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Assessment and Analysis of Dental Pulp Stem Cells (DPSCs) Biomarkers and Viability Following Cryopreservation Reveals Novel Association with MiR-218 Expression

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Abstract

Introduction: Recent efforts have demonstrated the clinical use and application for dental-pulp derived stem cells or DPSCs, which have been demonstrated to function in many dental and non-dental, tissue-based applications. Despite these advances, few studies have evaluated the effects of cryopreservation However, as most dentally-derived DPSC are banked during orthodontic treatment in the adolescent and teenager years, recent studies have begun to evaluate the effects of long-term cryopreservation on DPSC for longer periods of time. The primary objective of this study was to determine the effects of long-term cryopreservation on DPSC viability and pluripotency over time intervals extending up to ten years.

Methods: This retrospective study was reviewed and approved by the UNLV Institutional Review Board (IRB). Using an existing DPSC repository, sixteen DPSC isolates were thawed, cultured and screened for viability. RNA was extracted from each cell line and screened for mesenchymal stem cell biomarkers and microRNA expression.

Results: Six (n = 6) rapidly doubling time (rDT), three (n = 3) intermediate (iDT), and seven (n = 7) slow (sDT) isolates were successfully thawed and cultured. Viability ranged from 17.6% to 49.3%, which did not exhibit a clear, linear association with length of cryopreservation or doubling time. Screening of RNA revealed all DPSC isolates expressed the mesenchymal stem cell biomarker Nestin, with variable expression of Sox-2, Oct-4 and NANOG also observed. However, microRNA screening revealed differential expression among the rDT (miR-21, miR-27), iDT and sDT (miR-124, miR-135, miR-218) DPSC isolates - with the highest viability observed among the miR-218 expressing DPSC cells.

Conclusions: Although many studies have focused on the potential for DPSC use in biomedical and bioengineering applications, few studies have evaluated the long-term effects of cryopreservation or methods for identifying which DPSC isolates may be most likely to survive this procedure. This study is among the first to evaluate DPSC cryopreservation for periods up to ten years, as well as to identify potential biomarkers (such as miR-218) that may identify DPSC isolates with high survival potential.

Keywords: *Dental Pulp Stem Cells (DPSC); Cryopreservation; Biomarker; Microrna; QPCR*

Introduction

Many researchers are focused on the potential for mesenchymal stem cells, which are non-embryonic tissue-specific progenitor cells capable of repair and regeneration [1,2]. Areas of focused research interest have included clinical and translational applications for skin and cutaneous diseases, bone regeneration, lung disease, and even metabolic disorders such as Type II diabetes [3-7]. Recent efforts have demonstrated the clinical use and application for dental-pulp derived stem cells or DPSCs, which have been demonstrated to function in many dental, as well as a growing list of non-dental, tissue-based applications [8,9].

Although many studies have traditionally focused on DPSC for the treatment of dental- and periodontal-related disorders, this new evidence suggests clinical and translational applications for DPSC in many other tissues and organ systems [10-12]. Despite these advances, the few studies that have evaluated the effects of cryopreservation have focused on short periods of time - usually up to one year [13,14]. However, as most dentally-derived DPSC are banked during orthodontic treatment in the adolescent and teenager years, recent studies have begun to evaluate the effects of long-term cryopreservation on DPSC for periods of up to five years [15-18].

Based upon these studies, preliminary work from this group has attempted to increase the length of cryopreservation time under study beyond five years in order to more accurately determine any potential effects on DPSC viability [18,19]. This work has recently expanded to include the biomarkers and microRNA expression profiles of DPSC, which may correlate with viability, proliferation and differentiation potential [20-22]. As more information becomes available, the need to understand the potential ramifications of long-term cryopreservation and storage of DPSCs and the associated effects on their differentiation and proliferation potential becomes evident [23,24].

Moreover, as the effects of cell culture, passing and doubling time are now considered integral to the understanding of DPSC proliferation capacity - new studies that incorporate these phenotypes into the research methodology are needed to fully understand these relationships [25-27]. Furthermore, the assessment of how DPSC are amplified, characterized and the methods used to ultimately cryopreserve these cells for future use are becoming increasingly important to our understanding of their full potential [28,29].

