (Invitrogen Corp., Carlsbad, CA, USA). To assess the transcriptional activity of Runx2/Osf2, we generated reporter plasmid *p6OSE2-Luc*, in which luciferase expression is controlled by six copies of the Cbfa1 binding OSE2 site of *osteocalcin* promoter followed by the minimal promoter, as described previously (Ducy and Karsenty, 1995).

Transfection and reporter assays

Cells (5×10^4 cells per 35 mm dish) were transfected with various plamid DNAs using Fugene-6 (Roche Diagnostics) according to the manufacturer's recommendations. Twenty-four hours after transfection, cells were harvested using the Reporter Lysis Buffer (Promega Corporation, Madison, WI, USA) for analysis of luciferase and β-galactosidase activities. The luciferase and β-galactosidase assays were performed with the PicaGene system (Wako Pure Chemical Industries, Ltd.) and AURORA Gal-XE kit (Wako Pure Chemical Industries, Ltd.), respectively. All luciferase activities are normalized for transfection efficiency against the corresponding β-galactosidase activities from the cotransfected *pCMV-SPORT-β-gal* plasmid (Gibco BRL). Each assay was performed in duplicate or triplicate, and the same experiment was repeated at least twice.

Results

ALPase activity and gene expression in the periodontal ligament

To establish a PDL cell line, it is necessary to identify a marker specific to the PDL cells. However, no such marker is available at present. Since it is believed that PDL cells are osteogenic in

nature, we selected osteoblast-specific markers, including ALPase, *type I collagen*, *periostin*, *Runx2/Cbfa1/Osf2*, *BSP* and *osteocalcin*, in the hope that possible distinct expression of some of these genes may allow differentiation of these two types of cell and may allow for the characterization of PDL cells. We examined the expression of these markers in vivo.

ALPase activity

Fig. 1A and 1B depict ALPase staining of the mandibula. It is apparent that the majority of fibroblastic cells are positive for this enzyme activity, although the intensity of the staining of each cell varies widely.

Type I collagen

In terms of *type I collagen* expression, approximately 80% of cells in the PDL were positive (Fig. 1C); however, the expression level was lower than those in osteoblasts.

Periostin

Periostin was isolated as one of the osteoblast-specific genes (Takeshita et al., 1993), and the physiological role of this gene is still unknown. Surprisingly, strong expression of the *periostin* gene was detected in most of the PDL cells, whereas expression in the osteoblasts was weaker (Fig. 1D). A similar finding was recently reported (Horiuchi et al., 1999).

Runx2/Cbfa1/Osf2

Ranx2/Cbfa1/Osf2 is known to be a transcription factor essential for osteoblast differentiation (Ducy et al., 1997; Komori et al., 1997). As expected, *Runx2/Cbfa1/Osf2* expression was detected in the osteoblasts (Fig. 1E). Similarly, intense expression was evident in a large percentage (60 to 70%) of the PDL cells (Fig. 1E). The expression was higher in odontoblasts than in osteoblasts.

Osteocalcin

Osteocalcin is a marker of late stage osteoblast differentiation, and a lack of this gene reportedly causes an increase in bone formation in mice (Ducy et al., 1996). Expression of the *osteocalcin* gene was most intense in the odontoblasts, followed by osteoblasts and then by cementoblasts. No expression was detected in the PDL cells (Fig. 1F).

