

8-1-2013

## Adult Permanent (Extracted) Teeth as a Source of Dental Pulp-Derived Mesenchymal Stem Cells (DPSC): A Pilot Study

Charles Hill

University of Nevada, Las Vegas, nedh@cox.net

Follow this and additional works at: <https://digitalscholarship.unlv.edu/thesesdissertations>



Part of the [Dentistry Commons](#)

---

### Repository Citation

Hill, Charles, "Adult Permanent (Extracted) Teeth as a Source of Dental Pulp-Derived Mesenchymal Stem Cells (DPSC): A Pilot Study" (2013). *UNLV Theses, Dissertations, Professional Papers, and Capstones*. 1931.

<https://digitalscholarship.unlv.edu/thesesdissertations/1931>

This Thesis is protected by copyright and/or related rights. It has been brought to you by Digital Scholarship@UNLV with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you need to obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself.

This Thesis has been accepted for inclusion in UNLV Theses, Dissertations, Professional Papers, and Capstones by an authorized administrator of Digital Scholarship@UNLV. For more information, please contact [digitalscholarship@unlv.edu](mailto:digitalscholarship@unlv.edu).

ADULT PERMANENT (EXTRACTED) TEETH AS A SOURCE  
OF DENTAL PULP-DERIVED MESENCHYMAL STEM  
CELLS (DPSC): A PILOT STUDY

By

Charles Kennedy Hill

Bachelor of Science in Psychology  
Arizona State University  
1992

Doctor of Dental Medicine  
Southern Illinois University  
1996

Master of Public Health  
University of Nevada, Las Vegas  
2011

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  
December 2013**

Copyright by Charles Kennedy Hill, 2013  
All Rights Reserved



**THE GRADUATE COLLEGE**

We recommend the thesis prepared under our supervision by

**Charles Kennedy Hill**

entitled

**Adult Permanent (Extracted) Teeth as a Source of Dental Pulp-Derived Mesenchymal Stem Cells (DPSC): A Pilot Study**

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., Committee Chair

Rhonda Everett, M.P.H., Committee Member

Cliff Seran, D.M.D., Committee Member

James Mah, D.D.S., Committee Member

Timothy Bungum, Ph.D., Graduate College Representative

Kathryn Hausbeck Korgan, Ph.D., Interim Dean of the Graduate College

**December 2013**

## ABSTRACT

### **Adult Permanent (Extracted) Teeth as a Source of Dental Pulp-Derived Mesenchymal Stem Cells: A Pilot Study**

by

Charles Kennedy Hill

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

Mesenchymal stem cells (MSC) may be derived from a variety of adult human tissues, including dental pulp from extracted or exfoliated teeth. Evidence suggests differences in both the quality and quantity of the dental pulp-derived mesenchymal stem cells (DPSC) obtained from different sources, such as primary or “baby teeth” and adult, permanent teeth. This study aimed to evaluate the potential to obtain DPSC from intact, vital permanent teeth, and characterize the potential isolates using a randomized selection of active dental patients from the University of Nevada, Las Vegas School of Dental Medicine (UNLVSDM). DPSC were extracted, isolated, cultured and characterized using microscopy and RT-PCR analysis of extracted RNA. DPSC isolates were derived from 30/31 (96.8%) of tissue explants using the direct outgrowth (DO) method; mainly giving rise to uncommitted MSC progenitors with rapid doubling times (rDT, n=25/30 or 83.3%) and positive mRNA expression of MSC markers CD44, CD24, NANOG, Oct-4 and Sox2. DPSC isolates with slower doubling times (sDT, n=3/30 or 10%) and more limited differentiation potentials resembled neural or odontoblastic progenitor cells (sDT: NPC or sDT: OPC). Cell lines expressed neural differentiation markers CD133 and

$\beta$ III tubulin or the odontoblastic differentiation marker, dentin sialophosphoprotein precursor (DSPP), and had lower survival and viability rates following freezing, long-term storage and thawing. The need to identify potential sources of MSC to better treat age-related illnesses in the current population makes it necessary to more fully explore the feasibility and potential of DPSCs extracted from adult human teeth for this newly developing field of regenerative medicine.

## TABLE OF CONTENTS

|  |     |
|--|-----|
| ABSTRACT .....   | iii |
| TABLE OF CONTENTS.....   | v   |
| CHAPTER 1 INTRODUCTION.....  | 1   |
| Background and Significance.....   | 1   |
| Statement of Purpose .....   | 2   |
| Research Questions and Hypotheses .....  | 3   |
| CHAPTER 2 LITERATURE REVIEW .....  | 4   |
| CHAPTER 3 METHODOLOGY .....  | 9   |
| Research Design and Sampling Procedure... ..                                     | 9   |
| Protection of Human Subjects .....   | 9   |
| DPSC Isolation and Culture.....  | 10  |
| Cell Proliferation and Doubling Time .....                                       | 11  |
| Microscopy of Cell Morphology, Survival, and Viability.....                      | 12  |
| RNA Isolation, Concentration, and Yield.....                                     | 12  |
| Reverse-Transcription Polymerase Chain Reaction (RT-PCR).....                    | 13  |
| Statistical Analysis.....  | 15  |
| CHAPTER 4 RESULTS .....  | 16  |
| Cell Proliferation and Doubling Time .....                                       | 16  |
| Research Question #1 .....   | 18  |
| Research Question #2 .....   | 18  |
| CHAPTER 5 DISCUSSION AND CONCLUSION .....  | 19  |
| Isolation and Characterization of DPSC from Adult Permanent Extracted Teeth..... | 19  |
| Significance to Dentistry at the UNLVSODM .....                                  | 23  |
| Limitations of the Study.....  | 24  |
| Recommendation for Further Research .....  | 26  |
| APPENDICES .....   | 29  |
| Appendix A Figures Related to the Research Questions .....                       | 29  |
| Appendix B Patient Consent Forms.....  | 32  |
| Appendix C Office for Protection of Research Subjects Approval .....             | 36  |
| Appendix D Manuscript.....   | 37  |
| Appendix E In Press Notification .....   | 49  |
| Appendix F Further Research in DPSC De-differentiation and Demographics .....    | 50  |
| BIBLIOGRAPHY .....   | 57  |
| CURRICULUM VITAE.....  | 63  |

## CHAPTER 1

### INTRODUCTION

#### Background and Significance

Stem cells are distinguished from other cells because they may renew themselves through cell division after long periods of inactivity. Stem cells are unspecialized and may give rise to specialized types of cells like chondroblasts or odontoblasts. When a stem cell divides, each new cell remains either a stem cell or becomes a different type of cell with a more specialized function like a muscle, blood, or brain cell. Stem cells are able to replenish other cells as long as the body is still alive. There are two types of stem cells, embryonic and adult. In 1981, embryonic stem cells were first isolated from mice and in 1998 they were isolated from human embryos and grown in a laboratory setting [1]. Adult stem cells are multipotent while embryonic stem cells are pluripotent. Adult stem cells are less controversial in terms of their source than embryonic stem cells but they are unable to differentiate into as many cell types as embryonic stem cells. Adult stem cells can become adipocytes, chondroblasts, osteoblasts, myocytes, neural cells, and fibroblasts. Embryonic stem cells may become all cell types in the human body. Adult stem cells have been found in many tissues including the brain, heart, gut, liver, blood vessels, skeletal muscle, and teeth [2,3]. Teeth represent a relatively accessible source of stem cells compared to other tissues.

Stem cell research has shown promise in furthering our understanding of their isolation, storage, and use for future regenerative medicine. Many debilitating diseases such as diabetes, multiple sclerosis, and heart disease affect human beings as the aging process occurs. Because of their unique regenerative abilities, stem cells offer



replacement for cells lost through normal wear, injury, or disease. Adult stem cells are found in various tissues throughout the body to maintain or repair the tissue in which they reside. Science is just beginning to understand stem cell differentiation and the signals involved inside and outside the cells that trigger each step of the process. Control of adult stem cell differentiation on the laboratory setting is the basis for future transplantation-based therapies [1-3].

This research project examined whether dental pulp-derived mesenchymal stem cells could be isolated from adult permanent extracted teeth and if so, could the potential isolates be characterized.

#### Statement of Purpose

One purpose of this research project was to elucidate the potential to obtain DPSC from intact, vital permanent teeth of adult patients. A randomized selection of active dental patients requiring extractions were sampled and DPSC were extracted, isolated, cultured and characterized. The other purpose was to see if the potential DPSC isolates could be characterized with regard to their morphology, confluence and doubling time, and mRNA expression.

Some patients who seek care at the University of Nevada, Las Vegas School of Dental Medicine (UNLVSODM) clinics have one or more teeth extracted to aid in their dental treatment. It was anticipated that a certain percentage of dental pulp from extracted permanent teeth would yield stem cells and those stem cell isolates would vary in morphology, confluence and doubling time, and mRNA expression. Information from the study will be used to develop further research questions regarding potential uses of adult permanent extracted teeth from dental school patients at the UNLVSODM. This

will aid the development of school protocol and patients will be better informed of their options should they be interested in storage of dental pulp from their extracted teeth for possible future use in regenerative medicine.

### Research Questions and Hypotheses

1. Can dental pulp-derived mesenchymal stem cells (DPSC) be isolated with >50% viability from adult permanent extracted teeth?

H<sub>0</sub>: No, DPSC cannot be isolated with >50% viability from adult permanent extracted teeth.

H<sub>A</sub>: Yes, DPSC can be isolated with >50% viability from adult permanent extracted teeth.

2. Can the potential DPSC isolates be characterized regarding morphology, confluence and doubling time, and mRNA expression?

H<sub>0</sub>: No, potential DPSC isolates cannot be characterized regarding morphology, confluence and doubling time, and mRNA expression.

H<sub>A</sub>: Yes, potential DPSC isolates can be characterized regarding morphology, confluence and doubling time, and mRNA expression.

## CHAPTER 2

### LITERATURE REVIEW

Mesenchymal stem cells (MSC), or multipotent progenitors, may be derived from a variety of adult human tissues. Recent clinical studies demonstrate that dental pulp may provide a rich supply of multipotent, highly proliferative MSCs capable of regenerating a variety of human tissues including, but not limited to, bone and dental structures [4-6]. Dental-pulp derived stem cells (DPSC) may differentiate into many different lineages, including osteoblasts, chondroblasts, adipocytes, as well as vascular and neural tissues [7,8]. Only recently has research involving human dental pulp stem cells been established in the literature. In 2000, Gronthos et al. published work using dentin sialophosphoprotein precursor (DSPP) as a stem cell marker to differentiate between osteoblasts and their stem cell progenitors [9]. One of the ultimate goals of their research was tooth regeneration to replace lost natural teeth. Their existing knowledge of dentin sialophosphoprotein (DSP) and its ability to detect odontoblasts combined with the unique capability of dental pulp cells to form reparative dentin *in vivo* led them to query if dental pulp contained stem cells. The DPSC were identified on the basis of their ability to form single colonies in culture, self-renew *in vivo*, and multi-differentiate *in vitro* [9]. One of the most important advances in DPSC research came in 2003 when Shi and Gronthos revealed the presence of a stem cell niche in the perivascular region of the pulp [9]. This allowed researchers to better understand the location of the stem cells in tissue. In 2005, Shi et al. described techniques using cell markers, stromal-derived factor 1 (STRO-1), vascular cell adhesion molecule 1 (VCAM-1), and melanoma-associated antigen/mucin 18 (MUC-18) to characterize *in vitro* stem cell populations of smooth

muscle cells [9]. Since then, more cell markers like CD24, CD44, CD133, NANOG, Oct-4, and Sox-2 have been used to identify DPSC in laboratory settings *in vitro* [2,5,8]. Recently, banking of dental pulp stem cells has become an option for individuals wishing to save their samples for possible future use in regenerative medicine. National Dental Pulp Lab and Genecell are two organizations which provide such services.

Mesenchymal stem cells (MSC) are resident in, and may be obtained from, a variety of adult tissues [10,11]. Many studies have demonstrated waste tissue from medical procedures such as liposuction and childbirth can yield MSC, although harvesting may be costly and involve controversial ethical considerations when obtained from *in vitro* fertilization (IVF) or other embryonic sources [12,13]. However, recent clinical studies have demonstrated that dental pulp may also provide a rich supply of multi-potent, highly proliferative MSCs obtained less invasively, more cost effectively, and with fewer ethical considerations [4-8].

DPSC originate during human development when embryonic cells migrate from the neural crest to strengthen mesenchyme of the head and neck. During the sixth week of development, the ectoderm covering the stomodeum begins to proliferate and development of the dental laminae begins. A thick ectodermal structure develops into tooth germs where cells from the neural crest will differentiate into the dental germ containing dental papilla and follicle. Dental pulp is composed of ecto-mesenchymal structures containing neural crest-derived cells and multipotent stem cells [2].

The dental pulp chamber acts as a sealed container for DPSC. Stem cells reside in a specific area or niche of the dental pulp and remain quiescent for long periods of time until activated to replenish old cells due to injury, disease, or normal wear [5]. DPSC

may represent less than 1% of cell populations but are capable of producing multiple specialized cells in response to extracellular signaling. In order to be considered a stem cell, the cell must possess the abilities to produce a genetically identical cell (even after years of inactivity), and differentiate into a more specialized type of cell like a chondroblast or osteoblast [14]. DPSC are capable of regeneration of a dentin-pulp like complex with a mineralized matrix containing odontoblasts and fibrous tissue similar to the dentin-pulp complex found in teeth [14]. One goal for DPSC in regenerative medicine is the ability to re-grow lost natural teeth.

Many factors influence both the quantity and quality of DPSC tissue available for storage and future clinical or bioengineering applications. One of the primary factors is the tooth type or source of dental pulp. DPSC derived from human exfoliated deciduous teeth (SHED) or primary teeth may have substantially higher growth and differentiation potential, as well as greater survival rates after freezing and storage when compared with DPSCs derived from vital, permanent adult teeth [14-16]. Commercial companies, such as National Dental Pulp Lab ([ndpl.net](http://ndpl.net)) and GeneCell ([genecell.com](http://genecell.com)), now offer services for banking and long-term storage of dental pulp from primary teeth (SHED), focused primarily on parents with young children.

Other factors influence tissue quality and quantity, including specific methods used to isolate, collect, concentrate and store DPSCs. Some research has suggested the isolation of DPSCs by enzymatic dissociation (DPSC-ED) produces heterogeneous populations of faster-growing cells while the isolation technique of direct outgrowth (DPSC-OG) gives rise to largely homogenous populations, but with more limited differentiation potential [17,18]. Recent advances in cryobanking, cryopreservation, and storage of dental pulp

have significantly increased the recovery and long-term quality of dental pulp tissues – thereby increasing the possibility of new sources of MSCs for future clinical applications [19,20]. Two studies demonstrated cryopreservation of DPSC for two years. The resultant stem cells retained their multipotent differentiation potential and became osteoblasts [2,21]. Other studies have shown the choice of low- or no-serum media and the selection of early- versus late-passage populations for storage mediate the differentiation potential and proliferative capacity of DPSC isolates [22-24].

