

5-1-2019

Correlation Between Pluripotent Stem Cell Markers and microRNA Expression in Dental Pulp Stem Cells

Mark Whiting

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<http://dx.doi.org/10.34917/15778569>

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CORRELATION BETWEEN PLURIPOTENT STEM CELL MARKERS AND MICRORNA
EXPRESSION IN DENTAL PULP STEM CELLS

By

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A thesis submitted in partial fulfillment
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Master of Science - Oral Biology

School of Dental Medicine
Division of Health Sciences
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May 2019

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Thesis Approval

The Graduate College
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February 19, 2019

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Correlation between Pluripotent Stem Cell Markers and Microrna Expression in Dental Pulp Stem Cells

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Abstract

**Correlation Between Pluripotent Stem Cell Markers and microRNA
Expression in Dental Pulp Stem Cells**

By

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In human development there are many factors that cause our cells to form certain organs and systems with specific functions. These cells original start as stem cells before biological processes take over and cause differentiation into different cell lines. Since these undifferentiated stem cells have been discovered, many studies have been done to try and find the exact mechanism that causes a cell to change into another specific cell. Due to the multiple sources that stem cells can be harvest from the human body, this knowledge of how differentiation works could lead to breakthroughs in regenerative medicine.

Traditional biomarkers have been the focus of much research in identifying characteristics of stem cells. Biomarkers include Oct-4, Sox-2, NANOG, and Nestin. The

aforementioned biomarkers have been shown to identify stem cells that have pluripotent properties with potential to differentiate.

MicroRNA (miRNA) are small nucleotide chains that have been the topic of research in recent years. These chains have been shown to be involved in affecting gene expression and silencing. This fact has led to further studies to try and identify miRNA's relationship with stem cell's potential for differentiation.

The data for the following two studies provide evidence of the existence of certain miRNAs in a unique source of stem cells, specifically dental pulp stem cells (DPSC). The miRNA molecules investigated in these DPSCs include miR-16, miR-27, miR-124, miR-135, and miR-218. Evidence suggests the presence of specific miRNA in certain DPSC lines which include miR-16, miR-27, miR-124 and miR-218. With differential expression of miR-27, miR-124 and miR-218 in our different DPSC lines, evidence suggest these miRNA may impact the cells potential for differentiation.

Furthermore, these miRNA molecules were cross-referenced with the expression of certain characteristics of these DPSC line. Characteristics include the presence of biomarkers Oct-4, Sox-2, NANOG and Nestin. In addition, we also looked at their relation to the DPSC lines viability and proliferation rates. Our results have shown that biomarkers Oct-4, Sox-2 and Nanog correlated more with total live cell count whereas miRNAs miR-27, miR218, miR-124 and miR-16 were more closely related to cellular viability. Further research will be needed to more fully understand the relationship.

Acknowledgments

Thank you to my committee chair, Dr. Karl Kingsley, for introducing me to this topic. His support and dedication of time throughout my research project has been invaluable. Also, thank you my committee members, Dr. Joshua Polanski, Dr. Brian Chrzan, and Dr. Jennifer Pharr for your support.

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Chapter 1: Introduction

Background and Significance

Stem cells have been the topic of much research in recent years due to their unique biological properties. Their stemness and pluripotent properties make them ideal tools in regenerative medicine and scientists have found many sources of stem cells in the human body.[1-3] These sources include bone marrow, adipose tissue, periodontal tissue, heart tissue, peripheral blood and dental pulp cells.[4-8] Dental pulp stem cells (DPSC) are unique in their ease of access compared to other stem cells and are the focus of our research.

Past research has been done focusing on the analysis of several key biomarkers as they relate to pluripotency such as Oct-4, Sox-2, and NANOG.[9-10] Although the study of these biomarkers has helped to better understand DPSC, there is still much that is unknown. We hoped to expand on our knowledge through the evaluation of microRNA as they pertain to DPSC.

MicroRNA molecules are small non-coding RNA molecules involved in repression and activation of transcriptional factors in stem cells.[11-12] MicroRNA molecules have been shown to be involved in cellular differentiation and in altering expression of certain cell lineage. Their inclusion in this study will hopefully help to better understand DPSC and their usefulness in regenerative medicine.

Research Question

Based upon the paucity of evidence regarding miRNA expression among DPSC, the primary goal for this study was to evaluate expression of several key miRNAs, including miR-16, miR-27, miR-124, and miR218

1. Can microRNAs be detected in RNA isolated from primary DPSC explant?

H₀: No microRNA will be detectable from RNA isolated from DPSC explant.

H_A: MicroRNA will be detectable from RNA isolated from DPSC explants.

2. Is there a correlation between microRNA expression and DPSC biomarkers or cellular phenotypes?

H₀: There are no correlations between microRNA expression and DPSC biomarkers or cellular phenotypes.

H_A: There are correlations between microRNA expression and DPSC biomarkers or cellular phenotypes.

