Investigation of multipotent postnatal stem cells from human periodontal ligament

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Summary

Background **Periodontal diseases that lead to the destruction of periodontal tissues—including periodontal ligament (PDL), cementum, and bone—are a major cause of tooth loss in adults and are a substantial public-health burden worldwide. PDL is a specialised connective tissue that connects cementum and alveolar bone to maintain and support teeth in situ and preserve tissue homoeostasis. We investigated the notion that human PDL contains stem cells that could be used to regenerate periodontal tissue.**

Methods **PDL tissue was obtained from 25 surgically extracted human third molars and used to isolate PDL stem cells (PDLSCs) by single-colony selection and magnetic activated cell sorting. Immunohistochemical staining, RT-PCR, and northern and western blot analyses were used to identify putative stem-cell markers. Human PDLSCs were transplanted into immunocompromised mice (n=12) and rats (n=6) to assess capacity for tissue regeneration and periodontal repair.**

Findings **PDLSCs expressed the mesenchymal stem-cell markers STRO-1 and CD146/MUC18. Under defined culture conditions, PDLSCs differentiated into cementoblast-like cells, adipocytes, and collagen-forming cells. When transplanted into immunocompromised rodents, PDLSCs showed the capacity to generate a cementum/PDL-like structure and contribute to periodontal tissue repair.**

Interpretation **Our findings suggest that PDL contains stem cells that have the potential to generate cementum/PDL-like tissue in vivo. Transplantation of these cells, which can be obtained from an easily accessible tissue resource and expanded ex vivo, might hold promise as a therapeutic approach for reconstruction of tissues destroyed by periodontal diseases.**

Introduction

The periodontal ligament (PDL) is a soft connective tissue embedded between the cementum (a thin layer of mineralised tissue covering the roots of the teeth) and the inner wall of the alveolar bone socket, to sustain and help constrain teeth within the jaw. PDL not only has an important role in supporting teeth, but also contributes to tooth nutrition, homoeostasis, and repair of damaged tissue.[1–3] PDL contains heterogeneous cell populations[4,5] that can differentiate into either cementum-forming cells (cementoblasts) or bone-forming cells (osteoblasts).[6–9] Recent findings suggest that PDL cells have many osteoblast-like properties, including the capacity to form mineralised nodules in vitro, expression of the bone-associated markers alkaline phosphatase and bone sialoprotein, and response to bone-inductive factors such as parathyroid hormone, insulin-like growth factor 1, bone morphogenetic protein 2, and transforming growth factor β1.[2,4,10–14] The presence of multiple cell types within PDL has led to speculation that this tissue might contain progenitor cells that maintain tissue homoeostasis and re-generation of periodontal tissue.[6,15–17] However, to date, there is no direct evidence that a putative stem-cell population exists within PDL.

Periodontal diseases are infectious diseases that are characterised by destruction of periodontium (supporting tissue for tooth) including PDL, cementum, alveolar bone, and gingiva. Periodontal diseases are the main cause of tooth loss and are a substantial public health burden worldwide.[18,19] The reconstruction of healthy periodontium destroyed by periodontal diseases is a major goal of periodontal therapy. On the basis of recent advances in postnatal stem-cell biology, we postulated that PDL might contain multipotent stem cells that could be used to generate cementum and periodontal ligament in vivo. We report the isolation and characterisation of a unique stem-cell population from PDL tissue.

Methods

Samples and cell culture

Normal impacted third molars (n=25) were collected from 16 individuals aged 19–29 years at the Dental Clinic of the National Institute of Dental and Craniofacial Research, USA, following approved guidelines set by the National Institutes of Health Office of Human Subjects Research. PDL was gently separated from the surface of the root and then digested in a solution of 3 mg/mL collagenase type I (Worthington Biochem, Freehold, NJ, USA) and 4 mg/mL dispase (Roche, Mannheim, Germany) for 1 h at 37°C. PDL samples from different individuals were pooled and single-cell suspensions were obtained by passing the cells through a 70 μm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA).

To identify putative stem cells, single-cell suspensions (1×10^4 cells) were seeded into 10-cm culture dishes (Costar, Cambridge, MA, USA) with alpha-modification of Eagle's medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 15% fetal calf serum (Equitech-Bio Inc, Kerrville, TX, USA), 100 μmol/L ascorbic acid 2-phosphate (WAKO, Tokyo, Japan), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Biofluids, Rockville, MD, USA), and then incubated at 37°C in 5% carbon dioxide.

