RESEARCH REPORTS
Biological

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Characterization of a Novel Periodontal Ligament-specific Periostin Isoform

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APPENDIX

MATERIALS & METHODS

RNA Extraction and Real-time PCR Analysis

RNA Bee (Tel-Test, Friendswood, TX, USA) was used to extract total RNA from cultured cells and from human PDL tissues isolated from freshly extracted first premolar teeth from patients undergoing tooth extraction. Human tissue total RNAs (heart, skin, kidney, bone marrow, spleen, testis, lung, liver, skeletal muscle, brain, and thymus) were purchased from Bio Chain (Hayward, CA, USA). cDNA was synthesized from total RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was performed with Power PCR SYBR Master Mix (Applied Biosystems), and conventional RT-PCR was performed with Taq DNA Polymerase (Takara Bio, Shiga, Japan). Gene-specific primers are listed in the Appendix Table.

Tissue Preparation and Immunohistochemical Staining

A goat anti-periostin polyclonal antibody (#S-15; 1:1000; Santa Cruz Biotech, Santa Cruz, CA, USA), which recognizes the common region of mouse periostin, was used as the primary antibody for the detection of all mouse periostin isoforms. Normal goat IgG (Vector Laboratories, Burlingame, CA, USA) served as a control. Biotinylated anti-goat IgG antibody was used as a secondary antibody (Vector Laboratories). A Vectastain Avidin-Biotin Complex Kit (Vector Laboratories) and DAB solution (20 mg DAB, 50 mL PBS, 4.5 μL H_2O_2) were used for signal detection.

Western Blot Analysis

A rabbit anti-periostin antibody (#14041; 1:1,000; Abcam, Cambridge, MA, USA), which recognizes the common region of human and mouse periostin, a rabbit anti-integrin αv antibody (1:1,000; Santa Cruz Biotech), a mouse anti-FLAG antibody conjugated with horseradish peroxidase (1:2,000; Sigma, St. Louis, MO, USA), a rabbit anti-FAK antibody (1:1,000; Cell Signaling Technology, Danvers, MA, USA), and a rabbit anti-phosphorylated FAK (Tyr397) antibody (1:1,000; Cell Signaling Technology) were used as primary antibodies. HRP-conjugated donkey anti-rabbit IgG antibody (1:1,500; GE Healthcare, Piscataway, NJ, USA) was used as a secondary antibody.

