Original Article

Characteristics of Adipose-derived Stem Cells Isolated from Buccal Fat Pads Using CD 271 Cell Sorting Technique

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Abstract

Buccal fat pad is a suitable intra-oral source of Adipose-Derived Stem Cells (ADSCs) for bone tissue engineering. CD271 is one of the most specific cell surface markers used to isolate mesenchymal stem cells from various tissues. However, there has not been a study done that has used the cell-sorting technique with this marker to isolate the ASC from buccal fat tissue. The aim of this study was to compare the characteristics of ADSCs isolated from intraoral buccal fat pads using CD 271+ magnetic-activated cell sorting (MACS) and plastic adherence (PA). Buccal fat tissue was harvested from ten patients who underwent orthognathic surgeries. ADSCs were isolated from the tissue using PA (Group A) and MACS; CD 271+ (Group B) and CD271- (Group C), (5 participants per group). The characteristics of the cells including colony forming unit fibroblast (CFU-F), immunophenotyping markers, and multi-differentiation into tri-lineages were comparatively assessed. Gingival fibroblast served as the negative control group. The results demonstrated that (CFU-F) formed in the Group B cells, but were not detected in the other groups. The cells of groups A-C expressed the mesenchymal stem cell including CD 73, 90 and 105. No statistical difference was detected among the groups. It was noted that CD 73 was detected at the highest levels followed by CD 105 and CD 90 respectively. The cells of the control group expressed those markers remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $p<0.05$). In addition, the cells of all groups expressed hematopoietic stem cell markers including CD 14, 20, 34 and 45 at very low levels. The cells of groups A-C demonstrated adipogenic, chondrogenic and osteogenic differentiation when cultured in the inductive conditions. There was no significant difference of those properties among the groups. In conclusion, CD 271 is considered as a proper marker for sorting ADSCs from buccal fat tissue. However, it cannot be used as the sole marker. Although the ADSCs expressed CD 90 at the lowest levels, they still had osteogenic differentiation capacity. Therefore, they can be used as a stem cell source to repair bone defects.

Keywords: Buccal fat stem cells, Adipose-derived stem cells, Stromal vascular fraction, Mesenchymal stem cell, CD271, surface markers, cell sorting technique

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Introduction

Tissue engineering has become a popular alternative method in the field of reconstructive surgery. The bone tissue engineering triad includes scaffolds, cells, and signaling substances. A combination of scaffolds with bone forming cells as cell-scaffold constructs is a good strategy to enhance bone regeneration. Several studies[1-5] obtained better results in promoting new bone when the scaffolds were combined with several cell types, such as primary osteoblasts, mesenchymal stem cells (MSCs) from bone marrow (BM) (BM-MSCs), and dental pulp. Another source of MSCs is fat tissue from which adipose-derived stem cells (ADSCs) are obtained. ADSCs were found to express immunophenotyping markers similar to the BM-MSCs. Moreover, they can be differentiated toward various cell types, especially bone forming cells. Some studies[6-10] revealed that the buccal fat pads are suitable intra-oral sources of the ADSCs which provide a large amount of fat tissue that is easily harvested in routine intraoral surgical fields. Plastic adherence (PA) capacity of stem cells is commonly used to isolate the ADSCs from other cell types since non-adherent cells can be washed out after periods of culture. Although this technique is very simple and cheap, the amount of stem cells obtained is only about one cell per 105 of adherent cells.[11,12] A new method to purify stem cell populations is magnetic-activated cell sorting (MACS) which uses antibody-binding. The specific markers are conjugated to iron oxide microbeads that can retain the desired cells in the column containers in a magnetic field, whereas unlabeled cells are eluted.[13] Some studies[14-17] suggest using low-affinity nerve growth factor receptor (CD271,p75NTR) antibody as a positive selection marker of MSCs. The CD271+ cells obtain high purity of a MSC population that supports self-renewal capacity and multi-differentiation potential. However, there are no comparative results of characterization of the ADSCs isolated from the intraoral buccal fat pads using the conventional PA method or MACS. In this study, CD271 was used as the specific marker of the MSCs and the positive and negative cells to the marker were included for the investigation.

Materials and Methods

Patient enrollment

Ten volunteer patients were enrolled in the study. All patients underwent orthognathic surgeries to correct skeletal discrepancies at the Oral & Maxillofacial Surgery Clinic, Dental Hospital, Faculty of Dentistry, Prince of Songkla University. The protocol of this study was approved by the ethics committee of the Faculty of Dentistry, Prince of Songkla University (EC5909-38-P-LR). The inclusion criteria of the participants included ASA class I, age >20 years old, weight >50 kg and hematocrit ≥35 %. The excluded patients were those with systemic diseases including hereditary blood diseases, disorders of the blood and blood components, blood transmitted diseases, and diabetes.

Isolating ADSCs from fat tissue

The ADSCs of the participants were consecutively isolated using two different methods and divided into three groups. In Group A, five samples of cells were isolated from five patients using PA.[18] The other five patients were the sources of cells for Group B (five samples) and Group C (five samples). In Group B, the cells which were positive to CD 271 (CD271+) were isolated using MACS. In Group C, the cells which were negative to CD271 (CD271-) were isolated using MACS. Each participant underwent orthognathic surgery under general anesthesia. During the operations of Lefort I osteotomy of the maxilla or sagittal split osteotomy of the mandible, some parts of the fat pads were excised, then immediately placed into DMEM (Dulbecco's Modified Eagle Medium, Gibco, USA) and stored at 4°C until the isolation process. The fat tissue was washed several times with sterile phosphate buffered saline (PBS) to remove contaminating debris and red blood cells. The volumes were then measured using a 5 mL sterile disposable syringe. Afterwards, the tissues were minced into small

pieces and enzymatically digested using 0.75 % collagenase type I (Gibco, USA) in PBS at 37°C with gentle agitation for 60 min. The supernatants were collected, and then centrifuged at 400 g for 10 min to exclude the remaining adipocytes and lipid droplets. The cellular pellets were suspended in DMEM supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), and then filtered through a 100 µm filter (Corning, USA). In group A the cell suspension was plated onto 6-well culture plates (Corning, USA) and cultured in a humidified atmosphere with 5 % CO_2 at 37°C. In groups B and C, the cells were isolated using CD271 antibody for MACS (CD271 MicroBead Kit human, Miltenyi Biotec, Germany). In brief, the cell suspension was re-centrifuged at 300 g for 10 min and the cell pellets were resuspended in 60 µL of buffer (PBS with 0.5 % FBS, and 2 mM EDTA). The cell suspensions were incubated in 20 µL of FcR blocking reagent and 20 µL of CD271 MicroBeads for 15 min at 4°C. Afterwards, the cells were washed by adding 1-2 mL of buffer, and then centrifuged at 300 × g for 10 min. Re-suspended cell solutions were made by adding 500 µL of buffer, and then loaded onto a column which was placed in a MiniMACS separator (Miltenyi Biotec, Germany). The magnetically labeled CD271+ cells of group B were retained in the column, whereas the unlabeled CD 271- cells of group C were run through. The cells of Groups B and C were collected and cultured in DMEM supplemented with 10 % FBS in a humidified atmosphere with 5 % CO_2 at 37°C. The cells of all groups at passages 1-4 were used for the following experiments (five samples/ group/test).

