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Expression of microRNA miR-27, miR-124 and miR-218 Among Dental Pulp Stem Cell (DPSC) Isolates

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Abstract: Dental Pulp Stem Cells (DPSCs) are non-embryonic, mesenchymal stem cells that may have significant potential for therapeutic and regenerative biomedical applications. MicroRNAs are small non-coding RNA molecules that can act as transcriptional activators and repressors in many types of mesenchymal stem cells. To date, few studies have evaluated the expression or activity of microRNAs among dental pulp stem cells. Using eight previously isolated and characterized DPSC lines, RNA was extracted and examined using PCR to determine expression of several key miRNAs, including miR-16, miR-27, miR-124, miR-135, miR-143 and miR-218. These data demonstrated that at least four of these microRNAs are active among some of these DPSC isolates, including miR-16, miR-27, miR-124 and miR-218. Although the transcriptional targets of these miRNAs are not yet known, it is evident that the differential expression of some of these miRNAs (miR-27, miR-124, miR-218) may correlate (or even contribute) to differentiation status of these isolates. More research will be needed to determine the precise function and targets of these microRNAs to determine their effects on DPSC differentiation, which may foster biotechnology applications for DPSC bioengineering applications.

Keywords: Dental Pulp Stem Cell (DPSC), microRNA, Cellular Differentiation, Bioengineering

Introduction

Dental Pulp Stem Cells (DPSCs) are non-embryonic, mesenchymal stem cells that may have significant potential for therapeutic and regenerative biomedical applications (Hollands *et al*., 2018; Ledesma-Martínez *et al*., 2016; Kabir *et al*., 2014). Many of these research studies regarding these potential treatments have focused on dental and oral tissues (Hernández-Monjaraz *et al*., 2018; Aurrekoetxea *et al*., 2015; Duncan *et al*., 2016). However, other research has suggested that DPSC may be useful to biomedical engineering and tissue regeneration efforts for tissues outside of the oral cavity or head and neck tissues (Daltoé *et al*., 2014; Collart-Dutilleul *et al*., 2015; Mead *et al*., 2017; Victor and Reiter, 2017).

Although much has been discovered regarding the regenerative potential of DPSC, many facets of DPSC isolation and differentiation have yet to be elucidated (Zainuri *et al*., 2018; Bakopoulou *et al*., 2017; Bakkar *et al*., 2017). For example, some evidence may suggest the method of isolation may influence the stem cell properties and alter the differentiation potential of DPSC isolates (Hilkens *et al*., 2013; Karamzadeh *et al*., 2012; Rodríguez-Lozano *et al*., 2012). However, due to the recent discovery of DPSCs and their regenerative potential, much remains to be discovered regarding the mechanisms that may control differentiation, such as epigenetic regulation-which have been more extensively studied in other types of Mesenchymal Stem Cells (MSC) (Saidi *et al*., 2017; Ozkul and Galderisi, 2016; Deng *et al*., 2015).

MicroRNAs are small non-coding RNA molecules that can act as transcriptional activators and repressors in many types of mesenchymal stem cells (Katsuda and

Ochiya, 2015; Utikal *et al*., 2015; Huang *et al*., 2016; Martin *et al*., 2016) Some microRNAs (miR), such as miR-21 and miR-16 appear to be significant biomarkers and modulators of MSC potential and differentiation (Sekar *et al*., 2015; Clark *et al*., 2014; Fakhry *et al*., 2013). To date, few studies have evaluated the expression or activity of microRNAs among dental pulp stem cells (Tu *et al*., 2018; Sun *et al*., 2017; Li *et al*., 2015).

Based upon the paucity of evidence regarding miRNA expression among DPSC, the primary goal of this study was to evaluate expression of several key miRNAs, including miR-16, miR-27, miR-124, miR-135, miR-143 and miR-218.

Methods

Study Approval

The review and approval for this project was facilitated by the Office for the Protection of Research Subjects (OPRS) and Institutional Review Board (IRB) OPRS#763012-1 "Retrospective analysis of Dental Pulp Stem Cells (DPSC) from the University of Nevada Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and adult clinical population". The original isolation and collection of the DPSC samples was approved by the IRB and OPRS under protocol OPRS#0907-3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp".

