

REVIEW ARTICLE

Determinants of Dental Pulp Stem Cell Heterogeneity

Sulette Nel, BChD, MSc,* Chrisna Durandt, BSc, MSc, PhD,†

Candice Murdoch, MSc, MedSc,† and Michael S. Pepper, MBChB, PhD, MD, PD†

*Department of Oral Pathology and Oral Biology, School of Dentistry, and

†Institute for Cellular and Molecular Medicine (ICMM), Department of Immunology, and SAMRC Extramural Unit for Stem Cell Research and Therapy, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

Address requests for reprints to Sulette Nel, Department of Oral Pathology and Oral Biology, School of Dentistry, Faculty of Health Sciences, University of Pretoria, Private Bag X323, Arcadia, 0007, Pretoria, South Africa. E-mail address: sulette.nel@up.ac.za

ABSTRACT

Introduction: The aim of this review is to provide a narrative review on the determinants of dental pulp stem cell (DPSC) heterogeneity that may affect the regenerative properties of these cells.

Methods: PubMed, Scopus and Medline (Ovid) literature searches were done on human dental pulp stem cell (hDPSC) heterogeneity. The focus was on human dental pulp stem cells (hDPSCs) with a primary focus on DPSC heterogeneity.

Results: DPSCs display significant heterogeneity as illustrated by the various subpopulations reported, including differences in proliferation and differentiation capabilities and the impact of various intrinsic and extrinsic factors.

Conclusions: The lack of consistent and reliable results in the clinical setting may be due to the heterogeneous nature of DPSC populations. Standardization in isolation techniques and in criteria to characterize DPSCs should lead to less variability in results reported and improve comparison of findings between studies. Single-cell RNA sequencing holds promise in elucidating DPSC heterogeneity and may contribute to the establishment of standardized techniques.

KEY WORDS

Human dental pulp stem cells, heterogeneity

INTRODUCTION

Human dental pulp is unique non-mineralized tissue housed within the mineralized structure of the tooth and consists of various cell types including multipotent dental pulp stem cells (DPSCs). The self-renewal clonogenicity and multipotent differentiation potential of DPSCs are well described. Although differentiation into osteoblasts, adipocytes, chondrocytes, myocytes, odontoblasts and neural cells has been shown¹⁻⁷, there is a lack of consistent and reliable results when it comes to reproducing these studies *in vitro* and translating them to the clinical setting⁸. DPSC differentiation efficiency and regenerative dentine formation are inconsistent in *in vitro* and *in vivo* studies⁹⁻¹¹. This may be due to the heterogeneous nature of DPSC populations. The difficulty in establishing homogenous primary cell cultures has led investigators to consider the subpopulations that occur within the dental pulp to identify and ultimately select for those subpopulations with optimal regenerative properties. This review provides an overview of the current understanding of the determinants of DPSC heterogeneity.

Dental pulp stem cells (DPSCs)

DPSCs were first discovered in 2000 by Gronthos and co-workers¹². The terms “dental pulp stem cells”, “undifferentiated ectomesenchymal cells” and “mesenchymal stem cells (MSCs)” are used interchangeably. However, it is unclear whether these terms refer to one population of cells, subpopulations of a particular cell type or different cell populations. Stem cells are obtained from various components of the tooth including the dental follicle (dental follicle stem cells, DFSCs), periodontal ligament (periodontal ligament stem cells, PDLSCs), dental pulp of permanent teeth (DPSCs), dental pulp of deciduous teeth (stem cells from human exfoliated deciduous teeth, SHEDs) and stem cells from the apical papilla (SCAPs)¹³⁻¹⁵. Heterogeneous populations of stem cells are available in and around the primary and permanent teeth, and care should be taken to isolate one particular type and exclude contamination with cells from other origins. In this review we focus on DPSCs and the heterogeneity in this cell type.

Multipotent DPSCs are mostly isolated from third molars, exhibit a fibroblast-like morphology and reside in distinct niches in the dental pulp, including the perivascular¹⁶, subodontoblastic and apical regions¹⁷. DPSCs meet the minimal criteria for MSCs proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) as they show plastic adherence, express MSC-associated markers (CD73, CD90, CD105) and exhibit multipotent differentiation potential¹⁸. Nakajima and co-workers (2019) found that the dental pulp cells of only 14 of the 20 teeth studied showed successful

adhesion, while cells obtained from the other 6 teeth failed to adhere to the plastic surface and therefore were not considered to be DPSCs¹⁹. This translates to an isolation success rate of 70%.

DPSCs are multipotent cells with self-renewal capability and have characteristics similar to other stem cell populations including bone marrow derived mesenchymal stem cells (BM-MSCs). One key feature that DPSCs share with other MSC populations is their ability to differentiate into developmentally diverse phenotypes, such as adipocytes and neural cells⁹. The multipotent characteristics of DPSCs include their ability to differentiate into osteoblasts, adipocytes, chondrocytes, myocytes, odontoblasts and neural cells¹⁻⁷. Furthermore, DPSCs have distinct properties compared to other MSCs as they have increased proliferation potential, greater tissue generation capabilities, plasticity and immunomodulatory (anti-inflammatory) properties^{7,20-22}. Other advantages of DPSCs include the relative ease of isolating these cells as the source (extracted teeth) is generally discarded as biological waste and consequently isolation of these cells is associated with minimal ethical concerns.

DPSC heterogeneity

Heterogenous proliferation rates of DPSCs

DPSCs have a higher proliferation rate when compared to BM-MSCs^{12,23}. Several studies have however reported a decrease in the proliferation rate of DPSCs with increased rounds of *in vitro* expansion^{21,24}. Gronthos and co-workers (2002) showed that 80% of the progeny derived from single DPSC colonies proliferated for less than 20 population doublings (PDs) indicating that the majority of clonogenic DPSCs do not proliferate extensively *ex vivo*. These observations suggest that only a minority (20%) of DPSCs are represented at later PDs⁹. In addition, only 67% of the highly proliferative single-colony derived DPSC strains could form abundant amounts of dentine when compared to the parental multi-colony derived cultures *in vivo*. Gronthos and co-workers (2002) therefore postulated the existence of a hierarchy of progenitors in adult dental pulp where the majority of DPSCs seem to be committed progenitors that proliferate slowly, while a highly proliferative minor population exhibits self-renewal and multipotent stem cell characteristics⁹. Even though highly proliferative hDPSCs, with retained stemness and multipotent differentiation potential seem to be the ultimate candidates for regenerative therapies, it has been suggested that subpopulations with decreased proliferation and restricted differentiation potential may be more effective in regeneration of specialized tissues²⁵.

