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THE EFFECTS OF BONE MORPHOGENIC PROTEIN 2 ON THE VIABILITY AND PROLIFERATION OF DENTAL PULP STEM CELL ISOLATES

By

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2012

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

School of Dental Medicine
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University of Nevada, Las Vegas
May 2019

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Thesis Approval

The Graduate College
The University of Nevada, Las Vegas

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This thesis prepared by

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entitled

The Effects of Bone Morphogenic Protein 2 on the Viability and Proliferation of Dental Pulp Stem Cell Isolates

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

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Abstract

The Effects of Bone Morphogenic Protein 2 on the Viability and Proliferation of Dental Pulp Stem Cell Isolates

By

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Growth factor is an umbrella term used to describe a signaling molecule present in organisms that serves the purpose of influencing a stimulatory or inhibitory response from the target cell it acts on. Growth factors are categorized and classified by the types of tissues they act on and the cellular responses they illicit. For example, there are growth factors that act primarily in connective tissue on fibroblasts and they are aptly classified as the fibroblast growth factor family. Similarly, there is another family of growth factors that act on bone forming cells, among others, and they are known as bone morphogenic proteins. One member from this group of growth factors, BMP-2, is the focus of the current research.

Bone Morphogenic Proteins are part of the Transforming Growth Factor-beta superfamily of growth factors. Recently they have been implicated in a variety of pathological processes including colon cancer and Barrett's esophagus. BMP-2 is known for its ability to stimulate bone

formation and is the most successful bone modulator in the family. Its involvement in cellular differentiation into bone forming cells has been shown in many studies. BMP-2 acts on cell surface receptors which, through a series of reactions, enhances the likelihood of differentiating into a bone forming cell. This induction can be demonstrated in a variety of cell types. Those of particular interest are stem cells.

There are three main types of stem cells including: embryonic stem cells, adult non-embryonic stem cells, induced pluripotent stem cells. Adult stem cells are the most abundant and are less controversial than embryonic stem cells. Currently adult stem cells are being isolated from a variety of tissues including those of mesenchymal tissue origin. The cell type in the current study are dental pulp stem cells (DPSC) which have been shown to display phenotypic changes in response to various growth factors. The use of multiple growth factors concomitantly on DPSC has not been studied in great depth, and thus it is not known if multiple stimulatory growth factors will act synergistically or antagonistically with one another.

The data from the two following studies provide evidence that BMP-2 provides a stimulating influence on at least one dental pulp stem cell subset. When used concomitantly, BMP-2 and Vascular Endothelial Growth Factor (VEGF) enhances sDT DPSC phenotype (viability and growth) beyond that of either growth factor independently. Future studies may be needed to evaluate the potential for BMP-2 and other growth factors to induce DPSC differentiation and lineage-specific phenotypic changes for bioengineering applications or tissue regeneration.

Acknowledgments

Thank you to my committee chair, Dr. Karl Kingsley, for introducing me to this topic. His support and dedication of time throughout my research project has been invaluable. Also, thank you my committee members, Dr. Joshua Polanski, Dr. Brian Chrzan, and Dr. Jennifer Pharr for your support. I would like to thank Erica Nguyen, Eric Mullins, Louisa Heske for their time and help in the laboratory.

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Chapter 1: Introduction

Background and Significance

Tissue regeneration and biomedical engineering are goals of modern research that have made tremendous strides in recent years. Stem Cell research, popularized circa 2000, began with the focus primarily on embryonic stem cells. The promise of this realm of research lay in the cells' pluripotency - the ability of these cells to differentiate into a variety of cell types. More recently, stem cells have been isolated from a number of different tissue types including the pulp tissue within teeth. These stem cells are aptly called dental pulp stem cells (DPSC). DPSCs possess the trait of multipotency – a more restricted pedigree of potential cell derivatives than those that are pluripotent.

DPSCs are cells of mesenchymal tissue origin and are known for their potential to differentiate into a variety of cell types [4]. These changes are brought about through induction by stem cell exposure to growth factors [1]. A number of studies have been conducted that investigate the inductive potential of dentin matrix protein 1, VEGF, EGF, FGF, and BMP and the various cell lineages they yield [2, 3]. The field of bone synthesis and grafting is of great therapeutic importance in dentistry. Dental pulp stem cells have been shown to be able to be differentiated into osteoblast precursor cells by BMP-7 [5]. Understanding the biochemical process of cellular differentiation can allow for scientists to guide and possibly eventually control the cell's destiny. If able to guide the cell down a specific pathway of differentiation into a specific cell line, such as bone precursor cells, therapeutic benefits would be possible. This process is the focus of this study, specifically the BMP family of growth factors which is comprised of many variants, and the one that will be the focus of this project is the BMP-2 homodimer.

Research Questions

The goal of this study was to investigate the ability of bone morphogenic protein (BMP-2) to induce proliferation and differentiation of DPSC isolates into mineral forming bone cell precursor lineages.

1. Are dental pulp stem cells able to be induced into a mineral forming bone cell precursor lineage via BMP-2 stimulation?

   H_0: BMP-2 will have no effect on the induction of dental pulp stem cell differentiation into a mineral forming bone cell precursor lineage

   H_A: BMP-2 will affect the induction of dental pulp stem cell differentiation into a mineral forming bone cell precursor lineage.

2. Can BMP-2 effect the viability and proliferation of DPSC subtypes?

   H_0: BMP-2 will have no effect of the viability or proliferation of DPSCs.

   H_A: BMP-2 will affect the viability or proliferation of DPSCs.

3. Can VEGF effect the viability and proliferation of DPSC subtypes?

H_0: VEGF will have no effect on the viability or proliferation of DPSCs.

H_A: VEGF will affect the viability or proliferation of DPSCs.

4. Can BMP-2 in combination with VEGF effect the viability and proliferation of DPSC subtypes?

H_0: The combination treatment will have no effect on the viability or proliferation of DPSCs.

H_A: The combination treatment will affect the viability or proliferation of DPSCs.

Approval

The protocol for this study was reviewed and approved by the Office for the Protection of Research Subjects (OPRS) and Institutional Review Board (IRB) OPRS#763012-1 “Retrospective analysis of dental pulp stem cells (DPSC) from the University of Nevada Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and adult clinical population”. The original protocol for the collection and isolation of DPSC was approved by the IRB and OPRS#0907-3148 “Isolation of Non-Embryonic Stem Cells from Dental Pulp”.

Research Design

The DPSC that were used and analyzed in this study were previously obtained randomly from the UNLV-SDM pediatric patient population before the commencement of this study. The inclusion criteria for this project were patients – pediatric or adult – who were pre-orthodontic, of at least 7 years of age (mainly 12 – 15 years of age) and needed extraction of vital, non-carious teeth – mainly to relieve crowding of the anterior dentition. Both pediatric assent and parental consent were required to partake in the study. Informed consent was required for all adult patients. The exclusion criteria comprised of any individual who was not a patient of record at UNLV-SDM, patients whose teeth were extracted for any reason other than elective extraction including trauma, caries, or other pathology and any patients who declined to participate.

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Chapter 2

Assessment of Dental Pulp Stem Cell (DPSC) Biomarkers Following Induction with Bone Morphogenic Protein 2 (BMP-2)

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Role of Authors:
This work was carried out in collaboration between all authors. Authors Karl Kingsley and Joseph Cinelli designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors Joseph Cinelli and Erica Nguyen managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Abstract

Introduction: Tissue regeneration and biomedical engineering are the goals of modern research that have made tremendous strides in recent years. Dental pulp stem cells (DPSCs) have been demonstrated to exhibit functional multipotency, differentiating into neurons, adipocytes, and other cell types. The primary goal of this study was to investigate the ability of bone morphogenic protein (BMP-2) to induce proliferation and differentiation of DPSC isolates into mineral forming bone cell precursor lineages.

Study Design: This was a prospective study with the non-randomized experimental design.

Place and Duration of Study: This study was conducted at the University of Nevada, Las Vegas – School of Dental Medicine between May 2017 and August 2018.

Methodology: Eight previously isolated dental pulp stem cell (DPSC) isolates were grown in culture and treated with bone morphogenic protein (BMP-2) to evaluate any effects on growth, viability or biomarker expression.

Results: BMP-2 induced significant changes in cellular growth among a subset of DPSC with slow doubling times (sDT), which corresponded with similar increases in cellular viability. Also, BMP-2 was sufficient to induce mRNA expression of alkaline phosphatase (ALP) and other differentiation markers among the sDT isolates – although no significant changes were observed among the DPSC isolates with rapid or intermediate DTs (rDT, iDT).

Conclusions: This study may be the first to demonstrate not only the differential responsiveness of DPSC isolates to BMP-2, but also to identify the MSC biomarkers that may affect initial DPSC responsiveness to this stimulus. Although many studies have evaluated the role of the biomarkers NANOG, Sox-2 and Oct-4 in DPSC isolate, no other study of DPSC multipotency has evaluated the role of Nestin – which may be one of the key factors that potentiate or limits the responsiveness to BMP-2 and osteogenic potential among DPSCs.

