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Characteristic changes of periodontal ligament-derived cells during passage

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Background and Objective: Although periodontal ligament-derived cells are expected to be a useful source of cells for periodontal tissue engineering, the characteristic changes of primary cultured cells have not been well studied. Therefore, the aim of this study was to investigate the characteristics of periodontal ligament-derived cells and their changes during passage.

Material and Methods: Human periodontal ligament tissue was obtained from extracted third molars. Cells were subcultured until passage 6 and the cell characteristics from early to late passages were evaluated using immunofluorescence microscopy, alkaline phosphatase activity analyses, reverse transcription–polymerase chain reaction and quantitative real-time polymerase chain reaction. To examine the function of periodontal ligament-derived cells further, cells were transplanted into the renal subcapsule of an immunocompromised rat.

Results: Immunofluorescence results showed relatively uniform expression of MSX-2 and osteonectin from passage 1 until passage 6. The STRO-1-positive fraction was 33.5% at passage 0, which was reduced to 14.7% at passage 3. Cultured cells at passage 1 expressed mRNA for collagen type I, collagen type XII, Runx2, alkaline phosphatase, osteonectin, osteopontin, scleraxis, tenomodulin, Msx2, GDF5 and GDF7 genes, but not for bone sialoprotein. The level of mRNA expression from tenomodulin and collagen type XII genes decreased after passage 3. Alkaline phosphatase activity decreased in cells at later passages. Osteogenic induction of periodontal ligament-derived cells resulted in a down-regulation of the tenomodulin gene. Transplanted cells from both early and late passages produced dense collagen fiber bundles without calcified tissue.

Conclusion: Cultured periodontal ligament-derived cells were a morphologically homogeneous population, although expression of STRO-1 was limited in primary culture. Cultured cells showed de-differentiation during passage for both osteogenesis- and tendo/ligamentogenesis-related genes.

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Periodontal disease leads to tooth loss through the destruction of attachment apparatus and tooth-supporting structures such as bone, cementum and periodontal ligament. Periodontal ligament cells are believed to play an important role in the repair of damaged tooth-supporting structures in periodontal disease (1,2). Accordingly, periodontal ligament-derived cells have been used as a material for periodontal tissue engineering (3–5). Indeed, it has been reported that the periodontal ligament contains a stem cell population which can differentiate into various cell lineages (6). Recently, a gene-expression profile of cloned or immortalized periodontal ligament-derived cells was

reported (7). This gene-expression profile was unique from other osteogenic cells, which may imply the necessity of periodontal ligament-derived cells for the repair of periodontal tissue.

As a result of the limited availability of periodontal ligament tissue, cultured and nonselected periodontal ligament cells have been used for tissue engineering. Periodontal ligament contains a heterogeneous cell population, including fibroblast-like and osteoblast-like cells (7–10). It is not clear how these populations are maintained during primary cell culture or how these populations change over time. It was our aim, in this study, to investigate the character and the characteristic changes of primary cultured periodontal ligament-derived cells during passage as it is important to establish cell characteristics prior to implementing efficient periodontal tissue engineering.

Material and methods

Tissue preparation and cell culture

Periodontal ligament tissues were obtained from extracted teeth ($n = 5$; three male and two female donors, of average age 32 years) of impacted, healthy third molars. The procedure used to obtain extracted teeth from humans conformed to the tenets of the Declaration of Helsinki, and the experimental protocol was approved by the Ethical Committee at Nagoya University School of Medicine. Informed consent was obtained from each subject prior to donation of the tissue. Each tooth sample was rinsed twice in phosphate-buffered saline containing 1000 units/mL of penicillin G sodium, 1 mg/mL of streptomycin sulfate and 2.5 μg/mL of amphotericin B (Invitrogen Corporation, Grand Island, NY, USA) for 5 min at 25°C. Periodontal ligament tissue was mechanically removed from the root surface using a scalpel. To avoid contamination with gingival and apical tissue, tissue from the apical and coronal regions of the tooth was avoided. Tissue was digested with 2 mg/mL of collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 h in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St Louis, USA) containing 10% fetal bovine serum (Thermo Trace Ltd, Melbourne, Australia) and antibiotic–antimycotic solution on a shaker at 37°C. The cell suspension was then centrifuged for 5 min at 225 ***g***, and the cell pellet was resuspended in culture medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotic–antimycotic solution). Isolated cells were seeded in a 24-well culture dish. When cells became 80–90% confluent, they were subcultured (1×10^4 cells in a 10-cm dish) until passage 6 (population doubling level 24). At each passage, some cells were harvested and processed for RNA extraction, immunostaining and alkaline phosphatase activity analysis.

Reverse transcription–polymerase chain reaction and quantitative real-time polymerase chain reaction

Cells were rinsed twice with phosphate-buffered saline and total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Following extraction with phenol/chloroform and then with chloroform, RNA was precipitated with isopropanol in the presence of 0.2 M sodium acetate and redissolved in diethyl pyrocarbonate-treated water. The RNA concentration was determined from the absorption at 260/280 nm.

