Original Research Paper

Expression of Exosome-Derived MicroRNAs miR-21 and miR-135 are Differentially Regulated Among Dental Pulp Stem Cells

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Abstract: Dental Pulp Stem Cells (DPSCs) are a type of mesenchymal stem cell that has the potential to differentiate into various types of cells and tissue. Previous research has demonstrated that DPSCs can be easily accessed and isolated from both permanent and deciduous teeth, such as wisdom teeth or third molars, although there is less information available about the mechanisms and factors that regulate the growth and proliferative phenotypes and responses of DPSCs. Recent studies have revealed that a type of non-coding RNA known as microRNA can modulate these characteristics among many types of stem cells, although much is still unknown about how DPSC phenotypes may be regulated by microRNAs. Due to this lack of knowledge, the primary objective of this study was to evaluate microRNA expression and determine any correlations with DPSC phenotypes, such as proliferation or growth. Six DPSC isolates were retrieved from an existing repository and cultured using an existing approved protocol. Exosomes and extracellular vesicles were extracted from each DPSC isolate, which ranged in size from 50 to 250 nm. Exosome isolation was confirmed using Western blots for CD63 and Bradford protein assays. In addition, RNA was extracted, cDNA was synthesized, and qPCR was performed, which revealed that all DPSC isolates expressed miR-124, miR-133, and miR-224. However, differential expression of miR-21 among rapid and intermediate doubling time (rDT, iDT) isolates was observed, with expression of miR-135 found only among the intermediate and slow (iDT, sDT) DPSC isolates. This study provides some of the first evidence of associations between miRNA expression and specific DPSC growth phenotypes. Further studies will be needed to confirm these results and determine the mechanisms associated with the expression of miR-21 among rapidly growing DPSCs and miR-135 expression among more slowly growing DPSCs.

Keywords: Dental Pulp Stem Cells (DPSC), Micro RNA (miR), Exosomes

Introduction

Multiple research groups have demonstrated the potential range of uses for Dental Pulp Stem Cells (DPSC), which are composed of heterogeneous populations of pluripotent stem cells that can differentiate into a limited variety of cells and tissue types (Kadar *et al*., 2009; Ranganathan and Lakshminarayanan, 2012). Many early studies of DPSC focused on the question of whether DPSC should be obtained from human exfoliated teeth or SHED, which evaluated the potential utility of isolation and expansion from primary, deciduous teeth (Rosaian *et al*., 2020; Daltoe *et al*., 2014).

However, many studies now recognize the potential of DPSC from extracted permanent teeth including third molars or "wisdom teeth" that are routinely removed to relieve crowding before orthodontic treatment (Hadaegh *et al*., 2014; Atari *et al*., 2012; Karbanová *et al*., 2011).

Although several studies have demonstrated methods and protocols for DPSC extraction from both primary and permanent or adult teeth, including third molars-less information is available regarding the mechanisms that mediate and modulate pluripotency and responsiveness among these DPSC ex vivo (Karamzadeh *et al*., 2012; Tatullo *et al*., 2019). For example, previous research from

this institution has evaluated the effect of specific growth factors, including Vascular Endothelial Growth Factor (VEGF) and Bone Morphogenic Protein (BMP), in addition to other functional biomatrix materials such as Mineralized Trioxide Aggregate (MTA) (Forgues *et al.*, 2019; Bae *et al.*, 2021; Cinelli *et al.*, 2019). However, as the number of studies regarding DPSC responsiveness to growth factors grows, more evidence has come to suggest that additional factors such as transcriptional regulators may influence these phenotypes and behaviors (Bakhtiar *et al.*, 2018).

New research has revealed that short, non-coding microRNAs are capable of mediating specific aspects of differentiation and self-renewal among some different types of stem cell lineages, including stromal and hematopoietic cells (Kulthanaamondhita *et al.*, 2022; Kearney and Duncan, 2023; Shi *et al.*, 2019). Additional studies have demonstrated microRNAs may induce similar types of effects among mesenchymal stem cells to modulate osteogenic differentiation (Xie *et al.*, 2020; Wang *et al.*, 2019; Liu *et al.*, 2020). However, only a few studies have evaluated these mechanisms and effects in DPSC (Liang *et al.*, 2022; Fu *et al.*, 2016; Wei *et al.*, 2022). Pilot studies from this group have demonstrated differential expression of some microRNAs among DPSC isolates, including miR-217, miR-124, and miR-27 (Whiting *et al.*, 2019). In addition, this microRNA expression was correlated with mRNA expression of MSC biomarkers that include Sox-2, Oct-4, and NANOG-as well as cellular viability, which may suggest more complex transcriptional regulations may be involved (Whiting and Kingsley, 2019).