To assess colony-forming efficiency, day 10 cultures were fixed with 4% formalin, and then stained with 0·1% toluidine blue. Aggregates of 50 or more cells were scored as colonies. The proliferation rate of sub-confluent cultures (first passage) of stem cells was assessed by bromodeoxyuridine incorporation for 24 h, with a Zymed Laboratories BrdU staining kit (Vector Laboratories, Burlingame, CA, USA). Calcium accumulation was induced as reported previously,[20] and was detected by staining with 2% alizarin red S (pH 4·2). Calcium concentration was measured with a Sigma calcium kit 587-A (Sigma Diagnostics, St Louis, MO, USA). Induction of adipogenesis was done as previously reported.[21]

Dental pulp stem cells (DPSCs) and bone marrow stromal stem cells (BMSSCs) were isolated and cultured as previously described.[21–23] In some experiments, PDL stem cells (PDLSCs) and DPSCs were obtained from the same donor or donors. BMSSCs were obtained from a commercially available resource (AllCells LLC, Berkeley, CA, USA). All primary cells used in this study were at 2–4 passages. For each experiment, the same passage of PDLSCs, DPSCs, and BMSSCs was used.

Antibodies

Rabbit antibodies used included anti-HSP90 and TGFβRI (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-human-specific mitochondria (Chemicon, Temecula, CA, USA); and anti-alkaline phosphatase (LF-47), anti-osteocalcin (LF-32), anti-matrix extracellular protein (LF-155), and anti-type I collagen (LF-67) from Larry Fisher at the National Institute of Dental and Craniofacial Research, National Institutes of Health.[20] Mouse antibodies included anti-bone sialoprotein (LF-25, from Larry Fisher);[20] anti-CD146/MUC18, and anti-STRO-1.[23] Rabbit and mouse isotype-matched negative control antibodies were obtained from Caltag Laboratories (Burlingame, CA, USA).

RT-PCR

The primers used in RT-PCR included PPARγ2, LPL, and GAPDH (panel). Total RNA isolation, first-strand cDNA synthesis, and PCR processes were done as described previously.[24]

Northern blot analysis

Total RNA (15 μg) from primary PDLSC, DPSC, and BMSSC cultures was electrophoresed and then transferred to a nylon membrane. Probe was generated from purified PCR products with scleraxis primers (panel) by random labelling with (γ-^{32}P) deoxycytidine triphosphate (Dupont New England Nucleotide) by use of the Stratagene Prime It II labelling kit (Stratagene). After prehybridisation in QuickHyb hybridisation solution (Stratagene) at 68°C for 15 min, the filters were hybridised with scleraxis probe at 68°C for 1 h. The filters were washed twice in 2×standard sodium chloride and sodium citrate solution, 0·1% (weight per volume) SDS for 15 min at room temperature, followed by one wash in 0·1% standard sodium chloride and sodium citrate solution and 0·1% (weight per volume) SDS at 68°C for 30 min. The membranes were then exposed to a PhosphoImager cassette (Amersham Bioscience, Sunnyvale, CA, USA) for 16–72 h.

Immunohistochemistry

PDLSCs were subcultured into 8-chamber slides (2×10^4 cells/well, NUNC, Naperville, IL, USA). The cells were fixed in 4% paraformaldehyde for 15 min and then blocked and incubated with primary antibodies (at dilutions ranging from 1:200 to 1:500) for 1 h. The samples were subsequently incubated with goat secondary antibodies of either IgG-rhodamine red or IgG-Cy2 (Jackson ImmunoResearch, West Grove, PA, USA), for 45 min. Isotype-matched control antibodies were used under the same conditions. For enzymatic immunohistochemical staining, the Zymed broad spectrum immunoperoxidase AEC kit (Zymed Laboratories, South San Francisco, CA, USA) was used according to the manufacturer's protocol.

Western blot

Primary antibodies used for western blot were the same as those used in immunohistochemical staining at dilutions ranging from 1:200 to 1:1000. Western blot analyses were carried out as previously reported.[20]

***Panel:* Primers**

PPARγ2
Sense 5′-CTCCTATTGACCCAGAAAGC-3′ (114–133),
Antisense 5′-GTAGAGCTGAGTCTTCTCAG-3′ (441–460)
GenBank accession number: AY157024

LPL
Sense 5′-ATGGAGAGCAAAGCCCTGCTC-3′ (175–195),
Antisense 5′-GTTAGGTCCAGCTGGATCGAG-3′ (718–738)
GenBank accession number: NM_000237