Key Words: Dental pulp stem cells (DPSC); bone morphogenic protein (BMP-2); bioengineering; biotechnology.

Abbreviations: Dental pulp stem cell (DPSC), insulin-like growth factor (IGF), bone morphogenic protein (BMP), Office for the Protection of Research Subjects (OPRS), Institutional Review Board (IRB), University of Nevada Las Vegas (UNLV), School of Dental Medicine (SDM), cementoenamel junction (CEJ), phosphate buffered saline (PBS), bone morphogenic protein (BMP), doubling time (DT), Total RNA isolation reagent (TRIR), polymerase chain reaction

(PCR), deoxyribonucleic acid (DNA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), ANOVA (Analysis of variance), vascular endothelial growth factor (VEGF).

1. Introduction

Tissue regeneration and biomedical engineering are the goals of modern research that have made tremendous strides in recent years [1-3]. At the forefront of these efforts has been the use of stem cell-based therapies, which have demonstrated tremendous potential in these areas [4-6]. Although many studies have focused on embryonic and perinatal stem cells, the use of adult or mesenchymal stem cells may represent readily available, widely applicable and less controversial alternatives [7,8].

Many types of mesenchymal stem cells exist in a variety of tissues, including bone marrow, adipose tissue, and dental pulp [9-11]. Dental pulp stem cells (DPSCs) have been demonstrated to exhibit functional multipotency, differentiating into neurons, adipocytes, and other cell types [12,13]. Recent evidence has demonstrated considerable progress in new areas of research, such as DPSC use in the tissue engineering of bone [14-17].

Much of the research focusing on DPSC induction into osteoblast cells or precursors has focused on the isolation and identification of DPSCs with strong osteogenic potential [18-20]. Another important area of research has been concentrated on the stimulus to direct DPSC differentiation towards these osteogenic lineages, including insulin-like growth factor (IGF) and bone morphogenic proteins (BMPs) [21-23]. Although BMPs have been known to facilitate dentin formation and regeneration among DPSCs, more recent evidence suggests these effects may also induce osteoblastic differentiation and bone regeneration potential [23-25].

Based on this information, the primary goal of this study was to investigate the ability of BMP-2 to induce proliferation and differentiation of DPSC isolates into mineral forming bone cell precursor lineages expressing the appropriate biomarkers

2. Methodology

2.1 Study Approval

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

2.2 Study Design

The DPSC that were used and analyzed in this study were previously obtained randomly from the UNLV-SDM pediatric patient population before the commencement of this study. The inclusion criteria for this project were patients – pediatric or adult – who were pre-orthodontic, of at least 7 years of age (mainly 12 – 15 years of age) and needed extraction of vital, non-carious teeth – mainly to relieve crowding of the anterior dentition. Both pediatric assent and parental consent were required to partake in the study. Informed consent was required for all adult patients. The exclusion criteria comprised of any individual who was not a patient of record at UNLV-SDM, patients whose teeth were extracted for any reason other than elective extraction including trauma, caries, or other pathology and any patients who declined to participate.

2.3 DPSC Collection (Initial)

Collection of DPSC began with participants of the study undergoing extractions – primarily third molars. Following extraction, the teeth were decoronated at the cemento-enamel junction (CEJ) using a high-speed dental handpiece and bur. The dental pulp was then removed from the canal with an endodontic broach and placed in a sterile microcentrifuge tube pre-filled with 1X phosphate-buffered saline (PBS) on ice for transport to a biomedical laboratory for further processing and screening.

De-identification of the samples through the use of randomly generated, non-duplicated numbers assigned to each sample and related patient demographic information was de-identified to eliminate the possibility of revealing any information that could identify a participant or biasing the research. The researchers were not made privy to any of the identifying participant information at any point during the study.

2.4 Culture and Propagation

In brief, two primary methods for establishing DPSC isolates are the enzymatic digestion and direct outgrowth methods [26,27]. Although methods were utilized, no results were found using the enzymatic digestion method to separate DPSC from the dental pulp, while n=31/40 or 77.5% of DPSC isolates were established using the direct outgrowth method – as previously described [28,29]. All viable samples were derived from patients aged 31 years and younger, which were equally distributed between males and females [26,27]. The rate of growth and doubling time (DT) were obtained by culturing and propagated over ten passages.

The split (passage ratio) for each DPSC sample was 1:2 and trypan blue and a BioRad TC20 automated cell counter (Hercules, CA) was used to determine confluence of the cell lines when used following the protocol established by the manufacturer. The data is comprised of total and live cell counts allowing for calculation of the percentage of viable cells available for analysis. The DPSC cell lines were further sorted based on the doubling time (DT) as either rapid or rDT (~2 days) n=3 (dpsc-3882, dpsc-5653, dpsc-7089), intermediate or iDT (4-6 days) n=2 (dpsc-8124, dpsc-17322), and slow or sDT (10-12 days) n=3 (dpsc-11418, dpsc-11750, dpsc-11836). These doubling times may be functionally related to the proliferation and differentiation potential of the DPSC isolates, as previously described [26-29].

Rapid Doubling Time (rDT): dpsc-3882, dpsc-5653, dpsc-7089

Intermediate Doubling Time (iDT): dpsc-8124, dpsc-17322

Slow Doubling Time (sDT): 11418, dpsc-11750, dpsc-11836

2.5 Experimental Protocol

The various DPSC lines were plated in concentrations of $1.2x10^4$ cells/mL into 96-well tissue culture treated plates to assess the effects of bone morphogenic protein 2 (BMP-2), if any, on DPSC. The experimental cells, those dosed with BMP-2 from Fischer Scientific (RP-8638) at a concentration of 10 ng/mL, were compared to the control cells (non-dosed), similar to other studies of BMP-2 among MSC and DPSC [23-25]. A total of three experimental trials (n=24) were performed – eight DPSC isolates for each experimental condition and repeated three times throughout three weeks.

2.6 RNA Isolation

Total RNA was obtained from every sample through the use of Total RNA isolation reagent (TRIR) from Molecular Research Center (Cincinnati, OH) and following the manufacturer's protocol. Absorbance at wavelengths of 260 and 280nm (A260/A280 ratio) was used to screen the collected RNA for quality and quantity.

2.7 Polymerase Chain Reaction (PCR)

Evaluation of DPSC isolates for differences in the levels of mRNA expression was done using the ABgene Reverse-iT One-Step RT-PCR protocol and reagent kit under the following provisions: initial reverse transcription at 47C for 30mins followed by 30 cycles of denaturation at 95C for 10 minutes then annealing for 30 seconds at the appropriate temperature for each primer set and final extension at 60C for one minute. The following primers from Eurofins MWG Operon (Huntsville, AL) were synthesized:

Housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glycolytic pathway

Forward primer-

GAPDH, ATCTTCCAGGAGCGAGATCC; 20 nt, 55% GC, Tm 66C

Reverse primer-

GAPDH, ACCACTGACACGTTGGCAGT; 20 nt, 55% GC, Tm 70C

Annealing temperature 67C; Optimal temperature T(opt): Lower temperature – 5C = 61C

Osteogenic biomarker, Alkaline Phosphatase (ALP)

Forward primer-

ALP, CACTGCGGACCATTCCCACGTCTT;24 nt, 58% GC, Tm 74C

Reverse primer-

ALP, GCGCCTGGTAGTTGTTGTGAGCAT; 24 nt, 54% GC, Tm 72C

Annealing temperature: 72C; Optimal temperature T(opt): Lower temperature – 5C = 67C

Differentiation biomarker, dentin sialophosphoprotein (DSPP)

Forward primer-

DSPP, CAACCATAGAGAAAGCAAACGCG;23 nt, 48% GC, Tm 67C

Reverse primer-

DSPP, TTTCTGTTGCCACTGCTGGGAC; 22 nt, 55% GC, Tm 70C

Annealing temperature: 68C; Optimal temperature T(opt): Lower temperature – 5C = 62C.

2.8 Statistical Analysis

Descriptive statistics, including counts and percentages, for basic proliferation and viability of the isolated DPSC were compiled and analyzed. The appropriate tests for parametric data analysis, t-tests, were used to calculate and compare changes in viability and proliferation. To limit Type I errors, the t-test results were confirmed via multiple ANOVA (Analysis of variance).

3. Results

An assessment of the quality and quantity of RNA obtained from these assays under both control and experimental conditions was performed (Table 1). These data demonstrated that the average RNA concentration isolates from the rDT DPSC isolates under both conditions was similar and not significantly different (611.3, 618.2 ng/uL respectively), p= 0.588. Similar results were observed with the iDT (632, 628.1 ng/uL respectively) and sDT DPSC isolates (599.7, 649.4 ng/uL respectively), p=0.214. The quality of RNA assessed by the absorbance ratio of A260: A280 also demonstrated similar values between the control and experimental DPSC isolates: rDT (1.67, 1.69 respectively), iDT (1.72, 1.66 respectively) and sDT (1.75, 1.76 respectively).

