Isolation and characterization of human gingiva-derived mesenchymal stem cells using limiting dilution method

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Abstract  Background/purpose: Gingiva-derived mesenchymal stem cells (GMSCs) are attractive alternative MSC sources because of their relative abundance of sources and ease of accessibility. However, the isolation method for harboring GMSCs remains under discussion. The aim of the study was to isolate and explore in vitro characterization of human GMSCs, and compare stem cell properties with bulk-cultured gingival fibroblasts (GFs).

Materials and methods: GMSCs were isolated with limiting dilution method. Tissue-matched bulk-cultured GFs and GMSCs were evaluated in terms of their colony-forming abilities, population doubling capacities, cell surface epitopes, and multilineage differentiation potentials.

Results: GMSCs showed a significantly higher number of colony-forming units-fibroblast (P < 0.001) than bulk-cultured GFs, while the population doubling capacity of GMSCs reduced. Both types of cells were uniformly positive for MSC-associated markers CD44, CD73, CD90, CD105, and CD166, and were negative for hematopoietic markers CD14, CD34, and CD45. The only distinct marker was STRO-1, which was more highly expressed in GMSCs (13.4%) than in bulk-cultured GFs (0.02%). Upon induction, GMSCs displayed the capacity to undergo osteogenic, adipogenic, and chondrogenic differentiation. Real-time polymerase chain reaction showed related gene levels were significantly upregulated (P < 0.001). By contrast, bulk-cultured GFs lacked the capacity to undergo multilineage differentiation, and related gene levels showed no significant difference when compared with control groups.
Introduction

Stem cell biology has become an important field in regenerative medicine and tissue engineering therapy since the discovery and characterization of mesenchymal stem cells (MSCs). MSCs represent a population of multipotent stem cells that can be isolated from many tissues, including bone marrow, adipose tissue, placenta, and umbilical cord blood.1–4 All of these MSCs display fibroblast-like cell morphology, have self-renewal capacities, and multilineage differentiation potentials, such as giving rise to osteogenic, adipogenic, and chondrogenic lineages.5–7

MSC-like populations have also been isolated from human dental tissues, including dental pulp stem cells, stem cells from human exfoliated deciduous teeth, stem cells from apical papilla, dental follicle progenitor cells, and periodontal ligament stem cells.7–11 Those dental tissue-derived stem cells possess potent capacities to differentiate into odontogenic cells and generate reassembly dental tissue structures. Given the innate capacity of dental-derived MSC-like cells to ectopically generate structures resembling the tissues from which they are derived in vivo, these progenitor cell populations represent promising candidates for oral tissue regeneration.12–14 However, there are some drawbacks in using these stem cells for cell therapy, such as their limited tissue sources and the requirement for tooth extraction. Comparatively, a population of stem cells within gingival tissue, termed gingiva-derived mesenchymal stem cells (GMSCs), constitutes more appealing alternatives to other dental-derived MSCs for the accessibility and availability of human gingival tissues. GMSCs can be obtained from gingival tissue that are easily accessible from the oral cavity with minimal discomfort.12,13 Gingival tissues also exhibit scarless wound healing properties and a regenerative capability with rapid constitution of the tissue architecture. Interestingly, GMSCs display stable phenotype and telomerase activity in long-term cultures, and are not tumorigenic.14 Notably, GMSCs have demonstrated the capacity for self-renewal and in vitro inductive medium to differentiate into osteogenic, adipogenic, and chondrogenic lineages.5–6

