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Original Article

Comparison of the characteristics of human gingival fibroblasts isolated by tissue explants and by enzyme digestion methods

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Abstract: Objective: To compare the characteristics of human gingival fibroblasts (hGFs) obtained by tissue-explant and enzyme-digestion methods. Methods: The hGFs were isolated using tissue explants and enzyme digestion methods and identified by immunocytochemical analysis. Cell-surface molecules were detected by flow cytometry analysis. The colony-forming activity was examined by CFU-F assay. Oil red O staining and alizarin red staining were performed to identify the adipogenic and osteogenic differentiation of hGFs and the differentiation capacity was quantified using spectrophotometric method. Results: Flow cytometry analysis showed that hGFs expressed CD44, CD29, Strol-1, but not CD34 and CD45. Strol-1 was significantly higher in enzyme-digestion hGFs [(14.84±2.00)%] than in tissue-explant hGFs [(6.64±0.95)%] ($P<0.05$). CFU-F assay showed that the self-renewal capacity of hGFs in enzyme-digestion group [(21±8)%] was significantly higher than that in tissue-explant group [(14±8)%] ($P<0.05$). The quantification test of adipogenic differentiation capacity showed the absorbance of enzyme-digestion group [0.66±0.03] was much higher than that tissue-explant group [0.45±0.04] ($P<0.05$). And the similar result was showed in the osteogenic differentiation capacity test that the absorbance of enzyme-digestion group [0.14±0.01] was obviously higher than that tissue-explant group [0.12±0.01] ($P<0.05$). Conclusions: Compared with tissue-explant hGFs, enzyme-digestion hGFsshowed much more mesenchymal stem cell-like properties with significantly higher self-renewal capacity and adipogenic and osteogenic differentiation ability.

Keywords: Gingival fibroblasts, cell isolation, biological characteristics

Introduction

Gingiva seals the interface between teeth and oral mucosa, dynamicly participates in immune defense and has one of the fastest tissue turnover rates in body [1, 2]. As the predominant cell type in Gingival connective tissue, gingival fibroblasts (GFs) originate from the neural crest and possess distinct properties, such as ease of isolation and expansion in vitro, multipotent differentiation potential, anti-inflammatory andimmunomodulatory capacity, though being functionally heterogeneous. It is also demonstrated that skin wound repair with rapid re-epithelialization and angiogenesis was found in a mouse model with systemic infusion of gingival cells [3]. Interestingly, mesenchymal stem cells (MSCs) have been isolated from gingival tissue using the same isolation methods as GFs. As novel postnatal stem cells, Gingiva-derived mesenchymal stem cells (GMSCs) have been paid great attention for therapeutic potential in tissue regeneration [4, 5] and autoimmune disease treating [6], thus, considered as an ideal candidate cell resource for tissue engineering and cell-based therapies [7]. Though it is currently unclear whether the reported immunomodulatory capacity and multipotent differentiation potential of gingival fibroblast cultures depends on the presence of a relatively high proportion of MSCs in the cultures, the fact that GFs possess mesenchymal stem cell-like properties shows GFs have a promising prospect in cell-based therapies.

Human gingival fibroblasts (hGFs) are basically obtained from the gingival tissue by tissue-explant orenzyme-digestion methods. From

these cultures, MSC colonies can be separated and enriched by the limited dilution and colony-forming unit-fibroblastic cells (CFU-F) techniques, or isolated based on expression of MSC surface markers using flow cytometry or other separation techniques. As we know, GFs that isolated from the same biopsy are not all alike but compose different phenotypic subpopulations with distinct properties and functions [8]. And 90% of GMSCs isolated from mice were reported positive for the neural crest-associated surface markers, while 10% were from mesoderm [9]. These findings indicate that GFs obtained by different culture methods may isolate different populations from the gingival tissue. As other studies have reported that periodontal ligament cells (PDLCs) obtained by different isolation methods showed different chatacteristics [10] and GMSCs generated under a 3D spheroid culture condition possessed better therapeutic efficacy thantheir adherent cells [11], we speculate the heterogeneous populations of GFs may influence their multipotent differentiation potentials, self-renewal capacity and other stem cell-like properties. The aim of this study was to compare the characteristics of human gingival fibroblasts (hGFs) obtained from tissue-explant and enzyme-digestion methods.

