Research Article

Stem Cell Research International

Human Gingival Fibroblast: Single Colonies vs. Heterogeneous Culture, Characterization and Osteogenic Differentiation

Samira Diar-Bakirly*, Harmanpreet Kaur, Douglas N. Dederich, Paul Major and Tarek El-Bialy

Faculty of Medicine and Dentistry, University of Alberta Senate, Canada

***Corresponding author**
Samira Diar-Bakirly, Faculty of Medicine and Dentistry, 14758 47 Ave NW T6H5L4, Canada, E-mail: diarbaki@ualberta.ca

Submitted: 10 Jan 2018; **Accepted**: 17 Jan 2018; **Published**: 21 Jan 2018

Abstract

Cells with mesenchymal stem cell (MSC) properties were successfully isolated and characterized from different dental tissues. Amongst these cells are the human gingival fibroblast cells (GFs). Their use in tissue engineering is promising. However, choosing the right cellular population is one of several factors that are necessary for a successful tissue engineering approach. In order to better choose which population of cells to use, we isolated the GFs single colonies. We identified them, osteogenically induced them and compared them to the heterogeneous culture of these cells.

Materials and methods: *GFs cells were extracted from human gingival tissue; incubated to confluency. After which they were counted, serially diluted and seeded in 6 well plates. The cells were observed daily to locate the first formed colonies. Borosilicate cylinders were used to pick up the colonies. Flow cytometry was used to identify Stem cells surface markers to compare single colonies and heterogeneous cultures. The cells were then osteogenically induced for 21 days. The following assays were performed to compare the osteogenic potential between single colonies and heterogeneous cultures; Calcium assay, ALP/DNA specific activity, RT-qPCR for osteogenic related genes (OPN, OCN, ALP) and western blot analysis.*

Results: *All the assays results were consistent in revealing an increased osteogenic differentiation potential of the heterogeneous culture of the GFs over the single colonies cultures. These results indicate that the heterogeneous cultures of GFs have a higher stem cell population and subsequent osteogenic differentiation potential than the single cell colonies' cultures.*

Keywords: Gingival Fibroblasts, Osteogenic Differentiation, Dental stem cells

Introduction

The predominant cellular component of the gingival connective tissue is fibroblast. Their role in maintenance, development and remarkably good healing and regenerative capacity of the gingival tissues is conspicuous [1].

Cells with properties were successfully isolated and characterized from different dental tissues, such as dental pulp stem cells (DPSC), cells from exfoliated deciduous teeth SHED, periodontal human stem cells (PDLSC), stem cells from apical papilla (SCAP), dental follicle progenitor cells (DFPC) and human gingival fibroblast cells (hGFs) [2-7]. These cells were capable of differentiating into osteo/odontogenic, adipogenic, neurogenic and chondrogenic cellular phenotypes. In addition, they express stem cell surface markers indicating their mutlipotency [2-4].

Human gingival fibroblasts' embryonic origin from neural crest cells is believed to be the reason behind their distinct phenotypic characteristics [1]. Several recent studies have highlighted the potential of the hGFs to be readily reprogrammed into induced pluripotent stem cells too [8-10]. This potential of using the hGFs' self-renewal and pluripotency has a great implication of their use in future clinical tissue engineering purposes. Thus, overcoming ethical concerns of using the embryonic stem cells besides immune rejection.

However, despite the fact that the human gingival fibroblasts excrete stem cell factors, their stemness needs further investigation, as it remains unclear [11]. Several *in-vivo* and *in-vitro* studies have shown that tissue-engineered hGFs were successfully used to augment gingival tissue intra-orally or used for extra-oral regenerative applications [12,13]. Furthermore, using GFs stem cells for periodontal and gingival tissue regeneration is a great promise in tissue engineering over the traditional approaches of using surgical treatments and guided tissue membranes [14,15]. Many reasons were identified to prove that GFs are the first choice for oral tissue engineering purposes such as easy accessibility and less donor site morbidity besides less patient discomfort [5].

Tissue engineering aims at providing the right stimulus to the stem cells to differentiate and provide tissues internally in an *in-vivo* or externally in an *ex-vivo* environment [16]. Choosing the right cellular population is one of several factors that are necessary for a successful tissue engineering approach However, isolation of specific cell types out of the heterogeneous culture of cells besides the detailed identification is still an issue to overcome [17,18].