Reverse transcription from total RNA was performed using a reverse transcription kit (SuperScript™ First-Strand Synthesis System for RT-PCR; Invitrogen). Total RNA (1 μg), 1 μL of Oligo (dT), 1 μL of dNTP mixture (10 mM) and diethyl pyrocarbonate-treated water (total volume 12 μL) were mixed and pre-incubated at 70°C for 10 min. After placing on ice for 1 min, the incubated sample (12 μL) was mixed with 1 μL of Super Script reverse transcriptase (50 units/μL), 4 μL of 5× First-Strand Buffer (containing 375 mM KCl and 15 mM $MgCl_2$) and 2 μL of dithiothreitol and then incubated at 42°C for 50 min to allow the synthesis of first-strand cDNA. Reverse transcription–polymerase chain reaction (RT-PCR) analyses for the following genes were performed: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), collagen type I (*Col I*), collagen type XII (*Col XII*), *Runx2*, alkaline phosphatase (*ALP*), osteonectin (*ON*), osteopontin (*OP*), bone sialoprotein (*BSP*), *scleraxis*, tenomodulin (*TeM*), *Msx2*, growth differentiation factor 5 (*GDF5*) and growth differentiation factor 7 (*GDF7*). The primer sequences for those genes are listed in Table 1. These primers were designed from sequences in the Entrez Nucleotide database at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/sites/entrez/query.fcgi?db=Nucleotide).

One microgram of cDNA was amplified with 0.5 μM each primer, 0.2 mM dNTP mixture, 10× PCR buffer (containing $MgCl_2$) and 0.1 μL (5 units/μL) of Taq polymerase (Takara Bio Inc., Otsu, Shiga, Japan) in a 20-μL reaction mix. After 2 min of pre-incubation at 94°C, amplification was performed for 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C, except for *BSP*, where the annealing temperature was 62°C, and extension at 72°C for 1 min. PCR products were run on a 2% agarose gel and visualized after staining with ethidium bromide by using an image-analyzing system (Chemi Doc; Bio-Rad Laboratories, Hercules, CA, USA).

Quantitative real-time PCR analyses were performed on the ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), following the instructions recommended by the manufacturer. Expression of the *GAPDH*, *scleraxis*, *TeM* and *Col XII* genes were analyzed using quantitative real-time PCR. The primers used for quantitative real-time PCR are listed in Table 1. These sequences were also designed from sequences in the Entrez Nucleotide database at the National Center for Biotechnology Information website. The results were analyzed using ABI sequence detection system software.

Table 1. Sequences of the forward and reverse primers for reverse transcription–polymerase chain reaction (RT-PCR) and real-time quantitative polymerase chain reaction (q-PCR)

Gene	Sense primer	Antisense primer
RT-PCR		
GAPDH	5′-CGACCACTTTGTCAAGCTCA-3′	5′-AGGGGAGATTCAGTGTGGTG-3′
Runx2	5′-CTCTTCCCAAAGCCAGAGTG-3′	5′-CAGCGTCAACACCATCATTC-3′
Col I	5′-CTGGCAAAGAAGGCGGCAAA-3′	5′-CTCACCACGATCACCACTCT-3′
Col XII	5′-CTGCAATTTTCAAACCAAAG-3′	5′-TATGCCTTCAAACTCCTCAA-3′
ALP	5′-CCACGTCTTCACATTTGGTG-3′	5′-AGACTGCGCCTGGTAGTTGT-3′
ON	5′-AAATCCACTCCTTCCACAGT-3′	5′-CAAATGGCAAGAGAAAAATG-3′
OP	5′-TTGCAGTGATTTGCTTTTGC-3′	5′-CTCATGGTTTGGTTGGACT-3′
BSP	5′-CTATGGAGAGGACGCCACGCCTGG-3′	5′-CATAGCCATCGTAGCCTTGTCCT-3′
Scleraxis	5′-TGCGAATCGCTGTCTTTC-3′	5′-GAGAACACCCAGCCCAAA-3′
TeM	5′-CCCAGCAGAAAAGCCTATTG-3′	5′-GCGTGACGGGTCTTCTCTAC-3′
Msx2	5′-ACAGAGACCCAGGTCAAAAT-3′	5′-TAGCAGAGCAGGAGTACTGG-3′
GDF5	5′-TACACGGTCTTATCGTCCTG-3′	5′-GTTCGACATCTGGAAGCTCT-3′
GDF7	5′-GACTACGAGGCATACCACTG-3′	5′-ACCACCATGTCTTCGTACTG-3′
q-PCR		
GAPDH	5′-GCACCGTCAAGGCTGAGAAC-3′	5′-ATGGTGGTGAAGACGCCAGT-3′
Scleraxis	5′-TGCGAATCGCTGTCTTTC-3′	5′-GAGAACACCCAGCCCAAA-3′
TeM	5′-TTGAAGACCCACGAAGTAGA-3′	5′-ATGACATGGAGCACACTTTC-3′
Col XII	5′-CTGCAATTTTCAAACCAAAG-3′	5′-TATGCCTTCAAACTCCTCAA-3′

Alkaline phosphatase activity analyses

At passages 1, 3 and 6, cells were cultured in a six-well plate (Greiner Bio-Science, Frickenhousen, Germany) under the conditions stated above and used for biochemical analysis at 3, 7, 14 and 21 d. After washing with phosphate-buffered saline, alkaline phosphatase activity was measured using FAST *p*-Nitrophenyl Phosphate Tablet Sets (Sigma), according to the manufacturer's protocol. Absorbance was read at 415 nm using a microtiter plate reader (SmartSpeck^{TM}3000; Bio-Rad, Tokyo, Japan). At the same passage, alkaline phosphatase staining was performed on six-well plates, using Leukocyte Alkaline Phosphatase (Sigma) according to the manufacturer's protocol, when the cells reached 100% confluence.

