European Journal of Oral Sciences

Eur J Oral Sci 2012; 120: 303–310
DOI: 10.1111/j.1600-0722.2012.00975.x
Printed in Singapore. All rights reserved

© 2012 Eur J Oral Sci

European Journal of Oral Sciences

Identification of multipotent stem cells from adult dog periodontal ligament

Wen-Jun Wang*, Yu-Ming Zhao*, Bi-Chen Lin, Jie Yang, Li-Hong Ge

Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, China

*These authors contributed equally to this work.

Wang W-J, Zhao Y-M, Lin B-C, Yang J, Ge L-H. Identification of multipotent stem cells from adult dog periodontal ligament.
Eur J Oral Sci 2012; 120: 303–310. © 2012 Eur J Oral Sci

Periodontal diseases, which are characterized by destruction of the connective tissues responsible for restraining the teeth within the jaw, are the main cause of tooth loss. Periodontal regeneration mediated by human periodontal ligament stem cells (hPDLSCs) may offer an alternative strategy for the treatment of periodontal disease. Dogs are a widely used large-animal model for the study of periodontal-disease progression, tissue regeneration, and dental implants, but little attention has been paid to the identification of the cells involved in this species. This study aimed to characterize stem cells isolated from canine periodontal ligament (cPDLSCs). The cPDLSCs, like hPDLSCs, showed clonogenic capability and expressed the mesenchymal stem cell markers STRO-1, CD146, and CD105, but not CD34. After induction of osteogenesis, cPDLSCs showed calcium accumulation in vitro. Moreover, cPDLSCs also showed both adipogenic and chondrogenic potential. Compared with cell-free controls, more cementum/periodontal ligament-like structures were observed in CB-17/SCID mice into which cPDLSCs had been transplanted. These results suggest that cPDLSCs are clonogenic, highly proliferative, and have multidifferentiation potential, and that they could be used as a new cellular therapeutic approach to facilitate successful and more predictable regeneration of periodontal tissue using a canine model of periodontal disease.

Li-Hong Ge, Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, Beijing, China

Telefax: +86-10-62173402
E-mail: gelh0919@yahoo.com.cn

Key words: canine periodontal ligament stem cells; clonogenic capability; multidifferentiation potential; periodontal regeneration

Accepted for publication May 2012

Periodontal diseases are inflammatory disorders of the periodontium characterized by the destruction of periodontal tissues, including periodontal ligament (PDL), cementum, alveolar bone, and gingiva. Periodontal diseases are a major cause of tooth loss (1). The ultimate goal of periodontal therapy is to regenerate affected tissues to their original form, architecture, and function. However, this type of treatment is beyond current technologies. Successful periodontal regeneration requires the coordination of many events at both cellular and molecular levels (2).

The PDL is a specialized soft connective tissue embedded between the cementum and the inner wall of the alveolar bone socket; it sustains teeth and helps them to withstand natural chewing forces. The PDL has an important role in supporting tooth function, maintaining homeostasis, and repairing damaged tissue in response to periodontal disease or mechanical trauma (2–4). It has been demonstrated that the PDL contains heterogeneous cell populations which can differentiate into either cementum-forming cells (cementoblasts) or bone-forming cells (osteoblasts) (5). Recent studies have reported the isolation and characterization of mesenchymal stem cells (MSCs) from PDL stem cells (PDLSCs) (6–9).

The PDLSCs represent a unique MSC population, as demonstrated by their capacity to generate a cementum/PDL-like tissue ex vivo, which appeared to be different from typical bone/marrow structures generated by bone marrow stromal stem cells and dentin/pulp-like structures generated by dental pulp stem cells (6). Because of their capacity for self-renewal and multilineage differentiation, PDLSCs have been proposed as important position in the field of tissue engineering (10). Given ethical considerations, the feasibility and practicability of tissue engineering will require extensive investigations on large-animal models before human trials. Stem cells and subpopulations of progenitor cells in the PDLs of various different species, such as sheep (11) and rat (12), have already been isolated and characterized. However, dog is a preferred animal model and has been widely used in PDL regeneration because of its very similar dental tissue structure and pathophysiology to humans (13, 14).

To understand, in more detail, the potential of dog as an animal model in periodontal regeneration, we successfully isolated canine PDLSCs (cPDLSCs) and identified their stem cell properties. The properties of cPDLSCs, including clonogenic and growth characteristics, the expression of MSC surface markers, and the multidifferentiation potential, were evaluated in vitro and ex vivo.

Material and methods

Animals

Ten-month-old beagle dogs were obtained from the Experimental Animal Center of the Peking University Health Science Center. Care and handling of the animals were performed according to the guidelines of the Institutional Authority for Laboratory Animal Care, Peking University. This study was reviewed and approved by the Health Science Center, Peking University (LA2011-045).

