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A Review of Stem Cell Attributes Derived from the Oral Cavity

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ABSTRACT

Oral cavity stem cells (OCSCs) have been the focus of intense scientific efforts due to their accessibility and stem cell properties. The present work aims to compare the different characteristics of 6 types of dental stem cells derived from the oral cavity: dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), stem cells from the apical papilla (SCAP), bone marrow mesenchymal stem cells (BMSC), and gingival mesenchymal stem cells (GMSC). Using immunofluorescence and real-time polymerase chain reaction techniques, we analysed the cells for stem cell, differentiation, adhesion, and extracellular matrix markers; the ability to proliferate in vitro; and multilineage differentiation potential. Markers such as vimentin, CD44, alkaline phosphatase, CD146, CD271, CD49f, Oct 3/4, Sox 9, FGF7, nestin, and BMP4 showed significant differences in expression levels, highlighting the heterogeneity and unique characteristics of each cell type. At the same time, we confirmed that all cell types successfully differentiated into osteogenic, chondrogenic, or adipose lineages, with different readiness. In conclusion, our study reveals the distinct properties and potential applications of various dental-derived stem cells. These findings contribute to a deeper understanding of OCSCs and their significance in future clinical applications.

Introduction

Bone marrow mesenchymal stem cells (BMSCs) exhibit high proliferation ability and multilineage differentiation potential. They were the first mesenchymal stem cells (MSCs) to be described, isolated from adults' bone marrow aspirate.[1] They have been extensively researched in human and animal model systems using in vivo and in vitro cell cultures. Further, they have bridged the divide and are integrated into various trials for clinical applications, with mixed success.[2,3] BMSCs are not easily accessible, and an invasive procedure is required. Therefore, further research in the field, aiming at identifying alternative stem cell sources, has led to the discovery of oral cavity stem cells (OCSCs).

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Currently, several different types of OCSCs are well known, including dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), stem cells from the apical papilla (SCAP), dental follicle stem cells (DFSC), and gingival mesenchymal stem cells (GMSC).

During embryonic development, teeth are formed through a vital interaction between oral ectodermal epithelial cells—which contribute to the enamel, apical papilla, and dental follicular structures—and MSCs responsible for dentin, pulp, cementum, and periodontal ligaments.[4] Most of the dental MSCs isolated have ectomesenchymal origin, which gives them vast options for proliferation and specialisation. Through their organ function, they have key roles in the development and preservation of teeth and the surrounding tissues.[5] DPSC are cells that specialise in regenerating tissue-like complexes, which can mineralise matrix with tubules lined with odontoblasts, and fibrosis tissue containing blood vessels in an arrangement similar to the dentin–pulp complex. Periodontal ligament (PDL) is a specialised connective tissue that connects cementum and alveolar bone to maintain and support teeth in situ and preserve tissue homeostasis.[6] In developing teeth, root formation starts and epithelial cells from the cervical loop proliferate apically and influence the differentiation of odontoblasts forming the apical papilla. This apically extending 2-layered epithelial wall forms Herwig's epithelial root sheath, which is responsible for tooth root and PDL formation.[7]

The first dental MSCs to be identified were DPSCs, isolated by Gronthos et al[8] in 2000, followed by SHED, as described by Miura et al[9] in 2003. Subsequently, PDLSC, SCAP, and DFSCs were also described.[10-12] Previous reports have demonstrated the multilineage differentiation ability of MSCs derived from dental tissues, as well as a higher proliferation rate when compared with BMSCs.[13]

MSCs are found in very low numbers in tissues. For example, it is known that BMSCs represent only 0.001% to 0.01% of the cellular components in the bone marrow.[14] Nevertheless, these cells can be cultivated and expanded in vitro to identify their specific properties and assess their potential applications in regenerative medicine. Therefore, it is imperative to obtain sufficient knowledge about their properties.

Prior investigations within this domain have explored the subject matter. Vasandan et al[15] conducted a comparative analysis of DPSC and PDLSC regarding their differentiation potential, whilst Kunimatsi et al[16] examined bone differentiation and gene expression levels of alkaline phosphatase (ALP), BMP2, Runx2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in SHED, DPSC, and BMSC. Lei et al[17] explored the similarities and differences between SCAP and dental follicles and the proteomics and molecular mechanisms of both. Further, Smeda et al[18] have characterised DPSC and SCAP, with MSC markers CD73, CD90, and CD105. They have also performed real-time quantitative polymerase chain reaction (qRT-PCR), but it was exclusively focussed on osteogenic regulation. GMSCs are well known for their regenerative abilities, with great implications in periodontology and oral and maxillofacial surgery. Cao et al[19] compare GMSC only to BMSC and DPSC.

Typically, researchers focus on comparing a limited selection of 2 or 3 cell types, and when the range of cell types expands, the number of markers and genes tested for is usually small. The testing has also been done not only on human cells in vitro but also on a variety of animal species, such as mice and dogs, in vitro and in vivo.[20,21]

Further, the true significance of our research lies in its comprehensive methodology, which involves culturing and scrutinising all cell types simultaneously within the same controlled environment and time frame. This meticulous approach aims to eliminate potential variations that may arise from disparate conditions, thereby ensuring the robustness and reliability of our findings.

Despite the existence of previous research and reviews on the topic,[22,23] our study introduces a novel aspect by conducting identical experiments in vitro with large panel of markers simultaneously. Our research endeavours to identify, characterise, and compare the properties of odontogenic cell-derived stem cells (OCSCs). This innovative approach holds implications for understanding differences in gene expression, extracellular, stem cell, and differential markers.

Materials and methods

The study was approved by the Ethics Committee of Medical University–Sofia, Bulgaria. Cells were obtained from the following structures: dental pulp from deciduous and permanent teeth, periodontal ligament, apical papilla, gingiva, and alveolar bone of humans younger than 40 years without systemic diseases and healthy oral status. The inclusion criteria for this research required intact teeth without inflammation or caries infiltration, regardless of whether they had fully developed roots, were fully erupted, or were retained. These teeth are typically extracted as part of orthodontic treatment and may contribute to complications in occlusal development. Exclusion criteria encompassed teeth with cavities or fillings, various inflammations like pulpitis and periodontitis (both acute and chronic), as well as periapical infections, which resulted in the exclusion of the respective teeth from the study. All patients (or their guardians) participating in the study signed an informed consent form prior to sample extraction, following the decision of the Ethical Committee of Medical University, Sofia's Council of Medical Science (No. 4770\11.12.2018). All teeth extractions were carried out at the Department of Dental, Oral, and Maxillofacial Surgery within the Faculty of Dental Medicine, Medical University Sofia, following thorough orthodontic and surgical consultations. No teeth were extracted solely for this study.