Due to the breadth and depth of research regarding microRNA involvement in mesenchymal stem cells, it is evident that more information will be needed to determine the mechanisms behind microRNA modulation of specific DPSC characteristics and phenotypes (Javed *et al.*, 2010; Mazziotta *et al.*, 2021; Zhang *et al.*, 2017; Xu *et al.*, 2019). Based upon the lack of knowledge in this area, the primary objective of this project was to perform an extensive screening of microRNA expression among DPSC and to evaluate if this expression is associated with specific DPSC characteristics or phenotypes, including proliferation and differentiation. The remainder of this report outlines the techniques and methods used, including a brief overview of the establishment of the DPSC repository and original extraction protocol, followed by the technical details and analysis of the cellular and exosomal RNA isolation and cDNA synthesis, as well as the microRNA screening protocol and results.

Materials and Methods

Human Subjects Study Approval

This study was a retrospective analysis of an existing DPSC biorepository. This study was reviewed and approved by the University of Nevada, Las Vegas (UNLV) Office for the Protection of Research Subjects (OPRS) and Institutional Review Board (IRB) under Protocol 1717612-1 "Retrospective analysis of dental pulp stem cells from the University of Nevada, Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and clinical population" on February 21, 2021.

Original DPSC Collection Protocol

The original study to create the DPSC biorepository was reviewed and approved by the UNLV IRB and OPRS under protocol OPRS#0907-3148 titled "Isolation of Non-Embryonic Stem Cells from Dental Pulp on February 5, 2010. All the study participants were voluntary and provided Informed Consent at the time of collection. In brief, the inclusion criteria were UNLV-SDM clinic patients that were scheduled for routine extractions of wisdom teeth or third molars as part of ongoing Orthodontic therapy spacing. Exclusion criteria for the original study included any patients that declined participation, any patients not being treated at UNLV-SDM, and any patients requiring extraction for other reasons (e.g., periodontal disease, fractured tooth, extreme dental decay). After teeth were extracted and sectioned, the dental pulp was removed using an endodontic broach and transferred in sterile microcentrifuge tubes with Phosphate Buffered Saline (PBS) to a biomedical laboratory for separation and culturing. The direct outgrowth method was used in sterile cell culture-treated flasks and a cell culture incubator. Cell culture conditions included Roswell Park Memorial Institute (RPMI) media with the addition of Fetal Bovine Serum (FBS) and penicillin-streptomycin antibiotic solution. All DPSC isolates were cultured for at least ten passages before cryopreservation.

DPSC Culture

Six ($n = 6$) existing DPSC isolates were selected from the biorepository for use in the current study. Proliferation and growth rate or Doubling Time (DT) for each DPSC isolate was determined and compared with the rate during the initial ten passages, which has been correlated related to differentiation potential. DPSC with rapid (rDT) Doubling Time (1-2 days) may suggest low differentiation and high pluripotency, intermediate (iDT) Doubling Time (5-6 days) may indicate the loss of some pluripotency factors, while slow (sDT) Doubling Time (10-12 days) may indicate partial or incomplete differentiation:

- Two DPSC isolates (DPSC-3924, -9765) were originally characterized as rDT
- Two DPSC isolates (DPSC-5423, -8604) were originally characterized as iDT
- Two DPSC isolates (DPSC-4595, -9500) were originally characterized as sDT

Cellular RNA Isolation

RNA was extracted from all DPSC isolated with the TRIzol RNA extraction kit from Fisher Scientific (Fair Lawn, NJ). In brief, culture media was removed and cells were lysed with TRIzol reagent and moved to a sterile microcentrifuge tube. Using the manufacturer-recommended protocol, chloroform was added and triturated before centrifugation at 12,000 × g or Relative Centrifugal Force (RCF) for 15 min. The RNA-containing upper phase was removed and placed in a new, sterile microcentrifuge tube with isopropanol and mixed. The solution was centrifuged for ten minutes and the RNA-containing pellet was then washed with ETHANOL (EtOH) and centrifuged for an additional five minutes. After aspirating the supernatant, each pellet was subsequently resuspended using nuclease-free water. Quantification and analysis of RNA purity were performed using the Nano Drop 2000 Spectrophotometer and absorbance readings at both A260 and A280 nm.

cDNA Synthesis and qPCR Screening

The isolated RNA was transformed into cDNA with the ABgene Reverse-iT One-Step RT-PCR kit from Fisher Scientific (Fair Lawn, NJ) and a Mastercycler from Eppendorf (Hamburg, Germany). Reaction settings included reverse transcription at 47°C for 30 min and 40 cycles of denaturation, annealing, and final extension. All DPSC isolates were screened according to the International Society of Cellular Therapy (ISCT) for stem cell markers, which included CD34 and CD45 (negative control), as well as CD73, CD90, and CD105 (positive control). Additional MSC pluripotency markers were used, including Sox-2, Nestin, and NANOG, synthesized by SeqWright from Fisher Scientific (Fair Lawn, NJ):

