ORIGINAL ARTICLE

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 makers 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.

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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.[5,6]

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.[6–8] 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 the formation of connective tissue-like structures *in vivo*.[15] A few recent studies also demonstrated that GMSCs possessed osteogenic potential *in vivo* after incubation under osteo-inductive medium *in vitro*.[16,17] These properties indicate that the clinical use of GMSCs is an attractive therapeutic option for tissue regeneration and repair. However, it is still problematic as to which isolation method is to be favored when obtaining GMSCs. Various processes have been reported to isolate MSCs, while cell sorting technologies with certain surface markers by flow cytometry or magnetic activated cell sorting (MACS) are the most common methods.[18,19] However, to date, no specific cell surface markers have been available for isolating GMSCs. Recent reports indicated that there is 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[23] 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.

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/cm^2 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% CO$_2$). 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

were isolated using colony rings and expanded using individual tissue culture flask, as previously described.[30,31] Briefly, single colonies ($\geq$ 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-cm^2 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% CO$_2$). 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 $\geq$ 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.[32] Briefly, the two types of cells were seeded at 5×10^3 cells/cm^2 in 24-well plates respectively. After reaching 90% confluence, they were detached with 0.05% trypsin/EDTA. Cells were then reseeded at 5×10^3 cells/cm^2 into another well of a 24-well plate and cultured until in vitro cellular senescence was reached. Cell counts were performed at each passage and population doublings calculated using the formula (log$_2$ final cell number/seeding cell number). The final population doubling value for each type of cell was represented as the accumulated population doubling values obtained at each passage.

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 1×10^5 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 Stro-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 Epics-XL/MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Multilineage differentiation potential

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 8×10^3 cells per cm^2 as previously described.[6] 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 (KH$_2$PO$_4$; 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).

Adipogenic differentiation

For adipogenic differentiation, bulk-cultured GFs and GMSCs were cultured on six-well and 24-well plates in adipogenic inductive medium at a density of 8×10^3 cells per cm^2 as previously described.[6] Adipogenic inductive medium was αMEM supplemented with 10% FCS, 100mM L-ascorbate-2-phosphate, 1mM sodium pyruvate, 50-μg/mL streptomycin, 50-U/mL penicillin G, 2Mm L-glutamine, 0.1μM dexamethasone, and 60μM indomethacin (Sigma-Aldrich). 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 for 1 hour at room temperature. The presence of lipid drops was detected in 24-well plates by staining the cells with Oil Red O (Sigma-Aldrich). Six-well plates designated for real time-PCR were rinsed three times in PBS and RNA extracted using TRIzol according to manufacturer's instructions.

Chondrogenic differentiation

For chondrogenic differentiation, bulk-cultured GFs and GMSCs (1×10^6 cells) were transferred into 10-mL yellow-top polypropylene tubes and centrifuged at 600g at 4°C

for 5 minutes to form cell pellets at the bottom of the tube. Cell pellets were cultured in chondrogenic induction medium for 28 days as previously described.[14] Chondrogenic induction medium was high-glucose Dulbecco's modified Eagles's 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, 2Mm 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 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 RT^2 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 15 seconds, 60°C for 25 seconds, and 72°C for 10 seconds), and final extension at 72°C for 3 minutes. Primer sets examined included *RUNX2/CBFA1, OPN,* and *BSP2* (for osteogenic differentiation), *leptin, adipsin,* 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).

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 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