Once recovered from cryopreservation, the challenge to direct DPSC differentiation is keeping researchers busy. Several recent experiments have elucidated *in vitro* processes to direct uncommitted (and partially committed) DPSC isolates to differentiate using methods of cell-matrix adhesion molecules, growth factors, biomechanical scaffolding, tension and pressure [25,26]. DPSC migration and differentiation may be directed using extracellular matrix (ECM)-coated culture materials, including fibronectin, laminin, collagen, and fluorapatite [27-29]. The use of bioscaffolding, tension and pressure to induce DPSCs toward specific differentiated phenotypes has been elucidated [30-33]. Biochemical stimulation using growth factors to induce DPSCs into differentiated phenotypes, including insulin-transferrin-sodium selenite supplement (ITS), bone morphogenic protein 2 (BMP2), growth differentiation factor 11 (Gdf11), platelet-derived growth factor (PDGF-AB), transforming growth factor (TGF- $\beta$ 1), dexamethasone, and basic fibroblast growth factor (bFGF) has been shown [34-40].

The recent nature of these discoveries of DPSC from permanent teeth means our adult population with age-related diseases (for which DPSC therapy could be useful) may be aided by obtaining DPSC from their permanent teeth as primary teeth. Science is still

learning better methods to isolate, store, and manipulate dental pulp stem cells for use in patients that have debilitating conditions. Despite the growing body of evidence suggesting the potential for clinical applications and possible therapies, much remains to be discovered about the potential of DPSC from permanent teeth.

## CHAPTER 3

### METHODOLOGY

#### Research Design and Sampling Procedure

The design of the study was prospective and experimental. Subjects were randomly recruited by members of the UNLVSODM clinic during their dental visits between February and June, 2010. Informed consent was required and was conducted onsite. Inclusion criteria: subjects between eighteen (18) and sixty-five (65) years old who agreed to participate. In addition, all potential subjects must have sound, unrestored, vital teeth, requiring one or more extractions necessary for oral health, as determined by the clinical faculty member in charge. Exclusion criteria: Any subject under eighteen (18) or over sixty-five (65) years of age, subjects having dental extractions involving compromised pulp tissue, and any subject who refused to donate his or her extracted teeth. All patients who met the inclusion criteria were selected for the study.

Research questions were: 1. Can dental pulp-derived mesenchymal stem cells (DPSC) be isolated with >50% viability from adult permanent extracted teeth? 2. Can the potential DPSC isolates be characterized regarding morphology, confluence and doubling time, and mRNA expression?

Predictor Variables included: cell viability, morphology, confluence and doubling time, and mRNA expression. Outcome Variables included: presence of DPSC in dental pulp.

#### Protection of Human Subjects

The protocol for this study, "Isolation of Non-Embryonic Stem Cells from Dental Pulp" at the University of Nevada, Las Vegas - School of Dental Medicine (UNLV-



SODM) dental clinic was filed, amended, and approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#0907-3148) on February 5, 2010 (Appendix D). The author, Charles Kennedy Hill, was the lead clinical dental faculty member in charge of collection of dental pulp samples. Subjects were randomly recruited by members of the UNLVSODM clinic during their dental visits between February and June, 2010. Informed Consent was required and was conducted onsite. Patients were at minimal risk during the study. Chart numbers were used as identifiers instead of patient names. Patients were not contacted by any individual at the dental school to participate in the study and only the faculty advisor and graduate student researcher had access to the data. Data were stored in the researcher's computer and were maintained safely under password protection from other individuals.

Benefits from patient participation in the study included knowledge gained regarding the availability of mesenchymal stem cells across a segment of the dental school population. Results from the study may be shared with colleagues of the researcher to educate about dental pulp stem cells in the school's patient population and options for patients who have teeth extracted as part of their dental treatment. Protocol for informing patients of storage options for their extracted teeth may be developed.

#### DPSC Isolation and Culture

Dental pulp was extracted from the vital teeth of healthy adults who agreed to participate. The majority of the teeth included in this study were extracted due to severe periodontal disease, necessity for fabrication of complete dentures, and impaction or crowding (e.g., third molars or premolars for orthodontic reasons). Following extraction, teeth were placed into sterile solution and transported to the laboratory for sectioning.

Teeth were sectioned axially at the cemento-enamel junction (CEJ) using a diamond rotary disc in a dental handpiece and dental pulp removed with an endodontic broach. Dental pulp was immediately placed into sterile microcentrifuge tubes containing 1X phosphate buffered saline (PBS) and transferred to the laboratory for culture; any dental pulp not transferred within two hours was removed from the subsequent analysis. Tubes were pre-assigned a unique, randomly-generated number to prevent research bias. Demographic information regarding each sample was collected, which consisted of age, gender, race, and tooth type.

Dental pulp samples brought to the laboratory for culture were processed using direct outgrowth. Extracted dental pulp was vortexed for 10 – 30 seconds to dislodge cells and centrifuged for five (5) minutes at 2,100 relative centrifugal force (RCF) or *g*. Supernatant (PBS) was aspirated from the tube and dental pulp-derived cells were re-suspended in 1.0 mL of RPMI-1640 medium from Hyclone (Logan, UT) with 2mM L-Glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 µg/mL) solution and 10% fetal bovine serum (FBS), obtained from HyClone (Logan, UT). Cells were cultured in 75 cm<sup>2</sup> BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO<sub>2</sub> in humidified chambers. Media was changed every 48 hours until adherent cells reached 70% confluence. Cells were subsequently passaged at a 1:4 ratio.

#### Cell Proliferation and Doubling Time

Cell proliferation assays were performed in the appropriate complete media, as described above. In brief, cells reaching 70-80% confluence were trypsinized and plated

1:4 into new 75 cm<sup>2</sup> BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO<sub>2</sub> in humidified chambers and their confluence measured with a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). Doubling times (DT) from passages one (P1) through ten (P10) for each flask were recorded. Averages from the first five passages (P1-5), last five passages (P6-10) and overall average DT (P1-10) were then calculated. Data were analyzed and graphed using Microsoft Excel (Redmond, WA).

#### Microscopy of Cell Morphology, Survival, and Viability

During the process of passaging cells, small aliquots of trypsinized cells were stained using Trypan Blue (Sigma: St. Louis, MO), and live cells were enumerated by counting the number of Trypan-blue negative cells using a VWR Scientific Counting Chamber (Plainfield, NJ) and a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). Images were captured at 200X magnification with a Canon PowerShot G6 digital camera (Tokyo, Japan) and subsequently processed using Adobe Photoshop (San Jose, CA) Image Analysis tools. DPSCs from each passage were frozen for storage using a commercially available cryopreservation medium (Opti-Freeze) from Fisher Scientific (Fair Lawn, NJ), containing Dimethyl Sulfoxide (DMSO), using the procedure recommended by the manufacturer. Following six weeks in storage at -80°C, cells were thawed, re-suspended in the appropriate media, and live cells enumerated, as described above.

#### RNA Isolation, Concentration, and Yield

To determine if any cells derived from dental pulp were dental pulp stem cells (DPSC), RNA was isolated from  $1.5 \times 10^7$  cells of each of the experimental cell lines, using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) in accordance with the

procedure recommended by the manufacturer for RT-PCR analysis. RNA concentration and purity were calculated using UV spectroscopy. The absorbance of diluted RNA samples (10  $\mu$ L of RNA sample in 490  $\mu$ L nuclease-free water, pH 7.0) was measured at 260 and 280 nm. RNA purity was determined by calculating the ratio of A260:A280, which should be > 1.80. Concentration for RNA samples was determined by the A260 reading of 1 = 40  $\mu$ g/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water. Concentration was calculated as 40 x A260 absorbance measure x dilution factor (50). Total yield was determined by concentration x sample volume in mL.

Example: RNA standard

$$A_{260} = 0.75$$

$$\text{Concentration} = 40 \times 0.75 \times 50 = 1,500 \mu\text{g/mL}$$

$$\text{Yield} = 1,500 \mu\text{g/mL} \times 1.0 \text{ mL} = 1,500 \mu\text{g} \text{ or } 1.5 \text{ mg RNA}$$

#### Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

To quantify the expression of DPSC-specific mRNA, RT-PCR was performed on total RNA using the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following mesenchymal stem cell (MSC) primers synthesized by SeqWright (Houston, TX):

*ALP* FORWARD:            CACTGCGGACCATTCCCACGTCTT;

*ALP* REVERSE:            GCGCCTGGTAGTTGTTGTGAGCATA;

$\beta$ III TUBULIN FORWARD:    CTGCTCGCAGCTGGAGTGAG;

$\beta$ III TUBULIN REVERSE:    CATAAATACTGCAGGAGGGC;

*c-myc* FORWARD: TCCAGCTTGTACCTGCAGGATCTGA;  
*c-myc* REVERSE: CCTCCAGCAGAAGGTGATCCAGACT;  
*CD24* FORWARD: ACTCTCACTTGAAATTGGGC;  
*CD24* REVERSE: GCACATGTTAATTACTAGTAAAGG;  
*CD44* FORWARD: GAAAGGCATCTTATGGATGTGC;  
*CD44* REVERSE: CTGTAGTGAAACACAACACC;  
*CD133* FORWARD: CTCATGCTTGAGAGATCAGGC;  
*CD133* REVERSE: CGTTGAGGAAGATGTGCACC;  
*DSPP* FORWARD: CAACCATAGAGAAAGCAAACGCG;  
*DSPP* REVERSE: TTTCTGTTGCCACTGCTGGGAC;  
*GAPDH* FORWARD: ATCTTCCAGGAGCGAGATCC;  
*GAPDH* REVERSE: ACCACTGACACGTTGGCAGT;  
*NANOG* FORWARD: GCTGAGATGCCTCACACGGAG;  
*NANOG* REVERSE: TCTGTTTCTTGACTGGGACCTTGTC;  
*Oct4* FORWARD: TGGAGAAGGAGAAGCTGGAGCAAAA;  
*Oct4* REVERSE: GGCAGATGGTCGTTTGGCTGAATA;  
*Sox2* FORWARD: ATGGGCTCTGTGGTCAAGTC;  
*Sox2* REVERSE: CCCTCCCAATTCCCTTGTAT;

One  $\mu\text{g}$  of template (total) RNA was used for each reaction. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C. Thirty-five amplification cycles were run, consisting of 20-second denaturation at 94°C, 30 seconds of annealing at 58°C, and 6.5 minutes of extension at 72°C. Final extension was run for 5 minutes at 72°C. Reaction products were separated by gel electrophoresis using

Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY). Quantification of RT-PCR band densitometry and relative mRNA expression levels were performed using Adobe Photoshop (San Jose, CA) imaging software, Image Analysis tools.

#### Statistical Analysis

The differences between passages were measured using a *t* distribution,  $\alpha = 0.05$ . All samples were analyzed using two-tailed *t*-tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed *t*-test [41]. As long as the sample size is at least moderate (>20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses.

## CHAPTER 4

### RESULTS

#### Cell Proliferation and Doubling Time

Thirty-one (31) individual dental pulp samples were collected from twenty-four (24) different University of Nevada, Las Vegas School of Dental Medicine patients in the dental clinic between February and June, 2010 and processed for cell culture using direct outgrowth (DPSC-OG), as described. This resulted in thirty DPSC isolates, with at least one from each patient, yielding an overall success rate greater than 50% percent ( $n = 30/31$  or 96.8%).

During the initial growth phase (P0) each potential DPSC isolate reached 70% confluence or greater between 2.5 – 10.25 days. Average doubling time (DT) for the initial five passages P1 – 5 of each potential cell line was then established and calculated, revealing a characteristic average doubling time (DT) that varied from 2.5 to 10.25 days (Figure A1). The vast majority ( $n=27/30$  or 90%) exhibited a rapid DT (rDT), ranging from 2.5 to 4 days, while the remainder ( $n=3/30$  or 10%) exhibited slower DT (sDT) that ranged from 8 to 10.25 days. Although the DT remained fairly constant for most DPSC isolates, two cell lines exhibited a temporal decrease in DT observed between passages P6-10, resulting in an intermediate DT (iDT) of 5.4 and 6.4 days. The proliferation of iDT isolates was significantly faster than that of the sDT cells ( $p = 0.04$ ) but this change was not sufficient to be significantly different than rDT isolates ( $p = 0.11$ ).

To characterize these potential DPSC isolates, microscopy was performed (Figure A2). Analysis revealed cells with the fastest growth rates (rDT) had similar morphologies, which changed little during long-term passaging and culture; forming

tightly-packed dense colonies of flat, spindle-shaped cells commonly associated with uncommitted progenitor cells (UCP) (Figure A2 A). Both potential DPSC isolates that exhibited a temporal decrease to a more intermediate doubling time (iDT) appeared to have morphologies similar to rDT cells (Figure A2 B), although cell-cell junctions and cell boundaries appeared slightly more distinct. Cells with the slowest growth rates (sDT) gradually adopted more distinctive morphologies of long, axon-like projections suggesting the formation of a sub-population of neuronal progenitor cells (NPC) (Figure A2 C) or large, ovoid-shaped cells that appear to suggest to the formation of odontoblast progenitor cells (OPC) (Figure A2 D).

To provide more qualitative and quantitative assessments of these potential DPSC isolates, RNA was successfully isolated from all DPSC cell lines and RT-PCR was performed on equal concentrations of total RNA from each cell line (Figure A2 E). Expression of mRNA for mesenchymal stem cells (MSC) markers CD44, CD24, NANOG, Oct-4, and Sox2 was observed in all rDT and iDT cell isolates, but varied among sDT isolates. For example; sDT:OPC isolates expressed comparatively low levels of Oct-4, CD44, and NANOG, as well as the cell-cycle marker c-myc. They also expressed the odontoblast progenitor differentiation marker dentin sialophosphoprotein precursor (DSPP) but not alkaline phosphatase (ALP). In addition, sDT: NPC isolates did not express NANOG or CD44, but did express the neural progenitor markers CD133 and  $\beta$ III tubulin.

Any change in viability during cell passaging and culture was evaluated before and after freezing and storage (Figure A3). Results demonstrate the viability among rDT and iDT isolates remained consistently high, although a slight non-significant drop in



viability was apparent following freezing, storage and thawing ( $p > 0.05$ ). Viability of cells during P1 -10 was slightly, but not significantly, lower among sDT isolates, however these isolates exhibited a significant drop in viability following freezing and storage (sDT:ncp -32%, sDT:ocp – 29%,  $p < 0.01$ ).

#### Research Question One

The first research question was: Can dental pulp-derived mesenchymal stem cells (DPSC) be isolated with >50% viability from adult permanent extracted teeth? Yes, DPSC can be isolated from adult permanent extracted teeth with >50% viability.

#### Research Question Two

The second research question was: Can potential DPSC isolates be characterized regarding morphology, confluence and doubling time, and mRNA expression? Yes, potential DPSC isolates can be characterized regarding morphology, confluence and doubling time, and mRNA expression.

## CHAPTER 5

### DISCUSSION AND CONCLUSION

#### Isolation and Characterization of DPSC from Adult Permanent Extracted Teeth

Dental pulp-derived mesenchymal stem cells (DPSC) have the ability to self-renew and a multi-lineage potential in regenerative medicine [3]. This area of science is attracting noted attention due to the therapeutic potential of stem cells for the repair and replacement of tissues and organs that have lost function due to disease, aging, damage and defects. Adult stem cells (like DPSC from extracted permanent teeth) are less controversial in their isolation than embryonic stem cells and are the only dental stem cell source available for many adults who have not banked and stored their primary teeth.

The goals of this research project were to elucidate the potential to obtain DPSC from intact, vital permanent teeth of adult patients and characterize these potential isolates. The results demonstrate the feasibility of extracting, isolating and culturing DPSC isolates from intact vital permanent teeth derived from adult patients, with a success rate exceeding 50% ( $n = 30/31$  or 96.8%). The findings agree with previous studies that permanent teeth are a source of MSC uncommitted progenitors as well as cells forming tooth-like progenitors (sDT:OPC) [4-6].