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**Materials and methods**

**Cell isolation**

Human gingival tissues were obtained from three patients undergoing crown lengthening surgery with no history of periodontal disease at the School of Stomatology, Shandong University, Jinan, China. This study was approved by the Medical Ethics Committee of the Medical School, Shandong University (approval number: 2010015) and written informed consent was obtained from each individual patient. The gingival tissue samples were minced and digested in collagenase type I (Invitrogen, Carlsbad, CA, USA) and dispase II (Roche Diagnostics, Indianapolis, IN, USA) for 2 hours at 37°C. After that, the single cell suspension was filtered through a 70-μm cell strainer. Half of the single cell suspensions were plated at a concentration of 60 cells/cm² in 10-cm tissue culture dishes for the selection of single cell-derived colonies in α-minimal essential medium (α-MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA), 2mM l-glutamine (Sigma-Aldrich), 100μM l-ascorbate-2-phosphate (Wako Pure Chemical Industries Ltd, Osaka, Japan), 1mM sodium pyruvate (Sigma-Aldrich), 50-μg/mL streptomycin with 50-U/mL penicillin G (JRH Biosciences Inc., Lenexa, KS, USA), and 2.5-μg/mL amphotericin B (Life Technologies, Grand Island, NY, USA) in a humidified atmosphere (37°C, 5% CO₂). The nonadherent cells were removed 3 days later and the basic medium was changed three times per week. Individual plastic-adherent, MSC-like colonies grown for 10–14 days in 10-cm tissue culture dishes showed a decrease in the level of cell surface marker expression after MACS.20–22 Previous studies have indicated that human bone marrow-derived MSCs generate single cell-derived colonies if plated at extremely low densities.23–25 Digirolamo et al.26 showed that the replicative potential of the cells in culture was best predicted by a simple colony-forming assay when cells were plated at low densities, and the samples with the highest colony-forming efficiency also exhibited the greatest replicative potential. In addition, single cell-derived colonies obtained with low-density plating were able to differentiate into osteocytes, adipocytes, and chondrocytes under defined culture conditions, and had the capacity to generate reassembly tissue structures in vivo.25–29

Therefore, in this study, we selected single cell colonies of GMSCs using a limiting dilution method and established tissue-matched bulk-cultured GFs. Our study aimed to compare GMSCs with bulk-cultured GFs with regard to the colony-forming ability, population doubling capacity, cell surface epitopes, multilineage differentiation potentials, and related gene expression levels.
were isolated using colony rings and expanded using individual tissue culture flask, as previously described. Briefly, single colonies (≥ 50 cells clusters) of primary cells were isolated used colony rings. A colony ring was made by cutting down one part of the 1000-µL pipette tip with the blade on fire. The ring was put into a big class plate with wax on the base and autoclaved. The location of the selected single colony was circled under a microscope (Olympus Optical Co. Ltd, Tokyo, Japan) and the ring was put around the colony. Cell clusters from the colony were then detached with 0.05% trypsin/EDTA (Gibco BRL, Rockville, MD, USA) and transferred into 96-well plates. Cells were then transferred in sequence from the 96-well plates to a 48-well, a 24-well, and a six-well plate until it was possible to continue cultivation in flasks. For the establishment of bulk-cultured GFs, the other half of the single cell suspensions were also seeded into 25-cm² tissue culture flasks in α-MEM with 10% FCS and supplemented as described above. Bulk-cultured GFs represent a heterogeneous population of cells derived from multiple primary clonal lines. Five GF lines and five clonal GMSC lines were established from each tissue. All of the 15 GF lines and 15 clonal GMSC lines were used in the present study.

Colony-forming unit-fibroblast assay
To assess their colony forming efficiency, bulk-cultured GFs and GMSCs were seeded at 500 cells and 1000 cells per well in six-well plate and cultured in basic medium as described above in a humidified atmosphere (37°C, 5% CO₂). Culture medium was changed three times per week. After 14 days, the cultures were washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich), stained overnight with 0.1% (weight/volume) toluidine blue in 1% paraformaldehyde solution (BDH Chemicals, Poole, UK) and counted using a microscope. Aggregates of ≥ 50 cells were scored as a colony-forming unit-fibroblasts (CFU-F) colony. The numbers of colonies were statistically evaluated.

Population doubling assay
Population doubling assessment was carried out as previously described in literature with minor modifications. Briefly, the two types of cells were seeded at 5 × 10⁵ cells/cm² in 24-well plates respectively. After reaching 90% confluence, they were detached with 0.05% trypsin/EDTA. Cells were then reseeded at 5 × 10⁵ cells/cm² into another well of a 24-well, a 48-well, and a six-well plate until it was possible to continue cultivation in flasks. The establishment of bulk-cultured GFs, the other half of the single cell suspensions were also seeded into 25-cm² tissue culture flasks in α-MEM with 10% FCS and supplemented as described above. Bulk-cultured GFs represent a heterogeneous population of cells derived from multiple primary clonal lines. Five GF lines and five clonal GMSC lines were established from each tissue. All of the 15 GF lines and 15 clonal GMSC lines were used in the present study.