Determining the Characteristics of the cells

Colony-forming unit fibroblast (CFU-F) assays

The cells of each group at passage 1 were plated at a density of 100 cells/well in a 6-well plate to define the numbers of CFU-F. Within 20 days, the cells were fixed with 4 % paraformaldehyde and stained with 0.1 % toluidine blue (Sigma, USA). The CFU-F was observed under a light microscope (Nikon, Japan). The numbers of colonies were counted if the aggregations were at least 50 cells or the colonies were >2 mm in diameters.[2] The cells of each patient were assessed in triplicate.

Flow cytometry analysis

The MSC immunophenotypes of the ADSCs were defined following the International Society for Cellular Therapy (ISCT) protocols.[19] The analysis was performed using a fluorochrome-conjugated monoclonal antibody cocktail in the MSC Phenotyping Kit human (Miltenyi Biotec, Germany). In brief, 5 x 10^5 cells from each group in passages 2 and 3 were incubated in the antibodies against the surface antigens CD73, CD90, and CD105 as the positive markers and CD14, CD20, CD34, and CD45 as the negative markers. At least 10,000 events were acquired for each sample using a fluorescent-activated cell sorting instrument (FACSCalibur, BD Biosciences) and the data were analyzed using CELLQUEST software (BD Biosciences).

Multi-differentiate potential of the ADSCs

Adipogenic differentiation

The cells at 1 x 10^4/well were cultured in adipogenic induction medium comprised of DMEM supplemented with 10 % FBS, 1 µM dexamethasone, 10 µg/mL insulin, 500 µM 3-isobutyl-1-methylxanthine and 200 µM indomethacin (Sigma, USA) for 21 days. The culture mediums were changed every two days. On day 21, the cells were fixed in 10 % formaldehyde for 1 h and stained with Oil Red O solution (20 mg/mL in isopropanol) (Sigma, USA) for 15 min. Lipid vacuoles were quantified by extracting with 100 % isopropanol for 10 min and reading with a microplate reader (Multiscan™Go, Thermo Fisher Scientific) at the absorbance of 540 nm.

Chondrogenic differentiation

The cells (5 x 10^5) were centrifuged at 600 g for 5 min to form cell pellets and re-suspended in 2 mL chrondogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco) in a 15-mL centrifugation tube (Corning, USA) for 21 days to induce chondrogenic differentiation.[20,21] The culture medium was changed every three days.[20] Determination of expression of

chondrogenic differentiation was performed after 21 days of culture using alcian blue staining.[20]

Osteogenic differentiation

The cells (1 x 10^4 cells/well) were cultured in osteogenic medium (DMEM supplemented with 10 % FBS, 5 mM beta-glycerophosphate, 100 nM dexamethasone, and 50 µg/mL ascorbic acid). Osteoblastic differentiation markers including alkaline phosphatase activity (ALP) and osteocalcin expression (OCN) at days 3, 7, 14, and 21 were assessed using ELISA (five samples/group/time point). On the days of the experiments, the cells were washed two times using PBS. After that, 200 µL of 1 % Triton X-100 in PBS was added into each well and then the cells were lysed by freezing and thawing in three cycles (30 min/cycle). The mixtures were transferred into microcentrifuge tubes and centrifuged at 2000 x g for 10 min. The supernatants were collected as cell lysis solutions and kept at -80°C for the analysis of total cellular protein content, ALP activity, and OCN expression. The quantification of total protein in the solutions were performed according to the manufacturer's instructions (Bio-Rad Protein Assay; Bio-Rad Laboratories, USA) based on the method of Bradford. Absorbance at 750 nm was read using the microplate reader. The ALP activities were measured according to the instructions using the commercial kit of Alkaline Phosphatase, AMP Buffer (HUMAN, Germany) according to the recommendation of the International Federation of Clinical Chemistry. The levels of activity were calculated per one milligram of the total cellular protein (U/L/mg protein). Quantification of OCN was performed according to the manufacturer instructions using the Osteocalcin ELISA kit (Biomedical Technologies Inc., USA). The solutions were read at 450 nm absorbance using the microplate reader and their concentrations were calculated with the serial diluted standard solution. The OCN levels were demonstrated as ng/mg protein.[22]

Statistical Analysis

The measured parameters were analyzed using statistics analysis software (SPSS, version 22.0, USA). One-way analysis of variance (ANOVA) and Tukey HSD were applied to compare the differences among the groups and time points. The level of statistical significance was set at $P<0.05$.

Results

The average volume of buccal fat tissue harvested from the patients was 3.9 ± 2.6 mL.

CFU-F

CFU-Fs were detected only in Group B during 20 days of culture, whereas CFU-Fs were not detected in Groups A or C (Fig. 1).

Flow cytometry analysis

Expression of the MSC immunophenotypes of the ADSCs is demonstrated in Figure 2 and Table 1. Among the groups, the profiles of the positive markers of MSC were not statistically different. The cells of all groups expressed CD73 at the highest levels, followed by CD105 and CD90. Expression of the hematopoietic markers of all groups was less than 1 %. The fibroblasts expressed CD73 and 105 significantly less than those of Groups A and B ($P<0.05$).