CD34 forward: 5'-CCT CAG TGT CTA CTG CTG GTC T-3'
CD34 reverse: 5'-GGA ATA GCT CTG GTG GCT TGC A-3'

CD45 forward: 5′-CAT ATT TAT TTT GTC CTT CTC CCA-3′
CD45 reverse: 5′-GAA AGT TTC CAC GAA CGG-3′

CD73 forward: 5'- AGT CCA CTG GAG AGT TCC TGC A-3'
CD73 reverse: 5'-TGA GAG GGT CAT AAC TGG GCA C-3'

CD90 forward: 5′-ATG AAC CTG GCC ATC AGC A-3′
CD90 reverse: 5′-GTG TGC TCA GGC ACC CC-3′

CD105 forward: 5′-CCA CTA GCC AGG TCT CGA AG-3′
CD105 reverse: 5′-GAT GCA GGA AGA CAC TGC TG-3′

Sox2 forward: 5′-ATG GGC TCT GTG GTC AAG TC-3′
Sox2 reverse: 5′-CCC TCC CAA TTC CCT TGT AT-5′;

Nestin forward, 5'-CGT TGG AAC AGA GGT TGG AG-3'
Nestin reverse, 5'-TCC TGA AAG CTG AGG GAA G-3'

NANOG forward: 5′-GCT GAG ATG CCT CAC ACG GAG-3′
NANOG reverse: 5′-TCT GTT TCT TGA CTG GGA CCT TGT C-3′

Exosome Isolation

The following thawing from cryopreservation and establishing viable cultures, DPSC isolates were placed into exosome-depleted media using exosome-depleted FBS from Gibco (Waltham, MA) for 24 h. Exosomes and extracellular vesicles were then secreted into the media by each corresponding DPSC isolate without contamination with FBS-endogenous exosomes. Removal of cells and cellular debris from the collected supernatant exosome-containing media was accomplished using centrifugation at 2,000 × g (RCF) for 30 min. Exosomes were isolated using the Invitrogen Exosome Isolation Kit, involving treatment with Total Exosome Isolation Reagent from Invitrogen (Carlsbad, CA), trituration, and then incubation for 12 h (overnight) at 4°C. Each sample was subsequently centrifuged for 60 min at 10,000 × g (RCF) and 4°C. The supernatant was removed by aspiration, which leaves the exosome-containing pellet Pellet resuspension was performed using 200 μL of sterile Phosphate Buffered Saline (PBS).

RNA Extraction from Exosome

RNA was extracted from the Extracellular Vesicles (EV) and exosomes with the total exosome RNA and Protein Isolation Kit from Invitrogen (Carlsbad, CA) using the manufacturer-recommended protocol. In brief, an aliquot of the exosome containing PBS was processed using an equal volume of denaturing solution. This was subsequently incubated on ice for a minimum of five minutes before the addition of phenol: Chloroform. Samples were then mixed and centrifuged at 10,000 × g (RCF). The supernatant was then aspirated and 100% ethanol (EtOH) was added before mixing. An aliquot of 700 μL from each sample was placed into the manufacturer-supplied filter cartridge, which was then placed into the manufacturer-provided collection tubes for centrifugation at 10,000 × g (RCF) for 15 sec. The sample-containing cartridges were then moved into fresh Collection Tubes and 50 μL of elution solution was added before centrifugation at 10,000 g × (RCF) for 15 sec for RNA elution. RNA purity and quantity were then analyzed at absorbances of A260 and A280 nm using a Nano Drop spectrophotometer.

microRNA cDNA Synthesis

RNA extracted from EVs and exosomes were then converted into cDNA with the TaqMan Advanced miRNA cDNA Synthesis Kit from Applied Biosystems (Waltham, MA) using the manufacturer-recommended protocol. Briefly, PolyA Reaction Mix (containing 10*X* PolyA Reaction Buffer, PolyA enzyme, ATP, and RNase-free water) was prepared and mixed with a small aliquot of sample RNA and placed into a thermal cycler for 45 min at 37°C, followed by an additional 10 min at 65°C. Ligation Reaction Mix was then assembled according to the manufacturer's protocol and added to each sample. The solution was then mixed and centrifuged before thermal cycling at 16°C for one hour. Finally, Reverse Transcription (RT) Reaction mix was prepared as specified by the manufacturer protocol, and 15 μL was placed with each sample and mixed thoroughly before centrifugation and thermal cycling at 42°C for 15 min-with an additional five min at 85°C.