Materials and methods

Isolation of hGFs

Human gingival tissues were obtained as remnants of discarded tissues from systemically and periodontally healthy donors (18-25 years old) with informed consent and approval of the clinical research ethics committee of Qingdao University. The fresh tissue was washed several times with phosphate-buffered saline (PBS) containing 400 μg/ml streptomycin and 400 U/ml penicillin. For collection of tissue-explant hGFs, the epithelial layer of the gingival tissue was discarded and the connective tissue was sliced into small pieces (1 mm^3) and placed into 25 cm^2 culture flask. The culture flask was inverted for tissue pieces adherence and carefully turned over 2 h later with 3 ml of culture medium containing α-MEM medium (Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37°C in 5% CO_2. Cells were subcultured at 80% confluency by 0.25% trypsin/EDTA solution (Gibco).

For collection of enzyme-digestion hGFs, the gingival tissue was incubated in a medium containing 2 mg/ml dispase (Sigma) at 4°C overnight. Then the epithelial layer was easily separated from connective tissue. Following mincing, the connective tissue pieces (1 mm^3) were subsequently digested with a solution of 2 mg/ml collagenase (Sigma) for 50 min at 37°C and then filtered through a 70-μm cell strainer. The pass-through was centrifuged at 1000 rpm for 5 min. The cells were resuspended in 3 ml of α-MEM containing 10% FBS and placed into 25 cm^2 culture flask. Cells were subcultured at 80% confluency by 0.25% trypsin/EDTA solution.

Immunocytochemistry staining

hGFs obtained by both of tissue-explant and enzyme-digestion at passage 2 were fixed and identified by Immunocytochemistry staining with cytokeratin and vimentin antibodies (Boster, China). Briefly, Cells were incubated with primary antibodies against cytokeratin and vimentin for 30 mins at room temperature followed by incubation with the biotinylated secondary antibody using a LSAB2 system-HRP Kit (Dako) according to the manufacturer's instructions. DAB was used as peroxidase substrate and counter staining was performed with hematoxylin.

Flow cytometry

A total of 10^6 h GFs at passage 1 were washed with PBS and incubated with antibodies against human CD44, Strol-1, CD34, CD45 (BioLegend), CD29 (eBioscience) at 4°C for 30 min in the dark. Cells were then centrifuged at 1300 rpm for 10 min and resuspended in 1% paraformaldehyde for flow cytometry analysis with the use of CXP Analysis 2.1 software (Beckman Coulter).

Colony-forming unit-fibroblast (CFU-F) assay

To assess the colony forming efficiency of the two kinds of GFs, a total of 500 GFs at passage 1 were seeded into 6-CM cell culture dish and cultured in a-MEM with 10% FBS. The culture medium was replaced every 3 days. After 12 days of cultivation, all cultures were washed twice with PBS, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet (Sigma-Aldrich), washed twice with distilled water, and counted using a phase-contrast inverted microscope (Olympus, Japan). A CFU-F was defined as a cluster of more than 50 cells.

In vitro multipotent differentiation

Adipogenic differentiation: The second-passage hGFs were seeded at 2×10^3 cells/well in