Immunofluorescence microscopy

To confirm the presence of STRO-1, ON, and Msx2 proteins in periodontal ligament-derived cells, immunofluorescent staining was performed. Periodontal ligament-derived cells were cultured in a 24-well plate (Greiner Bio-Science, Frickenhousen, Germany) until 100% confluent. The dishes were rinsed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 30 min. Then, the cells were rinsed three times with phosphate-buffered saline. After incubation for 30 min with phosphate-buffered saline containing 2% bovine serum albumin as a blocking reagent and 0.25% Triton X-100, the cells were incubated with monoclonal anti-human STRO-1 (diluted 1:400) (R&D Systems, Minneapolis, MN, USA), monoclonal anti-human ON (diluted 1:400) (Alexis Corporation, San Diego, CA, USA) or polyclonal anti-human Msx2 (diluted 1:400) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 25°C. The dishes were then washed three times with phosphate-buffered saline and incubated for 30 min at 25°C with a secondary antibody (diluted 1:200) [Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) for osteopontin and Msx2; or fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Zymed Laboratories, South San Francisco, CA, USA) for STRO-1]. After washing three times with phosphate-buffered saline, the dishes were cover slipped with a mounting reagent containing 4′,6-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Digital images were acquired using DP CONTROLLER 1.2.1 and DP MANAGER 1.2.1 software (Olympus).

Transplantation

The following experiments were approved by the Animal Experiment Advisory Committee of Nagoya University School of Medicine, and conducted in accordance with the Guidelines for Animal Experimentation of Nagoya University. Cultured cells (1×10^5) at passages 1 or 6 were seeded on the surface of human tooth dentin and preshaped as a thin sheet (approximately 2×2 mm in size, and 0.2 mm in height) in a 10-cm plastic tissue culture dish. After 1 d, the dentin specimen was transplanted into the renal subcapsule of an immunocompromised rat (10–15 wk of age, F344/N Jcl-rnm; Chubu Kagaku Shizai Corporation, Nagoya, Japan), which was under general anesthesia induced by an intraperitoneal injection of sodium pentobarbital (15 mg/kg). Six weeks after the transplantation, the specimen was removed and fixed with 4% formalin, then decalcified and embedded in paraffin. Sections were deparaffinized and stained with hematoxylin and eosin or azan.

Effect of osteogenic induction on the differentiation of periodontal ligament-derived cells *in vitro* and *in vivo*

For assessment of the effects of osteogenic induction on the differentiation of periodontal ligament-derived cells, cells were treated with osteoinduction medium [Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, antibiotic–antimycotic solution, 50 μg/mL of ascorbic acid, 10 mM sodium β-glycerophosphate and 10^{-8} M dexamethasone (Sigma Chemical Co.)] for 2 wk at passages 1, 3 and 6. Total RNA was extracted from the specimen at each passage and expression of the marker genes was analyzed. Induced cells at passages 1 and 6 were seeded on a flat human dentin sheet and transplanted into the renal subcapsule of an immunocompromised rat as described above.

Statistical analysis

One-way repeated-measures analysis of variance was used for quantitative real-time PCR analyses, and two-way repeated-measures analysis of variance was used for alkaline phosphatase activity and to detect any significant difference within each group. When a significant difference was detected, the difference between any selected group and passage 1 was confirmed using Dunnett's test. Experimental values are presented as mean ± standard error. A p-value of < 0.05 was considered to be statistically significant.

Results

Primary culture of periodontal ligament-derived cells

On primary culture, periodontal ligament-derived cells extended and proliferated from the dissociated piece of tissue, and spindle-shaped cells covered the dish (Fig. 1A). Staining for alkaline phosphatase activity was performed and the spindle-shaped cells covering the dish were mostly positive (Fig. 1B).

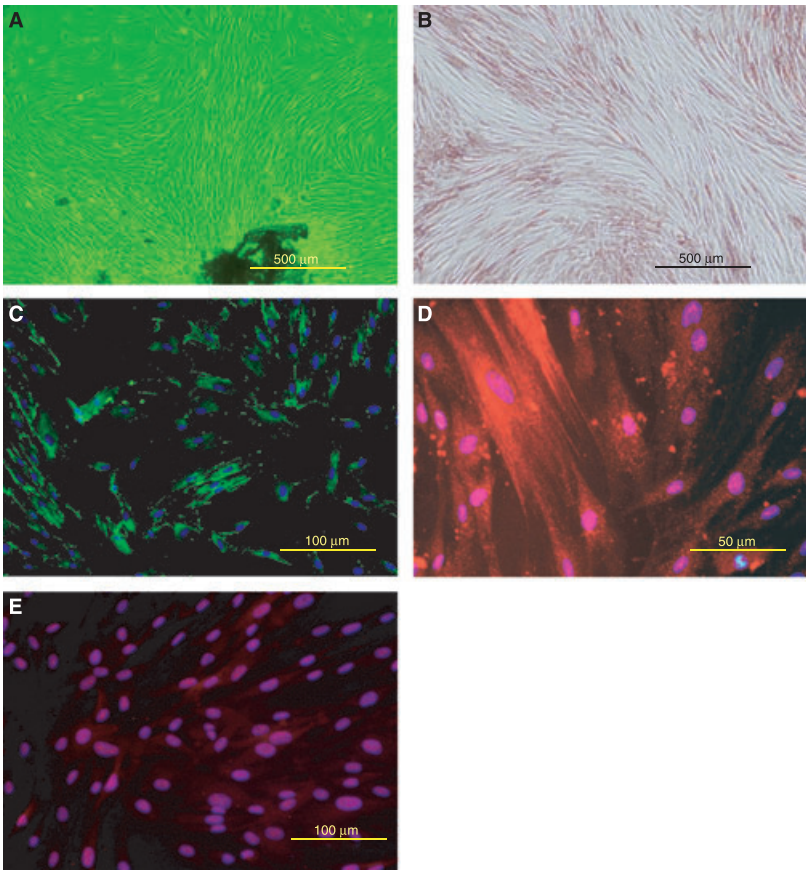

Fig. 1. Morphology and immunofluorescence of periodontal ligament-derived cells. (A) Phase-contrast photomicrographs of cultured periodontal ligament-derived cells in primary culture. Cells extended from a piece of periodontal ligament tissue and spindle-shaped cells covered the dish. (B) Almost all periodontal ligament-derived cells in primary culture stained positive for alkaline phosphatase. Fluorescent photomicrographs show STRO-1-positive (C), ON-positive (D) and *Msx*2-positive (E) cells analyzed using a fluorescence microscope.

