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ORIGINAL ARTICLE

Establishing a technique for isolation and characterization of human periodontal ligament derived mesenchymal stem cells

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Abstract Mesenchymal stem cells (MSCs) are extensively used in tissue regenerative procedures. One source of MSCs is the periodontal ligament (PDL) of teeth. Isolation of MSCs from extracted teeth is reasonably simple, being less invasive and presenting fewer ethical concerns than does the harvesting of MSC's from other sites. The objectives of this study were to isolate and characterize the PDL stem cells (PDLSC) from healthy adults' extracted teeth and then to characterize them by comparing them with bone-marrow derived MSCs (BMMSC).

Methods: The PDL tissue was scraped from the roots of freshly extracted teeth to enzymatically digest using collagenase. The cells were sub-cultured. Flow-cytometric analysis for the MSC surface-markers CD105, CD73, CD166, CD90, CD34, CD45 and HLA-DR was performed. To confirm the phenotype, total RNA was extracted to synthesize cDNA and which was then subjected to RT-PCR. The gene-expression for Oct4A, Sox2, NANOG and GAPDH was determined by gel-electrophoresis. To assess their multilineage potential, cells were cultured with osteogenic, chondrogenic and adipogenic medium and then stained by Alizarin-red, Alcian-blue and Oil-Red-O respectively. MSCs from the bone-marrow were processed similarly to serve as controls.

Results: The cells isolated from extracted teeth expanded successfully. On flow-cytometric analysis, the cells were positive for CD73, CD90, CD105, CD166 and negative for CD34, CD45 and HLA-DR. The PDLSCs expressed Oct4A, Sox2, and NANOG mRNA with GAPDH expression.

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Cells cultured in the osteogenic, chondrogenic and adipogenic media stained positive for Alizarin-red, Alcian-blue and Oil- Red-O respectively. The surface marker expression and the trilineage differentiation characteristics were comparable to those of the BMMSCs.

Conclusions: The periodontal ligament tissue of extracted teeth is a potential source of therapeutically useful MSCs. Harvesting them is not invasive and are a promising source of MSC as the PDLSCs showed characteristics similar to those of the highly regarded MSC's derived from bone-marrow.

1. Introduction

Many chronic non-healing wounds and persistent inflammatory conditions including periodontitis, a widespread condition of the supporting-tissues of the teeth, have been hypothesized to be treated potentially by the use of stem cells (Ennis et al., 2013). Specifically, the MSCs used for that purpose have been found useful in numerous clinical and in-vitro studies and are proven to be beneficial. There are many sources from which such progenitor-cells can be isolated for use in regenerative procedures. Traditionally, the MSCs are isolated from diverse adult and embryonic tissues like the bone-marrow, brain, peripheral-blood, skeletal muscles, liver, Wharton's Jelly, cutaneous-tissue, blood vessels, etc. (Park et al., 2011). However, several limitations of such stem cell therapy includes difficulty in their isolation, complex methods involved in their preparation, need for large number of required cells for a desired clinical effect and the ethics of ways they are harvested and delivered (Ennis et al., 2013; Niibe et al., 2017). One of the reasons for difficulty of their isolation is the invasive-nature of the procedures involved in the process of isolation.

Roberts and Jee et al. in their paper on cell-kinetics reported that the PDL contains a variety of cells (Roberts and Jee, 1974) and it has been suggested by researchers that these cells can produce various kinds of tissues under appropriate conditions. The cells that are present around the vasculature in the PDL have been identified as ''progenitor-cells'' by Park et al. (2011). These PDL stem cells belong to the 'adult-stem cells' category since they are isolated from postnatal tissues. They are currently considered to be potentially highly suitable for tissue regeneration.

However, not all the cells present within the PDL are the stem cells and their identification and isolation in a tissue sample is a difficult task. Identification must be done based on indirect properties like their surface-protein expression, cell-cycle, clonogenicity and their tri-lineage differentiation capabilities (Park et al., 2011). Since Seo et al. (2004) first isolated the PDSLCs many researchers have repeated such isolations and studied the characteristics of the resulting PDLSCs. Wang et al. (2011) compared the PDLSC's isolated from the alveolar-sockets after extraction with root-derived PDLSC's. Their observations indicated the socket-derived cells were comparable with root-derived PDSLSC's. However, the socket-derived cells had a better proliferative ability, osteogenic and adipogenic capabilities than the root-derived cells.

Many researchers (Giovani et al., 2016; Silvério et al., 2010; Ji et al., 2013) isolated PDLSC's from the deciduous teeth (d-PDLSC's) and concluded they had higher proliferation and stronger osteogenic and adipogenic potential that those derived from permanent teeth. However, Song et al. (2012) did not observe significant differences between the two groups of cells. Li et al. (2014) isolated the d-PDSLC from the root-resorbed deciduous teeth and observed high expression of RUNX2 and RANKL. They considered that the root resorption seen when such d-PDSLC come in contact with the root surface of the permanent teeth explained the presence of RUNX2 and RANKL as those molecules can lead to osteoclastic differentiation. This may be a hindrance if d-PDLSC's are used in periodontal regenerative procedures. Song et al. (2012) isolated PDLSC's from supernumerary teeth and observed higher colony-forming abilities. Differences in the methodology used during the isolation procedure including the culture-conditions are considered to be responsible for the variations seen in the cells investigated in the above mentioned studies. To try to clarify the differences between the MSCs Zhu and Liang (2015) in their review on PDL stem cells have proposed a set of optimized-conditions for isolation and culture of those cells but thus far no studies have been conducted comparing PDLSC's with the BMMSC's. Hence the primary aim of our research was to isolate and characterize the PDL derived stem cells and compare their surface expression and the trilineage differentiation potential to those of the more commonly used BMMSCs.

2. Materials and methods

Experiments were performed in accordance with the guidelines for medical ethics and research of the International Medical University, Malaysia. The study-design and experiments were approved by the Ethics Committee. All participants provided written informed consent to participate in this study.