**Table 1** Primer sequences for osteogenic, adipogenic, and chondrogenic markers.

| Gene	Product size (bp)	Forward primer 5′−3′	Reverse primer 3′−5′
Runx2	137	GTGGACGAGGCAAGAGTTTCA	CATCAAGCTTCTGTCTGTGCC
OPN	92	GCAGACCTGACATCCAGTACC	GATGGCCTTGTATGCACCATTC
BSP2	123	ACATCCAGTACCCTGATGCTACAG	GTGGGTTTCAGCACTCTGGT
Aggrecan	191	CTGCTTCCGAGGCATTTC	GCTCGGTGGTGAACTCTAGG
COL-II	181	GTTGGGAGTAATGCAAGGACC	ACCATCATCACCAGGCTTTC
SOX9	219	AGGTGCTCAAAGGCTACGAC	GCTTCTCGCTCTCGTTCAGA
Leptin	146	GGCTTTGGCCCTATCTTTTC	ACCGGTGACTTTCTGTTTGG
PPARγ2	160	CTCCTATTGACCCAGAAAGC	TCAAAGGAGTGGGAGTGGTC
Adipsin	127	GACACCATCGACCACGAC	CCACGTCGCAGAGAGTTC
β-actin	157	GATCATTGCTCCTCCTGAGC	GTCATAGTCCGCCTAGAAGCAT
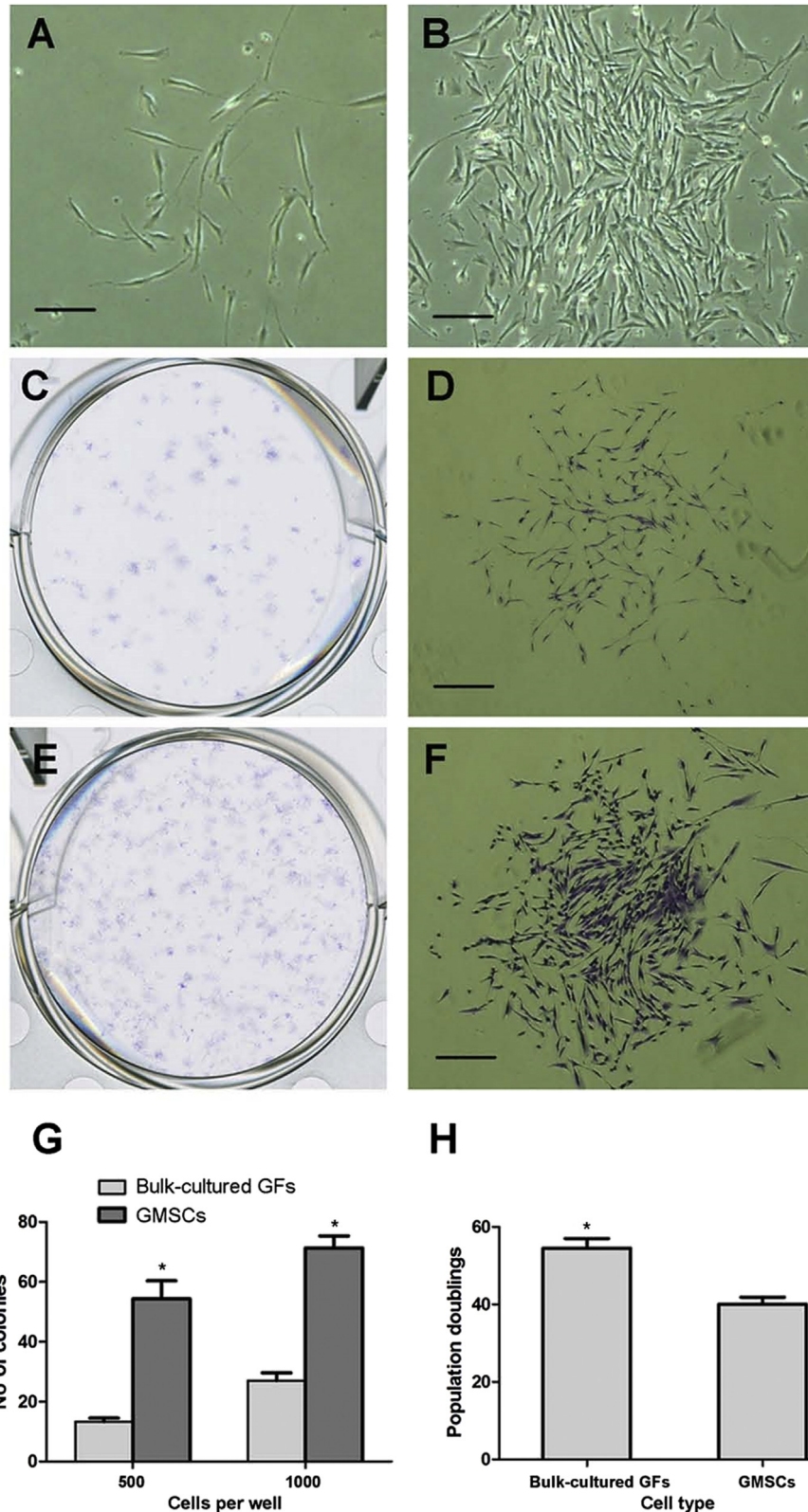
Figure 1 Characterization of human bulk-cultured gingival fibroblasts (GFs) and gingiva-derived mesenchymal stem cells (GMSCs). (A) Cultured primary cells derived from human gingival tissue exhibited typical fibroblast-like