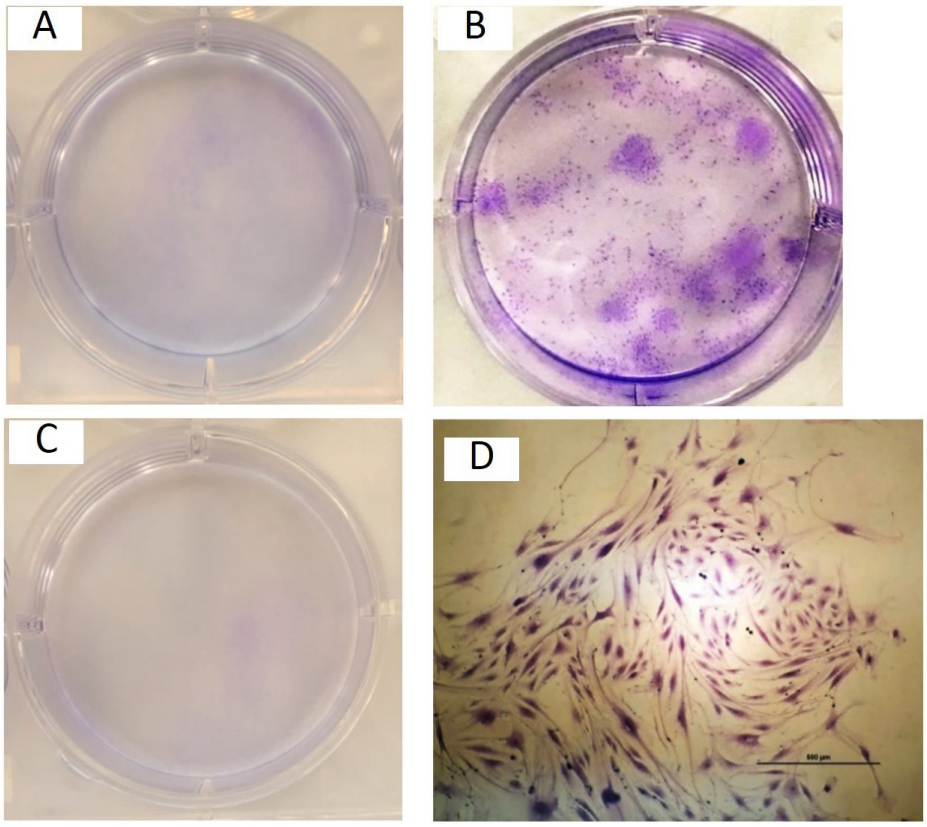

Figure 1 *Toluidine blue staining of CFU-F after 20 days of culture. A: Group A, B: Group B and C: Group C. D: The magnified image of Group B CFU-F.*

Table 1 *The percentages of immunophenotyping markers were demonstrated.*

CD Markers (%)	Groups	A (PA)	B (CD271+)	C (CD271-)	Gingival Fibroblasts (control)
MSCs markers	CD 90	54.5±27.4	48.7±16.7	58.1±12.6	56.4±9.3
	CD 105	78.7±12.1	60.6±9.7****	62.7±11.7*****	44.2±13.7##
	CD 73	88.8±5.3*	89.9±6.3**	86.7±13.5***	33.9±5.4#
Hematopoietic markers	CD 14, 20, 34, 45	0.71±0.4	0.72±0.4	0.99±0.1	0.2±0.17

*The percentages of CD73 of groups A-C were significantly higher than CD90 (*p=0.03, **p=0.002, ***p=0.006). The percentages of CD105 of groups B and C were significantly higher than CD90*
*(****p=0.018, *****p=0.02). The percentages of CD90, CD105 and CD73 of the control group were not significantly different. CD 73 and CD 105 of this group were significantly less than those of group A and B (#p =0.002, ##p=0.007))*

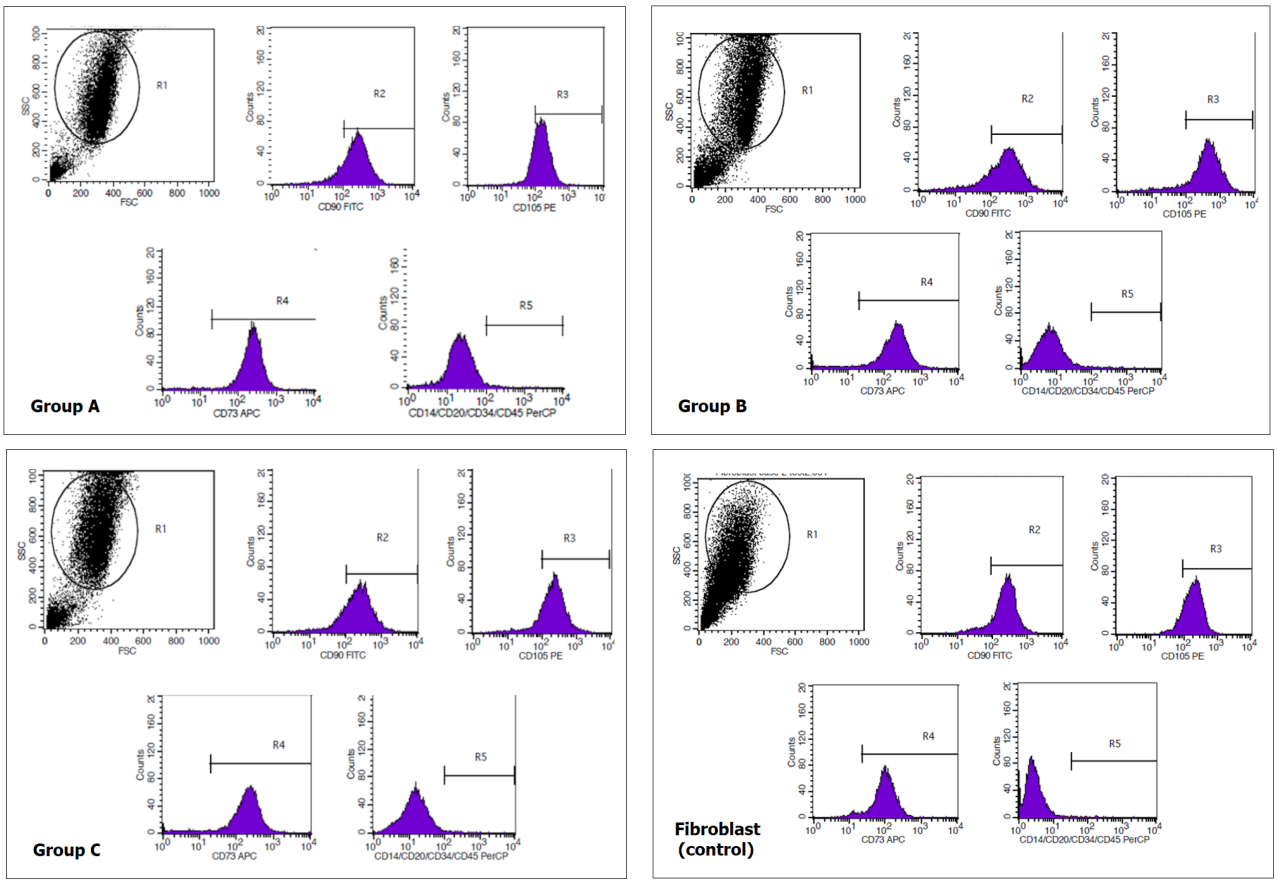

Figure 2 *The pictures of flow cytometry analysis show the profiles of the MSC markers and the hematopoietic markers of the experiment groups.*

Multi-differentiate potential of the ADSCs

Adipogenic differentiation

After 21 days of culture, lipid vacuoles were detected in red (Fig. 3A). The quantitative measurement of the extracted lipid vacuoles is demonstrated in Figure 3B. There was no statistical difference among the groups ($P<0.05$).