Approval

This project was reviewed and approved by the Institutional Review Board (IRB) office and the Protection of Human Subject (OPRS) under protocol OPRS#763012-1 “Retrospective analysis of dental pulp stem cells (DPSCs) from at the University of Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population.” Samples were originally collected using protocol OPRS#0907-3148 “Isolation of Non-Embryonic Stem Cells from Dental Pulp.”

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Chapter 2

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

This chapter has been published in “Current Research in Dentistry”, *An Int. Journal*, and is presented in the style of that Journal. The complete Citation is:

Whiting M, Kingsley K. Expression of microRNA miR-27, miR-124 and miR-218 Among Dental Pulp Stem Cell (DPSC) Isolates. *Current Research in Dentistry* vol 9: 12-18, 2018; DOI:10.3844/crdsp.2018.12.18.

Role of Authors:

This work was carried out in collaboration between all authors. Author Mark Whiting was responsible for sample preparation, RNA isolation and experimental protocol. Authors Dr. Karl Kingsley and Dr. Mark Whiting were responsible for project design, funding and manuscript preparation. All authors read and approved the final manuscript.

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.

Key words: Dental pulp stem cell (DPSC), microRNA, cellular differentiation, bioengineering

Abbreviations: dental pulp stem cells (DPSC), mesenchymal stem cells (MSC), Institutional Review Board (IRB), Office for the Protection of Human Subjects (OPRS), University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM), microRNAs (miR), cemento-enamel junction (CEJ), total RNA isolation reagent (TRIR), Polymerase chain reaction (PCR), doubling time (DT), rapid doubling times (rDT), slow doubling times (sDT), intermediate doubling times (iDT), phosphate buffed saline (PBS)

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.

Methodology

Project 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 ($A_{260}:A_{280} > 1.7$) and quantity ($> 1 \text{ ng}/\mu\text{L}$) 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 validated miR-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'-ATGAATTCTCGCCAGCTTTTCTT-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'-CGATATGGCTTTTATTCCTA -3'; (21 nt) Tm: 54.8°C
miR-135 reverse: 5'-GAGCAGGGTCCGAGGT -3'; (16 nt) Tm: 61.8°C

Optimal T_m (PCR): 49.8°C

miR-143 forward: 5'-AGTGCGTGTCGTGGAGTC-3'; (18 nt) T_m: 59.6°C

miR-143 reverse: 5'-GCCTGAGATGAAGCACTGT-3'; (19 nt) T_m: 70.7°C

Optimal T_m (PCR): 54.6°C

miR-218 forward: 5'-TCG GGC TTG TGC TTG ATC T-3'; (19 nt) T_m: 67°C

miR-218 reverse: 5'-GTG CAG GGT CCG AGT G-3'' (16 nt) 66°C

Optimal T_m (PCR): 61°C

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.

Table 1. RNA isolation and concentration from DPSC isolate

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	

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 ($A_{260}:A_{280}>1.65$), 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 ($A_{260}:A_{280} = 1.54$).

Table 2. RNA purity from DPSC isolates

DPSC isolate	RNA purity ($A_{260}:A_{280}$)	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	

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.

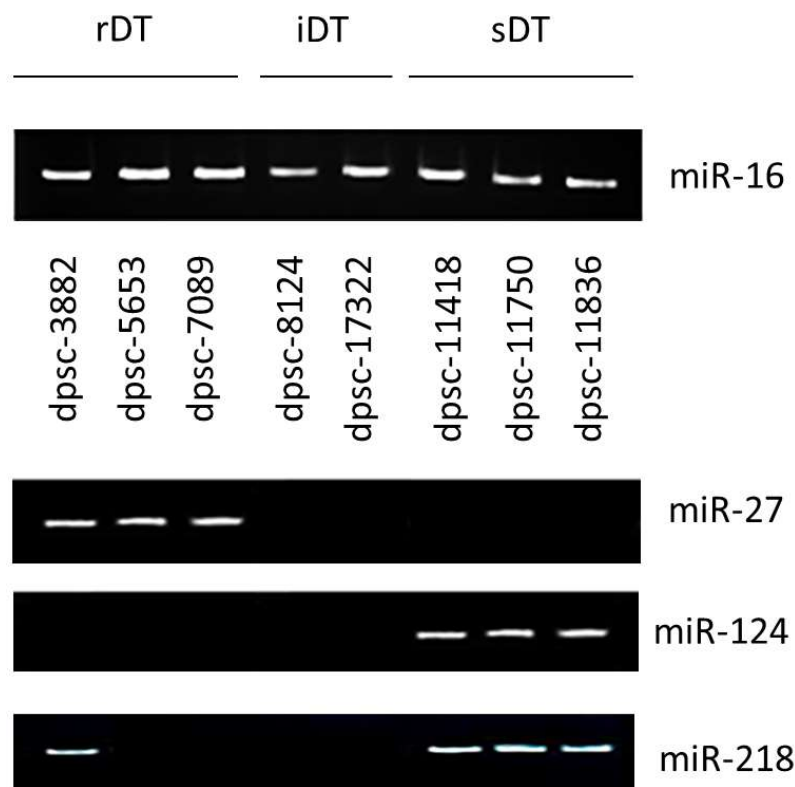


Fig1. Expression of miRNA among DPSC isolates. Screening for miRNA among eight DPSC isolates revealed expression of miR-16 (positive control) among all DPSC isolates and differential expression of miR-27, miR-124 and miR-218. No expression was observed for miR-135 or miR-143 (data not shown)

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-catenin 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.

