

Dental Pulp Stem Cell Biomarkers for Cellular Viability Following Long-Term Cryopreservation

Research Article

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Abstract

Many studies have demonstrated clinical applications for the use of dental pulp stem cells (DPSC) for the treatment of various conditions, which have driven medical and scientific interest in the collection, isolation and banking of DPSC tissues for research into these potential therapies. Few studies to date have evaluated the viability of DPSC following long-term cryopreservation. Based upon the paucity of information regarding long-term viability and biological markers for DPSC, the current aims of this study were to characterize and evaluate the effects of long-term cryopreservation, as well as to identify biomarkers that may be useful for future potential screening and applications. Using previously collected DPSC isolates, growth and viability over a period of four years were examined, revealing an overall decline in viability at each time point that did not appear to be linear. In addition, the analysis of specific intracellular biomarkers, including Nestin, NANOG, Sox-2 and Oct4 revealed that Oct4 and Sox-2 may be the most important variable factors associated with both DPSC growth rate and viability during cryopreservation. This information may be useful for future applications and therapies that could screen and sort DPSC using predetermined biomarkers to improve both efficiency and feasibility.

Keywords: Biomarkers; Human Dental Pulp-Derived Stem Cells; Cryopreservation.

Introduction

Many recent studies using animal models have demonstrated clinical applications for the use of dental pulp stem cells (DPSC) for the treatment of various conditions, including oral and maxillo-facial reparation, retinal disorders, neuropathies and central nervous system disorders [1-4]. New evidence has elucidated several potential mechanisms for inducing DPSC differentiation prior to implantation or clinical use, including induction into neural, osteogenic and odontoblastic precursors [5-7]. These developments have led to considerable scientific interest in DPSC and their potential to generate novel and innovative treatments for common, as well as intractable, disease states [8-10].

These advances have driven broad medical and scientific interest in the collection, isolation and banking of DPSC tissues for research into these potential therapies [11-13]. For example, stud-

ies from this institution have demonstrated the feasibility and potential for the collection, isolation and *in vitro* mechanisms for culture-induced differentiation and de-differentiation of DPSCs [14-16]. However, despite these achievements, much remains unknown regarding the parameters, including biological characteristics and biomarkers that influence not only differentiation, but long-term viability following extended cryopreservation [17-19].

Although some prior efforts have evaluated the effects of cryopreservation on DPSC, the majority of these studies have evaluated only short-term effects - less than six months [2, 12, 20, 21]. The few studies that have investigated the effects of long-term cryopreservation and storage are providing critical knowledge towards the advancement and ultimate development of DPSC-based therapies [19, 22, 23]. Based upon the paucity of information regarding long-term viability and biological markers for DPSC, the current aims of this study were to characterize and

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evaluate the effects of long-term cryopreservation, as well as to identify biomarkers that may be useful for future potential screening and applications.

Materials and Methods

Human subjects

Original approval for the collection, isolation and storage of dental pulp stem cells (DPSC) from teeth was granted for protocol OPRS#0907-3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp" in February, 2010 [14]. Approval for the current study to analyze retrospectively collected biological specimens was granted for protocol OPRS#763012-1 in August, 2015. In brief, adult patients that were scheduled for an extraction in the clinic were asked to provide Informed Consent in order to participate. The majority of patient participants had healthy, vital intact teeth extracted prior to Orthodontic treatment [24]. Patients having teeth extracted due to injury (fracture) or compromised dental pulp, including pulp infection or disease, were excluded.

Collection and isolation

The original protocol for the collection and isolation of DPSC from vital, intact teeth involved isolation of the dental pulp from the pulp chamber following extraction. In brief, this involved cross sectioning of the tooth at the cemento-enamel junction (CEJ), following by extraction of the dental pulp with an endodontic broach which was then placed into sterile 1.5 microcentrifuge tubes with phosphate buffered saline (PBS) for transfer to the biomedical laboratory for culture.

Culture and Cryopreservation

The original study protocol allowed for the isolation of dental pulp stem cells (DPSC) using the direct outgrowth method [14, 25]. In brief, cells were allowed to grow for ten passages and the rate of growth or doubling time (DT) was evaluated and assessed as the interval between 1:4 passaging and achieving confluence, as previously described [14, 16, 22]. This allowed for the identification of three distinct classes of DPSC, those with rapid doubling times (rDT) less than three days, those with relatively slow doubling times (sDT) of greater than one week (8-10 days), and a smaller subset with intermediate doubling times (iDT). These phenotypes were noted for each isolate prior to cryopreservation at (-80°C) using OptiFreeze Cryopreservation media from Fisher Scientific (Fair Lawn, NJ), as previously described [14-16, 22-24].