GAPDH
Sense 5′-AGCCGCATCTTCTTTTGCGTC-3′ (12–32)
Antisense 5′-TCATATTTGGCAGGTTTTTCT-3′ (807–827)
GenBank accession number: M33197

Primer for northern blot probe
Sense 5′-CTGGCCTCCAGCTACATCTC-3′ (900–919)
Antisense 5′-CTTTCTCTGGTTGCTGAGGC-3′ (1090–1109)
GenBank accession number: BK000280

Immunomagnetic isolation

This procedure has been reported previously.[23] Briefly, single-cell suspensions of PDLSCs were incubated with STRO-1 supernatant (mouse anti-human BMSSCs, IgM) for 1 h on ice. The cells were then washed with phosphate buffered saline containing 1% bovine serum albumin, and resuspended with rat anti-mouse IgM-conjugated Dynal beads at four beads per cell (Dynal, Oslo, Norway) for 45 min on a rotary mixer at 4°C. Bead-positive cells were isolated with a Dynal MPC-1 magnetic particle concentrator according to the manufacturer's recommendations.

Transplantation

About $4{\cdot}0\times10^6$ of in-vitro expanded PDLSCs were transplanted subcutaneously into the dorsal surfaces of 12 10-week-old immunocompromised beige mice (NIH-bg-nu-xid, Harlan Sprague Dawley, Indianapolis, IN, USA) as previously described.[21,22] The same number of in-vitro expanded DPSCs and BMSSCs were used as controls. These procedures were done in accord with specifications of an approved animal protocol (NIDCR #02–222).

PDLSCs were transplanted into the periodontal area in six immunodeficient rats as described previously.[25] Briefly, $2{\cdot}0\times10^6$ PDLSCs were mixed with 40 mg of hydroxyapatite/tricalcium phosphate ceramic particles (Zimmer, Warsaw, IN, USA) and transplanted into two 2 mm^2 periodontal defects that had been surgically created on the buccal cortex of the mandibular molar in the rats (NIH-rnu, Taconic, Germantown, NY, USA). These procedures were done in accord with specifications of an approved small-animal protocol (NIDCR #03–264). The transplants were recovered at 6–8 weeks post-transplantation, fixed with 4% formalin, decalcified with buffered 10% edetic acid (pH 8·0), and then embedded in paraffin. Sections were deparaffinised and stained with haematoxylin and eosin.

Statistical analysis

Wilcoxon rank-sum test was used to analyse the significance between the two groups. p values of less than 0·05 were judged to be statistically significant.

Role of the funding source

The sponsors had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

To identify putative stem cells, single-cell suspensions were generated from human PDL (figure 1A and 1B). The ability of PDL-derived cells to form adherent clonogenic cell clusters of fibroblast-like cells, similar to those recorded for different mesenchymal stem-cell populations, was shown by the formation of about 170 single colonies (figure 1C), generated from 10^5 single cells cultured at low density (figure 1I). This colony-forming cell population, which we termed periodontal ligament stem cells (PDLSCs), had a high uptake rate of bromodeoxyuridine, similar to the rate seen with DPSCs (figure 1D).

Ex-vivo expanded PDLSCs expressed the cell surface molecules STRO-1 and CD146/MUC18, two early mesenchymal stem-cell markers also present on BMSSCs and DPSCs (figure 1E and 1F). STRO-1-positive cells were also shown to be located in the PDL tissue by immunohistochemical staining (figure 1G and 1H). When anti-STRO-1 antibody was used to isolate PDLSCs released from freshly collected PDL tissue, most colony-forming cells were found to be contained within the STRO-1-positive cell population, confirming STRO-1 as an early progenitor marker for PDLSCs (figure 1I).

We assessed the expression level of scleraxis, a tendon-specific transcription factor, in PDLSCs, since PDL is similar to tendon with respect to its dense collagen fibre structure and its ability to absorb mechanical stress during normal physiological activity. PDLSCs expressed a