Analysis of surface markers using flow cytometry
Surface markers for bulk-cultured GFs and GMSCs were quantified using flow cytometry. After reaching confluence, cells were detached by 0.05% trypsin/EDTA and resuspended in blocking buffer containing Hanks’ Balanced Salt Solution (Sigma-Aldrich) supplemented with 5% FCS. Approximately 1 × 10⁵ cells were incubated with fluorescein isothiocyanate-conjugated mouse monoclonal antibodies (10 µg/mL) specific for human CD73, CD166 (Becton Dickinson Biosciences, San Jose, CA, USA), CD90, and Str-1 (R&D Systems, Inc., Minneapolis, MN, USA), CD44, CD105, CD14, CD34, CD45 (eBioscience, San Diego, CA, USA), or isotype-matched control immunoglobulin Gs for 1 hour on ice. Isotype-matched controls were then incubated with fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G (Southern Biotech, Birmingham, AL, USA) for 45 minutes on ice. After washing, cells were fixed in fluorescence-activated cell sorting fix solution. The samples were then subjected to flow cytometry using an Epic-XL/MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Osteogenic differentiation
For osteogenic differentiation, bulk-cultured GFs and GMSCs were cultured on six-well and 24-well plates in an osteogenic inductive medium at a density of 8 × 10³ cells per cm² as previously described. Osteogenic inductive medium was αMEM supplemented with 5% FCS, 100mM L-ascorbate-2-phosphate, 1mM sodium pyruvate, 50-µg/mL streptomycin, 50-U/mL penicillin G, 2mM L-glutamine, 0.1µM dexamethasone (Sigma-Aldrich), and 1.8mM inorganic phosphate (KH₂PO₄; BDH Chemicals). As controls, bulk-cultured GFs and GMSCs were cultured in basic medium. The media were refreshed twice weekly, and after 28 days incubation, cells were rinsed three times with PBS and fixed with 10% neutral buffered formalin (Sigma-Aldrich) for 1 hour at room temperature. Mineral deposition was detected in 24-well plates with Alizarin Red S staining (Sigma-Aldrich). Six-well plates designated for real time-polymerase chain reaction (PCR) were rinsed three times in PBS and RNA extracted using TRIzol according to manufacturer’s instructions (Life Technologies).

Chondrogenic differentiation
For chondrogenic differentiation, bulk-cultured GFs and GMSCs (1 × 10⁶ cells) were transferred into 10-µL yellow-top polypropylene tubes and centrifuged at 600g at 4°C.
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for 5 minutes to form cell pellets at the bottom of the tube. Cell pellets were cultured for chondrogenic induction medium for 28 days as previously described. Chondrogenic induction medium was high-glucose Dulbecco’s modified Eagles’ medium (Sigma-Aldrich) supplemented with 1 × insulin-transferrin-selenium + premix [insulin (6.25 μg/mL), transferring (6.25 μg/mL), selenious acid (6.25 ng/mL), linoleic acid (5.35 μg/mL); Becton Dickinson Biosciences], 100μM L-ascorbate-2-phosphate, 50-μg/mL streptomycin, 50-U/mL penicillin, 2M L-glutamine, 10μM dexamethasone, 0.125% bovine serum albumin (Sigma-Aldrich), and 10-ng/mL human transforming growth factor-β3 (Pepro Tech, Inc., Somerset, NJ, USA). As controls, cell pellets were cultured in basic medium. The media were refreshed twice weekly. After 28-days incubation, the cell pellets designated for histological assessment were fixed in 4% paraformaldehyde at 4°C overnight, then embedded in paraffin and sectioned for immunohistochemical staining with mouse antihuman collagen type II antibody (1:100 dilution; Chemicon International, Temecula, CA, USA). Cell pellet cultures designated for real time-PCR were rinsed three times in PBS and RNA extracted using TRIzol according to the manufacturer’s instructions.

