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Dental pulp stem cell (DPSC) pluripotency enhanced by Transforming growth factor (TGF-β1) *in vitro* may be inhibited by differentiation-inducing factors Laminin-5 and Dexamethasone

Kelcey Loveland[1], Aubrey Young[2], Mehrnaz Khadiv[1], Marah Culpepper[1], Karl Kingsley[3]

[1]Department of Clinical Sciences, University of Nevada, Las Vegas – School of Dental Medicine
[2]Department of Orthodontic and Dentofacial Orthopedics, University of Nevada, Las Vegas – School of Dental Medicine
[3]Department of Biomedical Sciences, University of Nevada, Las Vegas – School of Dental Medicine

Email address
Kelcey.Loveland@sdm.unlv.edu (K. Loveland), Aubrey.Young@sdm.unlv.edu (A. Young), Mehrnaz.Khadiv@sdm.unlv.edu (M. Khadiv), Marah.Culpepper@sdm.unlv.edu (M. Culpepper), Karl.Kingsley@unlv.edu (K. Kingsley)

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Abstract
Mesenchymal stem cells are a derived from a variety of human tissues and are being bioengineered and studied for possible uses in the advancement of medicine. Recent efforts are being focused on Dental Pulp Stem Cells (DPSC's) due to the accessibility of this tissue. The objective of this study was to evaluate the potential to induce differentiation of DPSC isolates *in vitro* by the adding of exogenous growth factors (GF), and by the coating of specific extracellular matrix molecules (ECM) onto the surface of tissue-culture dishes. Photomicroscopy and mRNA analysis demonstrated the addition of TGF- β 1 noteably increased pluripotency biomarkers in DPSC lines. The addition of Dexamethasone (Dex) or plating on Laminin-5 (LN5) was correlated with changes to cellular morphology and cell size in different subsets of cells. RNA isolated from these DPSCs for relative endpoint (RE) reverse transcription polymerase chain reaction (RT-PCR) revealed mRNA DPSC specific intracellular biomarkers (Klf4, Sox-2, Bin-1, Rnf12, Oct-4 and NANOG) and the cell surface marker (CD133) were enhanced following the administration of TGF- β 1 and were differentially down-regulated following Dexamethasone and Laminin-5 administration. This study provides some initial evidence that randomly selected DPSC isolates may be induced by established protocols to change phenotype and expression of pluripotent biomarkers with variable susceptibility between differing types of DPSCs. More studies will be needed to determine the range of cell types that can be successfully re-engineered in laboratory settings.

1. Background

The isolation of mesenchymal stem cells (MSC) is an important scientific endeavor in the health sciences.[1,2] Although much is known about embryonic stem cells and their regenerative capacity, recent efforts have focused on MSC because they are far less controversial, and more importantly, may be derived directly from children and adult patients.[3,4]Many tissues, including bone marrow, peripheral blood, heart tissue, and lung tissue, harbor populations of viable MSCs but more recent efforts have focused on more accessible sources – such as adipose tissue and dental pulp from extracted teeth.[5-9]

A growing body of evidence has suggested that dental pulp-derived stem cells (DPSC) are among the most accessible of human stem cell populations.[10]DPSCs are originally formed from both epithelial and mesenchymal stem cell progenitors, the epithelial-derived ameloblasts and the mesenchymal-derived dentin and bone and soft tissues of the periodontium.[11]Although they cannot form all cells and tissues of the body, these DPSCs are capable of differentiation into more than one cell type and are therefore classified as multipotent stem cells.[12] Studies have now confirmed DPSC, under specific conditions and stimuli, may be capable of differentiating into adipocytes, neurons, osteoblasts and chondrocytes – although these specific methods could vary by whether the DPSC were derived from the apical part of the papilla (AP-DPSC), the dental follicle (DF-DPSC) surrounding third molars, or the periodontal ligament (PDL-DPSC).[13,14]

Two nation-wide commercial companies now offer a service for processing and storing DPSC from either primary teeth or extracted adult teeth, but little information is known about the differentiation potential of these DPSC isolates.[15]Hung E. et al. found that premolars and other intact teeth extracted from orthodontic patients ages 18-25 exhibited the greatest likelihood for obtaining viable DPSC isolates.[16]These studies, however, did not evaluate the potential to induce differentiation in these uncommitted DPSC lines using established methods and protocols.