Based upon this information, the primary objective of this study was to determine the effects of long-term cryopreservation on DPSC viability and pluripotency over time intervals up to ten years. In addition, pluripotent MSC markers previously identified, such as OCT4, NANOG, Sox-2, and Nestin, which may relate to survival and viability were also evaluated. Finally, newly discovered non-coding microRNA has also been shown to both promote and inhibit pluripotency, such as miR-21, 133, and 135 were also included in the current study.

Materials and Methods

Human subjects study approval

This study was a retrospective analysis of dental pulp stem cell (DSPC) lines from an existing repository. The Institutional Review Board (IRB) and Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas (UNLV) approved the current study under Protocol 1717612-1 titled "Retrospective analysis of dental pulp stem cells (DPSC) 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 repository was approved 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 on-going Orthodontic therapy spacing. The exclusion criteria were any patients who declined to participate, any person not a patient of record at UNLV-SDM, and any patient requiring extraction for other reasons (e.g. periodontal disease, fractured tooth, extreme dental decay).

Following extraction, all teeth were subsequently sectioned at the cementum-enamel junction (CEJ) to allow for the extraction of dental pulp using an endodontic broach. Each sample was then placed into sterile microcentrifuge tubes containing phosphate buffered saline (PBS) and then transferred to a biomedical laboratory for further processing. DPSC isolates were obtained using the direct out-growth method in sterile cell culture-treated flasks and a cell culture incubator using Roswell Park Memorial Institute (RPMI) media with 10% fetal bovine serum (FBS) and 1% penicillin - streptomycin antibiotic solution added. DPSC isolates were then grown for a minimum of ten passages prior to cryopreservation.

DPSC lines

A series of Dental Pulp Stem Cell (DPSC) isolates had been previously cryopreserved at -80˚C in a deep freezer. Sixteen DPSC isolates were thawed and cultured in a RPMI growth media containing 1% penicillin-streptomycin and 10% fetal bovine serum. The cells were incubated at 37˚C with 5% CO2 inside a level 2 humidified biosafety incubator. Each individual lineage had been categorized as either having a slow doubling time (sDT) of 10 - 14 days, intermediate doubling time (iDT) of 5 - 7 days, or a rapid doubling time (rDT) of 2 - 3 days. These cell lines included N = 6 rDT lines (dpsc-3882, dpsc-3924, dpsc-5423, dpsc-5653, dpsc-7089, dpsc-9765), N = 3 iDT lines (dpsc-5243, dpsc-8604, dpsc-9894), and N = 7 sDT lines (dpsc-4595, dpsc-8124, dpsc-9500, dpsc-11418, dpsc-11750, dpsc-11836, dpsc-17322).

Viability following cryopreservation

Immediately following cell culture, 500 µL of each solution was removed and placed in an equal amount of Trypan Blue. The combined solution was analyzed for cell viability using a TC 20 automated cell counter (Bio Rad). Values for the total number of cells, the total number of live cells and the percentage of live cells were recorded. The process of obtaining cell viability data was repeated every 3 days.

Cell growth analysis

Following one, two, and three days of incubation, each cell culture was mixed thoroughly before having 0.1 mL of the solution removed and placed into a 96-well plate. The cells were fixed to the bottom of each well by adding an equal volume of formalin in a fume hood for 24 hours. The formalin was then aspirated out and the cells were dyed with 100 µL of Gentian Violet from Fisher Scientific (Fair Lawn, NJ). The cells were processed for 10 minutes before the dye was aspirated. Excess dye was washed off by applying 100 µL of 1X Phosphate-buffered saline (PBS). Each well was aspirated and washed again one to two more times until all of the residual dye had been removed. Once the final amount of 1X PBS was removed, the plate was placed into an ELx808 microplate reader from Eppendorf (Hamburg, Germany) and analyzed using a wavelength of 630 nm. Data was acquired for the absorbance, which correlates with the number of cells and can be used to determine growth and proliferation.