morphology. Scale bar: 500 μm; (B) MSC-like colonies grown for 10—14 days culture. 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. * $P < 0.001$.
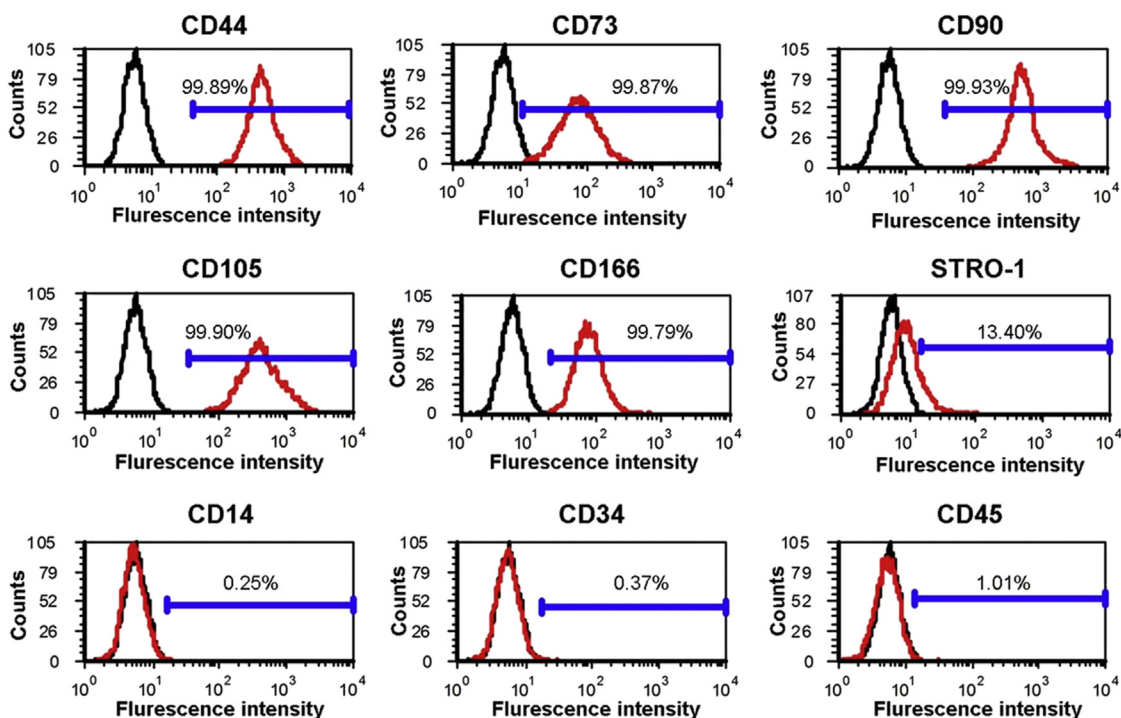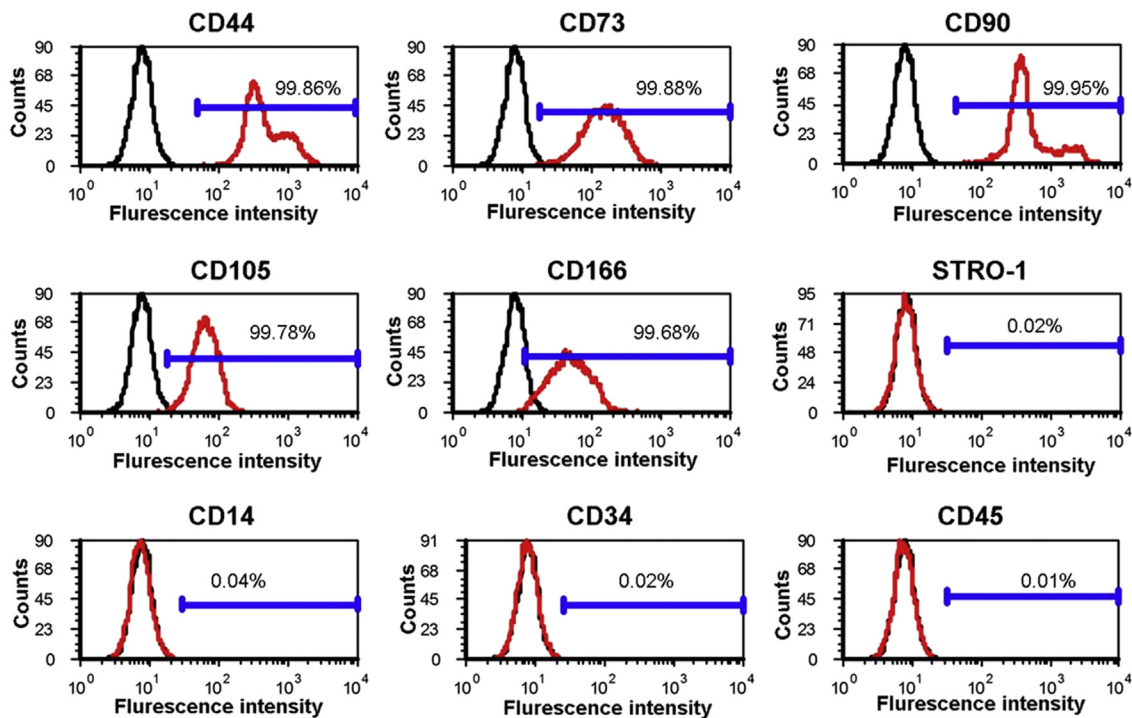
Figure 2 (A) Flow cytometry analyses of the expression of cell surface markers related to mesenchymal stem cells (CD44, CD73, CD90, CD105, CD166, and STRO-1); (B) flow cytometry analyses of the expression of cell surface markers