Real time-PCR

The expression of cell lineage-specific genes of bulk-cultured GFs and GMSCs were analyzed using real time-PCR. Total cellular RNA harvested from selected cells undergoing osteogenic, adipogenic, and chondrogenic differentiation after 28 days of culture using TRIzol was subjected to reverse transcription using Oligo dT primers and SuperScript III reverse transcriptase according to manufacturer’s instructions (Invitrogen). Real time-PCR was performed using RT2 SYBR Green/ROX qPCR Master Mix (SuperArray Bioscience, Frederick, MD, USA) with the Rotor-Gene RG-6000 real time-PCR machine. PCR conditions were hot start enzyme activation at 95°C for 15 minutes, 40 cycles of (95°C for 10 seconds), and 72°C for 15 seconds, then final extension at 72°C for 3 minutes. Primer sets examined included RUNX2/CBFA1, OPN, and BSP2 (for osteogenic differentiation), leptin, adipin, and PPARγ2 (for adipogenic differentiation), and aggrecan, COL-II, and SOX9 (for chondrogenic differentiation). Housekeeping gene β-actin was used as an internal control in applications (Table 1).

| Gene   | Primer sequences for osteogenic, adipogenic, and chondrogenic markers. | Product size (bp) | Reverse primer 3’–5’ |
|--------|--------------------------------------------------------------------------|-------------------|----------------------|
| Runx2  | GGAGGAGGCAAGAAGGTTCATACGTCAGTGCC                                       | 137               | CATCAAGCTTCTGCTGTCG |
| OPN    | AGGGTCAAGGTACCTCAGAGTACC                                                | 92                | GATGCTTCTGCTGTCGAGC |
| BSP2   | ACATCTCAGTACCTGATGCTAGCAC                                                | 123               | GTGGGTTCAGCACTCCTG7T |
| Aggrecan| CTCCTCGAGGAGCTTCC                                                       | 191               | GCTCGGATGTCAGACTCAGG |
| COL-II | GAGGTGGAGTATGAGGAGGACC                                                  | 181               | ACCATACACACGGCTTCC |
| SOX9   | AGGGTCAAGGTACCTCAGAGTACC                                                | 219               | CCACTTGCCAGAGGCTTCC |
| Leptin | GGCTTTGAGGCTATCTTTCC                                                    | 146               | ACCGTTGACTTCTGTTTGC |
| PPARγ2 | CTCCTTGTGCAAGCAGCCAGC                                                  | 160               | TCAAGAGGTTGAGGAGGTC |
| Adipin | GACACCATGGCAAGCAGCCAGCCAGC                                              | 127               | CCACTTGCCAGAGGCTTCC |
| β-actin| GATCAGTTAAGCTTCCTGAG                                                  | 157               | GATCAGTTAAGCTTCCTGAG |

Statistical analysis

CFU-F and population doubling data are presented as mean ± standard deviation. Real-time PCR are presented as mean ± standard error. SPSS software (version 17.0; IBM, Armonk, NY, USA) was used for this statistical analysis. Student t test for unpaired data was used with P < 0.05 considered to be statistically significant. All in vitro experiments were performed in triplicate.

Results

Growth and proliferation of human bulk-cultured GFs and GMSCs

Primary cultures of single cell suspensions from human gingival tissues exhibited a spindle-shaped fibroblast-like morphology (Figure 1A), and cells formed MSC-like colonies after 10–14 days culture in a low density (Figure 1B). All trials of a single-cell-derived colony forming efficiency were successfully performed. Fourteen days after seeding, CFU-Fs were observed in both types of cells. Bulk-cultured GFs established fewer new colonies and showed a more diffuse distribution compared with GMSCs seeded in the same conditions (Figures 1C and 1E). Cell clusters derived from a single colony of bulk-cultured GFs had lesser cell numbers and exhibited a more sparse appearance compared with GMSCs (Figures 1D and 1F). The number of CFU-Fs was significantly higher in the GMSCs cultures than bulk-cultured GFs at either seeding density (t test, P < 0.001; Figure 1G). Both bulk-cultured GFs and GMSCs exhibited long-term proliferation capacity exceeding 12 passages in culture. The total population doublings of GMSCs (40.95 ± 4.19) was significantly lower than that of bulk-cultured GFs (54.52 ± 2.51; t test, P < 0.001; Figure 1H).