2.1. Isolation and expansion of human PDLSC's

Four healthy human premolar teeth extracted for orthodontic reasons were collected immediately in a container containing Dulbecco's phosphate buffered saline (DPBS) with 1% antibiotic antimycotic (Gibco, Thermo Fischer Scientific, USA). The extracted teeth were then washed three to five times using DPBS, in a sterile petri dish in a bio-safety cabinet. The middle-third of the roots were scraped gently using a bard parker's blade number 15 into a sterile petri-dish. The tissues obtained were carefully examined and minced into smaller pieces of approximately 1 mm^3 when required. Enzymatic digestion was employed using freshly prepared 3 mg/ml collagenase type I solution (Gibco, Thermo Fischer Scientific, USA) and 4 mg/ml of dispase II (Gibco) with incubation at

37^0 in 5% CO2 for 45 min. By adding equal amount of knock out Dulbecco's modified eagle's medium (DMEM-KO, Life Technologies, USA) the action of the enzyme collagenase IV was neutralized. The mixture was then passed through a 70μmeter strainer (BD Biosciences, USA) to prepare single-cell suspension which was then transferred to a T25 flask (Corning, USA) containing complete culture media (88% KnockOut™ Dulbecco's Modified Eagle medium (Gibco, USA), 10% foetal bovine serum (Gibco, USA), 1% GlutaMAX™ L-glutamine (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), 10 ngm/ml basic fibroblast growth factor (Gibco, USA). The flask was placed in a humidified atmosphere of 37 °C and 5% CO_2 for three days. The medium was changed every three days. Floating cells were removed after 72 h and the adherent cells were further expanded and cryopreserved in liquid nitrogen for subsequent use. Experiments were performed using either passage(P) 4 or 5 cells.

2.2. Flow cytometric analysis for cell surface markers; characterization of isolated cells as MSC's

PDLSCs from P4 were used for these experiments. To characterize at a single-cell level, a multi-parametric flow-cytometry combining seven surface markers panel analysis was done. The antibodies CD105, CD73, CD166, CD90, CD34, CD45 and HLA-DR (BD biosciences, USA) were used to study the cell-surface characteristics. These markers were selected based on common MSC characteristics (Dominici et al., 2006). The combinations of CD34 FITC (Fluorescein isothiocyanate-fluorophore), CD 73 PE fluorophore, CD 34 FITC + CD73 PE, CD 45 FITC + CD 166 PE, HLADR FITC + CD 90 PE, CD 105 PE, for isotype control FITC (IgG1) + PE (IgG1), FITC (IgG2) + PE (IgG1) and one unstained tube was employed. This unstained tube served as negative control; and the isotype control tube had equal number of cells with corresponding antibody. Previously isolated and cryopreserved P4 BMMSCs served as positive control. The BMMSCs were obtained from the Biomedical Research Core Facility (BioRCF), Institute for Medical research (IMR), Kuala Lumpur, Malaysia. The steps were repeated for BMMSC. The PE conjugation was planned for all the expected positive markers and the FITC conjugation was planned for all the negative markers. The BD FACS Calibur software was used to analyse the marker expression.

2.2.1. Cell surface staining and analysis

The cells were trypsinized using TrypLE express (GIBCO, life sciences) and centrifuged for 5 min at 200*g*. The supernatant was pooled to harvest all the cells into one single tube and the cells were re-suspended in 1 ml of PBS. Cell-counting was done using a haemocytometer. The cells were then adjusted to the concentration of 0.7×10^6 cells in 700 μl PBS and equally divided into 7 separate flow-tubes. These cells were fixed with 4% paraformaldehyde and incubated with 3% bovine serum albumin and then incubated with primary antibodies against CD105, CD73, CD166, CD90, CD34, CD45 and HLA-DR for 45 min in ice. Following this, the cells were given a buffer-wash following which the secondary antibodies were added at room-temperature for 45 min. The mixture was washed three times prior to measurement using the flow-cytometer. The FACS Calibur software readings were recorded based on quadrant statistics for both the cell types and both histogram and density plots were considered.

2.3. Trilineage differentiation of the isolated cells:

PDLSCs from P3 were thawed and expanded in T75 flasks. Upon 80% confluence, the cells were trypsinized using trypsin enzyme (TrypLE Express, GIBCO) and counted using a haemocytometer. A total of 5.1×10^5 cells were plated into 13 wells at 3.9×10^4 cells per well at a cell density of 4.3×10^3 cells per cm^2 surface area of each 6-well plate. The cells were allowed to attach and reach 80% confluence. Approximately, equal number of BMMSC's were taken as a control.

In 4 days, the cells reached 80% confluence in all the 6-well culture plates. The media was changed from complete culture media to specific tri-lineage differentiation media (osteogenic, adipogenic and chondrogenic media – StemPro Osteocyte, Chondrocyte and Adipocyte differentiation kit, Thermo Fisher Scientific). The differentiation media was replenished every 4th day and photomicrographs of the cells were captured using the Nikon eclipse Ti-U inverted bright-field microscope attached to a Nikon 12-megapixel digital camera. The photomicrographs were captured under x10 and x20 magnifications. The control 6-well plate received regular complete culture media (CCM). The other 6-well plate that was used for cell counting was trypsinized using TrypLE express (GIBCO) and cell counting was performed to determine the number of cells that were present just before initiation of differentiation process. The cells were conditioned for 4 weeks (28 days) before analysing the osteogenic, adipogenic and chondrogenic differentiation potential of the cells by staining with Alizarin -Red, Oil-Red-O and Alcian-blue stains respectively. On day 28, the cells were fixed using 4% paraformaldehyde solution and stained appropriately.

2.3.1. Alizarin-red, Oil-red-O and Alcian-blue staining

The cells were fixed by adding 1 ml of 4% paraldehyde solution to each well for 45 min at room -temperature after aspirating the media. The fixative was carefully aspirated, and the wells were rinsed twice with water and each time aspirated. Two ml of Alizarin-red solution (EMD Millipore corporation, USA), Oil-Red-O solution (Merck) and 1% Alcian-blue solution (Merck) was added to appropriate wells and left for 30 min and 50 min at room-temperature for Alizarin-red/Alcian-blue and Oil-Red-O respectively. Following this, the stain was carefully aspirated, and the wells were washed four times with water and each time aspirated. Two ml of water was finally added to each well to prevent the cells from drying and to allow for visual inspection under the microscope and image acquisition.