RNA extraction

A phenol: chloroform extraction method was used to extract RNA from each stem cell. Initially, cellular media was removed from the cells by centrifugation and aspiration. TRIzol Reagent from Fisher Scientific (Fair Lawn, NJ) was applied to the remaining cells and pipetted for lysis. The lysate transferred to a sterile microfuge tube. A total of 200 µL of chloroform from Sigma-Aldrich (St. Louis, MO) was added for each 1 mL of TRIzol Reagent in the tubes. Each solution was mixed vigorously before being incubated at room temperature for 3 minutes. The samples were centrifuged at 12,000 x g at 4˚C for 15 minutes. The top aqueous phase of each sample contained the

desired RNA and was removed and combined with an equal volume of isopropanol from Sigma-Aldrich (St. Louis, MO) into a new sterile microfuge tube. Every tube was incubated on ice for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4˚ C. The supernatant layer was removed via aspiration while the remaining pellet was washed with 1 mL of 70% ethanol from Sigma-Aldrich (St. Louis, MO) per 1 mL of Trizol Reagent that was used earlier. Following centrifugation at 7500 x g for 5 minutes at 4˚ C, the supernatant was aspirated and the remaining pellet was resuspended in 100 µL of nuclease-free water. A nanodrop 2000 spectrophotometer from Fisher Scientific (Fair Lawn, NJ) was used to assess the concentration and purity of the RNA. Each tube was then stored at -20˚ C.

cDNA synthesis

In order to perform qPCR, the previously extracted RNA was converted into cDNA using an ABgene Reverse-iT One-Step RT-PCR kit from Fisher Scientific (Fair Lawn, NJ) and a Mastercycler gradient thermal cycler from Eppendorf (Hamburg, Germany). Each PCR tube contained 12.5 µL of 2X Reddy Mix (RT-PCR Master mix), 1.0 µg of DPSC RNA, 1.0 µL of both forward and reverse random primers from Invitrogen (Waltham, MA), 1.0 µL of an RTase blend, and enough nuclease-free water to bring each tube to the same volume. The thermocycler settings included 47˚ C for 30 minutes for reverse transcription and a final extension at 72˚ C for five minutes.

qPCR biomarker screening

Screening of total RNA converted into cDNA was performed using SYBR Green Real-Time PCR Master Mix from ThermoFisher Scientific (Fair Lawn, NJ) following the manufacturer recommended protocol. Briefly, each reaction included 12.5 ufL of 2X SYBR Green PCR Master Mix, 1.5 uL of forward and reverse primers specific for each target, 1.0 uL of cDNA (diluted to 10 ng/uL) and 8.5 uL of nuclease-free water. Real-time PCR was performed using QuantStudio 5 Real-Time PCR system from Fisher Scientific (Fair Lawn, NJ) using settings of 95˚C for ten minutes for initial activation, 40 cycles of denaturation at 95˚C for 15 seconds, with annealing and extension at the appropriate primer pair-specific temperature for one minute. Primers were synthesized by SeqWright from ThermoFisher Scientific (Fair Lawn, NJ):

GAPDH

GAPDH forward: 5′-ATC TTC CAG GAG CGA GAT CC-3′; 20 nt, 55% GC, Tm 66°C

GAPDH reverse: 5′-ACC ACT GAC ACG TTG GCA GT-3′; 20 nt, 55% GC, Tm 70°C

Optimal Tm: 61°C

Beta actin

Beta actin forward, 5′-GTG GGG TCC TGT GGT GTG-3′; 18 nt, 67% GC, Tm: 69°C

Beta actin reverse, 5′-GAA GGG GAC AGG CAG TGA-3′, 18 nt, 61% GC, Tm: 67°C

Optimal Tm: 61°C

miR-21

miR-21 forward: 5’-GCC ACC ACA CCA GCT AAT TT-3’; 20 nt; 50% GC, Tm: 66°C

miR-21 reverse: 5’-CTG AAG TCG CCA TGC AGA TA-3’; 20 nt; 50% GC; Tm: 65°C

Optimal Tm (PCR): 60°C

miR-133

miR-133 forward: 5'-CCG GTT AAC TCG AGC TCT GTG AGA G-3'; 25 nt, 56% GC Tm: 71°C

miR-133 reverse: 5'-CTA GCT AGG AAT TCT GTG ACC TGT G-'3'; 25 nt, 48% GC, Tm: 66°C