Isolation and culture of canine PDLSCs and human PDLSCs

The PDLSCs were isolated from freshly extracted, sound premolars ($n = 3$) of three, 10-month-old male beagle dogs, and the PDLSCs were isolated from freshly extracted, sound third molars ($n = 4$) of four human subjects following approved guidelines set by the Health Science Center, Peking University. The isolation procedure was performed as previously described (6), with minor modifications. Briefly, PDL was gently separated from the middle third of the root surface using forceps and then digested, for 1 h at 37°C, in a solution of 3 mg ml^{-1} of collagenase type I (Sigma, St Louis, MO, USA) and 4 mg ml^{-1} of dispase (Sigma). The 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 (1×10^4–1×10^5 cells) were seeded into 100-mm culture dishes containing α-modified Eagle's minimum essential medium (αMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mM glutamine, 100 U ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin, and then incubated at 37°C in 5% carbon dioxide. The cells used in this study were at passages 1–4. For each experiment, the same passage of cPDLSCs and human PDLSCs (hPDLSCs) was used.

Colony-forming unit fibroblastic assay

To assess colony-forming efficiency, single-cell suspensions of PDLSCs were cultured in 100-mm dishes for 9 d. Then, the cells were fixed with 4% paraformaldehyde and stained with 0.1% toluidine blue (Sigma). Aggregates of 50 or more cells were scored as colonies (passage 1).

Cell proliferation assay

The proliferation of subconfluent cultures (passage 1) of stem cells was assessed using a Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) from day 1 to day 9, according to the manufacturer's instructions.

Immunocytochemistry

Primary antibodies used in this study included anti-STRO-1, anti-CD146, anti-CD105, anti-CD34, anti-scleraxis, anti-collagen I, anti-alpha-smooth muscle actin (anti-α-SMA), anti-collagen II, and anti-osteopontin (anti-OPN). The primary antibodies were purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China), except for anti-STRO-1, which was obtained from R&D Systems (Minneapolis, MN, USA), anti-scleraxis and anti-OPN, which were from Abcam (Cambridge, UK), and anti-α-SMA, which was from Sigma. The PDLSCs (passage 3) were subcultured onto 24-chamber slides, fixed in 4% paraformaldehyde for 15 min, and blocked at room temperature for 30 min with PBS (pH 7.4) containing 10% normal goat serum. The cells were then incubated overnight at 4°C with diluted primary antibody, washed with PBS, and then incubated with secondary antibody at 37°C for 30 min. The Polymer Detection System for Immuno-Histological Staining (Zhongshan Goldenbridge Biotechnology) was subsequently used to detect immunoactivity, according to the manufacturer's instructions. The sections were counterstained with haematoxylin.

Flow cytometry

Single-cell suspensions of cPDLSCs and hPDLSCs ($>1 \times 10^6$ cells) were washed and resuspended in PBS. For indirect immunostaining, cells were incubated, for 20 min on ice, with 2.5 μg of STRO-1 antibody (R&D Systems) or isotype-matched immunoglobulins (eBioscience, San Diego, CA, USA). After washing, samples were incubated with phycoerythrin (PE)-conjugated secondary antibody (R&D Systems) for 20 min on ice, and then washed with PBS. For direct immunostaining, the cells were treated with 20 μl of PE-conjugated CD146 (BD Biosciences Pharmingen, San Jose, CA, USA) for 20 min on ice. After washing, all cells were analysed with a FACSCalibur Flow Cytometer (Becton Dickinson, Mountain View, CA, USA).

Multilineage differentiation in vitro

We tested the in-vitro multidifferentiation potential of the cPDLSCs and hPDLSCs towards osteogenesis, adipogenesis, and chondrogenesis, as described previously (15–17).

Osteogenic differentiation: Canine periodontal ligament stem cells and human periodontal ligament stem cells were seeded onto six-well plates, cultured to 80% confluence, and incubated for 2 wk in induction medium containing 10 nM dexamethasone, 10 mM β-glycerophosphate, 0.1 mM L-ascorbic acid-2-phosphate, 2 mM glutamine, and 15% FBS. After induction, the cells were fixed with 4% paraformaldehyde and evaluated by staining with 2% Alizarin Red S (pH 4.2) (Sigma) to show calcium deposition.

Adipogenic differentiation: Canine periodontal ligament stem cells and human periodontal ligament stem cells were seeded onto six-well plates, cultured to 80% confluence, and incubated for 3 wk with induction medium containing 1 μM dexamethasone, 10 mg l^{-1} of insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 60 mM indomethacin, 2 mM glutamine, and 15% FBS. At the end of the culture, the cells were fixed with 4% paraformaldehyde and lipid droplets were visualized by staining with 0.3% Oil Red-O (Sigma).

Chondrogenic differentiation: Canine periodontal ligament stem cells and human periodontal ligament stem cells were centrifuged at 350 ***g*** for 6 min. The tubes were tapped to detach the cell pellets and cultured in conical 15 ml polypropylene tubes at 37°C in chondrogenic medium containing 1% ITS^+ (ITS^+ is a mixture of recombinant human insulin, human transferrin, and sodium selenite.), 10^{-7} M dexamethasone, 50 μg ml^{-1} of L-ascor-

bic phosphate, 2 mM pyruvate, and 10 ng ml^{-1} of transforming growth factor-β1 (TGF-β1) (PeproTech, Rocky Hill, NJ, USA). The chondrogenic differentiation of PDLSCs was assessed by staining paraffin-embedded sections for type II collagen.