Cell isolation and cultivation

Dental stem cells were isolated as follows: gingival specimens (0.5 cm^2) were obtained during routine oral surgical procedures, followed by deepithelisation with a sterile scalpel blade, and apical papilla was gently separated from the apex of non–fully developed third molars, routinely extracted for orthodontic indications. Periodontal ligament was obtained via scraping with sterile scalpel blades from the middle third of the teeth roots; dental pulp was obtained from the pulp

chamber via small-size broach after cutting the tooth with sterile dental bur into the cementum–enamel junction, followed by crown removal; after tooth extraction, the deciduous pulp is isolated with enzymatic separation.[24-27] All tissue samples were immediately transported to the laboratory and washed 3 times with phosphate-buffered saline (PBS; Lonza). Soft tissue explants were then minced with a scalpel into small pieces (>3 mm^2) and enzymatically digested in 3 mg/mL collagenase type I and 4 mg/mL dispase (Sigma-Aldrich) for 60 minutes at 37 °C. After centrifugation at 1500 rpm for 5 minutes at 4 °C, the supernatant was discarded and the cell pellet was resuspended in cell culture media and seeded into 2 cm^2 culture dishes with Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1% antibioticantimycotic (Invitrogen) and 10% heat-inactivated foetal bovine serum (Sigma-Aldrich). Alveolar bone proper was fragmented into small pieces with a scalpel and seeded into 2 cm^2 culture dishes in the cell culture conditions described above.

The cell culture medium was replaced every second or third day. Cell migration and the formation of colonies were monitored via phase contrast microscopy (Leica DMRE, Leica Microsystems GmbH). After obtaining 80% confluency, explants were detached with 0.05% trypsin/ethylenediaminetetraacetic acid (Lonza) for 15 minutes, cells were counted using a hemocytometer and the total cell number was determined in 3 independent experiments. Cells were transferred to 25 cm^2 or 75 cm^2 tissue culture flasks (TPP).

Further cultivation was carried out until reaching 80% confluence. The following experiments were conducted with cells at fifth passage. All experiments were performed in triplicate.

Characterisation of dental stem cells

Characterisation of DPSC, SHED, PDLSC, SCAP, BMSC, and GMSC was performed via immunofluorescence. Multiple stem cell and differentiation marker expressions were identified. The following antibodies were used: Allophycocyanin-conjugated mouse anti-human CD271 (Miltenyi Biotec), Phycoerythrin-conjugated rat anti-human CD49f (Thermo Fisher Scientific Inc.), mouse anti-human STRO1, COL1a2, COL3a1, vimentin, nestin, CD44H, DSPP, Oct 3/4 (Santa Cruz Biotechnology), mouse anti-human ALP (Sigma-Aldrich), rabbit anti-human CK19 (Abcam), fluorescein-conjugated mouse anti-human CD146 (RnD Systems). Primary nonconjugated antibodies were at dilutions 1:250; conjugated antibodies in the experiment were at dilutions 1:1000.

High-throughput immunofluorescence analysis

Cells were incubated with the following conjugated antibodies: Allophycocyanin-conjugated mouse anti-human CD271 (Miltenyi Biotec), Phycoerythrin-conjugated rat anti-human CD49f (Thermo Fisher Scientific Inc.), Phycoerythrin-conjugated mouse anti-human CD90 (Beckman Coulter International SA), and Alexa Fluor 568-conjugated rabbit anti-human Sox 9 (Abcam). The following primary nonconjugated antibodies were used: mouse anti-human ALP (Sigma-Aldrich), mouse anti-human CD71, CD117, COL1a2, COL3a1, vimentin, nestin, CD44H, Oct 3/4, CD146 (Santa Cruz Biotechnology), mouse anti-human BMP4, and FGF7 (RnD Systems). Primary nonconjugated antibodies were at dilutions 1:250; conjugated antibodies in the experiment were at dilutions 1:1000.

For immunofluorescent analysis, cells were seeded into 96-well plates (TPP; 5000 cells/cm^2). After reaching subconfluence, cells were fixed with 4% formaldehyde for 30 minutes and then washed 3 times with PBS, followed by 30 minutes' incubation with 1% bovine serum albumin. A permeabilisation step consisting of cell incubation with 0.05% Tween20 (ICN Biomedicals Inc.) for 10 minutes and with 0.05% Triton *X*-100 (Calbiochem, Merck) for 30 minutes was performed to facilitate intracellular marker staining. Following incubation with antibodies and washing with PBS 3 times, the samples were subsequently counterstained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen) for 15 minutes to visualise the nuclei. Cell cultures were observed with IN Cell Analyzer 6000 imaging system/laser confocal slit system (GE Healthcare).

The cells were seeded in 96-well plates for high-content cellular analysis. Thirty-six fields of view were photographed (magnification ×20). Analysis was performed via IN Cell Analyzer Workstation 3.7.3 (GE Healthcare).

IN Cell Analyzer Workstation 3.7.3 recognised and analysed the nuclei, cytosolic, and background fluorescence between 2800 and 14,000 cells per marker. We counted in the cell channel the number of cells with cytosolic fluorescence/background fluorescence ratio values ≥1.1 (negative cells) and <3 (autofluorescence) and present the data in the Table as a percentage of the total cell number.

Real-time polymerase chain reaction (RT-PCR)

RNA was isolated from 80% confluent cells from 25 cm^2 flask with RNeasy Plus Mini Kit, then converted to cDNA with FastLane Cell cDNA Kit and analysed with RT2 Profiler PCR Array, Human Extracellular Matrix & Adhesion Molecules Array (PAHS 013ZE; QIAGEN). The expression of 84 individual genes important for cell–cell and cell–matrix interaction was measured following the manufacturer's instructions.

The cells were tested for cell adhesion molecules and extracellular matrix (ECM) molecules.

The cell adhesion molecules include transmembrane receptors: *ITGA1*, *ITGA2*, *ITGA3*, *ITGA4* (*CD49D*), *ITGA5*, *ITGA6*, *ITGA7*, *ITGA8*, *ITGAL*, *ITGAM*, *ITGAV*, *ITGB1*, *ITGB2*, *ITGB3*, *ITGB4*, *ITGB5*, *CD44*, *CDH1* (E-cadherin), *HAS1*, *ICAM1*, *MMP14*, *MMP15*, *MMP16*, *NCAM1*, *PECAM1*, *SELE*, *SELL* (*LECAM-1*), *SELP*, *SGCE*, *SPG7*, and *VCAM1*.

Cell–cell adhesion genes: *CD44*, *CDH1* (E-cadherin), *COL11A1*, *COL14A1*, *COL6A2*, *CTNND1*, *ICAM1*, *ITGA8*, and *VCAM1*.

Cell–extracellular matrix adhesion: *ITGA1*, *ITGA2*, *ITGA3*, *ITGA4* (*CD49D*), *ITGA5*, *ITGA6*, *ITGA7*, *ITGA8*, *ITGAL*, *ADAMTS13*, *CD44*, *ITGAM*, *ITGAV*, *ITGB1*, *ITGB2*, *ITGB3*, *ITGB4*, *ITGB5*, *SGCE*, *SPP1*, and *THBS3*.

