5-1-2019

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

Mark Whiting
vlcmboarder@gmail.com

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

By

Mark Whiting

Bachelor of Business Management
Brigham Young University
2010

Doctor of Dental Medicine
University of Nevada, Las Vegas
2016

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science - Oral Biology

School of Dental Medicine
Division of Health Sciences
The Graduate College

University of Nevada, Las Vegas
May 2019

UNLV | GRADUATE COLLEGE

Thesis Approval

The Graduate College
The University of Nevada, Las Vegas

February 19, 2019

This thesis prepared by

Mark Whiting

entitled

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

is approved in partial fulfillment of the requirements for the degree of

Master of Science - Oral Biology
School of Dental Medicine

Karl Kingsley, Ph.D.
Examination Committee Chair

Kathryn Hausbeck Korgan, Ph.D.
Graduate College Dean

Brian Chrzan, Ph.D.
Examination Committee Member

Joshua Polanski, Ph.D.
Examination Committee Member

Jennifer Pharr, Ph.D.
Graduate College Faculty Representative

Abstract

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

By

Mark Whiting

Dr. Karl Kingsley, Examination Committee Chair
Associate Professor of Biomedical Sciences
Director of Student Research
University of Nevada, Las Vegas
School of Dental Medicine

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.

Table of Contents

List of Tables

Chapter 2

Chapter 3

List of Figures

Chapter 2

Chapter 3

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?

H_0: 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?

   H_0: 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 (DPCS) 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."

References

1. Hollands P, Aboyeji D, Orcharton M. Dental pulp stem cells in regenerative medicine. Br Dent J. 2018 May 4. doi: 10.1038/sj.bdj.2018.348. [Epub ahead of print] PMID: 29725075

2. Ledesma-Martínez E, Mendoza-Núñez VM, Santiago-Osorio E. Mesenchymal Stem Cells Derived from Dental Pulp: A Review. Stem Cells Int. 2016;2016:4709572. doi: 10.1155/2016/4709572. Epub 2015 Dec 8. Review. PMID:26779263

3. Kabir R, Gupta M, Aggarwal A, Sharma D, Sarin A, Kola MZ. Imperative role of dental pulp stem cells in regenerative therapies: a systematic review. Niger J Surg. 2014 Jan;20(1):1-8. doi: 10.4103/1117-6806.127092. Review. PMID: 24665194

4. Bara JJ, Richards RG, Alini M, Stoddart MJ. Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic. Stem Cells. 2014 Jul;32(7):1713-23. doi: 10.1002/stem.1649. Review. PubMed PMID: 24449458

5. Chou SH, Lin SZ, Kuo WW, Pai P, Lin JY, Lai CH, Kuo CH, Lin KH, Tsai FJ, Huang CY. Mesenchymal stem cell insights: prospects in cardiovascular therapy. Cell Transplant. 2014;23(4-5):513-29. doi: 10.3727/096368914X678436. Review. PubMed PMID: 24816448

6. Leeman KT, Fillmore CM, Kim CF. Lung stem and progenitor cells in tissue homeostasis and disease. Curr Top Dev Biol. 2014;107:207-233. doi: 10.1016/B978-0-12-416022-4.00008-1. Review. PubMed PMID: 24439808; PubMed Central PMCID: PMC4038302

7. Lim MH, Ong WK, Sugii S. The current landscape of adipose-derived stem cells in clinical applications. Expert Rev Mol Med. 2014 May 7;16:e8. doi: 10.1017/erm.2014.8. Review. PubMed PMID: 24807467

8. La Noce M, Paino F, Spina A, Naddeo P, Montella R, Desiderio V, De Rosa A, Papaccio G, Tirino V, Laino L. Dental pulp stem cells: state of the art and suggestions for a true translation of research into therapy. J Dent. 2014 Jul;42(7):761-8. doi: 10.1016/j.jdent.2014.02.018. Epub 2014 Feb 28. Review. PubMed PMID: 24589847

9. Liu L, Wei X, Ling J, Wu L, Xiao Y. Expression pattern of Oct-4, Sox2, and c-Myc in the primary culture of human dental pulp derived cells. J Endod. 2011 Apr;37(4):466-72. doi: 10.1016/j.joen.2010.12.012. Epub 2011 Feb 25. PMID: 21419292

10. Kaukua N, Chen M, Guarnieri P, Dahl M, Lim ML, Yucel-Lindberg T, Sundström E, Adameyko I, Mao JJ, Fried K. Molecular differences between stromal cell populations from deciduous and permanent human teeth. Stem Cell Res Ther. 2015 Apr 18;6:59. doi: 10.1186/s13287-015-0056-7. PMID: 25927523

11. Amin ND, Bai G, Klug JR, Bonanomi D, Pankratz MT, Gifford WD, Hinckley CA, Sternfeld MJ, Driscoll SP, Dominguez B, Lee KF, Jin X, Pfaff SL. Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure. Science. 2015 Dec 18;350(6267):1525-9. doi: 10.1126/science.aad2509. PubMed PMID: 26680198; PubMed Central PMCID: PMC4913787

12. Wang T, Xu Z. miR-27 promotes osteoblast differentiation by modulating Wnt signaling. Biochem Biophys Res Commun. 2010 Nov 12;402(2):186-9. doi: 10.1016/j.bbrc.2010.08.031. Epub 2010 Aug 12. PubMed PMID: 20708603

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 (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

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	□□=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 (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).

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	

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.