Conclusions

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.

Acknowledgements

The authors would like to thank the Department of Advanced Education Program in Orthodontics and Dentofacial Orthopedics, as well as Dr. Jeffrey Ebersole and the Office of Research at the University of Nevada, Las Vegas – School of Dental Medicine for funding to support this project.

Ethics

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

Author Contributions

This work was carried out in collaboration between all authors. Authors Mark Whiting and Karl Kingsley were responsible for experimental protocols, including RNA isolation and PCR screening. Authors Dr. Karl Kingsley and Dr. Mark Whiting were responsible for project design, funding and manuscript preparation. All authors read and approved the final manuscript.

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Chapter 3

Pluripotent stem cell markers and microRNA expression may correlate with dental pulp stem cell viability and proliferation rates

This chapter has been submitted for review and publication in the journal *Asclepius Medical Research and Reviews* and is presented in the style of that Journal. The complete Citation is:

Whiting M, Kingsley K. Pluripotent stem cell markers and microRNA expression may correlate with dental pulp stem cell viability and proliferation rates. *Asclepius Medical Research and Reviews*

Role of Authors:

This work was carried out in collaboration between all authors. Author Mark Whiting was responsible for sample preparation, RNA isolation and experimental protocol. Authors Dr. Karl Kingsley and Dr. Mark Whiting were responsible for project design, funding and manuscript preparation. All authors read and approved the final manuscript.

Abstract

Background: Recent evidence has demonstrated that dental pulp-derived stem cells (DPSC) may represent a source of pluripotent progenitors capable of differentiating into many cell and tissue types. Although microRNAs are known to modulate differentiation and function in human dental tissues, much of this research has focused selectively on tooth development. The primary objective of this study was to evaluate the expression of microRNA in dental pulp stem cell isolates to compare with classical biomarkers of cellular phenotypes and pluripotency

Methods: Using eight previously isolated and characterized DPSC isolates, growth and viability were evaluated and RNA extracted for mRNA screening. DPSC biomarker and microRNA expression was analyzed for comparison with cellular phenotypes.

Results: Evaluation of the growth and proliferation rates of each cell line resulted in categorization of DPSC isolates into rapid, intermediate and slow doubling times, which demonstrated higher viability among the most rapidly proliferating DPSCs. Analysis of DPSC biomarkers (Oct-4, Sox-2, NANOG) revealed an association with total live cell count, while

microRNA expression (miR-27, miR-218, miR-124, miR-16) appeared to be more closely associated with cellular viability.

Conclusions: Although this study was limited to a small number of DPSC isolates, these results suggest a more thorough investigation and evaluation of biomarkers and microRNA expression may be necessary to elucidate the associations and complex interconnections with DPSC viability, proliferation, differentiation and pluripotency.

Key words: dental pulp stem cells (DPSC), microRNA, biomarkers, pluripotency

List of abbreviations: dental pulp stem cells (DPSC), mesenchymal stem cell (MSC), the Institutional Review Board (IRB), Office for the Protection of Research Subjects (OPRS), Doubling time (DT), rapid doubling time (rDT), intermediate doubling time (iDT), slow doubling time (sDT)

Background

Recent evidence has demonstrated that dental pulp-derived stem cells (DPSC) may represent a source of pluripotent progenitors capable of differentiating into many cell and tissue types [1,2] some of which may have therapeutic potential. Several key markers of pluripotency in DPSC have been identified, including the transcription factors Oct-4, Sox-2 and NANOG [3,4]. Although many studies have evaluated the functional effects of these transcriptional activators and repressors, many factors that contribute to maintaining pluripotency among DPSC remain undiscovered [5-7].

For example, recent evidence now suggests that mesenchymal stem cell (MSC) differentiation may be regulated not only by classical stem cell-associated transcription factors

(Sox-2, Oct-4, NANOG) – but also via transcriptional modulation by long, non-coding RNA known as microRNA [8,9]. In fact, there is some evidence that specific microRNAs may also be useful as biomarkers to identify and distinguish stem cells with differing therapeutic potential [10-12]. Although an extensive body of evidence has accumulated regarding the role of microRNAs and MSC, few (if any) studies have focused more specifically on the role of DPSC [13].

Although microRNAs are known to modulate differentiation and function in human dental tissues, much of this research has focused selectively on tooth development [14-16]. Developmental biology requires a thorough understanding of these mechanisms; however, recent studies now suggest that DPSC may also be useful in bioengineering and therapeutic applications for regenerative medicine [17-18]. Promising areas of interest include bone and neural tissue engineering using DPSC, – although much remains to be discovered regarding the mechanisms that control these processes [19,20].