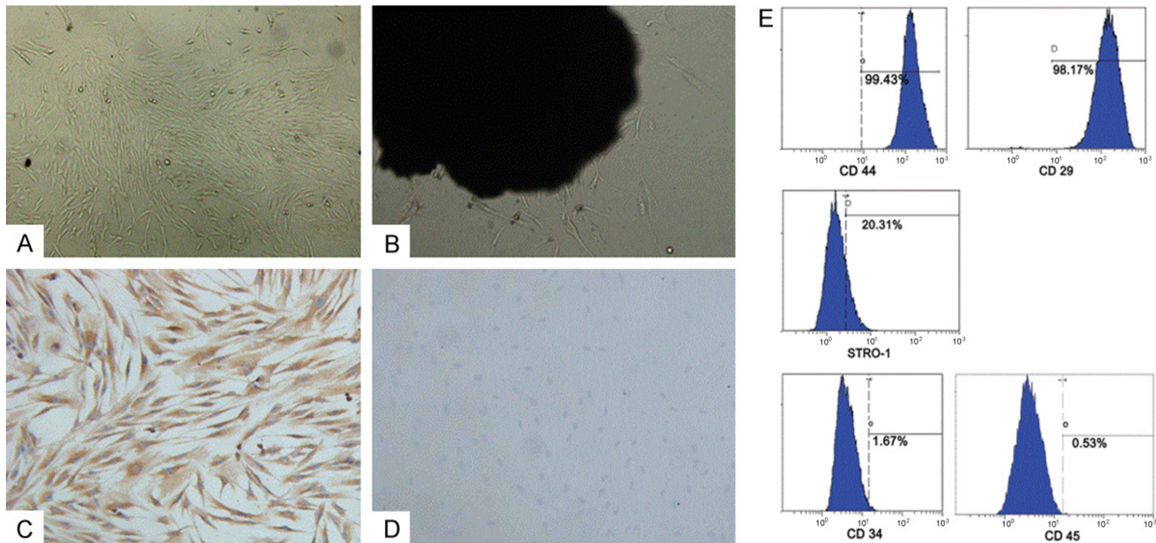

Figure 1. Characterization of hGFs. A. In vitro culture of enzyme-digestion hGFs at 5 days (×40); B. In vitro culture of tissue-explant hGFs at 7 days (×40); C. HGFs were detected positive for vimentin (×100); D. HGFs were detected negative for cytokeratin (×100); E. Flow cytometry analysis showed hGFs expressed CD44, CD29 and STRO-1 but not CD34, CD45.

Table 1. Flow cytometry analysis of tissue-explant and enzyme-digestion hGFs (%, mean±SD)

Surface molecule	Expression rate (%)		F	T	P
	Enzyme-digestion hGFs	Tissue-explant hGFs			
CD29	93.11±1.8	94.07±1.6	0.079	-0.402	0.701
CD44	97.04±1.16	95.46±0.57	1.715	1.255	0.256
Stro-1	14.84±2.00	6.64±0.95	1.139	3.679	0.01

96-well plates in α-MEM growth media, allowed to adhere overnight, and replaced with adipogenic induction medium supplemented with α-MEM containing 10% FBS, 1 μM dexamethasone (Sigma), 200 μM indomethacin (Sigma), 10 μM insulin (Sigma), 0.5 mM isobutyl-methylxanthine (Sigma), and 1% antibiotic/antimycotic. 14 days later, Oil Red O staining was performed to detect the formation of lipid-laden fat cells. The dye content was extracted by 60% isopropanol and quantified using microplate reader (Tecan Safire2, switzerland) at 510 nm [12].

Osteogenic differentiation: The second-passage hGFs were seeded at 1×10^3 cells/well in 96-well plates and incubated in α-MEM growth media for 24 h. Then the culture medium was replaced with osteogenic induction medium containing 5% FBS, 10 mM β-glycerophosphate (Sigma), 50 mg/ml ascorbate-2-phosphate (Sigma) and 0.1 μM dexamethasone. 28 days later, mineral deposition was identified by Alizarin Red S (Sigma-Aldrich) staining, then the dye content was extracted by an acetic acid extraction method and quantified using microplate reader at 405 nm [13].

Statistical analysis

Statistical analysis was performed using a statistical package (SPSS 19.0, SPSS Inc, USA). Data were expressed as mean±SD. Levene's Test was used to assess normality and homogeneity of variance. Differences between two groups were analyzed by an Independent-Samples t-test. The statistical significance chosen was $P<0.05$.