In brief, adult patients that were scheduled for an extraction in the clinic prior to Orthodontic treatment (mainly for spacing issues) were asked to provide Informed Consent in order to participate. Any patients having teeth extracted due to other reasons, which included injury (fracture) or compromised dental pulp, pulp infection or disease, were excluded from participation.

DPSC Isolation

The original isolation of the dental pulp from the pulp chamber following extraction involved cross sectioning of the extracted tooth (pre-molar or third molar) at the Cemento-Enamel Junction (CEJ), following by extraction of the dental pulp with an endodontic broach, that was subsequently placed into a sterile microcentrifuge tube containing Phosphate Buffered Saline (PBS) for transfer to the biomedical laboratory (Alleman *et al*., 2013; Hung *et al*., 2013).

In brief, each DPSC isolate was allowed to grow for ten passages using the direct outgrowth method and the rate of growth or Doubling Time (DT) was evaluated and assessed as the interval between 1:4 pass aging and achieving confluence. The analysis of DPSC isolate growth allowed for the identification of three distinct rates of DPSC growth, rapid Doubling Times (rDT) less than three days, slow Doubling Times (sDT) of greater than one week (8-10 days) and a smaller subset with intermediate Doubling Times (iDT) (Young and Kingsley, 2015; Tomlin *et al*., 2016). Each DPSC isolate was then cryopreserved at -80C for future analysis and experimentation.

RNA Isolation

For the current project, DPSC isolates were removed from storage and RNA was extracted from an aliquot of each DPSC isolate using 1.0×10^7 cells using the total RNA isolation reagent (TRIR) from Molecular Research Center, Inc. (Cincinnati, OH) using the protocol recommended by the manufacturer. The quantification of RNA concentration and purity was then assessed using spectrophotometric analysis of each sample at 260 and 280 nm. The ratio of A260:A280 measurements provide 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 n/guL) were processed and screened for microRNA biomarker expression as previously described (Petersen and Kingsley, 2016; Brennan *et al*., 2018). Mesenchymal Stem Cell (MSC) biomarkers used in this screening included several previously validatedmiR-27, miR-124, miR-135, miR-143 and miR-218, as well as the internal validation control, miR-16, as follows:

miR-16 forward: 5'-TAGCAGCACGTAAATATTGGCG-3'; (22 nt) Tm: 60.8°C miR-16 reverse: 5'-TGCGTGTCGTGGAGTC-3'; (16 nt) Tm: 59.3°C Optimal Tm (PCR): 54.3°C

miR-27 forward: 5'-ATATGAGAAAAGAGCTTCCCTGTG-3'; (24 nt) Tm: 61.2°C miR-27 reverse: 5'-CAAGGCCAGAGGAGGTGAG'3'; (19 nt) Tm: 64.5°C
Optimal Tm (PCR): 56.2°C

miR-124- forward: 5'-ATGAATTCTCGCCAGCTTTTTCTT-3'; (24 nt) Tm: 59.4°C miR-124 reverse: 5'-ATGAATTCATTTGCATCTGCACAAACCC-3'; (28 nt) Tm:63.2°C
Optimal Tm (PCR): 54.4°C

miR-135 forward: 5'-CGATATGGCTTTTTATTCCTA -3'; (21 nt) Tm: 54.8°C
miR-135 reverse: 5'-GAGCAGGGTCCGAGGT -3'; (16 nt) Tm: 61.8°C
Optimal Tm (PCR): 49.8°C
miR-143 forward: 5'-AGTGCGTGTCGTGGAGTC-3'; (18 nt) Tm: 59.6°C
miR-143 reverse: 5'-GCCTGAGATGAAGCACTGT-3'; (19 nt) Tm:70.7°C
Optimal Tm (PCR): 54.6°C

miR-218 forward: 5'-TCG GGC TTG TGC TTG ATC T-3'; (19 nt) Tm: 67°C
miR-218 reverse: 5'-GTG CAG GGT CCG AGT G-3'' (16 nt) 66°C
Optimal Tm (PCR): 61°C

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Results

To screen for the expression of specific non-coding RNA, total RNA was isolated from each DPSC and quantified to determine any differences in total RNA expression among the DPSC isolates (Table 1). These data revealed that total RNA obtained from DPSC isolates with rapid, slow and intermediate doubling times (rDT, iDT, sDT) were similar, p = 0.9646. More specifically, the average total RNA isolated from each type of DPSC isolate was not significantly different – although two isolates (dpsc-5653 rDT, dpsc-11418 sDT) had total RNA extraction values that were slightly lower than the majority of DPSC isolates.