qPCR Screening

Rapid, high-quality qPCR screening was then performed using the TaqMan fast advanced master mix kit from Applied Biosystems (Waltham, MA). In brief, master mix was prepared (containing 20*X* miRNA assay mix, 2*X* fast advanced master mix, and nuclease-free water) and 3*X* volumes were added to 15 μL added to 1:10-diluted cDNA template (5 μL), mixed, and then briefly centrifuged. Each plate was then processed in a quant studio real-time PCR machine using 20 sec at 95°C to activate the enzyme, with a subsequent 40 cycles involving denaturation for one second at 95°C and then annealing and extension at 60°C for 20 sec:

miR-16 forward: 5'-TAG CAG CAC GTA AAT ATT GGC G-3'
miR-16 reverse: 5'-TGC GTG TCG TGG AGT C-3';

miR-21 forward: 5'-GCC ACC ACA CCA GCT AAT TT-3'
miR-21 reverse: 5'-CTG AAG TCG CCA TGC AGA TA-3'
miR-27 forward: 5'-ATA TGA GAA AAG AGC TTC CCT GTG-3'
miR-27 reverse: 5'-CAA GGC CAG AGG AGG TGA G-'3'

miR-124 forward: 5'-TTC ACA GCG GAC CTT GA-3'
miR-124 reverse: 5'-GAA CAT GTC TGC GTA TCT C-3'

miR-128 forward: 5'-TCT CCT AAA GAG CCC GAA CA-3'
miR-128 reverse: 5'-TTG CAT TCA TAG CTG CAT CC-3'
miR-133 forward: 5'-CCG GTT AAC TCG AGC TCT GTG AGA G-3'

miR-133 reverse: 5'-CTA GCT AGG AAT TCT GTG ACC TGT G-'3'

miR-135 forward: 5'-CGA TAT GGC TTT TTA TTC CTA -3'
miR-135 reverse: 5'-GAG CAG GGT CCG AGG T -3';

miR-224 forward: 5'-GCG AGG TCA AGT CAC TAG TGG T-3'
miR-128 reverse: 5'-CGA GAA GCT TGC ATC ACC AGA GAA CG-3'

miR-410 forward: 5'-CCG CAC GAT ATA ACA CAG ATG-3'
miR-410 reverse: 5'-GTG CAG GGT CCG AGG TAT TC-3'

Results

Six DPSC isolates were retrieved from an existing biorepository and placed into culture (Fig. 1). Initial viability counts ranged between 61.5 and 71% with live cell counts ranging between 0.88×10^4 to 8.55×10^4. Initial data from cryopreservation regarding growth or Doubling Time (DT) was confirmed by culturing and passaging cells, which revealed that two DPSC isolates dpsc-3924 and dpsc-9765 exhibited rapid doubling time between 1.5-2.5 days (2.2 days, 1.5 days, respectively). More slowly dividing DPSC isolates included dpsc-5423 and dpsc-8604, which averaged doubling times between 4.5-5.5 days (5.1, 4.8 days) and were categorized as exhibiting intermediate doubling times, while the slowest doubling times were observed among dpsc-9500 and dpsc-4595 (10.2, 10.8 days, respectively).

To confirm the presence of mesenchymal and dental pulp stem cell biomarkers, isolation of RNA was performed with each DPSC cell culture (Table 1). This data showed RNA was successfully isolated from each DPSC culture, with average concentration observed at 604.3 ng/μL, ranging between 511-634 ng/μL-which falls within the acceptable range as noted in the manufacturer protocol (between 100-1000 ng/μL). Absorbance was measured at A260 nm and A280 nm, which revealed A260:A280 purity ratios averaging 1,71 that ranged between 1.69 - 1.74.

To verify the production of mesenchymal and DPSC-specific biomarkers by qPCR screening, the isolated cellular RNA was subsequently converted into cDNA (Table 2). These results demonstrated RNA was successfully converted into cDNA from each of the DPSC isolates, which averaged 1579.7 ng/μL and ranged between 1524.1 to 1634.2 ng/μL. The absorbance ratios that provide some measure of DNA purity averaged 1.83 and ranged between 1.80 and 189, which are acceptable for qPCR screening and analysis.