Research from our group has evaluated classical biomarkers and other factors that may influence the therapeutic potential of primary explants and DPSC isolates [21,22]. The most recent study from this group may have also identified a limited number of microRNAs that may influence DPSC pluripotency and differentiation, including miR-27, miR-124 and miR-218 [23]. Many other studies have examined the role of these microRNAs in MSC, although only a single, recent study has examined miR-218 in DPSC and none have evaluated the concomitant presence (or absence) of traditional biomarkers [24-26].

In order to advance the evidence in this area, the primary objective of this study was to evaluate the expression of microRNA in dental pulp stem cell isolates to compare with classical biomarkers of cellular phenotypes and pluripotency

Material and Methods

Human subjects

This project was reviewed and approved by the Institutional Review Board (IRB) and Office for the Protection of Research Subjects (OPRS) under protocol OPRS#763012-1 “Retrospective analysis of dental pulp stem cells (DPSC) from the University of Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population”. DPSC were originally collected using protocol OPRS#0907-3148 “Isolation of Non-Embryonic Stem Cells from Dental Pulp”.

In brief, patients scheduled for extraction of third molars for Orthodontic treatment (primarily indicated for spacing and crowding), were asked to participate. Informed Consent (and Pediatric Assent) were provided. Exclusion criteria included refusal to participate, provide Informed Consent/Assent, or compromised dental pulp due to injury, fracture, infection or disease.

Following cross sectioning of the extracted tooth at the cemento-enamel junction, DPSC were extracted from the pulp chamber using an endodontic broach and transferred to the laboratory for culture and analysis, as previously described [27,28]. Growth for a minimum of ten (10) passages were allowed for each DPSC isolate, as part of the direct outgrowth method [29,30].

Growth and Proliferation

Doubling time (DT) was established for each DPSC isolate using the growth rate measured between 1:4 passaging and achieving confluence. These data were used to characterize three distinct categories of DPSC growth, rapid doubling time (rDT) of two to three days,

intermediate doubling time (iDT) five to seven days, and slow doubling time (sDT) of ten to fourteen days [21,22].

Cellular viability and Photo microscopy

Analysis of cellular viability was performed using the Trypan Blue exclusion method and the BioRad TC20 automated cell counter, using the manufacturer recommended protocol. These data provided both live and total cell count, allowing for the calculation of the percentage of viable cells in each sample, as previously described [27,28,31]. Digital images of DPSC isolates were captured using the Lionheart LX Automated Microscope and 20X objective lens.

Statistical analysis

Rates of proliferation and cellular viability were measured and these descriptive statistics were collected and analyzed using the Student's two-tailed t-test. Statistical significance was set using an alpha level (α) = 0.05.

RNA isolation and Polymerase Chain Reaction (PCR)

RNA was extracted from each DPSC isolate using Total RNA isolation reagent (TRIR) from Molecular Research Center and the manufacturer recommended protocol [23,27]. RNA was analyzed to determine purity using spectrophotometric absorbance readings at 260 and 280 nm (A260:A280 ratio). All samples were required to meet the standard A260:A280 > 1.65.

Screening for mRNA expression was facilitated using the ABgene Reverse-iT One-Step RT-PCR kit and protocol. The basic specifications were an initial reverse transcription at 47C for 30 minutes and 30 cycles of the following: Denaturation 95C, annealing 30 seconds at selected

primer temperature (see below), Final extension 60C for 60 seconds. Results were obtained using gel electrophoresis and visualized using ethidium bromide with a Kodak Gel Logic 100 Imaging System. Primers were synthesized from Eurofins MWG Operon, as follows:

DPSC biomarker primers:

Oct-4 forward, 5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3'; 25 nt; 48% GC; Tm 70C

Oct4 reverse, 5'-GGCAGATGGTCGTTTGGCTGAATA-3'; 24 nt; 50% GC; Tm 70C

Optimal Tm: 71C

Sox2 forward, 5'-ATGGGCTCTGTGGTCAAGTC-3'; 20 nt; 55% GC; Tm 67C

Sox2 reverse, 5'-CCCTCCCAATTCCCTTGTAT-5'; 20 nt; 50% GC; Tm 64C

Optimal Tm: 65C

NANOG forward, 5'-GCTGAGATGCCTCACACGGAG-3'; 21 nt; 62% GC; Tm 71C

NANOG reverse, 5'-TCTGTTTCTTGACTGGGACCTTGTC-3'; 25 nt; 48% GC; Tm 69C

Optimal Tm: 70C

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

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

Optimal Tm: 65C

microRNA screening primers:

miR-27 forward, 5'-ATATGAGAAAAGAGCTTCCCTGTG-3'; 24 nt; 42% GC; Tm 64C

miR-27 reverse, 5'-CAAGGCCAGAGGAGGTGAG-3'; 19 nt; 63% GC; Tm 64C

Optimal Tm: 65C

miR-218 forward, 5'-TCGGGCTTGTGCTTGATCT-3'; 19 nt; 53% GC; Tm 67C

miR-218 reverse, 5'-GTGCAGGGTCCGAGTG-3'; 16 nt; 69% GC; Tm 66C

Optimal Tm: 67C

miR-124- forward, 5'-ATGAATTCTCGCCAGCTTTTCTT-3'; 24 nt; 38% GC; Tm 65C

miR-124 reverse, 5'-ATGAATTCATTTGCATCTGCACAAACCC-3'; 28 nt; 39% GC; Tm 65C

Optimal Tm: 66C

miR-16 forward, 5'-TAGCAGCACGTAAATATTGGCG-3'; 22 nt; 45% GC; Tm 65C

miR-16 reverse, 5'-TGCGTGTCGTGGAGTC-3'; 16 nt; 63% GC; Tm 65C

Optimal Tm: 66C

Results

Evaluation of the growth and proliferation rates of each DPSC isolate resulted in categorization of DPSC isolates into rapid, intermediate and slow doubling times (Table 1). The average doubling time (DT) for the rapid DPSC was 2.2 days, which was significantly less than the average doubling time for the intermediate (6.25 days) and slow DPSC isolates (12.1 days), $p=0.0241$.

Table 1. Analysis of DPSC growth rates.

DPSC isolate	Doubling Time	Categorization
dpSC-3882	2.6 days	rDT
dpSC-5653	2.1 days	rDT
dpSC-7089	1.9 days	rDT
	rDT average: 2.2 days range: 1.9 – 2.6 days	
dpSC 8124	5.9 days	iDT
dpSC-17322	6.6 days	iDT
	iDT average: 6.25 days range: 5.9 – 6.6 days	
dpSC-11418	10.2 days	sDT
dpSC-11750	13.1 days	sDT
dpSC-11836	12.9 days	sDT
	sDT average: 12.1 days range: 10.2 – 13.1 days	

Cellular viability was then measured for each DPSC isolate to determine if any correlations could be found with growth rates and doubling time (Table 2). In brief, the cellular viability for the rDT DPSC isolates averaged 56%, ranging between 52% - 59%. The average was considerable higher than those observed among the iDT DPSC isolates (37%; range 35% - 39%) or sDT DPSC isolates 31%; range 29-34%), $p=0.311$.

Table 2. Trypan Blue exclusion assay test for DPSC viability.

DPSC isolate	Cellular viability	Categorization
dpSC-3882	1.62 x 10 ⁵ cells/mL TC 0.84 x 10 ⁵ cells/mL LC Viable: 52%	rDT (2.6 days)
dpSC-5653	1.90 x 10 ⁵ cells/mL TC 1.12 x 10 ⁵ cells/mL LC Viable: 59%	rDT (2.1 days)
dpSC-7089	3.35 x 10 ⁵ cells/mL TC 1.90 x 10 ⁵ cells/mL LC Viable: 57%	rDT (1.9 days)
rDT	Ave: 2.29 x 10⁵ cells/mL TC Ave: 1.29 x 10⁵ cells/mL LC Average viability: 56%	
dpSC 8124	3.68 x 10 ⁵ cells/mL TC 1.45 x 10 ⁵ cells/mL LC Viable: 39%	iDT (5.9 days)
dpSC-17322	2.90 x 10 ⁵ cells/mL TC 1.00 x 10 ⁵ cells/mL LC Viable: 35%	iDT (6.6 days)
iDT	Ave: 3.29 x 10⁵ cells/mL TC Ave: 1.23 x 10⁵ cells/mL LC Average viability: 37%	
dpSC-11418	3.24 x 10 ⁵ cells/mL TC 0.948 x 10 ⁵ cells/mL LC Viable: 29%	sDT (10.2 days)
dpSC-11750	3.24 x 10 ⁵ cells/mL TC 1.00 x 10 ⁵ cells/mL LC Viable: 31%	sDT (13.1 days)
dpSC-11836	1.62 x 10 ⁵ cells/mL TC 0.558 x 10 ⁵ cells/mL LC Viable: 34%	sDT (12.9 days)
sDT	Ave: 2.70 x 10⁵ cells/mL TC Ave: 0.835 x 10⁵ cells/mL LC Average viability: 31%	

To more closely evaluate cellular phenotypes and morphology, as well as to evaluate the presence of both viable and non-viable cells, digital micrographs were obtained from each DPSC isolate (Figure 1). Briefly, these data demonstrated multiple, non-adherent DPSC cells with fewer cells displaying signs of apoptosis (intense blebbing) among the rDT isolates (Fig. 1A. – Fig. 1C). Higher proportions of these non-viable cells were observed among the iDT (Fig. 1D. Fig. 1H) and sDT isolates (Fig. 1. E – Fig. 1G.)