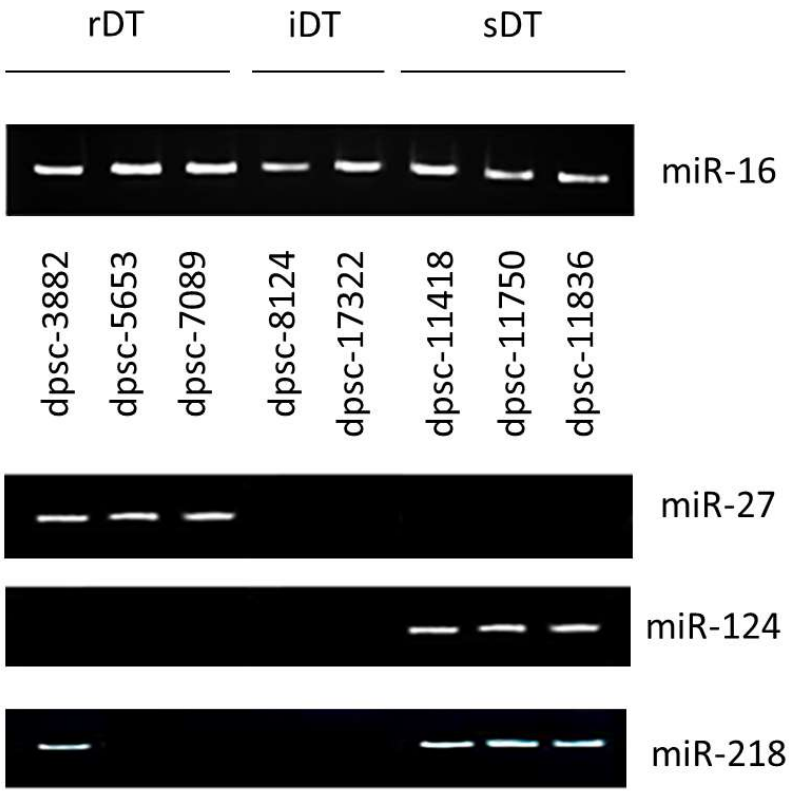

Fig1. Expression of miRNA among DPSC isolates. Screening for miRNA among eight DPSC isolates revealed expression of miR-16 (positive control) among all DPSC isolates and differential expression of miR-27, miR-124 and miR-218. No expression was observed for miR-135 or miR-143 (data not shown)

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-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.

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.

References

1. Hollands P, Aboyeji D, Orcharton M. Dental pulp stem cells in regenerative medicine. Br Dent J. 2018 May 4. doi: 10.1038/sj.bdj.2018.348. [Epub ahead of print] PMID: 29725075
2. Ledesma-Martínez E, Mendoza-Núñez VM, Santiago-Osorio E. Mesenchymal Stem Cells Derived from Dental Pulp: A Review. Stem Cells Int. 2016;2016:4709572. doi: 10.1155/2016/4709572. Epub 2015 Dec 8. Review. PMID: 26779263
3. Kabir R, Gupta M, Aggarwal A, Sharma D, Sarin A, Kola MZ. Imperative role of dental pulp stem cells in regenerative therapies: a systematic review. Niger J Surg. 2014 Jan;20(1):1-8. doi: 10.4103/1117-6806.127092. Review. PMID: 24665194
4. Hernández-Monjaraz B, Santiago-Osorio E, Monroy-García A, Ledesma-Martínez E, Mendoza-Núñez VM. Mesenchymal Stem Cells of Dental Origin for Inducing Tissue Regeneration in Periodontitis: A Mini-Review. Int J Mol Sci. 2018 Mar 22;19(4). pii: E944. doi: 10.3390/ijms19040944. Review. PMID: 29565801
5. Aurrekoetxea M, Garcia-Gallastegui P, Irastorza I, Luzuriaga J, Uribe-Etxebarria V, Unda F, Ibarretxe G. Dental pulp stem cells as a multifaceted tool for bioengineering and the

regeneration of craniomaxillofacial tissues. Front Physiol. 2015 Oct 16;6:289. doi: 10.3389/fphys.2015.00289. eCollection 2015. Review. PMID: 26528190

6. Duncan HF, Smith AJ, Fleming GJ, Cooper PR. Epigenetic modulation of dental pulp stem cells: implications for regenerative endodontics. Int Endod J. 2016 May;49(5):431-46. doi: 10.1111/iej.12475. Epub 2015 Jun 11. Review. PMID: 26011759

7. Daltoé FP, Mendonça PP, Mantesso A, Deboni MC. Can SHED or DPSCs be used to repair/regenerate non-dental tissues? A systematic review of in vivo studies. Braz Oral Res. 2014;28. pii: S1806-83242014000100401. Epub 2014 Aug 21. Review. PMID: 25166769

8. Collart-Dutilleul PY, Chaubron F, De Vos J, Cuisinier FJ. Allogenic banking of dental pulp stem cells for innovative therapeutics. World J Stem Cells. 2015 Aug 26;7(7):1010-21. doi: 10.4252/wjsc.v7.i7.1010. Review. PMID: 26328017

9. Mead B, Logan A, Berry M, Leadbeater W, Scheven BA. Concise Review: Dental Pulp Stem Cells: A Novel Cell Therapy for Retinal and Central Nervous System Repair. Stem Cells. 2017 Jan;35(1):61-67. doi: 10.1002/stem.2398. Epub 2016 Jun 16. Review. PMID: 27273755

10. Victor AK, Reiter LT. Dental pulp stem cells for the study of neurogenetic disorders. Hum Mol Genet. 2017 Oct 1;26(R2):R166-R171. doi: 10.1093/hmg/ddx208. Review. PMID: 28582499