The expression of International Society of Cellular Therapy (ISCT) positive and negative control biomarkers specific for MSC, qPCR screening was evaluated (Fig. 2).
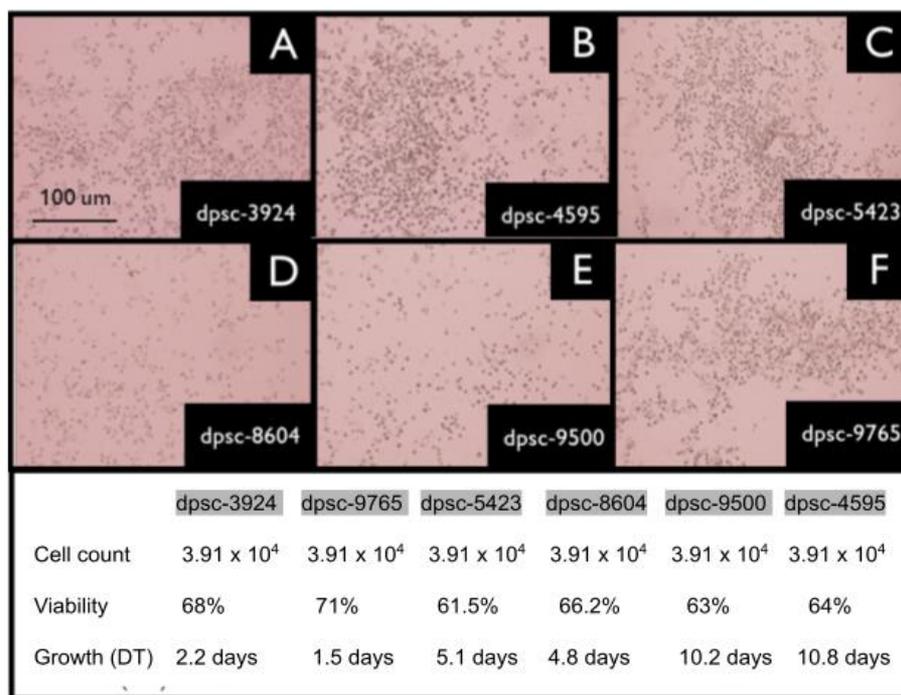
These data confirmed that none of the DPSC isolates expressed the negative controls CD34 or CD45, as outlined by the ISCT. In addition, all six DPSC isolates were observed expressing the ISCT positive control biomarkers CD90, CD73 and CD105. Finally, the expression of three pluripotency biomarkers, Sox-2, Nestin, and NANOG was also confirmed.

DPSC cultures were subsequently placed into exosome-free media and the Extracellular Vesicle (EV)- and exosome-containing supernatant was harvested (Fig. 3). Following the detailed procedure for processing and isolating exosomes, the analysis using particle metrix zeta view Nanoparticle Tracking Analysis (NTA) was used to determine the exosome and EV size and volume. These data revealed average exosome and EV diameter measurements ranging between 166.6 to 199.4 nm, with volumes ranging from 248 to 542.7 nm^3 corresponding to the well-established parameters of diameter (100-200 nm) and volume (200-600 nm^3).

The verification of extracellular vesicles and exosomes was performed using protein extracted from these samples using the Bradford Assay and Western blot analysis (Fig. 4). These data from the Bradford Assay demonstrated successful isolation of proteins with concentrations ranging from 1.42 to 1.63 ug/μL. Western blot analysis for CD63 (positive control for exosomes) demonstrated positive results from all DPSC samples within the normal expected range of 30-60 kDA.

To evaluate the expression of microRNAs, quantitative real-time qPCR was performed from the EVs and exosome-extracted RNA (Fig. 5). All of the DPSC isolates expressed miR-16 the positive control, as well as miR-124, miR-133 and miR-224. However, differential expression of microRNAs was observed between the DPSC isolates, with only three DPSC isolates expressing miR-21, including dpsc-3924, dpsc-8604, and dpsc-9765. In addition, four DPSC isolates were observed expressing miR-135, which included dpsc-4595, dpsc-5423, dpsc-8604, and dpsc-9500. None of these isolates evaluated expressed miR-410, miR-128, or miR-27.

Finally, to evaluate the differential expression of microRNAs among the DPSC isolates, results were sorted by proliferative phenotype (rapid Doubling Time or rDT, slow Doubling Time or sDT, and intermediate Doubling Time or iDT) (Table 3). These data revealed that miR-21 was differentially expressed only among the rDT (dpsc-3924, dpsc-9765) and one of the two iDT (dpsc-8604), but not among the sDT isolates. In addition, miR-135 was differentially expressed only among the iDT (dpsc-8604, dpsc-5423) and sDT (dpsc-9500, dpsc-4595), but not within the rDT samples. All DPSC isolates expressed miR-224, miR133, and miR-124, while no detection (n.d.) of miR-410, miR-128, or miR-27 among any of the DPSC isolates was observed.