Transplantation

About 2.0×10^6 in-vitro-expanded cPDLSCs were mixed with 40 mg of hydroxyapatite (HA) ceramic particles (Bio Osteon, Beijing, China) and transplanted subcutaneously into the dorsal surfaces of 8- to 10-wk-old immunocompromised mice (CB-17/SCID; Vitalriver, Beijing, China), according to the method reported by KREBSBACH *et al.* (18). The same number of hPDLSCs was used as a positive control, and a cell-free group served as a negative control. These procedures were performed in accordance with the specifications of an approved animal protocol of the Health Science Center, Peking University (LA2011-045). The transplants were harvested after 8 wk, fixed with 4% paraformaldehyde, decalcified with buffered 10% edetic acid (pH 8.0), and then embedded in paraffin. For histological analysis, 5-μm sections of implants were prepared and stained with haematoxylin and eosin (H&E) and with Masson's trichrome stain.

Results

Clonogenic capability of canine PDL-derived cells

To identify putative stem cells, we generated and cultured a single-cell suspension from beagle or human PDL. A portion of the cells adhered to the plate and remained quiescent for 5–6 d before they started dividing rapidly to form colonies. Colonies formed from single cells after 8–10 d in culture were visualized using toluidine blue stain (Fig. 1A). This colony-forming cell population was termed PDLSCs. About 20–25% of PDLSCs from beagle and human tissue formed adherent cell colonies (Fig. 1B). Compared with hPDLSCs, cPDLSCs showed a higher proliferation rate (Fig. 1C).

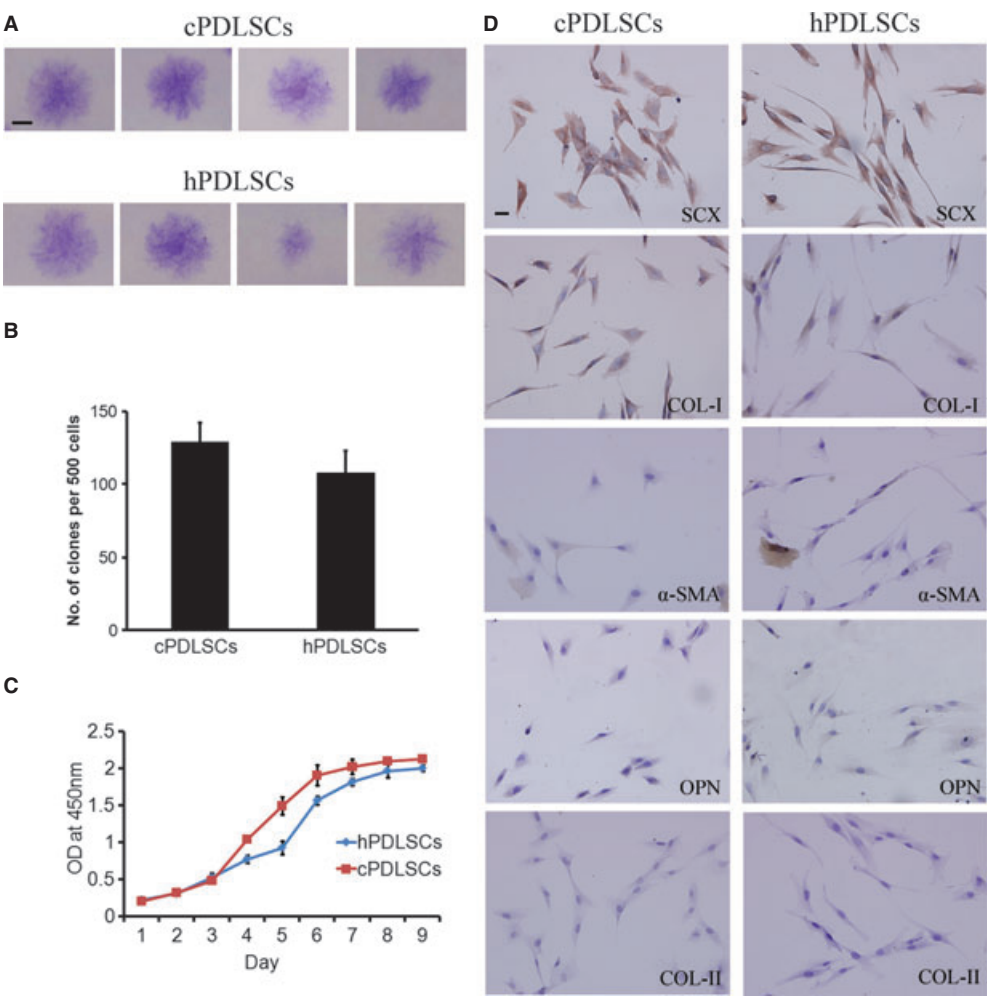

Fig. 1. Isolation and characterization of periodontal ligament stem cells (PDLSCs). (A) Representative colonies of fibroblast-like canine PDLSCs (cPDLSCs) and human PDLSCs (hPDLSCs). Bars, 500 μm. (B) Colony-forming efficiency of cPDLSCs and hPDLSCs. Results are shown as mean ± standard error of the mean of four dishes. (C) Growth curves of cPDLSCs and hPDLSCs. (D) Immunocytochemical staining for proteins related to periodontal ligament and cartilage in cPDLSCs and hPDLSCs. Bars, 100 μm. α-SMA, alpha-smooth muscle actin; COL-I (collagen I); COL-II (collagen II); OPN, osteopontin; SCX, scleraxis.