Flow cytometric analysis

By flow cytometry analyses, bulk-cultured GFs and GMSCs demonstrated similar expression levels of surface markers. The two types of cells were uniformly positive for MSC-associated makers CD44, CD73, CD90, CD105, and CD166, and were negative for the hematopoietic stem cell markers CD14, CD34, and CD45. The only distinct surface marker was...
Figure 1 Characterization of human bulk-cultured gingival fibroblasts (GFs) and gingiva-derived mesenchymal stem cells (GMSCs). (A) Cultured primary cells derived from human gingival tissue exhibited typical fibroblast-like morphology. Scale bar: 500 μm; (B) MSC-like colonies grown for 10–14 days culture. Scale bar: 500 μm; (C) single colonies formed after bulk-cultured GFs; (D) cell clusters derived from a single colony of bulk-cultured GFs; (E) GMSCs were plated at low density and cultured for 2 weeks; (F) GMSCs stained with 0.1% toluidine blue. Scale bar: 500 μm; (G) comparison of the number of colony-forming unit-fibroblasts derived from bulk-cultured GFs and GMSCs at 500 cells and 1000 cells plated per well (data were obtained from 6 individual experiments); (H) population doublings of bulk-cultured GFs and GMSCs following successive cell passages until cellular senescence was reached (data represent 15 GF lines and 15 clonal GMSC lines derived from 3 gingival tissues). Data are presented as mean ± standard deviation. * P < 0.001.
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Figure 2  (A) Flow cytometry analyses of the expression of cell surface markers related to mesenchymal stem cells (CD44, CD73, CD90, CD105, CD166, and STRO-1); (B) flow cytometry analyses of the expression of cell surface markers related to hematopoietic stem cells (CD14, CD34, and CD45) (red line). Isotype controls 1B5 (immunoglobulin G-1) and 1D4.5 (immunoglobulin G-2a) are represented by a black line. GF = gingival fibroblasts; GMSCs = gingiva-derived mesenchymal stem cells.
STRO-1, which was more highly expressed in GMSCs (13.4%) than in bulk-cultured GFs (0.02%; Figures 2A and 2B).

**Multilineage differentiation potential of bulk-cultured GFs and GMSCs**

The multilineage differentiation potential of bulk-cultured GFs and GMSCs was determined by culturing cells in lineage specific culture conditions. After 28 days of culture in osteogenic induction medium, GMSCs showed formation of mineralized nodules or aggregates. Calcium mineralization was confirmed by Alizarin Red S staining (Figure 3A). Bulk-cultured GFs showed no Alizarin Red S-positive mineralized nodules (Figure 3B). After 28 days of culture in adipogenic induction medium, GMSCs showed formation of lipid droplets that were positively stained with Oil Red O (Figure 3C) while no lipid droplets formed in bulk-cultured GFs (Figure 3D). After 28 days of culture in chondrogenic induction medium, cell pellets of GMSCs exhibited synthesis of collagen type II using immunohistochemistry (Figure 3E). Bulk-cultured GFs were negative for immunohistochemical staining of collagen type II (Figure 3F).

Quantitative real time-PCR analyses were performed to investigate the expression of differentiation-related genes by GMSCs and bulk-cultured GFs following culture in induction media for 28 days. GMSCs demonstrated in vitro osteogenic differentiation capacities, mRNA expression levels were significantly increased for osteogenic-associated markers RUNX2/CBFA1, OPN, and BSP2 after culture in osteogenic medium (t test, P < 0.001; Figure 4A). By contrast, bulk-cultured GFs lacked the capacity to undergo osteogenic differentiation, mRNA expression levels of