Then, to evaluate any potential effects on these DPSC isolates, 96-well growth assays were performed with and without the addition of BMP-2 (Fig. 1). These results demonstrated that the addition of BMP-2 (10 ng/mL) to the rapidly dividing (rDT) DPSC isolates (dpsc-3882, dpsc-5653, dpsc-7089) did not induce any significant effects on cellular proliferation over three days, p=0.388. Also, no measurable differences in cellular growth were observed with the intermediate doubling time (iDT) DPSC isolates (dpsc-8124, dpsc-17322) over this time period,

p=0.411. However, the addition of BMP-2 significantly increased the growth of the slow doubling time (sDT) DPSC isolates (dpsc-11418, dpsc-11750, dpsc-11836), p=0.039.

Table 1. RNA analysis of control and experimental DPSC isolates

	Control DPSC RNA analysis	Experimental DPSC RNA analysis
Rapid (rDT) DPSC	RNA concentration Average = 611.3 ng/uL A260:A280 (purity) Average = 1.67 Range 1.52 – 1.99	RNA concentration Average = 618.2 ng/uL A260:A280 (purity) Average = 1.69 Range 1.55 – 1.86
Intermediate (iDT) DPSC	RNA concentration Average = 632.0 ng/uL A260:A280 (purity) Average = 1.72 Range 1.55 – 1.94	RNA concentration Average = 628.1 ng/uL A260:A280 (purity) Average = 1.66 Range 1.58 – 1.91
Slow (sDT) DPSC	RNA concentration Average = 599.7 ng/uL A260:A280 (purity) Average = 1.75 Range 1.58 – 1.91	RNA concentration Average = 649.4 ng/uL A260:A280 (purity) Average = 1.76 Range 1.62 – 1.88

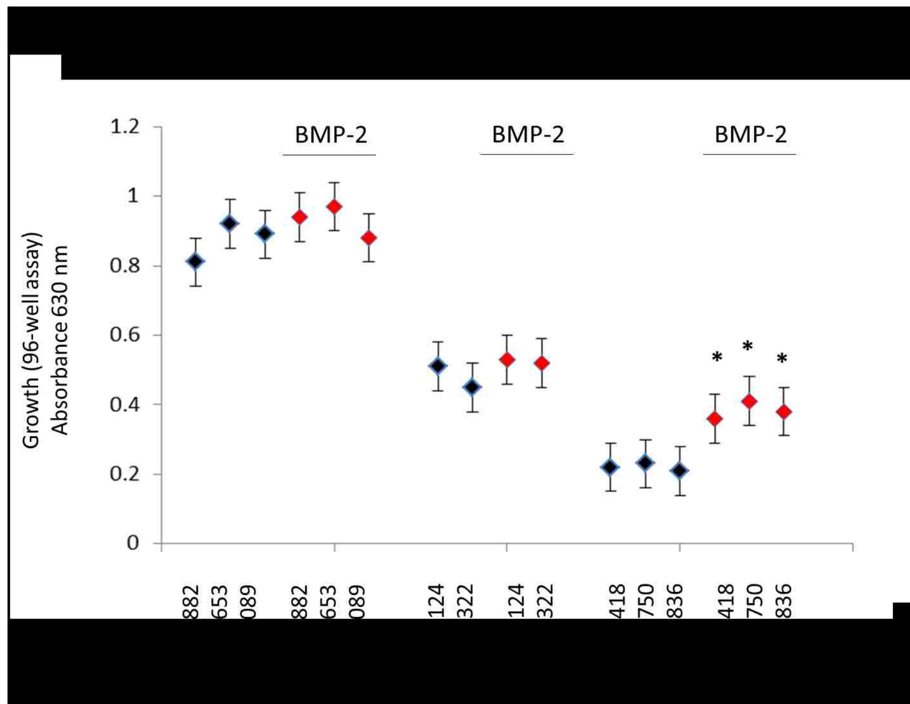

Figure 1. DPSC growth following BMP-2 treatment. BMP-2 administration exhibited strong, positive effects on growth of the slow doubling time (sDT) DPSC isolates (dpsc-11418, dpsc-11750, dpsc-11836), without any significant effects on intermediate (iDT; dpsc-8124, dpsc-17322) or rapid (rDT; dpsc-3882, dpsc-5653, dpsc-7089) DPSC isolates (p=0.411, p=0.388, respectively)

To examine if these changes in cellular growth following BMP-2 administration were associated with any changes to cellular viability, Trypan Blue assays were performed on each DPSC isolate at the end of each experimental assay (Fig. 2). In brief, these data demonstrated that the addition of BMP-2 did not significantly alter cellular viability among the rDT (dpsc-3882, dpsc-5653, dpsc-7089) or iDT (dpsc-8124, dpsc-17322) DPSC isolates (p=0.512, p=0.399,

respectively). However, distinct and significant positive effects were observed among the sDT DPSC isolates (dpsc-11418, dpsc-11750, dpsc-11836), p=0.022.

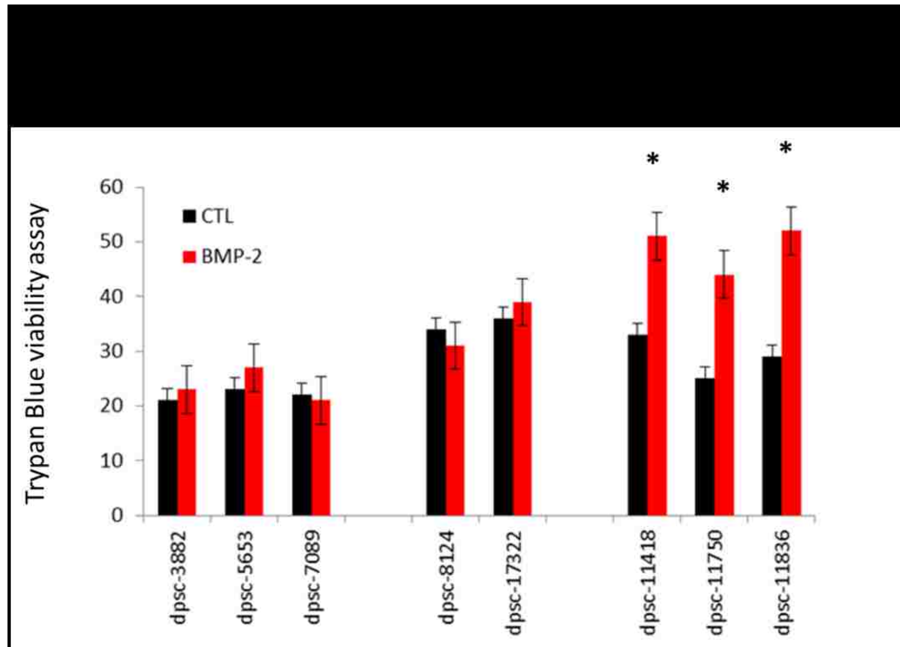

Figure 2. BMP-2 effects on DPSC viability in vitro. Administration of BMP-2 did not result in any significant effects on rapid (rDT; -3882, -5653, -7089) or intermediate (iDT; -8124, -17322) DPSC isolates (p=0.512, p=0.399, respectively) but significantly increased viability among sDT (-11418, -11750, -11836) , p=0.022

Due to the observed changes in both cell viability and growth following BMP-2 administration among the sDT DPSC isolates (-11418, -11750, -11836), an analysis of the DPSC biomarkers associated with osteoblastic differentiation were examined (Fig. 3). Total RNA isolated from all DPSC isolates following BMP-2 administration was screened using primers specific for alkaline phosphatase (ALP) and dentin sialophosphoprotein (DSPP) (Fig. 3A). This experiment revealed that one of the iDT DPSC isolates (-17322) and two of the sDT isolates (-11418-, -11750) exhibited differential mRNA production of these biomarkers. However, no

expression of either ALP or DSPP has observed among the remaining sDT and iDT DPSC isolates (-11836, -8124, respectively) or any of the rDT isolates. Photomicroscopy of the sDT isolates revealed that BMP-2 exerted broad proliferative effects, but morphologic changes were observed only in dpsc-11418 and dpsc-11750 (Fig. 3B).

An additional screening of mesenchymal stem cell (MSC) biomarkers were then performed to determine if any of these changes to cellular phenotype (viability, growth, morphology) or biomarker expression (ALP, DSPP) were associated with the expression (or lack) of MSC biomarkers (Fig. 4). More specifically, the expression of the MSC markers Nestin, NANOG, Oct-4 and Sox-2 were evaluated (Fig. 4A). This analysis revealed the concomitant expression of two or more MSC markers among the rDT and iDT DPSC isolates, but only Nestin among the sDT isolates. Moreover, the expression of Nestin mRNA strongly correlated with DPSC response to BMP-2 (dpsc-11418, dpsc-11750) with the absence of response observed in the sDT with a relatively lower expression of Nestin (dpsc-11836 (Fig. 4B).