The average doubling time (DT) for the initial five passages P1 – 5 of each potential cell line varied from 2.5 to 10.25 days (Figure A1). Despite this range, 90% of isolates reached the 70% level of confluence in 2.5 – 4 days (rapid doubling time or rDT). It is of interest to note these 90% of isolates in the rDT category also exhibit the lowest percentage reduction in viability after storage (Figure A3) of the three different doubling time categories of isolates (rDT, iDT, sDT). During passages P1 – 10, the rDT isolates

reduced in viability by only 3%. After storage using a cryopreservation medium containing Dimethyl Sulfoxide (DMSO) for six weeks at  $-80^{\circ}\text{C}$ , the thawed cells were re-suspended in the appropriate media, and live cells enumerated. The rDT isolates experienced only a 5% reduction in viability after cryopreservation which when compared to their reduction during passaging P1 – 10 of 3%, was not statistically significant ( $p = 0.18$ ). The 10% of remaining isolates in the slow doubling time (sDT) category ranged from 8 to 10.25 days to reach 70% confluence during passaging P1 – 10 and demonstrated a 5% - 6% reduction in viability. But after cryopreservation, they showed a reduction in viability by 29% - 32% which was statistically significant ( $p < 0.01$ ). These data confirm the previous observations that some DPSC isolates, particularly those that with slower growth rates and more limited differentiation potential (sDT:npc or sDT:opc), are less able to survive freezing, long-term storage and thawing for subsequent use; with a reduction of available cells by nearly 33%.

Two cell lines exhibited a temporal decrease in DT observed between passages P6-10, moving from the initial rDT category for passages P1 – 5 to an intermediate doubling time category (iDT) of 5.4 and 6.4 days. Viability of isolates in these two cell lines were reduced by 3% during passaging P1 – 10 but only reduced by 7% after cryopreservation which was not statistically significant ( $P = 0.12$ ). The proliferation of iDT isolates was significantly faster than that of the sDT cells ( $p = 0.04$ ) but was not significantly different than the rDT isolates ( $p = 0.11$ ). Although iDT cell lines may be headed toward sDT sooner than the other rDT cell lines, their viability has yet to be negatively reduced like the sDT cell lines. It is unknown when or if they will reach sDT status. It would be of interest to analyze the variables including age, gender, race, and tooth type to see if any

significant relationships in the three cell doubling time categories (rDT, iDT, sDT) were present.

Microscopy of the cell lines revealed cells with the fastest growth rates (rDT) had similar morphologies with tightly-packed dense colonies of flat, spindle-shaped cells commonly associated with uncommitted progenitor cells (UCP) (Figure A2 A). This is encouraging for future use of the DPSC-OG method of stem cell isolation as evidence from previous studies suggested direct outgrowth from tissue explants gave rise to slow-growing isolates with more limited differentiation potential, but in fact, data from this study suggested that many DPSC isolates derived from this method are rapidly proliferating uncommitted MSC progenitors (n=25/30 or 83.3%) [14,15,17,18]. Uncommitted MSC progenitor cells are preferred over cells that have already differentiated or partially differentiated. They offer greater potential to become cells the donor may need in the future. They also keep the cell line alive as uncommitted MSC progenitors may still be passaged to self-renew and form more stem cells. It was reported these cells may differentiate up to 35 population doublings *in vitro* [1].

Cells in the intermediate doubling time (iDT) category appeared to have flat, spindle-like shape similar to the rDT cells (Figure A2 B), although cell-cell junctions and cell boundaries appeared slightly more distinct. This was expected as the two groups showed similar rates of viability during passaging P1 – 10 and after cryopreservation. It appeared the two cell lines slowed in their doubling time but are not yet distinctly different cells than the rDT group. This is encouraging as they appear to be uncommitted MSC progenitors (which are preferred over cells that have begun differentiating).

Cells with the slowest growth rates (sDT) had different morphologies than the rDT and iDT groups. Long, axon-like projections suggestive of a sub-population of neuronal progenitor cells (sDT:NPC) (Figure A2 C) and large, ovoid-shaped cells appeared suggestive of odontoblast progenitor cells (sDT:OPC) (Figure A2 D). Encouraging was the plasticity of these cell lines which showed the ability to differentiate into a specialized cell type different from their tissue of origin. Future research may evaluate when the signals for differentiation may be controlled by the researcher to manipulate the cell into a specific cell type. Dental pulp is a source of uncommitted progenitor cells and knowing these cells are capable of becoming something other than osteoblastic or odontoblastic in nature is encouraging. The disappointing side of these sDT cell lines is their spontaneous differentiation has rendered them unable to continue the stem cell line. Their value for storage may also have decreased as they are more specialized cell types that showed decreased viability after storage and thawing in this study.

RNA was successfully isolated from all DPSC cell lines and RT-PCR was performed on equal concentrations of total RNA from each cell line (Figure A2 E). A total of eleven markers were used in this study. NANOG, Oct-4, and Sox-2 are transcription factors present on undifferentiated stem cells whose function is to allow stem cells to maintain their self-renewal capabilities. ALP is alkaline phosphatase which is located on stem cell membranes.  $\beta$ III Tubulin is a microtubule expressed in neural progenitor cells. C-myc codes for a transcription factor and is involved in stem cell proliferation. CD24 is a cell adhesion molecule present in neuroblastic cells. CD44 is a cell surface glycoprotein and is present in most cell types. CD133 is also a glycoprotein found in neural and hematopoietic cells. Dentin sialophosphoprotein (DSPP) is involved in tooth

development and is an odontoblastic progenitor. Finally, Glyceraldehyde 3-phosphate dehydrogenase (GADPH) is a transcription activator involved in the breakdown of glucose. These markers were chosen to detect undifferentiated stem cells as well as specialized cell types like neural and odontoblastic cells.

Expression of mRNA for mesenchymal stem cells (MSC) markers CD44, CD24, NANOG, Oct-4, and Sox2 was observed in all rDT and iDT cell isolates, but varied among sDT isolates. This was expected as rDT and iDT cell lines show minimal difference in their viability and morphology whereas sDT cell lines differed in both areas. The sDT cell lines showed different expression of different markers than the rDT and iDT cell lines and this matches the above findings. All cell lines expressed markers for MSC and this helped greatly toward confirmation of the presence of stem cells in the samples.

#### Significance to Dentistry at the UNLV SODM

The University of Nevada, Las Vegas School of Dental Medicine opened in 2002. Clinical treatment of patients began in 2004. Thousands of patients have been treated at the school presenting with a variety of dental diseases. This study analyzed findings regarding the isolation and characterization of dental pulp stem cells from extracted permanent teeth of dental patients. They will be used to develop future research projects within the dental school setting to further understanding of how to manage stem cells for future use and to make recommendations regarding stem cell storage for patients who plan to have teeth extracted at the school's clinic.

The dental school has the resources and commitment to provide dental care for patients in its clinic. Better understanding of the patient population by the school's

faculty and students should help increase the level of care delivered. Interaction between researchers, faculty and students allows for new clinic protocol to be implemented in the curriculum based on the increased understanding from studies performed at the school.

Various commercial entities like Genecell and National Dental Pulp Lab are now offering services which include processing and storage of dental pulp or DPSCs from exfoliated primary teeth or extracted permanent teeth. Less information is known about the viability and applications of DPSC. To provide evidence-based recommendations for patients (and parents) interested in banking teeth for future possible usage more research is needed. The initial processing fee combined with monthly storage fees now suggests that patients, or parents of these patients, may incur costs exceeding thousands of dollars for long-term storage before the DPSC are needed.

#### Limitations of the Study

Patient age, gender, race, and tooth type may have affected the results. Characteristics like cell viability may have been influenced by the age of the patient in which the extracted teeth were obtained. This was explored further in research related to this study [46]. In previous studies, primary teeth from patients under age 12 show higher numbers, growth, and differentiation potential of DPSC derived from human exfoliated deciduous teeth (SHED), as well as greater survival rates after freezing and storage when compared with DPSCs derived from vital, permanent adult teeth [15-17].

The method to obtain the DPSC involved a high-speed dental handpiece with a diamond disk for cutting the coronal portion of the crown from each tooth. Heat and debris during this process may have both affected the composition and viability of the extirpated dental pulp tissue. An alternative method to access pulp chambers involved

the use of cowhorn forceps to crack the tooth into pieces [42]. This method would have eliminated the heat and slurry of micro-particles that have the potential to contaminate the pulp samples. Damage to dental pulp is minimized as the tips of the forceps do not touch so the tooth is fractured but not the pulp.

The use of RT-PCR with cytometric markers such as CD44, NANOG, and Oct-4 aided in determination whether stem cells were present or not. Despite the positivity or negativity of the expression of the markers during cell analysis, it cannot be completely confirmed that stem cells were indeed present. The probability of stem cell presence and accuracy of the results reported were increased greatly with the use of multiple markers on multiple samples but do not 100% guarantee the presence of stem cells from dental pulp samples.

The method to culture and passage cells was direct outgrowth (DPSC-OG). Another method is enzymatic dissociation. It has been shown that enzymatic disassociation (DPSC-ED) yields different lineages in regards to phenotypic and differentiation characteristics [43]. Higher proliferation rates have been found with DPSC-ED as well as more heterogeneous cell populations. If used in this study, DPSC-ED may have yielded different results in dental pulp stem cell isolation and characteristics. Ultimately one method of cell isolation and culture was chosen for economic and convenience reasons.

Because the above mentioned limitations existed in the study, results indicate only if it is possible to isolate dental pulp-derived stem cells and discover some of their basic characteristics. It does not elucidate the ability to store stem cells long-term and recover them from cryopreservation for future use. The study was intended to be a pilot study and provide initial results and cell lineage for future research regarding viability of DPSC



after storage. The statistics run were basic and intended to be used to guide further research in the area if indeed a relationship did exist. The external validity of the study was lowered as only patients of the University of Nevada, Las Vegas School of Dental Medicine were included. The sample of 31 patients may or may not be an accurate representation of all dental patients especially as their ages were limited to 18 – 65 years.

Extracted teeth sometimes contain dental restorations and/or pulpal damage. Teeth exhibiting these traits were not collected and this limited the knowledge gained regarding the viability and characteristics of many potential sources of DPSC from patients.

#### Recommendation for Further Research

This study yielded results and dental pulp stem cell lineages that allowed for future research to continue our understanding of DPSC. It has been shown that isolation of viable DPSC from adult permanent extracted teeth is possible at the University of Nevada, Las Vegas School of Dental Medicine. It would be of interest to collect extracted teeth with dental restorations and/or pulpal damage to analyze their viability and characteristics. Stem cell isolates have been cultured and characterized and their viability measured after storage in cryopreservation media and thawing. Direct outgrowth was the isolation method chosen for this study but another method, enzymatic dissociation has also been used with success in other studies [17,18,43]. This method could be used on other extracted teeth samples to create different stem cell lineages which may bear alternative morphology and heterogeneity of cell populations than the DPSC-OG isolates. Cell capabilities regarding biomineralization potential and differentiation into osteoblasts and odontogenic progenitor cells might also differ with this isolation method.

Using the stem cell lineages from this study, demographic variables already acquired from subjects and samples such as age, gender, race, and tooth type could be involved in future research (and indeed were [46]). Correlation statistics could analyze any relationships between the variables and stem cell viability and their characteristics. Of particular interest is the variable of age as previous studies have shown primary teeth to have greater viability as well as growth and differentiation potential of stem cells than permanent teeth like those used in this study [15-17]. If found to be related to cell viability, recommendations to patients regarding their age would better assist clinicians in accurately predicting who would benefit from stem cell storage. It would be valuable to have a scientifically-based understanding of the demographics involved in DPSC viability to better target patients who might benefit from long-term storage of DPSC for future use.

Stem cell differentiation is still under investigation by researchers and is not always under the control of the researcher. In isolation during this study, some stem cells demonstrated the ability to spontaneously differentiate or partially differentiate into more specialized cell types. This is not advantageous as the ability to control what cell type they differentiate into has been lost. The reason to isolate and store stem cells is for their future use in regenerative medicine but cells must remain uncommitted so their differentiation into specialized cell types could be directed by scientists to target the specific tissue in need of repair by each donor. If a method could be used to prevent or even reverse the differentiation (de-differentiate) of DPSC it could increase the available number of cells for therapeutic use. This was investigated in related research from this study [45]. It is predicted that the longer stem cells are stored, the more likely they are to

spontaneously differentiate. If the ultimate goal of storage is to help patient years or even decades after initial isolation of the DPSC, it is in the best interest of science to understand the control mechanisms and methods to control stem cell differentiation. Although many technical issues still exist, numerous researchers have elucidated how these types of uncommitted (and some partially committed) DPSC isolates may be influenced to differentiate using various methods that include cell-matrix adhesion molecules, growth factors, biomechanical scaffolding, tension and pressure [22,24]. For instance, DPSC can be induced to migrate and differentiate using extracellular matrix-coated culture materials, including fibronectin, laminin, collagen, and fluorapatite [25-27]. Other studies have demonstrated the effects of growth factor stimulation to induce DPSCs into differentiated phenotypes, including Insulin-transferrin-sodium selenite supplement (ITS), bone morphogenic protein 2 (BMP2), growth differentiation factor 11 (Gdf11), platelet-derived growth factor (PDGF-AB), transforming growth factor (TGF- $\beta$ 1), dexamethasone or basic fibroblast growth factor (bFGF) [28,29,34-38]. Finally, other research has demonstrated the potential to use bioscaffolding, tension and pressure to induce DPSCs toward specific differentiated phenotypes [30,31,39,40].

The above-mentioned areas for future research would help dentists and other professionals better explain advantages and disadvantages of dental pulp stem cell isolation and storage to patients who have teeth extracted for dental and orthodontic reasons. What patients would benefit most, what teeth are most likely to contain viable stem cells, how their differentiation could be controlled or reversed, and which isolation and culturing method is best would be evidence-based and continue the goal of using DPSC for regenerative medicine.