Because of the lack of the specific surface marker for MSC characterization and heterogeneity of gingival MSC sub populations, more recent studies are seeking to identify a specific mesenchymal stem cell marker in order to target the population of gingival stem cells to be used in tissue engineering protocols [19-21]. In this pilot study, our attempt was to understand if there is a difference between single colonies of GFs and the remaining population of cells after differentiation. We hypothesized that single colonies from GFs cultures are enriched with mesenchymal stem cells. And they show higher expression of osteogenic related genes compared to the heterogeneous culture of these cells.

Materials and Methods

Gingival fibroblast isolation and culture

This project was approved by the ethics approval board at university of Alberta, protocol number (Pro00056111). This study is a pilot study, Gingival interdental papilla biopsy was obtained from dental patient during teeth extraction procedure n=1 the patient was a healthy female under the age of 30Y with no medical or dental history. The tissues were immediately placed in a medium containing DMEM with fetal bovine serum (FBS) and 100U/mL penicillin and 100µg/mL streptomycin (Thermofisher Gibco®). The tissues obtained were then diced and minced after which they were digested in a medium containing collagenase IV (2mg/mL; Sigma-aldrich pty. Ltd. Aus) for half an hour at 37˚C humidified incubator. The gingival tissue chunks were discarded and the cells were seeded with α-MEM containing 10% FBS and 1% Penicillin-Streptomycin Solution (10,000U/mL Pen 10,000ug/mL Strep) (Thermofisher Gibco®) in T-25 flask (Falcon® Tissue Culture Flasks, Sterile, Corning®) until the flask was confluent. The cells were then detached using Accutase® solution (Sigma-Aldrich Co.) and transferred to T-75 tissue culture flask where the cells were incubated to grow to confluency in 37˚C humidified incubator with 5% CO_2 using the latter medium prepared.

Serial dilution, seeding and colony harvest

Once confluent, the cells were de-attached using Accutase from T-75 flask. They were counted, and serially diluted to reach a seeding density of 500 cells/ well of a 6 well plate. The cells were observed daily to locate the first formed colonies; the colonies were marked and located under the light microscopy. A grid was used to count the number of cells in each colony; at day 8, the colonies that were distant from each other, with a cell count of 80-120 cells were selected for de-attachment. A borosilicate glass cylinders 6 mm ID x 5mm having an optically flat polished end that hydrostatically seals to another flat optical surface (Bioptechs Inc. Headquarters) were used.

In each cylinder, the medium was sucked out, 100µL of HBSS was used to wash each colony inside the cylinder before using 100µL of accutase to de-attach the colonies and pipet them out of the cylinder and then dispersed into one well of 6 well plates. 12 colonies were picked.

At the same time, the wells of equal seeding density of GFs were left to grow to confluency. The medium was changed every 3-4 days. Triplet wells of this heterogeneous culture of GFs were compared to the triplet samples of the single colony. The cells from P3-P5 were used for further analyses.

Flow Cytometric analysis

To investigate the differences in mesenchymal stem cells surface cell markers between single colonies and heterogeneous cultures, triplet samples of single colonies and heterogeneous cultures in the 6 well plates were used once confluent. Single cell suspensions were washed with HBSS, about 1 x 10^6 cells of GFs were incubated with fluorescence isothiocyanate (FITC) mouse clonal antibodies for human stem cells positive markers CD90, CD105, CD73 and negative hematopoietic markers CD45 and CD34 (BioLegend, San Diego, CA, USA). Cells were analyzed by flow cytometry at the FL1 channel (Quanta; Beckman Coulter), with unstained cells set to 1% as a background control.

Osteogenic differentiating medium

The single colonies wells and heterogeneous wells both were treated then by with osteogenic media to induce osteogenic differentiation using a prepared medium containing DMEM, 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin Solution, with the addition of 10 mM beta glycerol-phosphate (ß-GP), 50 µg/ml ascorbic acid and 10 nM Dexamethasone (Dex) to stimulate mineralization. The medium was changed every 3-4 days. After 21 days of osteogenic induction, the following assays were performed.