11. Zainuri M, Putri RR, Bachtiar EW. Establishing methods for isolation of stem cells from human exfoliated deciduous from carious deciduous teeth. Interv Med Appl Sci. 2018 Mar;10(1):33-37. doi: 10.1556/1646.10.2018.06. PMID: 30363356

12. Bakopoulou A, Apatzidou D, Aggelidou E, Gousopoulou E, Leyhausen G, Volk J, Kritis A, Koidis P, Geurtsen W. Isolation and prolonged expansion of oral mesenchymal stem cells under clinical-grade, GMP-compliant conditions differentially affects "stemness" properties. Stem Cell Res Ther. 2017 Nov 2;8(1):247. doi: 10.1186/s13287-017-0705-0. PMID: 29096714

13. Bakkar M, Liu Y, Fang D, Stegen C, Su X, Ramamoorthi M, Lin LC, Kawasaki T, Makhoul N, Pham H, Sumita Y, Tran SD. A Simplified and Systematic Method to Isolate, Culture, and Characterize Multiple Types of Human Dental Stem Cells from a Single Tooth. Methods Mol Biol. 2017;1553:191-207. doi: 10.1007/978-1-4939-6756-8_15. PMID: 28229417

14. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, Politis C, Lambrichts I, Bronckaers A. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell Tissue Res. 2013 Jul;353(1):65-78. doi: 10.1007/s00441-013-1630-x. Epub 2013 May 29. PMID: 23715720

15. Karamzadeh R, Eslaminejad MB, Aflatoonian R. Isolation, characterization and comparative differentiation of human dental pulp stem cells derived from permanent teeth by using two different methods. J Vis Exp. 2012 Nov 24;(69). pii: 4372. doi: 10.3791/4372. PMID: 23208006

16. Rodríguez-Lozano FJ, Insausti CL, Meseguer L, Ramírez MC, Martínez S, Moraleda JM. Tissue engineering with dental pulp stem cells: isolation, characterization, and osteogenic differentiation. J Craniofac Surg. 2012 Nov;23(6):e571-5. doi: 10.1097/SCS.0b013e31825e4e16. PMID: 23172482

17. Saidi N, Ghalavand M, Hashemzadeh MS, Dorostkar R, Mohammadi H, Mahdian-Shakib A. Dynamic changes of epigenetic signatures during chondrogenic and adipogenic differentiation of mesenchymal stem cells. Biomed Pharmacother. 2017 May;89:719-731. doi: 10.1016/j.biopha.2017.02.093. Epub 2017 Mar 6. Review. PMID: 28273634

18. Ozkul Y, Galderisi U. The Impact of Epigenetics on Mesenchymal Stem Cell Biology. J Cell Physiol. 2016 Nov;231(11):2393-401. doi: 10.1002/jcp.25371. Epub 2016 Apr 4. Review. PMID: 26960183

19. Deng P, Chen QM, Hong C, Wang CY. Histone methyltransferases and demethylases: regulators in balancing osteogenic and adipogenic differentiation of mesenchymal stem cells. Int J Oral Sci. 2015 Dec 18;7(4):197-204. doi: 10.1038/ijos.2015.41. Review. PMID: 26674421

20. Katsuda T, Ochiya T. Molecular signatures of mesenchymal stem cell-derived extracellular vesicle-mediated tissue repair. Stem Cell Res Ther. 2015 Nov 11;6:212. doi: 10.1186/s13287-015-0214-y. Review. PMID: 26560482

21. Utikal J, Abba M, Novak D, Moniuszko M, Allgayer H. Function and significance of MicroRNAs in benign and malignant human stem cells. Semin Cancer Biol. 2015 Dec;35:200-11. doi: 10.1016/j.semcancer.2015.07.001. Epub 2015 Jul 17. Review. PMID: 26192966

22. Huang C, Gou S, Wang L, Huang K, Liu L, Zhao W, Zheng L, Xiao J. MicroRNAs and Peroxisome Proliferator-Activated Receptors Governing the Differentiation of Mesenchymal Stem Cells. Curr Stem Cell Res Ther. 2016;11(3):197-207. Review. PMID: 26018230

23. Martin EC, Qureshi AT, Dasa V, Freitas MA, Gimble JM, Davis TA. MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: Perspectives on miRNA biogenesis and cellular transcriptome. Biochimie. 2016 May;124:98-111. doi: 10.1016/j.biochi.2015.02.012. Epub 2015 Feb 26. Review. PMID: 25726914

24. Sekar D, Saravanan S, Karikalan K, Thirugnanasambantham K, Lalitha P, Islam VI. Role of microRNA 21 in mesenchymal stem cell (MSC) differentiation: a powerful biomarker in MSCs derived cells. Curr Pharm Biotechnol. 2015;16(1):43-8. Review. PMID: 25564252

25. Clark EA, Kalomoiris S, Nolta JA, Fierro FA.MicroRNA function in multipotent mesenchymal stromal cells. Stem Cells. 2014 May;32(5):1074-82. Review. PMID: 24860868

26. Fakhry M, Hamade E, Badran B, Buchet R, Magne D. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World J Stem Cells. 2013 Oct 26;5(4):136-48. doi: 10.4252/wjsc.v5.i4.136. Review. PMID: 24179602

27. Tu S, Zheng J, Gao X, Guan C, Cai B, Xiang L. The role of Foxq1 in proliferation of human dental pulp stem cell. Biochem Biophys Res Commun. 2018 Mar 4;497(2):543-549. doi: 10.1016/j.bbrc.2018.02.077. Epub 2018 Feb 14. PMID: 29453987