Results

Morphological and immunocytochemical characteristics of hGFs

The first adherent cells appeared 2 h after the primary culture using enzyme digestion method and reached 80% of confluence at about day 10 (**Figure 1A**), while tissue-explant cells started to grow out from the gingival tissue at about day 7 and reached 80% of confluence at about day 20 (**Figure 1B**). Under optical microscopy, all the primary cells appeared spindle-shaped

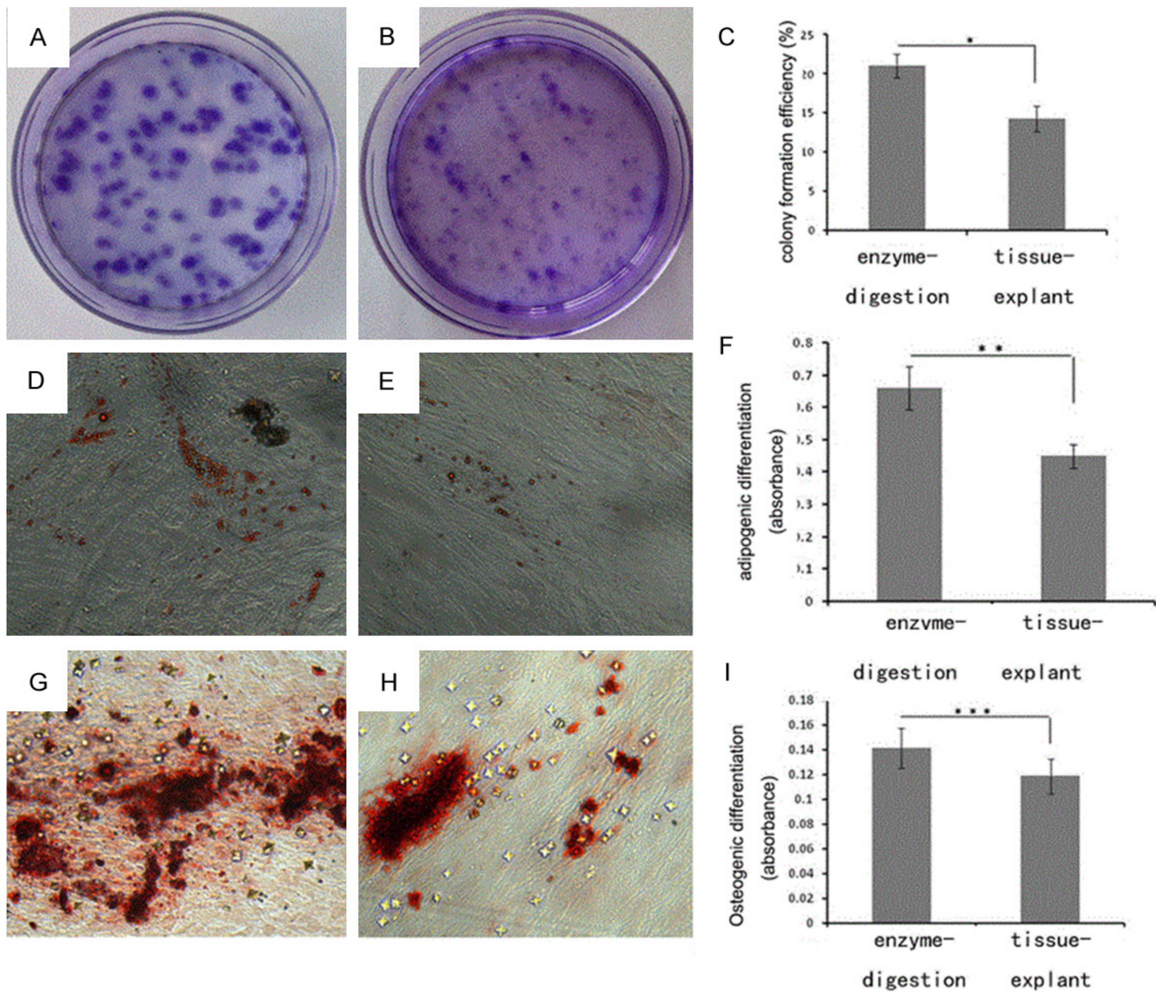

Figure 2. Colony formation and multipotent differentiation capacity of hGFs. A. CFU-F assay of enzyme-digestion hGFs. B. CFU-F assay of tissue-explant hGFs. C. Comparison of colony formation efficiency. D. Adipogenic differentiation of enzyme-digestion hGFs detected by Oil Red O staining (×100). E. Adipogenic differentiation of tissue-explant hGFs (×100). F. Comparison of adipogenic differentiation capacity. G. Osteogenic differentiation of enzyme-digestion hGFs identified by Alizarin Red S staining (×100). H. Osteogenic differentiation of tissue-explant hGFs (×100). I. Comparison of osteogenic differentiation capacity. *: P=0.001; **: P=0.000; ***: P=0.025.

fibroblast-like morphology. After subcultured, cells grew rapidly and appeared radial or whorled arrangement. Immunocytochemistry staining showed the cultured cells were detected positive for vimentin and negative for cytokeratin (**Figure1C** and **1D**).

Flow cytometry

Flow cytometry analysis showed that hGFs expressed CD44, CD29, Strol-1 surface markers, but not CD34 and CD45 (**Figure 1E**). Comparison of expression percentages of CD44, CD29 and Strol-1 was performed between enzyme-digestion and tissue-explant hGFs (**Table 1**). Strol-1 was significantly higher in enzyme-digestion hGFs [(14.84±2.00)%] than in tissue-explant hGFs [(6.64±0.95)%] (P=0.01). And there was no difference in comparison of CD44 and CD29 between the two groups (P>0.05).

CFU-F assay

Both types of cells displayed colony-forming ability with well-stained colonies (**Figure 2A**, **2B**). Enzyme-digestion hGFs formed a significantly large number of colonies [(21±8)%] compared with tissue-explant hGFs [(14±8)%] (**Figure 2C**).

Adipogenic differentiation capacity assay

Cultured in adipogenic induction medium for 2 weeks, lipid-rich cells were detected in both