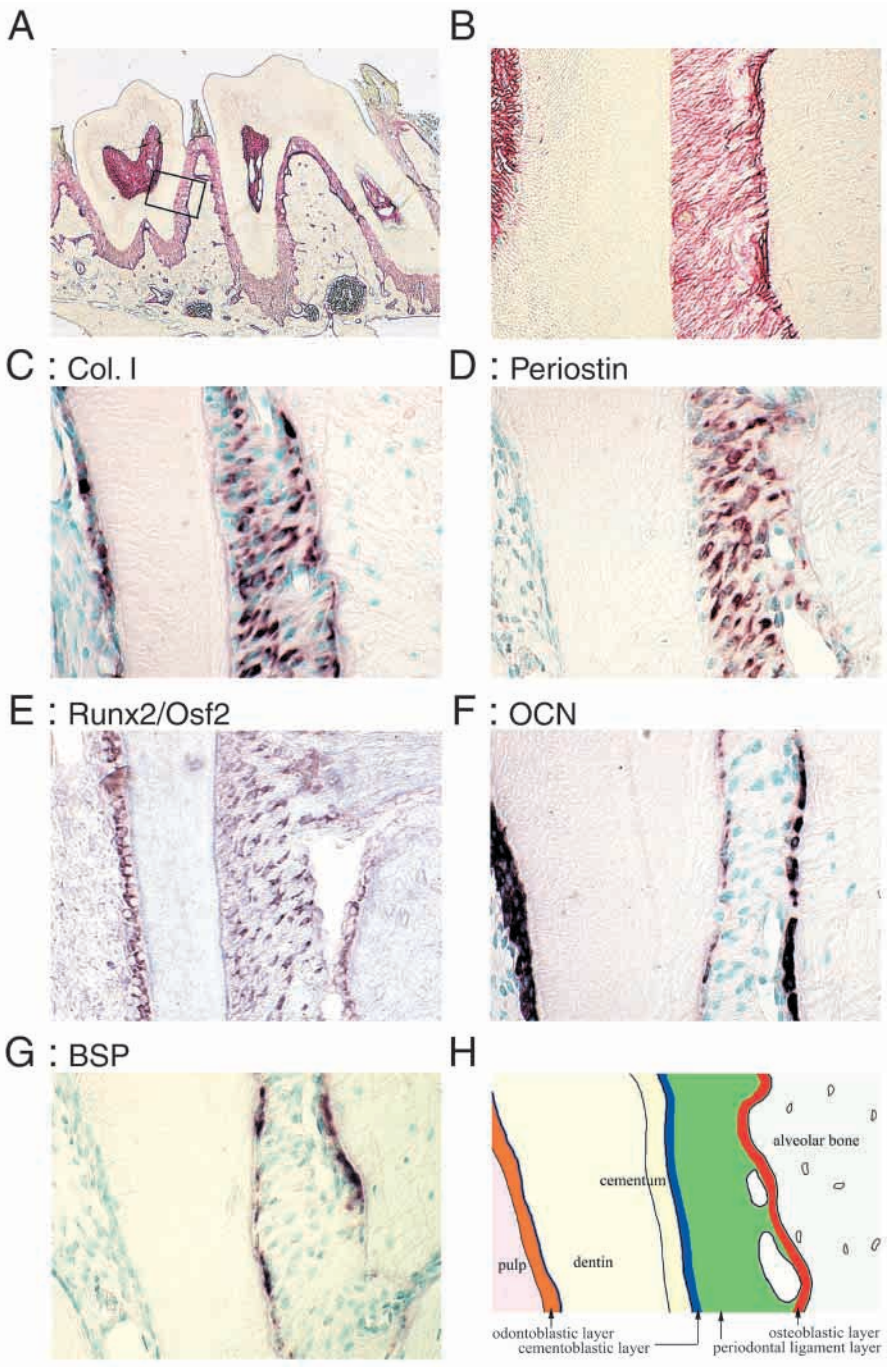

Fig. 1. Fibroblastic PDL cells express ALPase activity and genes for *type I collagen*, *periostin* and *Runx2/Osf2*, but not genes for *osteocalcin* and *BSP*. (A) ALPase staining of mouse mandibula. (B) Higher magnification of the inset in A. (C-G) In situ hybridization studies. The antisense probes used were C, *type I collagen*; D, *periostin*, E, *Runx2/Cbfa1/Osf2*; F, *osteocalcin* (*OCN*); and G, *BSP*. (H) A schematic drawing of the tissue section used. B-G show a tissue section roughly corresponding to the schematic drawing shown in H. Signals in C-G disappeared when the corresponding sense probes were used (data not shown). Note that expression of the *periostin* gene, originally cloned as an osteoblast-specific marker, in the fibroblastic PDL cells is much higher than in the osteoblasts lining the surface of alveolar bone (D). The expression level of *Runx2/Cbfa1/Osf2* gene was comparable in PDL, osteoblasts and odontoblasts, but was zero in the cementoblasts (E). The expression of the *OCN* gene is highest in the odontoblasts, followed by osteoblasts and then by cementoblasts. Expression of PDL cells is undetectable (F). *BSP* expression is detected in the cementoblasts and osteoblasts but not in PDL cells (G). Original magnification: A, ×8; B-G, ×40. See text for details.

BSP

BSP is a marker of osteoblasts at the middle stage of differentiation, and expression of this gene is undetectable or minimal in the PDL (MacNeil et al., 1994; MacNeil et al., 1996; D'Errico et al., 2000) in vivo. Consistent with the previous observation, expression of this gene was not detected in the PDL, whereas it was positive in the osteoblasts and cementoblasts. Odontoblasts were negative for BSP (Fig. 1G).

Cloning and selection of cell lines from the PDL explant culture

Using the dilution cloning method we obtained 19 cell lines from explant cultures of the PDL. Most of these cells were fibroblastic in appearance, although some looked polygonal at confluence. A cell line designated PDL-L2 was selected for further characterization on the basis of preliminary gene expression analyses using RT-PCR (see below).