28. Sun DG, Xin BC, Wu D, Zhou L, Wu HB, Gong W, Lv J. miR-140-5p-mediated regulation of the proliferation and differentiation of human dental pulp stem cells occurs through the lipopolysaccharide/toll-like receptor 4 signaling pathway. Eur J Oral Sci. 2017 Dec;125(6):419-425. doi: 10.1111/eos.12384. PMID: 29130547

29. Li D, Deng T, Li H, Li Y. MiR-143 and miR-135 inhibitors treatment induces skeletal myogenic differentiation of human adult dental pulp stem cells. Arch Oral Biol. 2015

Nov;60(11):1613-7. doi: 10.1016/j.archoralbio.2015.08.010. Epub 2015 Sep 5. PMID: 26351742

30. Alleman M, Low E, Truong K, Huang E, Hill CK, Chen TY, Deaton M, KINGSLEY K. Dental pulp-derived stem cells (DPSC) differentiation in vitro into odontoblast and neural progenitors during cell passaging is associated with alterations in cell survival and viability. International Journal of Medicine and Biomedical Research 2013, 2(2): 133-141.

31. Hung E, Lee S, Fitzgerald B, Hill CK, Kingsley K. Dental pulp-derived stem cell (DPSC) survival and viability may correlate with specific patient demographics. FDSRI Winter 2013, 1(3): 14-21.

32. A Young, K Kingsley. Dental Pulp Stem Cells: A review of factors that influence the therapeutic potential of stem cell isolates. Biomaterials and Biomedical Engineering June 2015, 2(2): 61-69.

33. Tomlin A, Sanders MB, Kingsley K. The effects of cryopreservation on human dental pulp-derived mesenchymal stem cells. Biomaterials and Biomedical Engineering 2016, 3(2).

34. Petersen B, Kingsley K. Differential expression of miR-21, miR-133 and miR-155 from exosome fractions isolated from oral squamous cell carcinomas in vitro. J Med Discov. 2016, 1(1): jmd6010.

35. Brennan D, Patel K, Howard N, Kingsley K, Howard K. Differential miRNA Expression in Oral Cancer Oncosomes: A Pilot In Vitro Study. International Research Journal of Oncology, 2018: 1(2): 1-9.

36. Yu B, Gong M, He Z, Wang YG, Millard RW, Ashraf M, Xu M. Enhanced mesenchymal stem cell survival induced by GATA-4 overexpression is partially mediated by regulation

of the miR-15 family. Int J Biochem Cell Biol. 2013 Dec;45(12):2724-35. doi: 10.1016/j.biocel.2013.09.007. Epub 2013 Sep 23. PMID: 24070634

37. Eguchi T, Watanabe K, Hara ES, Ono M, Kuboki T, Calderwood SK. OstemiR: a novel panel of microRNA biomarkers in osteoblastic and osteocytic differentiation from mesencymal stem cells. PLoS One. 2013;8(3):e58796. doi: 10.1371/journal.pone.0058796. Epub 2013 Mar 22. PMID: 23533592

38. Gao Y, Zhang Y, Lu Y, Wang Y, Kou X, Lou Y, Kang Y. TOB1 Deficiency Enhances the Effect of Bone Marrow-Derived Mesenchymal Stem Cells on Tendon-Bone Healing in a Rat Rotator Cuff Repair Model. Cell Physiol Biochem. 2016;38(1):319-29. doi: 10.1159/000438632. Epub 2016 Jan 27. PMID: 26824451

39. Yue Q, Zhang Y, Li X, He L, Hu Y, Wang X, Xu X, Shen Y, Zhang H. MiR-124 suppresses the chemotactic migration of rat mesenchymal stem cells toward HGF by downregulating Wnt/β-catenin signaling. Eur J Cell Biol. 2016 Sep;95(9):342-53. doi: 10.1016/j.ejcb.2016.06.005. Epub 2016 Jun 23. PMID: 27377850

40. Laine SK, Alm JJ, Virtanen SP, Aro HT, Laitala-Leinonen TK. MicroRNAs miR-96, miR-124, and miR-199a regulate gene expression in human bone marrow-derived mesenchymal stem cells. J Cell Biochem. 2012 Aug;113(8):2687-95. doi: 10.1002/jcb.24144. PMID: 22441842

41. Chen KD, Goto S, Hsu LW, Lin TY, Nakano T, Lai CY, Chang YC, Weng WT, Kuo YR, Wang CC, Cheng YF, Ma YY, Lin CC, Chen CL. Identification of miR-27b as a novel signature from the mRNA profiles of adipose-derived mesenchymal stem cells involved in the tolerogenic response. PLoS One. 2013 Apr 16;8(4):e60492. doi: 10.1371/journal.pone.0060492. Print 2013. PMID: 23613728

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'-ATGAATTCTCGCCAGCTTTTTCTT-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 10^5 cells/mL TC** **Ave: 1.29 x 10^5 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 10^5 cells/mL TC** **Ave: 1.23 x 10^5 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 10^5 cells/mL TC** **Ave: 0.835 x 10^5 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.)