APPENDIX A

FIGURES RELATED TO THE RESEARCH QUESTIONS

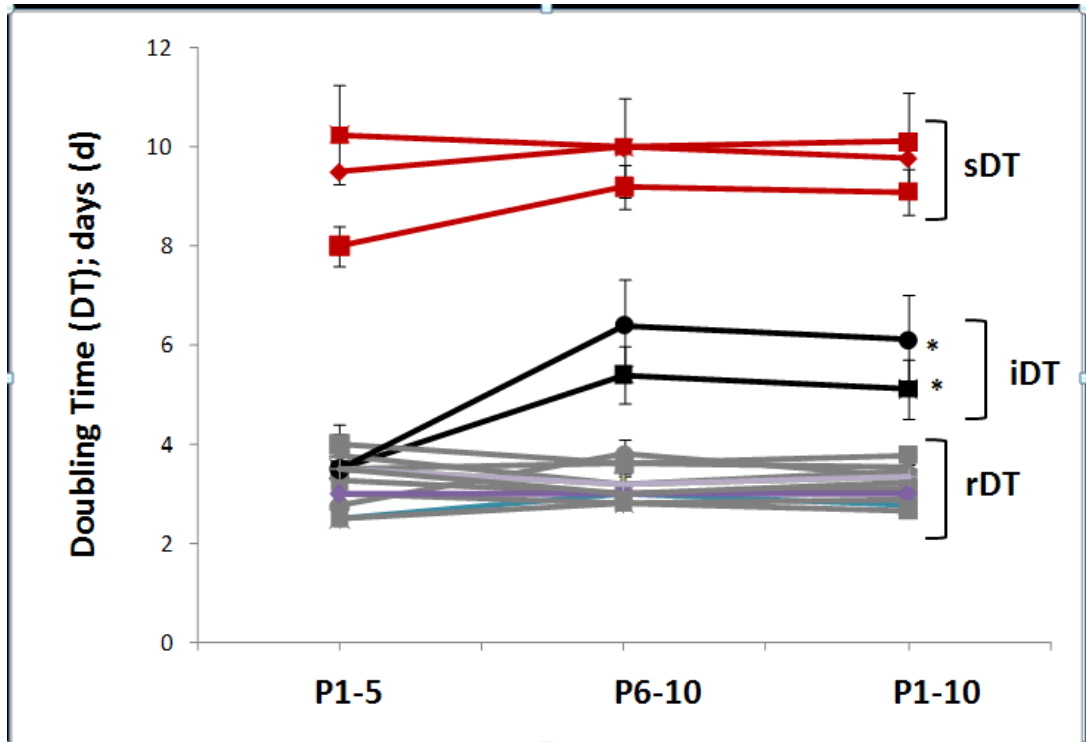


Figure A1. Cell proliferation and doubling time.

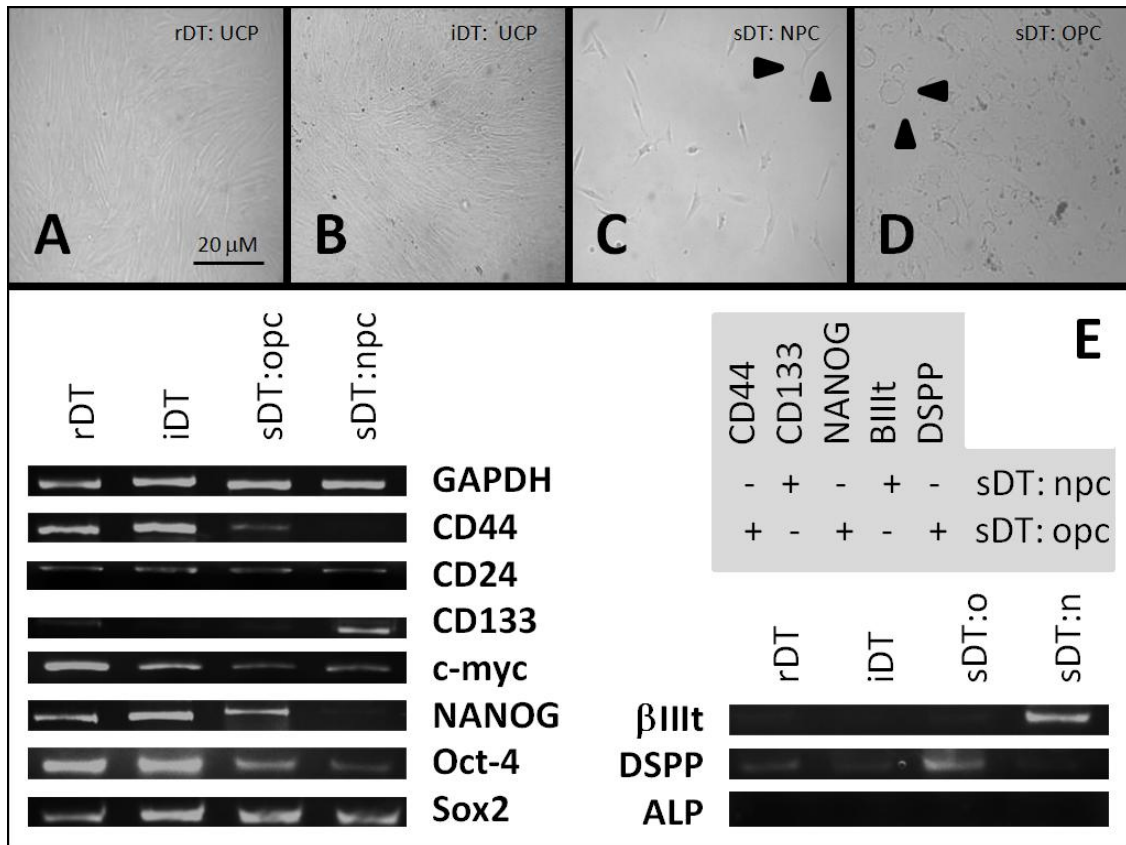


Figure A2. Cell microscopy and RT-PCR with mRNA expression.

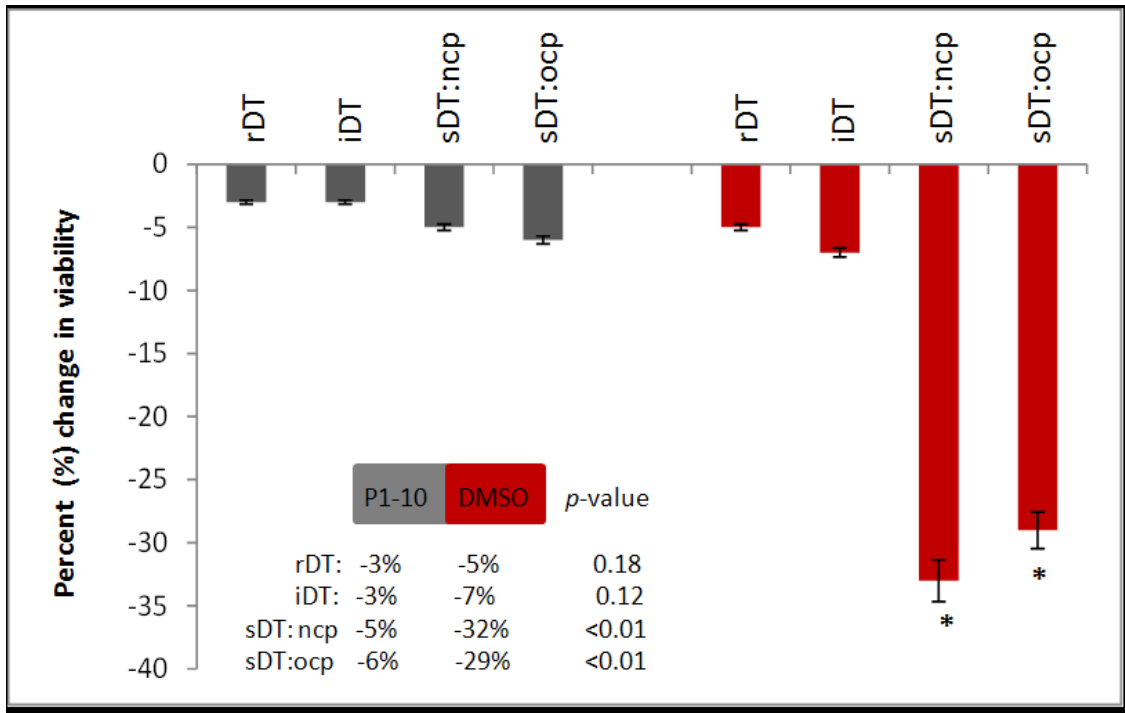


Figure A3. Viability during cell passaging and after storage.

## APPENDIX B

### PATIENT CONSENT FORMS

#### INFORMED CONSENT



Department of Clinical and Biomedical Sciences, UNLV School of Dental Medicine

---

**TITLE OF STUDY:** Isolation of Non-Embryonic Stem Cells from Dental Pulp

**INVESTIGATOR(S):** Charles Hill, DMD and Karl Kingsley, PhD

**CONTACT PHONE NUMBER:** (702) 774-2668 and (702) 774-2623

---

#### **Purpose of the Study**

You are invited to participate in a research study. The purpose of this study is to isolate non-embryonic stem cells from dental pulp of extracted teeth. These stem cells will be used as a positive control in an experiment to determine if oral tumors (cancers) also contain a sub-population of 'cancer stem cells'.

#### **Participants**

You are being asked to participate in the study because you are scheduled to have a tooth removed at the UNLV-SDM dental clinic and you fulfill the criteria for inclusion in this study.

Inclusion criteria: **Healthy adults 18-65 years old** must have sound, unrestored, vital teeth (teeth that have healthy pulp tissue), who need to have one or more extractions that are necessary for oral health.

Exclusion criteria: Any subject that refuses to donate their extracted teeth. There are no diseases or other conditions that would be exclusionary.

#### **Procedures**

If you volunteer to participate in this study, you will be asked to do the following: Donate your extracted tooth/teeth to the UNLV-SDM dental clinic for further study.

#### **Benefits of Participation**

There *may not* be direct benefits to you as a participant in this study. However, we hope to learn more about oral squamous cell carcinoma, a dangerous form of oral cancer that affects millions of people in the United States and across the world. By donating your extracted tooth/teeth you will allow us to search for cancer stem cells by comparing differences with dental pulp stem cells.

#### **Risks of Participation**

There are risks involved in all research studies. This study may include only minimal risks. *There is no anticipated risk by your participation in this study.* We will not divulge any private or personal information about you or your medical record nor will we allow any party other than the primary investigators to study the samples collected.

Your teeth, and the extracted dental pulp, will be studied for academic purposes only and no personal identifiers will be associated with your donated teeth once they are extracted.

Participant Initials \_\_\_\_\_

*Approved by the UNLV IRB. Protocol 1106-3856*

*Received: 06-23-11 Approved: 06-24-11 Expiration: 06-23-11*

1 of 2



School of Dental Medicine

Screening Information Sheet

In order that you fully understand the purposes and procedures of your screening appointment, PLEASE READ THE FOLLOWING:

The purpose of the screening appointment today is to determine your dental needs and to evaluate your suitability as a dental school patient. Students and faculty will examine your mouth. At this time the exam is only a screening exam not a comprehensive examination. If the faculty member determines that your problems are not the type that our students need to experience at this time or can successfully treat, they will explain this to you and suggest that you seek dental treatment elsewhere.

If your problems are of the type that our students can successfully treat and need to experience at this time, we will ask you to complete a comprehensive dental examination with radiographs (x-rays) at a later date. Following your examination we will try to match your dental needs with the needs of a particular student. This is not always possible. At times your care may be provided by several students working under the supervision of a Team Leader. In some cases you may need services beyond the capability of our students or beyond the scope of the school's mission. In those cases we will inform you as early as possible and recommend alternative sources of care.

Because all care is provided by students, treatment time is considerably longer than with a private dentist. Depending on how much care you need completing treatment with a student may require a year or longer. Patients must be available for their student-dentist a minimum of two half-days per month. If you do not make yourself available, cancel or break appointments, or uphold your responsibilities as a patient, your care may be terminated.

The School of Dental Medicine charges for all provided services. School fees are less than private practice. Payment is required when services are rendered. The approximate cost of your treatment will be determined after a full examination.

Our staff will call to make an appointment for you should you be accepted today. We cannot determine at this time when you will be called. You may contact the staff at 774-2400 should you have questions or comments. If you feel that your dental problems need more immediate treatment, we suggest that you seek dental treatment elsewhere.

We will be happy to answer any questions that you may have concerning your screening appointment.

THANK YOU FOR COMING IN TODAY!

I HAVE READ THE ABOVE, UNDERSTAND IT AND HAVE NO FURTHER QUESTIONS AT THIS TIME

Date Signed[Print\_Date\_US]:





**CONSENT FOR MEDICAL TREATMENT AND USES AND  
DISCLOSURES OF THE PATIENT HEALTH INFORMATION FOR  
TREATMENT, PAYMENT AND HEALTHCARE OPERATIONS (TPO) AT  
THE UNLV SCHOOL OF DENTAL MEDICINE**

I give my permission to the University of Nevada Las Vegas School of Dental Medicine ("Provider") and its employees, volunteers, agents and independent contractors to educate, interview, examine, perform laboratory procedures, make clinical photographs and to treat my dental condition, as they deem necessary. I understand that in case of a life-threatening emergency, this consent may be implied for the time of the emergency.

I understand that Provider is a teaching institution; therefore dental residents, post-doctoral dental students, pre-doctoral dental students, dental hygiene students and dental assisting students may participate in my care under the supervision of a physician/dentist. I understand that other outside medical professionals may also be consulted as deemed necessary for my care.

For coordination of my care and services, I understand that I may be provided with referrals to off campus specialists and the Provider may assist other treating physicians/dentists in the provision of my care.

- Informed Consent: If my condition requires an outpatient surgical procedure, the practitioner responsible for my care will explain to me the procedure to be performed, the general nature and extent of risks involved in such procedure and the alternative methods, if any.

- Consent for Minor Students: If you are a minor, we must have the signature of the parent or legal guardian (appointed by a court of law) on this form before any general treatment may begin, and such consent must be effective until you reach legal age in the State of Nevada (18 years old). Your parent or legal guardian must sign this consent form and receive a Notice of Privacy.

- Exemptions to this consent may be granted under NRS 129.030 for a life-threatening emergency or a serious health hazard; in other situations where a minor has been living apart from parents; to emancipated minors with court supporting documents; for family planning, contraceptive methods, and screening for sexually transmitted infections under NRS 442.255, NRS 129-060 and federal and state constitutional law; and counseling and treatment of alcohol and substance abuse under NRS 129.050.

**APPOINTMENT POLICY:**

- I agree to arrive at least fifteen (15) minutes prior to my appointment.
- I understand that my appointment may be cancelled if I'm late.
- I will check-in at the reception window upon my arrival.
- I understand I may be terminated from the program if I cancel three (3) appointments during the course of my treatment.
- I agree to call 48 hours in advance to cancel or reschedule an appointment.

**I understand and agree that Provider may use or disclose protected health information for treatment, payment and operations in accordance with the Notice of Privacy Practices that I have received, and any posted amendments to that Notice. I understand that Provider will not use or disclose protected health**



Patient Consent for Proposed Treatment Plan

1. A treatment plan has been presented to me and I have been informed of reasonable alternative treatments. Questions related to the material and treatment have been answered to my satisfaction. Expected risks and benefits of treatment and of no treatment have been explained. I understand that there are no guarantees related to any treatment.
2. I understand that the success of this treatment will be limited, or even fail, if I fail to progress through this treatment in a timely manner, or fail to maintain health and home care instructions, with routine maintenance and examination visits. I agree to cooperate completely with the recommendations of the student doctor while under their care, realizing that failure to do so could result in less than optimum results.
3. I understand that the fees quoted in the plan are estimates of the cost of treatment, may change at any time, and are payable at the time of treatment.
4. I understand that dental students will provide my treatment under the direction and supervision of School of Dental Medicine faculty.
5. I have read and freely consent to the above treatment plan subject to necessary changes as treatment progresses. My signature below indicates my understanding and consent.