The age of the donor or health of the extracted teeth (independent of age) appear to play a role in DPSC proliferation. Wu and co-workers (2015) reported an inverse correlation between the proliferation rate of DPSCs and donor age²⁶. These authors attributed the impaired proliferation rate of DPSCs isolated from aged donors to a higher apoptotic rate. It is well accepted that the telomere length of somatic cells decreases with age²⁷⁻²⁹. Alraies and co-workers (2017) associated variation in proliferative capacity between individual DPSC populations with changes in telomere length³. Telomere length analysis revealed that DPSC populations with high proliferative potential had much longer average telomere lengths³. These findings were irrespective of the cells being derived from the same or different patients as three samples obtained from one patient revealed different PDs. When differentiation potential was assessed, only the highly proliferative cells demonstrated multipotency and differentiated into osteogenic, chondrogenic and adipogenic cells. In contrast, the slowly proliferating cells were lineage-restricted to osteogenic differentiation. These authors also found that with increased *in vitro* expansion (beyond 30 PDs), osteogenic differentiation decreased while adipogenic differentiation increased³. Huang and co-workers (2014) also showed that DPSCs with more primitive characteristics exhibit an increased proliferation rate. These investigators showed that STRO-1⁺CD146⁺ primary DPSCs had greater colony-forming efficiency, i.e., proliferation rate, than STRO-1⁻CD146⁻ DPSCs. The expression of the transcription factors *OCT4* and *NANOG*, which play a role in maintaining pluripotency and self-renewal in primitive stem cells, was significantly higher in the STRO-1⁺CD146⁺ primary DPSCs compared to the STRO-1⁻CD146⁻ DPSCs^{30,31}. Huang and co-workers (2014) and Tsai and co-workers (2012) showed that knockdown of *OCT4* and *NANOG* resulted in decreased proliferation of DPSCs (STRO-1⁺CD146⁺) and BM-MSCs, respectively^{30,32}.

Sun and co-workers (2014) investigated the characteristics of DPSCs isolated from periodontally-compromised teeth. They illustrated that DPSCs isolated from adult teeth exposed to various degrees of inflammation-induced bone resorption, display a significantly slower proliferation rate when compared to DPSCs isolated from healthy, adult teeth. A significantly larger proportion of DPSCs isolated from healthy teeth expressed STRO-1⁺. STRO-1 has been suggested to be a marker for primitive, undifferentiated MSCs, again indirectly linking DPSC primitivity to increased proliferation³⁴. However, contradictory to Sun and co-workers (2014), Perreira and co-workers (2012) observed no difference in the proliferation rate of inflamed and healthy DPSCs³⁵.

Heterogenous surface protein expression and differentiation potential of DPSCs

According to the ISCT, MSCs should positively express CD73 (5'ectonucleotidase), CD90 (Thy-1) and CD105 (endoglin) ($\geq 95\%$), but should not express the following antigens: CD45 (a pan-leukocyte marker), CD34 (a marker for hematopoietic stem and progenitor cells and endothelial cells), CD11b or CD14 (expressed on monocytes and macrophages), CD19 or CD79 α (markers of B cells) and HLA-DR (human leukocyte antigen)¹⁸. CD31 (an endothelial marker) is often included in the panel of markers used to phenotype DPSC populations to monitor the level of endothelial cell contamination present in the cultures³⁶; DPSCs should be negative for CD31³⁷. There is currently no single marker that can be used to identify MSCs, including DPSCs. Furthermore, there is no consensus on the panel of markers that should be used to characterize DPSCs³⁸. Consequently, various marker combinations have been used to identify "DPSCs"³⁹ resulting in heterogenous populations isolated and used in DPSC regenerative studies. The individual markers commonly used in DPSC isolation and/or characterization panels are summarized in Table 1.

Table 1

A list of different markers (genes and cell surface proteins) used to identify DPSCs.

Markers used to identify DPSCs	
Mesenchymal stem cell surface markers	CD10 ^{73,74}
	CD13 ^{37,75}
	CD29 ^{37,75-79}
	CD31 ³⁶
	CD44 ^{37,51,73,77,78,80,81}
	CD56 ³⁶
	CD73 ^{4,37,44,46,51,73,75,76,78,80,82}
	CD90 ^{4,37,44,46,51,73,75,76,78-80,82,83}
	CD105 ^{4,37,44,46,51,73,76,78,80-83}
	CD106 ^{75,78}
	CD146 ^{36,44,75,77,78,81,83,84}
	CD166 ^{37,73,77}
	CD271 ^{36,73}
	MSCA-1/TNAP ³⁶
Stemness/embryonic markers/pluripotency-associated genes	<i>KLF4</i> ⁷⁶
	<i>NANOG</i> ^{32,76,77,83}
	<i>OCT4</i> ^{32,76,77,81,83}
	<i>SSEA4</i> ⁷⁷
	STRO-1 ^{36,43,44,75,78,81}