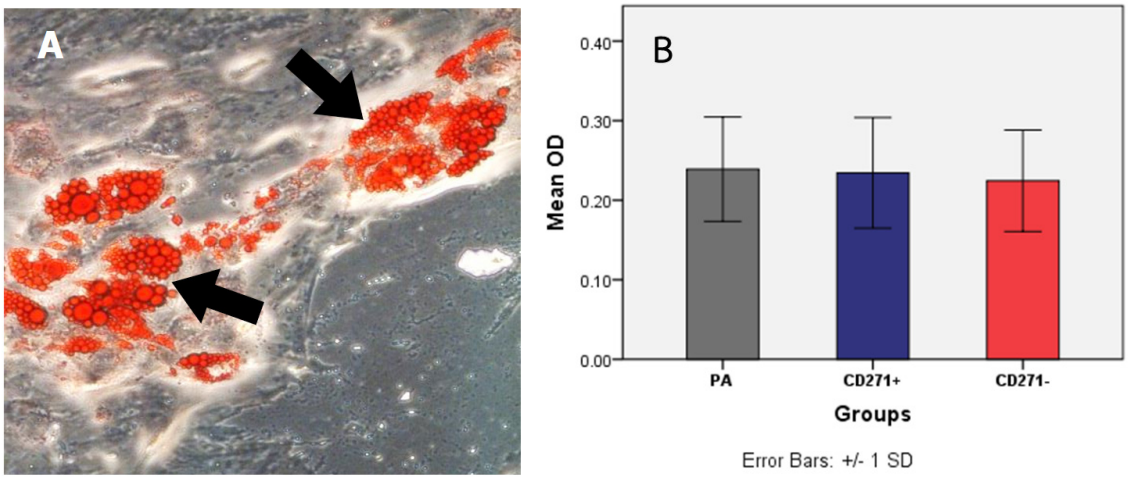

Figure 3 *(A) Oil Red O staining demonstrates the lipid vacuoles seen in red (arrows). (B) The bar graphs demonstrate the OD levels of the solubilized Oil Red O. No significant differences were detected among Groups A-C.*

Chondrogenic differentiation

After inductive culture, the cell pellets of Groups A-C could produce cartilaginous matrix (Fig. 4).

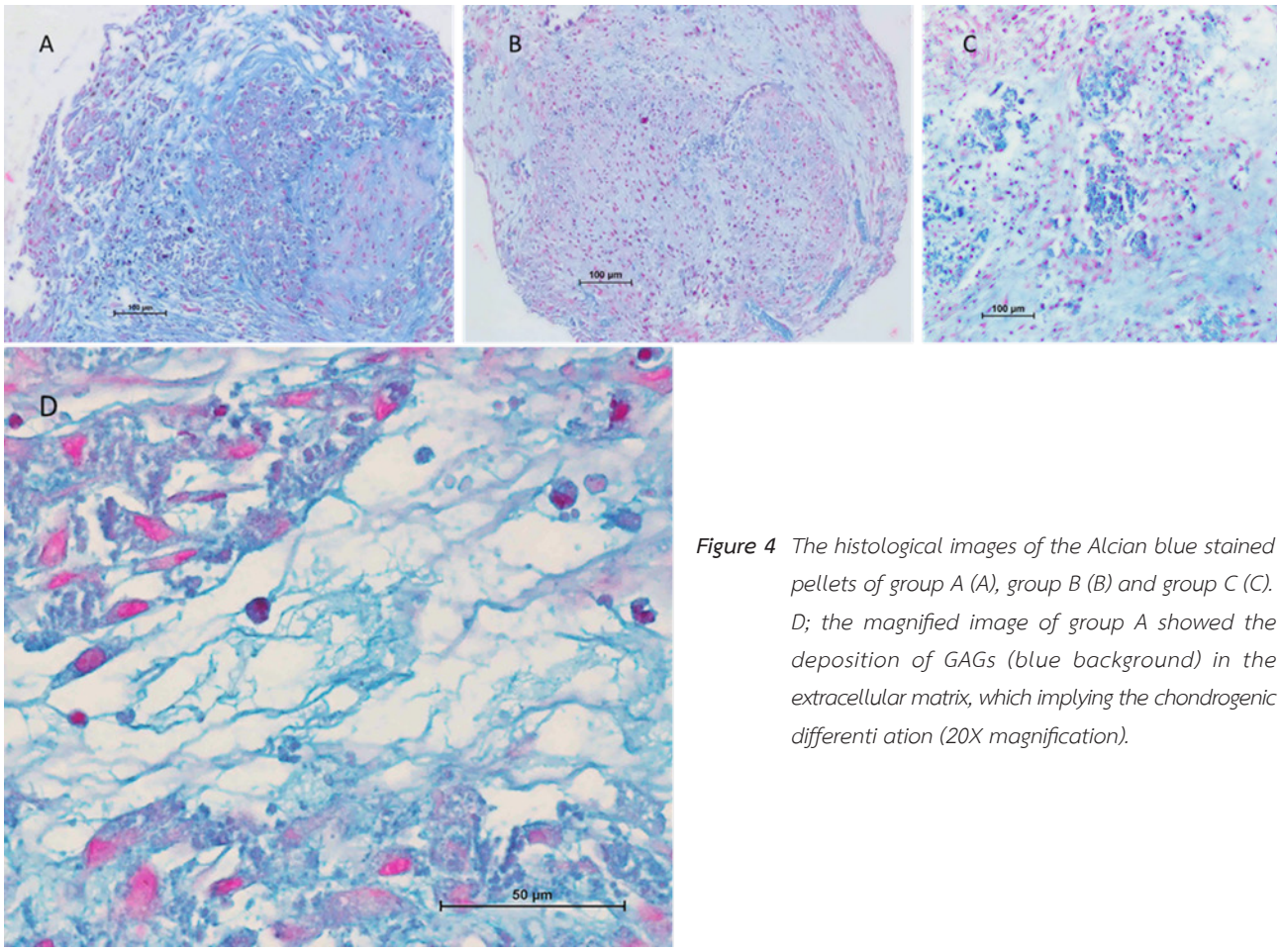

Figure 4 *The histological images of the Alcian blue stained pellets of group A (A), group B (B) and group C (C). D; the magnified image of group A showed the deposition of GAGs (blue background) in the extracellular matrix, which implying the chondrogenic differenti ation (20X magnification).*

Osteogenic differentiation

The ALP levels of Groups A-C are demonstrated in Figure 5. The levels of ALP of Groups A and B seemed to be stable during the first 14 days, and then they noticeably increased at day 21. On day 21, the ALP level of Group A was significantly greater than the other groups (P=0.000). In Group C, the highest ALP was detected at day 3 and then the levels decreased on the following days.

The OCN levels are shown in Figure 6. The levels of OCN in Groups A and B rapidly increased to reach the highest levels on day 7, and then decreased thereafter. The highest expression of OCN in Group C was detected at day 3, and then the levels rapidly decreased on the following days.