DNA content and ALP activity Assay

The wells were washed with HBSS, 2mL of ALP buffer was used in every well and incubated for 2 hours at room temperature. DNA standards were established and DNA content quantified using the CyQUANT® Assay kit (Thermofisher scientific). DNA concentration from every well was calculated using the fluorescent intensity readings (excitation and emission wavelengths of 485 nm and 527 nm respectively) from the 96 black well plate reader. In another 48 well plate 250 µL of the same samples from the wells of both single and heterogeneous groups were added to 250µL of the substrate, the absorbance was quantified at 405 nm. Finally, the ALP activity was normalized by the DNA content (µg/µL) of each sample to determine its specific ALP activity (ALP/DNA).

Calcium assay

The same wells containing lysed cells from ALP assay were washed with HBSS and then 1mL of HCL (0.5 M) was added overnight to dissolve the calcium mineral formed at 4˚C temperature overnight. In a 96 well plate 20 µL from each sample were added to 500µl solution of 2-amino-2-methyl-propan-1-ol (1.5% v/v) and o-cresolphthalein (37 mM) mixed with 50 µl 8-hydroxyquinoline (28 mM) and sulphuric acid (0.5% v/v). Optical absorbance was measured at 570nm using multi-reader plate calcium standards were used to form the standard curve and concentration was expressed in mg/dL.

RNA extraction, cDNA preparation and RT qPCR

The RNA was isolated according to the manufacture's instructions, from the GFs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was then measured using NanoDrop 2000C Spectrophotometer (Thermo Fisher). Primer sets of the marker genes selected (Table 1). The first strand of cDNA was synthesized from after reverse transcription reaction, 1 out of 10 dilutions was

made from the template to be used in real-time qPCR. 10 µl real-time reaction mixture consisting of 3 µl of cDNA, 1 µl each of forward and reverse primers and 5 µl of the master mix containing SYBR green dye. The mixtures were heated at 95º C for 2 min before going through 40 cycles of a denaturation step (15 sec at 95º C) and an annealing step (1 min at 60º C) using 7500 Real-Time PCR system during which the data were collected. Normalized fluorescence was plotted against cycle number (amplification plot), and the threshold suggested by the software was used to calculate Ct (cycle at threshold). Data were analyzed by the $2^{-\Delta\Delta C}{}_{T}$ method for quantification, with normalization to the Ct of the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Omitted Template cDNA from qPCR reaction was used as a negative control.

Table 1: Real-time PCR primers

Marker	Direction	Primer sequence 5'-3'
GAPDH	Forward primer	GAAGTCAGGTGGAGCGAGG
	Reverse primer	GCCCAATACGACCAAATCAGAG
OPN	Forward primer	GCAGCTTTACAACAAATACCCA
	Reverse primer	ACTTACTTGGAAGGGTCTGTGG
OCN	Forward primer	CCTCACACTCCTCGCCCTATT
	Reverse primer	GCTTGGACACAAAGGCTGCAC
ALP	Forward primer	TATCCTGGCTCCGTGCTCC
	Reverse primer	TAACTGATGTTCCAATCCTGCG

Western blot analysis

For immunoblotting analysis, the GFs cells were cultured for 21days with osteogenic differentiation medium. The cells were then washed with cold PBS twice and then lysed using RIPA buffer containing a protease inhibitor. The lysates were used as samples after centrifugation. Protein concentration was calculated from every sample using Pierce™ BCA Protein Assay Kit (Thermofisher) and following the manufacturer's instructions. Cell lysate 40µg out of each sample were resolved on 12% SDS polyacrylamide gel electrophoresis (PAGE). After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 5% Bovine serum albumin in 1% TBS-Tween (Sigma-Aldrich, St. Louis, MO, USA). The membrane was incubated in anti-OPN (diluted 1 to 1000 with 5% BSA in 1% TBS-Tween, Abcam) or anti ß-Actin (diluted 1 to 3000 with 5% BSA in 1% TBS-Tween, Cell signaling) overnight at 4˚C. After incubation with HRP-conjugated anti-rabbit IgG (1:3000, Cell Signaling Technology) for 1 hr at room temperature, the blots were detected with Amersham ECL Western Blotting detection reagent.