Table 2. Percentage of cells positive for STRO-1, osteonectin (ON), or Msx2 expression, determined by immunofluorescent staining, at passages 0 (P0) and 3 (P3)

	P0	P3
STRO-1 (strongly positive)	33.5 ± 5.4	14.7 ± 3.7
ON (weakly positive) (strongly positive)	96.7 ± 3.3 (83.8 ± 1.9) (12.8 ± 1.5)	100 ± 0.0
Msx2	100 ± 0.0	100 ± 0.0

Data are the mean ± standard error from three different fields. There was no statistically significant difference between P0 and P3.

Immunofluorescence microscopy

To confirm the phenotype of periodontal ligament-derived cells, the cells were incubated with antibodies to STRO-1 (Fig. 1C), ON (Fig. 1D) and Msx2 (Fig. 1E) following fixation. At the first passage, most of the cells were positive for ON and Msx-2, but the intensity of fluorescence for ON varied. The percentage of STRO-1-positive cells was 33.5% (Table 2). The percentages of weakly and strongly ON-positive cells are shown in Table 2. Cells that were weakly positive for ON had disappeared

almost completely by the third passage, and the intensity of the remaining ON-positive cells became almost uniform. The percentage of STRO-1-positive cells decreased markedly, and the percentage of Msx-2-positive cells remained unchanged, between the first and the third passages (Table 2).

Gene-expression analyses

The mRNA levels of osteogenic and tendo/ligamentogenesis-related genes were analyzed at every passage. At passage 1 (population doubling level 4), mRNA for all osteogenic markers (*Col I*, *Runx2*, *ALP*, *ON* and *OP*) was detected, except for *BSP* (Fig. 2A). mRNAs for tendo/ligamentogenic marker genes (*Col XII*, *scleraxis*, *TeM GDF5* and *GDF7*), and a transcriptional factor, *Msx*2, were also detected (Fig. 2A). Then, mRNAs at passages 1 and 6 (population doubling level 24) were compared. mRNA expression of *Col I*, *Col XII*, *Runx2*, *ALP*, *ON*, *OP*, *GDF5*, *GDF7* and *Msx2* were found at passage 6 (Fig. 2B). Similarly to the findings for passage 1, no *BSP* mRNA was detectable at passage 6. Interestingly, no mRNA for *TeM*, a tendo/ligamentogenesis-related gene located downstream of *scleraxis*, was detected at passage 6 (Fig. 2B). To investigate in greater detail the time course of expression of tendo/ligamentogenesis-related genes, the expression of *scleraxix*, *TeM* and *Col XII* genes was evaluated at every passage up to 6. Expression of *TeM* disappeared after passage 3 (population doubling level 12), whereas the expression of *scleraxis* was maintained in all samples. Although the expression of *Col XII* was maintained until passage 6, the level of expression decreased after passage 3 (Fig. 2C).