Following the successful isolation of RNA from all DPSC isolates with sufficient concentration for analysis, an assessment of the RNA quality was performed to determine the suitability of this RNA for subsequent PCR screening (Table 2). These data revealed the RNA purity (measured by the ratio of absorbance readings at 260 and 280 nm) was sufficient for PCR screening and analysis (A260:A280>1.65), with no significant differences observed between rDT, iDT and sDT averages, p = 0.4849. Only one DPSC isolate (dpsc-3882 rDT) was found to be slightly below the commonly accepted average purity standard (A260:A280 = 1.54).

Following the characterization of RNA obtained from each DPSC isolate, RT-PCR was utilized to screen the RNA for expression of non-coding microRNA (Fig. 1). These data revealed that all DPSC isolates expressed miR-16 (positive control), as expected. Screening for the additional microRNAs revealed differential expression of miR-27, miR-124 and miR-218. More specifically, miR-27 expression was observed among the rDT but not the iDT or SDTDPSC isolates. In contrast, miR-124 expression was observed only among the sDT but not the rDT or iDT DPSC isolates. However, miR-218 was expressed among all the sDT and one of the rDT DPSC isolates but not among the iDT isolates. No expression of miR-135 or miR-143 was observed among any DPSC isolate screened.

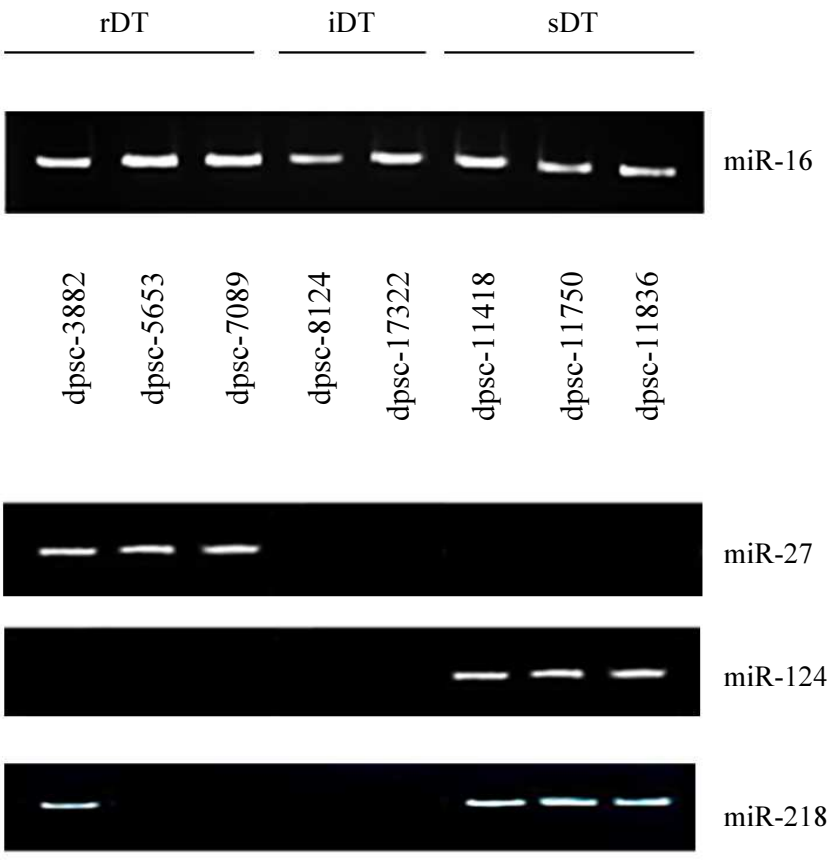

Fig. 1: Expression of miRNA among DPSC isolates. Screening for miRNA among eight DPSC isolates revealed expression of miR-16 (positive control) among all DPSC isolates and differential expression of miR-27, miR-124 and miR-218. No expression was observed for miR-135 or miR-143 (data not shown)