|	dpsc-3924	dpsc-9765	dpsc-5423	dpsc-8604	dpsc-9500	dpsc-4595
Cell count	3.91×10^4	3.91×10^4	3.91×10^4	3.91×10^4	3.91×10^4	3.91×10^4
Viability	68%	71%	61.5%	66.2%	63%	64%
Growth (DT)	2.2 days	1.5 days	5.1 days	4.8 days	10.2 days	10.8 days

Fig. 1: Culture of DPSC isolates from cryopreservation. Several DPSC isolates were identified and placed into culture, including (A) dpsc-3924, (B) dpsc-4595, (C) dpsc-5423, (D) dpsc-8604, (E) dpsc-9500 and (F) dpsc-9765. Viability ranged between 61.5-71% with growth or Doubling Time (DT) varying between rapid (1.5-2.5 days), intermediate (4.5-5.5 days), and slow (10-11 days)
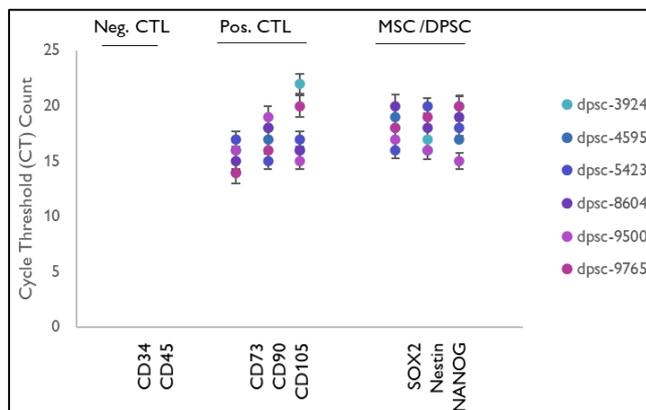
Fig. 2: Biomarker screening of DPSC isolates. Negative control (CD34, CD45) and positive control (CD73, CD90, CD105) aICST biomarkers and MSC pluripotency genes (SOX2, Nestin, NANOG) mRNA were confirmed by qPCR screening of cellular mRNA
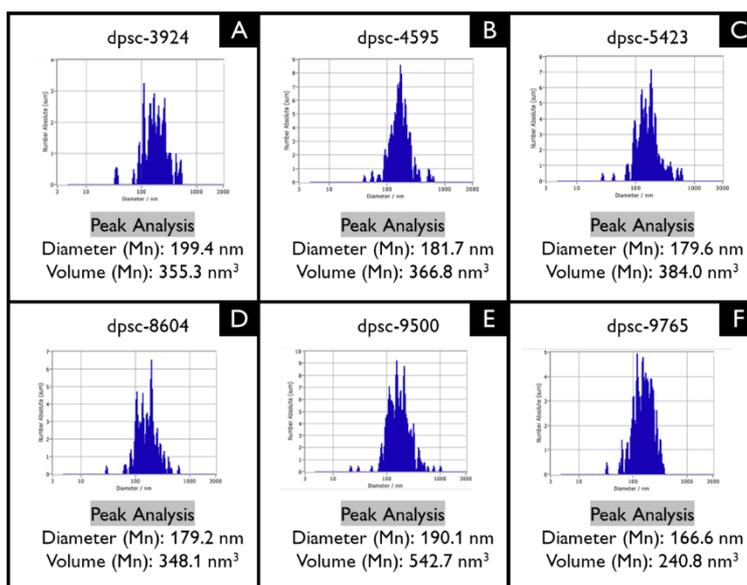
Fig. 3: Particle Metrix Zeta View Nanoparticle Tracking Analysis (NTA) of DPSC Extracellular Vesicles (EV). Mean or average EV diameter ranged from 166.6 to 199.4 nm, with volumes ranging from 248 to 542.7 nm^3
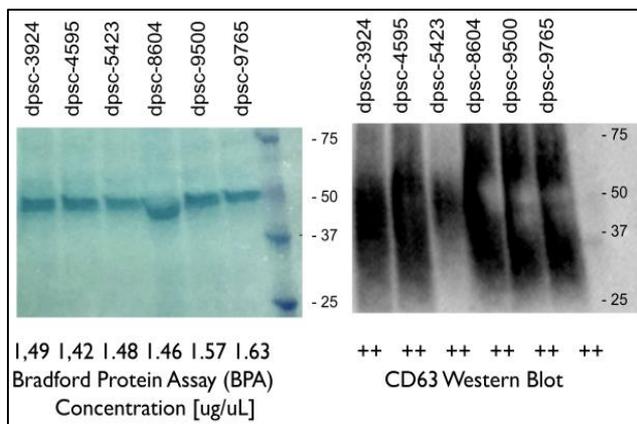
Fig. 4: Protein analysis from EVs and exosomes. Analysis of protein concentrations found similar levels among all DSPC isolates (1.42-1.63 ug/μL). Confirmation of CD63 expression by Western blot analysis was within the MW range of 30-60 kDA
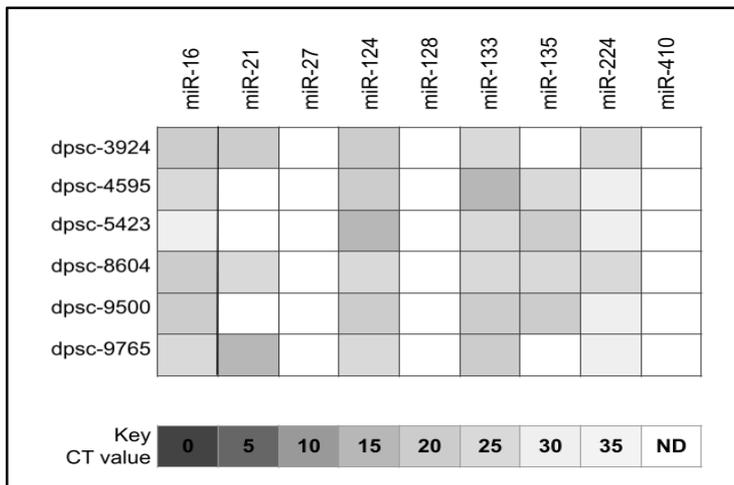
Fig. 5: Analysis of microRNA expression by qPCR screening. All DPSC isolates expressed miR-224, miR-133, miR-124, and miR-16. However, miR-21 and miR-135 were differentially expressed. None of the isolates expressed miR-27, miR-128 or miR-410