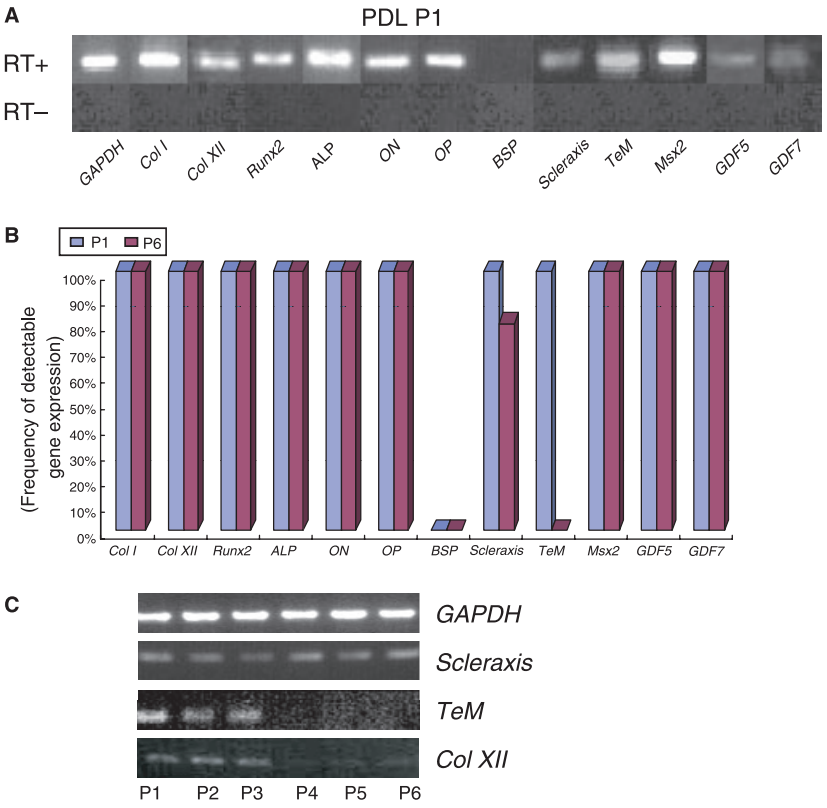

Fig. 2. Gene expression in periodontal ligament-derived cells. (A) The results from reverse transcription–polymerase chain reaction analyses showed expression of osteogenic markers (*Col I*, *Runx2*, *ALP*, *ON*, *OP*, *BSP)*, tendo/ligamentogenic markers (*scleraxis*, *TeM*, *Col XII*, *GDF5*, *GDF7)* and a transcription factor, *Msx2*, in periodontal ligament. Total RNA was extracted from periodontal ligament-derived cells at passage 1 (PDL P1). Messenger RNAs for *collagen type I*, *Runx2*, *ALP*, *ON* and *OP* were detected, whereas mRNA for *BSP* was not detected. Messenger RNAs for tendo/ligamentogenesis-related genes (*Col XII*, *scleraxis*, *TeM*, *GDF5* and *GDF7*) and for a transcription factor, *Msx2*, were also detected. (B) The frequency of gene expressions was analyzed in the cells at passages 1 and 6 ($n = 5$ for each group). Cultured cells at passage 1 (P1; population doubling level 4) and passage 6 (P6; population doubling level 24) expressed mRNA for *Col I*, *Col XII*, *Runx2*, *ALP*, *ON*, *OP*, *scleraxis*, *GDF5*, *GDF7* and *Msx2*. Expression of *TeM* was not observed in periodontal ligament-derived cells at passage 6. (C) Time course for the expression of tendo/ligamentogenesis-related genes. *Scleraxis* mRNA was detected at all passages. On the other hand, *TeM* mRNA disappeared after passage 3. Although *Col XII* mRNA was detected at all passages, the levels of gene expression showed a marked decrease after passage 3. P1, passage 1; P2, passage 2; P3, passage 3; P4, passage 4; P5, passage 5; P6, passage 6.

Real-time quantitative PCR analyses for tendo/ligamentogenesis-related genes

To confirm the change of mRNA levels during passages 1 to 6, real-time quantitative PCR was performed on three tendo/ligamentogenesis-related genes. In parallel with the results from RT-PCR, the expression of *TeM* and *Col XII* mRNAs decreased at passage 3 and remained low thereafter. The expression of *TeM* mRNA was almost undetectable at passage 6. Statistically significant differences were observed between the samples from passages 1 and 6 for both *TeM* and *Col XII* ($p < 0.05$) (Fig. 3B,C). On the other hand, mRNA for *scleraxis* did not show significant changes at any passage number (Fig. 3A).

Influence of osteogenic induction on the expression of tendo/ligamentogenesis-related genes

Next, the expression of tendo/ligamentogenesis-related genes was analyzed in periodontal ligament-derived cells under osteogenic induction at passages 1, 3 and 6. Expression of *scleraxis* was detected in the cells at passages 1, 3 and 6. Conversely, gene expression of *TeM* was absent in the