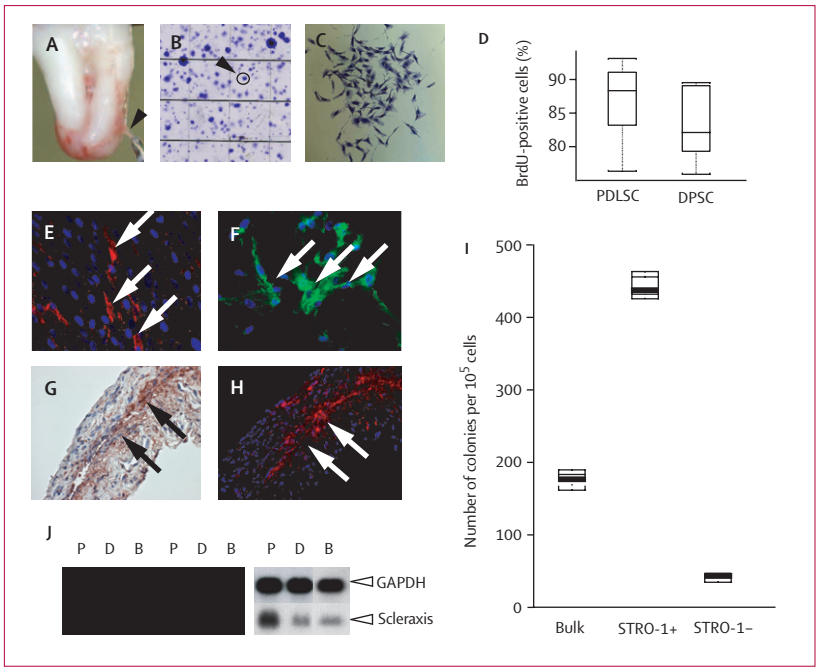

Figure 1: **Isolation of adult human PDLSCs**

(A) Extracted human third molar showing PDL attached to the surface of the roots (arrow). (B) Single colonies formed after PDLSCs were plated at low density and cultured as described in Methods. (C) Cell clusters derived from PDL formed a single colony stained with 0·1% toluidine blue. (D) Bromodeoxyuridine (BrdU) labelling efficiency of PDLSCs and DPSCs were assessed by BrdU incorporation for 24 h. The number of BrdU-positive cells was expressed as a percentage of total number of cells counted from six replicate cultures as shown in boxplot. PDLSCs showed a higher uptake rate than did DPSCs, but there was no significant statistical difference (p=0·294). Horizontal lines are median values. Bars show maximum and minimum values. (E–F) Immunocytochemical staining showed that cultured PDLSCs were expressed STRO-1 (E) and CD146/MUC18 (F), two early mesenchymal progenitor markers. (G–H) PDL tissue was positive for STRO-1 antibody with immunohistochemical (G) and fluorescence (H) staining. (I) Freshly isolated single-cell suspensions of human PDL reacted with the STRO-1 antibody after immunoselection with magnetic Dynal beads as described in the Methods. Clonogenic assays were subsequently done with unfractionated (bulk), STRO-1 negative (STRO-1–) and STRO-1 positive (STRO-1+) cell fractions. Data obtained from five individual PDL samples are shown in boxplot. Highlighted horizontal lines in boxplots are median values. (J) RT-PCR (left) and northern blot analysis (right) showed that cultured PDLSCs (P) expressed higher levels of scleraxis, a transcription factor specifically expressed in tendon cells, compared with DPSCs (D) and BMSSCs (B) . GAPDH=glyceraldehyde phosphate dehydrogenase (control).

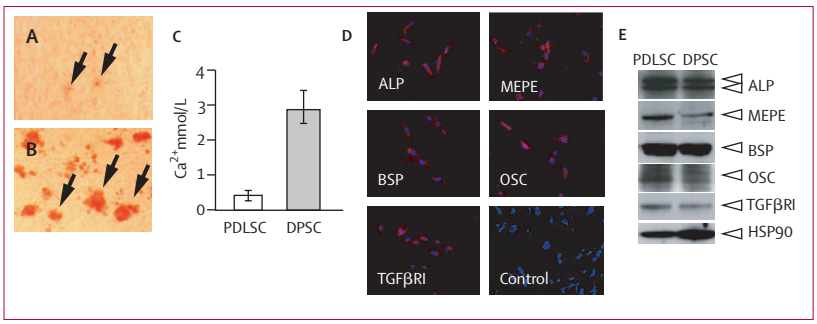

Figure 2: **Expression of cementoblastic/osteoblastic phenotype by PDLSCs**
Alizarin red staining showed limited amounts of mineralised nodule formation in PDLSC cultures (A). Compared with induced DPSC cultures (B), PDLSC cultures accumulated smaller amounts of calcium than did DPSCs (p=0·0026, C). (D) Immunocytochemical staining showed that PDLSCs expressed cementoblastic/osteoblastic markers, including alkaline phosphatase (ALP), matrix extracellular protein (MEPE), bone sialoprotein (BSP), osteocalcin (OSC), and TGFβ receptor type I (TGFβR1). (E) Western blot analysis confirmed the expression of the cementoblastic/ osteoblastic markers, HSP90 was used to assess the amount of protein loaded per sample.

higher level of scleraxis than did BMSSCs and DPSCs (figure 1J), suggesting that PDLSCs might belong to a unique population of postnatal mesenchymal stem cells.