2.4. Gene analysis - RNA extraction and reverse transcriptase (RT) polymerase chain reaction (PCR)

The total RNA from the isolated PDLSCs was extracted using the PureLink RNA extraction mini kit, Invitrogen, USA (Catalogue number - 12183018A) following the manufacturer's instructions. The extracted RNA was used for synthesis of cDNAs. Then the RT PCR was conducted with an obtained

1ng of RNA. The primers used in the experiment are listed in the Table 1. The cDNAs were expanded using about 30–35 PCR cycles. Every cycle had denaturation for about 30 s, temperature being set at 94 °C and annealing and extension at 72 °C for about 30s. The PCR products were read by gel-electrophoresis.

3. Results

3.1. Cells isolation and expansion

Cells isolated from the scrapings of the PDL appeared in the culture dishes as early as in Day 5 and cell colonies started to form between Days 7–21. The cells were small, thin (initially-Day 3) and appeared spindle shaped with a fibroblast like morphology (Fig. 1). Initially, the cells appeared in isolated focal areas and later to formed colonies and showed confluence in the cell culture flasks. The isolated PDLSCs reached 80% confluence in around 18 days.

3.2. Flow cytometric analysis

Following the quadrant statistics based on the X and Y parameters in the FACS Calibur software, for the combinations used, Isolated cells were positive and negative for the cell surface markers as depicted in the Table 2. FACS analysis showed very similar expression profiles for both the PDLSC's and the BMMSC's (Fig. 2).

3.3. Tri-lineage differentiation – cell fixation and staining characteristics

The cells through Day 16 and Day 24 (Fig. 1) and at the end of 28 days (Fig. 3) after staining with appropriate dyes, demonstrated differentiation into osteogenic, adipogenic and chondrogenic lineage. This was confirmed by the cells showing calcium deposits staining orange red (Fig. 3 panel 1) for osteocytes, by adipocytes containing lipid droplets stained red (Fig. 3 panel 2) and by developing chondrogenic cells demonstrated by the presence of glycosaminoglycans as indicated by positive staining by 1% Alcian-blue solution (Fig. 3 panel 3). Visual examination of all the culture plates was done, and the staining characteristics were qualitatively comparable to those of cells derived from the bone marrow.

Table 1 Primers employed in the reverse-transcription PCR experiment.

Gene	Primers
OCT-4A	Forward: 5′-TGGGCCAGGCTCTGAGGTGT -3′
	Reverse: 5′- TCCTGCTTCGCCCTCAGGCT -3′
SOX-2	Forward: 5′- GAGAACCCCAAGATGCACAAC -3′
	Reverse: 5′- CGCTTAGCCTCGTCGATGA -3′
GADPH	Forward: 5′- GTCTCCTCTGACTTCAACAGCG -3′
	Reverse: 5′- ACCACCCTGTTGCTGTAGCCAA -3′
NANOG	Forward: 5′- GCCTCACACGGAGACTGTCTC -3′
	Reverse: 5′- AGTGGGTTGTTTGCCTTTGG -3′

3.4. Gene analysis by polymerase chain reaction (PCR)

In addition to the cells surface marker characteristics and their osteogenic, chondrogenic and adipogenic capabilities, their expression profile for pluripotency markers was evaluated by PCR. These markers are pluripotent (Hardiany et al., 2018) as these are the transcription factors commonly expressed by the embryonic stem cells. Upon gel electrophoresis the cells expressed a set of pluripotency markers including Oct4A, Sox2, and NANOG. In the gels the gene GAPDH acted as the control. Among the selected markers, the PDLSC's expressed GAPDH and Sox2 more intensely than Oct4A and NANOG. (Fig. 3, panel 4)

4. Discussion

Currently, there are numerous sources of MSCs including the bone-marrow, Wharton's Jelly, fat, muscle salivary glands, synovial-fluid etc. However, isolation of MSC's from any of these sources is very technique sensitive and requires clinically invasive procedures that can cause severe discomfort to the patient. This indicates the desirability of an MSC source that has easy access, is less invasive and consequently may be relatively inexpensive. The prevalence of patients with malocclusion (Gudipaneni et al., 2018) who need extraction of the premolars and patients with impacted third molars (Al-Anqudi et al., 2014) is quite high. Therefore, this source would seem to be much greater than any other previously known source of MSC's.

In our study, the extracted teeth were transferred to the laboratory immediately in PBS solution. This was used as transport-media because it has been shown that PBS that is widely used in biological research fields maintains an optimal near-neutral pH in order to preserve the cells and maintain their osmolarity. Chen et al. (2015) evaluated effects of storage temperature on the viability of PDL fibroblasts after dental-trauma. They concluded that room-temperature is adequate for storing the avulsed teeth if they are in Hank's balanced salt-solution, DMEM, or Milk. However, Sigalas et al. (2004) observed that a greater number of cells survived and proliferate when they were stored at 0 °C than at room-temperature. Hence, in this study, the samples were transferred in cold conditions. Upon arrival of teeth in the laboratory, cold PBS was used to wash the samples for the same reasons mentioned above.

During tissue (PDL) recovery, exclusively the middle-portion of the root was scraped rather than the cervical or the apical-portions. This is because the proliferating mesenchymal cells are reported to be rich around the blood vessels in the middle-portions of the root than other locations (Sayaniwas et al., 1999). The harvested periodontal tissue was digested enzymatically to separate the cells. Enzymatic digestion was preferred over the other alternative, namely use of Trypsin/EDTA because enzymatic method is observed to be more efficient (Chopra et al., 2018) by about 20% in dispersing the cells. The isolated cells demonstrated fibroblastic morphology during their proliferation. The cells appeared spindle, elongated and they began to develop into colonies in 7–21 days. This in accordance with observations made by many researchers (Park et al., 2011; Sayaniwas et al., 1999; Tomar et al., 2010)