4. Discussion

The primary goal of this study was to investigate the ability of BMP-2 to induce proliferation and differentiation of DPSC isolates into mineral forming bone cell precursor lineages expressing the appropriate biomarkers. These results demonstrated that some, but not all, DPSC isolates were capable of responding to BMP-2 with corresponding changes to growth, viability, and cellular morphology. Moreover, these changes were associated with sDT DPSC isolates not expressing multiple MSC biomarkers, but rather one specific MSC marker – Nestin [30,31].

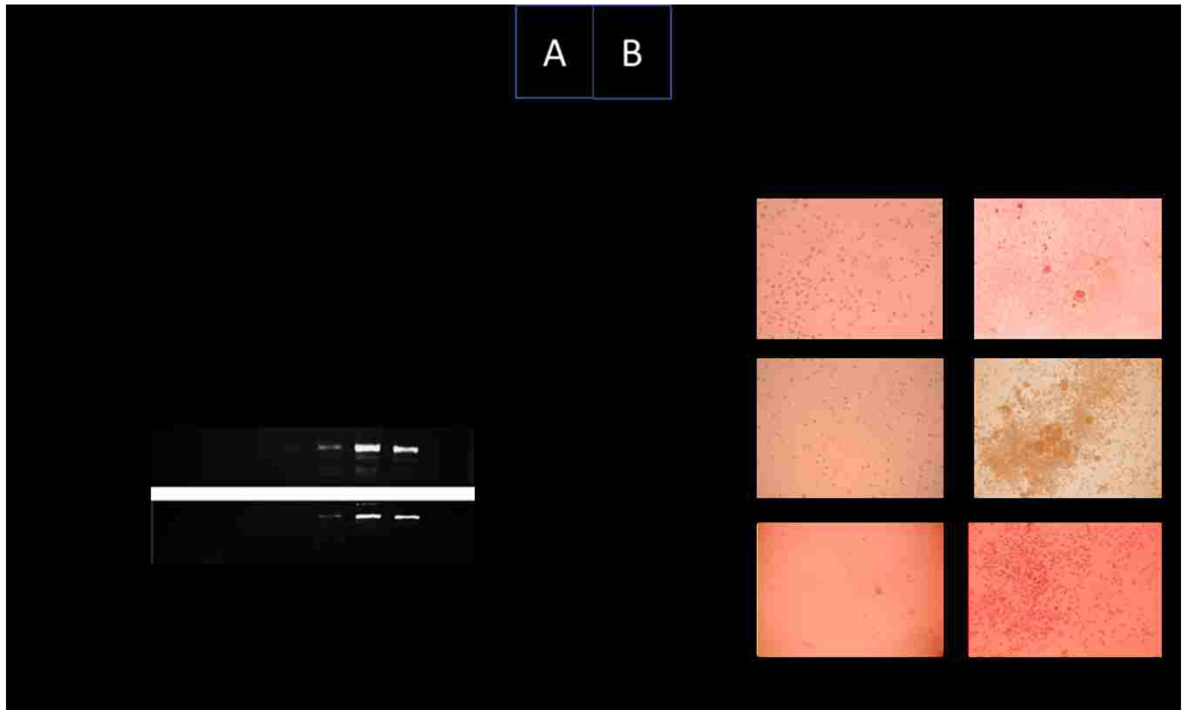

Figure 3. DPSC mRNA biomarker induction following BMP-2 treatment. A) BMP-2 administration induced alkaline phosphatase (ALP) and dentin sialophosphoprotein (DSPP) mRNA expression among one iDT (dpsc-17322) and two sDT (dpsc-11418, dpsc-11750) DPSC isolates, but not all (iDT dpsc-8124 and sDT dpsc-11836 were both negative). No changes were observed among the rDT isolates (dpsc-3882, dpsc-5653, dpsc-7089). B) Photo microscopy of the sDT isolates revealed proliferative effects of BMP-2 with morphologic changes observed only in dpsc-11418 and dpsc-11750. All photomicroscopy was performed taking images from the center of each well to minimize researcher selection bias. DPSC are mainly non-adherent, and their number may vary at different locations in each well as part of the normal experimental variation.

These data appear to confirm other experimental evidence that BMP-2 may exhibit the potential to induce ALP expression among some DPSC isolates [5,32]. However, there is a lack of experimental and observational evidence to evaluate the specific phenotypes and biomarkers associated with DPSC responsiveness – as few studies have compared the effects of BMP-2, DPSC differentiation, and MSC biomarkers [33]. The few studies to have evaluated these phenomena have also demonstrated differential results, with some DPSC isolates responding to BMP-2 (and others not) – although only Runx-2 and MEF2, a member of the myocyte enhancer factor-2 (MEF-2) box family appeared to be enhanced upon BMP-2 administration and ALP induction – although insufficient data were available to ascertain if these were upstream or downstream (cause or effect) changes [34,35].

Although these data provide novel insights into the properties and characteristics of DPSC isolates that may be responsive to BMP-2 administration, there are several limitations associated with this study that must also be considered. For example, new evidence has suggested that improved methods of culture may exist to differentially affect multipotency and stem cell-like properties of DPSC towards osteoblastic and osteogenic lineages [36,37]. Besides, some evidence has also suggested that the timing and administration of multiple stimuli (including BMP-2 in combination with vascular endothelial growth factor or VEGF) may also preferentially affect DPSC responsiveness to BMP-2 - although financial and timing constraints limited the scope of this initial study [33,35,38].

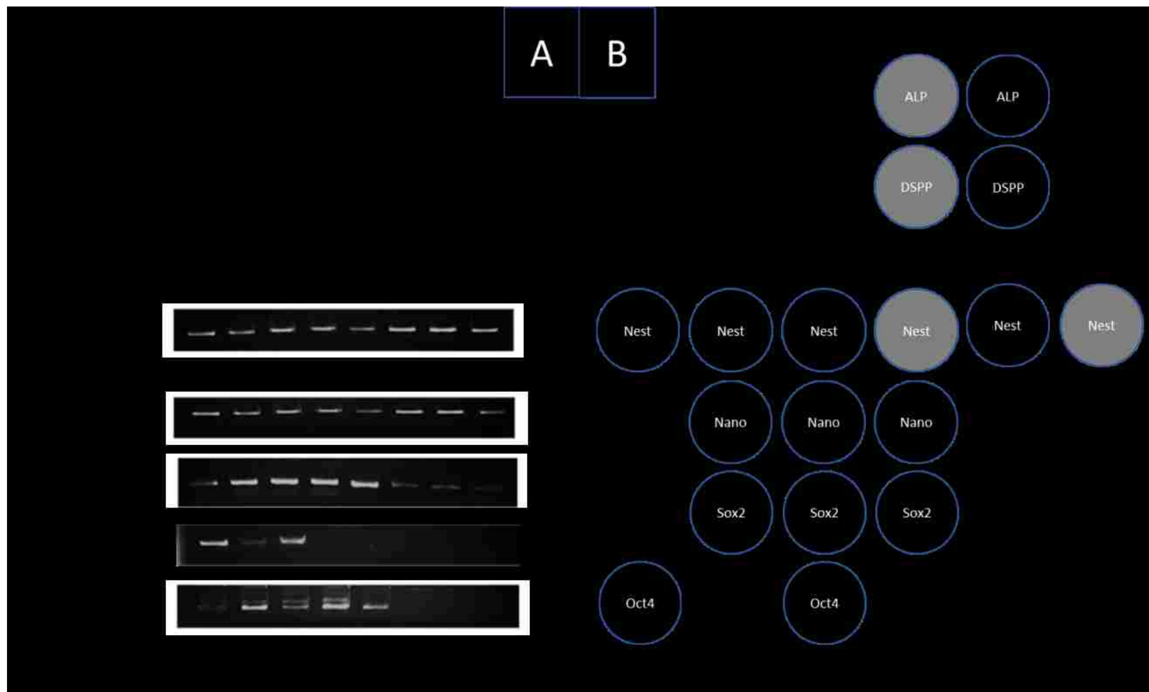

Figure 4. Analysis of MSC biomarker expression among DPSC isolates. A) mRNA expression of Nestin, NANOG, Oct-4 and Sox-2 were differentially expressed among the DPSC isolates, with multiple biomarkers expressed among the rDT and iDT isolates but only Nestin expressed among the sDT isolates. B) Expression of ALP and DSPP induced by BMP-2 was associated with the sDT DPSC isolates with high Nestin expression. Weak or transient expression of Nestin (and the absence of other MSC biomarkers) was observed in the sDT isolate not responsive to BMP-2 treatment (dpsc-11836). Graphic representation of these results: Black circles denote strong mRNA expression and grey circles denote moderate mRNA expression

Finally, the selection of MSC and DPSC biomarkers to evaluate should also be carefully considered [39]. For example, many other studies of DPSC differentiation have evaluated NANOG, Sox-2 and Oct-4, which are known transcription factors that may directly influence specific pathways related to cellular phenotypes [40,41]. However, the role of Nestin appears only to have been evaluated peripherally in studies of DPSC and neural differentiation without

evaluation of this biomarker among studies of osteogenic differentiation and BMP administration [42,43].