related to hematopoietic stem cells (CD14, CD34, and CD45) (red line). Isotype 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
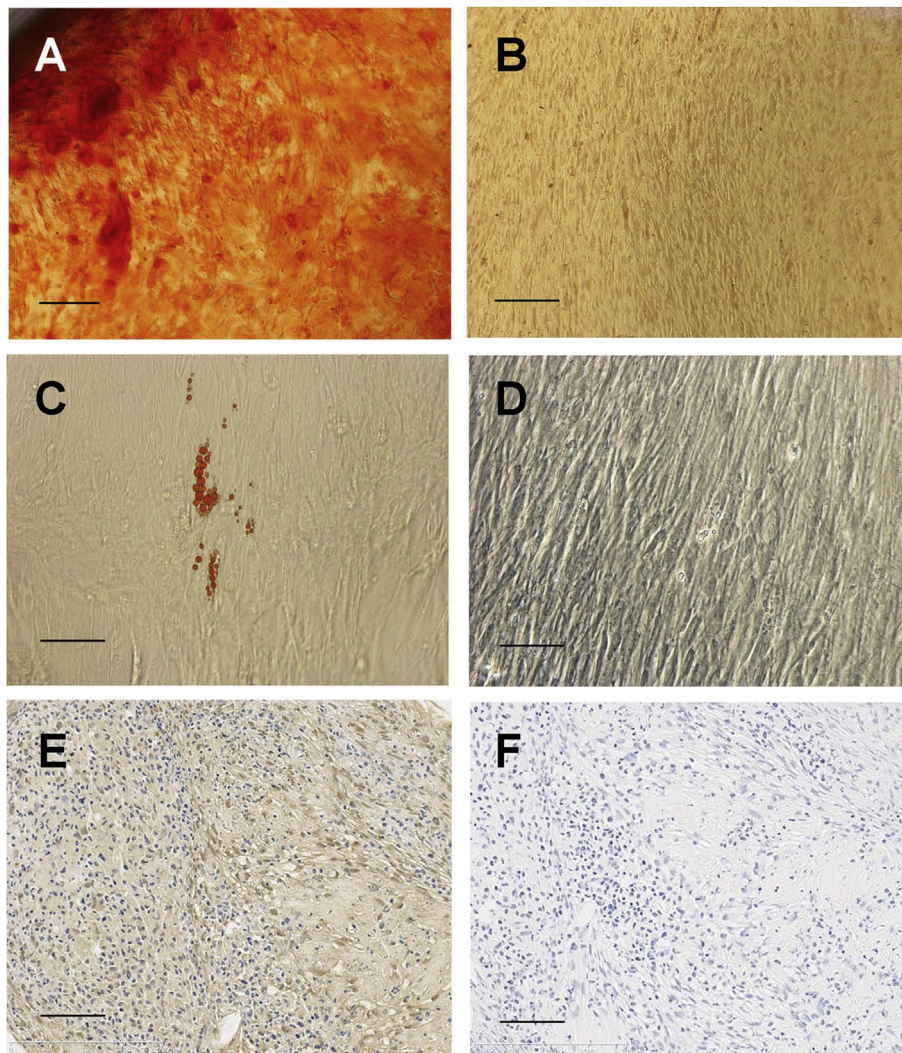
Figure 3 Representative imagines demonstrated multilineage differentiation of bulk-cultured gingival fibroblasts (GFs) and gingiva-derived mesenchymal stem cells (GMSCs). (A) Alizarin Red staining of the osteogenically stimulated GMSCs; (B) Alizarin Red staining of the osteogenically stimulated