To investigate the potential of PDLSCs to undergo cementoblastic/osteoblastic differentiation, established secondary PDLSC cultures were supplemented with L-ascorbate-2-phosphate, dexamethasone, and inorganic phosphate to induce mineralisation in vitro as previously described.[21] Small round alizarin red-positive nodules formed in the PDLSC cultures after 4 weeks of induction, indicating calcium accumulation in vitro (figure 2A). However, compared with DPSCs, PDLSCs formed fewer mineralised nodules, which correlated with lower concentrations of calcium in the extracellular matrix (figure 2A–2C). Immunohistochemical staining (figure 2D) and western blot analysis (figure 2E) showed that cultured PDLSCs expressed an array of cementoblastic/osteoblastic markers, including alkaline phosphatase, MEPE, bone sialoprotein, osteocalcin, and TGFβ receptor type I.

We assessed whether PDLSCs, like DPSCs, had the potential to differentiate into other cell lineages such as adipocytes. After 3 weeks of culture with an adipogenic inductive cocktail, PDLSCs developed into oil red O-positive lipid-laden fat cells (figure 3A and 3B). This development correlated with an upregulation in the expression of two adipocyte specific transcripts, PPARγ2 and lipoprotein lipase, as detected by RT-PCR (figure 3C).

To validate the capacity of PDLSCs to differentiate into functional cementoblast-like cells, ex-vivo-expanded PDLSCs were transplanted into immunocompromised mice. A typical cementum/PDL-like structure was regenerated, in which a thin layer of cementum-like tissue formed on the surface of the carrier, along with condensed collagen fibres with sparse cells that resembled PDL structures (figure 4A). The cemetum/PDL-like structures appeared totally different from typical bone/marrow structures generated by BMSSCs and dentin/pulp-like structures generated by DPSCs (figure 4B and 4C). These findings showed the difference in capacity for tissue regeneration between PDLSC and BMSSC/DPSC in vivo. The PDLSC transplants contained human-specific mitochondria-positive cementum-forming cells and a PDL-like structure containing human PDLSCs as well as recipient cells (figure 4D). Of 13 single-colony-derived PDLSC clones transplanted into immunocompromised mice, eight (61%) showed a capacity to form a cementum/PDL-like tissue, equivalent to multicolony-derived PDLSCs (figure 4E). The remaining five clones did not form cementum/PDL-like tissues (figure 4F).

Transplanted human PDLSCs were able to form a dense type I collagen-positive PDL-like tissue within the transplants (figure 5A to 5D). More importantly, collagen fibres generated in vivo were able to connect with newly formed cementum-like structures that mimicked physiological attachment of Sharpey's fibre (figure 5E), which is needed to form functional attachment of cementum/PDL structures. These results infer that PDLSCs might contain a subpopulation of cells capable of differentiating into cementoblasts/cementocytes and collagen-forming cells in vivo. Human PDLSCs were responsible for collagen fibre formation within the transplants, as shown by the reactivity of these cells with human-specific anti-mitochondria antibody (figure 5F to 5H).

To assess whether PDLSCs were able to contribute to periodontal tissue repair, we transplanted human PDLSCs into surgically created defects at the periodontal area of mandibular molars in immunocompromised rats. Transplanted human PDLSCs integrated into the PDL compartment in two of six examined samples (figure 6A), and occasionally attached to surfaces of alveolar bone and teeth (figure 6B and 6C), dependent on the areas we examined. These findings imply a potential functional role of human PDLSCs for periodontal tissue regeneration.

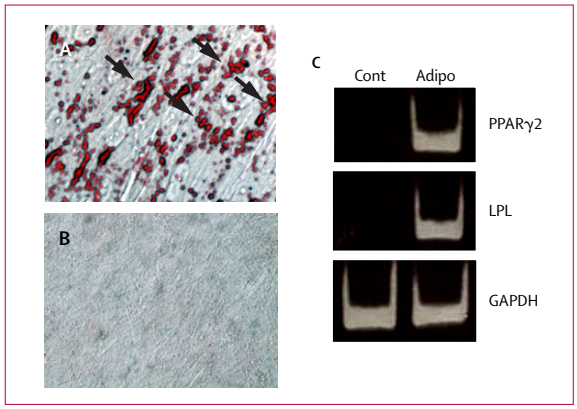

Figure 3: **Adipogenic differentiation of PDLSCs**
(A) Cultured PDLSCs formed oil red O positive lipid clusters after 3 weeks of induction in the presence of 0·5 mM isobutylmethylxanthine, 0·5 μM hydrocortisone, and 60 μM indomethacin. (B) Standard culture medium did not induce any oil red O positive lipid clusters. (C) Substantial upregulation of adipogenic markers PPARγ2 and lipoprotein lipase (LPL), was observed in the group induced with the adipogenic cocktail (Adipo) compared with the control group (Cont) by RT-PCR.