Statistical analysis

All the abovementioned assays were performed in triplicate for the 2 groups (Single colonies and Heterogeneous culture), Two-tailed independent Student's t-test and Mann-Whitney test were utilized using SPSS version 12.0 software package (SPSS, Chicago, IL, USA) whenever needed. Statistical significance was marked when p-values < 0.05.

Results

Flow cytometry

The flow cytometry was used to detect and compare CD90, CD105 and CD73 positive stem cells markers and the lack of the hematopoietic cell markers in both the single and heterogeneous cultures. Single colonies and the heterogeneous cultures expressed CD90 and CD105. They did not express CD34, CD45 and CD73 (Figure 1 A, B). The CD90 positive mesenchymal stem cell marker was more statistically significance in the heterogeneous than the single colonies culture.

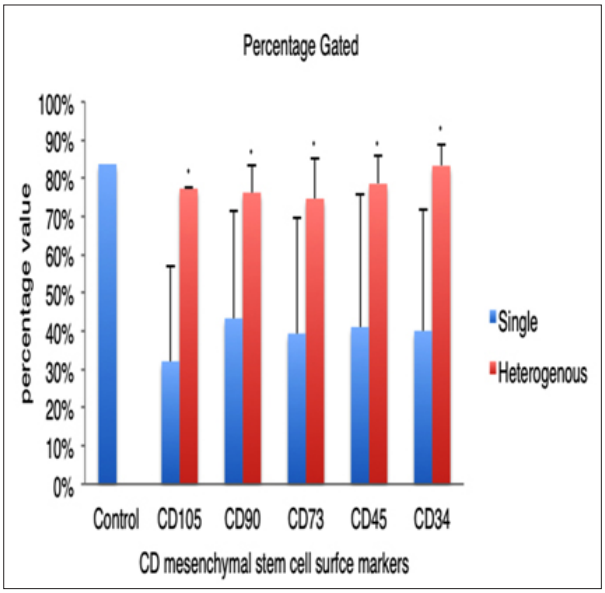

Fig 1A

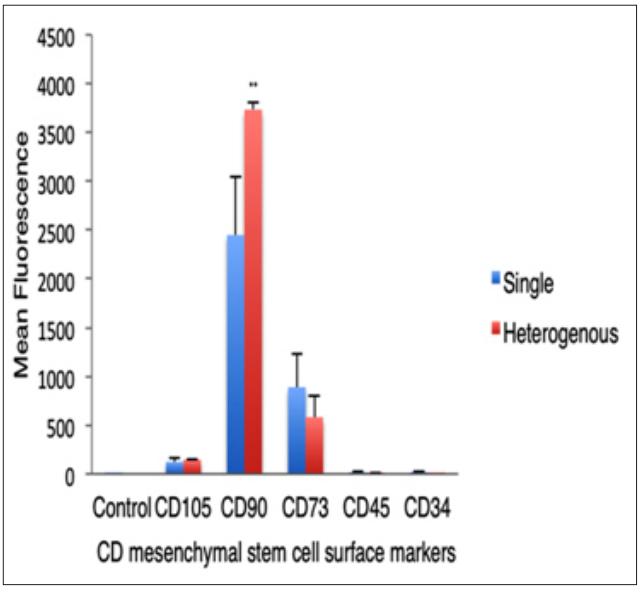

Fig 1B

Figure 1A: *Flow cytometry displaying the percentage gating of each of the cell surface markers and 1B, mean fluorescence intensity between single colonies and heterogeneous culture; *P<0.05, **P<0.01*

ALP/DNA Assay

To determine the difference between the osteogenic induction of both single and hetero cultures, ALP/DNA ratio was compared between the osteogenically induced single colonies and heterogeneous culture of cells. ALP/DNA specific activity ratio in heterogeneous culture of the GFs was higher than that of single colonies. The difference was statistically significant (Figure 2 A, B).