The cPDLSCs and the hPDLSCs showed high expression of scleraxis (SCX), a tendon-specific transcription factor, which demonstrated that the isolated cPDLSCs and hPDLSCs originated from the PDL tissue (Fig. 1D). In addition, both cPDLSCs and hPDLSCs expressed type I collagen, whereas only a certain population of these cells expressed α-SMA and OPN. Neither cPDLSCs nor hPDLSCs expressed type II collagen, a marker related to cartilage.

MSC marker expression

The MSC surface markers were detected by immunocytochemical staining (Fig. 2A). A small proportion of cPDLSCs expressed STRO-1, but the majority expressed CD146. In addition, we also detected moderate expression of CD105 in both cPDLSCs and hPDLSCs. However, these cells were negative for the haematopoietic stem cell marker, CD34.

A lower proportion of cPDLSCs (3.48%) than of hPDLSCs (5.58%) expressed STRO-1. CD146, which was highly expressed in hPDLSCs (64.98%), was moderately expressed in cPDLSCs (29.78%) (Fig. 2B).

cPDLSCs are multipotent in vitro

The multidifferentiation potential of the cPDLSCs towards osteogenesis, adipogenesis, and chondrogenesis was determined and compared with that of hPDLSCs. Small, round Alizarin Red-positive nodules formed in the PDLSC cultures after 2 wk of induction, indicating calcium accumulation in vitro (Fig. 3B,F). Compared with hPDLSCs, cPDLSCs formed fewer mineralized nodules. Oil Red-O staining of the lipid droplets within the adipocytes, an indicator of adipogenesis, was of a lower intensity in cPDLSCs than in hPDLSCs after 4 wk of culture in adipogenic induction medium (Fig. 3C,G). Chondrogenic differentiation after induction in chondrogenic medium was assessed in pellet culture by staining for type II collagen. After induction, cPDLSCs expressed type II collagen, as did hPDLSCs (Fig. 3D,H). Like hPDLSCs, cPDLSCs showed heterogeneous differentiation potential towards osteogenesis, adipogenesis, and chondrogenesis.

Generation of cementum-like and PDL-like structures by transplanted cPDLSCs

To determine whether cPDLSCs were able to differentiate into functional cementoblast-like cells, we transplanted cPDLSCs with HA carrier into immunocompromised mice. Both hPDLSC (Fig. 4D) and cPDLSC (Fig. 4A,B) transplants could form cementum-like tissue on the surface of the carrier. After decalcification, HA disappeared, leaving gaps. Transplanted cPDLSCs were able to form dense collagen fibres connecting with newly formed cementum-like structures that mimicked physiological attachment of Sharpey's fibres (Fig. 4B,C). No mineralized tissue was observed in the cell-free control group (Fig. 4E).

Discussion

Mesenchymal stem cells are multipotent stem cells that were first discovered and characterized from bone mar-

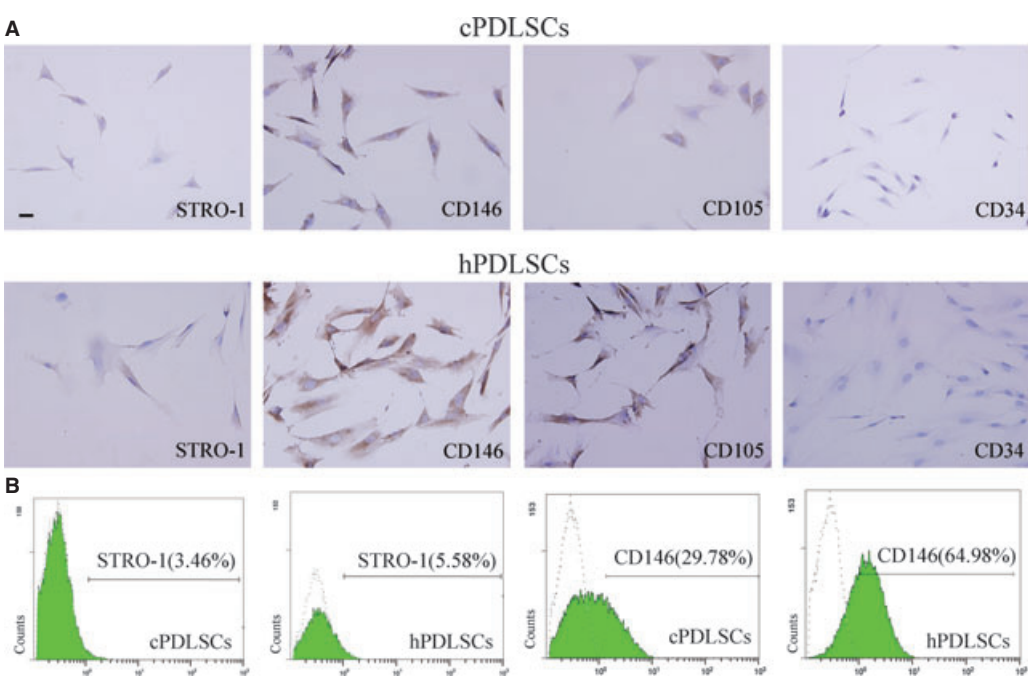

Fig. 2. Expression of mesenchymal stem cell (MSC) markers. (A) Immunocytochemical staining of cell-surface markers related to MSCs in canine periodontal ligament stem cells (cPDLSCs) and human PDLSCs (hPDLSCs). Bars, 100 μm. (B) Flow cytometric analysis of the expression of cell-surface markers related to stem cells and endothelial cells on cPDLSCs and hPDLSCs.