[Full\_Patient\_Name][Patient\_No]  
Date Signed:[Print\_Date\_US]

## APPENDIX C

### OFFICE FOR THE PROTECTION OF RESEARCH SUBJECTS APPROVAL



### Biomedical IRB – Expedited Review Approval Notice

**NOTICE TO ALL RESEARCHERS:**

*Please be aware that a protocol violation (e.g., failure to submit a modification for any change) of an IRB approved protocol may result in mandatory remedial education, additional audits, re-consenting subjects, researcher probation, suspension of any research protocol at issue, suspension of additional existing research protocols, invalidation of all research conducted under the research protocol at issue, and further appropriate consequences as determined by the IRB and the Institutional Officer.*

**DATE:** June 24, 2011  
**TO:** Dr. Karl Kingsley, School of Dental Medicine  
**FROM:** Office of Research Integrity - Human Subjects  
**RE:** Notification of IRB Action by /Charles Rasmussen/ Dr. Charles Rasmussen, Co-Chair  
Protocol Title: Isolation of Non-Embryonic Stem Cells from Dental Pulp  
Protocol #: 1106-3856  
Expiration Date: June 23, 2012

---

This memorandum is notification that the project referenced above has been reviewed and approved by the UNLV Biomedical Institutional Review Board (IRB) as indicated in Federal regulatory statutes 45 CFR 46 and UNLV Human Research Policies and Procedures.

The protocol is approved for a period of one year and expires June 23, 2012. If the above-referenced project has not been completed by this date you must request renewal by submitting a Continuing Review Request form 30 days before the expiration date.

**PLEASE NOTE:**

Upon approval, the research team is responsible for conducting the research as stated in the protocol most recently reviewed and approved by the IRB, which shall include using the most recently submitted Informed Consent/Assent forms and recruitment materials. The official versions of these forms are indicated by footer which contains approval and expiration dates.

Should there be *any* change to the protocol, it will be necessary to submit a **Modification Form** through ORI - Human Subjects. No changes may be made to the existing protocol until modifications have been approved by the IRB. Modified versions of protocol materials must be used upon review and approval. Unanticipated problems, deviations to protocols, and adverse events must be reported to the ORI – HS within 10 days of occurrence.

If you have questions or require any assistance, please contact the Office of Research Integrity - Human Subjects at [IRB@unlv.edu](mailto:IRB@unlv.edu) or call 895-2794.

Office of Research Integrity - Human Subjects  
4505 Maryland Parkway • Box 451047 • Las Vegas, Nevada 89154-1047  
(702) 895-2794 • FAX: (702) 895-0805

## APPENDIX D

### MANUSCRIPT

#### **Dental pulp-derived stem cells (DPSC) differentiation in vitro into odontoblast and neuronal progenitors during cell passaging is associated with alterations in cell survival and viability.**

Matthew Alleman<sup>1</sup>, Erik Low<sup>1</sup>, Khanh Truong<sup>1</sup>, Ellen Huang<sup>1</sup>, Charles K. Hill<sup>1</sup>, Tzu Yuan Chen<sup>2</sup>, Michael Deaton<sup>2</sup>, and Karl Kingsley<sup>3§</sup>,

<sup>1</sup>Department of Clinical Sciences, School of Dental Medicine, University of Nevada, Las Vegas, USA

<sup>2</sup>School of Life Sciences, College of Sciences, University of Nevada, Las Vegas, USA

<sup>3</sup>Department of Biomedical Sciences, School of Dental Medicine, University of Nevada, Las Vegas, USA

§Corresponding author

Email addresses:

MA: matthew.alleman@sdm.unlv.edu

EL: erik.low@sdm.unlv.edu

KT: khanh.truong@sdm.unlv.edu

EH: ellen.huang@sdm.unlv.edu

CKH: charles.hill@unlv.edu

TYC: chent8@unlv.nevada.edu

MD: mddeaton@gmail.com

KK: karl.kingsley@unlv.edu

#### **Abstract**

**Background:** Mesenchymal stem cells (MSC) can be derived from a variety of adult human tissues, including dental pulp from extracted or exfoliated teeth. Some evidence suggests differences in both the quality and quantity of the dental-pulp stem cells (DPSC) obtained from differing sources, such as primary or “baby teeth” and adult, permanent teeth.

**Aim:** To evaluate the potential to obtain DPSC from intact, vital permanent teeth, using a randomized selection of active dental patients.

**Methods:** DPSC were extracted, isolated, cultured and characterized using microscopy and RT-PCR analysis of extracted RNA.

**Results:** DPSC isolates were derived from 30/31 (96.8%) of tissue explants using direct outgrowth (DO); mainly giving rise to uncommitted MSC progenitors with rapid doubling times (rDT, n=25/30 or 83.3%) and positive mRNA expression of MSC markers CD44, CD24, NANOG, Oct-4 and Sox2. DPSC isolates with slower doubling times (sDT, n=3/30 or 10%) and more limited differentiation potentials resembled neural or odontoblastic progenitor cells (sDT:npc or sDT:opc), expressed neural differentiation markers CD133 and  $\beta$ III tubulin or the odontoblastic differentiation marker, dentin sialophosphoprotein (DSPP), and had lower survival and viability rates following freezing, long-term storage and thawing.

Conclusions: The need to identify potential sources of MSC to treat age-related illnesses in the current population makes it necessary to more fully explore the feasibility and potential of DPSCs extracted from adult human teeth for this newly developing field of regenerative medicine.

### **Key Words**

Dental pulp stem cells, mesenchymal stem cells, *in vitro* culture

### **Introduction**

Mesenchymal stem cells (MSC), or multipotent progenitors, can be derived from a variety of adult human tissues. Recent clinical studies demonstrate that dental pulp may provide a rich supply of multipotent, highly proliferative MSCs capable of regenerating a variety of human tissues including, but not limited to, bone and dental structures.<sup>[1-3]</sup>

Dental-pulp derived stem cells (DPSC) can differentiate into many different lineages, including osteoblasts, chondroblasts, adipocytes, as well as vascular and neural tissues.<sup>[4,5]</sup>

Many factors influence both the quantity and quality of DPSC tissue available for storage and future clinical or bioengineering applications. One of the primary factors may be the tooth type or source of dental pulp, as recent evidence suggests that DPSCs derived from human exfoliated deciduous teeth or primary teeth may have substantially higher growth and differentiation potential, as well as greater survival rates after freezing and storage when compared with DPSCs derived from vital, permanent adult teeth.<sup>[6-8]</sup> In fact, many commercial companies, such as National Dental Pulp Lab (ndpl.net) and GeneCell (genecell.com), now offer services to banking and provide long-term storage of dental pulp from primary teeth, with the marketing and advertising focused primarily on parents with young children in the process of tooth exfoliation.

Other factors may also influence tissue quality and quantity, including the specific methods used to isolate, collect, concentrate and store DPSCs. For example, some research has suggested that the isolation of DPSCs by enzymatic dissociation (DPSC-ED) may produce heterogeneous populations of faster growing cells while the isolation technique of direct outgrowth (DPSC-OG) from tissue explants may give rise to largely homogenous populations, but with more limited differentiation potential.<sup>[9,10]</sup>

Additionally, recent advances in cryobanking, cryopreservation, and storage of dental pulp have significantly increased the recovery and optimized long-term quality of dental pulp tissues – thereby increasing the possibility for new sources of MSCs for potential future clinical applications.<sup>[11,12]</sup> Finally, other studies have suggested that the choice of low- or no-serum media and the selection of early- versus late-passage populations for storage may mediate the differentiation potential and proliferative capacity of DPSC isolates.<sup>[13-15]</sup>

Most importantly, the recent nature of these discoveries means that for the majority of the population, the plethora of age-related diseases for which DPSC therapy could be useful can only be addressed by obtaining DPSC from vital, permanent adult teeth.<sup>[1,2,16]</sup>

Despite the growing corpus of evidence suggesting the potential for clinical applications and possible therapies, much remains to be discovered about the potential of DPSC from healthy permanent dentition.

Therefore, the primary purpose of this research project was to elucidate the potential to obtain DPSC from intact, vital permanent teeth of adult patients. A randomized selection of active patients requiring extractions were sampled and DPSC were extracted, isolated, cultured and characterized. The working hypothesis was that dental pulp isolates from permanent teeth would yield lower percentages of viable DPSCs, which would also exhibit comparatively slower growth and lower survival rates after freezing and storage.

## **Materials and Methods**

### **Human subjects**

The protocol for this study titled “Isolation of Non-Embryonic Stem Cells from Dental Pulp” at the University of Nevada, Las Vegas - School of Dental Medicine (UNLV-SDM) dental clinic was filed, amended, and approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#0907-3148) on February 5, 2010. In brief, subjects were randomly recruited by members of the UNLV-SDM clinic during their dental visits between February and June, 2010. Informed Consent was required and was conducted onsite. Inclusion criteria: subjects had to be between eighteen (18) and sixty-five (65) years old and must agree to participate. In addition, all potential subjects must have sound, unrestored, vital teeth (teeth that have healthy pulp tissue), and need to have one or more extractions that are necessary for oral health, as determined by the clinical faculty member in charge. Exclusion criteria: Any subject under eighteen (18) or over sixty-five (65) years of age, any subjects having dental extractions involving compromised pulp, and any subject that refuses to donate his or her extracted teeth.

### **DPSC isolation and culture**

In brief, dental pulp was extracted from the vital teeth of healthy adults who agreed to participate. The majority of the teeth included in this study were extracted due to severe periodontal disease, necessity for fabrication of complete dentures, or impaction and/or crowding (e.g., third molars). Following extraction, teeth were placed into sterile 1X phosphate buffered saline (PBS) solution and transported to the laboratory for sectioning. The teeth were sectioned axially at the cemento-enamel junction (CEJ) using a diamond rotary disc in a dental handpiece and the dental pulp was removed with an endodontic broach. The dental pulp was then immediately placed into sterile microcentrifuge tubes containing 1X PBS solution and transferred to the laboratory for culture; any dental pulp not transferred within two hours was removed from the subsequent analysis. Tubes were pre-assigned a unique, randomly-generated number to prevent research bias.

Demographic information regarding the sample was concurrently collected, which consisted of age, gender, and ethnicity only.

Subsequently, extracted dental pulp was vortexed for 10 – 30 seconds to dislodge cells and centrifuged for five (5) minutes at 2,100 relative centrifugal force (RCF) or g. Supernatant (PBS) was aspirated from the tube and dental pulp-derived cells were re-suspended in 1.0 mL of RPMI-1640 medium from Hyclone (Logan, UT) with 2mM L-Glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 µg/mL) solution and 10% fetal bovine serum (FBS), obtained from HyClone (Logan, UT). Cells were cultured in 75 cm<sup>2</sup> BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO<sub>2</sub> in humidified chambers.

Media was changed every 48 hours until adherent cells reached 70% confluence. Cells were subsequently passaged at a 1:4 ratio.

#### Cell proliferation and doubling time

Cell proliferation assays were performed in the appropriate complete media, as described above. In brief, cells reaching 70-80% confluence were trypsinized and plated 1:4 into new 75 cm<sup>2</sup> BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO<sub>2</sub> in humidified chambers and their confluence was measured with a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). Doubling times (DT) from passages one (P1) through ten (P10) for each flask were recorded. Averages from the first five passages (P1-5), last five passages (P6-10) and overall average DT (P1-10) were then calculated. Data were analyzed and graphed using Microsoft Excel (Redmond, WA).

#### Microscopy of cell morphology, survival and viability

During the process of passaging cells, small aliquots of trypsinized cells were stained using Trypan Blue (Sigma: St. Louis, MO), and live cells were enumerated by counting the number of Trypan-blue negative cells using a VWR Scientific Counting Chamber (Plainfield, NJ) and a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). Images were captured at 200X magnification with a Canon PowerShot G6 digital camera (Tokyo, Japan) and subsequently processed using Adobe Photoshop (San Jose, CA) Image Analysis tools. At each time point (d1-d3), several flasks were also processed using the Trypan stain, and live cells were enumerated using this procedure. Finally, DPSCs from each passage were also frozen for storage using a commercially available cryopreservation medium (Opti-Freeze) from Fisher Scientific (Fair Lawn, NJ), containing Dimethyl Sulfoxide (DMSO), using the procedure recommended by the manufacturer. Following six weeks in storage at -80°C, cells were thawed, re-suspended in the appropriate media, and live cells enumerated, as described above.

#### Statistical analysis

The differences between passages were measured using a t distribution,  $\alpha = 0.05$ . All samples were analyzed using two-tailed t-tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed t-test.<sup>[17]</sup> As long as the sample size is at least moderate (>20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses.

#### RNA isolation, concentration, and yield

To determine if any cells derived from dental pulp were dental pulp stem cells (DPSC), RNA was isolated from  $1.5 \times 10^7$  cells of each of the experimental cell lines, using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) in accordance with the procedure recommended by the manufacturer for RT-PCR analysis. RNA concentration and purity were calculated using UV spectroscopy. The absorbance of diluted RNA samples (10  $\mu$ L of RNA sample in 490  $\mu$ L nuclease-free water, pH 7.0) was measured at 260 and 280 nm. RNA purity was determined by calculating the ratio of A<sub>260</sub>:A<sub>280</sub>, which should be > 1.80. Concentration for RNA samples was determined by the A<sub>260</sub> reading of 1 = 40  $\mu$ g/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water. Concentration was calculated as 40 x A<sub>260</sub> absorbance measure x dilution factor (50). Total yield was determined by concentration x sample volume in mL.

Example: RNA standard

$$A_{260} = 0.75$$

Concentration = 40 x 0.75 x 50 = 1,500 µg/mL

Yield = 1,500 µg/mL x 1.0 mL = 1,500 µg or 1.5 mg RNA

Reverse-transcription polymerase chain reaction (RT-PCR)

To quantify the expression of DPSC-specific mRNA, RT-PCR was performed on total RNA using the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following mesenchymal stem cell (MSC) primers synthesized by SeqWright (Houston, TX):

ALP FORWARD:

CACTGCGGACCATTCCCACGTCTT;

ALP REVERSE:

GCGCCTGGTAGTTGTTGTGAGCATA;

βIII TUBULIN FORWARD:

CTGCTCGCAGCTGGAGTGAG;

βIII TUBULIN REVERSE:

CATAAATACTGCAGGAGGGC;

c-myc FORWARD:

TCCAGCTTGTACCTGCAGGATCTGA;

c-myc REVERSE:

CCTCCAGCAGAAGGTGATCCAGACT;

CD24 FORWARD:

ACTCTCACTTGAAATTGGGC;

CD24 REVERSE:

GCACATGTTAATTACTAGTAAAGG;

CD44 FORWARD:

GAAAGGCATCTTATGGATGTGC;

CD44 REVERSE:

CTGTAGTGAAACACAACACC;

CD133 FORWARD:

CTCATGCTTGAGAGATCAGGC;

CD133 REVERSE:

CGTTGAGGAAGATGTGCACC;

DSPP FORWARD:

CAACCATAGAGAAAGCAAACGCG;

DSPP REVERSE:

TTTCTGTTGCCACTGCTGGGAC;

GAPDH FORWARD:

ATCTTCCAGGAGCGAGATCC;

GAPDH REVERSE:

ACCACTGACACGTTGGCAGT;

NANOG FORWARD :

GCTGAGATGCCTCACACGGAG;

NANOG REVERSE:

TCTGTTTCTTGACTGGGACCTTGTC;

Oct4 FORWARD:

TGGAGAAGGAGAAGCTGGAGCAAAA;



Oct4 REVERSE:

GGCAGATGGTCGTTTGGCTGAATA;

Sox2 FORWARD:

ATGGGCTCTGTGGTCAAGTC;

Sox2 REVERSE:

CCCTCCCAATTCCCTTGAT;

One  $\mu\text{g}$  of template (total) RNA was used for each reaction. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C. Thirty-five amplification cycles were run, consisting of 20-second denaturation at 94°C, 30 seconds of annealing at 58°C, and 6.5 minutes of extension at 72°C. Final extension was run for 5 minutes at 72°C. Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY). Quantification of RT-PCR band densitometry and relative mRNA expression levels were performed using Adobe Photoshop (San Jose, CA) imaging software, Image Analysis tools.