The objective of this study was to evaluate the potential to induce differentiation of DPSC isolates originally derived from extracted teeth of adult orthodontic patients. The working hypothesis for this project was that any DPSC isolate, derived from vital, intact permanent adult teeth, could be induced towards differentiation *in vitro* using pre-plated cell-matrix adhesion molecules and the administration of exogenous growth factors.[17,18]

2. Methods

2.1. Human Subjects

This original protocol for this study titled "Isolation of Non-Embryonic Stem Cells from Dental Pulp" at the University of Nevada, Las Vegas - School of Dental Medicine (UNLV-SDM) dental clinic was approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#0907-3148) in February 2010.[15,16]In brief, the samples for this study were isolated from patients that were randomly recruited by members of the UNLV-SDM clinic during their dental visits between February and June 2010. Informed Consent was required and was conducted onsite at the time of study recruitment. Inclusion criteria: All patients were required to be consenting adults (> 18 years old) who agreed to participate. In addition, all dental pulp samples were collected from subjects with sound, unrestored, vital teeth (teeth that have healthy pulp tissue), who were already scheduled to have one or more extractions that were necessary for oral health, as determined by the clinical faculty member in charge. Exclusion criteria: Any subject under eighteen (18), any subjects scheduled for dental extractions involving compromised pulp, and any subject that refused to donate their extracted teeth or participate in this study.

2.2. DPSC Isolation and Culture

The teeth included in this retrospective study were originally extracted due to impaction (e.g., third molars) or crowding(e.g., premolars extracted for orthodontic treatment).Following extraction, the teeth were sectioned at the cemento-enamel junction (CEJ) using a diamond rotary disc and the dental pulp was removed with an endodontic broach and then immediately placed into sterile microcentrifuge tubes containing 1X phosphate buffered saline (PBS) and transferred to the laboratory for culture. Tubes were pre-assigned a unique, randomly-generated number to maintain patient confidentiality and to prevent research bias.

The dental pulp samples were then transferred to a biomedical laboratory for processing and culture using the direct outgrowth (DPSC-OG) method.[19,20] In brief, the PBS containing extracted dental pulp was centrifuged at 2.1 relative centrifugal force (RCF) and then resuspended in 1.0 mL of RPMI-1640 medium from Hyclone (Logan, UT) with 2mM L-Glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 μg/mL) solution and 10% fetal bovine serum (FBS), obtained from HyClone (Logan, UT). Cells were cultured in 75 cm^2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO_2 in humidified chambers. Media was changed every 48 hours until adherent cells reached 70% confluence. Cells were subsequently passaged at a 1:4 ratio for a minimum of ten passages.

2.3. RNA Isolation and RT-PCR

To determine if the cells from each dental pulp isolate (remaining after ten passages) were dental pulp stem cells

(DPSC), RNA was isolated from 1.5 x 10^7 cells of each of the experimental cell lines, using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) in accordance with the procedure recommended by the manufacturer.[21]RNA concentration and purity were calculated using UV spectroscopy. RT-PCR was then performed on total RNA using the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the mesenchymal stem cell (MSC) primers for CD44, CD133, NANOG, Oct4, Sox-2, Bin1, Rnf12, and Klf4 synthesized by SeqWright (Houston, TX), as previously described.[15,16,22] Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY). Quantitation of RT-PCR band densitometry and relative mRNA expression levels were performed using Adobe Photoshop (San Jose, CA) imaging software, Image Analysis tools. All four (4) cell lines used in this study were found at baseline to express intracellular (NANOG, Oct4, Sox-2, Klf4, Rnf12, Bin1) and cell surface markers (CD44, CD133) that are used to identify and characterize DPSC isolates.

2.4. Baseline Growth and Doubling Time

Assays to ascertain doubling time (DT) were performed in the appropriate complete media. In brief, cells at 70% confluence were trypsinized and plated 1:4 into new 75 cm^2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO_2 in humidified chambers and their confluence was measured with a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). Three separate, independent replications of each experiment were performed to determine doubling time for each DPSC isolate.

2.5. Experimental Assays

Proliferation and differentiation assays were performed in the appropriate complete media, with and without the addition of exogenous growth factors (GF) or extracellular matrix (ECM), in Corning Costar 12-well assay plates (Corning, NY) at a concentration of 1.2 x 10^4 cells per well, and proliferation was measured over twenty four (24) days. Cultured cells were fixed at five time points, at the initial plating (T1), after six (6) days (T2), after twelve (12) days (T3), after 18 days (T4) and after twenty four (24) days (T5), using 50 μL of 10% buffered formalin. For experimental plates fixed at each time point, the formalin was aspirated after twenty four (24) hours and each cell well was then stained with crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ). The stain was then aspirated and wells washed with 1X phosphate buffered saline (Fisher Scientific: Fair Lawn, NJ) and aspirated. The relative absorbance was then measured at 630 nm using a Bio-Tek ELx808 microplate reader (Winooski, VT). Data were analyzed and graphed using Microsoft Excel (Redmond, WA). Three separate, independent replications of each experiment were performed.