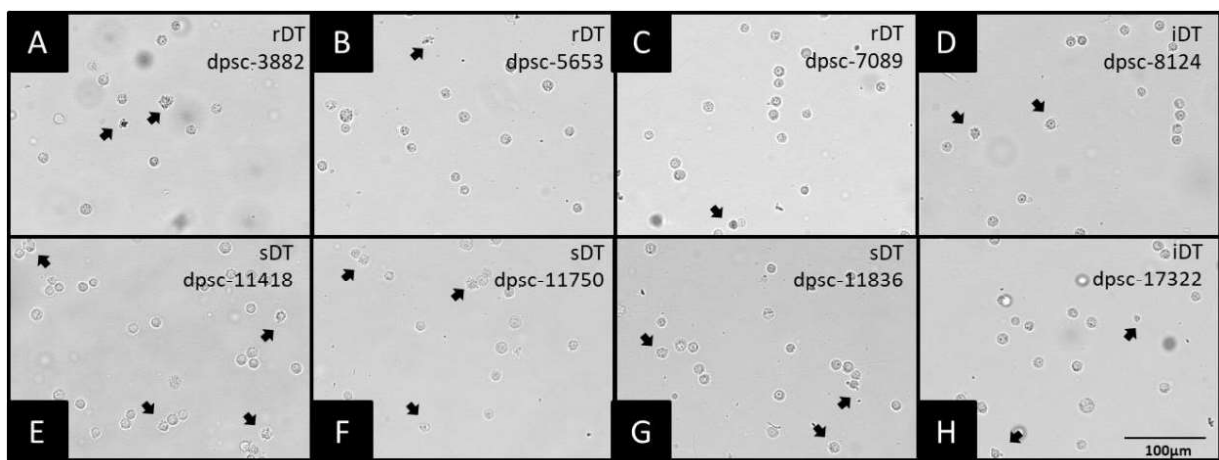


Figure 1. DIC digital photomicrographs of freshly dispersed and cultured dental pulp stem cells (DPSC's). (A-C) rapidly dividing (rDT) DPSC, (E-G) slowly dividing (sDT) DPSC, D+H intermediately dividing DPSC. Arrows showing intense cellular blebbing (apoptosis), scale bar = 100 μ m.

To evaluate the association between growth and viability, DPSC biomarkers (Oct-4, Sox-2, NANOG, Nestin) and microRNAs (miR-27, miR-218, miR-124, miR-16) were evaluated using RT-PCR for comparison with graphs of live and viable cells (Figure 2). More specifically, the total live counts from each cell line were plotted, which revealed the highest numbers among the rDT and iDT isolates (Fig. 2A). These data appear to correlate with the expression of

NANOG, Sox-2 and Oct-4 among the rDT and iDT isolates – although some variability in mRNA expression was observed (Fig. 2B).

Although mRNA expression of at least two DPSC biomarkers was noted in all rDT and iDT isolates, the expression of all DPSC biomarkers was noted in only one DPSC isolate (dpSC-7089), which was observed to have the highest overall live cell count and cell viability (Fig. 2B and Fig 2C). In addition, Oct-4 expression was not observed in either of the iDT isolates (dpSC-8124, dpSC-17322). However, the expression of Sox-2 and NANOG was observed in all of the iDT isolates, unlike the variable expression observed among the rDT isolates.

The highest overall viability was verified among the rDT isolates (dpSC-3882, dpSC-5653, dpSC-7089) with an average exceeding 50% (Fig. 2C). These data appear to correlate with the expression of miR-27, which was observed only among the rDT isolates (Fig. 2D). Expression of miR-124 was restricted to the sDT isolates, which appeared to have the lowest overall viability (approximately 30%), with variable expression observed with miR-218. It was noted that only miR-16 (control) expression was observed among the iDT isolates, with no expression of miR-27, miR-124 or miR-218 observed.