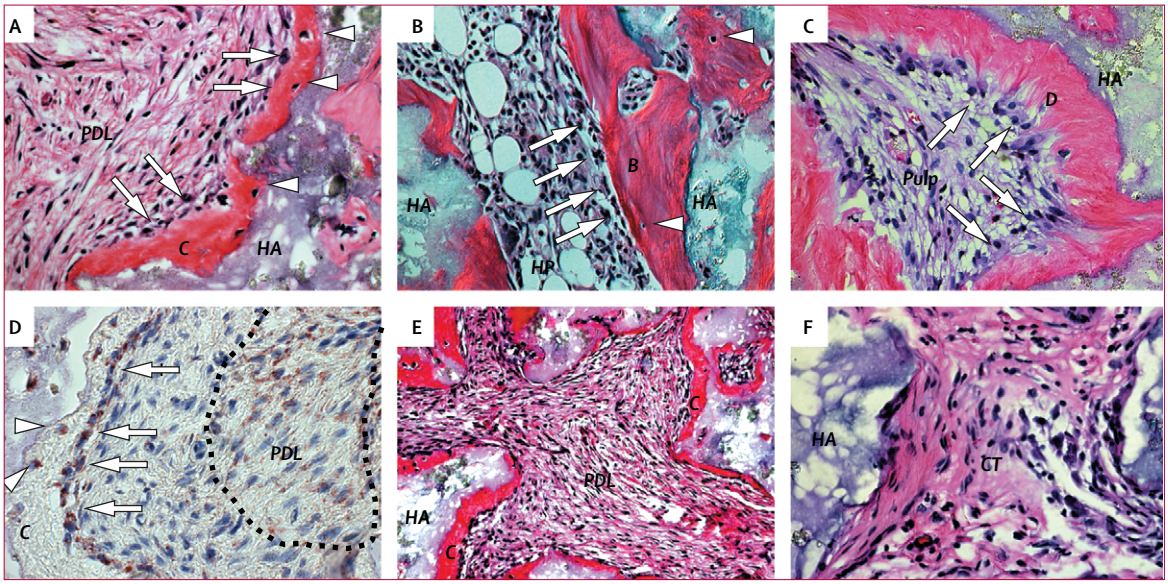

Figure 4: **Generation of cementum-like and PDL-like structures in vivo by PDLSCs**
(A) After 8 weeks of transplantation, PDLSCs differentiated into cementoblast-like cells (arrows) that formed a cementum-like structure (*C*) on the surface of the hydroxyapatite tricalcium phosphate (*HA*) carrier; cementocyte-like cells (triangles) and PDL-like tissue (*PDL*) were also generated. (B) BMSSC transplant was used as control to show the formation of a bone/marrow structure containing osteoblasts (arrows), osteocytes (triangles), and elements of bone (*B*) and haemopoietic marrow (*HP*). (C) DPSC transplant was also used as a control to show a dentin/pulp-like structure containing odontoblasts (arrows) and dentin-like (*D*) and pulp-like (*Pulp*) tissue. (D) Immunohistochemical staining showed that PDLSCs generated cementum-like structure (*C*) and differentiated into cementoblast-like cells (arrows) and cementocyte-like cells (triangles) that stained positive for human-specific mitochondria antibody. Part of the PDL-like tissue (*PDL*) also stained positive for human specific mitochondria antibody (within dashed line). (E) Of 13 selected strains of single-colony derived PDLSC, only eight (61%) generated cementum/PDL-like structures in vivo as shown at lower magnification (approximately ×20). New cementum-like structure (*C*) formed adjacent to the surfaces of the carrier (*HA*) and associated with PDL-like tissue (*PDL*). (F) The other five strains did not generate mineralised or PDL-like tissues in vivo.