Other cell adhesion molecules: *ANOS1*, *CCN2*, *CLEC3B*, *CNTN1*, *COL12A1*, *COL15A1*, *COL16A1*, *COL5A1*, *COL6A1*, *COL7A1*, *COL8A1*, *CTNNA1*, *CTNNB1*, *CTNND2*, *FN1*, *LAMA1*, *LAMA2*, *LAMA3*, *LAMB1*, *LAMB3*, *LAMC1*, *THBS1* (*TSP-1*), *THBS2*, *TNC*, *VCAN*, *VTN*.

ECM molecules include basement membrane constituents: *COL4A2*, *COL7A1*, *LAMA1*, *LAMA2*, *LAMA3*, *LAMB1*, *LAMB3*, *LAMC1*, and *SPARC*.

Collagens and ECM structural constituents: *COL1A1*, *COL4A2*, *COL5A1*, *COL6A1*, *COL6A2*, *COL7A1*, *COL8A1*, *COL11A1*, *COL12A1*, *COL14A1*, *COL15A1*, *COL16A1*, *ANOS1*, and *FN1*.

ECM proteases: *ADAMTS1*, *ADAMTS13*, *ADAMTS8*, *MMP1*, *MMP2*, *MMP3*, *MMP7*, *MMP8*, *MMP9*, *MMP10*, *MMP11*, *MMP12*, *MMP13*, *MMP14*, *MMP15*, *MMP16*, *SPG7*, and *TIMP1*.

ECM protease inhibitors: *ANOS1*, *COL7A1*, *THBS1* (*TSP-1*), *TIMP1*, *TIMP2*, and *TIMP3*.

Other ECM molecules: *CCN2*, *CLEC3B*, *ECM1*, *HAS1*, *SPP1*, *TGF-β1*, *THBS2*, *THBS3*, *TNC*, *VCAN*, and *VTN*.

Adipogenic, chondrogenic, osteogenic in vitro differentiation

All cell cultures were subdued to adipogenic, chondrogenic, and osteogenic differentiation conditions using previously described methods.[28,29] Cells of all 6 types were seeded in 6-well plates (TPP; 5000 cells/cm^2). After reaching 70% to 80% confluence, the standard cell culture medium was replaced with the following differentiation media: osteogenic medium consisting of 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μM ascorbic acid; adipogenic medium consisting of 50 μM dexamethasone, 500 nM isobutyl methyl xanthine, and 1 μg/mL insulin; and chondrogenic medium consisting of 100 nM dexamethasone, 100 μM ascorbic acid, and 5 ng/mL transforming growth factor-β1 (TGF-β1). The cells were incubated for 10 days with the differentiation media replaced every second or third day, followed by fixation with 4% paraformaldehyde for 20 minutes and rinsing with 1X PBS. The samples were then incubated with 2 g/mL Alizarin red S (AppliChem GmbH) with pH 4.2 to determine osteogenic differentiation, Alcian blue (Sigma Aldrich) for chondrogenic differentiation, and oil red O (Sigma Aldrich) for adipogenic differentiation. Cells were stained for 10 to 20 minutes at room temperature; the excess dyes were subsequently removed by washing 3 times with 1X PBS. Cells were observed using phase contrast microscopy.

Statistical analysis

The threshold cycle (Ct) for each well was determined by real-time cycle software. Statistical significance differences in mean Ct values were determined with RT2 Profiler PCR Array Data Analysis Software (The GeneGlobe Data Analysis Center available in the public domain at https://geneglobe.qiagen.com/bg/analyze). The difference was considered significant ($P < .05$) and greater than 2.0-fold change. Reference genes for the normalisation of RT-PCR data were GAPDH and β-2 microglobulin (B2M).

For high-content cellular analysis, the data for a total number around 10000 cells were analysed using SigmaPlot 13.0 (Systs Software Inc.). The fluorescent fraction determined by dividing the cytosolic fluorescence with the background fluorescence was used in all analyses. Unstained cells were compared to stained cells using the Mann–Whitney *U* test (nonparametric, unpaired), and $P < .05$ was considered statistically significant.

For cell counting, analysis of variance (ANOVA) followed by Bonferroni post hoc analysis with $\alpha < 0.05$ as significant was performed using IBM SPSS 23 (SPSS Inc.).

Results

Six types of OCSCs were successfully isolated and cultivated in standard cell culture conditions. The isolated cells demonstrate a typical stem cell–like appearance microscopically: plastic adherence and fibroblast-like shape with 1 or 2 projections. After passaging and further cultivation, cells were capable of reaching 80% to 90% confluence for a period of 7 to 10 days. All 6 cell types were cultivated up to fifth passage.

Immunofluorescence

The isolated cells were subjected to immunofluorescence analysis to determine the expression of intracellular and cell surface markers. The expression of markers was determined qualitatively via immunofluorescence. It was revealed that all investigated cell types express mesenchymal stem cell markers (nestin, vimentin, CD44H, CK19, ALP, STRO1, CD271, CD49f, CD146, Oct 3/4) and specific differentiation markers (Col1a2, Col3a1, DSPP) associated with dental tissues (Figure 1).

The isolated cells underwent high-throughput immunofluorescence analysis to assess the expression of intracellular and cell surface markers. Semiquantitative evaluation of marker expression via high-throughput immunofluorescence analyses indicated different levels of expression of mesenchymal stem cell markers in all tested cell types. Vimentin was uniformly highly expressed across all cell types, and *CD44* expression was observed in all cell types except SCAP. Differences of expression of other markers were more characteristic, as follows:

DPSC, SCAP, PDLSC, and SHED displayed higher expression levels of CD146, CD271, CD49f, Oct 3/4, and Sox 9. Notably, PDLSC exhibited the highest CD271 expression, SCAP showed elevated levels of CD49f, and DPSC displayed increased expression of CD146 and Sox 9. Oct 3/4 was significantly expressed in all groups except the GMSC. FGF7 exhibited a unique expression pattern, being exclusively expressed in PDLSC. Nestin was found to be expressed in both PDLSC and DPSC, whilst BMP4 showed predominant expression in SCAP and, to a lesser extent, in DPSC (Table). Raw data are included in Table S1 in Supplementary Materials.

RT-PCR

In our study, we conducted a comprehensive analysis of 6 different cell groups, each evaluated for the expression of 84 specific genes. The control group was BMSC. Amongst the examined genes, we observed significant overexpression of *LAMA2* and *LAMB1* in OCSCs compared to BMSCs; on the other hand, ITGA7 was significantly downregulated.