Optimal Tm (PCR): 60°C

miR135

miR-135 forward: 5'-CGA TAT GGC TTT TTA TTC CTA -3'; 21 nt, 33% GC, Tm: 56°C

miR-135 reverse: 5'-GAG CAG GGT CCG AGG T -3'; 16 nt, 69% GC, Tm: 67°C

Optimal Tm (PCR): 51°C

Sox2 forward: 5′-ATGGGCTCTGTGGTCAAGTC-3′; 20 nt: 55% GC; Tm 67 °C

Sox2 reverse: 5′-CCCTCCCAATTCCCTTGTAT-5′; 20 nt; 50% GC; Tm 64 °C

Optimal Tm: 65 °C

Nestin forward, 5'-CGTTGGAAC AGAGGTTGGAG-3'; 20 nt; 55% GC; Tm 66 °C

Nestin reverse, 5'-TCCTGAAAGCTGAGGGAAG-3'; 19 nt; 53% GC; Tm 64 °C

Optimal Tm: 65 °C

NANOG forward: 5′-GCTGAGATGCCTCACACGGAG-3′; 21 nt; 62% GC; Tm 71 °C

NANOG reverse: 5′-TCTGTTTCTTGACTGGGACCTTGTC-3′; 25 nt: 48%GC; Tm 69 °C Optimal Tm: 70 °C

Oct-4 forward: 5′-TGGAGAAGGAGAAGCTGGAGCAAAA-3′; 25 nt: 48% GC; Tm 70 °C

Oct4 reverse: 5′-GGCAGATGGTCGTTTGGCTGAATA-3′; 24 nt; 50% GC; Tm 70 °C

Optimal Tm: 71 °C

Results

Sixteen DPSC isolates were successfully thawed and cultured (Table 1). The doubling times were evaluated and confirmed the previous observations of rapid doubling times or rDT for dpsc-3882 (2.6 days), dpsc-3924 (1.9 days), dpsc-5423 (2.2 days), dpsc-5653 (2.1 days), dpsc-7089 (1.9 days), and dpsc-9765 (2.3 days). In addition, these data confirmed the previous observations of intermediate doubling times or iDT for dpsc-5243 (4.2 days), dpsc-8124 (5.9 days), dpsc-8604 (5.5 days), dpsc-9894 (5.1 days), and dpsc-17322 (6.6 days). These results also confirmed slow doubling times or sDT for dpsc-4595 (11.2 days), dpsc-9500 (10.4 days), dpsc-11418 (10.2 days), dpsc-11750 (13.1 days), and dpsc-11836 (12.9 days). Finally, current viability (2022) was assessed, which ranged from 17.6% (dpsc-11418; 2011) to 49.3% (dpsc-8124; 2014). The average viability of all DPSC isolates was 28.8%, which has decreased significantly -29% since last recorded (2018 DPSC average viability 40.5%).

Line	Doubling Time (days)	Rate	Viability (2022)	Viability (2018)
dpsc-11750 (2011)	13.1 days	slow	37.9%	31%
dpsc-11418 (2011)	10.2 days	slow	17.6%	29%
dpsc-17322 (2011)	6.6 days	intermediate	39.1%	35%
dpsc-11836 (2011)	12.9 days	slow	24.7%	34%
dpsc-3924 (2011)	1.9 days	rapid	21.6%	46%
dpsc-7089 (2012)	1.9 days	rapid	20.33%	57%
dpsc-5653 (2012)	2.1 days	rapid	31%	59%
dpsc-3882 (2012)	2.6 days	rapid	31%	52%
dpsc-9765 (2013)	2.3 days	rapid	25.9%	43%
dpsc-9500 (2013)	10.4 days	slow	20.7%	34%
dpsc-9894 (2013)	5.1 days	intermediate	25.6%	35%
dpsc-8124 (2014)	5.9 days	intermediate	49.3%	39%
dpsc-8604 (2014)	5.5 days	intermediate	28.7%	39%
dpsc-5423 (2014)	2.2 days	rapid	23.1%	31%
dpsc-5243 (2015)	4.2 days	intermediate	33.7%	41%
dpsc-4595 (2015)	11.2 days	slow	30.7%	44%

Table 1: *Dental pulp stem cell (DPSC) doubling time and viability.*

To more easily visualize these data and the wide range of viability observed, the data for each DPSC was plotted and graphed (Figure 1). These data demonstrate the broad range of viabilities observed, from a low of approximately 17.6% (dpsc-11418; 2011) to a high of 49.3% (dpsc-8124; 2014)). The lack of consistent pattern within these data sorted by year might suggest additional variables, such as doubling time (DT) or biomarker expression may also modulate these observations.