RT-PCR analysis demonstrates that gene expression in the PDL-L2 cells is parallel with that seen in the PDL in vivo

To assess the differences between the cloned cell lines and osteoblasts, expression of genes specific to osteoblasts was studied by RT-PCR. As shown in Fig. 2A, PDL-L2 cells were positive for *type I collagen*, *periostin* and *Runx2/Cbfa1/Osf2*, whereas they were negative for *osteocalcin* and *BSP*. *Osteocalcin* expression was detected only in the differentiated osteoblasts. Intense *BSP* expression was observed in the differentiated osteoblasts, whereas much weaker expression was seen in the undifferentiated osteoblasts. *BSP* is a marker of osteoblasts at the middle stage of differentiation, whereas *osteocalcin* is a marker of osteoblasts at the late stage of differentiation. A fibroblastic NIH3T3 cell line expressed *type I collagen* only at a much lower level than those of PDL-L2 and MC3T3-E1 cells. These data, together with those in Fig. 1, clearly demonstrate that the established cell line PDL-L2 shares identical expression of the genes examined. Furthermore, PDL-L2 expressed the *EGFR* genes (data not shown), which have previously been shown to be present in the PDL (Matsuda et al., 1993; Cho and Garant, 1996). Since Runx2/Cbfa1/Osf2 is a transcription factor that is specifically expressed and functions in osteogenic cells, western blot analysis was also performed to examine whether or not this protein is produced in PDL-L2 cells. As shown in Fig. 2, Runx2/Cbfa1 was detected in the nuclear extracts of the PDL-L2 cells. The expression level of Runx/Cbfa1 in PDL-L2s is approximately one-sixth of that in MC3T3-E1s, and the difference is much smaller compared with the 60-fold difference of gene expression (Fig. 2A). C3H10T1/2 cells, in which Runx/Cbfa1 is undetectable, were transfected with *Runx/Cbfa1* plasmid, and the cell lysates were analyzed in the same way as the control.

PDL cells respond to rhBMP-2 and mineralize in vitro

When PDL-L2 cells were incubated in normal medium, they grew until they reached confluence and remained healthy for up to 7 days. However, they became detached from the dish as a thin sheet-like structure after 7 days and could not grow any more (data not shown). By contrast, the osteoblastic cell line, MC3T3-E1, grew further and eventually formed nodules that ultimately calcified. Although PDL-L2 cells express the *ALPase* gene, its enzyme activity was barely detected under the conditions shown in Fig. 3. Since the cell line expresses *BMP*

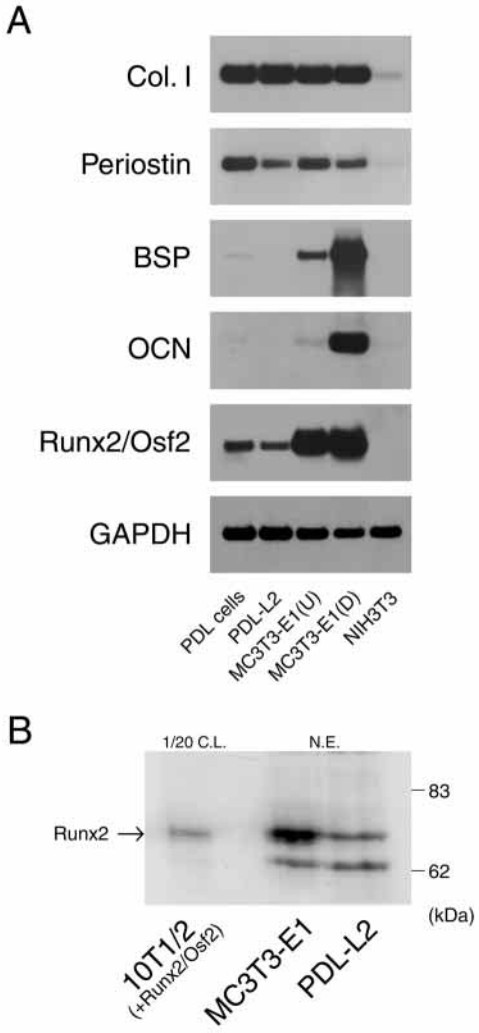

Fig. 2. RT-PCR analysis demonstrates that the PDL-L2 has gene expression identical to that of PDL cells in vivo. **(**A) MC3T3-E1 (U) represents MC3T3-E1 cells at 80% confluence; MC3T3-E1 (D) represents MC3T3-E1 cells at a fully differentiated stage (mineralization stage); PDL-L2 and NIH3T3 represent cells at confluence. Note that the expression level of *periostin* decreases with maturation of osteoblastic MC3T3-E1 cells, and it is higher in PDL-L2. The data are consistent with Fig. 1D. *Runx2/Cbfa1/Osf2* is expressed in both PDL-L2 and MC3T3-E1 cells, whereas *OCN* and *BSP* are absent in PDL-L2 but present in MC3T3-E1. These are also consistent with Fig. 1E-G. (B) Nuclear extracts (N.E.) of PDL-L2 and MC3T3-E1 or cell lysates (C.L.) of C3H10T1/2 were electrophoresed on 8% SDS-PAGE and then subjected to immunoblot analysis with an antibody against Runx/Cbfa1/Osf2. Runx2/Osf2 is detected in the PDL-L2 cells, although its expression level is one-sixth of that in MC3T3-E1 cells. Since C3H10T1/2 cells do not express Runx2/Osf2, cells transfected with *pcDNA3-Runx2/Osf2* were used for the control. Note that 20-fold dilution of cell lysate gave a comparable expression level of protein to the endogenous Runx2/Osf2 in the nuclear extract of PDL-L2. See text for details.