Gene expressions fall into 6 functional groups:

(1) ECM and basement membrane components. Notably, *LAMA2*, *LAMB1*, *VCAN*, and *COL14A1* were overexpressed in DPSC, SCAP, PDLSC, SHED, and GMSC. DPSC underexpressed

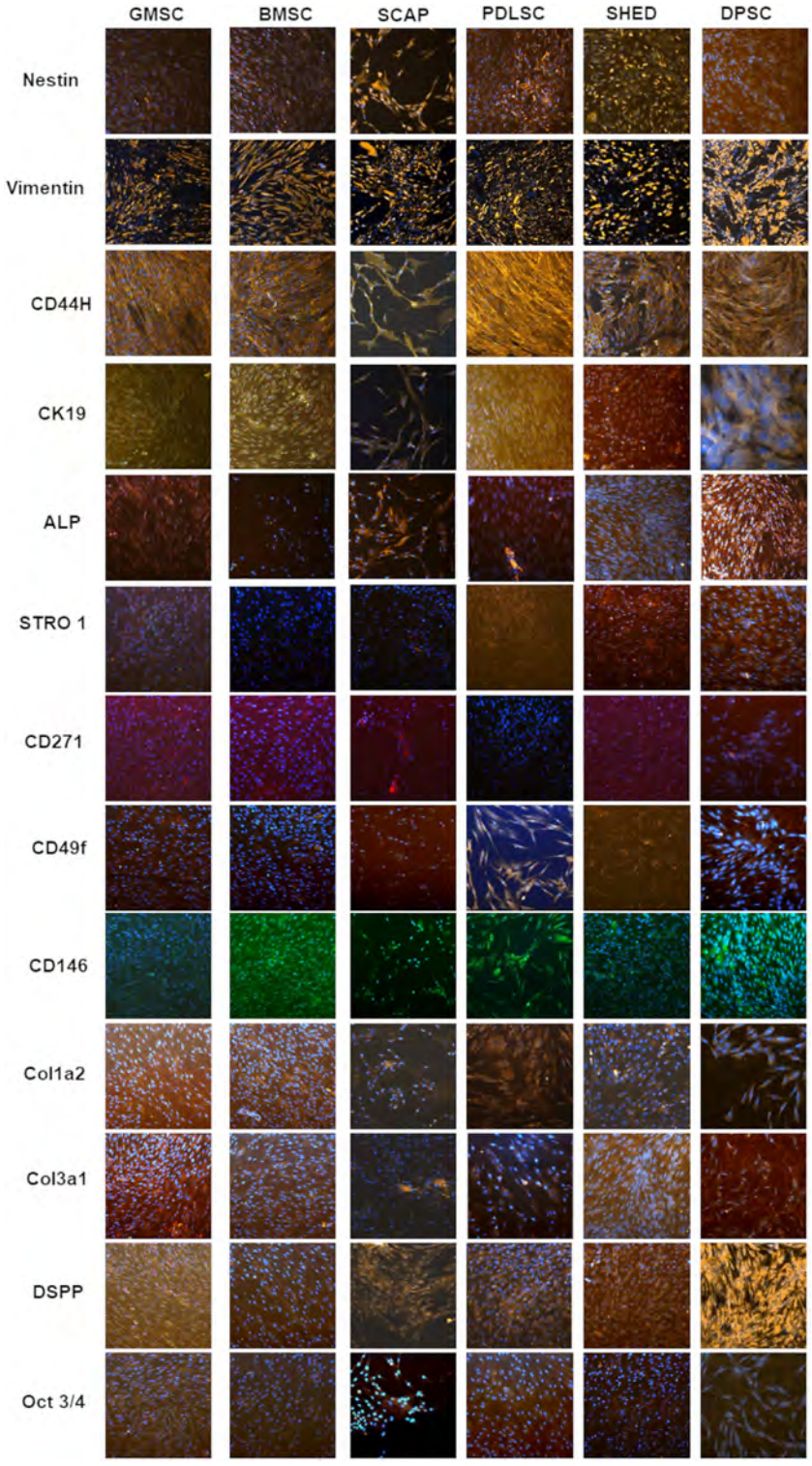

Fig. 1 – Characterisation of mesenchymal stem cell cultures of oral origin.

Confocal microscopic images of various surface markers antigens expressed on dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), periodontal ligament stem cells (PDLSC), bone marrow mesenchymal stem cells (BMSC), and gingival mesenchymal stem cells (GMSC). Magnification ×20.

COL4A, SHED underexpressed *LAMA3*, and SCAP underexpressed *LAMB3*.

(2) Cell adhesion molecules and integrins. DPSC overexpressed *ITGA4, ITGA6, ITGB3*, and *ITGA8*, whilst SHED overexpressed *ITGA4, ITGB3*, and *ITGA1*. SCAP displayed increased expression of *ITGA4, ITGA6, ITGB4*, and *ITGA8*. PDLSC overexpressed *ITGA6* and *ITGB3*, and GMSC overexpressed *ITGA4, ITGA8*, and *ITGA2*. Underexpressed in all cell types were *ITGA7*, and SCAP, PDLSC, and GMSC underexpressed *ITGA3*. Further, GMSC underexpressed *ITGB2*.

(3) Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). DPSC overexpressed *MMP10, MMP11, MMP12*, and *MMP13*, whilst SHED overexpressed *MMP1* and *MMP3* and underexpressed *TIMP1*. SCAP overexpressed *MMP8, MMP10, MMP11*, and *MMP13*. PDLSC displayed overexpression of *MMP8, MMP11, MMP13*, and *MMP16*, and GMSC overexpressed *MMP1, MMP3, MMP10*, and *MMP11*.

(4) Cell adhesion molecules and cell–cell interaction genes. DPSC overexpressed *CNTN1, NCAM1*, and *VCAM1*. SHED was the only group that overexpressed *CLEC3B* and also showed increased expression of *CNTN1* and *VCAM1*. SCAP overexpressed *CNTN1, NCAM1*, and *VCAM1*, whilst PDLSC overexpressed *CNTN1* and *VCAM1*, with the highest *NCAM1* overexpression. GMSC overexpressed *CNTN1, NCAM1*, and *VCAM1*.

(5) Cell signalling and transduction genes. SCAP overexpressed *CTNNB1* and underexpressed *CTNND2*. GMSC overexpressed *CTNND2*.

(6) Other participating components. DPSC overexpressed *RPLP0, COL16A1, THBS2, THBS3*, and *SGCE*. SHED only exhibited overexpression of *THBS2* and underexpressed *HAS1* and *TIMP1*. SCAP overexpressed *RPLP0, COL16A1, THBS2*, and *THBS3*. PDLSC overexpressed *RPLP0* and *COL16A1* and had increased *SGCE* expression. PDLSC underexpressed *HAS1*. GMSC did not show overexpression of these genes; however, it has shown underexpression of *TGF-β1*, *HAS1*, and *ANOS1* (Figure 2). Raw data are included in Table S2 in Supplementary Materials.

Proliferation profile

All cells expressed similar proliferation profiles, typically higher than BMSC. DPSC, SHED, and PDLSC increased their proliferation with a peak at fourth passage. SCAP and GMSC showed the highest proliferative capacity at passage 2, and BMSC showed constant proliferation through all passages. Statistical analysis of significant differences amongst all the groups was performed using ANOVA. Statistical significant differences were found amongst all the groups ($P < .05$; Figure 3).