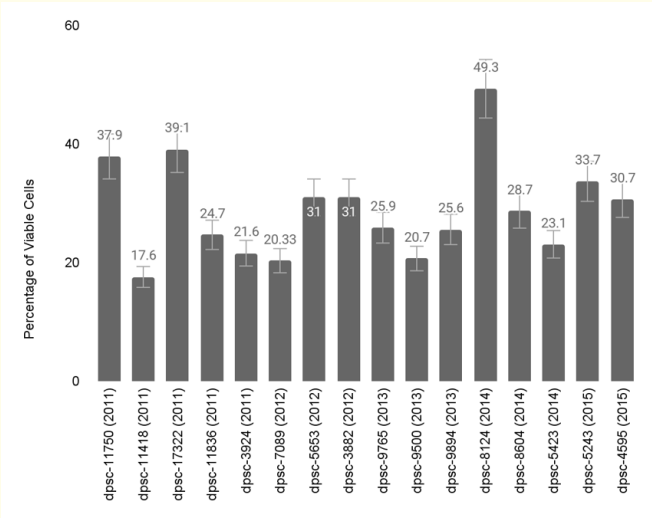

Figure 1: *Average viability of DPSC isolates upon thawing and culture. DPSC isolates sorted by year of isolation and cryopreservation (2011 - 2015) demonstrated a range of viability from a low of 17.6% to a high of 49.3%, with an overall average of 28.8%.*

Due to the heterogeneity of viability results observed with the individual isolates sorted by length of cryopreservation, the data were then sorted by averaging the DPSC isolates from each year to remove the potential for bias resulting from any individual cell line or the associated doubling times (Figure 2). These data demonstrated that the viability of the older DPSC isolates from 2011 - 2013 (24.06% - 28.18%) was somewhat lower than the viability of the more recent isolates from 2014 - 2015 (32.2% - 33.7%). However, due to the large heterogeneity of results within each year, these differences were not statistically significant, p = 0.234.

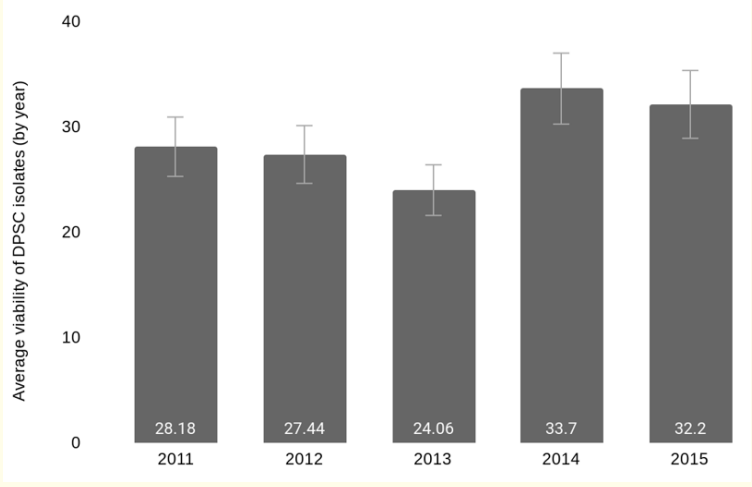

Figure 2: *Combined average viability of DPSC isolates by year. DPSC viability averages combined by year of isolation and cryopreservation (2011 - 2015) demonstrated lower viability among older isolates (2011 - 2013) than more recent isolates (2014 - 2015), but were not significantly different, p = 0.234.*

Based upon the lack of strong, direct association between length of cryopreservation and DPSC viability, these data were sorted and graphed to evaluate if DPSC doubling times were the most significant factor determining cellular viability (Figure 3). These results demonstrated that although the viability of rDT (average 25.5%) was slightly lower than that of the iDT (average 31.7%), this was not statistically significant, p = 0.081. In addition, the differences between iDT and sDT (26.3%) were also not statistically significant, p = 0.145.