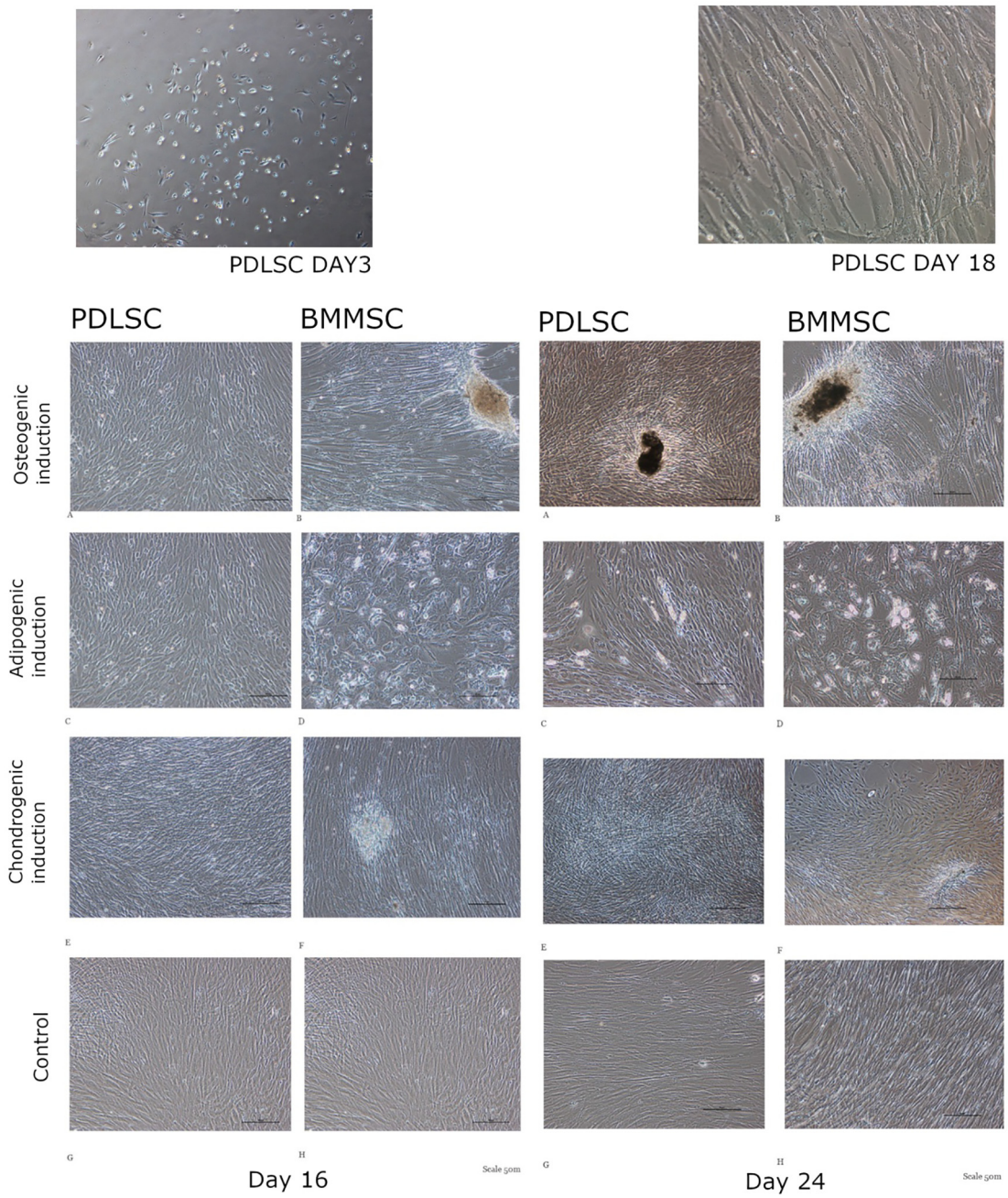

Fig. 1 Cell proliferation of human periodontal ligament derived MSCs (Top panel) at Day 3 and Day 18 after seeding. Spindle like morphology was observed during the cell proliferation. The cells started to from colonies and 80% confluence was observed around Day 18. Bottom panel represents PDLSC's (A, C, E, G – all unstained) and BMMSC's (B, D, F, H – all unstained) under the induction of Osteogenic (A and B) adipogenic (C and D) and chondrogenic (E and F) medium on Day 16 and Day24. G and H depicts cells cultured under regular culture medium that served as negative control.

who have isolated and cultured the PDLSC's from the extracted teeth.

The isolated cells showed typical MSC characteristics, demonstrating self-renewing properties along with tri-lineage differentiation capabilities. Szepesi et al. (2016) characterized MSC's derived from adipose-tissue, PDL and Wharton's Jelly by demonstration of induction of trilineage differentiation and by cell-surface marker expression. The authors employed the alizarin-red stain after 14 days of osteogenic induction. Their observations indicated adipose-tissue and PDL derived MSC's formed more calcific nodules than the Wharton's Jelly derived MSC's. They concluded that mineralizing abilities of Wharton's Jelly derived cells was less than that of the other two cell types. However, the Wharton's Jelly cells demonstrated significantly higher amount of adipogenic induction compared to other two MSC sources. The chondrogenic differentiation did not show significant differences between the cell types. The authors also reported a significant correlation between cal-

Table 2 The expression profile of the cell surface markers of the PDLSC's and BMMSC's derived mesenchymal stem cells.

Cell surface marker	Percentage of PDLSC's positive	Percentage of BMMSC's positive
CD73	95.06%	97.05%
CD90	98.69%	98.23%
CD105	88.25%	91.48%
CD166	89.24%	91%
CD34	0.93%	2.35%
CD45	9.53%	8.80%
HLA-DR	0.90%	0.65%

cium formation and CD90 expression. We consider that since periodontal regeneration is more an osteogenic process rather than an adipogenic one, the relatively reduced adipogenic abilities of PDLSC's observed in their study is not an impediment to their eventual periodontal therapeutic use. Similarly, Ma et al. (2019) compared the tri-lineage differentiation abilities of MSC's derived from amniotic-membrane, umbilical-cord and chorionic-plate in serum-free conditions. Their observations indicated similar morphology and Immunophenotypic marker expression profiles among the cell types while the amniotic-membrane derived cells demonstrated significantly high osteogenic potential than the other cells.