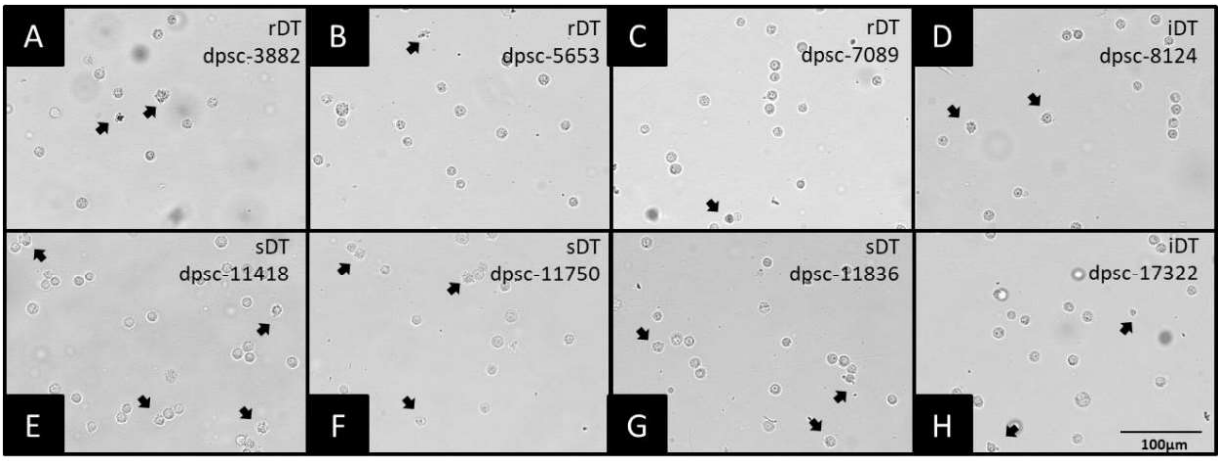

Figure 1. DIC digital photomicrographs of freshly dispersed and cultured dental pulp stem cells (DPSC's). (A-C) rapidly dividing (rDT) DPSC, (E-G) slowly dividing (sDT) DPSC, D+H intermediately dividing DPSC. Arrows showing intense cellular blebbing (apoptosis), scale bar = 100 µm.

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.

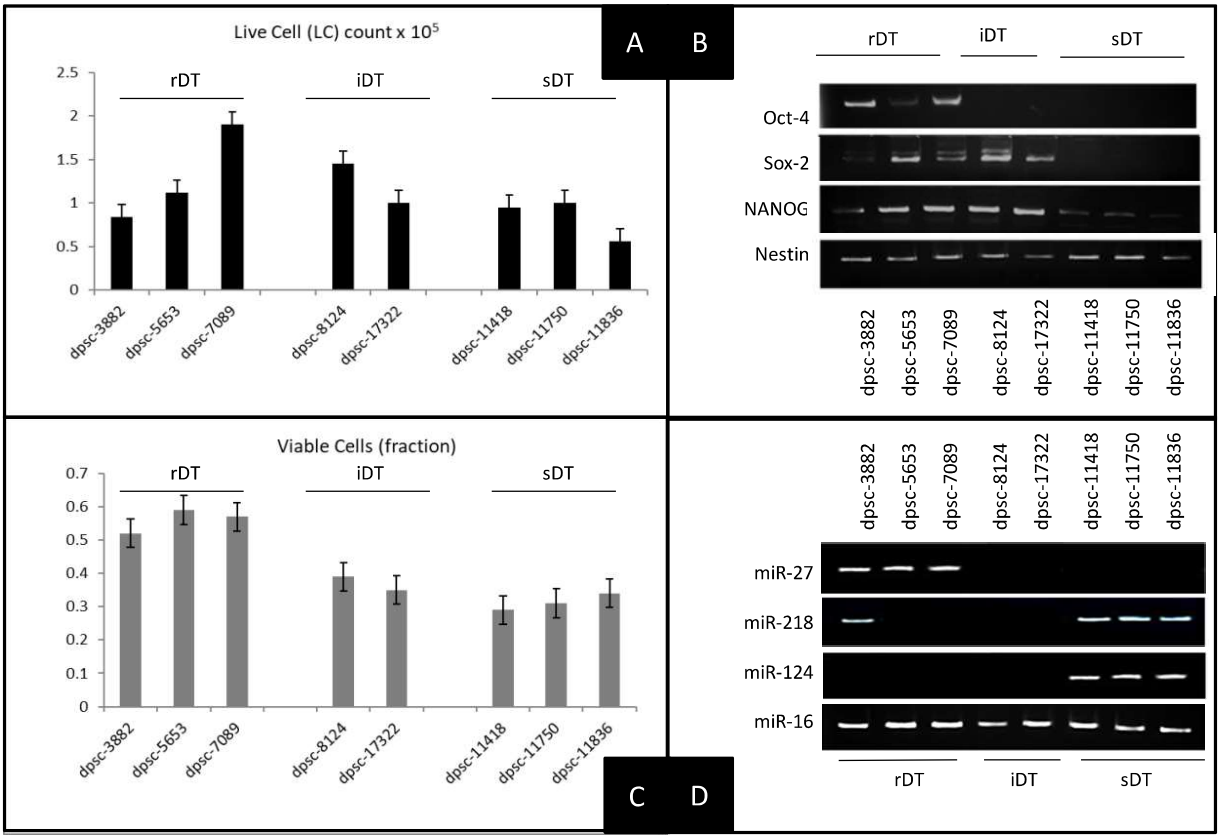

Figure 2. Correlation of DPSC markers with microRNA expression. A) Graphed live cell counts from DPSC isolates demonstrated highest levels among rDT and iDT isolates. B) mRNA expression of DPSC biomarkers revealed Oct-4, Sox-2 and NANOG among rDT isolates (variable) and only Nestin expression among sDT 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

References

1. Lima P, Aboyeji D, Orcharton M. Dental pulp stem cells in regenerative medicine. Br Dent J. 2018 May 4. doi: 10.1038/sj.bdj.2018.348. [Epub ahead of print] PMID: 29725075