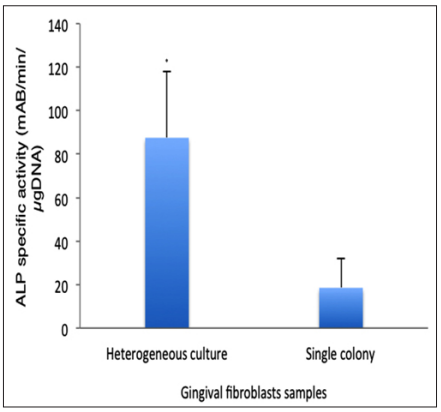

Fig 2A

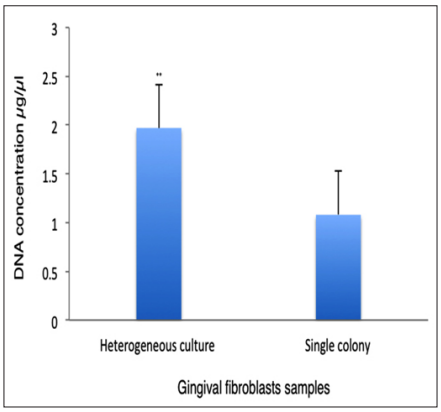

Fig 2B

Figure 2A: *The heterogeneous culture cells shows a significantly higher ALP specific activity and 2B, DNA concentration than the single colony cultures of cells; *P<0.05, **P<0.01*

Calcium Assay

The calcium concentration was high in the heterogeneous culture, whereas the single colonies were not expressing the same mineral content. There was a statistical significant difference between them (Figure 3).

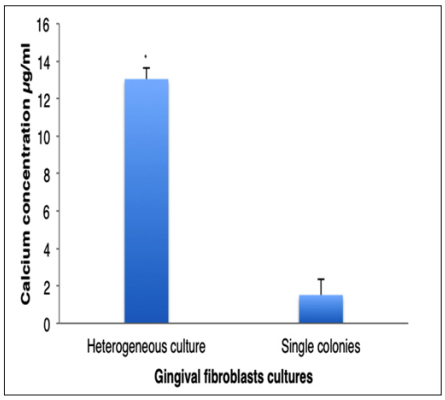

Figure 3: *Calcium content quantified, showing statistically significance between heterogeneous cultures and single colonies*

Real-time quantitative polymerase chain reaction (RT-qPCR)

Differences in gene expression after osteogenic induction were investigated using RT-qPCR analysis. The osteogenic related genes analyzed were OPN, OCN in addition to ALP. RT-qPCR analysis showed an increase of expression of all the studied genes in heterogeneous cultures over single colonies of the hGFs (Figure 4).

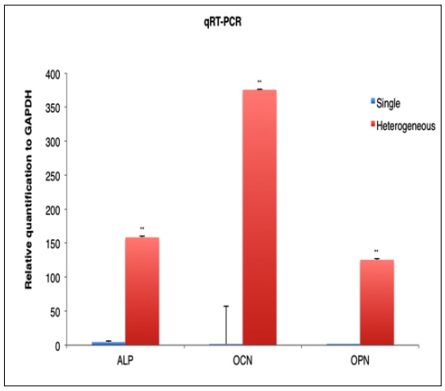

Figure 4: *Changes in gene expression after osteogenic differentiation at day 21. Real time PCR results showing gene expression of ALP, OCN and OPN; **p < 0.01*

Western blot

The bands clearly demonstrate the expression of OPN more pronounced in the heterogeneous cultures compared to the single colonies bands (Figure 5).

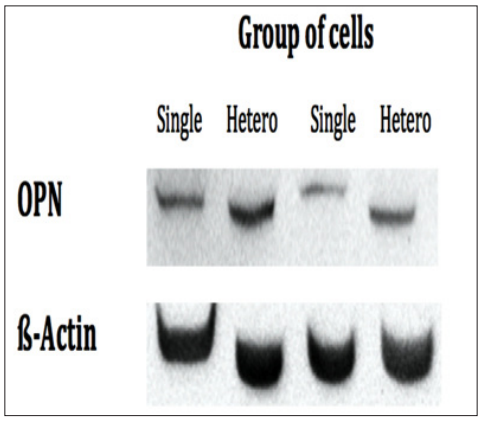

Figure 5: *OPN western blot bands showing high expression of the osteogenic protein OPN in the heterogeneous cultures more than the single colonies.*

Discussion

Human GFs in many studies have satisfied the criteria of mesenchymal stromal progenitor /stem cells [2,3,16,22]. Different methods to isolate GFs cells or their subpopulation out of that niche to serve certain therapeutic goals is necessary for tissue engineering purposes. However, it is not achieved yet [22]. It has been reported that every fibroblast culture contains small proportion of MSC without undergoing enrichment selection procedure [12,22]. However, the available means of identification of the MSCs population through certain markers allows only identifying the population of these cells rather than complete isolation and subsequent purification. There is a need to overcome the lack of specific MSC markers [23].