These biomarkers may be critical indicators not only of differentiation status and may also directly or indirectly affect other phenotypic behaviors observed in this study, such as doubling time. For example, it was observed that rapid and intermediate doubling times of specific DPSC isolates were associated with the expression of mRNA for MSC biomarkers including Nestin, NANOG, Sox-2 and Oct-4 – confirming previous observations in these DPSC isolates [26,27]. Also, DPSC isolates with slow doubling times were associated with the expression of Nestin but not NANOG, Sox-2 or Oct-4 indicating the potential for partial differentiation – also confirmed in previous studies [28,29]. Although these observations must be confirmed by other studies using other DPSC isolates.

5. Conclusions

Based upon this information, this study may be the first to demonstrate not only the differential responsiveness of DPSC isolates to BMP-2, but also to identify the MSC biomarkers that may affect initial DPSC responsiveness to this stimulus. Although many studies have evaluated the role of the biomarkers NANOG, Sox-2 and Oct-4 in DPSC isolate, no other study of DPSC multipotency has evaluated the role of Nestin – which may be one of the key factors that potentiate or limits the responsiveness to BMP-2 and osteogenic potential among DPSCs. These results suggest more research into these phenomena may be needed to further the understanding of DPSC differentiation and bioengineering.

6. Consent

Both pediatric assent and parental consent were required to partake in the study. Informed consent was required for all adult patients.

7. Competing Interests

The authors have declared that no competing interests exist.

8. Acknowledgements

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Chapter 3

Differential effects of Bone Morphogenic Protein (BMP) and Vascular Endothelial Growth Factor (VEGF) on Dental Pulp Stem Cell (DPSC) subpopulations.

This chapter has been accepted for publication in the journal EC Biosciences and Biomedical Engineering (ECBBE) and is presented in the style of that Journal. The complete Citation will be:

Cinelli J, Kingsley K. Differential effects of Bone Morphogenic Protein (BMP) and Vascular Endothelial Growth Factor (VEGF) on Dental Pulp Stem Cell (DPSC) subpopulations. EC Biosciences and Biomedical Engineering

Role of Authors:
This work was carried out in collaboration between all authors. Authors Karl Kingsley and Joseph Cinelli designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author Joseph Cinelli managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript

Abstract

Introduction: Recent work has demonstrated therapeutic and clinical applications for autologous dental pulp stem cells (DPSC) for bone tissue regeneration and bioengineering. Bone morphogenic protein (BMP) is a powerful growth factor that may be sufficient to modulate and regulate Mesenchymal Stem Cell (MSC) (and potentially DPSC) differentiation. Therefore, the primary objective of the current study was to evaluate the potential for BMP (alone and in combination) to induce changes to specific DPSC phenotypes.

Methods: Using previously isolated DPSC, six (n=6) isolates were treated with either BMP-2, Vascular Endothelial Growth Factor (VEGF) or both. Trypan blue viability assays and 96-well growth and proliferation assays were performed to determine any changes to DPSC phenotypes.

Results: The data clearly demonstrated that one subset of DPSC isolates (slow doubling time) exhibited significant increased viability under both BMP-2 and combined VEGF-BMP-2 administration. Differential and variable increases in cell growth were observed among the corresponding slow DPSC isolates, which suggests more complex relationships between growth and growth factor administration may be present. No similar effects were observed with the other subset of DPSC isolates (intermediate doubling time) and no effects on either growth or viability were observed under VEGF administration alone.

Conclusions: Although only a limited number of DSPC isolates could be evaluated, the data from this study revealed a strong and significant change to sDT DPSC phenotypes (growth and viability) under BMP-2 administration (either alone or in combination with VEGF). These changes appear to be restricted to this subpopulation of DPSC, which may provide some insight into the mechanisms and underlying biology responsible for these observations. Future studies may be needed to evaluate the potential for BMP-2 and other growth factors to induce DPSC differentiation and lineage-specific phenotypic changes for bioengineering applications or tissue regeneration.

Key words: BMP-2, VEGF, Bone morphogenic protein, vascular endothelial growth factor, stem cell, bioengineering, differentiation

Introduction

Stem cell research has recently described many new sources and clinical applications that are now available with many new potentially exciting discoveries [1,2]. For example, many researchers have demonstrated therapeutic and clinical applications for autologous dental pulp

stem cells (DPSC) for a number of disease states [3-5]. These efforts have placed renewed focus on the mechanisms responsible for directional and specific differentiation for DPSC explants and isolates [6-8].

Many methods have been evaluated for lineage specific differentiation cues specific to DPSC, including three-dimensional bioscaffolds and modulated laser irradiation [9,10]. However, due to the specialized nature of these approaches, more practical and widespread technologies have been deployed to facilitate these applications [11-13]. New research has revealed that more pragmatic approaches to DPSC expansion and bioengineering may have the potential to revolutionize these methods and approaches using more commonly available biomolecules and growth factors [14-16].

Many new studies now describe the potential to use DPSC for bone tissue regeneration and bioengineering [17-19]. These studies describe the use of bone morphogenic protein (BMP) as a powerful growth factor that can modulate and regulate MSC (and potentially DPSC) differentiation [20-22]. In fact, osteoblastic, odontoblastic, and osteogenic differentiation of DPSC using BMP has now become a more focused research strategy [23-25].

Recent efforts from this group have revealed that specific subpopulations of DPSC may be responsive to BMP-induced phenotypic changes [26]. Moreover, other growth factor stimuli have also been demonstrated to induce similar changes to DPSC in vitro [27]. Based upon these observations, the primary objective of the current study was to evaluate the potential for BMP (alone and in combination) to induce changes to DPSC phenotypes.

Material and Methods

Study approval

This study was retrospective in nature, utilizing previously isolated and characterized DPSC explants and isolates [28-30]. The original protocol for the collection and storage of DPSC was reviewed and approved by the Institutional Review Board (IRB) and the Office for the Protection of Research Subjects (OPRS) under OPRS#0907-3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp". The current retrospective analysis was reviewed and approved under OPRS#763012-1 "Retrospective analytsis of dental pulp stem cells (DPSC) from the UNLV School of Dental Medicine (SDM) pediatric and clinical population".

DPSC culture

DPSC explants and isolates were cryopreserved in 10% dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) at -80C. Each DPSC isolate was originally given an non-duplicated numerical identifier to prevent research bias and to remove any patient identifying information associated with the original sample collection protocol. No patient information was available to any research team member. Cells were thawed and cultured using Roswell Park Memorial Institute (RMPI) medium, supplemented with 10% FBS and 1% antibiotic solution consisting of Penicillin-Streptomycin in a humidified tissue culture incubator at 37C with 5% CO2.

In brief, DPSC cells were screened according to the International Society for Cellular Therapy (ISCT) criteria and were found to express CD90 or CD105 and did not express CD45 or CD34 [31]. Each DPSC was cultured with the frequency of passage or doubling time noted, as previously described [28-30]. In brief, DPSC were characterized as exhibiting a rapid doubling time (rDT) of approximately 1-2 days, an intermediate doubling time (iDT) of 4-6 days, or a

slow doubling time (sDT) of approximately 10-12 days. The previous work from this group established that sDT DPSC isolates were responsive to BMP, whereas rDT and iDT DPSC isolates were not [26]. Based upon this information, sDT DPSC isolates were selected for inclusion in the current study with iDT DPSC isolates as negative controls.

Cell viability

Cells were plated in 96-well assay plates with and without the addition of BMP-2 at a concentration of 10 ng/mL for three days. Parallel experiments were also plated using VEGF at this same concentration, with additional wells containing both BMP-2 and VEGF in combination. Cell viability was assessed using the Trypan Blue exclusion assay and a BioRad TC20 cell counter. Total and live cell number (as well as percentage of viable cells) were noted for each experimental condition and control. Each experiment was done in triplicate (n=3 rows of n=8 wells, total per experimental condition n=24).

Cell proliferation

Following viabilty testing, cells were fixed in 10% formalin and stained using Gentian Violet. Each plate was examined using a BioTek 808x 96-well plate reader at 630 nm to evaluate the total confluence and cell number in each experimental condition (control, growth factor). Results were plotted and graphed and differences between control and experimental conditions were evaluated using two-tailed t-tests, with an alpha level, a=0.05 to determine statistical significance.