Multilineage differentiation potential

To further investigate the stem cell properties of the isolated cells, we assessed their multilineage differentiation ability. All investigated OCSCs underwent specific differentiation. Figure 4 shows at higher magnification the differentiation state of the cells after 10 days of cultivation with specific cell culture media to which active substances were added. Even though all investigated cell cultures in the present study were capable of multilineage differentiation, we found that the investigated cell types were undergoing specific differentiation with different readiness (Figure 4).

Osteogenic differentiation ability in the cell cultures was confirmed through Alizarin red S staining after 10 days' incubation with differentiation media. Amongst all the investigated stem cell types, SCAP and BMSC, followed by GMSC, exhibited the highest capacity for calcium nodule formation. DPSC and SHED also displayed osteogenic capabilities, but to a lesser extent. In PDLSC culture, only single-cell clusters demonstrated the ability for calcium nodule formation. Adipogenic cell differentiation for 10 days led to a transformation of cell shape from spindle-like to polygonal, along with the formation of intracellular lipid vacuoles that stained positively with oil red O. In the present study, the most robust adipocyte-like cell induction was observed in PDLSC and SCAP cultures. Chondrogenic cell differentiation was assessed using Alcian blue staining after 10 days' incubation with differentiation cell culture media. All cell types were capable of differentiating into chondroblast-like cells, although SHED cultures underwent chondrogenic differentiation to a lesser extent.

Discussion

In recent decades, there has been a growing interest in OCSCs due to their easy accessibility and possession of typical stem cell properties.[30] However, comprehensive literature data describing the properties of all oral cavity–derived stem cells is still lacking. MSCs obtained from dental tissues have often been insufficiently classified and are typically compared to well-established stem cells like BMSCs.[31] For a significant period, it was widely accepted that only one cell type was predominantly characterised as fibroblasts.[32] To the best of our knowledge, there are currently very few reports aimed at comparing OCSCs and their key properties: that is, Yang et al[33] compare GMSC with the dental follicle and Qu et al[34] show the difference between DPSC and PDLSC, as well as BMSC and dental follicle cell types.

In our current in vitro culture conditions, which include the presence of FBS, MSCs demonstrate comparable growth potential, corresponding to the literature data.[35]

Our primary objective was to conduct a comprehensive characterisation of these stem cells to enhance our understanding of their functions and properties as stem cells. Typically, studies compare the properties of up to 3 types of stem cells from the oral cavity. However, during our investigation and observation of all OCSCs and the various protocols employed for their isolation and differentiation, we uncovered both minor and major distinctions amongst all 6 of them. In our current research, our focus was on isolating and investigating DPSC, SCAP, SHED, PDLSC, and GMSC. We used BMSC as control groups in each experiment, following prior work.[36,37] By revealing and comparing the properties of all the isolated cell types, we aim to provide comprehensive information for potential clinical applications.

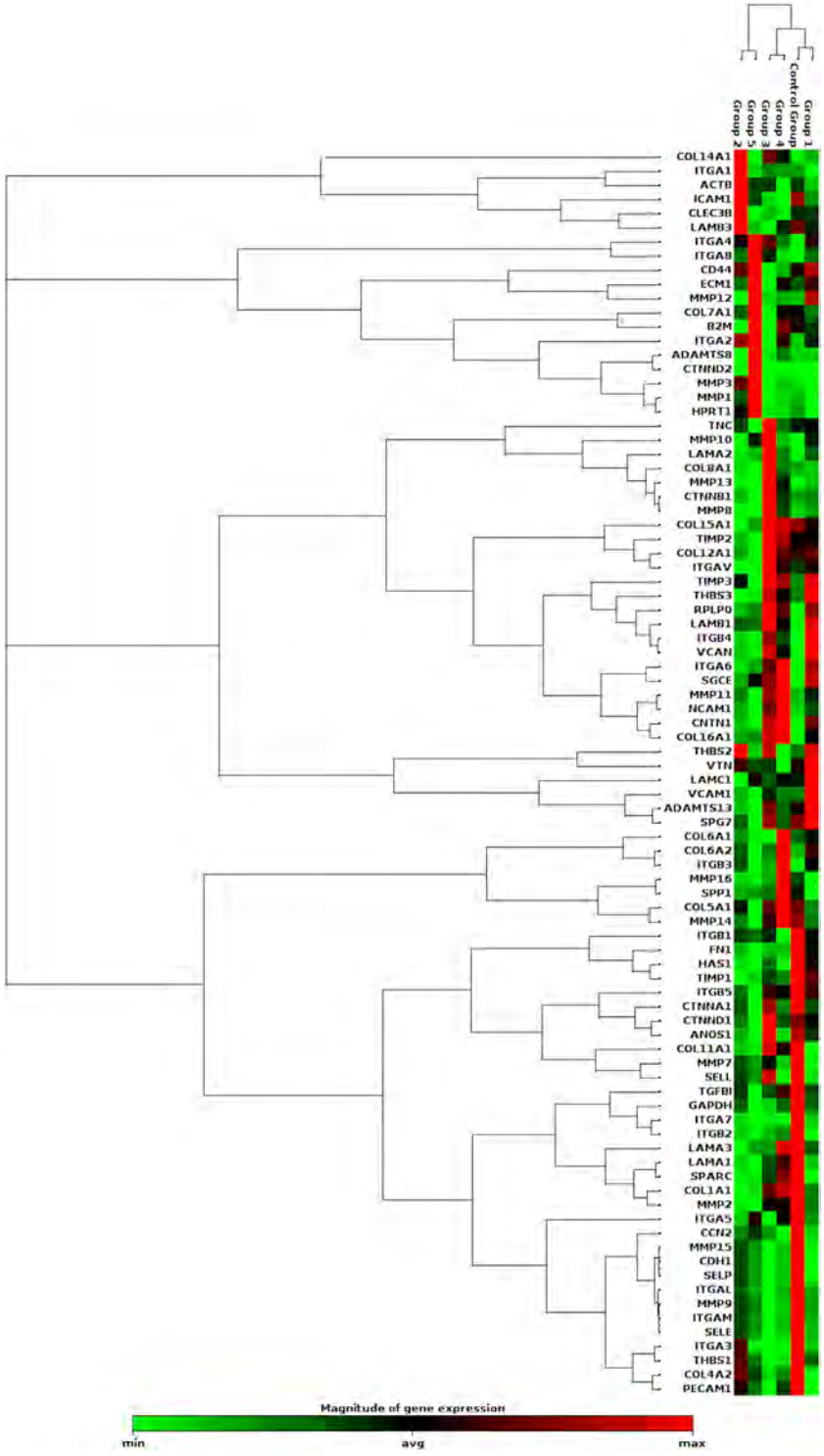

Fig. 2 – Nonhierarchical and hierarchical cluster analysis in the gene expression amongst the 6 groups of cells.

Group 1: dental pulp stem cells (DPSC), group 2: stem cells from human exfoliated deciduous teeth (SHED), group 3: stem cells from the apical papilla (SCAP), group 4: periodontal ligament stem cells (PDLSC), group 5: gingival mesenchymal stem cells (GMSC), control group: bone marrow mesenchymal stem cells (BMSC).