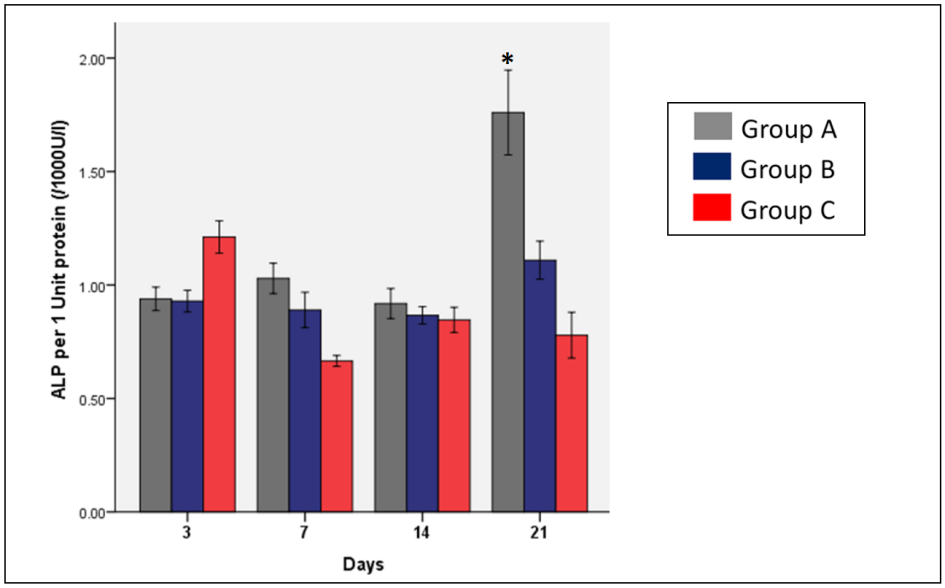

Figure 5 *The bar graphs demonstrate ALP activities of group A-C. The data showed that there was no statistically difference of the ALP levels among the groups on the first 14 days of culture. On day 21, the level of group A was significantly greater than the other groups (*p=0.001).*

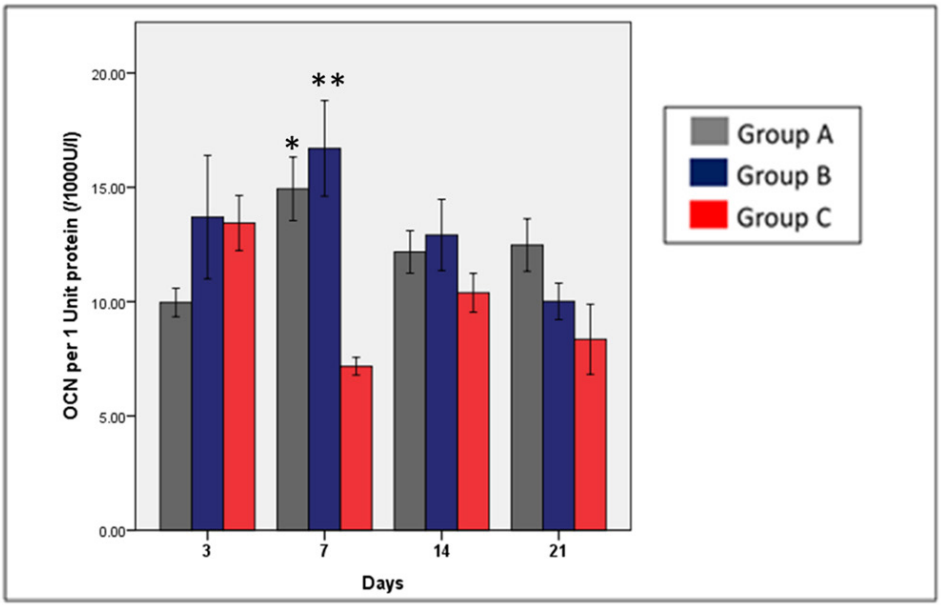

Figure 6 *Bar graphs demonstrate the OCN levels of Groups A-C. The maximum levels of Groups A and B were detected on day 7, whereas the maximum level was detected in Group C on day 3. The osteocalcin levels of Groups A and B were significantly higher than group C (*p=0.004,**p=0.001 respectively).*

Discussion

In the field of bone tissue engineering, adipose tissue is an alternative potential source of mesenchymal stem cells which have the ability to differentiate toward the lineage of osteoprogenitor cells.[6,23,24] Buccal fat pads are excellent intra-oral sources of adipose tissue that provide greater volumes of tissue compared with dental pulp and periodontal ligament. Moreover, the harvesting technique of the tissue is easily performed under local anesthesia which is less invasive compared with bone marrow sources. Plastic adherence is a conventional

technique used to isolate stem cells from tissue. The technique is routine and easy, but after enzymatic digestion, adipose tissue generates a pellet of stromal vascular fraction (SVF) which contains a heterogeneous cell population. At the cellular level, SVF is composed of mature adipocytes, fibroblasts, nerve cells, endothelial cells, immune cells, and preadipocytic cells.[25,26] Those cells usually have various protein and cytokine expressions and potency of differentiation.[25] Therefore, methods to purify them are still interesting. Cell sorting with specific surface markers to isolate the cells can obtain a more homogenous cell population. MACS would be the optimum method for identification of stem cells in clinical practices due to the fact that it can be done as a chairside procedure and the isolation processes can be finished within two hours. Moreover, it remarkably reduces cultivation time, and avoids contamination of the cell culture reagents, when compared with the conventional plastic adherence method. However, the amounts of stem cells from buccal fat tissue, retained in the column of MACS might be low and they should be further assessed. Several markers have been used to isolate MSCs from various sources. Nevertheless, a standard acceptable definition has not reached a consensus. The two types of CD surface markers of stem cells are sole markers and stemness markers. A sole marker is considered to be sufficient to identify stem cells from their in vivo environment, whereas stemness markers are used to identify subsets of cells with high CFU-Fs and trilineage potential. In principle, the sole markers are highly expressed, while the stemness markers may be moderately detected.[27] CD271 is considered to be one of the most specific markers to isolate MSCs from bone marrow, dental pulp, and adipose tissue.[14-16,28-32] A recent study[31] stated that CD271 is the best single marker to isolate dental pulp mesenchymal stem cells with the greatest differentiation potential. However, there has not been a study done which has used this marker to isolate ADSCs from buccal fat pads. Our study is the first to demonstrate the characteristics of the CD 271+ ADSCs isolated from buccal fat pads using the cell sorting method in terms of expression of MSC markers and the capacity to exhibit trilineage differentiation. Their properties were compared with those isolated with conventional plastic adherence and the gingival fibroblasts.