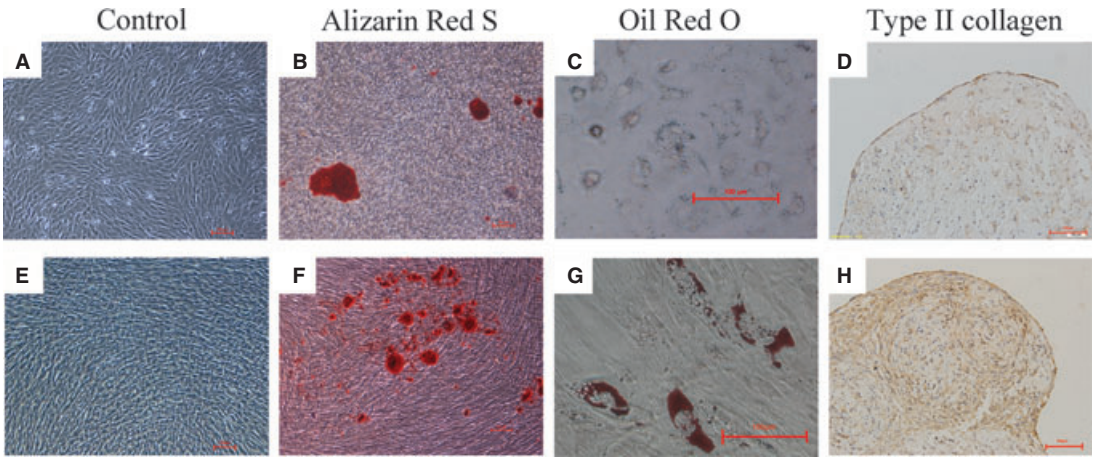

Fig. 3. Multidifferentiation potential of putative canine periodontal ligament stem cells (cPDLSCs) and human PDLSCs (hPDLSCs) in vitro. (A–D) cPDLSCs and (E–H) hPDLSCs. (A,E) Unstimulated controls. (B,F) Alizarin Red S staining, showing osteogenic differentiation. (C,G) Oil Red-O staining, showing adipogenic differentiation. (D,H) Chondrogenic differentiation, assessed by the expression of type II collagen. Figures show representative data from several independent experiments. Bars, 100 μm.