## Results

Thirty-one (31) individual dental pulp samples were collected from twenty-four different (24) UNLV-SDM patient clinic between February and June, 2010 and processed for cell culture using direct outgrowth (DPSC-OG), as described. This resulted in thirty DPSC isolates, with at least one from each patient, yielding an overall success rate greater than 95% percent ( $n = 30/31$  or 96.8%).

Cell proliferation and doubling time

During the initial growth phase (P0) each potential DPSC isolate reached 70% confluence or greater between 2 - 12 days. The average doubling time (DT) for the initial five passages P1 – 5 of each potential cell line was then established and calculated, revealing a characteristic average doubling time (DT) that varied from 2.5 to 10.25 days (Figure 1). The vast majority ( $n=27/30$  or 90%) exhibited a rapid DT (rDT), ranging from 2.5 to 4 days, while the remainder ( $n=3/30$  or 10%) exhibited a much slower DT (sDT) that ranged from 8 to 10.25 days. Although the DT remained fairly constant for most DPSC isolates, two cell lines exhibited a temporal decrease in DT observed between passages P6-10, resulting in an intermediate DT (iDT) of 5.4 and 6.4 days. The proliferation of iDT isolates was significantly faster than that of the sDT cells ( $p = 0.04$ ) but this change was not sufficient to be significantly different than the rDT isolates ( $p = 0.11$ ).

To characterize and assess these potential DPSC isolates, microscopy was performed (Figure 2). This analysis revealed that cells with the fastest growth rates (rDT) had similar morphologies, which changed little during long-term passaging and culture; forming tightly-packed dense colonies of flat, spindle-shaped cells commonly associated with uncommitted progenitor cells (UCP) (Fig. 2A). Both potential DPSC isolates that exhibited a temporal decrease to a more intermediate doubling time (iDT) appeared to have morphologies similar to rDT cells (Fig 2B), although cell-cell junctions and cell boundaries appeared slightly more distinct. Cells with the slowest growth rates (sDT) gradually adopted more distinctive morphologies, such as long, axon-like projections suggesting the formation of a sub-population of neuronal progenitor cells (NPC) (Fig.

2C) or large, ovoid-shaped cells that appear to suggest to the formation of odontoblast progenitor cells (OPC) (Fig. 2D).

To provide more qualitative and quantitative assessments of these potential DPSC isolates, RNA was successfully isolated from all DPSC cell lines and RT-PCR was performed on equal concentrations of total RNA from each cell line (Fig. 2E).

Expression of mRNA for mesenchymal stem cells (MSC) markers CD44, CD24, NANOG, Oct-4, and Sox2 was observed in all rDT and iDT cell isolates, but varied among sDT isolates. For example; sDT:OPC isolates expressed comparatively lower levels of Oct-4, CD44, and NANOG, as well as the cell-cycle marker c-myc, also expressed the odontoblast progenitor differentiation marker dentin sialophosphoprotein (DSPP) but not alkaline phosphatase (ALP). In addition, sDT: NPC isolates did not express NANOG or CD44, but did express the neural progenitor markers CD133 and  $\beta$ III tubulin.

Finally, any change in viability during cell passaging and culture was evaluated before and after freezing and long-term storage (Figure 3). These results clearly demonstrate that viability among rDT and iDT isolates remained consistently high, although a slight non-significant drop in viability was apparent following freezing, storage and thaw ( $p > 0.05$ ). Viability was slightly, but not significantly, lower among sDT isolates, however these isolates exhibited a significant drop in viability following freezing, storage and thaw (sDT:npc -32%, sDT:ocp - 29%,  $p < 0.01$ ).

### Tables and Figures

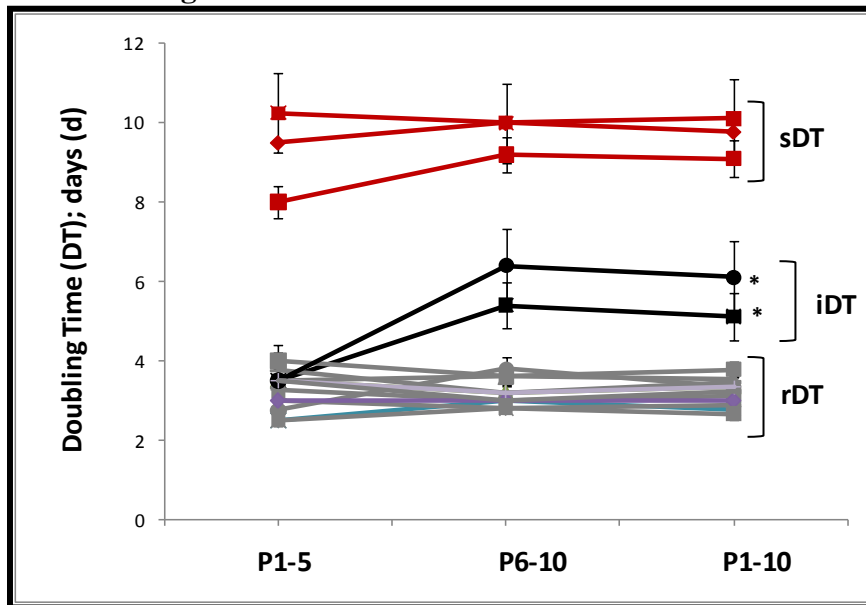


Figure 1. Cell proliferation and doubling time.

Average doubling time (DT) for the initial five passages P1 – 5 of each potential cell line varied from 2.5 to 10.25 days. Most ( $n=27/30$  or 90%) exhibited a rapid DT (rDT): 2.5 - 4 days, while some ( $n=3/30$  or 10%) exhibited a slower DT (sDT): 8 - 10.25 days. Two cell lines decreased DT between passages P6-10, resulting in an intermediate DT (iDT): 5.4 - 6.4 days. iDT growth was significantly faster than that of the sDT cells ( $p = 0.04$ ) but not significantly different than rDT isolates ( $p = 0.11$ ).

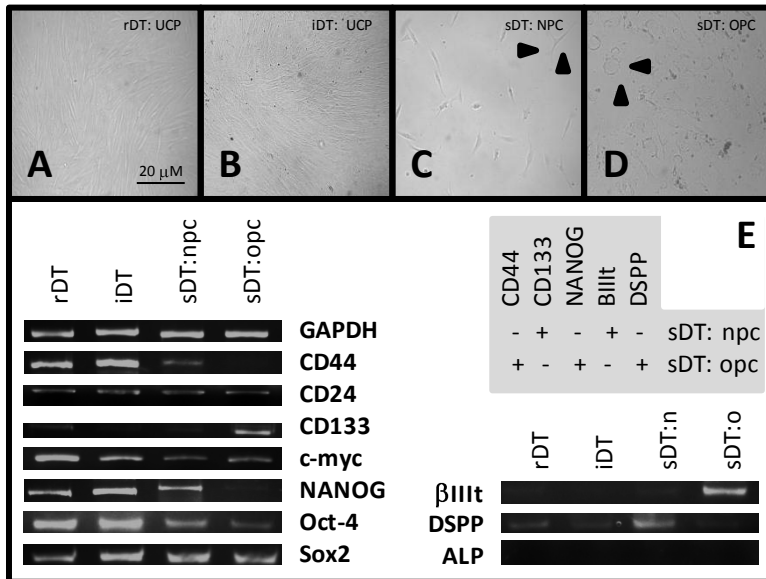


Figure 2. Microscopy and RT-PCR.

A. Rapid-growth isolates (rDT) had similar morphologies; tightly-packed dense colonies of flat, spindle-shaped cells. commonly associated with uncommitted progenitor cells (UCP) (Fig. 2A). B. Cells with intermediate doubling time (iDT) exhibited morphologies similar to rDT cells. C. Slow-growth isolates (sDT) adopted distinctive morphologies, including long, axon-like projections resembling neuronal progenitor cells (NPC) or D. Large, ovoid-shaped cells resembling odontoblast progenitor cells (OPC). E. mRNA expression for mesenchymal stem cells (MSC) markers CD44, CD24, NANOG, Oct-4, and Sox2 was observed in all rDT and iDT cell isolates, but varied among sDT isolates; sDT:OPC isolates expressed comparatively lower levels of Oct-4, CD44, NANOG, and c-myc and also expressed the odontoblast progenitor differentiation marker dentin sialophosphoprotein (DSPP) but not alkaline phosphatase (ALP). sDT: NPC isolates did not express NANOG or CD44, but did express the neural progenitor markers CD133 and  $\beta$ III tubulin.

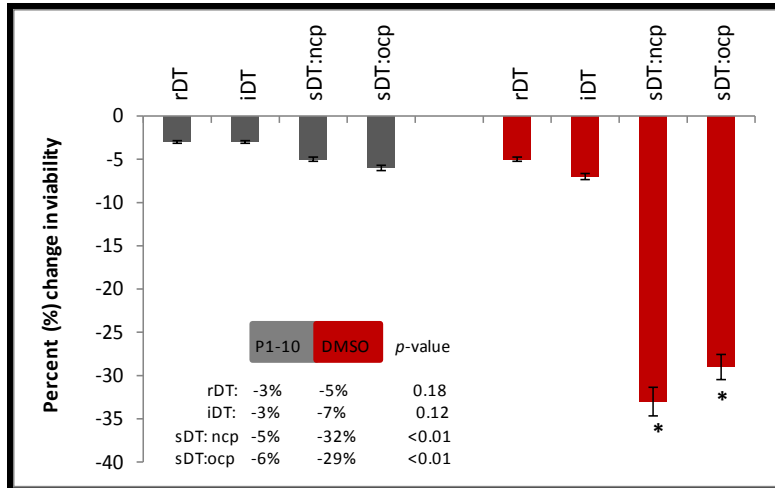


Figure 3. Viability during cell passaging and after freezing and long-term storage. Viability among rDT and iDT isolates remained high, with a small, non-significant drop in viability following freezing, storage and thaw ( $p>0.05$ ). Viability was slightly, but not significantly, lower among sDT isolates, however these isolates exhibited a significant drop in viability following freezing, storage and thaw (sDT:ncp -32%, sDT:ocp -29%,  $p<0.01$ ).

## Discussion

The goals of this research project were to elucidate the potential to obtain DPSC from intact, vital permanent teeth of adult patients. These results clearly demonstrate the feasibility of extracting, isolating and culturing DPSC isolates from intact vital permanent teeth derived from adult patients, with a success rate exceeding 95%. In addition, although some evidence may suggest direct outgrowth (DPSC-OG) from tissue explants may give rise to slow-growing isolates with more limited differentiation potential, these data suggest that many DPSC isolates derived from this method are, in fact, rapidly proliferating uncommitted MSC progenitors ( $n=25/30$  or 83.3%).<sup>[9,10]</sup> However, these data do confirm the previous observations that some DPSC isolates, particularly those that with slower growth rates and more limited differentiation potential (sDT:npc or opc), are less able to survive following freezing, long-term storage and thawing for subsequent use – reducing available cells by nearly one-third.

Although many technical issues still exist, numerous researchers have elucidated how these types of uncommitted (and some partially committed) DPSC isolates may be influenced to differentiate using various methods that include cell-matrix adhesion molecules, growth factors, biomechanical scaffolding, tension and pressure.<sup>[16,18]</sup> For instance, DPSC can be induced to migrate and differentiate using extracellular matrix-coated culture materials, including Fibronectin, laminin, collagen, and fluorapatite.<sup>[19-21]</sup> Other studies have demonstrated the effects of growth factor stimulation to induce DPSCs into differentiated phenotypes, including Insulin-transferrin-sodium selenite supplement (ITS), bone morphogenic protein 2 (BMP2), growth differentiation factor 11 (Gdf11), platelet-derived growth factor (PDGF-AB), transforming growth factor (TGF- $\beta$ 1), dexamethasone or basic fibroblast growth factor (bFGF).<sup>[22-28]</sup> Finally, other research has demonstrated the potential to use bioscaffolding, tension and pressure to induce DPSCs toward specific differentiated phenotypes.<sup>[29-32]</sup>

## **Conclusions**

Although some commercial entities are now offering services which include processing and storage of dental pulp or DPSCs from exfoliated primary teeth or extracted third molars, less information is widely known about the viability and potential applications of DPSCs in order to provide evidence-based recommendations for patients (and parents) interested in banking these tissues for future possible usage. The initial processing fee combined with the additional monthly storage fees – now suggests that patients, or parents of these patients, may face costs that exceed thousands of dollars for long-term storage before (or if) these cells are needed. Despite these obstacles, the need to identify potential sources of MSC for age-related illnesses in our aging population makes it necessary for dental researchers and clinicians to more fully explore the feasibility and potential for isolating and culturing DPSCs extracted from adult human teeth in order to provide more accurate and informed advice for this newly developing field of regenerative medicine.

## **Acknowledgements**

This research was supported, in part, by an institutional research grant (IRG) from the American Cancer Society (ACS) to KK (ACS-IRG#103719), by Gillian Galbraith and the Department of Biomedical Sciences, and by the Advanced Education in Orthodontics and Dentofacial Orthopedics Residency at UNLV-SDM.

## **Conflict of Interest/Competing Interest**

The authors declare that they have no competing interests.

## **References**

1. Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S: The efficacy of mesenchymal stem cells to regenerate and repair dental structures. *Orthod Craniofac Res.* 2005, 8(3):191-199.
2. Sloan AJ, Waddington RJ: Dental pulp stem cells: what, where, how? *Int J Paediatr Dent.* 2009, 19(1):61-70.
3. Huang GT: Dental pulp and dentin tissue engineering and regeneration: advancement and challenge. *Front Biosci (Elite Ed).* 2011, 3:788-800.
4. Djouad F, Jackson WM, Bobick BE, Janjanin S, Song Y, Huang GT, Tuan RS: Activin A expression regulates multipotency of mesenchymal progenitor cells. *Stem Cell Res Ther.* 2010, 1(2):11.
5. Karbanová J, Soukup T, Suchánek J, Pytlík R, Corbeil D, Mokry J: Characterization of Dental Pulp Stem Cells from Impacted Third Molars Cultured in Low Serum-Containing Medium. *Cells Tissues Organs.* 2010 [Epub ahead of print]
6. Govindasamy V, Abdullah AN, Ronald VS, Musa S, Ab Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH: Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. *J Endod.* 2010, 36(9):1504-1515.
7. Suchánek J, Visek B, Soukup T, El-Din Mohamed SK, Ivancaková R, Mokry J, Aboul-Ezz EH, Omran A: Stem cells from human exfoliated deciduous teeth--isolation, long term cultivation and phenotypical analysis. *Acta Medica (Hradec Kralove).* 2010, 53(2):93-99.