The results of CFU-F assay revealed that self-renewal capacity of the cells was detected only in the CD 271+ cells, whereas that property was not detected in the PA cells or in the CD 271- cells. These results corresponded with some previous studies.[14,15,29,33] Poloni, *et al.* compared numbers of CFU-Fs generated by human BMSCs isolated using Ficoll gradient and CD271+ mononuclear cells isolated using MACS. After 14 days of culture, the authors found higher numbers of CFU-Fs of the CD271+ cells compared with the unsorted BM-MSCs[15] Quirici, *et al.* determined the clonogenic potential in three different populations from human adipose tissue including PA, CD271+ cells, and CD34+ cells. At less than 10 weeks of culture, there were no detectable significant differences in the numbers of CFU-Fs among the groups. However, after 20 weeks, the number of CFU-Fs in the PA group nearly disappeared which was significantly less than in the CD34+ and 271+ groups.[29] Kuçi, *et al.* demonstrated that CFU-F activity was found only in the CD271+ cells, while there were no CFU-Fs detected in the CD271– cells.[14] Jarocha, *et al.*[33] compared the capacity for CFU-Fs among various methods of isolating MSCs from bone marrow including PA, RosetteSep-isolation, and CD105+ and CD271+ selection. The results showed that the CD271+ fraction had the highest number of CFU-F colonies compared with the other groups.[33] It is possible that the heterogeneous cells isolated using PA and the CD271- cells were possibly contaminated with hematopoietic cells, endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocytes, and macrophages. The strong proliferation of cells can diminish the growth of progenitor cells.[34,35] Therefore, selective isolation using a specific marker can promote better self-renewal capacity.

Based on the minimum criteria of ISCT, the cells

that display the properties of PA, positive expression of CD90, CD105, CD73, and negative expression of hematopoietic markers, and multi-differentiation potency can be termed "MSC".[36] Our results demonstrated that the averages cell populations of PA, CD271+, and CD271- groups positively expressed CD90, CD105, and CD73 were less than 90 %, while the negatively expressed hematopoietic markers of CD14, CD20, CD34, and CD45 were less than 1 % without a significant difference among the groups. The positive cell numbers were less than the ISCT criteria to identify MSC, which purposes that the cell population should express the CD73, CD90 and CD105 ≥ 95 %.[36] However, our results corresponded to the consensus between ISCT and International Federation for Adipose Therapeutics and Science (IFATS)[35], which purposes that the ASC should be positive to CD13, CD29, CD44, CD73, CD90, and CD105 (>80 %) and negative to CD31, CD45, and CD235a (<2 %). Whereas, the SVF should express the primary markers of stromal cells including CD13, CD29, CD44, CD73, CD90 (>40 %), and CD34 (>20 %), but express the negative markers of CD31 (<20 %) and CD45 (<50 %).

Interestingly, the control group of fibroblasts could express the MSC markers, but the amounts were remarkably less than the experiment groups (significant differences were found in CD 73 and CD 105, $P<0.05$). In addition, they negatively expressed the hematopoietic markers less than the other groups. This character corresponded to previous studies,[27,37] which reported that the fibroblasts resembled many behaviors of MSCs such as cell morphology, self-renewing capacity, and cell surface protein expression, but they lacked multipotency.

It was noted that the CD271+ cells expressed CD73 at the highest levels of 89.9±6.3 % followed by CD105 at 60.6±9.7 % and CD90 at 48.7±16.7 %. Therefore, our results also demonstrated that the CD 271+ cells were co-expressed with CD105, 73, and 90, whereas they negatively expressed the hematopoietic stem cell markers. The result was similar to some previous studies which found that 49.6±1.7 % of CD271+ ADSCs co-expressed with CD90[17] and 99 % of CD 217+ cells co-expressed with CD90 and CD105[38], but contrasted to other studies, which found that only 10-20 % of CD271+ BMSCs co-expressed with CD90.[14,16] Several surface markers have been investigated as co-expression markers of the CD271+ cells. Some studies found that 82–85 % of the CD271+ cells from adipose tissue co-expressed with CD34.[29,38] Maria, *et al.*[39] reported the usefulness of using CD271 combined with CD45 to isolate fresh bone marrow MSCs. Mabuchi, *et al.*[40] suggested that a combination of markers using CD271, CD90, and CD106 for the isolation achieved the most potent and genetically stable MSC.

For differentiation of the cells, the results of our study demonstrated that the ADSCs could differentiate into three lineages including adipogenesis, chondrogenesis, and osteogenesis. Several previous studies investigated the correlations between the CD markers of the cells and their ability to differentiate. Some studies correspondingly demonstrated that MSCs, which are positive to the CD271, 73, and 105, have the potency of chondrogenic differentiation,[41-44] whereas those that are positive to CD90, have more potency for osteogenic differentiation in both *in vitro* and *in vivo*.[45-47] Arufe, *et al.*[41] investigated the differentiation of CD73+ and CD271+ synovial membrane cells and found that the CD271+ cells had higher potency of chondrogenic differentiation compared with CD73+ cells. Ruth, *et al.*[31] found that about 10.6 % of cultured dental pulp cells were positive for CD271 and they had promising odontogenic and chondrogenic potential. The CD105+ cells showed significantly greater chondrogenic potential *in vitro* even when cultured on tissue culture plastic, gel-embedded sheets[42] and biodegradable scaffolds.[43] Kavan, *et al.*[25] reported that CD90+ADSCs underwent improved osteogenic differentiation over CD90-, CD105+, and unsorted cells. *In vitro*, the authors found that co-selection of CD105low+/CD90high+ cells had more osteogenic phenotype compared with CD105low+/CD90low+ cells. Our results showed that although the amounts of the

CD 271+/90+ADSCs were remarkably less than those of the CD271+/73+ and 271+/105+cells, they could differentiate into osteoblastic lineage after culturing in the osteoblastic inductive conditions. Moreover, the levels of osteogenic differentiation markers of the CD271+cells were not statistically different to those of the PA cells. Therefore, it is presumed that the osteogenic differentiation capacity of the CD 271+ADSCs was similar to that of the PA ADSCs.

Various kinds of progenitor cells are present in the perivascular niche of adipose tissue, including tissue-resident mesoderm-derived cells, circulating bone marrow-derived cells, and neural crest (NC)-derived cells.[48] However, the developmental origin of ADSCs still remains unclear. Several literature reports hypothesize that some subsets of bone marrow, dental pulp, and adipose mesenchymal stem cells originate from the neural crest.[49-53] It is found that the neural crest-derived stem cells colonize earlier, but are largely replaced by non-NC derivatives. Therefore, the contribution of NC cells to either BM-MSCs or adipogenic progenitors sharply declines with age and a very small proportion of the NC-derived cells exist in adults.[54-56] Wrage *et al.*[57] reported that approximately 2 % of ADSCs are NC-derived and they do not contribute to neural differentiation under culture conditions. Cuevas-Diaz Duran, *et al.* and Quirici, *et al.,* reported that the amounts of CD271+ cells isolated from fresh human adipose tissue were approximately 2.89 % and 4.4 % respectively.[29,38] Correspondingly, Yoshihiro, *et al.*[58] demonstrated that a minor subpopulation of ADSCs was derived from NC cells and they exhibited an adipocyte-restricted differentiation potential, whereas chondrogenic potential was markedly attenuated. Another theory believes that the perivascular zone is the *in vivo* niche of mesenchymal stem cells which arise from a fibroblastic or pericytic origin. The cells are recognized as pericytes or perivascular cells which reside in the innermost layer of stromal cells contacting vessel endothelium.[27,58-60] CD146 is considered to be an early surface marker of MSCs derived from perivascular cells.[61] CD146+ perivascular cells can express general MSC surface antigens of CD73, CD90, and CD105, and they commonly negatively express CD31, CD34, and CD45.[25,62,63] On the other hand, CD146 is also highly expressed in MSCs, but not in dermal fibroblasts.[63] Feng-Juan, *et al.*[27] suggest that CD146 is another appropriate stem cell marker for universal detection of MSC populations from various tissues. The authors suggested that CD271+/CD146- cells are bone-lining cells, whereas, CD271+/146+ cells have perivascular localization. On the contrary, Yoshihiro, *et al.*[58] identified p75NTR-positive NC-derived cells along the vessels in the trunk fat tissue and found that almost none of them were positive to the pericyte markers. Therefore, the amounts of CD146 should be investigated as the co-expressed marker of CD271+ADSCs in future experiments.