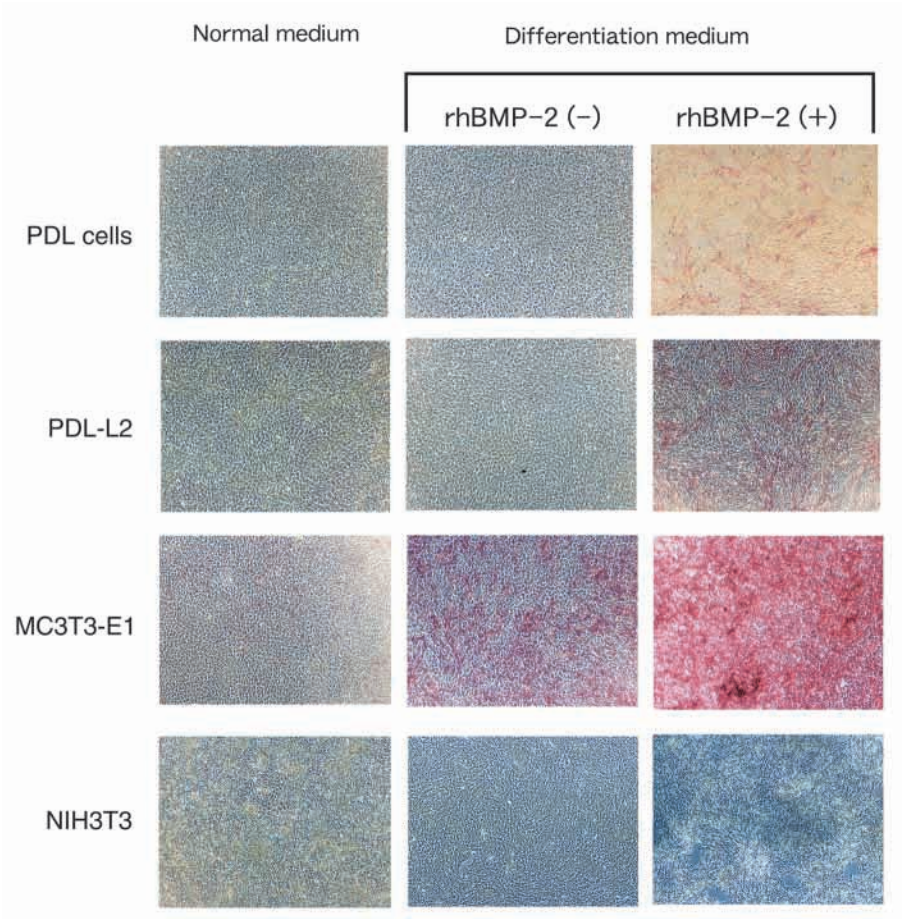

Fig. 3. rhBMP-2 induces ALPase activity in PDL-L2 cells in vitro. Cells were cultured either in the normal medium or the differentiation medium. After 3 days of incubation, cells were given 250 ng/ml rhBMP-2 or vehicle and incubated for an additional 3 days. Cells were then fixed and subjected to ALPase staining. Consistent with previous reports, MC3T3-E1 expressed more ALPase activity as the incubation increased, and this was markedly enhanced by rhBMP-2. ALPase staining of PDL-L2 is faint and scarce in the absence of rhBMP-2. However, addition of rhBMP-2 is markedly induced to a level close to that of unstimulated MC3T3-E1 cells. Note that fibroblastic NIH3T3 cells did not respond to rhBMP at all. Original magnification was ×4. See text for details.