The expression of cell surface proteins *in vitro* is affected by various factors including extensive proliferation cycles, dense culture conditions and diverse culture media^{3,24,40}. Conflicting results have been obtained in an attempt to elucidate the relationship between the phenotypic profile of DPSCs and their differentiation potential^{1,2,41-43}. Ducret and co-workers (2019) confirmed that stem cells obtained from dental pulp differ in their self-renewal, lineage commitment and differentiation potential³⁶. This observation was supported by Kobayashi and co-workers (2020) as they showed that a single specimen of dental pulp tissue contained clones with restricted differentiation potential as well as multipotent stem-like clones⁴⁴. Furthermore, these authors reported the loss of adipogenic differentiation potential followed by loss of chondrogenic differentiation potential as the number of hDPSC doublings increased⁴⁴. Harrington and co-workers (2014) also observed that not all DPSC clones were able to undergo adipogenic and chondrogenic differentiation⁴⁵. These authors were unable to link the differences of differentiation potential of DPSC clones to a specific mesenchymal marker. Kobayashi and co-workers (2020) showed that the expression of stem cell markers, including CD146, persisted despite the loss of differentiation potential during long-term culture. These authors concluded that the expression of stem cell markers by DPSCs is not necessarily a prediction of the differentiation potential of these cells⁴⁴. Recently, Perczel-Kovach and co-workers (2021) suggested that STRO-1 cannot be considered as a reliable marker for stemness in DPSCs as the STRO-1⁺ cell fraction expanded significantly during osteogenic differentiation⁴³. In a recent review, Bashir (2021) suggested that DPSCs have limited differentiation potential and are able to differentiate into odontoblasts and neurocytes but not into osteoblasts and chondrocytes³⁷. Lee and co-workers (2022) recently suggested that hDPSCs comprise of mainly neurogenic and osteogenic cell populations with decreased adipose differentiation potential⁴⁶. This highlights the complexity of DPSC isolation and subsequent translation of reported regenerative potential to the clinical setting.

General factors influencing DPSC heterogeneity

Intrinsic factors

The differences observed in the regenerative properties of DPSCs may be the result of inherent factors including mesodermal versus neuro-ectodermal origin², cells being at different stages within the proposed hierarchical model for adult stem cells⁴⁷, or originating from different stem cell niches within the dental pulp⁴⁸ (Fig. 1).

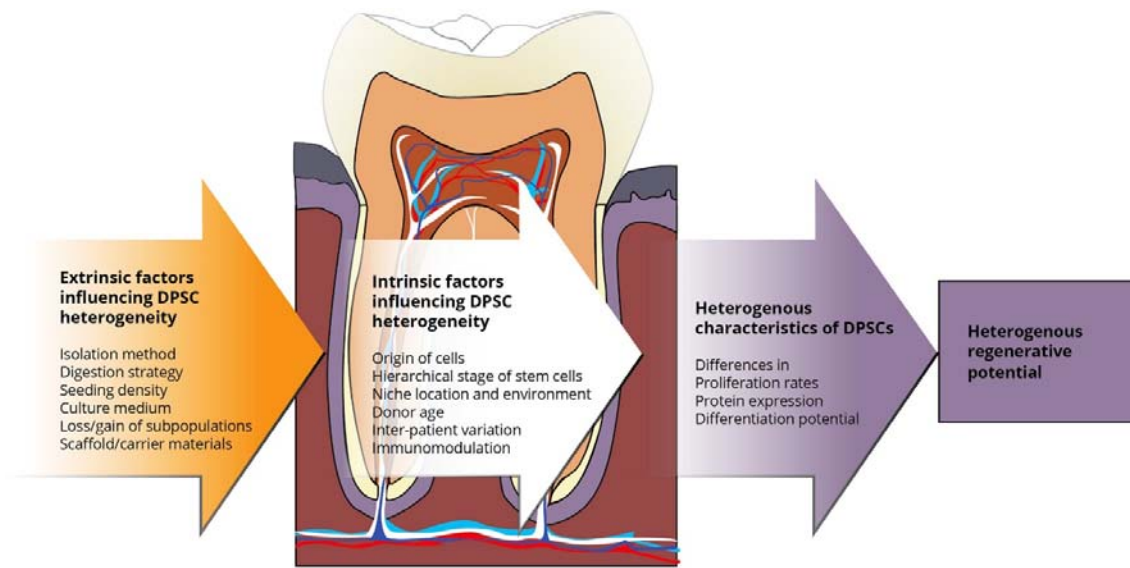


Figure 1

A summary of factors influencing DPSC heterogeneity.

As mentioned earlier, DPSCs reside within distinct niches in dental pulp tissue where they either self-renew or differentiate. The micro-environment of the niche influences stem cell behaviour and the cell's ability to either participate in the maintenance, repair and/or regeneration of the tissue involved^{49,50}. The function of adult stem cells outside the niche is often limited¹⁷. The variable niche environments may further contribute to DPSC heterogeneity⁴⁸. Recently Cui and co-workers (2021) confirmed that DPSCs are mostly located in the perivascular area⁵¹ and the cells residing in peri-vascular niches may display different properties to those obtained from subodontoblastic or apical regions^{16,17}. It is however not clear whether tissue-specific MSCs originate from local mesenchymal tissues and migrate toward the ingrown vasculature or if they originate from the vasculature and are then influenced by local signals that result in tissue specificity⁴⁸.

It is generally accepted that an increase in donor chronological age impairs the regenerative capabilities of stem/progenitor cells, and doubling time increases with increasing age^{52,53}. However, Alraies and co-workers (2017) reported inherent differences in telomere length and differentiation capabilities of individual DPSC populations from within a young donor age group³. Thus factors, not completely elucidated and still to be identified, other than age, seem to be involved in determining the differentiation capacity of DPSCs obtained from a single individual. Inter-patient variation complicates the diverse regenerative outcomes even further⁵⁰.

Immunomodulation has also been hypothesized to play a role in tissue regeneration and may contribute to the heterogeneous differentiation potential of stem cells⁵⁴. hDPSCs have been suggested to have anti-inflammatory properties via paracrine mechanisms associated with T-cells as pro-inflammatory cytokines [interleukin-2 (IL-2), interleukin-6-receptor (IL-6-R), interleukin-12 (IL-12), interleukin-17A (IL-17A), interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α)] were decreased and levels of anti-inflammatory cytokines [inducible protein 10 (IP-10)] increased from PHA-CD3(+) T cells co-cultured with DPSCs in a transwell system⁵⁵. Ozdemir and co-workers (2016) have shown that hDPSCs suppress T helper-1 (Th1) and T helper-2 (Th2) cells but stimulate the T helper 17 (Th17) and T regulatory (Treg) subsets during co-culture with CD4 positive T cells⁵⁶. The immunosuppressive activity of DPSCs has also been linked to DPSC-mediated activation of T cells that secrete IFN- γ , which then primes DPSCs to release TFG- β ⁵⁷. Interferon gamma (IFN- γ) has been shown to inhibit MSC proliferation and to alter osteogenic, neural and adipogenic differentiation⁵⁸. Interestingly, Laing and co-workers (2018) proposed that immunomodulation is induced via DPSC apoptosis after *in vivo* systemic injection⁵⁹.