Recently, Owston et al. (2019) assessed the differentiation characteristics of MSC's from periosteum and BMMSC's. The osteogenic and chondrogenic potential were similar with reduced adipogenic ability in the periosteum derived MSC's. Researchers have also tried to evaluate the trilineage potential in animal derived MSC sources. Heidari et al. (2013) compared the proliferative and the multi-lineage potential of bone-marrow, spleen and liver tissue derived MSC's from sheep. Their observations reported identical multilineage potential of all the three sources. In line with the literature review, the

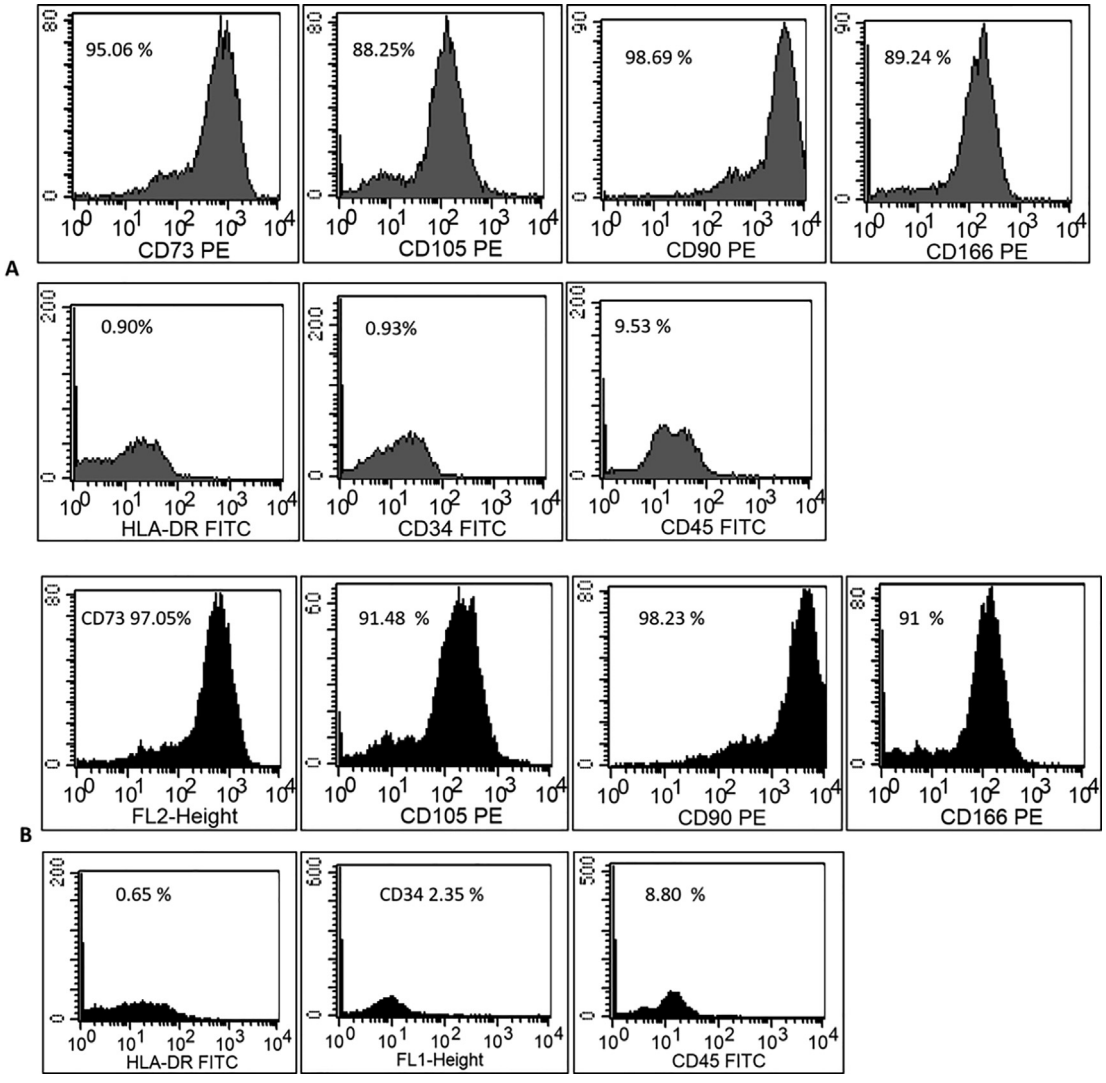

Fig. 2 Immunophenotypic analysis of PDLSC's (A) and BMMSC's (B) by flow cytometry. Cultured cells were incubated with specific antibodies against the cell surface antigens CD73, CD105, CD90, CD166, HLA-DR, CD34 and 45. Both PDLSC's and BMMSC's showed similar expression profile.