2.6. Materials

Two direct methods for induction of DPSC isolate differentiation *in vitro* were utilized: 1) the addition of exogenous growth factors (GF), and 2) the coating of specific extracellular matrix molecules (ECM) onto the surface of tissue-culture dishes, which were performed for each of the experimental assays described above. As previous research has demonstrated transforming growth factor (TGF-β1; M.W. 44.3 kDA) may, in fact, be critical to maintaining DPSC pluripotency, TGF-β1 was obtained from Calbiochem (La Jolla, CA) and the cellular media supplemented to a final concentration of 2.5 ng/mL or 0.56 uM.[18]In addition, one of the primary methods for GF-induced differentiation of DPSC and MSC isolates, Dexamethasone (Dex.) was obtained from Fisher Scientific (Fair Lawn, NJ) and added to cellular media for a final concentration of 10 nM.[23,24] Finally, the control for the GF experiments was the MEK1 inhibitor (40 ng/mL or 50 μM), a cell-cycle and growth factor inhibitor, obtained from Calbiochem/EMD Biosciences/Millipore, M.W. 267.3 (Darmstadt, Germany).[22] Extracellular matrix (ECM) molecules were obtained from Fisher Scientific (Fair Lawn, NJ) and tissue culture wells were coated with purified ECM at a 20 ug/mL protein concentration for one hour (60 minutes) at room temperature (25 C), as previously described. Poly-L-lysine (34-382-0001), Collagen-1 (50-361-599) and Laminin-5 (NC9992259).[24-27] Data were analyzed and graphed using Microsoft Excel (Redmond, WA).

2.7. Statistical Analysis

The differences between treatments were measured using a *t* distribution, $\alpha = 0.05$. All samples were analyzed using two-tailed *t*-tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed *t*-test.[28]As long as the sample size is at least moderate (>20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses.

3. Results

Four (4) previously characterized dental pulp stem cell lines were thawed and subsequently grown in culture to assess their baseline growth and doubling time (Figure 1).[15, 16] The average doubling time was 2.83 days, which ranged between 2.05 -3.87 days. For the *in vitro* differentiation experiments, each cell line (DPSC-3882, 5653, 9765, and 11418) was then plated into 96-well tissue culture treated plates and evaluated using photomicroscopy to determine

any changes to cellular morphology, as well as cellular number and doubling time. In these 24-day assays, from the initial time point (T1) to the final endpoint (T5),each of the four DPSC cell lines grew from less than 5% confluence (Fig. 1A-D) to approximately 34% confluence (Fig. 1I).

The addition of TGF-β1, recently demonstrated to maintain or increase pluripotency in DPSC, was associated with accelerated growth of approximately 55% in these cell lines (Fig. 1J).[23,24] The average doubling time decreased by 27.3% from 2.83 days to 2.2, ranging between 1.1 – 4.09 days. Although doubling time in one cell line (DPSC-5653) did not exhibit a significant change (3.87 to 4.09 days), doubling time in DPSC-9765 (2.18 to 1.14 days), DPSC-3882 (3.24 to 2.22 days), and DPSC-11418 (2.05 to 1.35 days) decreased markedly ($n=24$, $p<0.05$). Although significant changes to the rates of cellular growth were observed with the addition of TGF-β1, no overt changes to cellular morphology or size were observed in any cell line (Fig. 1A-H).