The immunomodulating potential of DPSCs is generally not observed in quiescent DPSCs but is influenced by the surrounding microenvironment⁶⁰. The immunosuppressive function of DPSCs *in vitro* is dependent on priming with cytokines like IL-1 β , TNF- α and IFN- γ ⁵⁴ and can therefore be influenced by extrinsic factors. Most of the immunomodulatory studies have been done *in vitro* or in animal models, which does not fully replicate the complex immune response^{54,60}. Furthermore, the relationship between regeneration and immunomodulation has not been established⁵⁴, and more research is needed to elucidate the mechanism of immunosuppression of DPSCs *in vivo*⁶⁰. One should also keep in mind that mild inflammation of the dental pulp is conducive of tertiary dentine formation; therefore the ideal environment and interplay between DPSCs and immune cells would have to be determined and established for successful dentine regeneration.

Extrinsic factors

Extrinsic factors may also influence the heterogeneity of the cultured population (Fig.1). As mentioned, there is currently no single marker that can be used to identify DPSCs. Therefore the different combinations of markers used to isolate “DPSCs” would result in heterogeneous DPSC populations with variable regenerative potential.

In vitro culturing induces dramatic alterations of transcriptional profiles and phenotype compared to freshly isolated DPSCs⁵¹. Methods of obtaining the cultured cell population also vary, and therefore the cell population can differ according to the isolation method. The two

most common methods used are outgrowth and enzyme digestion methods^{61,62}. Stem cells obtained from the outgrowth method seem to be more differentiated progenitor cells while DPSCs isolated through enzymatic digestion exhibit stronger stemness with increased STRO-1 expression^{62,63}.

Digestion strategies used to dissociate the dental pulp into single cells have also been shown to affect the type of DPSC subpopulations isolated. DPSCs isolated using a combination of collagenase I and dispase have significantly increased numbers of CD146⁺ cells with increased osteogenic and chondrogenic differentiation, while DPSCs obtained by collagenase only contain a lower proportion of CD146⁺ cells and are associated with increased adipogenic potential¹. Inflamed DPSCs isolated via enzymatic digestion strategies showed increased osteo-/odontogenic differentiation potential compared to cells obtained through the outgrowth method⁶⁴.

Seeding density also seems to have an impact on DPSC culture characteristics. Noda and co-workers (2019) have demonstrated that the proportion of CD73⁺ and CD105⁺ cells is significantly decreased within densely seeded DPSC cultures when compared to cultures in which the DPSCs were seeded sparsely⁴⁰. The seeding density also seems to influence cell differentiation potential as mineralized nodule formation was increased in the densely seeded cultures⁴⁰.

Culture medium can also influence DPSC characteristics. When Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) was compared to serum-free culture medium [basal DMEM supplemented with 1% insulin transferrin selenium (ITS), 25 µg/mL ascorbic acid, 25 ng/mL basic fibroblast growth factor (bFGF), 50 µg/mole β-mercaptoethanol and 30 µg/mL cholesterol], the serum-free medium resulted in increased proliferation. The serum-free medium upregulated stemness markers (*OCT4*, *SOX2* and *NANOG*) while maintaining the potential to differentiate into adipocytes and chondroblasts⁶⁵. Marrazzo and co-workers (2016) reported that the use of culture medium supplemented with human platelet lysate increased *SOX2* transcripts when compared to FBS supplemented samples⁶⁶. More recently, Qu and co-workers (2020) showed that stem cell marker expression, specifically CD105, decreased when DPSCs were cultured in a xeno-free/serum-free medium compared to FBS cultured cells²⁴. The xeno-free/serum-free cultures also resulted in a decreased proliferation rate at later passages and a decreased ability to form colonies²⁴. The composition of culture medium used to induce differentiation also impacts the differentiation potential of DPSCs⁶⁷.

One can assume that highly proliferative DPSCs are responsible for the extensive expansion potential of the initial heterogeneous populations *in vitro*, and that less proliferative DPSCs would be selectively lost during extended subculture. However, external *in vitro* associated factors may influence the heterogeneous population, resulting in various subpopulations that are not necessarily physiologically relevant, especially in cultures that have been extensively sub-cultured⁵¹. Loss or gain of certain subpopulations during *ex vivo* expansion may therefore influence the differentiation and regenerative potential of these cells.

DPSC heterogeneity has also been shown in human DPSCs cultured in 3D type I collagen gels *in vitro*. Collagen gels are used as cell carriers and mechanical support when DPSCs are transplanted into wound sites. High proliferative/multipotent and low proliferative/unipotent DPSCs obtained from human third molars showed heterogeneous characteristics regarding gel contraction and matrix metalloproteinase (MMP) expression/activity. MMPs degrade biomaterial scaffolds and as different subpopulations of DPSCs result in variable MMP activities, the respective populations may have different therapeutic outcomes⁶⁸. According to Ducret and co-workers (2021) the exact mechanism as to how DPSCs degrade and replace hydrogel scaffolds still needs to be elucidated and the addition of bioactive molecules to scaffold materials will also influence the differentiation potential of these cells⁶⁹.

Advances towards standardized techniques

The lack of appropriate research tools and the techniques currently used are the main challenges researchers face in their quest to better understand the heterogeneous nature of DPSCs. Flow cytometry is often used to characterize the cells phenotypically. Although it is a powerful technique, it is accompanied by several technical challenges. The use of the appropriate controls during flow cytometric analysis is vital to ensure the accuracy of analysis and optimal differentiation between antigen-positive and antigen-negative cells³⁶. Multi-parameter flow cytometric analysis allows for determining co-localization of several molecular markers on the surface of an individual cell and should be encouraged as it can potentially assist regarding inconsistent results reported currently.