Table 1: Analysis of total cellular RNA extraction and isolation

DPSC isolate	RNA concentration	RNA purity (A260:A280)
dpsc-3924	631.30 ng/μL +/- 22.40	1.72
dpsc-4595	518.40 ng/μL +/- 31.82	1.69
dpsc-5423	615.10 ng/μL +/- 49.10	1.71
dpsc-8604	629.30 ng/μL +/- 47.10	1.74
dpsc-9500	511.30 ng/μL +/- 41.20	1.70
dpsc-9765	634.10 ng/μL +/- 27.50	1.72
Average	604.28 ng/μL	1.71
Range	511.3-634.1 ng/μL	1.69-1.74

Table 2: cDNA synthesis from cellular RNA

DPSC isolate	cDNA concentration	DNA purity (A260:A280)
dpsc-3924	1550.1 ng/μL	1.86
dpsc-4595	1613.3 ng/μL	1.89
dpsc-5423	1549.1 ng/μL	1.82
dpsc-8604	1632.2 ng/μL	1.81
dpsc-9500	1524.1 ng/μL	1.85
dpsc-9765	1571.4 ng/μL	1.80
Average	1579.7 ng/μL	1.83
Range	1524.1-16342.2 ng/μL	1.80-1.89

Table 3: MicroRNA expression among DPSC isolates

microRNA	rDT (rapid)	iDT (intermediate)	sDT (slow)
miR-21	dpsc-3924		
	dpsc-9765	dpsc-8604	n.d.
miR-135	n.d.	dpsc-5423	dpsc-4595
		dpsc-8604	dpsc-9500
miR-124	dpsc-3924	dpsc-5423	dpsc-4595
	dpsc-9765	dpsc-8604	dpsc-9500
miR-133	dpsc-3924	dpsc-5423	dpsc-4595
	dpsc-9765	dpsc-8604	dpsc-9500
miR-224	dpsc-3924		
	dpsc-9765	dpsc-5423	
dpsc-8604	dpsc-4595	dpsc-9500	
miR-27	n.d.	n.d.	n.d.
miR-128	n.d.	n.d.	n.d.
miR-410	n.d.	n.d.	n.d.

Discussion

Due to the evolving nature of microRNA regulation of MSC and DPSC phenotypes, the main aim of this study was to expand the number and range of microRNAs evaluated and determine any associations with DPSC phenotypes, such as proliferation rate or differentiation status. This study successfully demonstrated the establishment of DPSC isolates in culture, the harvesting of EVS and exosomes, and the qPCR screening of microRNA expression. These results demonstrated several important observations, such as the expression of some microRNAs among all DPSC isolates, the differential expression of some microRNAs specific to particular DPSC isolates, and the lack of expression of specific microRNAs in all DPSC isolates.