groups by Oil Red O staining, and enzyme-digestion hGFs formed more lipid vacuoles than tissue-explant hGFs (**Figure 2D**, **2E**). The absorbance of the dye content in enzyme-digestion group [0.66±0.03] was much higher than that in tissue-explant group [0.45±0.04] (P=0.000) (**Figure 2F**).

Osteogenic differentiation capacity assay

After 4 weeks of osteogenic induction, hGFs showed formation of mineralized nodules which were identified by Alizarin Red S staining (**Figure 2G**, **2H**). The absorbance of Alizarin Red S in enzyme-digestion group [0.14±0.01] was obviously higher than that in tissue-explant group [0.12±0.01] (P=0.025) (**Figure 2I**).

Discussion

GFs have been considered as a new seed cell source in periodontal tissue engineering for advantages such as ease of isolation and expansion in vitro, self-renewal and multipotent differentiation potential in the past years. Recently, MSCs have been isolated from gingival tissue using the same isolation methods as GFs. As novel postnatal stem cells, gingiva-derived mesenchymal stem cells (GMSCs) possess the basic characteristics of MSCs, anti-inflammatory and extraordinary immunomodulatory capacities, and have been paid great attention for therapeutic potential in tissue regenerationand autoimmune disease treating. In our previous studies, GMSCs implanted into class III furcation defectes created in beagle dogs significantly enhanced the regeneration of damaged periodontal tissue [4], and GMSCs transplanted via the tail vein into C57BL/6J mice were found not only home to the mandibular defects but also promote bone regeneration [5].

Given the promising application prospects of GFs and GMSCs in in tissue engineering and cell-based therapies, primary cultured GFs are needed in more and more researches. As a conventional culture method for obtaining GFs, tissue explant method is simple and economic, however, with low cell emigration and long culture period. To obtain large numbers of GFs rapidly, enzyme digestion method is confirmed as an ideal technique which gets a single suspension of GFs by digesting the gingival tissues using enzymes. However, no studies have mentioned before whether the characteristics of the GFs obtained by different culture methods are different or not.