8. Tandon S, Saha R, Rajendran R, Nayak R: Dental pulp stem cells from primary and permanent teeth: quality analysis. *J Clin Pediatr Dent.* 2010, 35(1):53-58.
9. Huang GT, Sonoyama W, Chen J, Park SH: In vitro characterization of human dental pulp cells: various isolation methods and culturing environments. *Cell Tissue Res.* 2006, 324(2):225-236. [Epub 2006 Jan 27]
10. Bakopoulou A, Leyhausen G, Volk J, Tsiftoglou A, Garefis P, Koidis P, Geurtsen W: Assessment of the impact of two different isolation methods on the osteo/odontogenic differentiation potential of human dental stem cells derived from deciduous teeth. *Calcif Tissue Int.* 2011, 88(2):130-141. [Epub 2010 Dec 14]
11. Woods EJ, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS: Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. *Cryobiology.* 2009, 59(2):150-157. [Epub 2009 Jun 16]
12. Lee SY, Chiang PC, Tsai YH, Tsai SY, Jeng JH, Kawata T, Huang HM: Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells. *J Endod.* 2010, 36(8):1336-1340. [Epub 2010 Jun 23]
13. Jo YY, Lee HJ, Kook SY, Choung HW, Park JY, Chung JH, Choung YH, Kim ES, Yang HC, Choung PH: Isolation and characterization of postnatal stem cells from human dental tissues. *Tissue Eng.* 2007, 13(4):767-773.
14. Hirata TM, Ishkitiev N, Yaegaki K, Calenic B, Ishikawa H, Nakahara T, Mitev V, Tanaka T, Haapasalo M: Expression of multiple stem cell markers in dental pulp cells cultured in serum-free media. *J Endod.* 2010, 36(7):1139-1144. [Epub 2010 Apr 10]
15. Yu J, He H, Tang C, Zhang G, Li Y, Wang R, Shi J, Jin Y: Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging. *BMC Cell Biol.* 2010, 11:32.
16. Kadar K, Kiraly M, Porcsalmy B, Molnar B, Racz GZ, Blazsek J, Kallo K, Szabo EL, Gera I, Gerber G, Varga G: Differentiation potential of stem cells from human dental origin - promise for tissue engineering. *J Physiol Pharmacol.* 2009, 60(Suppl 7):167-175.
17. Hays WL: Inferences about population means. In: *Statistics* (5th edition). International Thomson Publishing 1994. 311-342.
18. Mori G, Centonze M, Brunetti G, Ballini A, Oranger A, Mori C, Lo Muzio L, Tetè S, Ciccolella F, Colucci S, Grano M, Grassi FR: Osteogenic properties of human dental pulp stem cells. *J Biol Regul Homeost Agents.* 2010, 24(2):167-175.
19. Waddington RJ, Youde SJ, Lee CP, Sloan AJ: Isolation of distinct progenitor stem cell populations from dental pulp. *Cells Tissues Organs.* 2009, 189(1-4):268-274. [Epub 2008 Aug 14]
20. Howard C, Murray PE, Namerow KN: Dental pulp stem cell migration. *J Endod.* 2010, 36(12):1963-1966. [Epub 2010 Oct 8]
21. Liu J, Jin TC, Chang S, Czajka-Jakubowska A, Clarkson BH: Adhesion and growth of dental pulp stem cells on enamel-like fluorapatite surfaces. *J Biomed Mater Res A.* 2011, 96(3):528-534. doi: 10.1002/jbm.a.33002. [Epub 2011 Jan 4]
22. Nakashima M, Mizunuma K, Murakami T, Akamine A: Induction of dental pulp stem cell differentiation into odontoblasts by electroporation-mediated gene delivery of growth/differentiation factor 11 (Gdf11). *Gene Ther.* 2002, 9(12):814-818.

23. Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A: Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2. *J Dent Res.* 2004, 83(8):590-595.
24. Huang GT, Shagramanova K, Chan SW: Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro. *J Endod.* 2006, 32(11):1066-1073.
25. Király M, Porcsalmy B, Pataki A, Kádár K, Jelitai M, Molnár B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G: Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. *Neurochem Int.* 2009, 55(5):323-332. [Epub 2009 Apr 5]
26. Morito A, Kida Y, Suzuki K, Inoue K, Kuroda N, Gomi K, Arai T, Sato T: Effects of basic fibroblast growth factor on the development of the stem cell properties of human dental pulp cells. *Arch Histol Cytol.* 2009, 72(1):51-64.
27. Suchanek J, Soukup T, Visek B, Ivancakova R, Kucerova L, Mokry J: Dental pulp stem cells and their characterization. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* 2009, 153(1):31-35.
28. Lee JY, Nam H, Park YJ, Lee SJ, Chung CP, Han SB, Lee G: The effects of platelet-rich plasma derived from human umbilical cord blood on the osteogenic differentiation of human dental stem cells. *In Vitro Cell Dev Biol Anim.* 2011, 47(2):157-164. [Epub 2010 Nov 17]
29. Zhang W, Walboomers XF, Van Kuppevelt TH, Daamen WF, Van Damme PA, Bian Z, Jansen JA: In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages. *J Tissue Eng Regen Med.* 2008, 2(2-3):117-125.
30. Yu V, Damek-Poprawa M, Nicoll SB, Akintoye SO: Dynamic hydrostatic pressure promotes differentiation of human dental pulp stem cells. *Biochem Biophys Res Commun.* 2009, 386(4):661-665. [Epub 2009 Jun 23]
31. Riccio M, Resca E, Maraldi T, Pisciotta A, Ferrari A, Bruzzesi G, De Pol A: Human dental pulp stem cells produce mineralized matrix in 2D and 3D cultures. *Eur J Histochem.* 2010, 54(4):e46.
32. Yang X, Yang F, Walboomers XF, Bian Z, Fan M, Jansen JA: The performance of dental pulp stem cells on nanofibrous PCL/gelatin/nHA scaffolds. *J Biomed Mater Res A.* 2010, 93(1):247-257.

APPENDIX E  
IN PRESS NOTIFICATION



**IJMBR:ARTICLE UPDATE**  
editor to: karl.kingsley

05/07/2013 02:08 PM

---

History: This message has been forwarded.

---

MICHAEL JOANNA PUBLICATIONS

---

Subject: Acceptance Letter  
Title: Dental pulp-derived stem cells (DPSC) differentiation in vitro into odontoblast and neuronal progenitors during cell passaging is associated with alterations in cell survival and viability

It's a great pleasure to inform you that the mentioned manuscript has been accepted for publication in the International Journal of Medicine and Biomedical Research (IJMBR) based on the recommendations of the reviewers.

You will receive a copy edited version within the next 8 weeks.

Best Regards

Professor Sofola O.A,  
Editor-in-Chief, IJMBR

---

Karl Kingsley, PhD  
Assistant Professor of Biomedical Sciences



## APPENDIX F

### Further Research in DPSC De-differentiation and Demographics

Patient demographic information was taken during sampling. Of interest was the possible relationship between viability of DPSC isolates, derived from permanent adult teeth and one or more patient characteristics. Using the same methods but different extracted teeth, forty-one (41) individual dental pulp samples were collected and processed for cell culture using direct outgrowth (DPSC-OG) [45]. The categorical demographic characteristics associated with viable DPSC lines from each patient (gender, ethnicity), as well as tooth type were analyzed using chi-square ( $\chi^2$ ), Fisher's exact test. The continuous demographic variable (age) was analyzed using Pearson's correlation. The result was thirty (30) DPSC, yielding an overall success rate of 73.2% (n = 30/41). The proportion of males (64%) in this study was higher than females (36%), (Table F1). The proportion of minority patients (70.7%) was also higher than non-minority (29.3%). The average age of participants was 37.5 years, which ranged between 19 and 63 years.

The proportion of viable DPSC lines derived from females (33.3%) did not differ significantly from the overall proportion in the overall study sample (36.5%). In addition, proportion of viable DPSC from minority patients (63.6%) did not differ significantly from the overall percentage in the study sample (70.7%). However, a much larger proportion of viable DPSC lines came from premolars (96.6%), which was significantly different than the proportion from the overall study sample (48.8%). To evaluate if age was related to this finding, the average age of viable DPSC lines (23.7 years) was compared with the overall average (37.5 years) and the non-viable average age (42.1 years). More in depth analysis revealed the age range for viable DPSC was primarily

between 19 and 25 years old, with one outlier at 35 years old. With the exception of the 35 year old, all viable DPSC lines came from premolars from these younger individuals.

| <u>Demographic Variable</u> | <u>Sample Population</u>   | <u>Viable DPSC line</u>   | <u>Statistics</u>       |
|-----------------------------|----------------------------|---------------------------|-------------------------|
| Gender                      | Females, n=16<br>(39.0%)   | Females, n=10<br>(33.3%)  | $\chi^2=0.686$ , d.f.=1 |
|                             | Males, n=25 (61.0%)        | Males, n=20 (66.7%)       | $p = 0.4074$            |
| Race/Ethnicity              | White, n=12 (29.3%)        | White, n=8 (36.3%)        | $\chi^2=2.380$ , d.f.=1 |
|                             | Minority, n=29<br>(70.7%)  | Minority, n=22<br>(63.6%) | $p = 0.1229$            |
| Tooth type                  | Premolar, n=29<br>(70.7%)  | Premolar, n=29<br>(96.6%) | $\chi^2=791.7$ , d.f.=1 |
|                             | Other, n=12 (29.3%)        | Other, n=1 (3.4%)         | $p < 0.0001$            |
|                             | Molar, n=7 (17.1%)         | Molar, n = 1 (3.4%)       |                         |
|                             | Third molar, n=3<br>(7.3%) |                           |                         |
|                             | Canine, n=2 (4.9%)         |                           |                         |
| Age                         | Overall ave. = 37.5        | Viable ave. = 23.7        | $R = -0.685$            |
|                             |                            | Non-viable ave. =<br>42.1 | $p < 0.0001$            |

Table F1. Demographic variables.

The goal was to elucidate any demographic characteristics that might influence the potential to obtain viable DPSCs from intact, vital permanent teeth of adult patients. Findings suggest that premolars, obtained from younger patients (19-25 years old) exhibited the highest success rate, exceeding 95%. Also demonstrated was no gender bias or racial and ethnic component was associated with survival or viability of DPSCs. These findings suggest that DPSC can be obtained from viable, intact permanent teeth in adult patients, however, there may also be an age-related component that further limits or restricts the possibilities for extraction and tissue culture.

During this study, an incidental finding was some DPSC spontaneously differentiated in storage. Although few studies have evaluated the possibility of de-differentiating DPSC that have spontaneously committed to a particular cell lineage, some evidence suggests the administration of DCA may be one potential mechanism for achieving this goal. The same methods used to isolate, passage, and characterize DPSC were used to conduct further research which provided evidence that DCA administration not only inhibits the growth and proliferation of uncommitted DPSC isolates, but more specifically reduces the expression of neuronal-specific biomarkers associated with specific DPSC isolates that appear to be neuronal progenitors [46]. Future research in this area, however, will be needed for scientists and clinicians to more fully explore the feasibility and potential for isolating, culturing, and re-directing differentiation of DPSC extracted from adult human teeth in this new, rapidly developing field of regenerative medicine.

## Materials

Methyl dichloroacetate (DCA) MW= 42.97, d=1.381 g/cm<sup>3</sup> (10.8M) was obtained from Acros Organics – Thermo Fisher Scientific (Fair Lawn, NJ). Media were supplemented with DCA in cell culture media for final concentrations ranging between 10 and 1000 nM DCA.

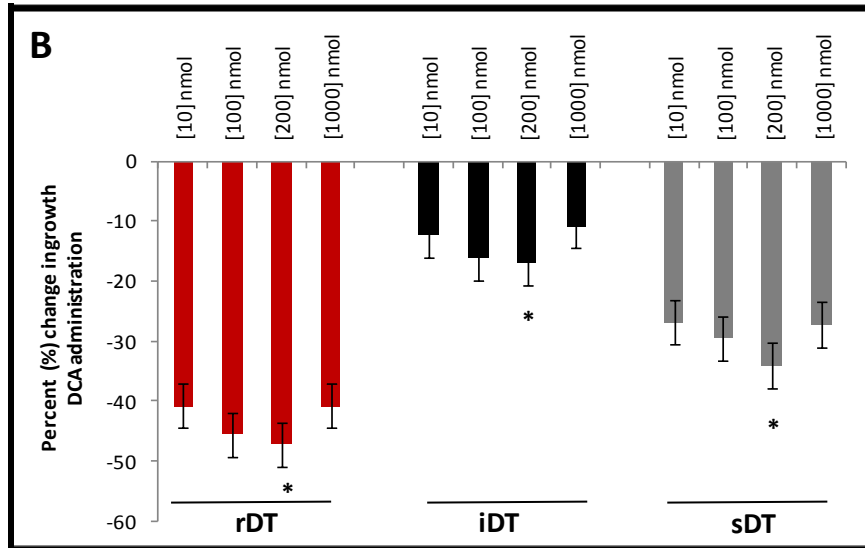


Figure F1. Viability Change with DCA Administration.

DPSC isolates were plated in 96-well assay plates and their proliferation measured to determine if the administration of DCA was sufficient to alter cellular proliferation (Figure F1). A dose-dependent relationship was observed in among rDT isolates, with increasing concentrations of DCA resulting in a more robust inhibition of cell growth. The lowest concentration of DCA evaluated (10 nmol) was sufficient to inhibit the growth of rDT isolated by 40.8%, with increasing concentrations exhibiting greater proliferation inhibition effects up to the growth inhibitory maximum ( $GI_{MAX}$ ) observed at

200 nmol (-47.2%); higher concentrations elicited less robust proliferation-inhibiting effects extending to 1000 nmol (-39%).

Administration of DCA was also sufficient to inhibit iDT proliferation, although equivalent concentrations were less effective at inhibiting iDT cell growth than rDT cells. More specifically, the lowest concentrations of DCA (10 nmol) were sufficient to inhibit the growth of iDT cells by 12.3%, up to a  $GI_{MAX}$  of -16.9% (200 nmol), with decreasing effects observed up to 1000 nmol (-10.8%). The DCA-induced proliferation inhibiting effects were also observed among sDT cells, inhibiting sDT cell growth by 26.9% at 10 nmol up to the  $GI_{MAX}$  of -34.1% (200 nmol), with decreasing effects observed up to 1000 nmol (-27.2%).

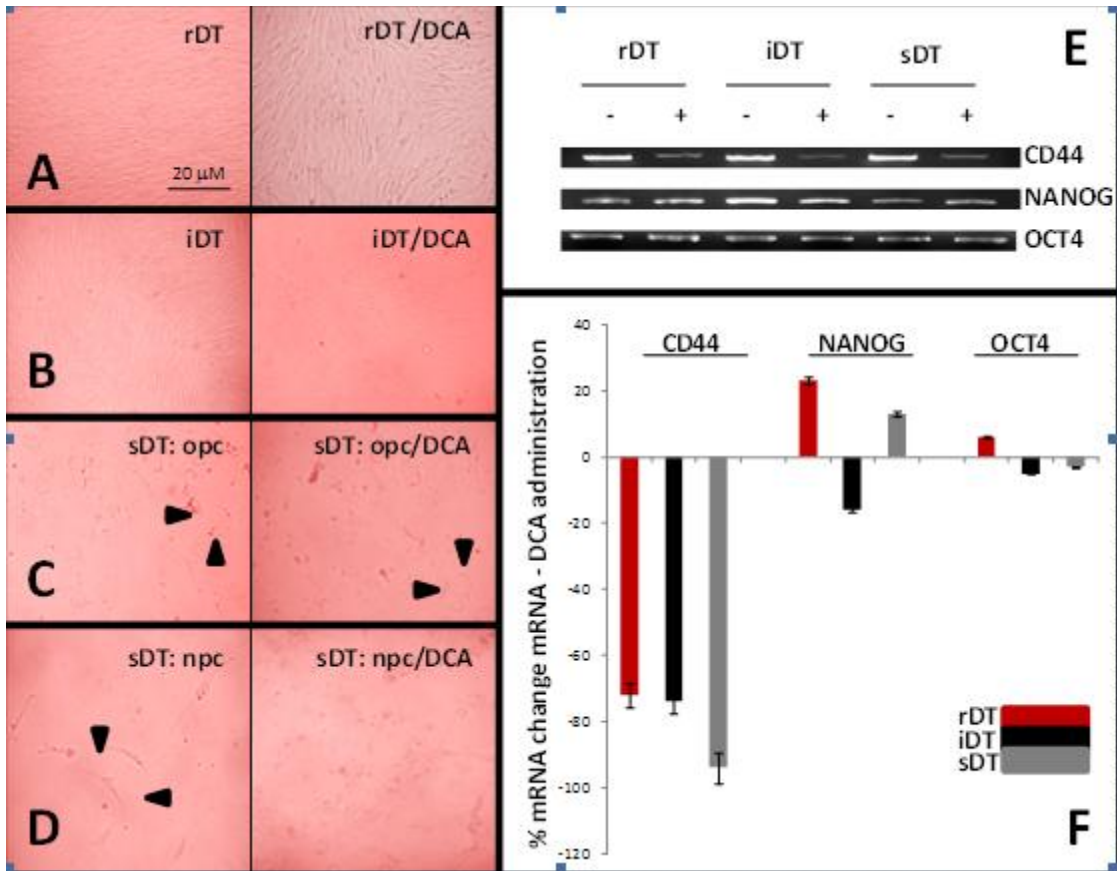


Figure F2. DPSC morphology and RNA marker expression.