Cellular viability

Upon thawing at each time point (1 week, 1 month, 6 months, 12 months, 24 months, 36 months and 48 months), viability was assessed using the Trypan Blue exclusion assay as previously described [22-24]. In brief, thawed cells were centrifuged and resuspended with cell culture media RPMI-1640 with 2 mM L-Glutamine containing 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium Pyruvate, and 1% Penicillin-Streptomycin (10,000 unit/mL). Aliquots of 20uL cell suspension were then mixed with Trypan Blue and placed into hemocytometer counting slides for analysis using a BioRad TC20 automated cell counter (Hercules, CA) using the protocol recommended by

the manufacturer. These data include total cell number, total live cells (used to calculate viability) and percentage of viable cells. Three measurements were taken for each DPSC isolate for statistical analysis and averaging.

RNA isolation and RT-PCR

RNA was isolated from an aliquot of each DPSC isolate using 1.0×10^7 cells at each of the previous time points, including baseline (T0) prior to cryopreservation, and at each of the subsequent one year time points (T1-T4). RNA was isolated using the total RNA isolation reagent (TRIR) from Molecular Research Center, Inc. (Cincinnati, OH) using the protocol recommended by the manufacturer. RNA quality and quantity was assessed using spectrophotometric analysis of each sample at 260 and 280 nm. The ratio of A260:A280 measurements provided a measurement of RNA purity (acceptable range between 1.7 – 2.0) and a general estimate of quantity.

All isolates with sufficient quality (A260:A280 > 1.7) and quantity (> 1 ng/uL) were processed and screened for DPSC biomarker expression as previously described [14-16, 22-24]. Mesenchymal stem cell (MSC) and DPSC biomarkers used in this screening included several previously validated cell surface (CD24, CD44 and CD133) and intracellular markers (Nestin, NANOG, Sox-2, Oct4) [26-28], as well as the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) or GAPDH, as follows:

CD24 FORWARD: ACTCTCACTTGAAATTGGGC;
CD24 REVERSE: GCACATGTTAATTACTAGTAAAGG;

CD44 forward primer, 5'-GAAAGGCATCTTATGGATGTGC-3'
CD44 reverse primer, 5'-CTGTAGTGAAACACAACACC-3'

CD133 forward primer, 5'-CTCATGCTTGAGAGATCAGGC-3'
CD133 reverse primer, 5'-CGTTGAGGAAGATGTGCACC-3'

Nestin FORWARD: CGTTGGAACAGAGGTTGGAG;
Nestin REVERSE: TCCTGAAAGCTGAGGGAAG;

NANOG forward primer, 5'-GCTGAGATGCCTCACACGGAG-3'
NANOG reverse primer, 5'-TCTGTTTCTTGACTGGGACCTTGTC-3'

Oct4 forward primer, 5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3'
Oct4 reverse primer, 5'-GGCAGATGGTCGTTTGGCTGAA-TA-3'

Sox2 forward primer, 5'-ATGGGCTCTGTGGTCAAGTC-3'
Sox2 reverse primer, 5'-CCCTCCCAATCCCTTGTAT-5'

GAPDH FORWARD: ATCTTCCAGGAGCGAGATCC;
GAPDH REVERSE: ACCACTGACACGTTGGCAGT

In brief, all reactions were standardized using 1ng/uL of extracted RNA and then processed using ABgene Reverse-iT One-Step RT-PCR protocol and reagents, as previously described [22-24]. Per standard procedures, reverse transcript was performed for 30

minutes at 47°C and then 30 amplification cycles were run, which included denaturation of 20 seconds, annealing of 30 seconds at the optimal temperature for each primer set, and five minutes of final extension at 72°C. Results were visualized using gel electrophoresis and ethidium bromide in a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Rochester, NY).

Statistics

Basic descriptive statistics for viability were derived from the viability averages and reported in tables. DPSC from different categories of growth rates (rDT, iDT, sDT) were aggregated to create overall averages for these groups. Differences in viability at all time points between DPSC-rDT, -iDT, and -sDT were evaluated using two-tailed t-tests, which provide robust analysis even for samples with moderate sizes ($n \sim 20$) [29, 30].