In the present study, both groups of primary GFs appeared fibroblastlike morphology and flow cytometry analysis showed that hGFs expressed CD44, CD29, Strol-1 surface markers, but not CD34 and CD45, which indicated the mesenchymal origin of GFs. The results were also verified by immunocytochemistry staining. Though there is no specific molecular marker to define MSCs, Stro-1has been used in many researches for the selection and identification of dental MSCs [14, 15]. In this study, the finding that the expression of Strol-1 was significantly higher in enzyme-digestion hGFs than in tissue-explant hGFs suggested there might be differences in stem cell-like properties between the two group cells, though it could not accurately reflect the proportion of MSCs in primary hGFs.

As stem cells possess self-renewal capacity and multiple differentiation potential, in this study, both types of hGFs displayed colony-forming ability with well-stained colonies. And the enzyme-digestion hGFs formed a significantly large number of colonies compared with tissue-explant hGFs. We also found tissue-explant hGFs could form lipid vacuoles and mineralised nodules in multiple differentiation capacity assays, though the quantity was less than enzyme-digestion hGFs. These findings further confirmed our speculation that the stem cell-like properties of hGFs are different depending on the isolation method used. The differences may be explained from two aspects. On the one hand, enzyme-digestion hGFs were gotten by digesting the gingival tissues using enzymes and seeded into culture flask as a single-cell suspension. It was impossible for a single cell to contact with its neighbouring cells and be supported by paracrine regulation. Only those with high proliferation and self-renewal ability may grow into colonies and reached confluence [16]. Hence, compared with tissue-explant hGFs, enzyme-digestion hGFs had a higher proliferation rate and formed more lipid vacuoles and mineralised nodules. On the other hand, though MSCs can be isolated from either enzyme-digestion GFs or tissue-explant GFs, it is more likely there is a significant difference in the proportion of MSCs obtained by different isolation methods. Up to now, the tiss-

ue location of the gingival MSCs is unclear. Recently, many immunolocalization and cell culture studies have demonstrated that perivascular tissue appears to provide a distinct niche for MSCs or MSC like cells [17, 18]. As fibroblasts represent the major cell type in in gingival connective tissue, and only close to the edge of the tissue explant cells have a greater chance to move out, the primary cells isolated using tissue-explant method in this study were mostly fibroblasts. In contrast, there was a higher proportion of MSCs in enzyme-digestion hGFs isolated by digesting the whole gingival tissue pieces, which could explain the result in flow cytometry analysis that Strol-1 was significantly higher in enzyme-digestion hGFs than in tissue-explant hGFs.

Since MSCs were isolated from gingival tissue and studied in tissue regeneration and autoimmune disease treating, GMSCs have been considered as an ideal candidate cell resource for tissue engineering and cell-based therapies. With the development of study of GMSCs, it is reported 90% of GMSCs isolated from mice were originated from neural crest while 10% from mesoderm, and GMSCs from different origins show different stem cell properties. Future studies should be focused on identification of distinct phenotypic properties of GMSCs from different origins and verify specific molecular markers to identify and enrich appropriate cell populations for various treating applications in different areas.

In conclusion, GFs obtained by tissue-explant and enzyme-digestion methods showed different characteristics. Compared with tissue-explant hGFs, enzyme-digestion hGFs showed much more mesenchymal stem cell-like properties with significantly higher self-renewal, adipogenic and osteogenic differentiation capacity. Obtaining large numbers of GFs rapidly and then isolating GMSCs possessing different phenotypic properties are of great significance to further research of GFs and GMSCs in tissue engineering and cell-based therapies.

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Disclosure of conflict of interest

None.

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References

[1] Mak K, Manji A, Gallant-Behm C, Wiebe C, Hart DA, Larjava H and Hakkinen L. Scarless healing of oral mucosa is characterized by faster resolution of inflammation and control of myofibroblast action compared to skin wounds in the red Duroc pig model. J Dermatol Sci 2009; 56: 168-180.