2. Shetty H, Kakade A, Shetty S, Neelakantan P, Nagar S, Desai RS, Beri K. Immunohistochemical characterization of stem cell and differentiation markers of the dental pulp of human natal teeth. Future Sci OA. 2018 Oct 4;4(10):FSO342. doi: 10.4155/fsoa-2018-0062. eCollection 2018 Dec. PMID: 30450229 Free PMC Article

3. Liu L, Wei X, Ling J, Wu L, Xiao Y. Expression pattern of Oct-4, Sox2, and c-Myc in the primary culture of human dental pulp derived cells. J Endod. 2011 Apr;37(4):466-72. doi: 10.1016/j.joen.2010.12.012. Epub 2011 Feb 25. PMID: 21419292

4. Kaukua N, Chen M, Guarnieri P, Dahl M, Lim ML, Yucel-Lindberg T, Sundström E, Adameyko I, Mao JJ, Fried K. Molecular differences between stromal cell populations from deciduous and permanent human teeth. Stem Cell Res Ther. 2015 Apr 18;6:59. doi: 10.1186/s13287-015-0056-7. PMID: 25927523

5. Liu L, Wu L, Wei X, Ling J. Induced overexpression of Oct4A in human dental pulp cells enhances pluripotency and multilineage differentiation capability. Stem Cells Dev. 2015 Apr 15;24(8):962-72. doi: 10.1089/scd.2014.0388. Epub 2015 Jan 8. PMID: 25422984

6. Yuan J, Liu X, Chen Y, Zhao Y, Liu P, Zhao L, Han W. Effect of SOX2 on osteogenic differentiation of dental pulp stem cells. Cell Mol Biol (Noisy-le-grand). 2017 Jan 30;63(1):41-44. doi: 10.14715/cmb/2017.63.1.8. PMID: 28234623

7. Huang CE, Hu FW, Yu CH, Tsai LL, Lee TH, Chou MY, Yu CC. Concurrent expression of Oct4 and Nanog maintains mesenchymal stem-like property of human dental pulp cells.

Int J Mol Sci. 2014 Oct 15;15(10):18623-39. doi: 10.3390/ijms151018623. PMID: 25322154

8. Krstic J, Trivanovic D, Obradovic H, Kukolj T, Bugarski D, Santibanez JF. Regulation of Mesenchymal Stem Cell Differentiation by Transforming Growth Factor Beta Superfamily. Curr Protein Pept Sci. 2018;19(12):1138-1154. doi: 10.2174/1389203718666171117103418. PMID: 29150917
9. Huang C, Gou S, Wang L, Huang K, Liu L, Zhao W, Zheng L, Xiao J. MicroRNAs and Peroxisome Proliferator-Activated Receptors Governing the Differentiation of Mesenchymal Stem Cells. Curr Stem Cell Res Ther. 2016;11(3):197-207. Review. PMID: 26018230
10. Martin EC, Qureshi AT, Dasa V, Freitas MA, Gimble JM, Davis TA. MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: Perspectives on miRNA biogenesis and cellular transcriptome. Biochimie. 2016 May;124:98-111. doi: 10.1016/j.biochi.2015.02.012. Epub 2015 Feb 26. Review. PMID: 25726914
11. Sekar D, Saravanan S, Karikalan K, Thirugnanasambantham K, Lalitha P, Islam VI. Role of microRNA 21 in mesenchymal stem cell (MSC) differentiation: a powerful biomarker in MSCs derived cells. Curr Pharm Biotechnol. 2015;16(1):43-8. Review. PMID: 25564252
12. Clark EA, Kalomoiris S, Nolta JA, Fierro FA. Concise review: MicroRNA function in multipotent mesenchymal stromal cells. Stem Cells. 2014 May;32(5):1074-82. Review. PMID: 24860868
13. Collino F, Bruno S, Deregibus MC, Tetta C, Camussi G. MicroRNAs and mesenchymal stem cells. Vitam Horm. 2011;87:291-320. doi: 10.1016/B978-0-12-386015-6.00033-0. Review. PMID: 22127248

14. Sehic A, Tulek A, Khuu C, Nirvani M, Sand LP, Utheim TP. Regulatory roles of microRNAs in human dental tissues. Gene. 2017 Jan 5;596:9-18. doi: 10.1016/j.gene.2016.10.009. Epub 2016 Oct 8. Review. PMID: 27725267

15. Zhang R, Li T. Modulation of microRNAs in Tooth Root and Periodontal Tissue Development. Curr Stem Cell Res Ther. 2018;13(2):118-124. doi: 10.2174/1574888X12666170713123647. Review. PMID: 28707582

16. Jin Y, Wang C, Cheng S, Zhao Z, Li J. MicroRNA control of tooth formation and eruption. Arch Oral Biol. 2017 Jan;73:302-310. doi: 10.1016/j.archoralbio.2016.08.026. Epub 2016 Aug 27. Review. PMID: 27835837

17. Aurrekoetxea M, Garcia-Gallastegui P, Irastorza I, Luzuriaga J, Uribe-Etxebarria V, Unda F, Ibarretxe G. Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues. Front Physiol. 2015 Oct 16;6:289. doi: 10.3389/fphys.2015.00289. eCollection 2015. Review. PMID: 26528190

18. Daltoé FP, Mendonça PP, Mantesso A, Deboni MC. Can SHED or DPSCs be used to repair/regenerate non-dental tissues? A systematic review of in vivo studies. Braz Oral Res. 2014;28. pii: S1806-83242014000100401. Epub 2014 Aug 21. Review. PMID: 25166769