Results

All DPSC were cultured for a minimum of ten passages to establish their growth rate, which varied within the range of 2.0 and 10.3 days. The doubling times were then used to group the DPSC into rapid doubling times (rDT < 3 days), intermediate doubling times (4-6 days) or comparatively slow doubling times (sDT > 8-10 days) - as previously established (Tomlin, 2016). Baseline viability was measured prior to the initial storage and cryopreservation following the initial ten passages. An aliquot from each DPSC line was retrieved from cryostorage at each of four time intervals and placed into cell culture (Figure 1). The analysis of cellular viability at each of the four time points (12 months - 48 months, T1 - T4) revealed an inverse relationship between the duration of DPSC cryopreservation and cellular viability upon thawing.

More specifically, DPSC with a rapid doubling time (rDT) exhibited an average decrease in cellular viability of -7.1% following 12 months in cryostorage, while DPSC with an intermediate doubling time (iDT) decreased an average of 6% over this time interval. DPSC with the slowest doubling time (sDT) exhibited the greatest decrease at this initial time point of -26.7%, which was statistically significant ($p < 0.01$). At each successive time point (T2-T4) all DPSC isolates exhibited decreasing viability, with the most significant declines observed between T1 and T2 - while the smallest occurred between T3 and T4.

At each time point and determination of cellular viability, mRNA expression was assessed for multiple specific DPSC biomarkers. Some DPSC biomarkers examined (ABCG, CD24, CD44, CD133) were not included in this analysis as they exhibited no differences in mRNA expression (data not shown) (Figure 2). Intracellular mesenchymal stem cell markers Oct-4, Sox-2, NANOG and Nestin did exhibit differences in mRNA expression and were examined (Figure 2A). This analysis revealed differential expression of mRNA among the three groups DPSC-rDT cell lines. For example, although all three groups were observed to express mRNA for Nestin and NANOG, only one DPSC-rDT expressed both Sox-2 and Oct4. The remaining DPSC-rDT exhibited differential expression of either Oct4 or Sox-2 but not both (Figure 2B). Both of the DPSC-iDT exhibited similar mRNA expression profiles, which included Nestin, NANOG and Sox-2 but not Oct4. However, all of the DPSC-sDT exhibited similar expression of Nestin and, to a limited extent, NANOG.

Due to the resulting overlap in the DPSC biomarker expression profiles of the DPSC-iDT and one of the rDT isolates, viability of each DPSC isolate was further evaluated based upon the individual expression profile result (Figure 3). Disaggregating the

Figure 1. Effects of cryopreservation on DPSC viability. Initial viability for DPSC isolates with slow, intermediate and rapid doubling times (sDT, iDT, rDT) was compared with results following cryostorage after 12 months (T1), 24 months (T2), 36 months (T3) and 48 months (T4). This revealed overall decreased viability, which varied by DPSC type. DPSC-rDT exhibited the least reduction in viability (-24%), while DPSC-sDT exhibited the greatest reduction (-51%) ($p < 0.01$).