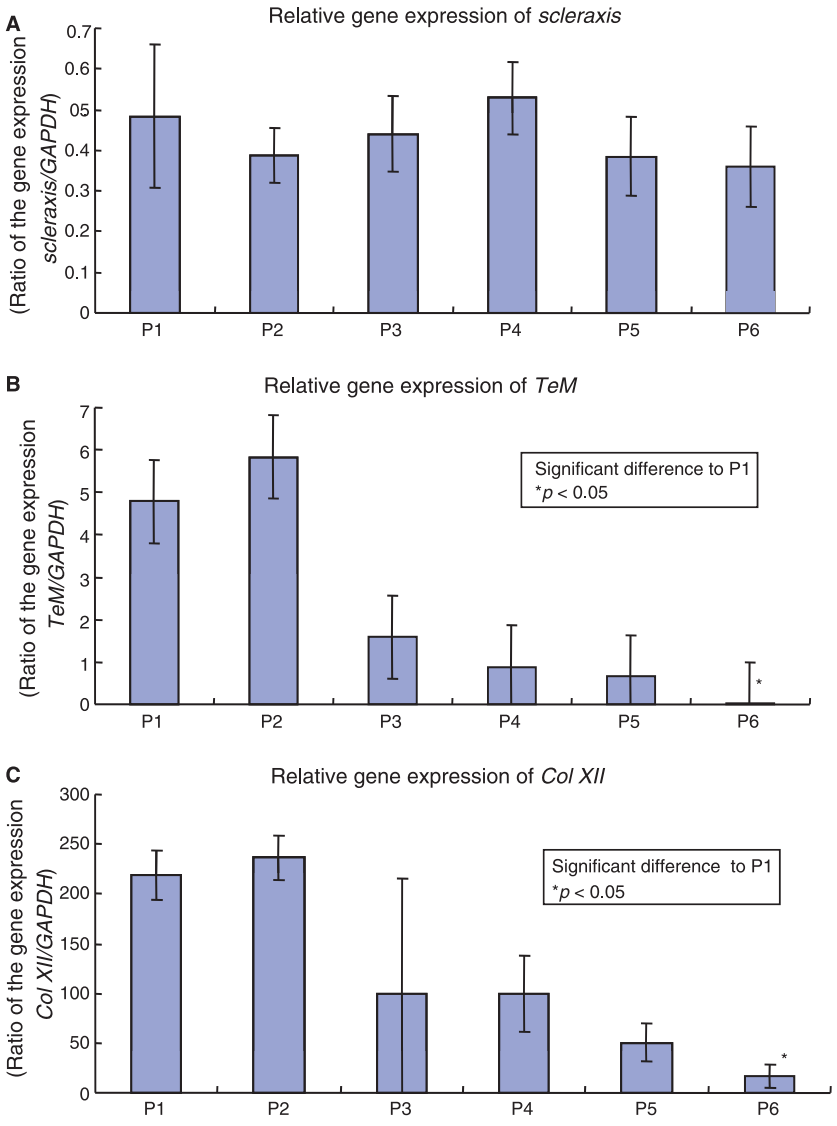

Fig. 3. Real-time quantitative polymerase chain reaction analyses of *scleraxis*, *TeM* and *Col XII* at passages 1, 2, 3, 4, 5 and 6. Expression of *TeM* and *Col XII* decreased at passage 3 and statistically significant differences were observed between the samples at passages 1 and 6 in the expression of *TeM* and *Col XII* ($p < 0.05$). Expression of *scleraxis* did not show a significant difference among any of the groups. P1, passage 1; P2, passage 2; P3, passage 3; P4, passage 4; P5, passage 5; P6, passage 6.

cells under osteogenic induction, even at passage 1. Expression of *Col XII* was detectable in the cells at passage 1 under osteogenic induction and showed a marked decrease at passage 3, which was almost identical to that in the IND(−) group (Fig. 4).

Alkaline phosphatase activity

To investigate the effects of passage on osteogenic capability, a time-course of alkaline phosphatase activity at passages 1, 3 and 6 was measured. Alkaline phosphatase activity clearly decreased as the passage number increased, and a statistically significant difference was observed in alkaline phosphatase activity at 3, 7 and 14 days of passage 6 ($p < 0.05$) compared with that at passage 1 (Fig. 5).

Transplantation into the renal subcapsular space

To investigate the function of periodontal ligament-derived cells and their changes during passage, periodontal ligament-derived cells at passages 1 or 6 were seeded onto the surface of a flattened human tooth dentin sheet and then transplanted into the renal subcapsular space of immunocompromised rats. Six weeks after transplantation, a thin, flat, fibrous tissue was observed on the surface of the dentin seeded with passage 1 cells (Fig. 6C–F), which was not found in the control group without periodontal ligament-derived cells (Fig. 6A,B). The fibrous tissue consisted of dense collagen fibers, but hard tissue, such as cementum or bone-like tissue, was not observed. When periodontal ligament-derived cells at passage 6 were transplanted, an almost identical structure was observed, but again cementum or bone-like tissue was not found (data now shown). Hard tissue formation was not observed when induced periodontal ligament-derived cells at passage 1 (Fig. 6G,H) and passage 6 were transplanted (data now shown).

Discussion

In this study, the characteristics of periodontal ligament-derived cells were analyzed, with an emphasis on the changes observed during passage. Most importantly, these cells constantly expressed both osteogenic/cementogenesis and tendo/ligamentogenesis-related genes. Regeneration of periodontal tissue is a complex process that includes the simultaneous regeneration of both hard and soft tissue. Periodontal ligament-derived cells are unique as they express genes involved in both hard and soft tissue regeneration, which is beneficial for periodontal ligament tissue engineering and suggests that the function of these cells cannot be completely replaced by other cell types.

On the other hand, the availability of periodontal ligament for tissue engineering is despairingly limited. Teeth extracted as a result of periodontal disease cannot be used because of the lack of healthy periodontal ligament and also because of the possible risk of bacterial contamination. Even healthy extracted teeth, such as third molars, may retain only a limited amount of periodontal ligament tissue.

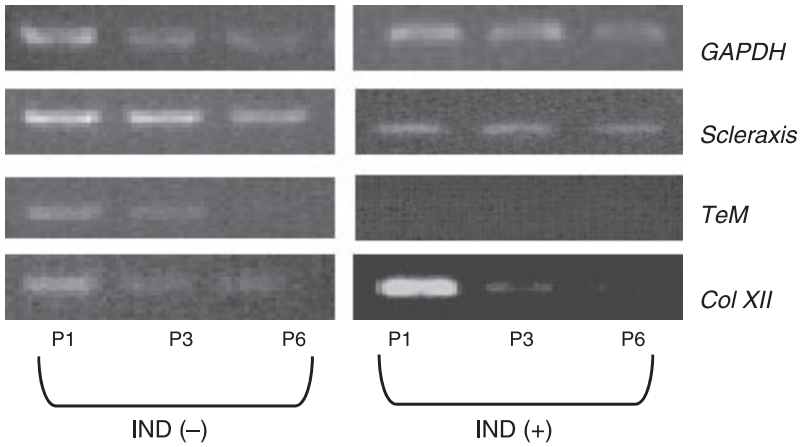

Fig. 4. Expression of tendo/ligamentogenesis-related genes by periodontal ligament-derived cells under osteogenic induction [Ind(+)] at passages 1, 3 and 6. Expression of *scleraxis* was observed at all passages examined. On the other hand, expression of *TeM* diminished, and almost disappeared, even at passage 1, under osteogenic induction. Expression of *Col XII* under osteogenic induction also diminished after passage 3 but was still detectable, even at passage 6, in both IND(−) and IND(+) groups. P1, passage 1; P3, passage 3; P6, passage 6.