Single-cell Raman spectroscopy (SCRM) has recently been shown to be a non-invasive method to discriminate between DPSCs with contrasting proliferative and differentiation capabilities *ex vivo*⁷⁰. Raman spectroscopy is a laser-based scattering technique and the spectra obtained for individual cells provide unique biochemical fingerprints of cellular status and function⁷⁰. SCRM profiles reflect increased DNA/protein content and the prevalence of

particular DNA and protein signature peaks which may distinguish high proliferative/multipotent DPSCs from low proliferative/unipotent populations⁷⁰. However, more research is required to confirm the use of SCRM as a viable technique for the selective screening, identification, and isolation of DPSCs for regenerative procedures.

Human dental pulp cell lines have recently been established that maintain the original stemness characteristics as well as differentiation potential. This may reduce the costs involved in pulp regeneration therapies²¹. However, the use of these immortalized cell lines is associated with several concerns from the clinical perspective and is likely to serve mainly as a research tool to investigate the characteristics and function of DPSCs *in vitro*.

Single-cell RNA sequencing was recently performed on human dental pulp cells and has shed some light on the existence of DPSC subpopulations^{46,50,51}. Single-cell whole transcriptome sequencing was first described in 2009⁷¹ and enables researchers to measure biological variation in heterogeneous cell populations based on gene expression levels at the single cell level⁷². The application of microfluidic single-cell technology revealed altered transcriptional and regenerative characteristics in DPSC subpopulations compared to freshly isolated dental pulp cells⁵¹. This technology also enabled Pagella and co-workers (2021) to identify three main subpopulations of MSCs in human dental pulp. The three subpopulations were identified based on differential expression of *MYH11* (encoding smooth muscle myosin heavy chain 11), *THY1* (encoding CD90) and *CCL2* (also known as monocyte chemoattractant and activating factor (MCAF)). More recently, single-cell RNA analysis showed an osteogenic and odontogenic cluster (expressing *DCN*, *COL1A1*, *COL1A2*, *FN1* and *VCAN*) as well as a neurogenic cluster (expressing *S100A4*, *NEFM*, *COL4A1* and neurofilament assembly bundle-associated gene sets) in hDPSCs⁴⁶. However, single-cell RNA sequencing revealed inter-patient variability which limits the reproducibility of results and contributes to diverse outcomes when the research is translated to the clinic⁵⁰.

CONCLUSION

It is well established that DPSCs display significant heterogeneity and subpopulations demonstrate differences in proliferation, protein expression and differentiation capabilities. Various intrinsic factors including stem cell origin, micro-environment of the stem cell niche, immunomodulation, donor age and inter-patient variation as well as external factors like stem cell isolation techniques, digestion strategies, seeding density, culture medium and cell carrier systems, influence the repertoire of subpopulations found within DPSC cultures, and may contribute to disparate results, thereby concealing the true regenerative properties of this stem cell source. Identification and selection of subpopulations with enhanced

regenerative properties remains a challenge and the interplay between proliferation, stem cell marker expression and differentiation potential is likely to be determinant. Inconsistencies in characterizing DPSCs (based on cell surface marker expression) and variable isolation techniques remain challenging. Standardization of these procedures could increase reproducibility. The use of high-resolution techniques, such as single-cell transcriptome analysis, may hold the key to unravelling the complexity of DPSC subpopulations and elucidating the heterogenous nature of these cells.

CRedit AUTHORSHIP CONTRIBUTION STATEMENT

Sulette Nel: Conceptualization, Resources, Methodology, Investigation, Writing – original draft. **Chrisna Durandt:** Methodology, Formal analysis, Validation, Writing – review & editing. **Candice Murdoch:** Resources, Methodology, Investigation. **Michael S. Pepper:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

ACKNOWLEDGMENTS

Approval was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria, South Africa. Ethics Reference number: 438/2018. The authors thank the South African Medical Research Council (Extramural Unit for Stem Cell Research and Therapy) and the University of Pretoria (via the Institute for Cellular and Molecular Medicine) for supporting this project. The authors deny any conflicts of interest related to this study.

REFERENCES

1. Ebrahimi Dastgardi M, Ejeian F, Nematollahi M, Motaghi A, Nasr-Esfahani MH. Comparison of two digestion strategies on characteristics and differentiation potential of human dental pulp stem cells. *Arch Oral Biol* 2018;93:74-79.
2. Pisciotto A, Carnevale G, Meloni S, Riccio M, De Biasi S, Gibellini L, et al. Human dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations. *BMC Dev Biol* 2015;15:14.
3. Alraies A, Alaidaroos NY, Waddington RJ, Moseley R, Sloan AJ. Variation in human dental pulp stem cell ageing profiles reflect contrasting proliferative and regenerative capabilities. *BMC Cell Biol* 2017;18(1):12.
4. Baldion PA, Velandia-Romero ML, Castellanos JE. Odontoblast-Like Cells Differentiated from Dental Pulp Stem Cells Retain Their Phenotype after Subcultivation. *Int J Cell Biol* 2018;2018:6853189.
5. Nuti N, Corallo C, Chan BM, Ferrari M, Gerami-Naini B. Multipotent Differentiation of Human Dental Pulp Stem Cells: a Literature Review. *Stem Cell Rev Rep* 2016;12(5):511-523.
6. Al-Habib M, Huang GT. Dental Mesenchymal Stem Cells: Dental Pulp Stem Cells, Periodontal Ligament Stem Cells, Apical Papilla Stem Cells, and Primary Teeth Stem