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**Figure 3** Representative images demonstrated multilineage differentiation of bulk-cultured gingival fibroblasts (GFs) and gingiva-derived mesenchymal stem cells (GMSCs). (A) Alizarin Red staining of the osteogenically stimulated GMSCs; (B) Alizarin Red staining of the osteogenically stimulated bulk-cultured GFs (scale bar: 200 μm); (C) Oil Red O staining of the adipogenically stimulated GMSCs; (D) Oil Red O staining of the adipogenically stimulated bulk-cultured GFs (scale bar: 50 μm); (E) Immunohistochemical staining with anticollagen type II antibody of the chondrogenically stimulated GMSCs; (F) Immunohistochemical staining with anticollagen type II antibody of the chondrogenically stimulated bulk-cultured GFs (scale bar: 100 μm).
Figure 4  Real time-polymerase chain reaction analyses of osteogenic, adipogenic, and chondrogenic-related gene expression after 28 days. (A) Representative gene expression levels of osteogenic markers RUNX2/CBFA1, OPN, and BSP2 in gingiva-derived mesenchymal stem cells (GMSCs) and bulk-cultured gingival fibroblasts (GFs) in nonosteogenic (control) and osteogenic medium; (B) representative gene expression levels of adipogenic markers leptin, PPARγ2, and adipsin in GMSCs and bulk-cultured GFs in nonadipogenic (control) and adipogenic medium; (C) representative gene expression levels of chondrogenic markers aggrecan, COL-II, and SOX9 in GMSCs and bulk-cultured GFs in nonchondrogenic (control) and chondrogenic medium. Data are presented as mean ± standard error. * P < 0.001.
osteoigenic-associated markers showed no significantly different when compared with control groups (t test, P > 0.05; Figure 4A). Similarly, adipogenic and chondrogenic differentiation of GMSCs resulted in the higher expression of adipogenic-associated markers leptin, adipin, and PPARγ2, and chondrogenic-associated markers aggregan, COL-II, and SOX9 (t test, P < 0.001; Figures 4B and 4C). Adipogenic differentiation or chondrogenic differentiation was not evident in bulk-cultured GFs by no significant difference of gene expression levels of related markers leptin, adipin, PPARγ2, aggregan, COL-II, and SOX9 (t test, P > 0.05; Figures 4B and 4C).

Discussion

Gingival tissue overlying the alveolar bone of tooth satchets plays an important role in the maintenance of oral health. It exhibits fetal-like scarless healing processes after wounding, suggesting that unique types of MSCs reside in gingival tissue. Zhang et al. firstly isolated a population of progenitor cells within gingival tissues termed GMSCs, which showed characteristics of self-renewal and multipotent differentiation and immunomodulatory in vivo and in vitro. Recent studies have also shown that proper manipulation of GMSCs is essential in tissue engineering. GMSCs represent a more widely available population for therapeutic applications because of the accessibility of human gingival tissues. The establishment of optimal methods for GMSCs collection is important for the application of GMSCs in stem cell based regeneration medicine and tissue engineering. Since GMSCs are collected from gingival tissues, they may contain various subpopulations of cells. The heterogeneous cell populations may impair the self-renewal capacity and multipotent differentiation potentials of GMSCs. Therefore, to isolate high purity GMSCs and prevent their contamination with fibroblasts is critically important for their applications. Cell sorting technologies with certain surface markers with MACS or flow cytometry are the most common methods, which can select a specific subpopulation enriched with certain surface makers. However, specific selection of GMSCs from such heterogeneous cell populations is thus not sufficient enough with these methods because no specific cell surface markers are available for isolating GMSCs and the surface markers applied to heterogeneous populations of stem cells are gradually lost during culture. Many other methods for generating GMSC subpopulations have been proposed recently in the literature, including the use of nonadherent dish and chitosan membranes. Although these methods may make MSC spheroids, they were unable to select a highly potent cell population from the heterogeneous populations of GFs or to affect the isolation effect partially via the regulation of different cadherin molecules for each subpopulation. In this study, we isolated clonal GMSC lines from one single primary cell using a limiting dilution method. The cell population can be easily selected and isolated by observing the cell viability and cell proliferation rate. The selected colonial GMSC populations were homogeneous and without contamination of fibroblasts or endothelial cells. GMSCs displayed stable morphology and did not lose MSC characteristics at higher passages. Upon induction, they could be differentiated into osteoblasts, adipocytes, chondrocytes, while tissue-matched bulk-cultured GFs lack many of the predefined stem cells characteristics.

The morphology of GMSCs showed no discernible difference compared with that of bulk-cultured GFs. They all displayed spindle-shaped, fibroblast cell morphology. The analysis of their growth characteristics showed remarkable differences. GMSCs have a higher number of CFU-Fs in vitro than bulk-cultured GFs, while a slight reduction in the population doubling capacity of GMSCs was observed. We speculate that our bulk-cultured GFs from gingival tissue are an abundance of highly proliferative cells that lack of stem/progenitor cells. The high proliferative abilities of gingiva-derived cells are also well in accordance with the fact that gingiva have high healing and regenerative abilities. In addition, the high proliferative and colony forming abilities for GMSCs are beneficial for the regenerative applications in terms of easy in vitro duplication.