[2] Winning TA and Townsend GC. Oral mucosal embryology and histology. Clin Dermatol 2000; 18: 499-511.

[3] Zhang QZ, Su WR, Shi SH, Wilder-Smith P, Xiang AP, Wong A, Nguyen AL, Kwon CW and Le AD. Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. Stem Cells 2010; 28: 1856-1868.

[4] Yu X, Ge S, Chen S, Xu Q, Zhang J, Guo H and Yang P. Human gingiva-derived mesenchymal stromal cells contribute to periodontal regeneration in beagle dogs. Cells Tissues Organs 2013; 198: 428-437.

[5] Xu QC, Wang ZG, Ji QX, Yu XB, Xu XY, Yuan CQ, Deng J and Yang PS. Systemically transplanted human gingiva-derived mesenchymal stem cells contributing to bone tissue regeneration. Int J Clin Exp Pathol 2014; 7: 4922-4929.

[6] Zhang Q, Shi S, Liu Y, Uyanne J, Shi Y, Shi S and Le AD. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol 2009; 183: 7787-7798.

[7] Tomar GB, Srivastava RK, Gupta N, Barhanpurkar AP, Pote ST, Jhaveri HM, Mishra GC and Wani MR. Human gingiva-derived mesenchymal stem cells are superior to bone marrow-derived mesenchymal stem cells for cell therapy in regenerative medicine. Biochem Biophys Res Commun 2010; 393: 377-383.

[8] Hassell TM and Stanek EJ 3rd. Evidence that healthy human gingiva contains functionally

heterogeneous fibroblast subpopulations. Arch Oral Biol 1983; 28: 617-625.
[9] Xu X, Chen C, Akiyama K, Chai Y, Le AD, Wang Z and Shi S. Gingivae contain neural-crest- and mesoderm-derived mesenchymal stem cells. J Dent Res 2013; 92: 825-832.
[10] Tanaka K, Iwasaki K, Feghali KE, Komaki M, Ishikawa I and Izumi Y. Comparison of characteristics of periodontal ligament cells obtained from outgrowth and enzyme-digested culture methods. Arch Oral Biol 2011; 56: 380-388.
[11] Zhang Q, Nguyen AL, Shi S, Hill C, Wilder-Smith P, Krasieva TB and Le AD. Three-dimensional spheroid culture of human gingiva-derived mesenchymal stem cells enhances mitigation of chemotherapy-induced oral mucositis. Stem Cells Dev 2012; 21: 937-947.
[12] Yu W, Chen Z, Zhang J, Zhang L, Ke H, Huang L, Peng Y, Zhang X, Li S, Lahn BT and Xiang AP. Critical role of phosphoinositide 3-kinase cascade in adipogenesis of human mesenchymal stem cells. Mol Cell Biochem 2008; 310: 11-18.
[13] Gregory CA, Gunn WG, Peister A and Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. Anal Biochem 2004; 329: 77-84.
[14] Yang X, Walboomers XF, van den Beucken JJ, Bian Z, Fan M and Jansen JA. Hard tissue formation of STRO-1-selected rat dental pulp stem cells in vivo. Tissue Eng Part A 2009; 15: 367-375.
[15] Xu J, Wang W, Kapila Y, Lotz J and Kapila S. Multiple differentiation capacity of STRO-1+/CD146+ PDL mesenchymal progenitor cells. Stem Cells Dev 2009; 18: 487-496.
[16] Fortier LA. Stem cells: classifications, controversies, and clinical applications. Vet Surg 2005; 34: 415-423.
[17] Crisan M, Corselli M, Chen CW and Peault B. Multilineage stem cells in the adult: a perivascular legacy? Organogenesis 2011; 7: 101-104.
[18] Fournier BP, Larjava H and Hakkinen L. Gingiva as a source of stem cells with therapeutic potential. Stem Cells Dev 2013; 22: 3157-3177.