- Cells-Isolation, Characterization, and Expansion for Tissue Engineering. *Methods Mol Biol* 2019;1922:59-76.
7. Gan L, Liu Y, Cui D, Pan Y, Zheng L, Wan M. Dental Tissue-Derived Human Mesenchymal Stem Cells and Their Potential in Therapeutic Application. *Stem Cells Int* 2020;2020:8864572.
8. Fawzy El-Sayed KM, Ahmed GM, Abouauf EA, Schwendicke F. Stem/progenitor cell-mediated pulpal tissue regeneration: a systematic review and meta-analysis. *Int Endod J* 2019;52(11):1573-1585.
9. Gronthos S, Brahimi J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;81(8):531-535.
10. Nakashima M, Iohara K, Murakami M, Nakamura H, Sato Y, Aiji Y, et al. Pulp regeneration by transplantation of dental pulp stem cells in pulpitis: a pilot clinical study. *Stem Cell Res Ther* 2017;8(1):61.
11. Min TJ, Kim MJ, Kang KJ, Jeoung YJ, Oh SH, Jang YJ. 3D Spheroid Formation Using BMP-Loaded Microparticles Enhances Odontoblastic Differentiation of Human Dental Pulp Stem Cells. *Stem Cells Int* 2021;2021:9326298.
12. Gronthos S, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000;97(25):13625-13630.
13. Lymperi S, Ligoudistianou C, Taraslia V, Kontakiotis E, Anastasiadou E. Dental Stem Cells and their Applications in Dental Tissue Engineering. *Open Dent J* 2013;7:76-81.
14. Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z. Stem cells: past, present, and future. *Stem Cell Res Ther* 2019;10(1):68.
15. Saito MT, Silverio KG, Casati MZ, Sallum EA, Nociti FH, Jr. Tooth-derived stem cells: Update and perspectives. *World J Stem Cells* 2015;7(2):399-407.
16. Rapino M, Di Valerio V, Zara S, Gallorini M, Marconi GD, Sancilio S, et al. Chitlac-coated Thermosets Enhance Osteogenesis and Angiogenesis in a Co-culture of Dental Pulp Stem Cells and Endothelial Cells. *Nanomaterials (Basel)* 2019;9(7).
17. Mitsiadis TA, Feki A, Papaccio G, Caton J. Dental pulp stem cells, niches, and notch signaling in tooth injury. *Adv Dent Res* 2011;23(3):275-279.
18. 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(4):315-317.
19. Nakajima K, Kunimatsu R, Ando K, Hiraki T, Rikitake K, Tsuka Y, et al. Success rates in isolating mesenchymal stem cells from permanent and deciduous teeth. *Sci Rep* 2019;9(1):16764.
20. Liu J, Yu F, Sun Y, Jiang B, Zhang W, Yang J, et al. Concise reviews: Characteristics and potential applications of human dental tissue-derived mesenchymal stem cells. *Stem Cells* 2015;33(3):627-638.
21. Orimoto A, Kyakumoto S, Eitsuka T, Nakagawa K, Kiyono T, Fukuda T. Efficient immortalization of human dental pulp stem cells with expression of cell cycle regulators with the intact chromosomal condition. *PLoS One* 2020;15(3):e0229996.
22. Ozgul Ozdemir RB, Ozdemir AT, Kirmaz C, Eker Sariboyaci A, Karaoz E, Erman G, et al. Age-related changes in the immunomodulatory effects of human dental pulp derived mesenchymal stem cells on the CD4(+) T cell subsets. *Cytokine* 2021;138:155367.
23. Aghajani F, Hooshmand T, Khanmohammadi M, Khanjani S, Edalatkhah H, Zarnani AH, et al. Comparative Immunophenotypic Characteristics, Proliferative Features, and Osteogenic Differentiation of Stem Cells Isolated from Human Permanent and Deciduous Teeth with Bone Marrow. *Mol Biotechnol* 2016;58(6):415-427.
24. Qu C, Brohlin M, Kingham PJ, Kelk P. Evaluation of growth, stemness, and angiogenic properties of dental pulp stem cells cultured in cGMP xeno-/serum-free medium. *Cell Tissue Res* 2020;380(1):93-105.
25. Kok ZY, Alaidaroos NYA, Alraies A, Colombo JS, Davies LC, Waddington RJ, et al. Dental Pulp Stem Cell Heterogeneity: Finding Superior Quality "Needles" in a Dental