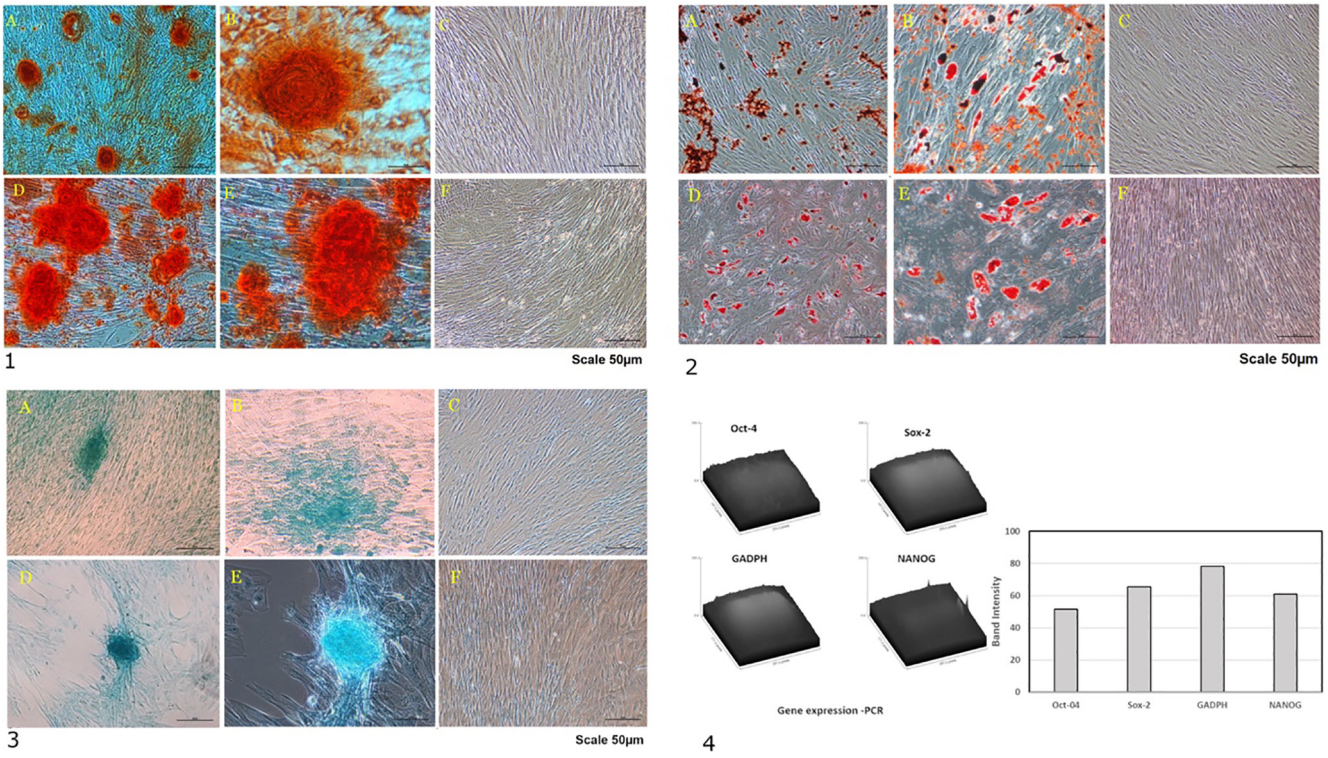

Fig. 3 Picture labelled 1, 2 and 3 represents osteogenic, adipogenic and chondrogenic differentiation of periodontal ligament mesenchymal stem cells respectively. Periodontal cells (A, B and C) stained with Alizarin Red solution (panel1), Oil Red O (panel2) and Alcian blue (panel3) after 28 days under ×10 and ×20 magnification. Pictures D, E and F in each panel (1,2 and3) depicts bone marrow derived cells stained with the same stain solution under ×10 and ×20 magnification. Differentiated osteoblasts and calcium deposits from both the cell types are stained red. Similarly, in panel 2 the differentiated cells demonstrate lipid droplets inside them that are stained bright red by Oil Red O solution while the undifferentiated cells are negative for the stain. Similarly, in panel 3 Cells demonstrate chondrogenic glycosaminoglycans that are stained blue by the Alcian blue solution. While the undifferentiated cells are negative for the stain. Pictures labelled C and F in panel 1,2 and 3 depict cells cultured under regular culture medium that served as negative control. (A, D, C and F - ×10 magnification and B, E-×20 magnification). Panel 4 depict the mRNA expression of pluripotency markers from the PDLSC's. Both SOX 2 and GADPH were highly expressed and Oct-4 and NANOG showed relatively a lower level of expression. Graph depicting the band intensity is also shown.

osteogenic differentiation that was observed in our cells was comparable between PDLSC's and BMMSC's. However, our evaluation was a qualitative comparison not a quantitative one.

Representative images (Fig. 3, panels 1,2 and 3) from our experiments show successful differentiation into osteogenic, chondrogenic and adipogenic lineage. In our research, the isolated cells also showed cell-surface markers and mRNA expression profile expected from MSC's of any origin. In 2018, Reis et al. (2018) compared a multi-panel set of cell-surface markers seen with platelet-lysate expanded MSC's to those seen with fetal-calf serum expanded MSC's. Their observations showed a high percentage of cells that were positive in platelet-lysate expanded MSC's for 48 different surface proteins of which 13 were significantly high. They also noted that the enriched surface proteins were relevant to increased chondrogenic and osteogenic differentiation abilities along with better cell proliferation and migration capacities. However, we employed the conventional serum-based cell expansion during multiple passages. Additional studies comparing the PDLSC's expansion under serum -free media are required to help understand the surface characterization better.

According to the 'International Society for Cellular Therapy' (Dominici et al., 2006) the phenotypic features that label an MSC among other cells are positive expression of CD105, CD90 and CD73 cell surface markers and absence of CD45, CD34 or CD14. The results of our experiments were consistent with that outcome with positive and negative marker on cells from P4. However, Grau-Vorster et al. (2019) conducted a retrospective analysis of HLA-DR expression in 130 BMMSC's from two independent Good Manufacturing Practice compliant laboratories. Their results indicated that HLA-DR positive cells can still maintain the fibroblastic morphology, phenotype identity, multipotency and immunomodulatory capacities. The type of serum used did not affect the observed results. Similarly, Gharibi and Hughes (2012) studied the MSC expansion characteristics after cytokine induction. Based on their results, they hypothesized that the "use of cell-surface markers as characteristic criteria for MSC might be misleading and not indicative of stemness". The authors recommended the use of cell-surface markers during the initial isolation and sorting (Gharibi and Hughes, 2012) and its avoidance for the cells from later passages. Based on our expression profile we hypothesize that initial passages do not alter the cell surface

marker expression in PDLSC's. Further studies on cell-lines from later passages are indicated.

The teeth most commonly used as a source of PDL fibroblast-cultures are premolars and third-molars and usually are extracted for orthodontic purposes (Marchesan et al., 2011). These teeth are usually free from the common dental diseases and that was the case in our experiments. Isolating the periodontal cells from infected teeth or the teeth that are affected by periodontal disease in controversial (Kapila et al., 1998; El-Awady et al., 2010). There are conflicting reports on the description and the viability of the PDLSCs isolated from healthy and inflamed periodontal conditions (Soheilifar et al., 2016; Park et al., 2011; Marcopoulou et al., 2003; Ibi et al., 2007). However, despite repeated attempts, we failed to isolate the PDLSCs from inflamed-periodontal tissues. Every attempt of ours to isolate the PDLSCS showed contamination and cells were not attaching to the cell-culture plastics even after repeated attempts. More studies are needed in this area and is one of the limitations of our study. We intend to continue to study the cells isolated from inflammatory conditions of the periodontium, sub-culture those cells under serum -free conditions and also perform a comparative quantitative study on animal derived PDLSC's.