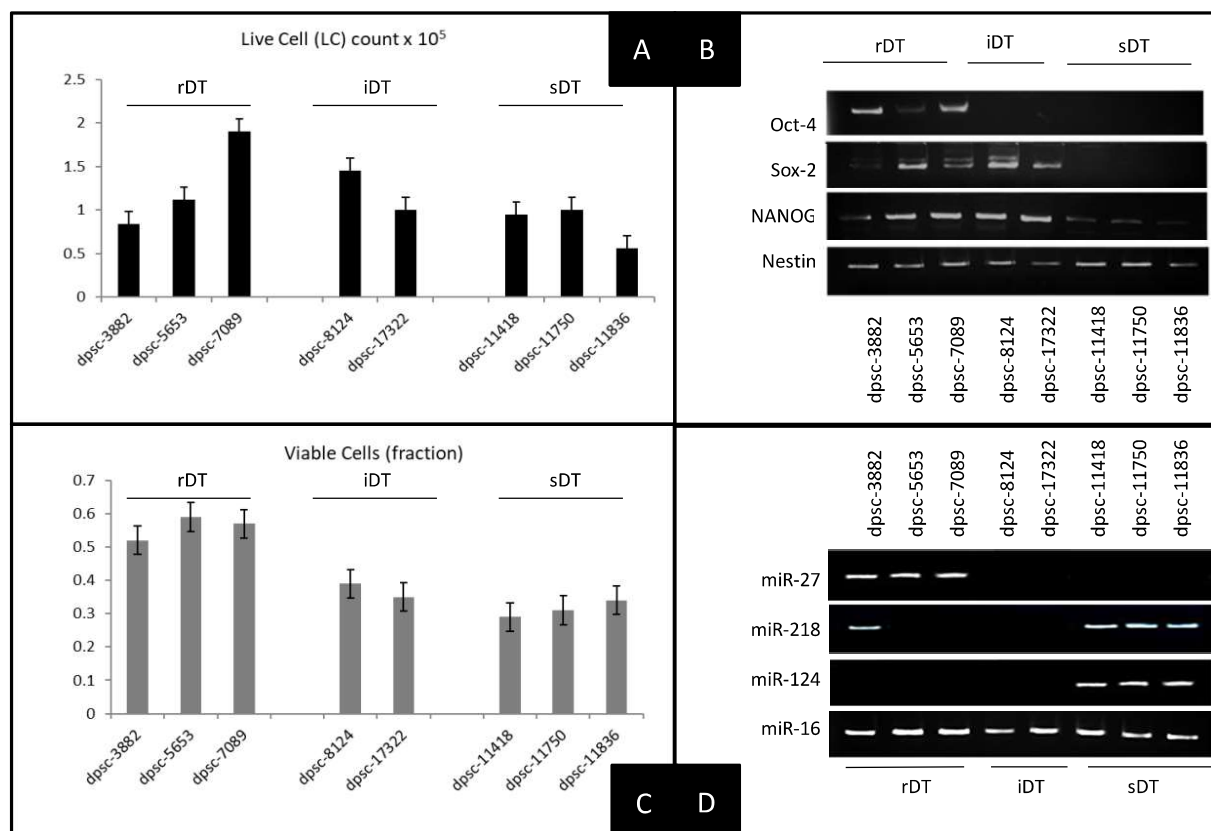


Figure 2. Correlation of DPSC markers with microRNA expression. A) Graphed live cell counts from DPSC isolates demonstrated highest levels among rDT and iDT isolates. B) mRNA expression of DPSC biomarkers revealed Oct-4, Sox-2 and NANOG among rDT isolates (variable) and only Nestin expression among sDT isolates. C) Percentage of viable cells revealed higher percentages among rDT isolates compared with either iDT or sDT. D) MicroRNA expression of miR-27 was observed among rDT only, while miR-124 and miR-218 was observed mainly among the sDT isolates.

Discussion

The primary objective of this study was to evaluate the expression of microRNA in dental pulp stem cell isolates to compare with classical biomarkers of cellular phenotypes and pluripotency. Evaluation of the growth and proliferation rates of each cell line resulted in

categorization of DPSC isolates into rapid, intermediate and slow doubling times, which demonstrated higher viability among the most rapidly proliferating DPSCs. Analysis of DPSC biomarkers (Oct-4, Sox-2, NANOG) revealed an association with total live cell count, while microRNA expression (miR-27, miR-218, miR-124, miR-16) appeared to be more closely associated with viability.

These data appear to confirm recent studies that demonstrated proliferation and growth potential among DPSC isolates to be closely linked with expression of Sox-2 and other DPSC biomarkers, such as Oct-4 [32-34]. The results of this study also support previous findings from this group regarding the association between doubling time (growth) and these biomarkers [21,22,27]. In addition, these data also confirm one of the only studies to demonstrate an association between microRNA expression (miR-218) and DPSC viability and differentiation potential [35].

Although only limited evidence regarding DPSC and microRNAs may be available, a growing body of evidence regarding microRNA expression and stem cell differentiation may suggest further research in this area is warranted [36,37]. For example, miR-124 may a key regulator of osteogenic, myogenic and neuronal differentiation in MSC – although this role has yet to be confirmed among DPSC [38-40]. miR-218 may also function in a similar capacity, as a key regulator of osteogenic differentiation in MSC [41].

The role of miR-27 in differentiation of other MSC may be the most thoroughly examined of these microRNAs [24,25]. For example, miR-27 has been shown to promote osteoblast differentiation by directly modulating transcription of several key components of the beta-catenin/Wnt pathway [42]. miR-27 may also function to promote myeloblast differentiation through direct and indirect modulation of Runx1 transcription [43,44]. However, miR-27 has

also been shown to inhibit adipose differentiation and mitochondrial function via multiple pathways in other types of adipose-derived MSC [45-47].

Conclusions

Although this study was limited to a small number of DPSC isolates, these results suggest a more thorough investigation and evaluation of biomarkers and microRNA expression may be necessary to elucidate the associations and complex interconnections with DPSC viability, proliferation, differentiation and pluripotency.

Declarations

This project was reviewed and approved by the Institutional Review Board (IRB) and Office for the Protection of Research Subjects (OPRS) under protocol OPRS#763012-1 “Retrospective analysis of dental pulp stem cells (DPSC) from the University of Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population”.

Competing interests

The authors declare there are no conflicts of interest.

Funding

This project was funded by the Office of Research and the Department of Advanced Education in Orthodontics and Dentofacial Orthopedics, at the University of Nevada, Las Vegas – School of Dental Medicine.