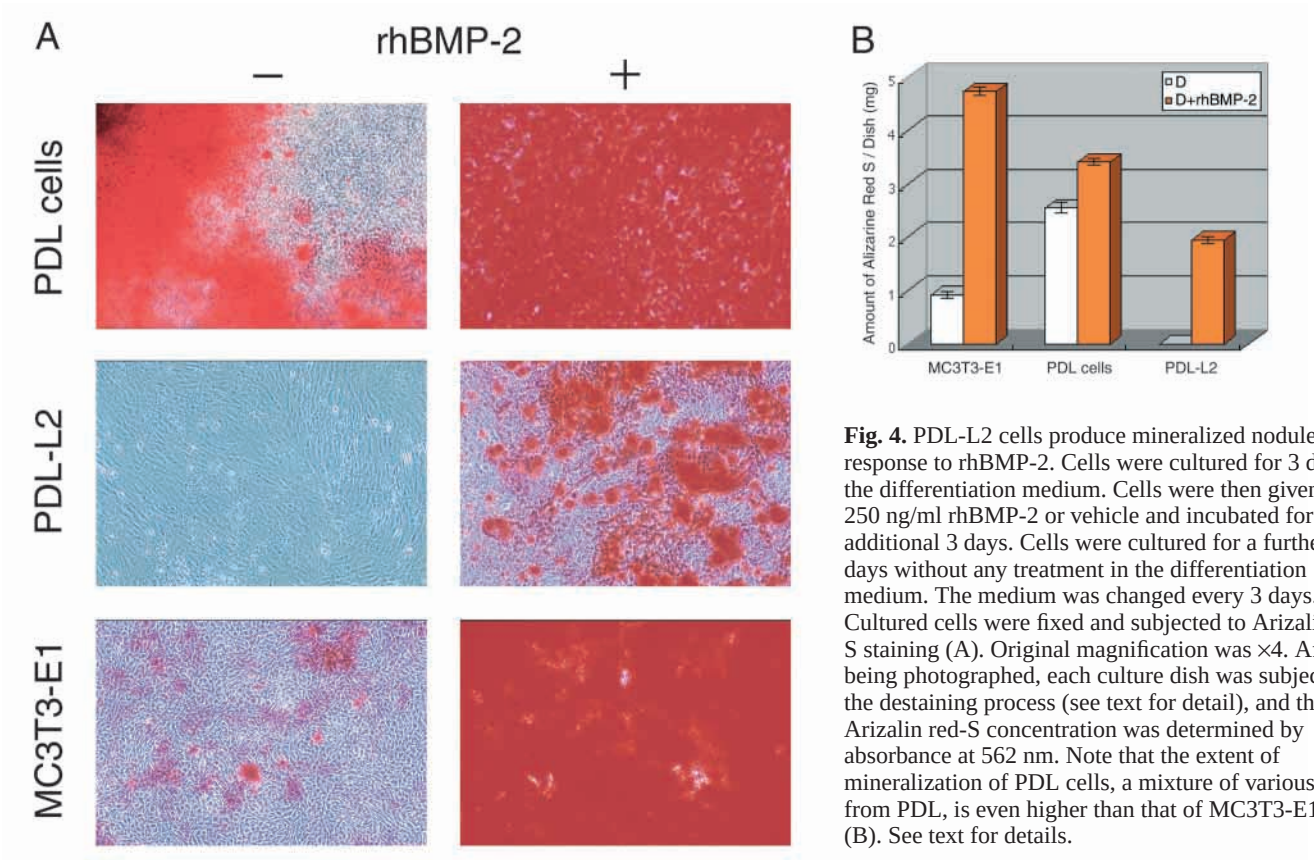

Fig. 4. PDL-L2 cells produce mineralized nodules in response to rhBMP-2. Cells were cultured for 3 days in the differentiation medium. Cells were then given either 250 ng/ml rhBMP-2 or vehicle and incubated for an additional 3 days. Cells were cultured for a further 25 days without any treatment in the differentiation medium. The medium was changed every 3 days. Cultured cells were fixed and subjected to Arizalin red-S staining (A). Original magnification was ×4. After being photographed, each culture dish was subjected to the destaining process (see text for detail), and the Arizalin red-S concentration was determined by absorbance at 562 nm. Note that the extent of mineralization of PDL cells, a mixture of various cells from PDL, is even higher than that of MC3T3-E1 cells (B). See text for details.

receptor type I and *II* (data not shown), however, stimulation by BMP may enhance alkaline ALPase activity and induce mineralization as is the case with C3H10T1/2 cell line (Katagiri et al., 1990). To test this possibility, PDL-L2 cells were incubated in the presence of rhBMP-2. As shown in Fig. 3, BMP-2 treatment enhanced ALPase activity not only in osteoblastic MC3T3-E1 cells but also in PDL-L2 cells, whereas fibroblastic NIH3T3 cells failed to respond (Fig. 3). BMP-2 also induced nodule formation and mineralization in PDL-L2 cells, although the extent of these phenomena was much less than those in MC3T3-E1 cells (Fig. 4). NIH3T3 cells again failed to respond to the BMP treatment. In contrast to the PDL-L2 cells, PDL cells, a mixed cell population from the PDL, readily mineralized in the absence of rhBMP-2, and the extent of mineralization was even higher than that of osteoblastic MC3T3-E1 cells (Fig. 4).