There are not many published studies that compare PDLSCs to BMMSCs. The present study focussed on the isolation of PDLSCs and comparing them with the most established MSC like the BMMSC's. Our study showed that the PDLSC showed similar cell-surface marker expression (percentage of cells positive and negative for MSC phenotype) when compared to the profile expressed by the BMMSCs. Similarly, the tri-lineage differentiation experiment also demonstrated similar staining characteristics between both cell types.

5. Conclusion

We were able to successfully isolate and characterized stem cells from the PDL of the freshly extracted teeth. The surface expression profile and the tri-lineage differentiation potential of the isolated cells were similar to those of BMMSC's. Hence, we consider that PDLSCs may be a good alternative source of MSC's. Further studies are required to elucidate if the regenerative potential of PDLSC's is similar or better than the BMMSC's.

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CRediT authorship contribution statement

Spoorthi Ravi Banavar: Writing - original draft, Investigation, Formal analysis, Validation, Data curation, Resources. **Swati Yeshwant Rawal:** Conceptualization, Funding acquisition. **Ian Charles Paterson:** Conceptualization, Methodology, Funding acquisition. **Gurbind Singh:** Methodology, Funding acquisition. **Fabian Davamani:** Data curation, Writing - review & editing, Formal analysis, Supervision. **Suan Phaik Khoo:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing, Supervision, Project administration. **Eng Lai Tan:** Methodology, Funding acquisition, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Al-Anqudi, S.M., Al-Sudairy, S., Al-Hosni, A., Al-Maniri, A., 2014. Prevalence and Pattern of Third Molar Impaction: A retrospective study of radiographs in Oman. Sultan Qaboos Univ. Med. J. 14, e388–e392.

Chen, F., Qi, S., Lu, L., Xu, Y., 2015. Effect of storage temperature on the viability of human periodontal ligament fibroblasts. Dent. Traumatol. 31, 24–28. https://doi.org/10.1111/edt.12124.

Chopra, H., Liao, C., Zhang, C.F., Pow, E.H.N., 2018. Lapine periodontal ligament stem cells for musculoskeletal research in preclinical animal trials. J. Transl. Med. 16, 174. https://doi.org/10.1186/s12967-018-1551-2.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D.S., Deans, R.J., Keating, A., Prockop, D.J., Horwitz, E.M., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317. https://doi.org/10.1080/14653240600855905.

El-Awady, A.R., Messer, R.L.W., Gamal, A.Y., Sharawy, M.M., Wenger, K.H., Lapp, C.A., 2010. Periodontal Ligament Fibroblasts Sustain Destructive Immune Modulators of Chronic Periodontitis. J. Periodontol. 81, 1324–1335. https://doi.org/10.1902/jop.2010.100054.

Ennis, W.J., Sui, A., Bartholomew, A., 2013. Stem Cells and Healing: Impact on Inflammation. Adv. wound care 2, 369–378. https://doi.org/10.1089/wound.2013.0449.

Gharibi, B., Hughes, F.J., 2012. Effects of medium supplements on proliferation, differentiation potential, and in vitro expansion of mesenchymal stem cells. Stem Cells Transl. Med. 1, 771–782. https://doi.org/10.5966/sctm.2010-0031.

Giovani, P.A., Salmon, C.R., Martins, L., Paes Leme, A.F., Rebouças, P., Puppin Rontani, R.M., Mofatto, L.S., Sallum, E.A., Nociti, F. H., Kantovitz, K.R., 2016. Secretome Profiling of Periodontal Ligament from Deciduous and Permanent Teeth Reveals a Distinct Expression Pattern of Laminin Chains. PLoS One 11,. https://doi.org/10.1371/journal.pone.0154957 e0154957.

Grau-Vorster, M., Laitinen, A., Nystedt, J., Vives, J., 2019. HLA-DR expression in clinical-grade bone marrow-derived multipotent mesenchymal stromal cells: a two-site study. Stem Cell Res. Ther. 10, 164. https://doi.org/10.1186/s13287-019-1279-9.

Gudipaneni, R.K., Aldahmeshi, R.F., Patil, S.R., Alam, M.K., 2018. The prevalence of malocclusion and the need for orthodontic treatment among adolescents in the northern border region of

Saudi Arabia: an epidemiological study. BMC Oral Health 18, 16. https://doi.org/10.1186/s12903-018-0476-8.

Hardiany, N.S., Huang, P., Dewi, S., Paramita, R., Wanandi, S.I., 2018. Analysis of pluripotency marker expression in human glioblastoma multiforme cells treated with conditioned medium of umbilical cord-derived mesenchymal stem cells. F1000 Research 7, 106. https://doi.org/10.12688/f1000research.13154.1

Heidari, B., Shirazi, A., Akhondi, M.M., Hassanpour, H., Behzadi, B., Naderi, M.M., Sarvari, A., Borjian, S., 2013. Comparison of proliferative and multilineage differentiation potential of sheep mesenchymal stem cells derived from bone marrow, liver, and adipose tissue. Avicenna J. Med. Biotechnol. 5, 104–117.

Ibi, M., Ishisaki, A., Yamamoto, M., Wada, S., Kozakai, T., Nakashima, A., Iida, J., Takao, S., Izumi, Y., Yokoyama, A., Tamura, M., 2007. Establishment of cell lines that exhibit pluripotency from miniature swine periodontal ligaments. Arch. Oral Biol. 52, 1002–1008. https://doi.org/10.1016/j.archoralbio.2007.04.009.

Ji, K., Liu, Y., Lu, W., Yang, F., Yu, J., Wang, X., Ma, Q., Yang, Z., Wen, L., Xuan, K., 2013. Periodontal tissue engineering with stem cells from the periodontal ligament of human retained deciduous teeth. J. Periodontal Res. 48, 105–116. https://doi.org/10.1111/j.1600-0765.2012.01509.x.

Kapila, Y.L., Lancero, H., Johnson, P.W., 1998. The Response of Periodontal Ligament Cells to Fibronectin. J. Periodontol. 69, 1008–1019. https://doi.org/10.1902/jop.1998.69.9.1008.