Authors contributions

Karl Kingsley and Mark Whiting were responsible for experimental protocols, including RNA isolation and PCR screening, as well as project design and funding. William J Hatton was responsible for photo microscopy and cell imaging. All authors participated in data analysis and manuscript preparation

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Chapter 4: Summary and Conclusions.

The goal of this project was to assess the presence of miRNA in DPSC lines. Due to the fact that certain miRNA have been shown to affect gene expression it is important to understand their role as it pertains to the pluripotent potential of DPSC and ultimately the differentiation of those specific cells.

Chapter 2 of this paper was a study of the presence of specific miRNA in our samples of DPSC. Eight different DPSC lines were evaluated using PCR to determine that presence of miR-16, miR-27, miR-124, and miR-218. Furthermore, it was determined there was differential expression of miR-27, miR-124, and miR-218. With further research these results may help further explain differentiation status of these cells.

Chapter 3 of this paper further evaluated the presence of miRNA presence in DPSC and its correlation with other characteristics. We again used the same eight cell lines for DPSC samples and classified them into three categories based upon proliferation rates. These categories were rapid, intermediate, and slow dividing times with higher viability being more closely associated with rapid proliferation. Furthermore, we analyzed the results as they correlated with the presence of the biomarkers Oct-4, Sox-2 and NANOG. Our conclusions were that the presence of biomarkers Oct-4, Sox-2 and NANOG were more closely connected with total live cell count whereas the presence of miRNAs miR-27, miR-124, and miR-218 were tied more closely to cellular viability.

Conclusions from these 2 chapters led us to believe that there is a presence of certain miRNA in our DPSC samples. Because of this presence we can reject our null hypothesis and accept our alternative hypothesis for questions 1 of this paper. In addition, our further analysis has shown correlation between miRNA expression and DPSC biomarkers with cellular viability

and total live count. Due to these results we move to reject our null hypothesis and accept our alternative hypothesis in question 2 of this paper.

1. Can microRNAs be detected in RNA isolated from primary DPSC explant?

H_A: MicroRNA will be detectable from RNA isolated from DPSC explants.

2. Is there a correlation between microRNA expression and DPSC biomarkers or cellular phenotypes?

H_A: There are correlations between microRNA expression and DPSC biomarkers or cellular phenotypes.

Limitations and Recommendations:

As mentioned in previous chapters, one limitation for this study is the limited number of DPSC samples that were analyzed. These limits were brought about by funding issues and viable cell lines available to use in this study since the original samples were collected in previous years. I would recommend gathering more samples to analyze so that we could run statistical analysis to confirm the importance of certain miRNA found in DPSCs.

In addition, there are a limited number of studies from other research outlets to compare our results with. Due to the vast number of different miRNA molecules that exist it is difficult narrow down which ones to include in the study. Further research needs to be done to determine the function of specific miRNA molecules before we look for their presence or absence in our DPSC samples.

Appendix A



UNLV Biomedical IRB - Administrative Review Notice of Excluded Activity

DATE: August 3, 2015

TO: Karl Kingsley, PhD
FROM: UNLV Biomedical IRB

PROTOCOL TITLE: [763012-1] Evaluation of the effects of cryopreservation on survival of dental pulp stem cells.
SUBMISSION TYPE: New Project

ACTION: EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE: August 3, 2015
REVIEW TYPE: Administrative Review

Thank you for your submission of New Project materials for this protocol. This memorandum is notification that the protocol referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The UNLV Biomedical IRB has determined this protocol does not meet the definition of human subjects research under the purview of the IRB according to federal regulations. It is not in need of further review or approval by the IRB.

We will retain a copy of this correspondence with our records.

Any changes to the excluded activity may cause this protocol to require a different level of IRB review. Should any changes need to be made, please submit a Modification Form.

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your protocol title and IRBNet ID in all correspondence.

Office of Research Integrity - Human Subjects
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Appendix B

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I, **Karl Kingsley**, holder of copyrighted material entitled **Expression of microRNA miR-27, miR-124 and miR-218 Among Dental Pulp Stem Cell (DPSC) Isolates**, authored by **Mark Whiting**, and **Karl Kingsley** originally published in **Current Research in Dentistry**.

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January 29, 2019

Signature

Date

Karl Kingsley, PhD, MPH

Professor

Name (typed)

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Appendix C

Permission to Use Copyrighted Material

University of Nevada, Las Vegas

RESERVED for second manuscript

Curriculum Vitae

Graduate College

University of Nevada, Las Vegas

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Bachelor of Business Management, 2010
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Doctor of Dental Medicine, 2016
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Thesis Title:

Correlation Between Pluripotent Stem Cell Markers and microRNA Expression in Dental Pulp Stem Cells

Thesis Examination Committee:

Chairperson, Karl Kingsley, Ph.D. M.P.H.
Committee Member, Joshua Polanski, M.S, PhD.
Committee Member, Brian Chrzan, D.D.S., Ph.D.
Graduate Faculty Representative, Jennifer Pharr Ph.D.
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