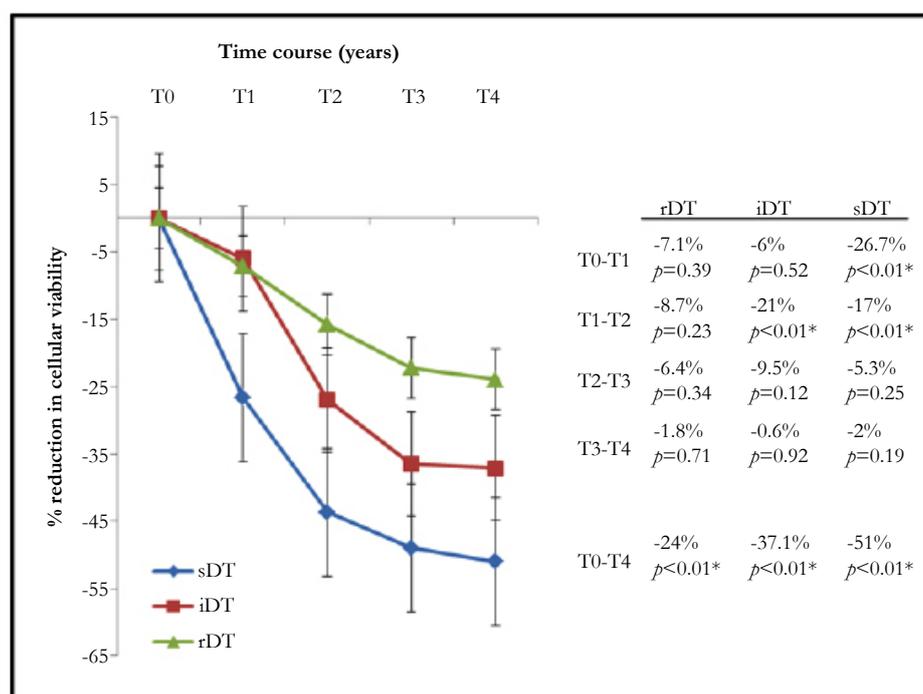


Figure 2. DPSC biomarker expression. A) Total RNA isolated from each DPSC line (rDT, iDT, sDT) was screened for expression of mRNA specific for DPSC biomarkers Oct4, Sox-2, NANOG, and Nestin – revealing differential expression among the rDT isolates. Differential expression was observed between sDT (Sox-2-, Oct4-), iDT (Oct4-) and rDT isolates. B) mRNA expression profiles were created using these biomarkers, revealing distinct patterns specific to sDT and iDT, which may overlap with one of the rDT isolates.

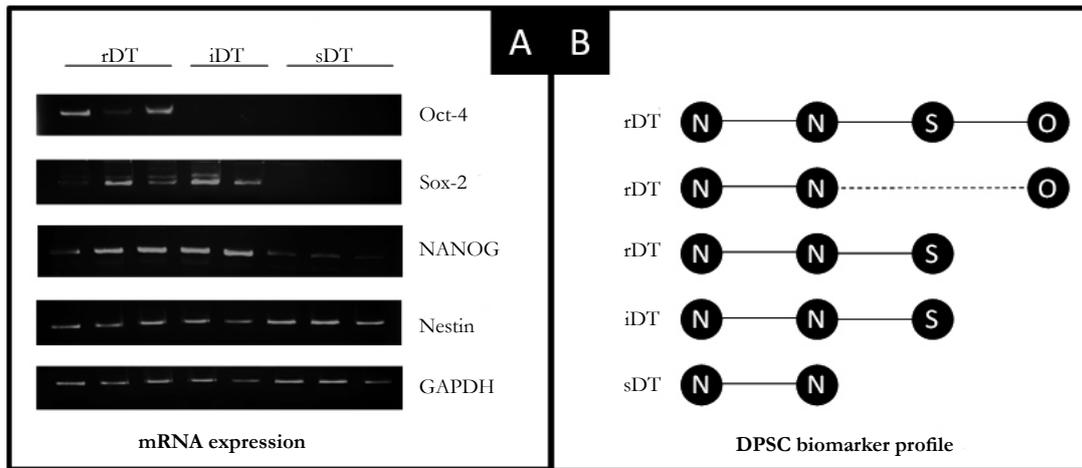
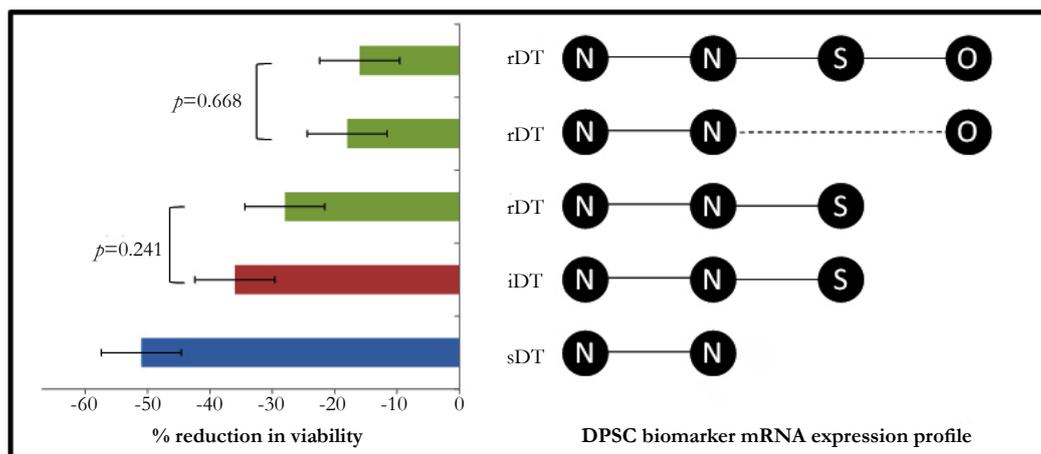


Figure 3. Analysis of cellular viability by DPSC expression profile. Using the biomarker expression profile, viability for DPSC-rDT isolates with differential expression (N-N-S-O, N-N-O, N-N-S) was compared with viability for DPSC-iDT isolates (N-N-S) revealing differential viability. Both DPSC-rDT isolates expressing Oct4 exhibited similar and smaller reductions in overall viability ($p = 0.668$), while rDT and iDT with similar profiles exhibited similar, but greater reductions in viability ($p = 0.241$).