Following previous research that demonstrated induction of MSC and DPSC differentiation *in vitro* using specific extracellular matrix (ECM) molecules and growth factors, these methods were utilized to ascertain any effects on cellular phenotype or growth in these DPSC isolates (Figure 2).[23-27] The addition of growth factors to the cell culture medium induced variable responses in the DPSC isolates (Fig. 2A). As previously noted, TGF-β1 increased the doubling time and proliferation in all DPSC isolates by approximately 50% (range: 1.32 – 1.91 fold increase), although no overt changes to cellular morphology were observed. The addition of the MEK1 inhibitor PD98059 was not sufficient to induce any overt changes to any of the DPSC isolates. However, the addition of Dexamethasone (Dex) was sufficient to induce a differential response in DPSC-9765 (Fig. 2B) and DPSC-11418 (Fig. 2C), but not in DPSC-3882 or DPSC-5653 (data not shown). More specifically, growth in DPSC-9765 was increased by 2.03-fold while proliferation of DPSC-11418 increased by 2.31-fold – significantly higher than the growth observed in DPSC-3882 or DPSC-5653 ($p<0.05$) – which was also correlated with changes to cellular morphology and the formation of dense accumulations of localized, site-specific aggregations of larger cells with visibly altered morphology.

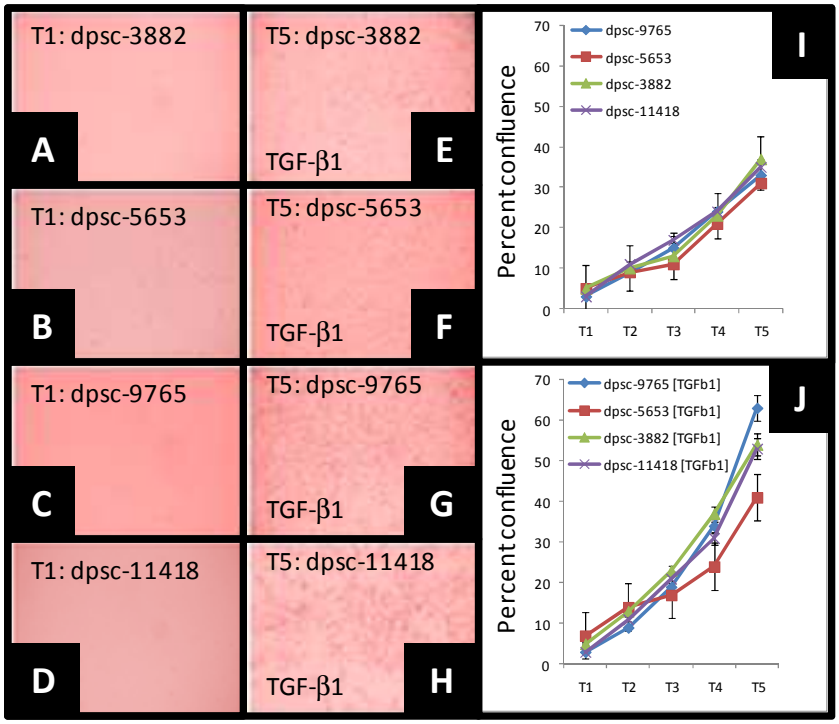

Figure 1. *Baseline DPSC proliferation is enhanced in vitro by the addition of TGF-βI. A-D. Initial plating of cells (T1) revealed an average confluence of approximately 5%. Baseline growth (without the addition of growth factors) demonstrated an average doubling time of 2.83 days (DPSC-9765: 2.18d; DPSC-5653: 3.87d; DPSC-3882: 3.24d; DPSC-11418: 2.05d) for a final confluence at the final time point (T5) of approximately 34% (I). The addition of TGF-βI increased growth at T5 to approximately 53% (J) and decreased average doubling time to 2.21 days (DPSC-9765: 1.14d; DPSC-5653: 4.09d; DPSC-3882: 2.22d; DPSC-11418: 1.35d).*