Table 1: RNA isolation and concentration from DPSC isolates

DPSC isolate	RNA quantification	Statistical analysis
rDT	912.8 ng/uL +/- 22.5	$\chi 2 = 0.072$
iDT	929.8 ng/uL +/- 5.9	d.f. = 2
sDT	913.5 ng/uL +/- 36.1	p=0.9646
dpsc-3882 (rDT)	921.1 ng/uL	
dpsc-5653 (rDT)	887.3 ng/uL	
dpsc-7089 (rDT)	930.1 ng/uL	
dpsc-8124 (iDT)	925.6 ng/uL	
dpsc-17322 (iDT)	933.9 ng/uL	
dpsc-11418 (sDT)	879.4 ng/uL	
dpsc-11750 (sDT)	910.0 ng/uL	
dpsc-11836 (sDT)	951.2 ng/uL	
	Range: 879.4-951.2 ng/uL	
	Average: 917.3 ng/uL	

Table 2: RNA purity from DPSC isolates.

DPSC isolate	RNA purity (A260:A280)	Statistical analysis
rDT	1.67	$\chi 2 = 1.447$
iDT	1.77	d.f. = 2
sDT	1.84	p = 0.4849
dpsc-3882 (rDT)	1.54	
dpsc-5653 (rDT)	1.77	
dpsc-7089 (rDT)	1.72	
dpsc-8124 (iDT)	1.65	
dpsc-17322 (iDT)	1.89	
dpsc-11418 (sDT)	1.91	
dpsc-11750 (sDT)	1.83	
dpsc-11836 (sDT)	1.79	
	Range: 1.54-1.91	
	Average: 1.76	

Discussion

Based upon the paucity of evidence regarding miRNA expression among DPSC, the primary goal of this study was to evaluate expression of several key miRNAs, including miR-16, miR-27, miR-124, miR-135, miR-143 and miR-218. The results of this pilot study have revealed that RNA could be successfully isolated and screened for microRNA expression among all the DPSC isolates. Furthermore, although the expression of the positive control microRNA (miR-16) was observed among all DPSC isolates, differential or lack of expression was observed among each of the remaining microRNAs (Yu *et al*., 2013; Eguchi *et al*., 2013).

These results are significant as the evidence for microRNA expression among DPSC isolates is in the very early stages of exploration and few studies to date have evaluated this phenomenon (Tu *et al*., 2018; Sun *et al*., 2017; Li *et al*., 2015). This study screened for miR-143 and miR-135 expression, which was demonstrated to function in the pathway for myogenic differentiation of DPSC (Li *et al*., 2015), although virtually no information is currently available about the normal function and expression of these microRNA among non-differentiated DPSC.

The results of this study greatly expand the range of microRNA expression profiles among DPSC to include several key regulators of MSC activity, such as miR-218 which is known to regulate proliferation and stem cell activity through the TOB1 (transducer of ERBB2, 1) pathway (Gao *et al*., 2016). In addition, the role of miR-124 which may function to modulate the Wnt/beta-catening pathway and MSC chemotaxis – although no study has yet confirmed the expression of miR-218 in DPSC (Yue *et al*., 2016; Laine *et al*., 2012). Finally, this study may be the first evidence of the expression of miR-27 among DPSC, which has been identified as a critical microRNA modulating the tolerogenic response of adipose-derived MSCs (Chen *et al*., 2013).

Despite the significance of these findings, some limitations must also be discussed. For example, this study represents a small number of individual DPSC isolates and may therefore not be representative of all DPSC isolates (Alleman *et al*., 2013; Hung *et al*., 2013). In addition, the differential expression of microRNAs in this study may be functionally related to other factors that have not yet been identified – although significant amounts of information and characterization regarding these DPSC has already been identified (Young and Kingsley, 2015; Tomlin *et al*., 2016). Finally, financial

and other temporal constraints limited the number of microRNAs that could be screened which may suggest additional microRNAs that mediate the expression of those newly identified from this study (miR-27, miR-124, miR-218) may be high priorities for future studies of these DPSC isolates.

Conclusion

Although the transcriptional targets of these miRNAs are not yet known, it is evident that the differential expression of some of these miRNAs (miR-27, miR-124, miR-218) may correlate (or even contribute) to differentiation status of these isolates. More research will be needed to determine the precise function and targets of these microRNAs to determine their effects on DPSC differentiation, which may foster biotechnology applications for DPSC bioengineering applications.

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Author's Contributions

All authors equally contributed in this work.

Ethics

The authors declare there are no conflicts of interest and no ethical issues to declare.

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