Cell Culture and Induction of PDL Cell Cytodifferentiation

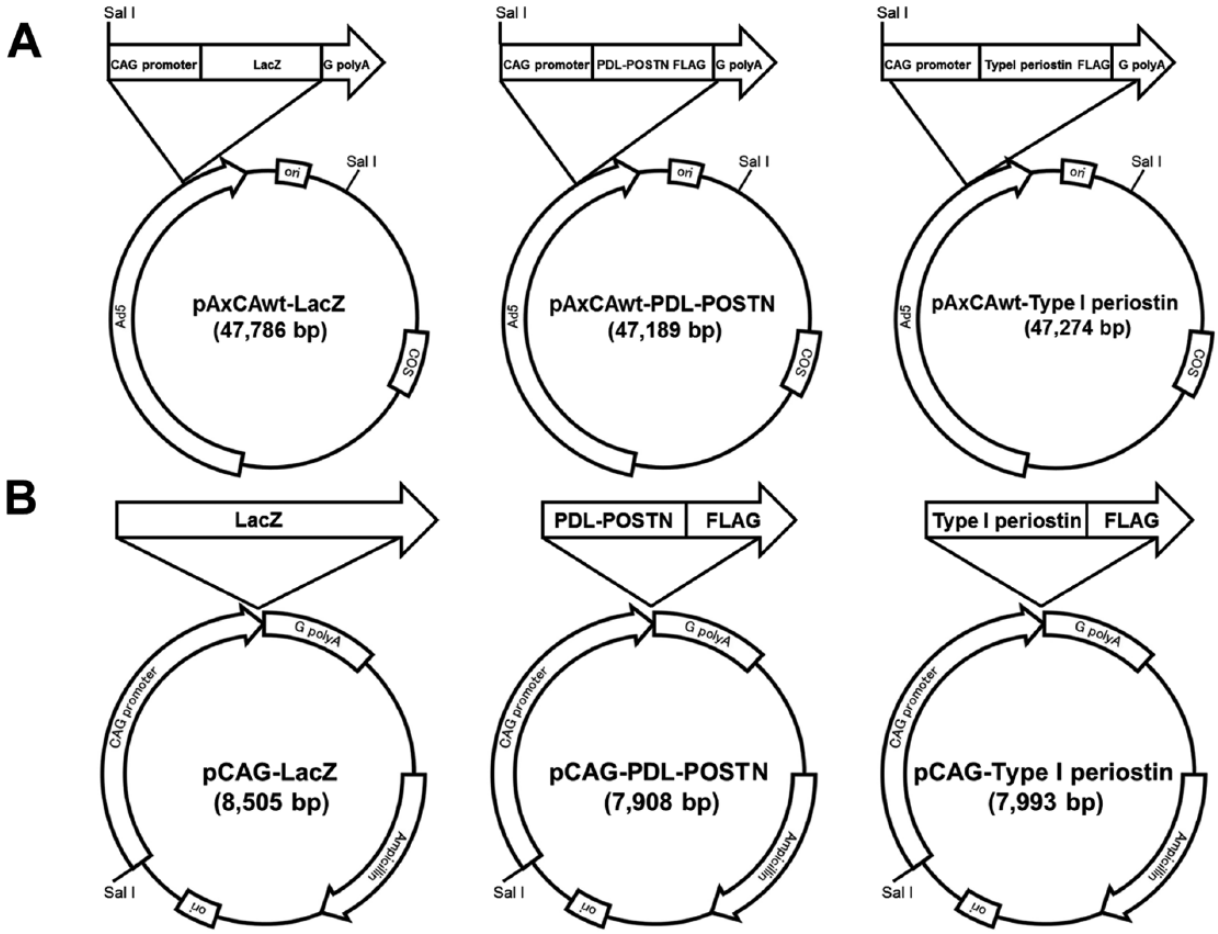
For the differentiation of human and mouse PDL cells into hard-tissue-forming cells *in vitro*, cells were cultured in 12-well plates until they reached confluence. The standard medium was then replaced with α-MEM supplemented with 10% FCS, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid (mineralization-inducing medium). A rat anti-integrin αv neutralizing antibody (Millipore, Billerica, MA, USA) was added to the mineralization-inducing medium at the indicated concentrations. Normal rat IgG (eBioscience, San Diego, CA, USA) served as a control.

Plasmids and Transfection

The open-reading frame of the FLAG-tagged full-length periostin isoform (Type I) and the FLAG-tagged PDL-POSTN was subcloned from pGEM-T plasmids into a pAxCAwt cosmid vector (Miyake *et al*., 1996). These cosmid vectors were then recombined with adenovirus by means of an adenovirus expression vector kit (Takara Bio), and adenovirus vectors were finally completed for recombinant FLAG-tagged periostin protein production. An adenovirus expressing *LacZ* was used as a control. The cosmid vectors were then digested with *Sal*I restriction enzyme (New England Biolabs, Ipswich, MA, USA) to remove the adenovirus component. The vectors were then self-ligated to obtain plasmid vectors, termed pCAG-Type I periostin and pCAG-PDL-POSTN, for FLAG-tagged full-length periostin isoform (Type I) and FLAG-tagged PDL-POSTN expression, respectively. MPDL22 cells were then independently transfected with each plasmid by means of an Amaxa Nucleofector device (Lonza, Basel, Switzerland). A plasmid vector, pCAG-LacZ, expressing *LacZ* was used as a negative control. The adenovirus and plasmid constructs are shown in the Appendix Fig.