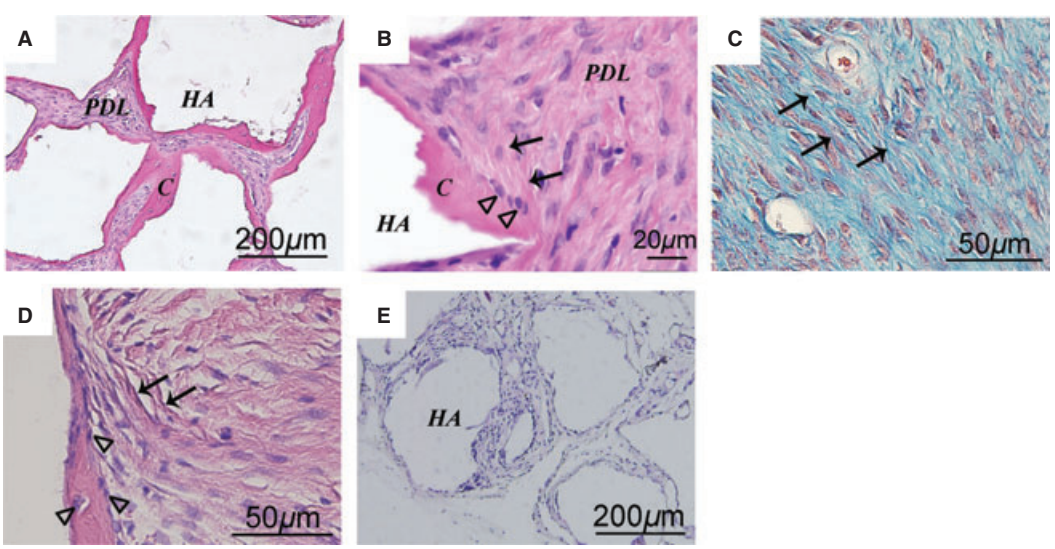

Fig. 4. Generation of cementum-like and periodontal ligament (PDL)-like structures ex vivo by canine periodontal ligament stem cells (cPDLSCs). (A) Eight weeks after transplantation, cPDLSCs generated cementum (*C*)/PDL-like structures (*PDL*) ex vivo on the surface of the hydroxyapatite (*HA*), as shown at lower magnification (approximately ×10). After decalcification, HA disappeared, leaving gaps. (B) Transplanted cPDLSCs differentiated into cementoblast-like cells (triangles) that formed cementum-like structures (*C*) connecting to newly formed collagen fibres (arrows), similar to the structure of Sharpey's fibres. (C) Masson's trichrome staining showed that transplanted cPDLSCs generated a substantial amount of collagen fibres (arrows). Collagen fibres are blue in Masson's trichrome-stained sections. (D) Human PDLSC (hPDLSC) transplants were used as a positive control to show a cementum-like structure (*C*) and PDL-like structures (*PDL*) ex vivo, and differentiation into cementoblast-like cells (triangles) and cementocyte-like cells (triangles) that connected to newly formed collagen fibres (arrows). (E) No mineralized tissue was observed in the cell-free control group.

row (19). Mesenchymal stem cell-like populations from other tissues have now been characterized based on the 'gold standard' criteria (20–22) of clonogenicity, multipotency, and self-renewal. Five different types of human dental stem/progenitor cells have been isolated and characterized: dental pulp stem cells (22), stem cells from exfoliated deciduous teeth (23), PDLSCs (6), stem cells from apical papilla (24), and dental follicle progenitor cells (25). Growing knowledge on the properties of these dental MSCs has accelerated the development of dental-tissue regeneration techniques and their implementation in regenerative medicine.

In general, large-animal models resemble human conditions in many aspects, such as anatomy, physiology, and pathology. Therefore, those models are preferable in dental research to small-animal models (26). Beagle

dogs are widely used for the study of periodontal regeneration owing to their docile temperament and natural susceptibility to periodontal disease (27, 28). Recently, many studies have focused on periodontal regeneration using PDL cells in canine models (13, 14, 29). However, to date, canine PDLSCs have not been clearly characterized. In this study, we successfully isolated and characterized MSCs from canine PDL. The tendon-specific transcription factor SCX is highly expressed in cPDLSCs, demonstrating that these cells originated from PDL tissue. In addition, cPDLSCs expressed type I collagen, in accordance with previous studies (30, 31). Moreover, these cells displayed many characteristics similar to those already reported for human PDLSCs, including clonogenic capability, expression of a set of MSC markers, and multidifferentiation potential.

One of the most prominent properties of MSCs is their ability to generate colonies after they are plated at a low density. The colony-forming unit fibroblastic (CFU-F) assay is a standardized assay for characterizing MSCs (31). In this study, cPDLSCs showed clonogenic capability, as did hPDLSCs. These colonies were heterogeneous in size and in cell density, reflecting a variety of proliferation potentials in the isolated cPDLSCs.

As with other postnatal MSCs (6, 22, 32), cPDLSCs expressed STRO-1, CD146, and CD105. STRO-1 is a putative stem-cell surface marker for the isolation of MSCs (32). Previous studies (33) suggested that STRO-1 was a marker of the perivascular niche of these stem-cell populations. Recent studies showed that blood-vessel walls harboured a reserve of progenitor cells that might be integral to the origin of MSCs and other related adult stem cells (34). Mesenchymal stem cells with greater differentiation potential express higher levels of CD146 on the cell surface (35). Because CD146 is known to be expressed on several cell types (e.g. MSCs and endothelial cells) and its expression may be linked to multipotency (36), we used CD146 to determine whether cPDLSCs were associated with blood vessels. The results showed a high level of expression of CD146, suggesting that cPDLSCs might derive from a population of perivascular cells. CD105 is a widely adopted marker used to detect human MSCs (37). In this study, CD105 was expressed in cPDLSCs and in hPDLSCs, demonstrating their MSC phenotype. Both cPDLSCs and hPDLSCs were negative for CD34, in agreement with previous studies (30).

The heterogeneity of STRO-1/CD146/CD105-positive MSCs in our study implies that cPDLSCs may represent a heterogeneous stem-cell-enriched population that contains some early uncommitted progenitor cells. However, there are still no specific molecules or genetic approaches that could identify cPDLSCs. We found that cPDLSCs showed lower levels of expression of STRO and CD146 than hPDLSCs. This may be partly because the STRO-1 and CD146 antibodies used in our study were mouse anti-human Igs, as there is no anti-canine Igs currently available. To test mouse anti-human STRO-1 Ig cross-reactivity with canine cells, we performed immunocytochemical staining on hPDLSCs, cPDLSCs, and lymphocytes (data not shown) and found that only hPDLSCs and cPDLSCs were specifically stained with the human STRO-1 antibody. Similarly, many other previous studies have also successfully used anti-human STRO-1 Ig to characterize stem cells isolated from canine tissues (38). However, this method lacks specificity compared with the use of species-specific antibodies.

The heterogeneous population of MSCs was contaminated with other progenitor cells (31, 39). Early PDL progenitor cells are capable of expressing α-SMA (5, 40) and OPN (41). Alpha-SMA is a marker for determining the degree of cell differentiation in the dental follicle and in periodontal tissues (42). Expression of α-SMA is generally associated with differentiation of fibroblasts into contractile myofibroblasts. Myofibroblasts are generally present in organs with a high remodelling capacity, such as the PDL, or during conditions of increased remodelling (43). Osteopontin is an osteogenesis marker found in the extracellular matrix of bone or cementum and is an early marker of periodontal tissue regeneration (44). Our results showed that a population of cPDLSCs expressed α-SMA and OPN, demonstrating that a small proportion of progenitor cells was present among the cPDLSCs.