To more accurately assess the qualitative effects DCA at  $GI_{MAX}$  concentrations, sufficient to inhibit proliferation of rDT, iDT and sDT DPSC isolates, microscopy and RT-PCR were performed (Figure F2). Although administration of the  $GI_{MAX}$  concentration of DCA (200 nmol) significantly inhibited both rDT (A) and iDT (B) cell growth, no significant alterations to cellular morphology or cell spreading were observed under experimental conditions. In addition, sDT cells established distinctive morphologies, such as large, ovoid-shaped cells (C) that were suggestive of odontoblast progenitor cells (sDT:opc), which did not exhibit any phenotypic changes in morphology. Two sDT isolates formed long, narrow cells with axon- or dendrite-like projections (D), suggestive of neuronal progenitor cells (sDT:npc).

To provide more qualitative assessments of the changes induced by DCA administration on these potential DPSC isolates, RNA was successfully isolated from all DPSC cultures and relative endpoint (RE) RT-PCR performed on equal concentrations of total RNA from each cell line (Figure F2E). Expression of mRNA for the mesenchymal stem cell marker CD44, NANOG and Oct4 was observed in all rDT, iDT and sDT isolates (representative samples shown). However, the addition of DCA at the  $GI_{MAX}$  concentration of 200 nmol significantly reduced expression of the cell surface MSC marker CD44, but not NANOG or Oct4. Densitometry measurements of band intensity using RE-RT-PCR for mRNA expression following DCA administration were compared to baseline expression from the untreated cells (Figure F2F), revealing a stark reduction in CD44 expression in all three types of isolates, rDT, iDT and sDT (-72%, -74%, -94%, respectively).

The results of this study suggest that DCA administration is sufficient to inhibit proliferation and growth of DPSCs at levels that are demonstrated to be non-toxic to normal, non-cancerous cells. However, DCA administration may also influence differentiation-specific markers in DPSC isolates that are partially committed to become neural progenitors; although no such effects were observed among the DPSC odontoblast progenitors.

Although these effects in this initial pilot study appear to have specificity for neural progenitors, they do not seem to extend to odontoblast progenitors – a result that may potentially limit the application and use of DCA for these purposes. Much work remains to be done to elucidate the possible mechanisms that might explain these effects and their specificity for particular types of DPSC isolates that are partially committed towards a specific cell lineage.

## BIBLIOGRAPHY

1. National Institutes of Health: **Stem cell basics**.  
<http://stemcells.nih.gov/staticresources/info/basics/SCprimer2009.pdf>.
2. D'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V, Papaccio G: **Human dental pulp stem cells: from biology to clinical applications**. *Journal of Experimental Zoology (Mol. Dev. Evol.)* 2009, 312B:408-415.
3. Govindasamy V, Ronald VS, Totey S, Din SB, Mustafa WM, Totey S, Zakaria Z, Bhonde RR: **Micromanipulation of culture niche permits long-term expansion of Dental pulp stem cells – an economic and commercial angle**. *In Vitro Cell Dev. Biol.* 2010, 46:764-773.
4. Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S: **The efficacy of mesenchymal stem cells to regenerate and repair dental structures**. *Orthod Craniofac Res.* 2005, 8(3):191-199.
5. Sloan AJ, Waddington RJ: **Dental pulp stem cells: what, where, how?** *Int J Paediatr Dent.* 2009, 19(1):61-70.
6. Huang GT: **Dental pulp and dentin tissue engineering and regeneration: advancement and challenge**. *Front Biosci (Elite Ed)*. 2011, 3:788-800.
7. Djouad F, Jackson WM, Bobick BE, Janjanin S, Song Y, Huang GT, Tuan RS: **Activin A expression regulates multipotency of mesenchymal progenitor cells**. *Stem Cell Res Ther.* 2010, 1(2):11.
8. Karbanová J, Soukup T, Suchánek J, Pytlík R, Corbeil D, Mokřý J: **Characterization of Dental Pulp Stem Cells from Impacted Third Molars Cultured in Low Serum-Containing Medium**. *Cells Tissues Organs*. 2010 [Epub ahead of print]



9. Gronthos S, Mankani M, Brahimi J, Robey PG, Shi S: **Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo.** Proc. Natl. Acad. Sci. USA 2000, 97:13625-13630.
10. Beane OS, Darling EM: **Isolation, characterization, and differentiation of stem cells for cartilage regeneration.** Ann Biomed Eng. 2012, 40(10): 2079-2097.
11. Wagey R, Short B: **Isolation, enumeration, and expansion of human mesenchymal stem cells in culture.** Methods Mol Biol. 2013, 946: 315-334.
12. Di Bernardo G, Piva R, Giordano A, Galderisi U: **Exploiting stem cell therapy: The 3rd meeting of stem cell research Italy.** J Cell Physiol. 2013, 228(4): 911-4.
13. Zhang ZY, Teoh SH, Hui JH, Fisk NM, Choolani M, Chan JK: **The potential of human fetal mesenchymal stem cells for off-the-shelf bone tissue engineering application.** Biomaterials. 2012, 33(9): 2656-72.
14. Suchánek J, Visek B, Soukup T, El-Din Mohamed SK, Ivancaková R, Mokry J, Aboul-Ezz EH, Omran A: **Stem cells from human exfoliated deciduous teeth-- isolation, long term cultivation and phenotypical analysis.** *Acta Medica (Hradec Kralove)*. 2010, 53(2):93-99.
15. Govindasamy V, Abdullah AN, Ronald VS, Musa S, Ab Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH: **Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth.** *J Endod.* 2010, 36(9):1504-1515.
16. Tandon S, Saha R, Rajendran R, Nayak R: **Dental pulp stem cells from primary and permanent teeth: quality analysis.** *J Clin Pediatr Dent.* 2010, 35(1):53-58.

17. Huang GT, Sonoyama W, Chen J, Park SH: **In vitro characterization of human dental pulp cells: various isolation methods and culturing environments.** *Cell Tissue Res.* 2006, **324**(2):225-236. [Epub 2006 Jan 27]
18. Bakopoulou A, Leyhausen G, Volk J, Tsiftoglou A, Garefis P, Koidis P, Geurtsen W: **Assessment of the impact of two different isolation methods on the osteo/odontogenic differentiation potential of human dental stem cells derived from deciduous teeth.** *Calcif Tissue Int.* 2011, **88**(2):130-141. [Epub 2010 Dec 14]
19. Woods EJ, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS: **Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use.** *Cryobiology.* 2009, **59**(2):150-157. [Epub 2009 Jun 16]
20. Lee SY, Chiang PC, Tsai YH, Tsai SY, Jeng JH, Kawata T, Huang HM: **Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells.** *J Endod.* 2010, **36**(8):1336-1340. [Epub 2010 Jun 23]
21. Papaccio G, Graziano A, D'Aquino R, Graziano MR, Pirozzi G, Menditti D, De Rosa A, Carinci F, Laino G: **Long-term cryopreservation of dental pulp stem cells: (sbp-dpscs) and their differentiated osteoblasts: a cell source for tissue repair.** *Journal of Cellular Physiology* 2006, 208:319-325.
22. Jo YY, Lee HJ, Kook SY, Choung HW, Park JY, Chung JH, Choung YH, Kim ES, Yang HC, Choung PH: **Isolation and characterization of postnatal stem cells from human dental tissues.** *Tissue Eng.* 2007, **13**(4):767-773.
23. Hirata TM, Ishkitiev N, Yaegaki K, Calenic B, Ishikawa H, Nakahara T, Mitev V, Tanaka T, Haapasalo M: **Expression of multiple stem cell markers in dental pulp cells cultured in serum-free media.** *J Endod.* 2010, **36**(7):1139-1144. [Epub 2010 Apr 10]

24. Yu J, He H, Tang C, Zhang G, Li Y, Wang R, Shi J, Jin Y: **Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging.** *BMC Cell Biol.* 2010, **11**:32.
25. Kadar K, Kiraly M, Porcsalmy B, Molnar B, Racz GZ, Blazsek J, Kallo K, Szabo EL, Gera I, Gerber G, Varga G: **Differentiation potential of stem cells from human dental origin - promise for tissue engineering.** *J Physiol Pharmacol.* 2009, **60**(Suppl 7):167-175.
26. Mori G, Centonze M, Brunetti G, Ballini A, Oranger A, Mori C, Lo Muzio L, Tetè S, Ciccolella F, Colucci S, Grano M, Grassi FR: **Osteogenic properties of human dental pulp stem cells.** *J Biol Regul Homeost Agents.* 2010, **24**(2):167-175.
27. Waddington RJ, Youde SJ, Lee CP, Sloan AJ: **Isolation of distinct progenitor stem cell populations from dental pulp.** *Cells Tissues Organs.* 2009, **189**(1-4):268-274.  
[Epub 2008 Aug 14]
28. Howard C, Murray PE, Namerow KN: **Dental pulp stem cell migration.** *J Endod.* 2010, **36**(12):1963-1966. [Epub 2010 Oct 8]
29. Liu J, Jin TC, Chang S, Czajka-Jakubowska A, Clarkson BH: **Adhesion and growth of dental pulp stem cells on enamel-like fluorapatite surfaces.** *J Biomed Mater Res A.* 2011, **96**(3):528-534. doi: 10.1002/jbm.a.33002. [Epub 2011 Jan 4]
30. Zhang W, Walboomers XF, Van Kuppevelt TH, Daamen WF, Van Damme PA, Bian Z, Jansen JA: **In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages.** *J Tissue Eng Regen Med.* 2008, **2**(2-3):117-125.
31. Yu V, Damek-Poprawa M, Nicoll SB, Akintoye SO: **Dynamic hydrostatic pressure promotes differentiation of human dental pulp stem cells.** *Biochem Biophys Res Commun.* 2009, **386**(4):661-665. [Epub 2009 Jun 23]

32. Riccio M, Resca E, Maraldi T, Pisciotta A, Ferrari A, Bruzzesi G, De Pol A: **Human dental pulp stem cells produce mineralized matrix in 2D and 3D cultures.** *Eur J Histochem.* 2010, **54**(4):e46.
33. Yang X, Yang F, Walboomers XF, Bian Z, Fan M, Jansen JA: **The performance of dental pulp stem cells on nanofibrous PCL/gelatin/nHA scaffolds.** *J Biomed Mater Res A.* 2010, **93**(1):247-257.
34. Nakashima M, Mizunuma K, Murakami T, Akamine A: **Induction of dental pulp stem cell differentiation into odontoblasts by electroporation-mediated gene delivery of growth/differentiation factor 11 (Gdf11).** *Gene Ther.* 2002, **9**(12):814-818.
35. Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A: **Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2.** *J Dent Res.* 2004, **83**(8):590-595.
36. Huang GT, Shagranova K, Chan SW: **Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro.** *J Endod.* 2006, **32**(11):1066-1073.
37. Király M, Porcsalmy B, Pataki A, Kádár K, Jelítai M, Molnár B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G: **Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons.** *Neurochem Int.* 2009, **55**(5):323-332. [Epub 2009 Apr 5]
38. Morito A, Kida Y, Suzuki K, Inoue K, Kuroda N, Gomi K, Arai T, Sato T: **Effects of basic fibroblast growth factor on the development of the stem cell properties of human dental pulp cells.** *Arch Histol Cytol.* 2009, **72**(1):51-64.
39. Suchanek J, Soukup T, Visek B, Ivancakova R, Kucerova L, Mokry J: **Dental pulp stem cells and their characterization.** *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* 2009, **153**(1):31-35.

40. Lee JY, Nam H, Park YJ, Lee SJ, Chung CP, Han SB, Lee G: **The effects of platelet-rich plasma derived from human umbilical cord blood on the osteogenic differentiation of human dental stem cells.** *In Vitro Cell Dev Biol Anim.* 2011, **47(2):157-164.** [Epub 2010 Nov 17]
41. Hays WL: **Inferences about population means.** **In: Statistics (5th edition).** International Thomson Publishing 1994. 311-342.
42. Agha-Hosseini F, Jahani MA, Jahani M, Mirzaii-Dizgah I, Ali-Moghaddam K: **In vitro isolation of stem cells derived from human dental pulp.** *Clinical Transplantation* 2010, **24:E23-E2.**
43. Bakopoulou A, Leyhausen G, Volk J, Tsiftoglou A, Garefis P, Koidis P, Geurtsen W: **Assessment of the impact of two different isolation methods on the osteo/odontogenic differentiation potential of human dental stem cells derived from deciduous teeth.** *Calcified Tissue International* 2010, **88:130-141.**
44. Chen YK, Huang AHC, Chan AWS, Shieh TY, Lin LM: **Human dental pulp stem Cells derived from different cryopreservation methods of human dental pulp tissues of diseased teeth.** *Journal of Oral Pathology and Medicine* 2011, **40:793-800.**
45. 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.** In Review, Submitted for Publication 2013.
46. Burnett A, Kumar R, Westphal JD, Hill CK, Kingsley K: **Dichloroacetate (DCA) inhibited cell proliferation and promoted de-differentiation of dental pulp-derived mesenchymal stem cells in vitro.** In Review, Submitted for Publication 2013.

## CURRICULUM VITAE

Graduate College  
University of Nevada, Las Vegas

Charles Kennedy Hill

### Degrees:

Bachelor of Science in Psychology, 1992  
Arizona State University

Doctor of Dental Medicine, 1996  
Southern Illinois University

Master of Public Health, 2011  
University of Nevada, Las Vegas

Thesis Title: Adult Permanent (Extracted) Teeth as a Source of Dental Pulp-Derived Mesenchymal Stem Cells (DPSC): A Pilot Study

### Thesis Examination Committee:

Chairperson, Karl Kingsley, Ph.D, M.P.H.  
Committee Member, Clifford Seran, D.M.D.  
Committee Member, Rhonda Everett, D.D.S., M.P.H.  
Graduate Faculty Representative, Timothy Bungum, Dr.P.H.  
Graduate Coordinator, James Mah, D.D.S., M.S., D.M. Sc.