PDL-L2 has a mechanism by which the *Runx/Cbfa1* gene function is suppressed

As shown in Fig. 1, the expression level of *Runx2/Cbfa1* in the PDL fibroblasts is similar to or even higher than that in osteoblasts in vivo, but the PDL never mineralize, indicating the presence of a mechanism that inhibits mineralization of the tissue. This seems to be also true in vitro, since a superphysiological concentration of BMP-2 caused only a minimal mineralization nodules. When PDL-L2 cells were cultured in the differentiation medium without rhBMP-2, *Runx2/Osf2* expression markedly increased after 3 days, whereas expression of *osteocalcin* was barely detectable and that of *BSP* was not detected until 28 days of culture (Fig. 5A). Addition of rhBMP-2 increased the expression level of *Runx2/Osf2* to the maximum level, whereas the expression of *osteocalcin* and *BSP* were delayed and still suppressed compared to those seen in MC3T3-E1 cells (Fig. 2). Although the expression levels of these genes in PDL-L2 cells are lower than those in osteoblastic MC3T3-E1 cells, the pattern and sequence of change in each cell line was essentially the same. In contrast to those genes, the expression level of *periostin* transiently increased and returned to the control level at 28 days (Fig. 5A). This is also similar to the change in MC3T3-E1 cells (Fig. 2; data not shown). To further understand the underlying mechanism, we next compared the level of Runx2/Osf2 activity in cell lines using the cis-element OSE2, six of which are connected in tandem to a reporter plasmid. As depicted in Fig. 5B, transcriptional activation by Runx2/Osf2 in the rat osteoblastic ROS 17/2.8 cells was 60-fold more than that in C3H10T1/2; the latter is devoid of a significant amount of Runx2/Cbfa1 (Sasaki-Iwaoka et al., 1999). On the other hand, transcriptional activation in the PDL-L2 was minimal (Fig. 5B). When C3H10T1/2 cells were transfected with the *Runx2/Osf2* plasmid, the transcriptional activity dramatically increased (Fig. 5C). By contrast, the same treatment failed to stimulate the transcriptional activity in the PDL-L2. These data clearly indicate the presence of a mechanism responsible for suppressing the Runx2/Osf2 activity in PDL-L2 cells.

Discussion

We have cloned an immortalized PDL cell line from transgenic mice harboring SV40 large T antigen, and we have demonstrated that one of the cell line designated as PDL-L2 shares the gene expression profile seen in fibroblastic PDL cells in vivo and that it produces mineralized nodules in vitro when stimulated by rhBMP-2.

PDL cells have been suggested to be osteogenic in nature and to differentiate into either osteoblasts or cementoblasts depending on the need and the environment. Cho et al. demonstrated that PDL cells from rats formed mineralized nodules in vitro, although the nodules differed from those formed by osteoblasts (Cho et al., 1992). Arceo et al. also demonstrated that human PDL cells, but not gingival fibroblastic cells, formed mineral-like nodules in vitro (Arceo et al., 1991). However, the PDL cells used in those and other