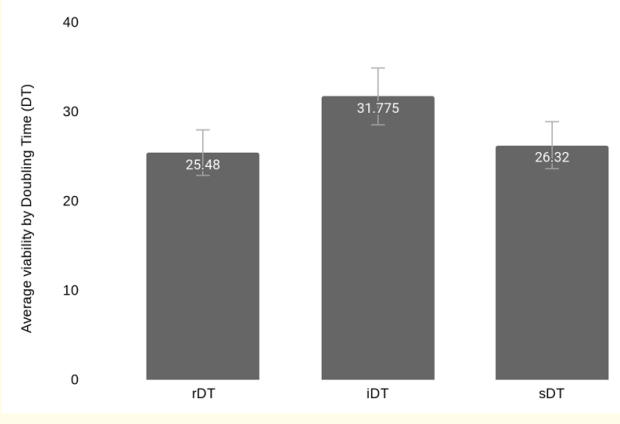

Figure 3: *Combined average viability of DPSC by doubling time (DT). The average viability of rDT (25.5%) was lower than the iDT average (31.7%), but was not statistically significant, p=0.081. Average viability between iDT and sDT (26.3%) was also not statistically significant, p=0.145.*

To evaluate whether these observations may be associated with a combination of both doubling time (DT) and length of cryopreservation, viability of the DPSC isolates were sorted by both year of cryopreservation and growth rate (Figure 4). These data also demonstrated significant levels of heterogeneity among samples from the same year, with no specific association between doubling time and viability within each year. For example, the rDT and sDT isolates from 2011 had similar viabilities (rDT, 21.6% and sDT, 24.7% and 17.6%), which were lower than the iDT from the same year (37.9% and 39.1%). However this pattern was not observed in other years, such as 2013 (rDT, 25.9%; iDT 25.6%, sDT, 20.7%). This analysis may suggest that additional factors may be responsible for the differences in viability, which may be separate and distinct from growth rate (doubling time) and year of cryopreservation.

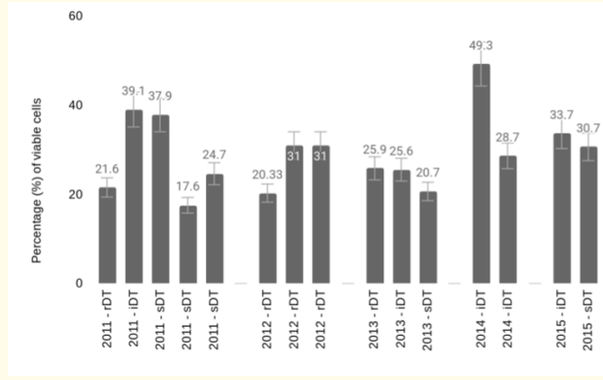

Figure 4: *Viability of DPSC was sorted by doubling time (DT) and year of cryopreservation. This analysis demonstrated significant levels of heterogeneity among samples from the same year, with no specific or direct association between doubling time and viability within each year.*

To evaluate whether viability was associated with biomarker or microRNA expression, RNA was extracted from all DPSC isolates and screened using qPCR (Figure 5). These data demonstrated that all DPSC isolates produce the positive control microRNA, miR-16. In addition, variable expression of miR-21, miR-27, miR-133, miR-135 and miR-218 was observed. Screening for biomarker expression revealed that Nestin was produced by all DPSC isolates, with variable expression observed with NANOG, Oct-4 and Sox-2.

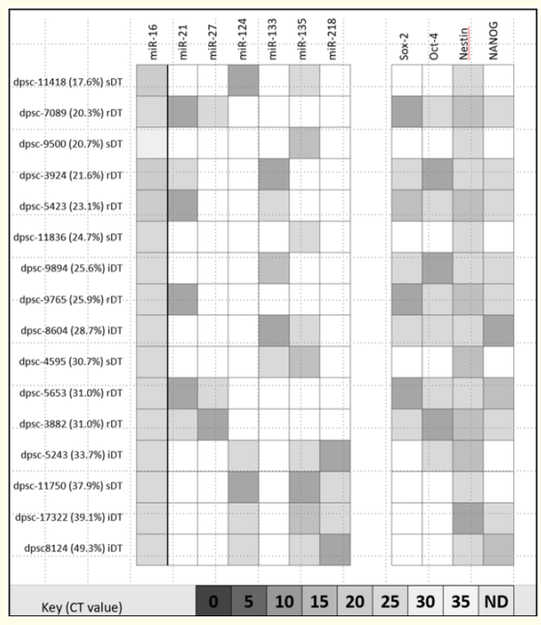

Figure 5: *qPCR screening results. MicroRNA expression was confirmed by positive control miR-16 among all DPSC isolates with differential expression of miR-21, miR-27, miR-133, miR-135 and miR-218. Biomarker expression confirmed Nestin among all DPSC isolates with differential expression of Sox-2, Oct-4 and NANOG.*