In our study, the surface markers used are agreed upon as universal MSC markers. However, not specific [24]. The heterogeneous cultures have demonstrated in our results an increased expression of the positive MSC markers CD90, 105 and the lack of hematopoietic MSC markers CD45, CD 34. Such results coincide with the findings of having MSCs within the GFs heterogeneous culture. Yet, it doesn't confirm that the colony forming units are all enriched with MSCs, as has been suggested. Bearing in mind the single colonies cultures were reported as phenotypically heterogeneous [23,25].

In a recent study, Alvarez et al isolated the periodontal ligament pure MSC using FACS according to three surface markers combinations, and their results demonstrated that CD271+ cells exhibited the greatest osteogenic differentiation potential [26]. Fournier et al isolated the GF colonies and reported their differentiation potential into three different lineages [27].

After osteogenic induction, our results were consistent with the flow cytometry findings, unsurprisingly, the cultures with higher expression of MSC surface markers showed higher calcium content, higher ALP/DNA specific activity, and eventually higher genetic activity for the specific osteogenic proteins (ALP, OPN and OCN). OPN protein was markedly higher expressed in the heterogeneous, confirming and consistent with all the previous assays done.

Growing the GFs into single colonies is by no means one of the simplest methods to obtain a pure set of highly enriched population of cells, if they show to possess higher MSC properties. We attempted in our study to compare the osteogenic differentiation of the heterogeneous culture of GFs over the isolated single colonies. The aim was to seek a pure or enriched population of the mesenchymal stem cells. The selected colonies were of very similar count, number/ size, and were de-attached and osteogenically induced under the same incubation conditions.

Our results suggest and don't conclude that the heterogeneous population of gingival fibroblast cells have higher osteogenic gene expression as compared to the isolated MSCs. A possible explanation for our findings is the abundance of the ECM molecules/ niche within the heterogeneous cultures. The GFs secrete certain growth factors, which bounds these growth factors [28]. In addition to the mechanical signals and the cell-cell interaction, which affects the fate of the gingival MSC differentiation and function [27,29]. These findings are undermined by few limitations; a major one is the sample size. We have used only one biopsy from one patient; this was an intention for a pilot study. Another limitation is the lack of a negative control with no osteogenic differentiation.

Thus we reject our hypothesis; Further investigations are needed to identify what specific population of GFs have multipotent stem cells characteristics in order to develop a consistent and reliable methods to utilize the GFs in future tissue regeneration protocols.

Funding sources

This research was supported by the University Hospital Fund (UHF) medical research grant competition grant.

Acknowledgements

Special thanks for Cezary kucharski for his help during lab work.

References

1. Newman MG, Takei H, Klokkevold PR, Carranza FA. Carranza's clinical periodontology. : Elsevier health sciences; 2011.
2. Bojic S, Volarevic V, Ljujic B, Stojkovic M (2014) Dental stem cells-characteristics and potential. Histol Histopathol 29: 699-706.
3. Dziubińska P, Jaskólska M, Przyborowska P, Adamiak Z (2013) Stem cells in dentistry–Review of literature. Polish journal of veterinary sciences 16: 135-140.
4. Mitrano TI, Grob MS, Carrion F, Nova-Lamperti E, Luz PA, et al. (2010) Culture and characterization of mesenchymal stem cells from human gingival tissue. J Periodontol 81: 917-925.
5. Mostafa NZ, Uludağ H, Varkey M, Dederich DN, Doschak MR, El-Bialy TH (2011) In vitro osteogenic induction of human gingival fibroblasts for bone regeneration. The open dentistry journal 5: 139-145.
6. Gao Y, Zhao G, Li D, Chen X, Pang J, Ke J (2014) Isolation and multiple differentiation potential assessment of human gingival mesenchymal stem cells. International journal of molecular sciences 15: 20982-20996.
7. Jin S, Lee J, Yun J, Kim I, Ko Y, et al. (2015) Isolation and characterization of human mesenchymal stem cells from gingival connective tissue. J Periodont Res 50: 461-467.
8. Egusa H, Okita K, Kayashima H, Yu G, Fukuyasu S, et al. (2010) Gingival fibroblasts as a promising source of induced pluripotent stem cells. PLoS One 15: e12743.