Results

The results of this analysis revealed that BMP-2 and VEGF have specific effects on sDT DPSC viability (Figure 1). More specifically, the baseline viability for the sDT DPSC isolates (average 27.1%, range 24 – 31%) was significantly increased with the administration of BMP-2 (average 49%, range 46.7% - 51.3%), p=0.00147. In addition, the combination of BMP-2 and VEGF was sufficient to increase viability to an even higher level (64%, range 57.7% - 70%), which was significantly higher than the baseline controls and the administration of only BMP-2, p=0.00412. However, the administration of VEGF in the absence of BMP-2 did not significantly affect cellular viability among any of the sDT DPSC isolates (average 27.7%, range 24.6% - 31.3%) compared with the negative control, p=0.688.

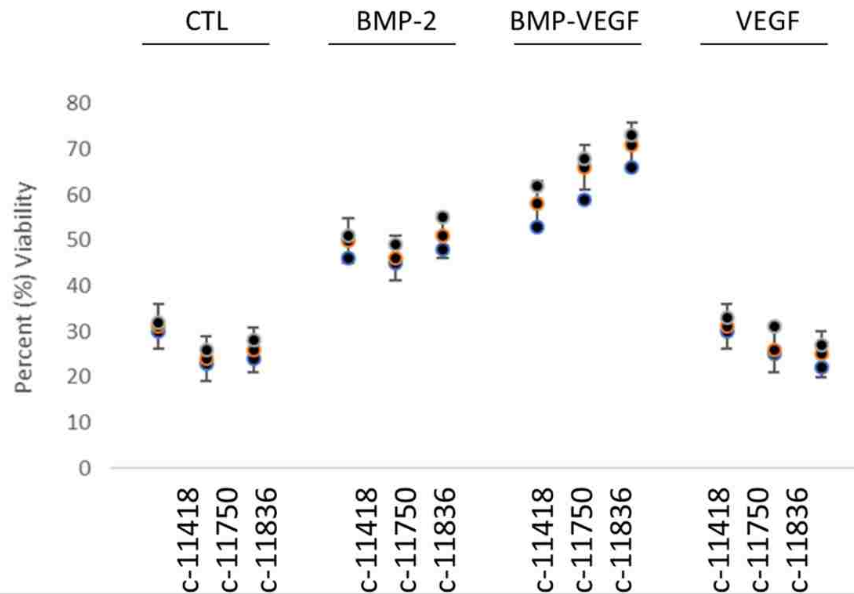

Figure 1. Viability of sDT DPSC isolates following growth factor administration. Average viability of sDT DSPC isolates 27.1% (dpsc-11418, dpsc-11750, dpsc-11836) was significantly higher under BMP-2 (49%), p=0.00147; with even higher viability with BMP-2 and VEGF in combination (64%), =0.00412. However, VEGF in the absence of BMP-2 did not affect viability (27.7%) compared with negative controls, p=0.688.

The analysis of these data with the iDT DPSC isolates revealed that neither BMP-2 or VEGF have specific effects on iDT DPSC viability (Figure 2). More specifically, the baseline viability for the iDT DPSC isolates (average 33.4%) was comparable with the administration of BMP-2 (average 32.4%), p=0.4313. In addition, the combination of BMP-2 and VEGF also had no significant effect on viability (34.3%), p=0.5557. Finally, the administration of VEGF in the absence of BMP-2 also did not significantly affect cellular viability among any of the iDT DPSC isolates (average 34.8%) compared with the negative control, p=0.2621.

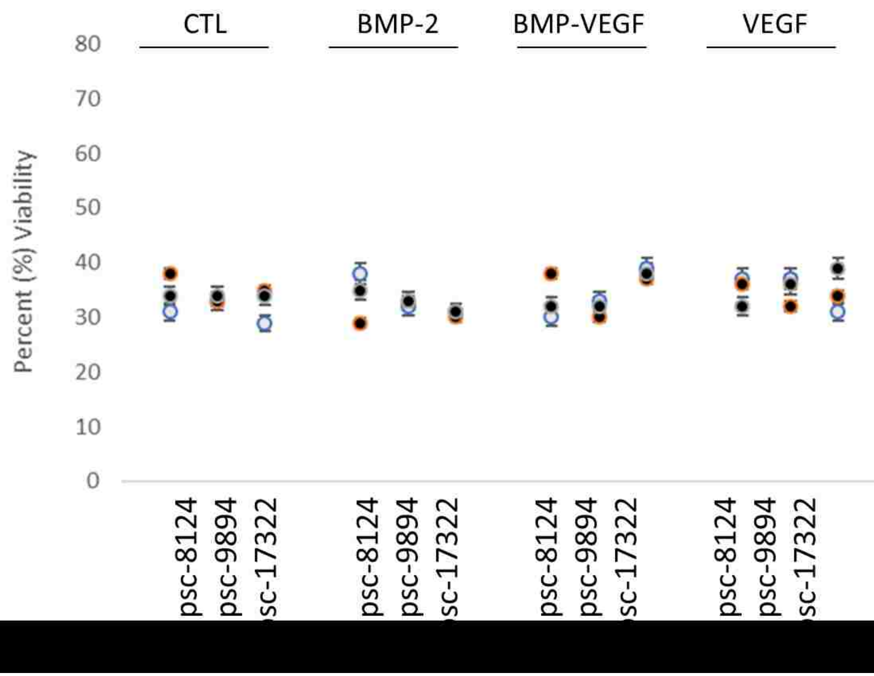

Figure 2. Viability of iDT DPSC isolates following growth factor administration. Average viability of iDT DPSC isolates (33.4%) was comparable with BMP-2 administration (32.4%), p=0.4313, VEGF administration (34.8%), p=0.2621 and the combination of BMP-2 and VEGF in combination (34.3%), p=0.5557.

To determine if the effects of BMP-2 and VEGF also induced changes to cellular proliferation, 96-well three-day proliferation assays were also performed (Figure 3). These data demonstrated that administration of BMP-2 was sufficient to increase growth among the sDT DPSC isolates by nearly two-fold (baseline average absorbance 0.325, BMP-2 0.651), which was statistically significant, p=0.00065. Furthermore, variable but significantly increased growth was also observed with the combined administration of BMP-2 and VEGF (average 0.895), p=0.00091. However, VEGF administration was not sufficient to induce any significant or

measurable changes to sDT growth among the sDT DPSC isolates (average absorbance 0.334), p=0.759.

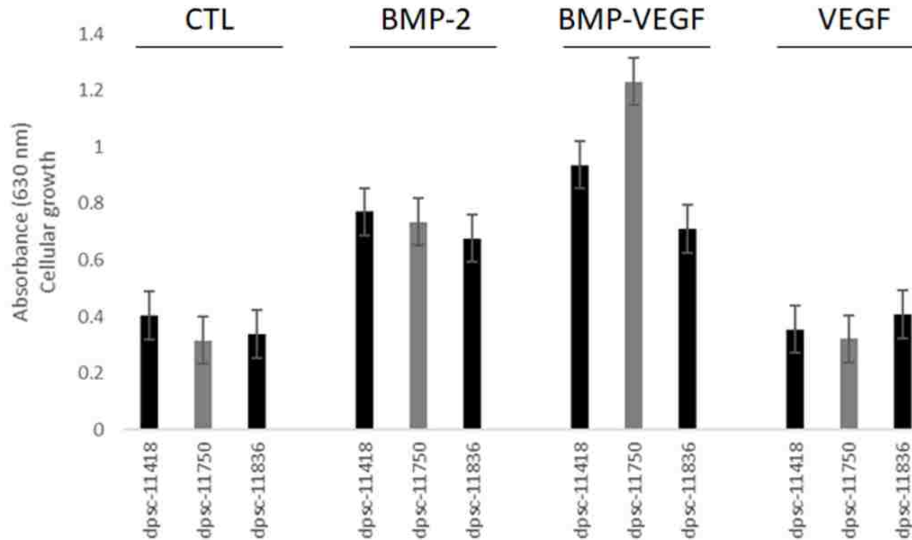

Figure 3. Changes to sDT DPSC growth following growth factor administration. Addition of BMP-2 was sufficient to induce significant increases in growth among the sDT DPSC (average: 0.651) compared with baseline controls (average: 0.325), p=0.00065. Variable increased growth was observed with BMP-2 and VEGF (average 0.895), p=0.00091 with no changes under VEGF administration (average 0.334), p=0.759.

To more closely evaluate the variable changes induced by concomitant BMP-2 and VEGF administration, these data were re-graphed to analyze these results sorted by sDT DPSC isolate (Figure 4). These data revealed a marked, variable response among the sDT DPSC isolates. For example, the administration of BMP-2 increased growth among dpsc-11418 by 91% while the combined administration of BMP-2 and VEGF increased growth by 139% - clearly suggesting an additive or synergistic effect.

However, the administration of BMP-2 increased growth among dpsc-11750 by nearly 131%, while the combined administration of BMP-2 and VEGF increased growth by only 71.3%. This may suggest VEGF exerts a contradictory or negative effect on the growth-enhancing effects of BMP-2 with this DPSC isolate.