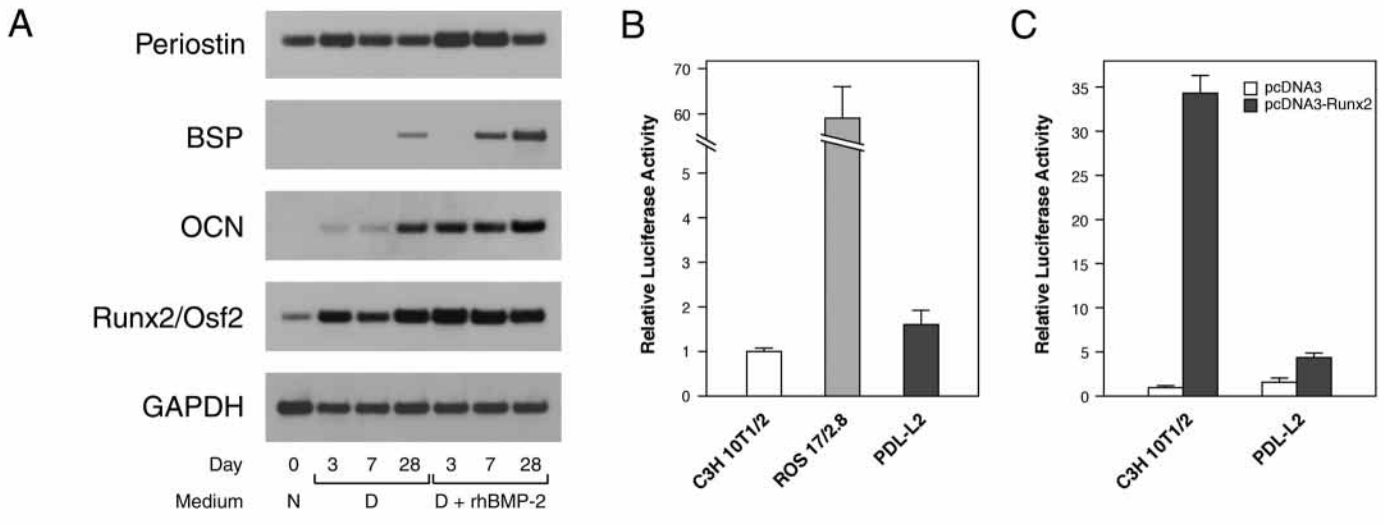

Fig. 5. Transcriptional activity of Runx2/Cbfa1 is suppressed in the PDL-L2 cells. (A) PDL-L2 cells were cultured in the differentiation medium (D) with or without 250 ng/ml rhBMP-2 and gene expression was examined by RT-PCR. (B) Each cell line was transfected with *p6OSE-2-Luc*, and luciferase activity was determined. (C) Each cell line was transfected with *p6OSE-2-Luc* together with *pcDNA3-Runx2/Osf2* or *pcDNA3*, and luciferase activity was determined. Each assay was carried out in a duplicate or triplicate. Each set of data is a representative of at least two independent experiments. See text for details.

studies were a mixture of heterogenous cell populations, and it was difficult to identify which cells, alone or interacting with other cell types, were responsible for the nodule formation. The present study further supports the notion that the PDL cells are closely related to the osteogenic cell lineage by demonstrating that the PDL cells express genes thought to be specific to osteoblasts including *periostin* and *Runx2/Cbfa1/Osf2* and that PDL cells alone can produce mineralized nodules in response to rhBMP, although the dose used was in a super-physiological range.

It may be argued that the PDL-L2 cell line originated from cementoblasts attached to the explants of PDL used for cloning. This seems unlikely, however, since cementoblasts abundantly expressed *BSP* gene in vivo, whereas fibroblastic PDL cells were negative for *BSP*, as shown in Fig. 1G, a finding that is consistent with those of previous reports (MacNeil et al., 1994; MacNeil et al., 1996; D'Errico et al., 1997). In addition, the *Runx2/Cbfa1/Osf2* gene is expressed in PDL cells but not in cementoblasts (Fig. 1E). Similarly, *periostin* is highly expressed in PDL cells but not in cementoblasts (Fig. 1D). The cloned PDL-L2 cells showed an identical expression pattern for these genes to in vivo fibroblastic PDL cells (Fig. 2). Furthermore, PDL-L2 cells did not produce mineralized nodules in the absence of exogenous BMP-2 (Fig. 4), whereas cementoblasts have been shown to be capable of producing mineralized nodules without BMP treatment (D'Errico et al., 2000). It is also unlikely that PDL-L2 cells originated from gingival fibroblasts, since neither of these genes is expressed in gingival fibroblasts in vivo (data not shown). To our knowledge, this is the first demonstration of a cloned PDL cell line that reflects the exact gene expression seen in vivo, although in a limited number of cells, and is capable of producing mineralized nodules in vitro.

PDL-L2 cells express genes for *periostin* and *Runx2/Osf2* but not *BSP* and *osteocalcin*, suggesting that PDL-L2s may be cells at a stage a little before the middle stage of osteoblast differentiation. However, the cells do not mineralize without BMP-2 treatment, which suggests that they do not yet appear to be committed towards osteoblastic cells. This is consistent with the previous observation that a pluripotent cell line C3H10T1/2 can differentiate into mature osteoblasts in response to rhBMP-2 treatment (Katagiri et al., 1990). However, a big difference exists between the two cell lines: while C3H10T1/2 cells fully mineralize in response to rhBMP-2 (Katagiri et al., 1990), the response of PDL-L2 cells is minimal (Fig. 4). This observation suggests that there may be a regulatory mechanism preventing differentiation of PDL-L2 cells toward osteoblasts; this notion is consistent with the fact that the expression level of the *Runx2/Osf2* gene in PDL cells in vivo appeared to be comparable to that in osteoblasts (Fig. 1E), and yet the PDL cells never mineralize. Since our data suggest that PDL-L2s may have a mechanism by which the Runx/Osf2 function in inducing *BSP* and *osteocalcin* is suppressed, we examined this possibility further.