For example, the expression of miR-124 and miR-133 among all DPSC isolates in this study has been previously reported in other studies of DPSC with observations supporting a relationship with DPSC multipotency (Ghahfarrokhi *et al.*, 2020; Iaculli *et al.*, 2017). This complements earlier studies from this group that also demonstrated miR-124 expression among other DPSC isolates, but also greatly enlarges the number and type of DPSCs previously screened. This includes the first DPSC microRNA screening for putative stem cell biomarkers miR-27, miR-128, and miR-410 (Whiting *et al.*, 2019; Whiting and Kingsley, 2019). Interestingly, this study also found expression of miR-224 among all DPSCs, which supports other observations of miR-224 expression in DPSC that correlated with DPSC protection from apoptosis and also the promotion of DPSC growth and proliferation-although these studies featured only heterogenous DPSC isolates without the extensive characterization of DPSC growth and proliferation phenotypes and biomarker expression accomplished in this study (Ke *et al.*, 2019; Qiao *et al.*, 2020).

However, this study also demonstrated the differential expression of specific microRNAs among DPSC isolates, revealing an association with miR-21 expression and more rapid growth and proliferation. This may support other observations of miR-21 and the role this microRNA plays in the development and progression of rapidly dividing cancers, however, most other previous studies in DPSC have only demonstrated correlations between miR-21 expression and anti-inflammatory pathways (Dioguardi *et al.*, 2022; Singh *et al.*, 2021; Nara *et al.*, 2019; Ayadilord *et al.*, 2021). Other researchers have recently reported that miR-21 may potentially function in DPSC via interactions with STAT3 to facilitate odontoblast differentiation (Xu *et al.*, 2018). This study may be among the first to show the association between growth and proliferation and miR-21 expression in DPSC.

This study also found differential expression of miR-135 among the DPSC isolates, specifically among the slower-growing (iDT and sDT) DPSC isolates. Although this is among the first studies to report this finding, two other recent studies have found expression of miR-135 may be associated with partial differentiation in DPSC, which may explain why this is found among the slower-growing and potentially partially differentiated DPSC isolates (Ghahfarrokhi *et al.*, 2020; Li *et al.*, 2015). Interestingly, most other studies of miR-135 have demonstrated associations with specific types cancer-including digestive system cancers, which may also suggest that some tissue-specific differentiation may influence the expression of this microRNA that is restricted to specific types of cells and tissues in very specific locations (Kadkhoda *et al.*, 2022).

This study did not observe the expression of miR-27, miR-128, or miR-410 among the DPSC isolates, which may support other observations of miR-128 and miR-410 being differentially expressed only in DPSC involving cases of pulpitis and severe inflammation (Brodzikowska *et al.*, 2019; Xia *et al.*, 2022). Although limited expression of miR-27 was observed in previous DPSC studies from this group (Whiting *et al.*, 2019; Whiting and Kingsley, 2019), emerging evidence has shown that this microRNA may be mostly involved in lipid metabolism and the development of atherosclerosis-which may explain the lack of expression among these DPSC isolates (Xie *et al.*, 2016; Chen *et al.*, 2012).

Although these findings are significant, due to the nature of this type of retrospective analysis, some limitations are associated with this type of analysis that should also be discussed. First, this is a retrospective study of previously collected DPSCs, which may have changed their microRNA expression profiles following long-term cryopreservation (Daltoe *et al.*, 2014). Studies from this group have also found differences in survival, viability, and biomarker expression following long-term cryopreservation, which may suggest that handling and storage of DPSCs may alter their growth, metabolism, and differentiation potential over time-further complicating the results of any studies using these DPSC isolates, including this one (Tomlin *et al.*, 2016; 2018). In addition, financial limitations only allowed for a small number of DPSCs to be evaluated and screened-which suggests that future studies of this nature might include a larger number and range of DPSCs to confirm these observations.

Conclusion

These data confirm the differential expression of miR-21 and miR-135 among some DPSCs. These data suggest this differential microRNA expression may correlate with differing DPSC growth and proliferation rates, which support other observations of DPSCs and other cell types. Further studies will be needed to confirm these results in additional DPSC isolates and to determine any functional

mechanisms associated with the expression of miR-21 among rapidly growing DPSCs and miR-135 expression among the more slowly growing DPSCs.

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Author's Contributions

Matthew Hunsaker: Responsible for methodology, investigation, formal analysis and writing-original draft preparation.

Adelle Fuller and Brandon Richards: Responsible for methodology, data curation, formal analysis and writing-original draft preparation.

Karl Kingsley and Katherine M. Howard: Responsible for conceptualization, methodology, resources, data curation, formal analysis, supervision, and writing review and edited.

Ethics

The authors declare the presentation of preliminary data included in this manuscript was done by AR and BR at the International Association for Dental Research (IADR) conference in 2020. In addition, MH used preliminary data from this manuscript for his Master of Oral Biology thesis at UNLV-SDM.

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