19. Kim BC, Bae H, Kwon IK, Lee EJ, Park JH, Khademhosseini A, Hwang YS. Osteoblastic/cementoblastic and neural differentiation of dental stem cells and their applications to tissue engineering and regenerative medicine. Tissue Eng Part B Rev. 2012 Jun;18(3):235-44. doi: 10.1089/ten.TEB.2011.0642. Epub 2012 Mar 6. Review. PMID: 22224548

20. Seong JM, Kim BC, Park JH, Kwon IK, Mantalaris A, Hwang YS. Stem cells in bone tissue engineering. Biomed Mater. 2010 Dec;5(6):062001. doi: 10.1088/1748-6041/5/6/062001. Epub 2010 Oct 6. Review. PMID: 20924139

21. A Young, K Kingsley . Dental Pulp Stem Cells: A review of factors that influence the therapeutic potential of stem cell isolates. Biomaterials and Biomedical Engineering June 2015, 2(2): 61-69. DOI: 10.12989/bme.2015.2.2.061

22. Tomlin A, Nelson B, Kingsley. K. Dental Pulp Stem Cell Biomarkers for Cellular Viability Following Long-Term Cryopreservation. Int J Cell Sys Dev Biol. 2018, 1(1): 1-6.

23. Whiting M, Kingsley K. Expression of microRNA among dental pulp stem cell (DPSC) isolates. Current Research in Dentistry, 2019. [Online first]. DOI : 10.3844/crdsp.2018.12.18

24. Ma Y, Yao N, Liu G, Dong L, Liu Y, Zhang M, Wang F, Wang B, Wei X, Dong H, Wang L, Ji S, Zhang J, Wang Y, Huang Y, Yu J. Functional screen reveals essential roles of miR-27a/24 in differentiation of embryonic stem cells. EMBO J. 2015 Feb 3;34(3):361-78. doi: 10.15252/embj.201489957. Epub 2014 Dec 17. PMID: 25519956

25. Xue Y, Qian H, Hu J, Zhou B, Zhou Y, Hu X, Karakhanyan A, Pang Z, Fu XD. Sequential regulatory loops as key gatekeepers for neuronal reprogramming in human cells. Nat Neurosci. 2016 Jun;19(6):807-15. doi: 10.1038/nn.4297. Epub 2016 Apr 25. PMID: 27110916

26. Chang K, Chen RS, Chang FH, Chen MH. Promoting dentinogenesis of DPSCs through inhibiting microRNA-218 by using magnetic nanocarrier delivery. J Formos Med Assoc. 2018 Nov 21. pii: S0929-6646(18)30456-X. doi: 10.1016/j.jfma.2018.10.018. [Epub ahead of print] PMID: 30472043

27. Alleman M, Low E, Truong K, Huang E, Hill CK, Chen TY, Deaton M, Kingsley K. Dental pulp-derived stem cells (DPSC) differentiation in vitro into odontoblast and neural progenitors during cell passaging is associated with alterations in cell survival and viability. International Journal of Medicine and Biomedical Research 2013, 2(2): 133-141.

28. Hung E, Lee S, Fitzgerald B, Hill CK, Kingsley K. Dental pulp-derived stem cell (DPSC) survival and viability may correlate with specific patient demographics. Forum for Dental Student Research and Innovation (FDSRI)/ Winter 2013, 1(3): 14-21.

29. Karamzadeh R, Eslaminejad MB, Aflatoonian R. Isolation, characterization and comparative differentiation of human dental pulp stem cells derived from permanent teeth by using two different methods. J Vis Exp. 2012 Nov 24;(69). pii: 4372. doi: 10.3791/4372. PMID: 23208006

30. Bakkar M, Liu Y, Fang D, Stegen C, Su X, Ramamoorthi M, Lin LC, Kawasaki T, Makhoul N, Pham H, Sumita Y, Tran SD. A Simplified and Systematic Method to Isolate, Culture, and Characterize Multiple Types of Human Dental Stem Cells from a Single Tooth. Methods Mol Biol. 2017;1553:191-207. doi: 10.1007/978-1-4939-6756-8_15. PMID: 28229417

31. Cinelli J, Ngueyn E, Kingsley K. Assessment of dental pulp stem cell (DPSC) biomarkers following induction with bone morphogenic protein (BMP-2). Journal of Advances in Biology and Biotechnology, 2018, 19(2): 1-12. Doi:10.9734/JABB/2018/44215

32. Xie H, Dubey N, Shim W, Ramachandra CJA, Min KS, Cao T, Rosa V. Functional Odontoblastic-Like Cells Derived from Human iPSCs. J Dent Res. 2018 Jan;97(1):77-83. doi: 10.1177/0022034517730026. Epub 2017 Sep 12. PMID: 28898112

33. Yang Y, Zhao Y, Liu X, Chen Y, Liu P, Zhao L. Effect of SOX2 on odontoblast differentiation of dental pulp stem cells. Mol Med Rep. 2017 Dec;16(6):9659-9663. doi: 10.3892/mmdlxr.2017.7812. Epub 2017 Oct 17. PMID: 29039570

34. Liu P, Cai J, Dong D, Chen Y, Liu X, Wang Y, Zhou Y. Effects of SOX2 on Proliferation, Migration and Adhesion of Human Dental Pulp Stem Cells. PLoS One. 2015 Oct 23;10(10):e0141346. doi: 10.1371/journal.pone.0141346. eCollection 2015. PMID: 26496354

35. Gay I, Cavender A, Peto D, Sun Z, Speer A, Cao H, Amendt BA. Differentiation of human dental stem cells reveals a role for microRNA-218. J Periodontal Res. 2014 Feb;49(1):110-20. doi: 10.1111/jre.12086. Epub 2013 May 13. PMID: 23662917