bulk-cultured 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).
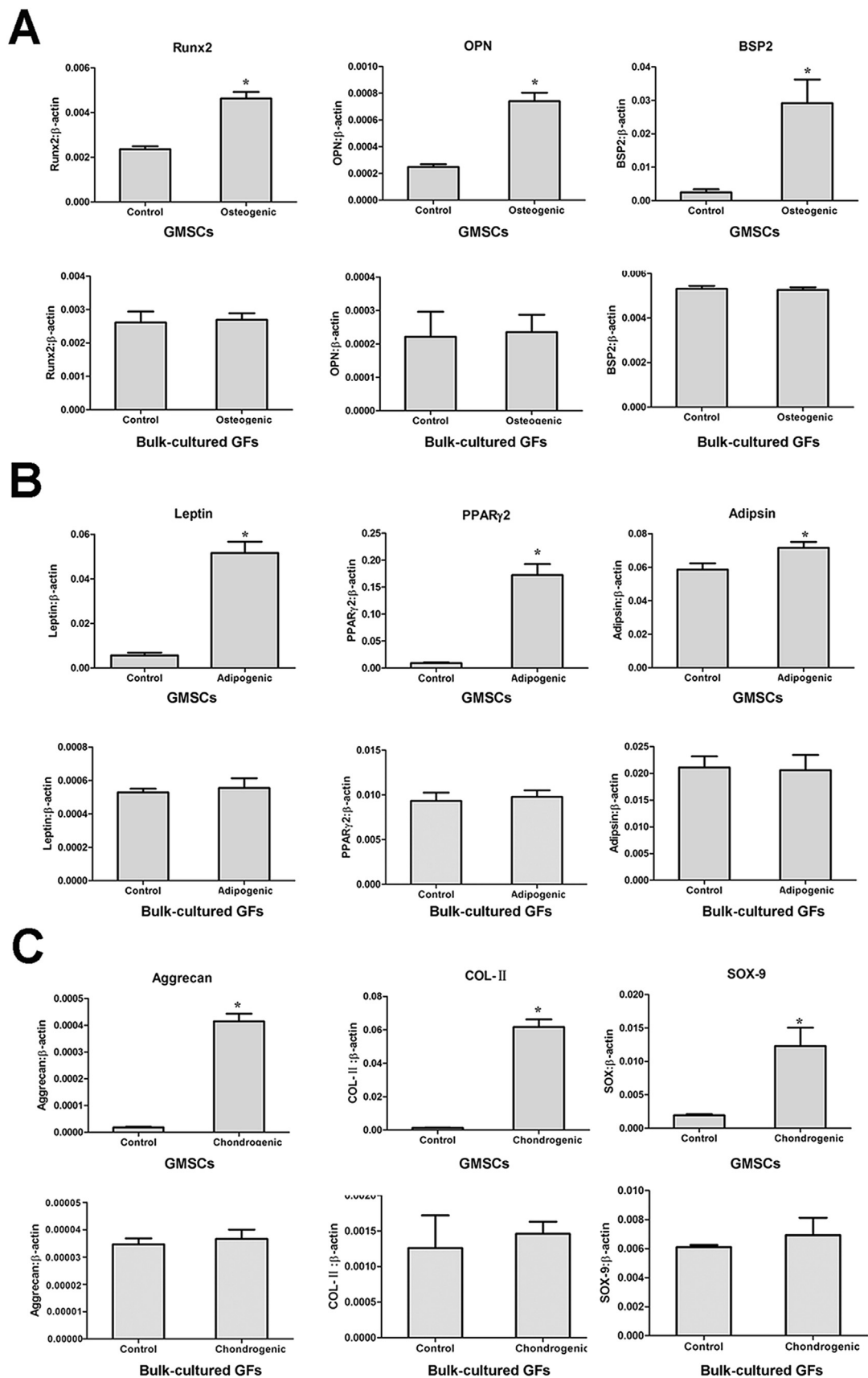
Figure 4 Real time-polymerase chain reaction analyses of osteogenic, adipogenic, and chondrogenic-related gene expression after 28 days. (A) Representative gene expression levels of osteogenic markers *RUNX2/CBFA1*, *OPN*, and *BSP2* in gingiva-derived mesenchymal stem cells (GMSCs) and bulk-cultured 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.