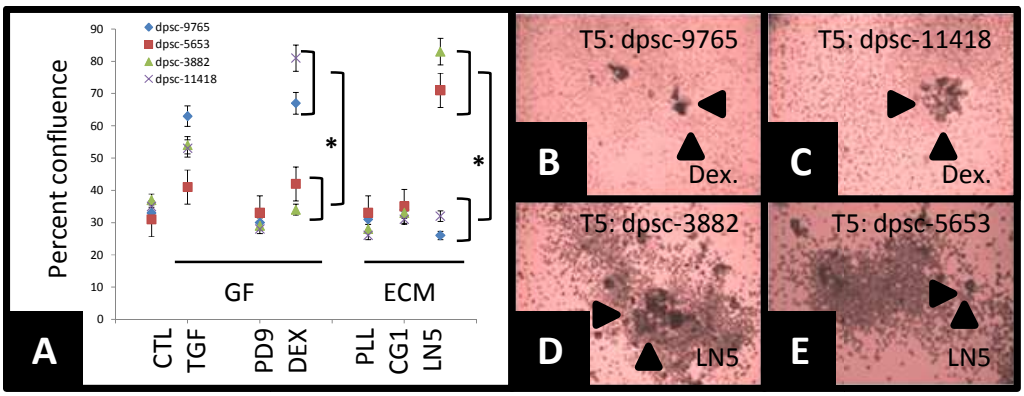

Figure 2. *DPSC cellular phenotypes altered by exogenous factors. A. The addition of extracellular matrix (ECM) coated wells or growth factor (GF) was sufficient to induce changes to some DPSC isolates. As previously noted, TGF-β1 increased growth in all four isolates with no changes observed in cellular morphology (A). The addition of the MEK1 cell-cycle inhibitor PD98059 was not sufficient to induce any phenotypic changes, although Dexamethasone (Dex) was sufficient to increase both growth and to alter cellular morphology in DPSC-9765 (B) and DPSC-11418 (C). Wells coated with Laminin-5 (LN5) but not poly-L-lysine or Collagen 1 (CG1), were sufficient to induce significant changes to cellular morphology and increased growth in only two DPSC isolates, DPSC-3882 (D) and DPSC-5653 (E).*

The wells coated with the ECM control poly-L-lysine (PLL) and the experimental wells coated with Collagen 1 (CG1) did not induce any changes to cell number or morphology in any of the four cell lines (Fig. 2A). However, a differential response was noted in the wells coated with Laminin-5 (LN5) with two DPSC isolates exhibiting both an increase in cell number, as well as significant changes to the morphology of some subsets of cells. More specifically, LN5 was sufficient to induce an increase in growth in DPSC-3882 (Fig. 2D) and DPSC-5653 (Fig. 2E) by more than two-fold (2.24 and 2.29, respectively), which was significantly different than the growth observed in the other DPSC isolates (data not shown) and from the baseline measurements ($p < 0.05$).

In order to evaluate and assess the differential phenotypic changes observed under Dexamethasone administration and Laminin-5 plating, RNA was successfully isolated from all DPSC isolates under these conditions and relative endpoint (RE) reverse transcription polymerase chain reaction (RT-PCR) was performed using equal concentrations of total RNA from each isolate (Figure 3). These analyses revealed that mRNA specific for the intracellular biomarkers for DPSC, Klf4 and Sox-2 – as well as the cell surface marker CD133, were expressed in DPSC-3882, DPSC-5653, DPSC-9765 and DPSC-11418 at baseline. Additional mRNA biomarkers, including Bin-1, Rnf12, Oct-4 and NANOG were also expressed (data not shown). The relative levels of these intracellular and extracellular biomarkers were enhanced following the administration of TGF-β1. However, the differential phenotypes observed under Dexamethasone and Laminin-5 administration were associated with differential expression of these same biomarkers.

More specifically, the altered the growth and phenotype of DPSC-9765 and DPSC-11418 following Dexamethasone administration was correlated with a loss of mRNA specific for the transcriptional control regulator Klf4 (as well as Bin1 and Rnf12). In addition, expression of mRNA specific for the downstream biomarkers Sox-2 (as well as Oct4 and NANOG) and CD133 was also down-regulated – consistent with previous observations of apical papilla (AP) - and dental follicle (DF)-derived DPSCs that are responsive to GF-induced methods for *in vitro* differentiation. However, no significant changes to these biomarkers were observed in either DPSC-3882 or DPSC-5653 – the DPSC isolates non-responsive to Dexamethasone.