We first compared the transcriptional activity of Runx2/Osf2 in osteoblastic cells and PDL-L2 cells. The activity in ROS 17/2.8 cells was 60-fold that in C3H10T1/2 cells, which are devoid of a significant amount of the *Runx2/Osf2* gene (Sasaki-Iwaoka et al., 1999). By contrast, the transcriptional activity in PDL-L2 cells was not much higher than that in C3H10T1/2 despite the fact that PDL-L2 cells expressed a significant amount of the *Runx2/Osf2* gene (Fig. 5B). When C3H10T1/2 cells were transfected with *Runx2/Osf2* plasmid, the transcriptional activity increased more than 30-fold, whereas the response of PDL-L2 cells was minimal (Fig. 5C). It may be argued that Runx2 protein production is suppressed in the PDL-L2 even though gene expression is relatively high. This possibility, however, was excluded since western blot analysis revealed that endogenous Runx2/Cbfa1 is detectable in the PDL-L2 cells. These data clearly indicate that there exists a mechanism by which the function of *Runx2/Osf2* is suppressed in PDL-L2 cells. More work is needed to clarify the exact mechanism underlying the unique character of the PDL-L2 cells.

The hypothesis that there may be a regulatory mechanism in vivo preventing differentiation of PDL cells toward osteoblasts is also consistent with the observation by Lang et al. that periodontal cell populations cultured in vitro failed to form cementum-like tissues in vivo when they were orthotopically re-implanted, whereas cell populations from alveolar bone did form cementum-like tissue in the same situation (Lang et al., 1995). The thesis that cementoblasts and osteoblasts in the periodontium commonly originate from precursor cells residing in the alveolar bone is also supported by previous observations (Gould et al., 1977; Melcher et al., 1986). Our data are also consistent with the data reported by Rajishankar and colleagues (Rajishankar et al., 1998). Using the periodontal window wound healing model, they demonstrated in vivo that PDL inhibited invasion of alveolar bone even when the damaged tissues were stimulated by a large amount of BMP-7. Meanwhile the wounds including bone and cementum as well as the PDL were completely healed by the treatment. They concluded that PDL has a mechanism for regulating PDL width and maintaining periodontal homeostasis that is resistant to the strong osteogenic stimulation by BMP. Our study supports this notion and suggests that the mechanism may be due to the suppression of the transcriptional activity of Runx2/Osf2, since the transcription factor is necessary for calcification of bone and cartilage (Ducy et al., 1997; Komori et al., 1997). Thus, whether or not PDL fibroblasts differentiate into osteoblasts and/or cementoblasts in vivo is still a matter of debate, our data on the PDL-L2, however, rather indicate that PDL fibroblastic cells is not osteogenic. Further studies are needed to clarify the underlying mechanism.

It should be noted that *Runx2/Osf2* was expressed in odontoblasts in addition to PDL cells and osteoblasts and that the expression level was much higher in odontoblasts than in PDL cells and osteoblasts (Fig. 1E). Our results were somewhat inconsistent with those of recent studies demonstrating *Runx2/Osf2* gene expression in odontoblasts during tooth development, but after completion of tooth development, the principal site of expression of this gene was restricted to the PDL (D'Souz et al., 1999; Jiang et al., 1999). The reason for the discrepancies between those two studies and the present study is not clear at present. Since the signal detected in our study was clear and consistent with the previous observations concerning the expression of this gene in osteoblasts and PDL cells (D'Souz et al., 1999; Jiang et al., 1999), and since the signal disappeared completely when a sense probe was used, it is unlikely that the signals were non-specific. Differences in the probes used and in the differentiation stage of the animals in these studies may

account for the discrepancies. Further studies are needed to resolve this issue.

In addition to the PDL-L2 cells, we established 18 more cell lines. Most of them (approximately 70%) showed characteristics similar to those of PDL-L2. This is in agreement with in situ hybridization data showing that *Runx2/Osf2*-positive cells occupy 60 to 70% of PDL fibroblasts (Fig. 1). Other cell lines showed patterns of gene expression different from those of PDL-L2 (data not shown). Further characterization of these cell lines will allow us to determine whether these cell lines exhibit all the osteogenic functions proposed for the PDL tissues, whether there are really heterogenous types of fibroblastic cells in the PDL and which cell types are responsible for one or all of these functions. It is important to identify a marker(s) for PDL cells, and our cell lines will also be useful for this purpose.

In conclusion, we have established an immortalized murine PDL cell line, PDL-L2, which appears to mimic gene expression observed in PDL cells in vivo. The cell line produces a limited amount of mineralized nodules when stimulated by exogenous BMP-2 and is equipped with a mechanism for suppressing the function of Runx2/Osf2. PDL-L2 cells together with other cloned cell lines will provide new tools with which to study the biological function of the PDL and new insights into the role of the PDL in tooth development and maintenance of teeth and alveolar bone of the mandibula. Our study also confirmed that a population of PDL cells expresses the *Runx2/Osf2* gene at a similar level to that in osteoblasts and demonstrated that odontoblasts express this gene at even higher levels in adult life. In addition, our study demonstrated that PDL cells express the *periostin* gene at a much higher level than do osteoblasts in mandibula.

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