Finally, the administration of BMP increased growth by nearly double, or 99%, among dpsc-11836, which was similar to the growth observed under combined administration of BMP-2 and VEGF (105%), suggesting that VEGF may not be exerting any additional effects on growth in this DPSC isolate.

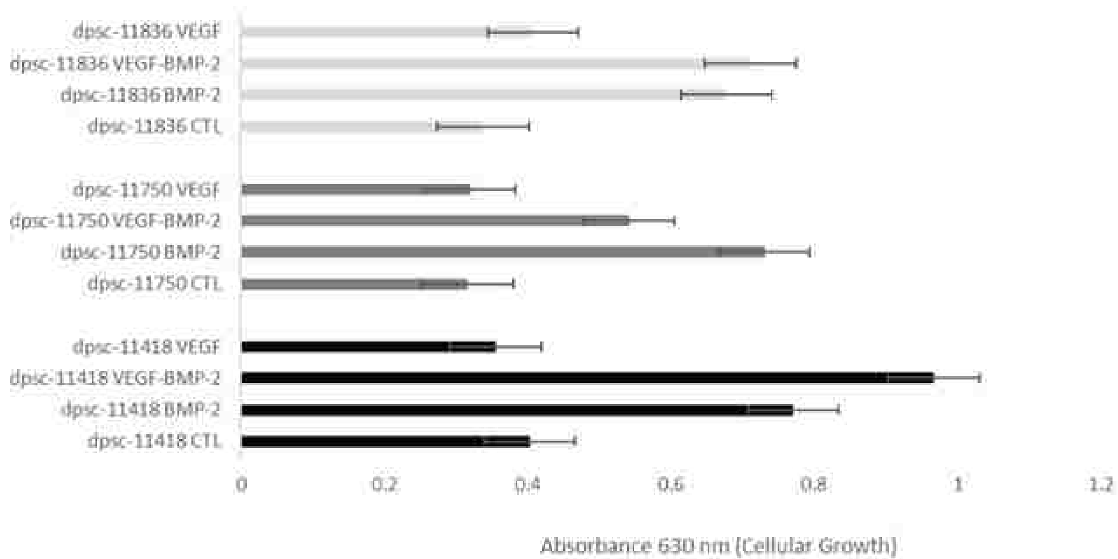

Figure 4. Variable growth induced by concomitant BMP-2 and VEGF administration sorted by sDT DPSC isolate. Among dpsc-11418 cells, BMP-2 increased growth by 91%, while BMP-2 and VEGF increased growth by 139%. Among dpsc-11750, BMP-2 increased growth by 131%, while BMP-2 and VEGF increased growth by only 71.3%. Finally, BMP increased growth by 99% among dpsc-11836, which was similar to combined administration of BMP-2 and VEGF (105%). No significant changes from controls were observed with the administration of VEGF.

These growth assays were also performed to evaluate whether growth factor administration affected iDT DPSC growth following growth factor administration (Figure 5). These data demonstrated that baseline growth or absorbance from negative controls (average 0.301) was not significantly different among the BMP-2 experimental group (average 0.303, $p=0.911$). Moreover, the combined administration of both BMP-2 and VEGF also did not exhibit any significant effects on iDT DPSC growth (average 0.287, $p=0.364$). Finally, the administration of VEGF alone did not induce any significant changes from baseline controls (average 0.312, $p=0.289$).

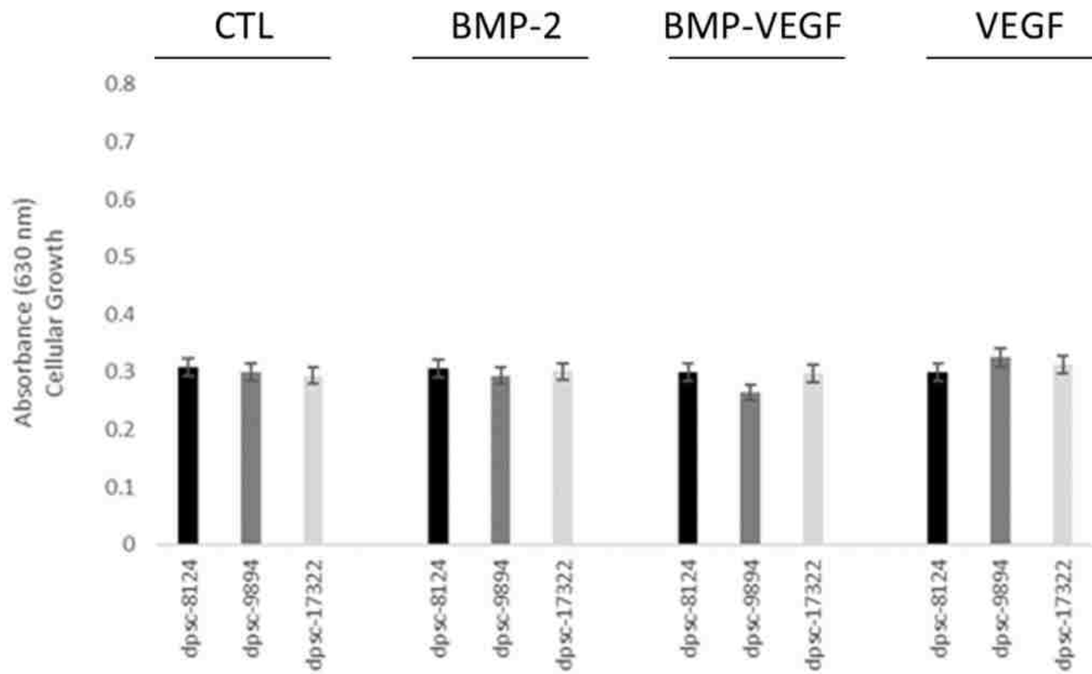

Figure 5. Analysis of iDT DPSC growth following growth factor administration. Baseline growth or absorbance from negative controls (average 0.301) was not significantly different among the BMP-2 (average 0.303, p=0.911), combined BMP-2 and VEGF (average 0.287, p=0.364), or VEGF (average 0.312, p=0.289) experimental groups.

To more closely evaluate these effects on sDT DPSC isolates photo microscopy was performed (Figure 6). These data demonstrated both the increased cell number under BMP-2 (C,G,K) and combined VEGF-BMP-2 administration (D,H,L). No obvious changes to cellular number or morphology was apparent among the sDT DPSC isolates under VEGF administration alone (B,F,J) compared with non-treated controls (A,E,I).

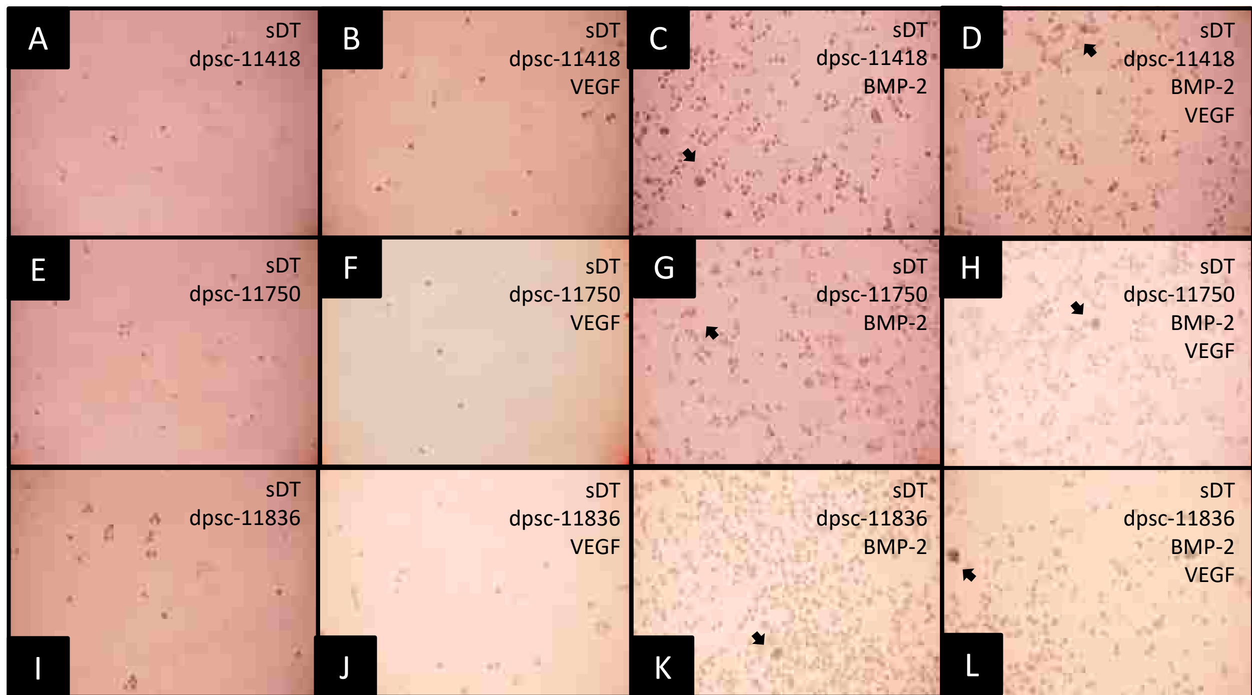

Figure 6. Microscopy of sDT DPSC under growth factor administration. No apparent differences were observed between non-treated (control) cells (A,E,I) and VEGF (B,F,J). However, increased cell number and morphologic changes were observed among sDT DPSC isolates under BMP-2 (C,G,K) and combined VEGF-BMP-2 administration (D,H,L).