rDT isolates in this analysis revealed that the rDT isolates expressing Nestin (N), NANOG (N), Sox-2 (S) and Oct4 (O) (N-N-S-O) were virtually indistinguishable from the rDT isolates that expressed Nestin, NANOG and Oct4, but not Sox-2 (N-N-O) ($p = 0.668$). In addition, the overall reduction in viability for the rDT isolates that expressed Oct4 (regardless of Sox-2) expression was significantly lower than the reductions in viability among the rDT isolates that expressed Nestin, NANOG, and Sox-2 (N-N-S) but not Oct4.

Analysis of viability from the DPSC-rDT and iDT isolates with similar biomarkers expression profiles of Nestin, NANOG and Sox-2 (N-N-S) revealed similar reductions in viability at most time points, but were statistically indistinguishable from one another ($p=0.241$). Finally, the analysis of DPSC-sDT isolates, which only expressed Nestin and NANOG (N-N) revealed the greatest reduction in cellular viability at each interim time and the largest reduction overall between T0 and T4. These findings were sig-

nificantly different from those of the DPSC-iDT and DPSC-rDT isolates evaluated.

Discussion

This study is among the first to provide evidence that long-term cryopreservation has significant effects on the viability of DPSC [22]. It is important to note that although previous studies have evaluated some of the biological effects of cryopreservation on DPSC, most evaluated these effects after a period of six months or less [2, 12, 21]. If clinical and therapeutic applications are to be a viable option for patients, more studies regarding the basic biology and feasibility of storage and cryopreservation will be needed to further elucidate the parameters that govern these observations and findings.

More importantly, this study may be the first to provide evidence that the reduced viability and long-term effects of cryopreserva-

tion may not be strictly dose-dependent. For example, although some studies evaluated and analyzed viability and growth following a short time interval (usually one to two weeks) compared with a longer time interval, such as six months [2, 21], this study may represent the first evidence to demonstrate that the declines in viability appear to be most striking within the first two years, with smaller changes observed in following years and almost no change in viability between years three and four - regardless of DPSC phenotype (sDT, iDT, rDT). Moreover, the magnitude of these changes in viability appeared to correlate with cellular phenotype or growth rate - the more rapidly growing DPSC-rDT exhibiting the smallest reduction in viability at all time points and the slowest growth DPSC-sDT exhibiting the largest overall reduction.

To more fully examine these observations, the evaluation of biomarkers from each DPSC isolate revealed similar expression of cell surface markers (CD24, CD44, CD133) but striking differential expression of key intracellular biomarkers (NANOG, Sox-2, Oct4) [22, 27, 28]. For example, although all sDT and iDT isolates had similar expression profiles to one another (N-N and N-N-S, respectively), the three rDT isolates exhibited differential expression (N-N-S-O, N-N-O, N-N-S). Interestingly, when the viability of each individual isolate was analyzed independently, this revealed that the rDT and iDT isolates with similar biomarker profiles (N-N-S) had similar viability following cryopreservation, which was lower and distinct from the rDT that also expressed Oct4 (N-N-S-O, N-N-O). This may suggest that Oct4 but not Sox-2, both associated with pluripotency in mesenchymal and dental pulp stem cells, may also be associated with (or an indicator of) one or more biological pathways involved in the regulation of cellular viability [27, 28].

Despite the significance of these findings, it is important to note that there are several limitations which must also be considered. First, this is a retrospective examination of previously collected DPSC isolates - therefore, the initial conditions of isolation, culture and storage were outside the parameters of this study and could not be subjected to change or experimentation. Also, this study was conducted using patients from a public University-based dental school patient population, which may be significantly different from the traditional orthodontic patient populations seeking treatment and potential DPSC cryopreservation [23]. Finally, differing methods or materials for cryopreservation were not studied - which may have influenced the outcomes observed in this study.

Conclusions

This study is among the first to provide evidence that long-term storage and cryopreservation of DPSC varies non-linear over time. This study is also among the first to provide evidence that phenotypic behaviors, such as doubling time or growth, may be one of the most important factors that determines long-term DPSC viability. Finally, this study also revealed that Oct4 and Sox-2 are among the most important variable factors that are associated with both growth and viability, which may be useful for future applications and therapies that could screen and sort DPSC using predetermined biomarkers.

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