Li, B., Zhang, Y., Wang, Q., Dong, Z., Shang, L., Wu, L., Wang, X., Jin, Y., 2014. Periodontal Ligament Stem Cells Modulate Root Resorption of Human Primary Teeth via Runx2 Regulating RANKL/OPG System. Stem Cells Dev. 23, 2524–2534. https://doi.org/10.1089/scd.2014.0127.

Ma, J., Wu, J., Han, L., Jiang, X., Yan, L., Hao, J., Wang, H., 2019. Comparative analysis of mesenchymal stem cells derived from amniotic membrane, umbilical cord, and chorionic plate under serum-free condition. Stem Cell Res. Ther. 10, 19. https://doi.org/10.1186/s13287-018-1104-x.

Marchesan, J.T., Scanlon, C.S., Soehren, S., Matsuo, M., Kapila, Y. L., 2011. Implications of cultured periodontal ligament cells for the clinical and experimental setting: a review. Arch. Oral Biol. 56, 933–943. https://doi.org/10.1016/j.archoralbio.2011.03.003.

Marcopoulou, C.E., Vavouraki, H.N., Dereka, X.E., Vrotsos, I.A., 2003. Proliferative effect of growth factors TGF-beta1, PDGF-BB and rhBMP-2 on human gingival fibroblasts and periodontal ligament cells. J. Int. Acad. Periodontol. 5, 63–70.

Niibe, K., Suehiro, F., Oshima, M., Nishimura, M., Kuboki, T., Egusa, H., 2017. Challenges for stem cell-based ''regenerative prosthodontics". J. Prosthodont. Res. 61, 3–5. https://doi.org/10.1016/j.jpor.2016.09.001.

Owston, H.E., Ganguly, P., Tronci, G., Russell, S.J., Giannoudis, P. V., Jones, E.A., 2019. Colony Formation, Migratory, and Differentiation Characteristics of Multipotential Stromal Cells (MSCs) from "Clinically Accessible" Human Periosteum Compared to Donor-Matched Bone Marrow MSCs. Stem Cells Int. 2019, 1–14. https://doi.org/10.1155/2019/6074245.

Park, J.-C., Kim, J.-M., Jung, I.-H., Kim, J.C., Choi, S.-H., Cho, K.-S., Kim, C.-S., 2011. Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed PDL tissue: in vitro and in vivo evaluations. J. Clin. Periodontol. 38, 721–731. https://doi.org/10.1111/j.1600-051X.2011.01716.x.

Reis, M., McDonald, D., Nicholson, L., Godthardt, K., Knobel, S., Dickinson, A.M., Filby, A., Wang, X., 2018. Global phenotypic characterisation of human platelet lysate expanded MSCs by high-throughput flow cytometry. Sci. Rep. 8, 3907. https://doi.org/10.1038/s41598-018-22326-5.

Roberts, W.E., Jee, W.S.S., 1974. Cell kinetics of orthodontically-stimulated and non-stimulated periodontal ligament in the rat. Arch. Oral Biol. 19, 17–21. https://doi.org/10.1016/0003-9969(74)90219-2.

Sayaniwas, M., Hilliges, M., Lindskog, S., 1999. Proliferative activity in the juxtaradicular human periodontal ligament. Dent. Traumatol. 15, 175–181. https://doi.org/10.1111/j.1600-9657.1999.tb00797.x.

Seo, B.-M., Miura, M., Gronthos, S., Mark Bartold, P., Batouli, S., Brahim, J., Young, M., Gehron Robey, P., Wang, C.Y., Shi, S., 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364, 149–155. https://doi.org/10.1016/S0140-6736(04)16627-0.

Sigalas, E., Regan, J.D., Kramer, P.R., Witherspoon, D.E., Opperman, L.A., 2004. Survival of human periodontal ligament cells in media proposed for transport of avulsed teeth. Dent. Traumatol. 20, 21–28. https://doi.org/10.1111/j.1600-4469.2004.00219.x.

Silvério, K.G., Rodrigues, T.L., Coletta, R. Dela, Benevides, L., Da Silva, J.S., Casati, M.Z., Sallum, E.A., Nociti, F.H., 2010. Mesenchymal Stem Cell Properties of Periodontal Ligament Cells From Deciduous and Permanent Teeth. J. Periodontol. 81, 1207–1215. https://doi.org/10.1902/jop.2010.090729.

Soheilifar, S., Amiri, I., Bidgoli, M., Hedayatipanah, M., 2016. Comparison of Periodontal Ligament Stem Cells Isolated from the Periodontium of Healthy Teeth and Periodontitis-Affected Teeth. J. Dent. (Tehran) 13, 271–278.

Song, J.S., Kim, S.-O., Kim, S.-H., Choi, H.-J., Son, H.-K., Jung, H.-S., Kim, C.-S., Lee, J.-H., 2012. In Vitro and In Vivo Characteristics of Stem Cells Derived from the Periodontal Ligament of Human Deciduous and Permanent Teeth. Tissue Eng. Part A 18, 2040–2051. https://doi.org/10.1089/ten.tea.2011.0318.

Szepesi, Á., Matula, Z., Szigeti, A., Várady, G., Szalma, J., Szabó, G., Uher, F., Sarkadi, B., Német, K., 2016. In Vitro Characterization of Human Mesenchymal Stem Cells Isolated from Different Tissues with a Potential to Promote Complex Bone Regeneration. Stem Cells Int. 2016, 1–9. https://doi.org/10.1155/2016/3595941.

Tomar, G.B., Srivastava, R.K., Gupta, N., Barhanpurkar, A.P., Pote, S.T., Jhaveri, H.M., Mishra, G.C., Wani, M.R., 2010. 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. 393, 377–383. https://doi.org/10.1016/j.bbrc.2010.01.126.

Wang, L., Shen, H., Zheng, W., Tang, L., Yang, Z., Gao, Y., Yang, Q., Wang, C., Duan, Y., Jin, Y., 2011. Characterization of Stem Cells from Alveolar Periodontal Ligament. Tissue Eng. Part A 17, 1015–1026. https://doi.org/10.1089/ten.tea.2010.0140.

Zhu, W., Liang, M., 2015. Periodontal Ligament Stem Cells: Current Status, Concerns, and Future Prospects. Stem Cells Int. 2015, 1–11. https://doi.org/10.1155/2015/972313.