36. Gu H, Xu J, Huang Z, Wu L, Zhou K, Zhang Y, Chen J, Xia J, Yin X. Identification and differential expression of microRNAs in 1, 25-dihydroxyvitamin D3-induced osteogenic differentiation of human adipose-derived mesenchymal stem cells. Am J Transl Res. 2017 Nov 15;9(11):4856-4871. eCollection 2017. PMID: 29218085

37. Hao Y, Ge Y, Li J, Hu Y, Wu B, Fang F. Identification of MicroRNAs by Microarray Analysis and Prediction of Target Genes Involved in Osteogenic Differentiation of Human Periodontal Ligament Stem Cells. J Periodontol. 2017 Oct;88(10):1105-1113. doi: 10.1902/jop.2017.170079. Epub 2017 Jun 9. PMID: 28598283

38. Qadir AS, Um S, Lee H, Baek K, Seo BM, Lee G, Kim GS, Woo KM, Ryoo HM, Baek JH. miR-124 negatively regulates osteogenic differentiation and in vivo bone formation of mesenchymal stem cells. J Cell Biochem. 2015 May;116(5):730-42. doi: 10.1002/jcb.25026. PMID: 25424317

39. Qadir AS, Woo KM, Ryoo HM, Yi T, Song SU, Baek JH. MiR-124 inhibits myogenic differentiation of mesenchymal stem cells via targeting Dlx5. J Cell Biochem. 2014 Sep;115(9):1572-81. doi: 10.1002/jcb.24821. PMID: 24733577

40. Mondanizadeh M, Arefian E, Mosayebi G, Saidijam M, Khansarinejad B, Hashemi SM. MicroRNA-124 regulates neuronal differentiation of mesenchymal stem cells by targeting Sp1 mRNA. J Cell Biochem. 2015 Jun;116(6):943-53. doi: 10.1002/jcb.25045. PMID: 25559917

41. Xu JF, Yang GH, Pan XH, Zhang SJ, Zhao C, Qiu BS, Gu HF, Hong JF, Cao L, Chen Y, Xia B, Bi Q, Wang YP. Altered microRNA expression profile in exosomes during osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. PLoS One. 2014 Dec 11;9(12):e114627. doi: 10.1371/journal.pone.0114627. eCollection 2014. PMID: 25503309

42. Wang T, Xu Z. miR-27 promotes osteoblast differentiation by modulating Wnt signaling. Biochem Biophys Res Commun. 2010 Nov 12;402(2):186-9. doi: 10.1016/j.bbrc.2010.08.031. Epub 2010 Aug 12. PMID: 20708603

43. Feng J, Iwama A, Satake M, Kohu K. MicroRNA-27 enhances differentiation of myeloblasts into granulocytes by post-transcriptionally downregulating Runx1. Br J Haematol. 2009 May;145(3):412-23. doi: 10.1111/j.1365-2141.2009.07632.x. Epub 2009 Mar 2. PMID: 19298589

44. Lozano-Velasco E, Contreras A, Crist C, Hernández-Torres F, Franco D, Aránega AE. Pitx2c modulates Pax3+/Pax7+ cell populations and regulates Pax3 expression by repressing miR27 expression during myogenesis. Dev Biol. 2011 Sep 1;357(1):165-78. doi: 10.1016/j.ydbio.2011.06.039. Epub 2011 Jul 1. PMID: 21749861

45. Chen KD, Goto S, Hsu LW, Lin TY, Nakano T, Lai CY, Chang YC, Weng WT, Kuo YR, Wang CC, Cheng YF, Ma YY, Lin CC, Chen CL. Identification of miR-27b as a novel signature from the mRNA profiles of adipose-derived mesenchymal stem cells involved in the tolerogenic response. PLoS One. 2013 Apr 16;8(4):e60492. doi: 10.1371/journal.pone.0060492. Print 2013. PMID: 23613728

46. Kang T, Lu W, Xu W, Anderson L, Bacanamwo M, Thompson W, Chen YE, Liu D. McroRNA-27 (miR-27) targets prohibitin and impairs adipocyte differentiation and mitochondrial function in human adipose-derived stem cells. J Biol Chem. 2013 Nov 29;288(48):34394-402. doi: 10.1074/jbc.M113.514372. Epub 2013 Oct 16. PMID: 24133204

47. Chen SZ, Xu X, Ning LF, Jiang WY, Xing C, Tang QQ, Huang HY. miR-27 impairs the adipogenic lineage commitment via targeting lysyl oxidase. Obesity (Silver Spring). 2015 Dec;23(12):2445-53. doi: 10.1002/oby.21319. Epub 2015 Nov 5. PMID: 26536844

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-R-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
4505 Maryland Parkway . Box 451047 . Las Vegas, Nevada 89154-1047
(702) 895-2794 . FAX: (702) 895-0805 . IRB@unlv.edu

Appendix B

Permission to Use Copyrighted Material

University of Nevada, Las Vegas

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. December 2018, 11(12-18)** hereby give permission for the author to use the above described material in total or in part for inclusion in a Master's thesis at the University of Nevada, Las Vegas.

I also agree that the author may execute the standard contract with ProQuest for storage and reproduction of the completed thesis, including the materials to which I hold copyright

January 29, 2019

Signature	Date
Karl Kingsley, PhD, MPH	Professor
Name (typed)	Title

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

Mark Whiting

Email: whitingmark@me.com

Degrees:

Bachelor of Business Management, 2010
Brigham Young University

Doctor of Dental Medicine, 2016
University of Nevada, Las Vegas

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.
Graduate Coordinator, Brian Chrzan, D.D.S., Ph.D.