The phenotypic characterization of GMSCs has been extensively documented. However, it is unknown whether these surface markers are present or absent in bulk-cultured GFs. A recent report demonstrated that human gingival margin-derived STRO-1/MACS cells positively expressed the hematopoietic markers CD34 and CD45, which are not normally expressed in mesenchymal stem/progenitor cells. GMSCs collected from gingival tissues may be contaminated with various types of cells such as hematopoietic stem cells because gingiva has a dense vasculature. In the present study, we detected the expression of CD44, CD73, CD90, CD105, CD166, CD14, CD34, and CD45 in GMSCs and GFs using flow cytometry, in accordance with the marker expression profile defined for multipotent stromal cells in the International Society for Cellular Therapy position statement. GMSCs showed all of the previously defined classical characteristics of multipotent postnatal stem/progenitor cells. The results showed that GMSCs were positive for CD44, CD73, CD90, CD105, CD166, (all above 95%), and STRO-1, and negative for CD14, CD34, and CD45. The immunophenotypic profiles further verified the stromal origin of our cell culture without hematopoietic precursor contamination. We compared the levels of the markers in human GMSCs and bulk-cultured GFs. None of the cell antigens, including CD44, CD73, CD90, CD105, CD166, CD14, CD34, and CD45 differed between GMSCs and bulk-cultured GFs. The only distinct surface marker was STRO-1, which was more highly expressed in GMSCs than in bulk-cultured GFs. STRO-1 antigen is one of the important early markers of MSCs, and it can serve as an index for sorting bone marrow-derived MSCs in high purity and it can also be used to isolate a homogeneous stem cell populations from periodontal ligament. A recent study indicated that a MSC-like subpopulation can be isolated from gingival tissues via STRO-1/MACS. However, the STRO-1 expression of heterogeneous populations of stem cells was gradually lost during culture expansion in vitro and the decreased level of STRO-1 expression after the magnetic sorting step has been previously described. Thus, a time dependent quantification of the shift in the marker expression profile could be clinically employed. In the present study, the expression level of STRO-1 in GMSCs was 13.4%, and bulk-cultured GFs showed STRO-1 expression at a low level (0.02%). Our results first demonstrated that the levels of these surface markers in bulk-cultured GFs were similar to those in GMSCs, and
indicated MSC populations isolated using these surface markers may be contaminated by GFs. Identification of specific markers for GMSCs distinguishing from fibroblasts should be developed and further identification using molecular and genetic approaches are required.

We then investigated whether GMSCs maintained the multilineage differentiation potential in the capacity to form mineral, fat, and cartilage-like matrix in vitro, compared with bulk-cultured GFs. Previous studies demonstrated that dental stem cells including human exfoliated deciduous teeth, dental pulp stem cells, and periodontal ligament stem cells, as mentioned, can give rise to odontoblasts, adipogenic, and chondrogenic tissues. GMSCs in this study showed multilineage differentiation potentials; by contrast, bulk-cultured GFs lacked the capacity to undergo osteogenic, adipogenic, or chondrogenic differentiation. The results were further supported by mRNA expression levels of osteogenic, adipogenic, and chondrogenic lineage-specific genes following induction in appropriate media for 28 days. It appears that the gingival tissue contains a minor subset of stem cells with multilineage differentiation potential. The results are consistent with recent reports that the percentages of MSC-like cells in heterogeneous cell populations of gingival tissue vary significantly and can be as low as 3–6%. We speculate that the differentiation potential of our bulk-cultured GFs is masked by the abundance of highly proliferative cells that lack the ability to undergo multilineage differentiation.

Our findings showed that human gingival tissues contain a population of multipotent postnatal stem cells that can be isolated with limiting dilution method and expanded in vitro, providing a unique reservoir of stem cells from an accessible tissue source. Whether a specific marker expression profile could be clinically employed to identify the GMSCs distinguishing them from fibroblasts and other stem/progenitor cells remains a very interesting point to be investigated.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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