- Pulpal "Haystack" for Regenerative Medicine-Based Applications. *Stem Cells Int* 2022;2022:9127074.
26. Wu W, Zhou J, Xu CT, Zhang J, Jin YJ, Sun GL. Derivation and growth characteristics of dental pulp stem cells from patients of different ages. *Mol Med Rep* 2015;12(4):5127-5134.
 27. Ghimire S, Hill CV, Sy FS, Rodriguez R. Decline in telomere length by age and effect modification by gender, allostatic load and comorbidities in National Health and Nutrition Examination Survey (1999-2002). *PLoS One* 2019;14(8):e0221690.
 28. Marquez-Ruiz AB, Gonzalez-Herrera L, Luna JD, Valenzuela A. DNA methylation levels and telomere length in human teeth: usefulness for age estimation. *Int J Legal Med* 2020;134(2):451-459.
 29. Sanders JL, Newman AB. Telomere length in epidemiology: a biomarker of aging, age-related disease, both, or neither? *Epidemiol Rev* 2013;35:112-131.
 30. Huang CE, Hu FW, Yu CH, Tsai LL, Lee TH, Chou MY, et al. Concurrent expression of Oct4 and Nanog maintains mesenchymal stem-like property of human dental pulp cells. *Int J Mol Sci* 2014;15(10):18623-18639.
 31. Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, et al. A protein interaction network for pluripotency of embryonic stem cells. *Nature* 2006;444(7117):364-368.
 32. Tsai CC, Su PF, Huang YF, Yew TL, Hung SC. Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Mol Cell* 2012;47(2):169-182.
 33. Sun HH, Chen B, Zhu QL, Kong H, Li QH, Gao LN, et al. Investigation of dental pulp stem cells isolated from discarded human teeth extracted due to aggressive periodontitis. *Biomaterials* 2014;35(35):9459-9472.
 34. Nasef A, Zhang YZ, Mazurier C, Bouchet S, Bensidhoum M, Francois S, et al. Selected Stro-1-enriched bone marrow stromal cells display a major suppressive effect on lymphocyte proliferation. *Int J Lab Hematol* 2009;31(1):9-19.
 35. Pereira LO, Rubini MR, Silva JR, Oliveira DM, Silva IC, Pocas-Fonseca MJ, et al. Comparison of stem cell properties of cells isolated from normal and inflamed dental pulps. *Int Endod J* 2012;45(12):1080-1090.
 36. Ducret M, Farges JC, Padeloup M, Perrier-Groult E, Mueller A, Mallein-Gerin F, et al. Phenotypic Identification of Dental Pulp Mesenchymal Stem/Stromal Cells Subpopulations with Multiparametric Flow Cytometry. *Methods Mol Biol* 2019;1922:77-90.
 37. Bashir NZ. The role of insulin-like growth factors in modulating the activity of dental mesenchymal stem cells. *Arch Oral Biol* 2021;122:104993.
 38. Ahmed GM, Abouauf EA, AbuBakr N, Dorfer CE, El-Sayed KF. Tissue Engineering Approaches for Enamel, Dentin, and Pulp Regeneration: An Update. *Stem Cells Int* 2020;2020:5734539.
 39. Ledesma-Martinez E, Mendoza-Nunez VM, Santiago-Osorio E. Mesenchymal Stem Cells Derived from Dental Pulp: A Review. *Stem Cells Int* 2016;2016:4709572.
 40. Noda S, Kawashima N, Yamamoto M, Hashimoto K, Nara K, Sekiya I, et al. Effect of cell culture density on dental pulp-derived mesenchymal stem cells with reference to osteogenic differentiation. *Sci Rep* 2019;9(1):5430.
 41. Mikami Y, Ishii Y, Watanabe N, Shirakawa T, Suzuki S, Irie S, et al. CD271/p75(NTR) inhibits the differentiation of mesenchymal stem cells into osteogenic, adipogenic, chondrogenic, and myogenic lineages. *Stem Cells Dev* 2011;20(5):901-913.
 42. Matsui M, Kobayashi T, Tsutsui TW. CD146 positive human dental pulp stem cells promote regeneration of dentin/pulp-like structures. *Hum Cell* 2018;31(2):127-138.
 43. Perczel-Kovach K, Hegedus O, Foldes A, Sangngoen T, Kallo K, Steward MC, et al. STRO-1 positive cell expansion during osteogenic differentiation: A comparative study of three mesenchymal stem cell types of dental origin. *Arch Oral Biol* 2021;122:104995.

44. Kobayashi T, Torii D, Iwata T, Izumi Y, Nasu M, Tsutsui TW. Characterization of proliferation, differentiation potential, and gene expression among clonal cultures of human dental pulp cells. *Hum Cell* 2020;33(3):490-501.
45. Harrington J, Sloan AJ, Waddington RJ. Quantification of clonal heterogeneity of mesenchymal progenitor cells in dental pulp and bone marrow. *Connect Tissue Res* 2014;55 Suppl 1:62-67.
46. Lee S, Chen D, Park M, Kim S, Choi YJ, Moon SJ, et al. Single-Cell RNA Sequencing Analysis of Human Dental Pulp Stem Cell and Human Periodontal Ligament Stem Cell. *J Endod* 2022;48(2):240-248.
47. Ratajczak MZ. A novel view of the adult bone marrow stem cell hierarchy and stem cell trafficking. *Leukemia* 2015;29(4):776-782.
48. Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res* 2009;88(9):792-806.
49. Huang X, Li Z, Liu A, Liu X, Guo H, Wu M, et al. Microenvironment Influences Odontogenic Mesenchymal Stem Cells Mediated Dental Pulp Regeneration. *Front Physiol* 2021;12:656588.
50. Pagella P, de Vargas Roditi L, Stadlinger B, Moor AE, Mitsiadis TA. A single-cell atlas of human teeth. *iScience* 2021;24(5):102405.
51. Cui Y, Ji W, Gao Y, Xiao Y, Liu H, Chen Z. Single-cell characterization of monolayer cultured human dental pulp stem cells with enhanced differentiation capacity. *Int J Oral Sci* 2021;13(1):44.
52. Kellner M, Steindorff MM, Stempel JF, Winkel A, Kuhnel MP, Stiesch M. Differences of isolated dental stem cells dependent on donor age and consequences for autologous tooth replacement. *Arch Oral Biol* 2014;59(6):559-567.
53. Yi Q, Liu O, Yan F, Lin X, Diao S, Wang L, et al. Analysis of Senescence-Related Differentiation Potentials and Gene Expression Profiles in Human Dental Pulp Stem Cells. *Cells Tissues Organs* 2017;203(1):1-11.
54. Andrukhov O, Behm C, Blufstein A, Rausch-Fan X. Immunomodulatory properties of dental tissue-derived mesenchymal stem cells: Implication in disease and tissue regeneration. *World J Stem Cells* 2019;11(9):604-617.
55. Demircan PC, Sariboyaci AE, Unal ZS, Gacar G, Subasi C, Karaoz E. Immunoregulatory effects of human dental pulp-derived stem cells on T cells: comparison of transwell co-culture and mixed lymphocyte reaction systems. *Cytotherapy* 2011;13(10):1205-1220.
56. Ozdemir AT, Ozgul Ozdemir RB, Kirmaz C, Sariboyaci AE, Unal Halbutogllari ZS, Ozel C, et al. The paracrine immunomodulatory interactions between the human dental pulp derived mesenchymal stem cells and CD4 T cell subsets. *Cell Immunol* 2016;310:108-115.
57. Kwack KH, Lee JM, Park SH, Lee HW. Human Dental Pulp Stem Cells Suppress Alloantigen-induced Immunity by Stimulating T Cells to Release Transforming Growth Factor Beta. *J Endod* 2017;43(1):100-108.
58. Croitoru-Lamoury J, Lamoury FM, Caristo M, Suzuki K, Walker D, Takikawa O, et al. Interferon-gamma regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3 dioxygenase (IDO). *PLoS One* 2011;6(2):e14698.
59. Laing AG, Riffo-Vasquez Y, Sharif-Paghaleh E, Lombardi G, Sharpe PT. Immune modulation by apoptotic dental pulp stem cells in vivo. *Immunotherapy* 2018;10(3):201-211.
60. Kwack KH, Lee HW. Clinical Potential of Dental Pulp Stem Cells in Pulp Regeneration: Current Endodontic Progress and Future Perspectives. *Front Cell Dev Biol* 2022;10:857066.
61. Huang GT, Sonoyama W, Chen J, Park SH. In vitro characterization of human dental pulp cells: various isolation methods and culturing environments. *Cell Tissue Res* 2006;324(2):225-236.