To more closely examine these results, the qPCR biomarker screening results were sorted by doubling time (DT) of the DPSC isolates, rDT, iDT and sDT (Table 2). This analysis of data revealed that the rDT DPSC isolates expressed all four biomarkers: Sox-2, Oct-4, Nestin and NANOG. In addition, two of the three iDT DPSC isolates expressed all four biomarkers (dpsc-9894, dpsc-8604) with only dpsc-5423 expressing Oct-4 and Nestin. Furthermore, all sDT DPSC isolates expressed Nestin, similar to the rDT and iDT DPSC isolates. However, none of the sDT DPSC isolates produced Sox-2 or Oct-4, while only dpsc-17322 and dpsc-8124 produced NANOG.

microRNA	rDT (rapid)	iDT (intermediate)	sDT (slow)
Sox-2	dpsc-7089 dpsc-3924 dpsc-5423 dpsc-9765 dpsc-5653 dpsc-3882	dpsc-9894 dpsc-8604	n.d.
Oct-4	dpsc-7089 dpsc-3924 dpsc-5423 dpsc-9765 dpsc-5653 dpsc-3882	dpsc-5423 dpsc-9894 dpsc-8604 dpsc-5423	n.d.
Nestin	dpsc-7089 dpsc-3924 dpsc-5423 dpsc-9765 dpsc-5653 dpsc-3882	dpsc-9894 dpsc-8604 dpsc-5423	dpsc-11418 dpsc-9500 dpsc-11836 dpsc-4595 dpsc-11750 dpsc-17322 dpsc-8124
NANOG	dpsc-7089 dpsc-3924 dpsc-5423 dpsc-9765 dpsc-5653 dpsc-3882	dpsc-9894 dpsc-8604	dpsc-17322 dpsc-8124

Table 2: *DPSC biomarker expression.*

Similarly, the qPCR microRNA screening results were also sorted by doubling time (DT) of the DPSC isolates, rDT, iDT and sDT, to provide more detailed analysis of these results (Table 3). This analysis of data revealed that all of the DPSC isolates expressed the positive control, miR-16. However, miR-21 and miR-27 expression was restricted to only the rDT DPSC isolates, with miR-21 expressed among all rDT DPSC isolates and miR-27 expressed among only dpsc-5653 and dpsc-3882. No expression of miR-21 or miR-27 was detected among any of the iDT or sDT isolates.

microRNA	Rdt (Rapid)	Idt (Intermediate	Sdt (Slow)
miR-16	dpsc-7089 dpsc-3924 dpsc-5423 dpsc-9765 dpsc-5653 dpsc-3882	dpsc-9894 dpsc-8604 dpsc-5423	dpsc-11418 dpsc-9500 dpsc-11836 dpsc-4595 dpsc-11750 dpsc-17322 dpsc-8124
miR-21	dpsc-7089 dpsc-3924 dpsc-5423 dpsc-9765 dpsc-5653 dpsc-3882	n.d.	n.d.
miR-27	dpsc-5653 dpsc-3882	n.d.	n.d.
miR-124	n.d.	dpsc-5423	dpsc-11418 dpsc-11750 dpsc-17322 dpsc-8124
miR-133	dpsc-3924 dpsc-5423	dpsc-9894 dpsc-8604	dpsc-4595
miR-135	n.d.	dpsc-5423 dpsc-8604	dpsc-11418 dpsc-9500 dpsc-11836 dpsc-4595 dpsc-11750 dpsc-17322 dpsc-8124
miR-218	n.d.	dpsc-5423	dpsc-11750 dpsc-17322 dpsc-8124

Table 3: *DPSC microRNA expression profile.*

Expression of miR-124 was differentially expressed among only iDT and sDT isolates, with only dpsc-5423 among the iDT DPSC isolates and dpsc-11418, dpsc-11750, dpsc-17322, and dpsc-8124 among the sDT DPSC isolates. Differential expression of miR-133 was also observed with miR-133 with only some of the rDT (dpsc-3924, dpsc-5423), iDT (dpsc-9894, dpsc-8604), and sDT (dpsc-4595) DPSC isolates. In addition, differential expression of miR-135 was observed with most of the iDT isolates (dpsc-8604, dpsc-5423) and all of the sDT isolates. Finally, differential expression of miR-218 was observed among some of the iDT (dpsc-5423) and sDT (dpsc-11750, dpsc-17322, dpsc-8124) isolates. No expression of miR-124, miR-135 or miR-218 was observed among the rDT DPSC isolates.