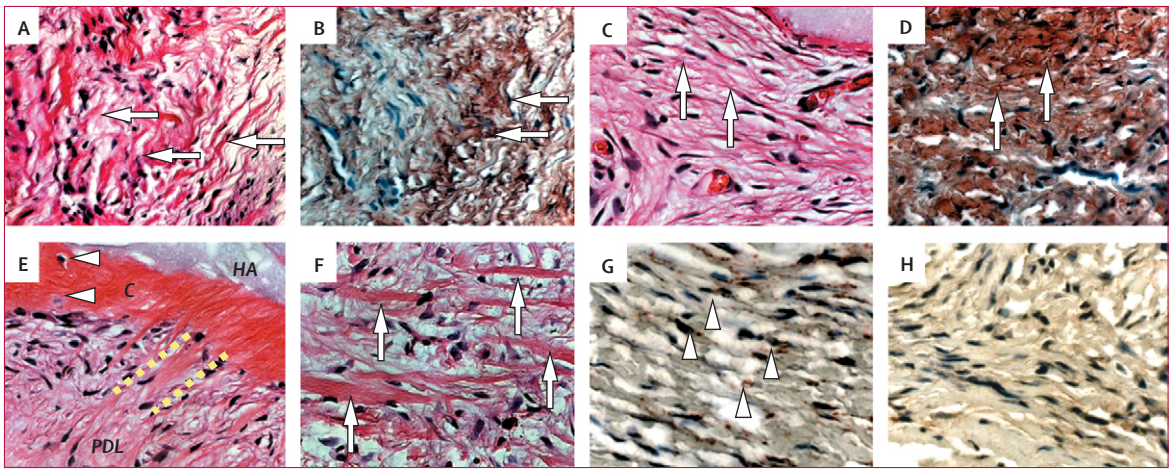

Figure 5: **Generation of collagen fibres by PDLSCs in vivo**
(A) Haematoxylin and eosin staining of human PDL tissue showing collagen fibres (arrows). (B) Collagen fibres of human PDL were positive for anti-type I collagen antibody staining (arrows). (C) Transplanted PDLSCs generated collagen fibres (arrows) along with the newly formed cementum-like structure (*C*). (D) These fibres were positive for anti-type I collagen antibody staining (arrows), similar to human PDL. (E) Transplanted PDLSCs formed cementum-like structures (*C*) that connected to newly formed collagen fibres (yellow dashed lines), similar to the structure of Sharpey's fibre. (F) Transplanted PDLSCs generated a substantial amount of collagen fibres (arrows). (G) These collagen fibres were positive for anti-human specific mitochondria antibody staining (triangles). (H) Pre-immunoserum was used as a negative control of PDLSC transplant for anti-human specific mitochondria antibody.

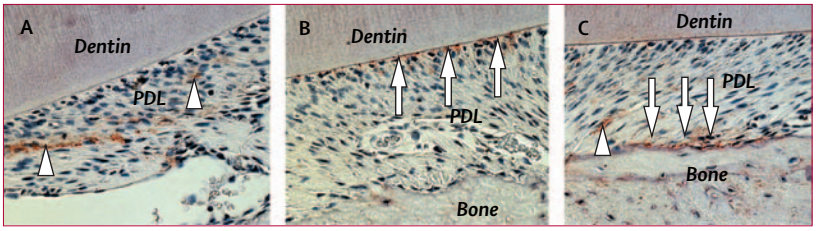

Figure 6: **PDLSCs in periodontal tissue repair in immunocompromised rats**
Immunohistochemical staining of recovered transplant tissue with human-specific anti-mitochondria antibody showed that human PDLSCs: (A) were located in the PDL compartment (triangles), (B) were involved in the attachment of PDL to the tooth surface (arrows), and (C) participated in repair of alveolar bone (arrows) and PDL (triangle).

Discussion

From the aspect of tooth development, PDL is derived from the dental follicle that surrounds developing teeth, providing a pool of cementum-forming cells.[26,27] Our findings show that human PDL contains a population of multipotent postnatal stem cells that can be isolated and expanded in vitro, providing a unique reservoir of stem cells from an accessible tissue resource. Importantly, PDL collected from one tooth can give rise to many stem cells, because of their capacity for proliferation ex vivo. Therefore, tissue regeneration mediated by human PDLSCs might have potential as a practical cellular-based treatment for periodontal diseases.