9. Wada N, Wang B, Lin N, Laslett AL, Gronthos S, et al. (2011) Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts. J Periodont Res 46: 438-447.
10. Yin X, Li Y, Li J, Li P, Liu Y, et al. (2016) Generation and periodontal differentiation of human gingival fibroblasts-derived integration-free induced pluripotent stem cells. Biochem Biophys Res Commun 473: 726-732.
11. Choi J, Hwang H, Jang Y (2015) The efficiency of the in vitro osteo/dentinogenic differentiation of human dental pulp cells, periodontal ligament cells and gingival fibroblasts. Int J Mol Med 35: 161-168.
12. Fournier BP, Larjava H, Hakkinen L (2013) Gingiva as a source of stem cells with therapeutic potential. Stem Cells Dev 22: 3157-3177.
13. McGuire MK, Scheyer ET (2007) A randomized, double-blind, placebo-controlled study to determine the safety and efficacy of cultured and expanded autologous fibroblast injections for the treatment of interdental papillary insufficiency associated with the papilla priming procedure. J Periodontol 78: 4-17.
14. Saczko J, Dominiak M, Kulbacka J, Chwiłkowska A, Krawczykowska H (2008) A simple and established method of tissue culture of human gingival fibroblasts for gingival augmentation. Folia Histochemica et Cytobiologica 46: 117-119.
15. Köseoğlu S, Duran İ, Sağlam M, Bozkurt SB, Kırtıloğlu OS, et al. (2013) Efficacy of collagen membrane seeded with autologous gingival fibroblasts in gingival recession treatment: a randomized, controlled pilot study. J Periodontol 84: 1416-1424.
16. Lymperi S, Ligoudistianou C, Taraslia V, Kontakiotis E, Anastasiadou E (2013) Dental Stem Cells and their Applications in Dental Tissue Engineering. Open Dent J 7: 76-81.
17. Han J, Menicanin D, Gronthos S, Bartold P (2014) Stem cells, tissue engineering and periodontal regeneration. Aust Dent J 59: 117-130.
18. Hynes K, Menicanin D, Gronthos S, Bartold PM (2012) Clinical utility of stem cells for periodontal regeneration. Periodontol 59: 203-227.
19. Zhang Q, Nguyen A, Yu W, Le A (2012) Human oral mucosa and gingiva: a unique reservoir for mesenchymal stem cells. J Dent Res 91: 1011-1018.
20. Xu X, Chen C, Akiyama K, Chai Y, Le A, et al. (2013) Gingivae contain neural-crest-and mesoderm-derived mesenchymal stem cells. J Dent Res 92: 825-832.
21. Fawzy El-Sayed KM, Dorfer CE (2016) Gingival mesenchymal stem/progenitor cells: a unique tissue engineering gem. Stem cells international 1-16.
22. Hakkinen L, Larjava H, Fournier BP (2014) Distinct phenotype and therapeutic potential of gingival fibroblasts. Cytotherapy 16: 1171-1186.
23. Augello A, Kurth TB, De Bari C (2010) Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. Eur Cell Mater 20: 121-133.
24. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317.
25. Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, et al. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19: 35-42.
26. Alvarez R, Lee H, Wang C, Hong C (2015) Characterization of the osteogenic potential of mesenchymal stem cells from human periodontal ligament based on cell surface markers. International journal of oral science 7: 213-219.
27. Fournier BP, Ferre FC, Couty L, Lataillade J, Gourven M, et al. (2010) Multipotent progenitor cells in gingival connective tissue. Tissue Engineering Part A 16: 2891-2899.
28. Reilly GC, Engler AJ (2010) Intrinsic extracellular matrix properties regulate stem cell differentiation. J Biomech 43: 55-62.
29. Macri L, Silverstein D, Clark RA (2007) Growth factor binding to the pericellular matrix and its importance in tissue engineering. Adv Drug Deliv Rev 59: 1366-1381.