Discussion

The primary goal of this study was to determine the effects of long-term cryopreservation on DPSC viability and pluripotency over time intervals up to ten years. In addition, pluripotent MSC markers and newly discovered non-coding microRNA were also screened in the current study to determine if expression of any of these were associated with DPSC viability over time. The results of this study demonstrated that although viability does vary with length of cryopreservation, this was not a strictly linear relationship between these variables, which confirms previous observations from this group [18-21]. Interestingly, some of the more recent isolates exhibited lower viability, while some of the oldest isolates exhibited high viability - which also supports other observations of differential viability - although most of the time frames observed in these studies were significantly less than those in the current analysis [13,15,17,30,31].

Although the relationship between doubling time (DT) and multipotency has been observed in other studies [20,21,25,26], this did not appear to be significantly associated with viability over time - an observation that has been made previously but without sufficient explanation for the mechanisms responsible [18,19,32,33]. This study also analyzed several biomarkers and specific microRNAs which may provide some functional information about growth and proliferation, as well as survival and viability similar to other research studies in this area [34,35]. More specifically this study found that the DPSC isolates with the highest viability expressed three specific microRNAs, miR-124, miR-135 and miR-218, which was only found among iDT and sDT DPSCs. In addition, while miR-124 and miR-135 were also expressed in DPSC isolates with lower viability - only miR-218 was expressed among the iDT and sDT DPSC with the highest viability.

Differential expression of miR-218 has been observed in other DPSC isolates and has been previously associated with differentiation potential [36-38]. More specifically, in undifferentiated DPSC isolates that express both Oct-4 and NANOG, osteogenic induction reduced miR-218 expression and one of the putative mir-218 transcriptional targets RUNX2 expression [36]. In other studies, long non-coding CCAT1 was sufficient to decrease miR-218 expression and to promote differentiation [38]. This information does not strictly conform with the results of the current study, which demonstrated the miR-218 expressing cells with highest viability (dpsc-11750, dpsc-17322, dpsc-8124) did not express Oct-4 (or Sox-2). This may be attributed to the fact that these DPSC isolates did not express Oct-4 and were therefore functionally distinct from those DPSC populations in previous studies.

In addition, this study also found differential expression of miR-124 and miR-125 among the high-viability, miR-218 expressing DPSC isolates - although this expression was not exclusive to these DPSCs. Interestingly, miR-124 has also been recently observed in DPSCs but has been more closely associated with neural differentiation following growth factor treatment [39,40]. This may be the first observation of miR-124 expression among non-differentiated DPSCs and may be closely associated with viability if co-expressed with miR-218. Similarly, expression of miR-135 has been observed among other stem cells but has been more closely associated with neuromuscular differentiation studies [41,42]. This may be among the first studies to associate miR-135 expression with miR-124 and miR-218 expression and the observation of increased viability.

Although these results are significant, there are some limitations of this study that should also be considered. First, this is a retrospective study of previously isolated and cryopreserved DPSCs. As with all retrospective studies, the methods of initial isolation and cell culture may have influenced the proliferative potential and viability of these DPSC isolates and must be considered when comparing these results with other studies of this nature [18-21]. In addition, this group was restricted to the availability of DPSC isolates within the existing repository. Future studies may wish to expand this analysis and incorporate DPSC isolates from other institutions and research groups to further expand the inferences that can be drawn from these results. Finally, due to financial constraints only a limited number of microRNAs could be screened and analyzed. Future studies with additional resources might expand the number of microRNAs to include additional targets that might influence the mechanisms associated with viability and survival.

Conclusions

Although many studies have focused on the potential for DPSC use in biomedical and bioengineering applications, few studies have evaluated the long-term effects of cryopreservation or methods for identifying which DPSC isolates may be most likely to survive this procedure. This study is among the first to evaluate DPSC cryopreservation for periods up to ten years, as well as to identify potential biomarkers (such as miR-218) that may identify DPSC isolates with high survival potential.

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Author Contributions

KK and KMH were responsible for the overall project design. PC, KL, MR, AT, and BT were responsible for data generation. KK and MH were responsible for analysis and the writing of this manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest to report.

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