Stem cell and differentiation markers

The present study employed a wide range of markers to fully characterise isolated OCSCs. Some markers are linked to stem cell properties, whilst others are associated with specific biological functions. The diverse marker expression amongst the isolated cell types confirmed their multilineage differentiation ability and revealed variations in stem cell properties.

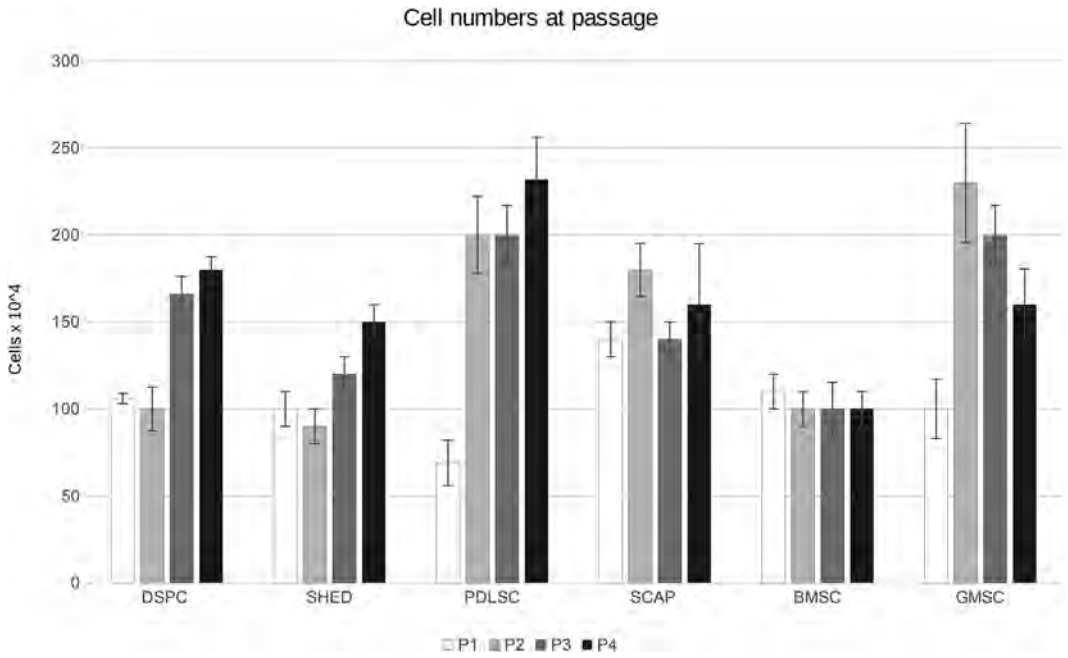

Fig. 3 – Cell count of dental pulp stem cells (DSPC), periodontal ligament stem cells (PDLSC), stem cells from the apical papilla (SCAP), bone marrow mesenchymal stem cells (BMSC), and gingival mesenchymal stem cells (GMSC) during passages 1 through 4.

Vimentin, a well-known MSC marker, showed high expression in the heterogeneous cultures, consistent with prior reports.[38] Vimentin is a type III intermediate filament responsible for maintenance of cellular shape, intracellular transport of molecules, cellular motility, focal adhesions, and so on. It is shown to enhance integrin activation and receptor for hyaluronic acid (*CD44*), binding it directly.[39] *CD44*, a mesenchymal marker crucial for adhesion in hyaluronian matrix, is also associated with the epithelial–mesenchymal transition.[40] It was highly expressed in GMSC, SHED, and PDLSC, but not in SCAP.[41] SHED and PDLSC displayed elevated *CD44* expression, possibly related to their role in the oral microenvironment, that is, connecting and remodelling extracellular fibrillar structures in processes of periodontal ligament structural adjustment and tooth root resorption. Along with high vimentin expression, *CD44* expression may signify a greater number of undifferentiated mesenchymal cells in the heterogeneous SHED and PDLSC cultures (Table). This may be related to synthesis of periodontal fibers and counteracting the masticatory forces, and for SHED dental resorption and exfoliation and permanent teeth eruption; ALP constitutes a group of proteins critical in the process of skeletal mineralisation.[42] Given that ectomesenchymal stem cells are present in both bone and embryonic tooth structures, it is anticipated that these cells isolated from non–fully developed third molars would express ALP to some extent. The data presented in the Table indicate that DPSC and SCAP exhibited the highest ALP expression, followed by PDLSC and BMC, whilst GMSC and SHED demonstrated the lowest expression of this marker. The elevated ALP expression in DPSC and SCAP likely relates to their involvement in mineralisation processes.[43] All tested markers in this study exhibited similar expression in the control groups, BMSCs, and GMSCs, with high vimentin expression and significant CD71 and collagen 3 expression. Collagen 3 is typically associated with collagen 1 and plays a significant role in connective tissue development.[44] Furthermore, our data shows differences in the expression of CD49f, CD146, CD271, CD117, BMP4, Sox 9, Oct 3/4, nestin, and FGF7 amongst the cell types. These variations are attributed to their embryologic origin and tissue-specific functions. CD49F (integrin $\alpha 6$) is expressed in various tissues and is associated with stem cell proliferation, including cancerous stem cells,[45] whilst CD146/MCAM plays a crucial role in osteogenic differentiation and bone formation.[32] CD271 is a specific marker for characterising and purifying human bone marrow MSCs,[46] and CD117 is considered an angiogenic stem cell marker expressed by all our cell types to varying degrees.[47] Sox 9, a transcription factor expressed during chondrogenesis, maintains its expression in adults, not limited to embryonic development.[48] Oct 3/4 is another stem cell marker associated with early cell development and pluripotent characteristics.[49] Nestin regulates intermediate filament protein production and influences progenitor cell division, particularly in neural progenitor cells.[50] BMP4 is responsible for angiogenesis and tissue mineralisation, influencing various cellular functions.[51] FGF7, a fibroblast growth factor, primarily affects mesenchymal cell proliferation, migration, and differentiation.[52]

Our findings revealed significant variations in marker expression amongst different cell types. CD146, CD271, CD49f, Oct 3/4, and Sox 9 displayed higher levels in DPSC, SCAP, PDLSC, and SHED. Notably, PDLSC exhibited the highest CD271 expression, surpassing that of BMSCs.[53] SCAP showed elevated CD49f levels crucial for osteogenic