62. Jeon M, Song JS, Choi BJ, Choi HJ, Shin DM, Jung HS, et al. In vitro and in vivo characteristics of stem cells from human exfoliated deciduous teeth obtained by enzymatic disaggregation and outgrowth. *Arch Oral Biol* 2014;59(10):1013-1023.
63. Park MK, Kim S, Jeon M, Jung UW, Lee JH, Choi HJ, et al. Evaluation of the Apical Complex and the Coronal Pulp as a Stem Cell Source for Dentin-pulp Regeneration. *J Endod* 2020;46(2):224-231 e223.
64. Gopinath VK, Soumya S, Jayakumar MN. Osteogenic and odontogenic differentiation potential of dental pulp stem cells isolated from inflamed dental pulp tissues (I-DPSCs) by two different methods. *Acta Odontol Scand* 2020;78(4):281-289.
65. Abdel Moniem EM, El-Batran MM, Halawa AM, Gomaa DH, Eldeen GN, Aly RM. Optimizing a serum-free/xeno-free culture medium for culturing and promoting the proliferation of human dental pulp stem cells. *Stem Cell Investig* 2019;6:15.
66. Marrazzo P, Paduano F, Palmieri F, Marrelli M, Tatullo M. Highly Efficient In Vitro Reparative Behaviour of Dental Pulp Stem Cells Cultured with Standardised Platelet Lysate Supplementation. *Stem Cells Int* 2016;2016:7230987.
67. Okajceková T, Strnadel J, Pokusa M, Zahumenská R, Janicková M, Halasová E, et al. A Comparative In Vitro Analysis of the Osteogenic Potential of Human Dental Pulp Stem Cells Using Various Differentiation Conditions. *Int J Mol Sci* 2020;21(7).
68. Alraies A, Waddington RJ, Sloan AJ, Moseley R. Evaluation of Dental Pulp Stem Cell Heterogeneity and Behaviour in 3D Type I Collagen Gels. *Biomed Res Int* 2020;2020:3034727.
69. Ducret M, Costantini A, Gobert S, Farges JC, Bekhouche M. Fibrin-based scaffolds for dental pulp regeneration: from biology to nanotherapeutics. *Eur Cell Mater* 2021;41:1-14.
70. Alraies A, Canetta E, Waddington RJ, Moseley R, Sloan AJ. Discrimination of Dental Pulp Stem Cell Regenerative Heterogeneity by Single-Cell Raman Spectroscopy. *Tissue Eng Part C Methods* 2019;25(8):489-499.
71. Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods* 2009;6(5):377-382.
72. Streets AM, Zhang X, Cao C, Pang Y, Wu X, Xiong L, et al. Microfluidic single-cell whole-transcriptome sequencing. *Proc Natl Acad Sci U S A* 2014;111(19):7048-7053.
73. Pisal RV, Suchanek J, Siller R, Soukup T, Hrebikova H, Bezrouk A, et al. Directed reprogramming of comprehensively characterized dental pulp stem cells extracted from natal tooth. *Sci Rep* 2018;8(1):6168.
74. Ferro F, Spelat R, Beltrami AP, Cesselli D, Curcio F. Isolation and characterization of human dental pulp derived stem cells by using media containing low human serum percentage as clinical grade substitutes for bovine serum. *PLoS One* 2012;7(11):e48945.
75. Aydin S, Sahin F. Stem Cells Derived from Dental Tissues. *Adv Exp Med Biol* 2019;1144:123-132.
76. Rodas-Junco BA, Canul-Chan M, Rojas-Herrera RA, De-la-Pena C, Nic-Can GI. Stem Cells from Dental Pulp: What Epigenetics Can Do with Your Tooth. *Front Physiol* 2017;8:999.
77. Anitua E, Troya M, Zalduendo M. Progress in the use of dental pulp stem cells in regenerative medicine. *Cytotherapy* 2018.
78. Zhou D, Gan L, Peng Y, Zhou Y, Zhou X, Wan M, et al. Epigenetic Regulation of Dental Pulp Stem Cell Fate. *Stem Cells Int* 2020;2020:8876265.
79. Zhang XM, Sun Y, Zhou YL, Jiao ZM, Yang D, Ouyang YJ, et al. Therapeutic effects of dental pulp stem cells on vascular dementia in rat models. *Neural Regen Res* 2021;16(8):1645-1651.
80. Manokawinchoke J, Nattasit P, Thongngam T, Pavasant P, Tompkins KA, Egusa H, et al. Indirect immobilized Jagged1 suppresses cell cycle progression and induces odonto/osteogenic differentiation in human dental pulp cells. *Sci Rep* 2017;7(1):10124.

81. Pedroni ACF, Sarra G, de Oliveira NK, Moreira MS, Deboni MCZ, Marques MM. Cell sheets of human dental pulp stem cells for future application in bone replacement. *Clin Oral Investig* 2019;23(6):2713-2721.
82. Lei T, Wang J, Liu Y, Chen P, Zhang Z, Zhang X, et al. Proteomic profile of human stem cells from dental pulp and periodontal ligament. *J Proteomics* 2021;245:104280.
83. Hollands P, Aboyeji D, Orchardson M. Dental pulp stem cells in regenerative medicine. *Br Dent J* 2018.
84. Ducret M, Fabre H, Degoul O, Atzeni G, McGuckin C, Forraz N, et al. Immunophenotyping Reveals the Diversity of Human Dental Pulp Mesenchymal Stromal Cells In vivo and Their Evolution upon In vitro Amplification. *Front Physiol* 2016;7:512.