We confirmed that cells derived from canine PDL had multidifferentiation potential in vitro and ex vivo. The osteogenic potential of hPDLSCs has been assessed previously (6). Our findings demonstrate that cPDLSCs have the capacity to form mineralized deposits in vitro. The adipogenesis and chondrogenesis capacities demonstrated the multidifferentiation potential of the cPDLSCs. In this respect, cPDLSCs seemed to be competent at a comparable level to hPDLSCs.

When transplanted into immunocompromised mice with the conductive biomaterial HA, cPDLSCs generated a cementum/PDL-like complex. The complex was characterized by a layer of aligned cementum-like tissues and by PDL-like tissues clearly associated with this layer. Most importantly, these transplanted cells could produce both mineralized and soft connective tissues, the morphological features of which were characterized as a cementum structure containing inserted ligament-like Sharpey's fibres. However, the cell-free control group failed to form mineralized tissue. These results demonstrated that the transplanted cPDLSCs, rather than the cells from immunocompromised mice, may have contributed to the generation of the mineralized tissue. The multidifferentiation potential in vitro and ex vivo supports the application of cPDLSCs in periodontal regeneration.

In conclusion, we demonstrated that cPDLSCs from canine PDL are a population of stem cells with the phenotypic characteristics of MSCs. The isolated cPDLSCs are clonogenic and highly proliferative cells, with multidifferentiation potential, and could form cementum-like tissue ex vivo. Therefore, cPDLSCs will be a useful source of MSCs for further periodontal-

regeneration research in a canine model before progression to human trials.

Acknowledgements – This work was supported by grants from the National Natural Science Foundation of China (Nos 81170928 and 81141011).

Conflicts of interest – The authors deny any conflicts of interest related to this study.