Appendix Table. Nucleotide Sequences of Primers Used for PCR Analysis

Gene	Sequence	Tm (°C)	GenBank Accession Number
human POSTN	5′-GCGAGATCATCAAGCCAGCA-3′ 5′-GGCACAAATAATGTCCAGTCTCCA-3′	60.0	NM_006475.2
human POSTN 3′-region	5′-AACTCCTCTATCCAGCAGACAC-3′ 5′-CCTTGAACTTTTTTGTTGGCTTGC-3′	56.0	NM_006475.2
human POSTN exon a-c′	5′-CCAGCAGACACACCTGTTGG-3′ 5′-CCTTGAACTTTTTTGTTGGC-3′	54.0	NM_006475.2
human HPRT	5′-CGAGATGTGATGAAGGAGATGGG-3′ 5′-GCCTGACCAAGGAAAGCAAAGTC-3′	63.8	NM_000194.2
mouse POSTN	5′-GGATTCGAACCCGGAGTCACTA-3′ 5′-CGACGTGGATGACACCATTTG-3′	60.0	NM_015784.3
mouse GAPDH	5′-TGTGTCCGTCGTGGATCTGA-3′ 5′-TTGCTGTTGAAGTCGCAGGAG-3′	63.0	NM_008084.2



Appendix Figure. Construction of the cosmid and plasmid vectors for periostin isoform expression. **(A)** Construction of cosmid vectors of LacZ, PDL-POSTN and Type I periostin isoform for recombination with adenovirus. **(B)** Construction of plasmid vectors expressing LacZ, PDL-POSTN and Type I periostin isoform in MPDL22 cells. The cosmid vectors were digested with SalI restriction enzyme to remove adenovirus component and then, ligated with SalI site to obtain the plasmid vectors. Ad5, adenovirus 5 genome sequences with deletions of E1 and E3; CAG, chicken β-actin; G polyA, rabbit β-globin polyA; ori, the signals for the origin of replication; COS, the signals for packaging.

RNA Interference of Periostin

The pSilencer RNA interference kit (Ambion, Carlsbad, CA, USA) was used to generate small hairpin RNAs (shRNA) as previously described (Yamada *et al*., 2007). We used a negative control shRNA oligonucleotide from the Ambion kit that is unrelated to periostin. The shRNA vectors were introduced into MPDL22 cells by means of an Amaxa Nucleofector device (Lonza), according to the manufacturer's protocol. We started drug selection with hygromycin (600 μg/mL) 24 hrs after transfection and established stable transfectants with periostin knockdown.

Conditioned Medium Containing Recombinant Periostin Isoforms

To prepare conditioned medium containing recombinant FLAG-tagged PDL-POSTN, full-length periostin, or β-galactosidase (as a control), we cultured human PDL cells in ten 10-cm dishes in 10 mL of α-MEM supplemented with 10% fetal calf serum, then infected with periostin isoform-expressing adenoviruses or *LacZ*-expressing adenovirus. Three days after infection, culture medium was collected (100 mL) and confirmed by Western blot analysis to contain comparative levels of recombinant PDL-POSTN and full-length periostin (data not shown).

^{3}H-thymidine Incorporation Assay

Cells were seeded into 24-well culture dishes (1×10^{4} cells/well). Medium was replaced the next day with FCS-free α-MEM. After serum deprivation for 24 hrs, the cells were stimulated for 24 hrs with α-MEM containing 10% FCS. We measured DNA synthesis by dosing wells with 2 μCi/well ^{3}H-thymidine for 4 hrs. The cells were then washed three times with PBS, and soluble radioactivity was extracted with 5% trichloroacetic acid. The incorporated radioactivity was determined in a liquid scintillation counter (Aloka, Tokyo, Japan).

APPENDIX REFERENCES

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