osteogenic-associated markers showed no significantly difference 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, adipsin*, and *PPARγ2*, and chondrogenic-associated markers *aggrecan, 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, adipsin, PPARγ2, aggrecan, COL-II*, and *SOX9* (*t* test, P > 0.05; Figures 4B and 4C).

Discussion

Gingival tissue overlying the alveolar bone of tooth sachets 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[15] 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.[16,17] 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.[20–22] Many other methods for generating GMSC subpopulations have been proposed recently in the literature, including the use of nonadherent dish and chitosan membranes.[33,34] 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 colonal 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, fibroblastic 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.[16,32] 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.[35] 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.[36] GMSCs showed all of the previously defined classical characteristics of multipotent postnatal stem/progenitor cells.[16,32,36] 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[37,38] and it can also be used to isolate a homogeneous stem cell populations from periodontal ligament.[11] A recent study indicated that a MSC-like subpopulation can be isolated from gingival tissues via STRO-1/MACS.[35] 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.[20–22] 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, adiopogenic, and chondrogenic tissues.[7,8,11] 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%.[12,15,33] 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.

Acknowledgments

References

1. Halleux C, Sottile V, Gasser JA, Seuwen K. Multi-lineage potential of human mesenchymal stem cells following clonal expansion. *J Musculoskelet Neuronal Interact* 2001;2:71—6.
2. Lee RH, Kim B, Choi I, et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* 2004;14:311—24.
3. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004;103:1669—75.
4. Yen BL, Huang HI, Chien CC, et al. Isolation of multipotent cells from human term placenta. *Stem Cells* 2005;23:3—9.
5. Caplan AI. Mesenchymal stem cells. *J Orthop Res* 1991;9:641—50.
6. Gronthos S, Zannettino AC, Hay SJ, et al. Molecular and cellular characterization of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* 2003;116:1827—35.
7. Graziano A, d'Aquino R, Laino G, Papaccio G. Dental pulp stem cells: a promising tool for bone regeneration. *Stem Cell Rev* 2008;4:21—6.
8. Wang J, Wang X, Sun Z, et al. Stem cells from human-exfoliated deciduous teeth can differentiate into dopaminergic neuron-like cells. *Stem Cells Dev* 2010;19:1375—83.
9. Ding G, Wang W, Liu Y, et al. Effect of cryopreservation on biological and immunological properties of stem cells from apical papilla. *J Cell Physiol* 2010;223:415—22.
10. Yao S, Pan F, Prpic V, Wise GE. Differentiation of stem cells in the dental follicle. *J Dent Res* 2008;87:767—71.
11. Seo BM, Miura M, Gronthos S, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;364:149—55.
12. Fournier BP, Ferre FC, Couty L, et al. Multipotent progenitor cells in gingival connective tissue. *Tissue Eng Part A* 2010;16:2891—9.
13. Mitrano TI, Grob MS, Carrión F, et al. Culture and characterization of mesenchymal stem cells from human gingival tissue. *J Periodontol* 2010;81:917—25.
14. Tomar GB, Srivastava RK, Gupta N, et al. 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—83.
15. Zhang Q, Shi S, Liu Y, et al. 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—98.
16. Wang F, Yu M, Yan X, et al. Gingiva-derived mesenchymal stem cell-mediated therapeutic approach for bone tissue regeneration. *Stem Cells Dev* 2011;20:2093—102.
17. Yang H, Gao LN, An Y, et al. Comparison of mesenchymal stem cells derived from gingival tissue and periodontal ligament in different incubation conditions. *Biomaterials* 2013;34:7033—47.
18. Hackett CH, Flaminio MJ, Fortier LA. Analysis of CD14 expression levels in putative mesenchymal progenitor cells isolated from equine bone marrow. *Stem Cells Dev* 2011;20:721—35.
19. Battula VL, Treml S, Bareiss PM, et al. Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. *Haematologica* 2009;94:173—84.
20. Fawzy El-Sayed KM, Paris S, Becker S, et al. Isolation and characterization of multipotent postnatal stem/progenitor cells from human alveolar bone proper. *J Craniomaxillofac Surg* 2012;40:735—42.
21. Boxall SA, Jones E. Markers for characterization of bone marrow multipotential stromal cells. *Stem Cells Int* 2012;2012:975871.
22. Ishii M, Koike C, Igarashi A, et al. Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts. *Biochem Biophys Res Commun* 2005;332:297—303.
23. Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* 1999;107:275—81.

24. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284: 143—7.

25. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 2002;20: 530—41.

26. Nagatomo K, Komaki M, Sekiya I, et al. Stem cell properties of human periodontal ligament cells. *J Periodont Res* 2006;41: 303—10.

27. Du L, Yang P, Ge S. Stromal cell-derived factor-1 significantly induces proliferation, migration, and collagen type I expression in a human periodontal ligament stem cell subpopulation. *J Periodontal* 2012;83:379—88.

28. 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—9.

29. Feng F, Akiyama K, Liu Y, et al. Utility of PDL progenitors for *in vivo* tissue regeneration: a report of 3 cases. *Oral Dis* 2010; 16:20—8.

30. Kuznetsov SA, Krebsbach PH, Satomura K, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation *in vivo*. *J Bone Miner Res* 1997;12: 1335—47.

31. Gronthos S, Brahim J, Li W, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;81:531—5.

32. Ge S, Mrozik KM, Menicanin D, Gronthos S, Bartold PM. Isolation and characterization of mesenchymal stem cell-like cells from healthy and inflamed gingival tissue: potential use for clinical therapy. *Regen Med* 2012;7:819—32.

33. Zhang Q, Nguyen AL, Shi S, et al. Three-dimensional spheroid culture of human gingiva-derived mesenchymal stem cells enhances mitigation of chemotherapy-induced oral mucositis. *Stem Cells Dev* 2012;21:937—47.

34. Hsu SH, Huang GS, Feng F. Isolation of the multipotent MSC subpopulation from human gingival fibroblasts by culturing on chitosan membranes. *Biomaterials* 2012;33:2642—55.

35. El-Sayed KM, Paris S, Graetz C, et al. Isolation and characterization of human gingival margin-derived STRO-1/MACS+ and MACS− cell populations. *Int J Oral Sci* 2015;7:80—8.

36. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8:315—7.

37. Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther* 2007;9:204.

38. 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.