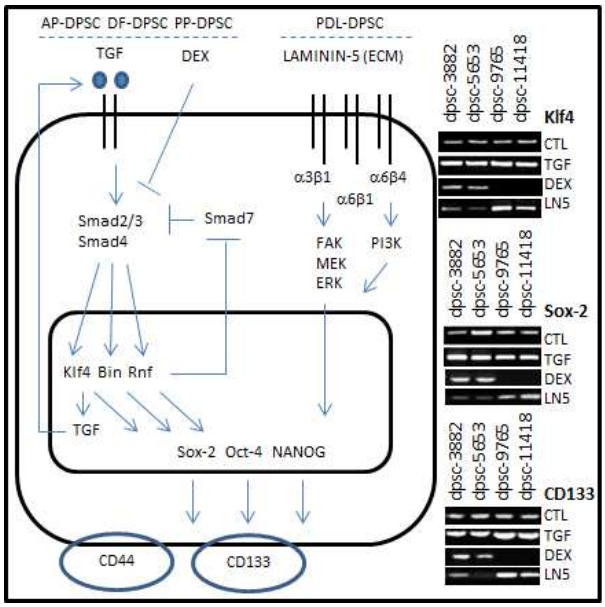

Figure 3. *DPSC intracellular and extracellular biomarkers differentially altered by exogenous factors. The addition of extracellular matrix (ECM) Laminin-5 coated wells was sufficient to inhibit Klf4 and Sox-2 intracellular mRNA expression, as well as the cell-surface marker CD133 in dpsc-3882 and dpsc-5653 cell lines; these data may be consistent with the PDL-DPSC phenotype. Dexamethasone was sufficient to induce similar changes in dpsc-9765 and dpsc-11418 isolate; these data may be consistent with AP-DPSC, DF-DPSC or PP-DPSC phenotypes .*

Conversely, the altered phenotypes observed in DPSC-3882 and DPSC-5653 following plating on Laminin-5 coated dishes were associated with a down-regulation (but not loss) of mRNA expression in both Klf4 and Sox-2 (as

well as Bin1, Rnf12, Oct4, and NANOG; data not shown). Expression of mRNA for the downstream cell surface DPSC biomarker CD133, however, was only slightly reduced in the DPSC-3882 isolate - although this expression was completely lost in DPSC-5653. These results appear to be consistent with previous observations that periodontal ligament (PD)-derived DPSC isolates may be primarily responsive to ECM-mediated methods for *in vitro* differentiation.

4. Discussion

The goal of this project was to evaluate the potential to induce differentiation among DPSC isolates from four (4) orthodontic patients using established MSC and DPSC protocols and methods. These results demonstrated that all four DPSC isolates exhibited phenotypes and expressed biomarkers at baseline consistent with MSC and DPSC and also responded appropriately to the pluripotency sustaining effects of *in vitro* TGFβ1 administration.[23,24] However, the two primary methods for inducing differentiation, ECM-plating and GF administration,[23-27] exhibited differential responses in these DPSC isolates.

That these DPSC isolates were susceptible to the effects of either ECM- or GF-induced alterations to growth, proliferation, morphology and DPSC biomarker expression, suggests that these methods may be sufficient to stimulate these responses in some DPSC isolates, although not universally. More importantly, that the specific type of DPSC isolate (influenced by the method of isolation and culture) may be, in fact, a critical component for a more complete understanding of how these methods could be employed to evaluate the potential lineages and usages for any given patient.[14,19,20]

For example, these findings complement the ever-expanding body of research that suggests DPSC isolates are often not heterogeneous aggregations of DPSCs but may, in fact, be clones derived from different and often very specific subsets of DPSCs, such as AP-DPSC, DF-DPSC or PDL-DPSC.[19,20] Many factors influence DPSC quality and quantity and type, including the methods used to isolate, collect, concentrate, and store these isolates.[1] For instance, isolation by enzymatic dissociation (DPSC-ED) may be more likely to produce heterogeneous populations of faster growing cells, as this process allows for dissociation of multiple cell types from the extracted dental pulp– although this process may limit viability and may also decrease overall yield.[11,14]In contrast, DPSC isolation by direct outgrowth (DPSC-OG), the method employed in this study, tends to produce largely homogenous populations of one DPSC type, with more limited differentiation potential due to their random selected clonal derivation.[15,16]

This study had several limitations that should be outlined, including a very limited sample size (n=4), which may restrict the overall ability to make inferences about these results. In addition, the DPSC isolates were derived from adult orthodontic patients, which may have different health outcomes and parameters than other dental patient populations, which could also influence the overall results and outcomes associated with this study.[21] Finally, this was a retrospective analysis of previously collected DPSC isolates – therefore, prospective studies that incorporate larger sample sizes will be needed to further elucidate the parameters that most likely influence the differentiation potential for DPSC isolates from dental patients.

5. Conclusions

Although some commercial enterprises now offer DPSC banking and storage services, much less is known about the viability or potential applications for DPSCs isolated from dental patients. Although many patients routinely have intact, adult teeth extracted for orthodontic and other dental-related issues, few studies have addressed the potential to characterize the potential for these isolates to be manipulated in laboratory settings. This study provides some initial evidence that randomly selected DPSC isolates from orthodontic patients may be induced by established protocols to change phenotype and expression of pluripotent biomarkers with variable susceptibility between differing types of DPSCs, although more studies will be needed to determine the range of cell types that can be successfully re-engineered in laboratory settings.

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