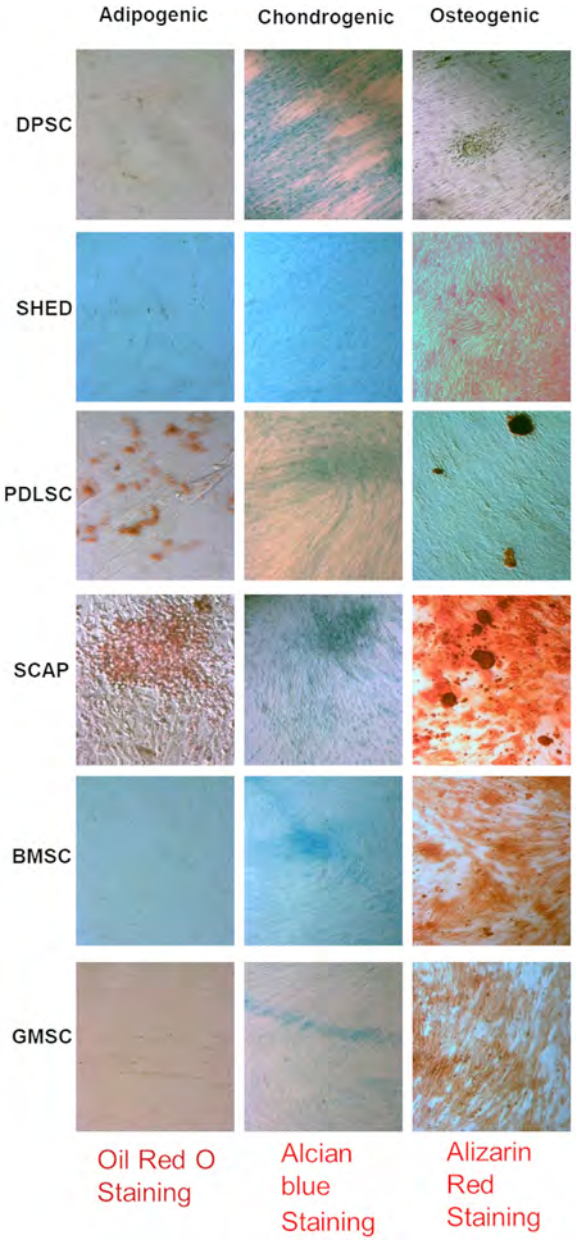

Fig. 4 – Alizarin red staining, after 10 days of osteogenic differentiation; Alcian blue staining after 10 days of chondrogenic differentiation; oil red O staining after 10 days of adipogenic differentiation of dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), stem cells from the apical papilla (SCAP), bone marrow mesenchymal stem cells (BMSC), and gingival mesenchymal stem cells (GMSC). Magnification ×5.

differentiation,[54] whilst DPSC had increased CD146 and Sox 9 expression linked to embryonic development and pluripotency.[55] Oct 3/4 had significant expression in all groups except BMSCs, indicating its role in early cell development and pluripotency, aligning with previous studies.[56] Furthermore, we discovered a unique FGF7 expression pattern, exclusive to PDLSC, implying its role in the periodontal ligament microenvironment, potentially supporting cells facing occlusal forces and environmental factors.[57] Nestin was present in both PDLSC and DPSC, whilst BMP4 was predominantly expressed in SCAP. All the cell types showed distinct and different expression profiles offering insights into the diverse properties of isolated oral-cavity derived stem cells.[58]

Gene expression analysis

ECM is a dynamic system of extracellular proteins, glycoproteins, and polysaccharides, providing tissue integrity and structure and providing cells with appropriate scaffolding for their function. Remodelling the ECM is associated with growth, tissue repair, oncogenesis and invasion, modulation of immune reactions, and inflammation.

Using native cells from an adult organism often results in heterogeneous cell cultures. This diversity is crucial for stem cell research, enabling the study of surface markers for specific cell subsets, including stem cells. Stem cells play a key role in healing by reforming the ECM, propagating, and integrating into newly formed tissue. To understand this process, we analysed RNA profiles, focussing on surface attachment proteins, ECM components, and remodelling enzymes. The results revealed distinct expression patterns in tested cells, categorised into 6 groups based on their roles in cell adhesion. The nonhierarchical and hierarchical cluster analysis indicated coregulated genes across the groups, which suggested pairings such as DPSC/BMSC, SHED/GMSC, and SCAP/PDLSC. These dental-derived stem cell groups may have specific characteristics and functions and embryonal development. Further, GMSC and SHED displayed similar expression profiles compared to BMSC.

The *LAMA2* gene, associated with laminin subunit alpha 2, has been extensively examined in various tissues. However, this is the first instance of its investigation in dental MSCs. Notably, all tested cell types exhibited *LAMA2* overexpression, with the lowest overexpression observed in SHED.

LAMB1 expression remained consistent across all tested cell types. *LAMB1*, a crucial component of the ECM, plays roles in cell adhesion, migration, and tissue organisation. Its shared expression amongst these diverse cells underscores its role in maintaining tissue integrity and function. This consistency amongst dental stem cells and fibroblasts positions them as promising candidates for regenerative therapies and tissue engineering applications, alongside well-established BMSCs.[59]

All tested cell types exhibited the presence of various MMPs, crucial enzymes involved in remodelling the ECM. These enzymes play essential roles that are not specific to stem cells but are fundamental in various physiologic processes. They are responsible for maintaining the structural integrity of the ECM and participate in tissue development, wound healing, and tissue repair.[60] Their functions extend beyond stem cells, contributing to the maintenance of tissue balance and response to both normal and pathologic conditions. Thus, they play essential roles in tissue physiology and pathology, similar to some of the functions exhibited by the tested stem cells. In the context of tooth and periodontal development, SCAP and PDLSC are responsible for forming

Table – High-throughput immunofluorescence analysis of BMSC, GMSC, SHED, PDLSC, DPSC, and SCAP expression of markers.

Percentage of expression of markers in OSCS						
%	BMSC	GMSC	SHED	PDL	DPSC	SCAP
Vimentin	85.14	83.97	91.75	87.92	91.97	92.56
CD44	33.40	16.67	57.10	56.66	18.67	0.50
CD71	30.78	21.16	26.95	41.46	31.95	33.90
Collagen 3	42.93	45.45	38.13	21.31	26.33	32.22
CD90	37.37	4.70	28.07	35.47	42.05	40.14
Collagen 1	15.41	43.74	35.14	26.91	23.45	18.57
CD49F	12.02	12.90	21.51	33.21	32.96	41.56
CD146	15.76	18.62	23.07	27.96	47.65	25.43
CD271	19.17	19.27	21.36	40.26	29.94	37.85
Oct3/4	11.44	5.49	22.33	31.09	33.79	21.51
Sox9	7.02	5.78	27.98	37.07	38.56	25.51
ALP	10.59	2.93	8.98	12.58	22.68	22.65
Nestin	11.09	7.12	11.18	26.54	25.94	12.13
FGF7	8.72	11.03	18.97	20.23	19.87	15.95
CD117	17.86	13.96	5.19	14.83	12.16	12.77
BMP4	5.80	4.70	6.67	8.93	19.91	21.68

Red: positive cell count > 10 < 20%; orange: positive cell count > 20 < 50%; green: positive cell count >50%. Cutoff for cytosolic fluorescence/background fluorescence is ≥ 1.1 (negative cells) < 3 (autofluorescence).
BMSC, bone marrow mesenchymal stem cells; DPSC, dental pulp stem cells; GMSC, gingival mesenchymal stem cells; PDLSC, periodontal ligament stem cells; SCAP, stem cells from the apical papilla; SHED, stem cells from human exfoliated deciduous teeth.

different structures, which may explain their significant overexpression of these MMPs.