Discussion

The goal of the current study was to evaluate the potential for BMP (alone and in combination) to induce changes to specific DPSC phenotypes. The results of this study clearly demonstrated that one subset of DPSC isolates (sDT) exhibited significant increased viability under both BMP-2 and combined VEGF-BMP-2 administration. Differential and variable increases in cell growth were observed among the corresponding slow DPSC isolates, which

suggests more complex relationships between growth and growth factor administration may be present.

However, no similar effects were observed with the other subset of DPSC isolates (iDT) and no effects on either growth or viability were observed under VEGF administration alone, which suggests these effects may be specific to the sDT DPSC isolates and may restricted to either BMP-2 or BMP-2 in combination with VEGF.

These data support other observations of diverse and differential effects of BMP-2 on other mesenchymal stem cells or MSCs [32,33]. In addition, the only other study to evaluate BMP-2 and VEGF administration in DPSC also found differential and diverse effects of these growth factors, both alone and in combination – which may suggest more complicated and complex relationships between the underlying DPSC biology and growth factor-induced phenotypes [34].

Although these data provide novel observations that may indicate future directions for research endeavors, there are some limitations that should also be discussed. First and most importantly, only a small number of DPSC isolates were available to this research team. A more thorough and comprehensive examination of other DPSC isolates may reveal other pertinent information regarding pluripotency and DPSC phenotypes, which could not be explored within the limited scope of this project [35,36].

Conclusions

Although only a limited number of DSPC isolates could be evaluated, the data from this study revealed a strong and significant change to sDT DPSC phenotypes (growth and viability) under BMP-2 administration (either alone or in combination with VEGF). These changes appear

to be restricted to this subpopulation of DPSC, which may provide some insight into the mechanisms and underlying biology responsible for these observations. Future studies may be needed to evaluate the potential for BMP-2 and other growth factors to induce DPSC differentiation and lineage-specific phenotypic changes for bioengineering applications or tissue regeneration.

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Chapter 4: Summary and Conclusions

The purpose of this project was to evaluate the effects of BMP-2 on DPSC phenotype expression. Since DPSC are the most readily available source of adult stem cells it is important to explore their therapeutic potential. In dentistry forming bone is a utilizable asset for practitioners in almost every clinical specialty in the field.

Chapter 2 of this document was a retrospective study which studied phenotypic factors including viability and proliferation of a variety of DPSC isolates which were subtyped based on their doubling times as either rDT, iDT, or sDT. The results showed that the sDT subtype of DPSC were significantly stimulated by BMP-2. The sDT DPSC showed significant increases in viability ($p=0.022$), growth ($p=0.039$), and proliferation and cellular morphology. Additionally, some cell lines began transcribing mRNA for ALP and DSPP in three of the cell lines (one of iDT and two of sDT subgroups). This study also elucidated a potential positive correlation between the biomarker Nestin and DPSC response to BMP-2.

Chapter 3 was a study with a more select sample group including only the sDT and iDT lines of DPSC ($n=6$). rDT DPSC were excluded because of their lack of phenotypic response to BMP-2 in the first study featured in this thesis. The study in this chapter examined the effects of BMP-2 and VEGF individually and when applied to the cell lines concomitantly. The study found that when applied individually, BMP-2 significantly increased the viability($p=0.00147$) and proliferation ($p=0.00065$) of sDT DPSC when compared to the control group but did not significantly affect the iDT subgroup. VEGF did not significantly affect either the viability or proliferation of either the sDT or iDT DPSC isolates. Concomitant exposure to BMP-2 and VEGF yielded significant increases in sDT DPSC viability ($p=0.00412$) and proliferation ($p=0.00091$) compared to the control group. Concomitant exposure to BMP-2 and VEGF did not

significantly affect the iDT DPSC isolates. These data may suggest that even within the DPSC subpopulations there may be varying levels of differentiation leading to a differing response to similar treatment as shown when comparing cell proliferation of line dpsc-11750 between the BMP-2 only and BMP-2 + VEGF therapies. Here it was observed that the concomitant treatment yielded less of a response than BMP-2 alone indicating that within this cell line VEGF may act as an inhibitory signal, whereas in the other two sDT DPSC lines VEGF appeared to work either synergistically or additively with BMP-2 to illicit a stronger response from the cells.

Conclusions from both chapters 2 and 3 demonstrated statistically significant differences in the phenotypic characteristics of viability, growth, proliferation, and cellular morphology. Additionally, cellular components such as mRNA and biomarkers were examined. It was found that certain subgroups of DPSCs were transcribing mRNA for ALP and DSPP which are indicators of bone formation, and that rDT and iDT DPSC tested positively for two or more of the MSC biomarkers tested and that the sDT DPSC tested positive for the biomarker nestin which had a strong correlation with the cell lines that responded most strongly to the BMP-2 treatment. When treated with BMP-2, VEGF, and both of them simultaneously, all sDT DPSC cell line viability and proliferation showed a positive response to BMP-2 alone and concomitantly with VEGF, while VEGF alone did not produce a significantly different response. iDT DPSC did not respond to BMP-2 or VEGF either alone or in tandem.

Based on the information uncovered in these chapters, the main question posed in this study can be answered by rejecting the null hypothesis and accepting the alternative hypothesis. The second question can be answered by rejecting the null hypothesis and accepting the alternative hypothesis, the third question can be answered by accepting the null hypothesis and

rejecting the alternative hypothesis, the final question can be answered by rejecting the null hypothesis and accepting the alternative hypothesis

1. Are dental pulp stem cells able to be induced into a mineral forming bone cell precursor lineage via BMP-2 stimulation?

   H_A: BMP-2 will affect the induction of dental pulp stem cell differentiation into a mineral forming bone cell precursor lineage.

2. Can BMP-2 effect the viability and proliferation of DPSC subtypes?

   H_A: BMP-2 will affect the viability or proliferation of DPSCs.

3. Can VEGF, effect the viability and proliferation of DPSC subtypes?

   H0: VEGF will have no effect on the viability or proliferation of DPSCs.

4. Can BMP-2, either alone or in combination with VEGF, effect the viability and proliferation of DPSC subtypes?

   HA: BMP-2 in combination with VEGF will affect the viability or proliferation of DPSCs

Limitations and Recommendations

As mentioned in the chapters above, one limitation of this study is that the method of DPSC culturing technique could be altered to a medium that may have the ability to differentially affect multipotency and stem cell-like properties of DPSC towards osteoblastic and

osteogenic lineages, which could enhance the cellular response to the BMP-2 stimulus. A study that uses multiple culturing media that have varying abilities may be useful to determine whether or not extracellular conditions can influence the response to the growth factor stimulus.

Also, the timing and sequence of the application of multiple stimuli (including BMP- 2 in combination with vascular endothelial growth factor) may also preferentially affect DPSC responsiveness to those stimuli. Expanding the study to examine the effects of differing timing and application sequence may produce useful data.

The sample size of DPSC isolates ideally would be larger to provide more accurate and reliable data. Repeating the experiment with an increased sample size may bolster the results collected in this study. In addition to using a larger sample size, it may be enlightening to compile and examine donor patient data such as age and systemic health condition in order to determine if a correlation between DPSC DT and viability exists.

Lastly, constraints of time and finances limited the scope of this study to only evaluating BMP-2 and VEGF and their effects on DPSC isolates, though there are a number of other growth factors that can affect the differentiation of the cells. More information on the effects of other growth factors could aid in determining which growth factors are most advantageous in the differentiation of DPSC.

Appendix A

UNLV Biomedical IRB - Administrative Review
Notice of Excluded Activity

DATE:	August 3, 2015
TO:	Karl Kingsley, PhD
FROM:	UNLV Biomedical IRB
PROTOCOL TITLE:	[763012-1] Evaluation of the effects of cryopreservation on survival of dental pulp stem cells.
SUBMISSION TYPE:	New Project
ACTION:	EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE:	August 3, 2015
REVIEW TYPE:	Administrative Review

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

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

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

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

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

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

Appendix B

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February 22, 2019

Signature	Date

Karl Kingsley, PhD, MPH — Professor

Name (typed)	Title

Appendix C

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Chapter 1:

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