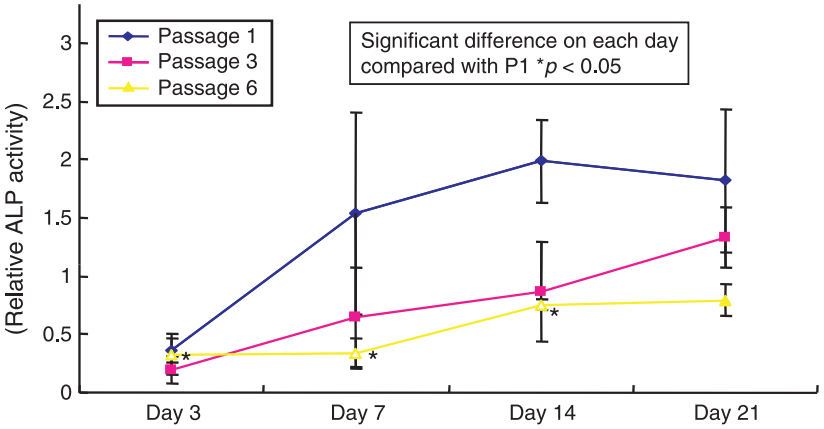

Fig. 5. Time-course of alkaline phosphatase activity of periodontal ligament-derived cells at passages 1, 3 and 6. Alkaline phosphatase activity gradually decreased as the passage number increased and a statistically significant difference was observed between passage-1 and passage-6 cells at 3, 7 and 14 days ($p < 0.05$).

In reality, there is not enough tissue available for selective cell culture, which may explain why nonselected periodontal ligament-derived cells have been used for the purpose of periodontal tissue engineering (3–5).

To use primary cultured periodontal ligament-derived cells for tissue engineering, the initial question that arises is heterogeneity. In this study, we cultured periodontal ligament-derived cells until passage 6. At the initial stages of primary culture, we observed at least two morphologically distinct populations (i.e. spindle-shaped cells, which grew rapidly, and more broad-shaped cells that grew relatively slowly), as described previously (11,12). During culture, both cell populations seemed to converge into almost a single population of spindle-shaped cells. Considering the fact that the spindle-shaped cells grew rapidly, it is not surprising that the fastest growing cells became dominant.

Human periodontal ligament tissue has been reported to contain STRO-1-positive cells. However, the ratio of STRO-1-positive cells varies (6,13). Fluorescence-activated cell sorter analysis of isolated human periodontal ligament cells showed 27% STRO-1-positive cells (6). By contrast, cultured human periodontal ligament cells were reported to contain only 1.2% STRO-1-positive cells, although 30% of the single-cell colonies displayed positive staining for STRO-1 (13). In this study, 33.5% of primary cultured cells were positive for STRO-1 when the cells were confluent, which was reduced to 14.7% by the third passage. The ratio of STRO-1-positive cells in this study was larger than that reported for cultured periodontal ligament cells (13), but smaller than that for isolated cells (6). The reason for this discrepancy is not clear. However, it has been reported that the ratio of STRO-1-positive cells may change during culture (13) as differentiation may reduce STRO-1 expression (7). Furthermore, the different techniques used (i.e. fluorescence-activated cell sorting and immunofluorescent analyses) have differential sensitivities. In this study, we used confluent cells, which may also affect STRO-1 expression.

It was our aim to investigate the characteristic changes of periodontal ligament-derived cells during passage. The results showed a remarkable change in the expression of the tendo/ligamentogenesis-related genes, *TeM and Col XII*, which were down-regulated as the passage number increased. *TeM and Col XII* gene expression rapidly decreased from passage 2 onwards, which was confirmed by real-time quantitative PCR. This finding may suggest the tendency of periodontal ligament-derived cells to dedifferentiate during passage, which might be more apparent for tendo/ligamentogenesis-related genes than for osteogenic genes. Interestingly, *scleraxis*, which is also a tendo/ligamentogenesis marker, was expressed at every passage. *TeM* is a late marker of tendon formation, which may be affected more severely by dedifferentiation during passage than an early marker, such as *scleraxis* (14,15). This finding was also supported by the gene expression of *Col XII* (also a late ligament marker), which decreased after