We explored the expression of various collagen genes, key components of the ECM. Amongst all the tested cell types, one gene consistently exhibited significant expression: *COL1A1*. Previous studies have already highlighted the crucial role of *COL1A1* in dental stem cells and its relevance in tissue regeneration.[61] *COL1A1* is particularly important in dental tissues, providing structural support, strength, and stability in the periodontal ligament and dentin. The robust expression of *COL1A1* underscores its paramount significance in dental stem cell biology.

Amongst the other tested collagen genes, *COL14A1*'s expression in PDLSC and SHED is noteworthy. This can be explained by the embryologic and developmental functions of these cells. Like *COL1A1*, *COL14A1* also plays a critical role in maintaining tissue integrity and structure, which relates to the functions of the periodontal ligament and its anchoring role in teeth. Additionally, the shared elevation of *COL16A1* expression in GMSC and SHED highlights their active involvement in supporting and preserving the structural integrity of oral and gingival tissues, which face constant mechanical stresses.

Integrins, important for cell adhesion and signalling, displayed varied expression patterns across cell types. *ITGA6* exhibited higher expression in PDLSC, DPSC, and SCAP, consistent with results from immunofluorescence. This elevated *ITGA6* expression in these dental stem cell populations holds significant implications for their functions. In PDLSC, emphasises the role of *ITGA6* in anchoring cells to the periodontal ligament, essential for tooth attachment and mobility. For DPSC and SCAP, heightened *ITGA6* likely mediates cell –matrix interactions, enhancing adhesion and potentially contributing to dental tissue repair and regeneration.[62] In addition, our findings indicate a diverse and distinctive integrin expression profile amongst dental stem cell populations, underscoring their unique roles in adhesion, signalling, and tissue regeneration within the oral environment.

Amongst the 5 groups of dental-derived stem cells studied, gingival mesenchymal stem cells (GMSC) stand out as the only group with high *CTNND2* expression. *CTNND2* (catenin delta 2) plays a crucial role in cell adhesion and tissue organisation.[63] The elevated expression of *CTNND2* in GMSC suggests its potential importance in maintaining cellular integrity and facilitating interactions with neighbouring cells. The differential *CTNND2* expression in GMSC compared to other dental stem cell groups underscores the unique characteristics and functional properties of GMSC.

Gene expression analysis showed an extensive ECM remodelling profile of the mesenchymal cell cultures. Although this result was expected, we found subtle but distinct differences in patterns of expression, which give us better understanding of dental tissues' intimate properties.

The following study has a potential limitation in its use of RT-PCR rather than more advanced techniques such as single-cell RNA sequencing. RT-PCR offers specificity to known

RNA sequences but is limited in discovering new RNA species and in its capacity for providing a broader image of the transcriptome. At the same time, RT-PCR's quantitative analysis is constrained by a limited dynamic range, necessitating careful normalisation, a challenge not as pronounced in RNA sequencing, which can more accurately measure gene expression across a wide range. One other limitation is the fact that all markers identified in this study were assessed in vitro. The inability to detect these markers in vitro decreases the possibility of using these conventional markers for therapeutic applications.

Multilineage differentiation potential

In our research, we investigated the differentiation readiness of dental-derived stem cells, with a primary focus on adipogenic, osteogenic, and chondrogenic pathways. We studied DPSC, SHED, PDLSC, SCAP, BMSC, and GMSC to gain insights into their regenerative capabilities. Our research shows distinct differences in the differentiation readiness of the different cell cultures after short-term of incubation in differentiating conditions. This comparison is crucial for understanding their pluripotency and reducing uncertainties in heterogeneous cultures of adult stem cells, including dental,[64] and for developing future clinical application protocols.

Despite the relatively brief differentiation period compared to traditional protocols, our research highlights that significant differentiation amongst the cells begins to manifest after just 10 days. This time frame serves as a crucial marker of their readiness for differentiation.[65] Notably, osteogenic differentiation has been observed across all cell types to varying degrees, alongside chondrogenic differentiation. In adipocyte differentiation, PDLSCs and SCAPs exhibited higher induction levels, yet given an extended duration, other cell types are likely to further engage in this differentiation process.

Whilst ongoing research primarily focusses on the regenerative applications of OCSCs in oral and maxillofacial tissue repair, it is crucial to acknowledge their broader mesenchymal characteristics.[66] These versatile traits extend OCSCs' relevance beyond dental and bone regeneration, offering potential in various tissue engineering application. Given their ability for multilineage differentiation and active involvement in tissue repair within the oral cavity, exploring further their clinical implications becomes crucial. As research progresses, attention shifts towards understanding not just OCSC differentiation pathways but also their regulatory mechanisms, paving the way for broader applications in mesenchymal differentiation contexts across various medical disciplines.[67,68]

Our research aimed to uncover stem cell properties within OCSC based on specific markers, a step towards advancing stem cell therapy and clinical integration. The present study is subject to several limitations, encompassing general concerns surrounding MSCs as well as particular issues that apply to the present study. A significant area of concern involves the prolonged in vitro culture of these cells, which may induce cell ageing, potentially altering their morphology and modifying the expression of stem cell markers. Subsequent studies should aim to explore telomerase activity, addressing the ageing process, exploring cell surface and intracytoplasmic expression of stem and differentiation markers, and conducting a more comprehensive assessment of their morphology across extended cell passages. Notably, this study solely focussed on 2D cultures, prompting the need to investigate the genotype, phenotype, and overall biological behaviour of the studied cells within 3D cultures in future research.

Conclusions

This analysis of oral cavity–derived stem cells has exposed distinct and specific expression patterns of surface markers, ECM components, ECM remodelling enzymes, collagen genes, integrins, and differentiation potential. These findings may provide insights into the functional properties of these cell populations. Our study aims to uncover the nuanced characteristics specific to each distinct cell type. Through experimentation and analysis, we anticipate to not only understand their individual functionalities but also gain deeper insights into their collective behaviour. Understanding both their shared characteristics and distinct attributes is an important step in using their regenerative potential in various tissue repair and regeneration contexts, thereby advancing the field of regenerative medicine and tissue engineering. By contributing meaningfully to the advancement of knowledge in regenerative medicine and tissue engineering, we aspire to pave the way for transformative breakthroughs in health care.

Conflict of interest

None disclosed.

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Author contributions

Conceptualisation: NI and BC. Methodology: MP and VM. Software: MM. Validation: NI, VD, and ZM. Formal analysis: VD, AV, and MM. Investigation: MM and EA. Resources: VM, EA, and PS. Data curation: NI, ZM, IC, and AV. Writing–original draft preparation: MM. Writing–review and editing: BC, NI, ZM, and VD. Visualisation: MM, AV, and BC. Supervision: MP, VM, PS, PP, and IC. Project administration: NI and MD. Funding acquisition: VM. All authors have read and agreed to the published version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.identj.2024.03.008.

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