Conclusion

The buccal fat pad is a suitable intra-oral source of mesenchymal stem cells. The CD271 surface marker seems not to be suitable to be used as the single marker for the sorting technique of ADSCs from buccal fat tissue.

Acknowledgements

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References

1. Chen W, Liu X, Chen Q, Bao C, Zhao L, Zhu Z, *et al.* Angiogenic and osteogenic regeneration in rats via calcium phosphate scaffold and endothelial cell coculture with hBMSCs, hUCMSCs, hiP-SC-MSCs and hESC-MSCs. ***J Tissue Eng Regen Med*** 2017. doi:10.1002/term.2395.[Epub ahead of print]

2. Wongsupa N, Nuntanaranont T, Kamolmattayakul S, Thuaksuban N. Biological characteristic effects of human dental pulp stem cells on poly-epsilon-caprolactone-biphasic calcium phosphate fabricated scaffolds using modified melt stretching and multilayer deposition. ***J Mater Sci Mater Med*** 2017;28:25.

3. Lendeckel S, Jodicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, *et al.* Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report. ***J***

Craniomaxillofac Surg 2004;32:370-3.
4. Diomede F, Zini N, Gatta V, Fulle S, Merciaro I, D'Aurora M, *et al.* Human periodontal ligament stem cells cultured onto cortico-cancellous scaffold drive bone regenerative process. ***Eur Cell Mater*** 2016;32:181-201.
5. Wongsupa N, Nuntanaranont T, Kamolmattayakul S, Thuaksuban N. Assessment of bone regeneration of a tissue-engineered bone complex using human dental pulp stem cells/poly(epsilon-caprolactone)-biphasic calcium phosphate scaffold constructs in rabbit calvarial defects. ***J Mater Sci Mater Med*** 2017;28:77.
6. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. ***Stem Cells*** 2006;24:1294-301.
7. Farre-Guasch E, Marti-Page C, Hernandez-Alfaro F, Klein-Nulend J, Casals N. Buccal fat pad, an oral access source of human adipose stem cells with potential for osteochondral tissue engineering: an *in vitro* study. ***Tissue Eng Part C Methods*** 2010;16:1083-94.
8. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, *et al.* Human adipose tissue is a source of multipotent stem cells. ***Mol Biol Cell*** 2002;13:4279-95.
9. Karantalis V, Hare JM. Use of mesenchymal stem cells for therapy of cardiac disease. ***Circ Res*** 2015;116:1413-30.
10. Suzuki E, Fujita D, Takahashi M, Oba S, Nishimatsu H. Adipose tissue-derived stem cells as a therapeutic tool for cardiovascular disease. ***World J Cardiol*** 2015;7:454-65.
11. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. ***J Cell Biochem*** 1997;64:278-94.
12. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. ***J Cell Physiol*** 2007;213:341-7.
13. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. ***Cytometry*** 1990;11:231-8.
14. Kuci S, Kuci Z, Kreyenberg H, Deak E, Putsch K, Huenecke S, *et al.* CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. ***Haematologica*** 2010;95:651-9.
15. Poloni A, Maurizi G, Rosini V, Mondini E, Mancini S, Discepoli G, *et al.* Selection of CD271(+) cells and human AB serum allows a large expansion of mesenchymal stromal cells from human bone marrow. ***Cytotherapy*** 2009;11:153-62.
16. Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. ***Exp Hematol*** 2002;30:783-91.
17. Yamamoto N, Akamatsu H, Hasegawa S, Yamada T, Nakata S, Ohkuma M, *et al.* Isolation of multipotent stem cells from mouse adipose tissue. ***J Dermatol Sci*** 2007;48:43-52.
18. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, *et al.* Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. ***Cell*** 2007;131:324-36.
19. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.***Cytotherapy*** 2006;8:315-7.
20. Arpornmaeklong P, Sutthitrairong C, Jantaramanant P, Pripatnanont P. Allogenic human serum, a clinical grade serum supplement for promoting human periodontal ligament stem cell expansion. ***J Tissue Eng Regen Med*** 2016:142-52.
21. Arpornmaeklong P, Brown SE, Wang Z, Krebsbach PH. Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. ***Stem Cells Dev*** 2009;18:955-68.
22. Thuaksuban N, Luntheng T, Monmaturapoj N. Physical characteristics and biocompatibility of the polycaprolactone-biphasic calcium phosphate scaffolds fabricated using the modified melt stretching and multilayer deposition. ***J Biomater Appl*** 2016;30:1460-72.
23. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. ***Tissue Eng*** 2001;7:211-28.
24. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and differentiation. ***Methods*** 2008;45:115-20.
25. Johal KS, Lees VC, Reid AJ. Adipose-derived stem cells: selecting for translational success. ***Regen Med*** 2015;10:79-96.
26. Zimmerlin L, Donnenberg VS, Pfeifer ME, Meyer EM, Peault B, Rubin JP, *et al.* Stromal vascular progenitors in adult human adipose tissue. ***Cytometry A*** 2010;77:22-30.
27. Lv FJ, Tuan RS, Cheung KM, Leung VY. Concise review: the surface markers and identity of human mesenchymal stem cells. ***Stem Cells*** 2014;32:1408-19.
28. Jones E, McGonagle D. Human bone marrow mesenchymal stem cells *in vivo.* ***Rheumatology*** 2008;47:126-31.
29. Quirici N, Scavullo C, de Girolamo L, Lopa S, Arrigoni E, Deliliers GL, *et al.* Anti-L-NGFR and -CD34 monoclonal antibodies identify multipotent mesenchymal stem cells in human adipose tissue. ***Stem Cells Dev*** 2010;19:915-25.
30. Alvarez-Viejo M, Menendez-Menendez Y, Otero-Hernandez J. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. ***World J Stem Cells*** 2015;7:470-6.
31. Alvarez R, Lee HL, Hong C, Wang CY. Single CD271 marker