References

1. Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR Jr, Papapanou PN, Sacco RL. Relationship between periodontal disease, tooth loss, and carotid artery plaque: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Stroke* 2003; **34**: 2120–2125.
2. Bartold PM, McCulloch CA, Narayanan AS, Pitaru S. Tissue engineering: a new paradigm for periodontal regeneration based on molecular and cell biology. *Periodontol 2000* 2000; **24**: 253–269.
3. Andreasen JO. A time-related study of periodontal healing and root resorption activity after replantation of mature permanent incisors in monkeys. *Swed Dent J* 1980; **4**: 101–110.
4. Kramer PR, Nares S, Kramer SF, Grogan D, Kaiser M. Mesenchymal stem cells acquire characteristics of cells in the periodontal ligament in vitro. *J Dent Res* 2004; **83**: 27–34.
5. Lekic P, Rojas J, Birek C, Tenenbaum H, McCulloch CA. Phenotypic comparison of periodontal ligament cells in vivo and in vitro. *J Periodontal Res* 2001; **36**: 71–79.
6. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004; **364**: 149–155.
7. Nagatomo K, Komaki M, Sekiya I, Sakaguchi Y, Noguchi K, Oda S, Muneta T, Ishikawa I. Stem cell properties of human periodontal ligament cells. *J Periodontal Res* 2006; **41**: 303–310.
8. Xu J, Wang W, Kapila Y, Lotz J, Kapila S. Multiple differentiation capacity of STRO-1+/CD146+PDL mesenchymal progenitor cells. *Stem Cells Dev* 2009; **18**: 487–496.
9. Iwata T, Yamato M, Zhang Z, Mukobata S, Washio K, Ando T, Feijen J, Okano T, Ishikawa I. Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. *J Clin Periodontol* 2010; **37**: 1088–1099.
10. Chen FM, Jin Y. Periodontal tissue engineering and regeneration: current approaches and expanding opportunities. *Tissue Eng Part B Rev* 2010; **16**: 219–255.
11. Gronthos S, Mrozik K, Shi S, Bartold PM. Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. *Calcif Tissue Int* 2006; **79**: 310–317.
12. Techawattanawisal W, Nakahama K, Komaki M, Abe M, Takagi Y, Morita I. Isolation of multipotent stem cells from adult rat periodontal ligament by neurosphere-forming culture system. *Biochem Biophys Res Commun* 2007; **357**: 917–923.
13. Tsumanuma Y, Iwata T, Washio K, Yoshida T, Yamada A, Takagi R, Ohno T, Lin K, Yamato M, Ishikawa I, Okano T, Izumi Y. Comparison of different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model. *Biomaterials* 2011; **32**: 5819–5825.
14. Akizuki T, Oda S, Komaki M, Tsuchioka H, Kawakatsu N, Kikuchi A, Yamato M, Okano T, Ishikawa I. Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. *J Periodontal Res* 2005; **40**: 245–251.
15. Bi Y, Stuelten CH, Kilts T, Wadhwa S, Iozzo RV, Robey PG, Chen XD, Young MF. Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. *J Biol Chem* 2005; **280**: 30481–30489.
16. Gimble JM, Morgan C, Kelly K, Wu X, Dandapani V, Wang CS, Rosen V. Bone morphogenetic proteins inhibit adipocyte differentiation by bone marrow stromal cells. *J Cell Biochem* 1995; **58**: 393–402.
17. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998; **238**: 265–272.
18. Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RV, Rowe DW, Robey PG. Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. *Transplantation* 1997; **63**: 1059–1069.
19. Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976; **4**: 267–274.
20. Caplan AI. Mesenchymal stem cells. *J Orthop Res* 1991; **9**: 641–650.
21. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997; **276**: 71–74.
22. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000; **97**: 13625–13630.
23. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* 2003; **100**: 5807–5812.
24. Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, Huang GT. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endodont* 2008; **34**: 166–171.
25. Morsczeck C, Moehl C, Gotz W, Heredia A, Schaffer TE, Eckstein N, Sippel C, Hoffmann KH. In vitro differentiation of human dental follicle cells with dexamethasone and insulin. *Cell Biol Int* 2005; **29**: 567–575.
26. Pellegrini G, Seol YJ, Gruber R, Giannobile WV. Pre-clinical models for oral and periodontal reconstructive therapies. *J Dent Res* 2009; **88**: 1065–1076.
27. Weinberg MA, Bral M. Laboratory animal models in periodontology. *J Clin Periodontol* 1999; **26**: 335–340.
28. Oz HS, Puleo DA. Animal models for periodontal disease. *J Biomed Biotechnol* 2011; **2011**: 754857.
29. Marques FM, Filomena BM, Lina C, Barbara O, Palmeirao CE. Histological evaluation of periodontal regeneration in autogenous tooth transplantation in the dog: a comparison between one and two-stage surgical techniques, a pilot study. *Dent Traumatol* 2010; **26**: 76–79.
30. Feng F, Akiyama K, Liu Y, Yamaza T, Wang TM, Chen JH, Wang BB, Huang GT, Wang S, Shi S. Utility of PDL progenitors for in vivo tissue regeneration: a report of 3 cases. *Oral Dis* 2010; **16**: 20–28.
31. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, Li L, Leet AI, Seo BM, Zhang L, Shi S, Young MF. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 2007; **13**: 1219–1227.
32. Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* 1991; **78**: 55–62.
33. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003; **18**: 696–704.
34. Crisan M, Casteilla L, Lehr L, Carmona M, Paoloni-Giacobino A, Yap S, Sun B, Leger B, Logar A, Penicaud L, Schrauwen P, Cameron-Smith D, Russell AP, Peault B, Giacobino JP. A reservoir of brown adipocyte progenitors in human skeletal muscle. *Stem Cells* 2008; **26**: 2425–2433.
35. Russell KC, Phinney DG, Lacey MR, Barrilleaux BL, Meyertholen KE, O'Connor KC. In vitro high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells* 2010; **28**: 788–798.
36. Covas DT, Panepucci RA, Fontes AM, Silva WA Jr, Orellana MD, Freitas MC, Neder L, Santos AR, Peres LC, Jamur MC, Zago MA. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. *Exp Hematol* 2008; **36**: 642–654.
37. Aslan H, Zilberman Y, Kandel L, Liebergall M, Oskouian RJ, Gazit D, Gazit Z. Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105 + cells. *Stem Cells* 2006; **24**: 1728–1737.

38. Dissanayaka WL, Zhu X, Zhang C, Jin L. Characterization of dental pulp stem cells isolated from canine premolars. *J Endod* 2011; **37**: 1074–1080.
39. Fujii S, Maeda H, Wada N, Tomokiyo A, Saito M, Akamine A. Investigating a clonal human periodontal ligament progenitor/stem cell line in vitro and in vivo. *J Cell Physiol* 2008; **215**: 743–749.
40. Fujii S, Maeda H, Tomokiyo A, Monnouchi S, Hori K, Wada N, Akamine A. Effects of TGF-beta1 on the proliferation and differentiation of human periodontal ligament cells and a human periodontal ligament stem/progenitor cell line. *Cell Tissue Res* 2010; **342**: 233–242.
41. Saito M, Handa K, Kiyono T, Hattori S, Yokoi T, Tsubakimoto T, Harada H, Noguchi T, Toyoda M, Sato S, Teranaka T. Immortalization of cementoblast progenitor cells with Bmi-1 and TERT. *J Bone Min Res* 2005; **20**: 50–57.
42. Hosoya A, Nakamura H, Ninomiya T, Yoshiba K, Yoshiba N, Nakaya H, Wakitani S, Yamada H, Kasahara E, Ozawa H. Immunohistochemical localization of alpha-Smooth muscle actin during rat molar tooth development. *J Histochem Cytochem* 2006; **54**: 1371–1378.
43. Henneman S, Bildt MM, Degroot J, Kuijpers-Jagtman AM, Von den Hoff JW. Relaxin stimulates MMP-2 and alpha-smooth muscle actin expression by human periodontal ligament cells. *Arch Oral Biol* 2008; **53**: 161–167.
44. Lekic P, Sodek J, McCulloch CA. Relationship of cellular proliferation to expression of osteopontin and bone sialoprotein in regenerating rat periodontium. *Cell Tissue Res* 1996; **285**: 491–500.