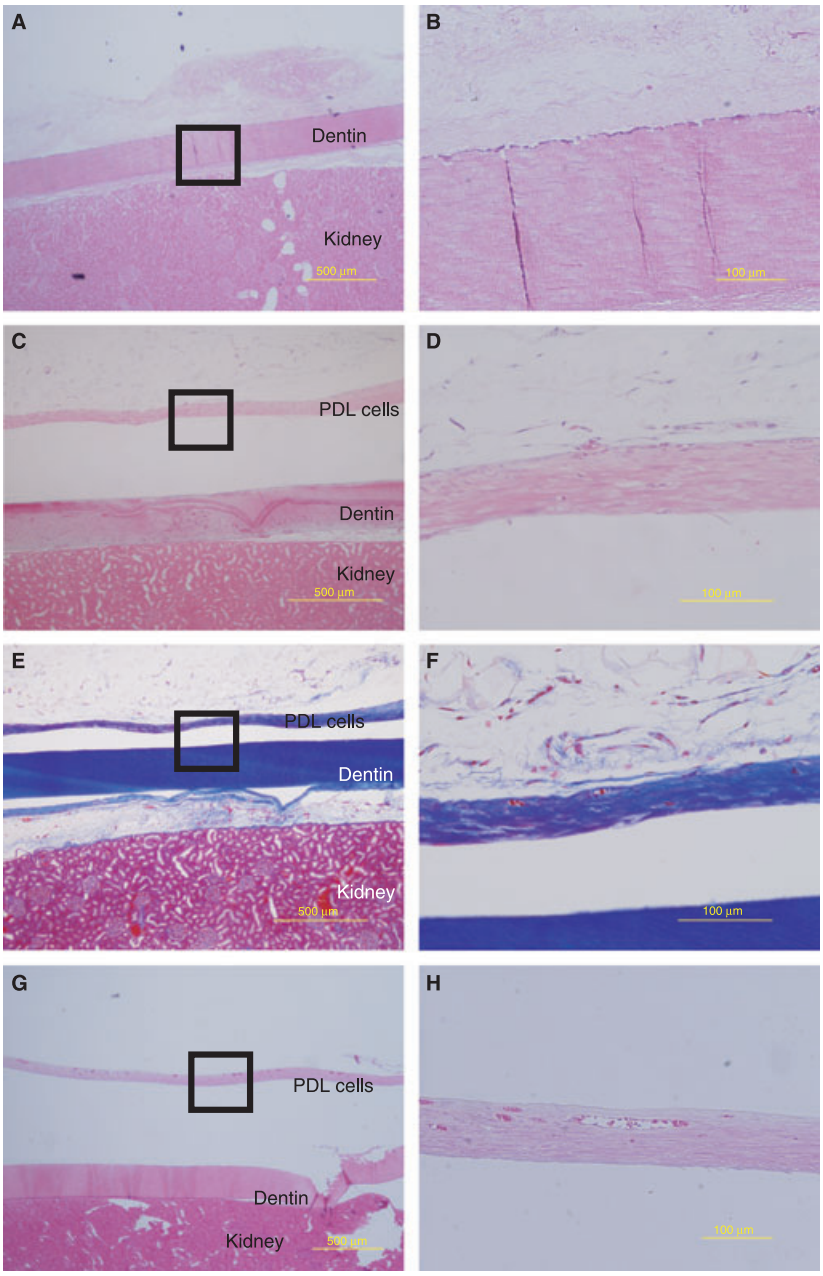

Fig. 6. Bright-field photomicrographs showing transplanted periodontal ligament-derived cells with dentin after 6 weeks. (A) Hematoxylin and eosin-stained section from the control group without cells. (B) A higher magnification of panel A. (C) Hematoxylin and eosin-stained section from the transplanted periodontal ligament-derived cells (passage 1) with dentin. (D) A higher magnification of panel C. Transplanted cells produced dense collagen fibers, but hard tissue such as cementum was not observed. (E, F) Azan-stained sections of the transplanted periodontal ligament-derived cells with dentin. (C–F) Sections from transplanted periodontal ligament-derived cells without osteogenic induction. (G, H) Sections with osteogenic induction of the cells at passage 1. Similarly to noninduced cells, induced cells did not form bone or cementum-like tissue. PDL, periodontal ligament.

passage 3. However, the tendency of dedifferentiation during passage was also confirmed by measuring alkaline phosphatase activity, which gradually decreased as passage number increased. Alkaline phosphatase is a known osteogenic marker and thus the osteogenic capability may also be reduced during passage of periodontal ligament-derived cells.

Another interesting finding of this study was a possible regulatory mechanism of tendo/ligamentogenic genes and their relationship with osteogenic induction. Periodontal ligament-derived cells do not usually differentiate into osteoblasts, which might be a mechanism used to maintain the periodontal ligament space. However, it is not known if expression of tendo/ligamentogenesis-related genes is affected by osteogenic induction. The results from this study showed the possibility that osteogenic induction can down-regulate tendo/ligamentogenic genes. *TeM* and *Col XII* are both known as late ligament markers. On the other hand, the difference after osteogenic induction was more clearly observed in *TeM*, which may suggest a specific regulatory role of *TeM* during periodontal tissue development/regeneration. Interestingly, the C-terminal domain of *TeM* exhibits some biological activity, such as anti-angiogenic and antitumor activities, when expressed in a secreted form (16). It will be interesting to investigate whether the forced expression of tendo/ligamentogenesis-related genes, such as *TeM*, can down-regulate the expression of osteogenesis-related genes, which might be a potential reciprocal mechanism of osteogenesis and tendo/ligamentogenesis-related gene regulation in the periodontal ligament. Further studies will be required to elucidate detailed regulatory mechanisms of osteogenic- and tendo/ligamento genesis-related genes.

Although periodontal ligament-derived cells showed expression of osteogenic marker genes, except for *BSP*, transplanted cells did not show any hard tissue formation such as of a cementum/periodontal ligament-like structure. Several studies have shown contrary findings and reported the formation of hard tissue besides dense periodontal ligament-like tissue (12,17). This discrepancy might be a result of the difference of cells used for transplantation. Those studies used cloned/selected cell population, while the cells used in this study were nonselected dominant cells from

periodontal ligament. Species and graft site may also affect the formation of hard tissue. Furthermore, it is conceivable that the osteogenic potential of periodontal ligament-derived cells is tightly regulated and might be suppressed by some genes specific to periodontal ligament-derived cells. It has been reported that *Msx2* is a potential candidate for inhibiting osteogenesis in periodontal ligament (18) and a more complex mechanism could exist for the regulation of periodontal ligament-derived cells. It will be important to investigate the detailed regulatory mechanisms of osteogenic/cementogenic induction, as well as the tendo/ligamentogenic induction of periodontal ligament-derived cells. This could lead to a more sophisticated methodology for periodontal tissue engineering. Further studies using cloned cells under stricter settings would also be beneficial.

In this study, we have shown the characteristics of primary cultured periodontal ligament-derived cells and their changes during passage. The results suggest the usefulness of primary cultured, nonselected, periodontal ligament-derived cells for tissue engineering, especially in their earlier passages. A basic understanding of periodontal ligament-derived cells is indispensable for successful periodontal tissue engineering. As partly shown in this study, a more detailed understanding of the regulatory mechanism of osteogenic and tendo/ligamentogenic genes in periodontal ligament-derived cells is an important future research target.

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