isolates mesenchymal stem cells from human dental pulp. ***Int J Oral Sci*** 2015;7:205-12.
32. Tomlinson MJ, Jones EA, Giannoudis PV, Yang XB, Kirkham J. CD271 Negative Human Dental Pulp Cells Yield Significantly More Adherent Colony Forming Cells than the Positive Phenotype. ***Int J Stem Cell Res Ther*** 2016;3:025.
33. Jarocha D, Lukasiewicz E, Majka M. Adventage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105+ and CD271+ cells. ***Folia Histochem Cytobiol*** 2008;46:307-14.
34. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, *et al.* Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. ***Stem cells*** 2006;24:376-85.
35. Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, *et al.* Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). ***Cytotherapy*** 2013;15:641-8.
36. Dominici M, Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. ***Cytotherapy*** 2006;8:315-7.
37. Alt E, Yan Y, Gehmert S, Song YH, Altman A, Gehmert S, *et al.* Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential. ***Biol Cell*** 2011;103:197-208.
38. Cuevas-Diaz Duran R, González-Garza MT, Cardenas-Lopez A, Chavez-Castilla L, Cruz-Vega DE, Moreno-Cuevas JE. Age-Related Yield of Adipose-Derived Stem Cells Bearing the Low-Affinity Nerve Growth Factor Receptor. ***Stem Cells Int*** 2013;2013:372164.
39. Alvarez-Viejo M, Menendez-Menendez Y, Blanco-Gelaz MA, Ferrero-Gutierrez A, Fernandez-Rodriguez MA, Gala J, *et al.* Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. ***Transplant Proc*** 2013;45:434-9.
40. Mabuchi Y, Morikawa S, Harada S, Niibe K, Suzuki S, Renault-Mihara F, *et al.* LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. ***Stem Cell Reports*** 2013;1:152-65.
41. Arufe MC, De la Fuente A, Fuentes I, de Toro FJ, Blanco FJ. Chondrogenic potential of subpopulations of cells expressing mesenchymal stem cell markers derived from human synovial membranes. ***J Cell Biochem*** 2010;111:834-45.
42. Ishimura D, Yamamoto N, Tajima K, Ohno A, Yamamoto Y, Washimi O, *et al.* Differentiation of adipose-derived stromal vascular fraction culture cells into chondrocytes using the method of cell sorting with a mesenchymal stem cell marker. ***Tohoku J Exp Med*** 2008;216:149-56.
43. Jiang T, Liu W, Lv X, Sun H, Zhang L, Liu Y, *et al.* Potent *in vitro* chondrogenesis of CD105 enriched human adipose-derived stem cells. ***Biomaterials*** 2010;31:3564-71.
44. Rada T, Reis RL, Gomes ME. Distinct stem cells subpopulations isolated from human adipose tissue exhibit different chondrogenic and osteogenic differentiation potential. ***Stem Cell Rev*** 2011;7:64-76.
45. Yamamoto M, Nakata H, Hao J, Chou J, Kasugai S, Kuroda S. Osteogenic Potential of Mouse Adipose-Derived Stem Cells Sorted for CD90 and CD105 *In Vitro*. ***Stem Cells Int*** 2014;2014:1-17.
46. Chung MT, Liu C, Hyun JS, Lo DD, Montoro DT, Hasegawa M, *et al.* CD90 (Thy-1)-Positive Selection Enhances Osteogenic Capacity of Human Adipose-Derived Stromal Cells. ***Tissue Eng Part A*** 2013;19:989-97.
47. Hosoya A, Hiraga T, Ninomiya T, Yukita A, Yoshiba K, Yoshiba N, *et al.* Thy-1-positive cells in the subodontoblastic layer possess high potential to differentiate into hard tissue-forming cells. ***Histochem Cell Biol*** 2012;137:733-42.
48. Majka SM, Fox KE, Psilas JC, Helm KM, Childs CR, Acosta AS, *et al.* De novo generation of white adipocytes from the myeloid lineage via mesenchymal intermediates is age, adipose depot, and gender specific. ***Proc Natl Acad Sci U S A*** 2010;107:14781-6.
49. Billon N, Iannarelli P, Monteiro MC, Glavieux-Pardanaud C, Richardson WD, Kessaris N, *et al.* The generation of adipocytes by the neural crest. ***Development*** 2007;134:2283-92.
50. Mao JJ, Prockop DJ. Stem cells in the face: tooth regeneration and beyond. ***Cell stem cell*** 2012;11:291-301.
51. d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, *et al.* Human dental pulp stem cells: from biology to clinical applications. ***J Exp Zool B Mol Dev Evol*** 2009;312b:408-15.
52. Navabazam AR, Sadeghian Nodoshan F, Sheikhha MH, Miresmaeili SM, Soleimani M, Fesahat F. Characterization of mesenchymal stem cells from human dental pulp, preapical follicle and periodontal ligament. ***Iran J Reprod Med*** 2013;11:235-42.
53. Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J, *et al.* Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. ***Stem Cells Dev*** 2008;17:1175-84.
54. Takashima Y, Era T, Nakao K, Kondo S, Kasuga M, Smith AG, *et al.* Neuroepithelial cells supply an initial transient wave of MSC differentiation. ***Cell*** 2007;129:1377-88.
55. Morikawa S, Mabuchi Y, Niibe K, Suzuki S, Nagoshi N, Sunabori T, *et al.* Development of mesenchymal stem cells partially originate from the neural crest. ***Biochem Biophys Res Commun***

2009;379:1114-9.
56. Lemos DR, Paylor B, Chang C, Sampaio A, Underhill TM, Rossi FM. Functionally convergent white adipogenic progenitors of different lineages participate in a diffused system supporting tissue regeneration. ***Stem cells*** 2012;30:1152-62.
57. Wrage PC, Tran T, To K, Keefer EW, Ruhn KA, Hong J, *et al.* The Neuro-Glial Properties of Adipose-Derived Adult Stromal (ADAS) Cells Are Not Regulated by Notch 1 and Are Not Derived from Neural Crest Lineage. ***PLoS One*** 2008;3:e1453.
58. Sowa Y, Imura T, Numajiri T, Takeda K, Mabuchi Y, Matsuzaki Y, *et al.* Adipose Stromal Cells Contain Phenotypically Distinct Adipogenic Progenitors Derived from Neural Crest. ***PLoS One*** 2014;8:e84206.
59. Feng J, Mantesso A, De Bari C, Nishiyama A, Sharpe PT. Dual origin of mesenchymal stem cells contributing to organ growth and repair. ***Proc Natl Acad Sci U S A*** 2011;108:6503-8.
60. Slukvin II, Vodyanik M. Endothelial origin of mesenchymal stem cells. ***Cell cycle*** 2011;10:1370-3.
61. Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? ***Arch Oral Biol*** 2012;57:1439-58.
62. Vaculik C, Schuster C, Bauer W, Iram N, Pfisterer K, Kramer G, *et al.* Human dermis harbors distinct mesenchymal stromal cell subsets. ***J Invest Dermatol*** 2012;132:563-74.
63. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. ***Cell stem cell*** 2008;3:301-13.