Previous experiments have shown that human bone marrow and dental pulp tissue contain postnatal stem cells that are capable of differentiating into osteoblasts/odontoblasts, adipocytes, and neuronal-like cells. These stem cells were characterised as STRO-1/CD146-positive progenitors derived from a perivascular niche within the bone marrow and dental pulp microenvironments.[21,23,24] In the present study, we found that the PDLSCs are similar to other mesenchymal stem cells with respect to their expression of STRO-1/CD146, implying that PDLSCs might also be derived from a population of perivascular cells.[28,29]

Our findings suggest that PDLSCs represent a novel population of multipotent stem cells, as shown by their capacity to develop into cementoblast-like cells, adipocytes in vitro, and cementum/PDL-like tissue in vivo, and by their high expression of scleraxis, a specific transcription factor associated with tendon cells.[30] PDLSCs also showed the capacity to form collagen fibres, similar to Sharpey's fibres, connecting to the cementum-like tissue, suggesting the potential to regenerate PDL attachment. These data lend further support to the notion that PDLSCs are a unique population of postnatal stem cells. However, because of the heterogeneity of STRO-1/CD146-positive mesenchymal stem cells,[23] it is possible that PDLSCs used in our experiments may represent a heterogeneous stem-cell-enriched population that contains some early uncommitted progenitor cells. We are now attempting to identify unique markers for PDLSCs, with molecular and genetic approaches.

The osteogenic potential of PDL cells has been assessed previously with several cell-culture methods, and the ability of such cultures to form a mineralised matrix has been noted.[4,11] Our data show the potential of PDLSCs to form calcified deposits in vitro, as previously shown with other mesenchymal stem-cell populations such as BMSSCs and DPSCs. However, PDLSCs formed sparse calcified nodules compared with their bone marrow and pulp tissue counterparts. Although PDLSCs were found to express an array of cementoblastic/osteoblastic markers, they did not form dentin or bone and its associated haemopoietic components in vivo. On the basis of these findings, future work should focus on identifying markers uniquely expressed by PDLSCs, to differentiate these cells from other types of stem cell.

Previous reports have described isolation from bovine dental follicles of progenitor cells capable of generating cementum-like tissue similar to that described in the present study.[26] However, in an earlier study, human PDL cells derived from third molars failed to generate cementum when transplanted into immunocompromised mice,[31] suggesting that the putative PDL stem cells remained constrained within the tissue. Our study used colony selection and STRO-1/CD146 markers to isolate PDLSCs from PDL. In-vivo transplantation showed that in-vitro-expanded PDLSCs generate a cementum/PDL-like complex characterised by a layer of aligned cementum-like tissues and clearly associated PDL-like tissues. PDLSCs, like DPSCs, show a higher number of population doublings than do BMSSCs in culture; the potential mechanisms contributing to the long lifespan of PDLSCs and DPSCs are not clear. BMSSCs, derived from the bone-marrow microenvironment, might need some marrow-associated growth factors for their continuous proliferation in vitro. By contrast, PDLSCs, derived from solid PDL tissue, might be less dependent on these factors. Even though PDLSCs, DPSCs, and BMSSCs are mesenchymal stem cells, and share some common protein expression profiles, PDLSCs differ significantly in their developmental potentials in vivo and their ability to develop into distinct tissues representative of the microenvironments from which they were derived in vivo.

Our findings show that postnatal PDLSCs are clonogenic, highly proliferative cells and capable of regenerating cementum/PDL-like tissues, properties that effectively define them as stem cells. Consequently, PDLSCs have potential for use in periodontal tissue regeneration. Many new approaches have been developed for treating periodontal defects, including guided tissue regeneration, growth factors, and enamel matrix proteins,[1,32–36] but so far, none of these treatments has provided consistently predictable outcomes.[37] Our results show that human PDLSCs participate in the process of periodontal tissue repair in immunocompromised rats. However, owing to the small size of the periodontal defects and the small number of animals used in our investigation, we were unable to demonstrate that PDLSCs are able to regenerate periodontal tissues. In future studies, the therapeutic capacity of these cells to repair larger

periodontal defects induced by periodontal disease should be assessed in large animal models.

Contributors

B M Seo and M Miura contributed equally to this work. B M Seo, M Miura and S Shi designed, planned, and co-ordinated the study, handled animals, did experiments, and wrote the manuscript. S Gronthos and P M Bartold participated in the study design, cell sorting experiments, and writing of the manuscript. S Batouli and J Brahim played a substantial part in the stem cell isolation experiments. M Young and P G Robey were involved in the Northern blot analysis and writing of the manuscript. C-Y Wang participated in the study design, interpretation of results, and writing of the manuscript.

Conflict of interest statement

The Department of Health and Human Service of the US Government has issued a provisional patent application entitled: "Multipotent postnatal stem cells from human periodontal ligament"; promotion of DHHS reference number E-033–2004/0 (patent pending; Shi S, Seo B, Miura M). The other authors have no conflict of interest.

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