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THE EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR ON THE CHARACTERISTICS OF PLURIPOTENT DENTAL PULP STEM CELLS

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Thesis Approval

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March 8, 2019

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The Effects of Vascular Endothelial Growth Factor on the Characteristics of Pluripotent Dental Pulp Stem Cells

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Abstract

The Effects of Vascular Endothelial Growth Factor on the Characteristics of Pluripotent Dental Pulp Stem Cells

By

Cale Forgues

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Many believe that stem cells hold the key to regenerative medicine. Therefore, research with stem cells has become increasingly popular over the years. Three specific groups of study have become present in literature. The first group has focused much of their research on harvesting and cultivating stem cell lines to retain their pluripotent ability. Stem cells can be tricky to cultivate and preserve over time as they will often differentiate or die. Another group examines the specific characteristics that a pluripotent stem cell has and how to retain, or even create, a cell with pluripotent potential. Lastly, the third group aims to focus on the ability to differentiate stem cells down specific cell lineages for therapeutic use. This study will draw upon

the latter two focuses and examine the specific characteristics of stem cells when combined with a cytokine during cultivation.

Vascular endothelial growth factors (VEGF) are a family of growth factors known to play an important part in embryonic development of vascular formation as well as dental development. The processes of neovascularization, formation of new vessels in adults, is highly reliant on the presence of VEGF for both the activation and attraction of undifferentiated bone marrow derived mesenchymal stem cells (MSCs). While this is the case with bone marrow derived MSCs, few studies have shown the effects VEGF has on other adult stem cell lines, such as dental pulp stem cells (DPSC).

Dental pulp stem cells have only recently been discovered. Another source for pluripotent stem cells, DPSCs are easily harvested from avulsed or extracted teeth. Further studies hope to determine the versatility of DPSCs and find specific uses for them in regenerative therapies.

Acknowledgments

A very big thank you to Dr. Karl Kingsley for allowing me to work with him throughout this research project. He was nothing but helpful and a joy to work with. His support has been invaluable. I would also like to thank my committee members, Dr. Fiona Britton, Dr. Brian Chrzan, and Dr. Jennifer Pharr.

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Chapter 1: Introduction

Background and Significance

Having been only recently been discovered, dental pulp stem cells (DPSCs) may have the potential to revolutionize oral regenerative therapies. Dental pulp stem cells (DPSCs) are non-embryonic, mesenchymal stem cells that are easily harvested, cultured, and maintained from extracted or avulsed teeth[1-3] For these reason DPSCs are ideal for studying the effects of growth factors on undifferentiated pluripotent cell lines. Being a stem cell with the ability to differentiation down any of the major embryonic stem cell lines, current studies are trying to determine which growth factors can be used to induce differentiation towards a desired cell lines.[4-5] One of these major growth factors known to be important to embryonic stem cell attraction and activation is vascular endothelial growth factor (VEGF).

Vascular endothelial growth factor (VEGF) is a family of growth factors known to play an important part in vascular formation in early endothelial development as well as bone formation in dental development. [6-7] The majority of studies to date have examined how the family of VEGF ligands act on specific tyrosine kinase receptors to create intracellular responses in differentiated endothelial cells. The cellular responses, and intracellular effects of, VEGF on various lineages of multipotent DPSCs remains relatively unknown.[8-9]

Research Question

Based upon the limited amount of information regarding DPSC and in vitro differentiation, as well as the limited information regarding VEGF and the potential to differentiate DPSC – the main objective of this study was to screen evaluate the effects of VEGF on several DPSC isolates.

1. Is there an effect on characteristics of the DPSC lines when cultured with VEGF?

   Null (H_0) hypothesis: There will be no effect on the characteristics of the DPSC isolates when cultured with VEGF

   Alternative (H_A) hypothesis: There will be an effect on the characteristics of the DPSC isolates when cultured with VEGF

2. Is there an effect on characteristics of the DPSC lines when cultured with VEGF in combination with BMP-2?

   Null (H_0) hypothesis: There will be no effect on the characteristics of the DPSC isolates when cultured with VEGF in combination with BMP-2

   Alternative (H_A) hypothesis: There will be an effect on the characteristics of the DPSC isolates when cultured with VEGF in combination with BMP-2

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

This retrospective study would involve the analysis of DPSCs previously isolated from clinical patients, recruited at random in the UNLV-SDM pediatric clinic. Eight cell lines will be used and replicated for three experimental trials (n=24). Cells will be cultured and propagated for ten passages to determine the rate of growth and doubling time (DT). Doubling time (DT) will be categorized as rapid or rDT (~2 days), intermediate or iDT (4-6 days), and slow or sDT (10-12 days). To assess any changes to differentiation, total RNA will be isolated from each isolate using the Total RNA isolation reagent (TRIR) from Molecular Research Center (Cincinnati, OH) using the protocol recommended by the manufacturer. Screening for changes to mRNA expression in each DPSC isolated will be accomplished using the ABgene Reverse-iT One-Step RT-PCR protocol and reagent kit. Comparisons of the changes to viability or proliferation will be calculated and compared using two-tailed t-tests, which are appropriate for parametric data analysis. Due to the potential for Type I error, all analyses will be subsequently confirmed using analysis of variance or multiple (ANOVA).

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Chapter 2

Effects of vascular endothelial growth factor (VEGF) on dental pulp stem cells (DPSC)

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This work was carried out in collaboration between all authors. Author Cale Forgues and Eric Mullins were responsible for sample processing. Authors Dr. Karl Kingsley and Dr. Cale Forgues were responsible for project design, funding and manuscript preparation. All authors read and approved the final manuscript.

Abstract

Background: Dental pulp stem cells (DPSCs) are non-embryonic, mesenchymal stem cells that may have significant potential for therapeutic and regenerative biomedical applications. Studies of DPSC differentiation have demonstrated the potential to form many tissue types, including neural, osteogenic, and vascular precursors using cytokines and growth factors, such as vascular endothelial growth factor (VEGF). Methods: Eight previously isolated dental pulp stem cell (DPSC) isolates were grown in culture and treated with VEGF to evaluate any effects on growth, viability or biomarker expression. Results: Administration of VEGF at 10 ng/mL significantly inhibited growth in two rapidly dividing or rDT DPSC isolates, with no other measurable effects noted among the intermediate (iDT) or slow (sDT) growing DPSC isolates. In addition, VEGF administration of had no significant effects on viability of the sDT or iDT DPSC isolates, however, all three of the rapidly dividing or rDT DPSC isolates exhibited significantly increased viability. Finally, mRNA expression of osteogenic biomarkers alkaline phosphatase

(ALP) and Dentin sialophosphoprotein (DSPP) was observed among the rDT isolates with specific combinations of DPSC biomarkers expressed (NANOG in combination with Sox-2 or Oct-4 but not both). Conclusions: The results of these data suggest that VEGF administration may be sufficient to induce partial differentiation of DPSC isolates, although this may be dependent upon the MSC biomarker expression of the DPSCs. These preliminary data may further research into the potential for tissue regeneration and bioengineering.

Key words: Dental pulp stem cell (DPSC), vascular endothelial growth factor (VEGF), cellular differentiation

Introduction

Dental pulp stem cells (DPSCs) are non-embryonic, mesenchymal stem cells than can be obtained, isolated, cultured, and cryopreserved with relative ease compared with other potential sources, which has driven recent scientific research into their potential for therapeutic applications [1-3]. Harvested from the dental pulp of primary teeth, extracted teeth, or avulsed teeth, DPSCs are multi-potent stem cells that may be useful to facilitate advanced regenerative therapies [4,5]. , These studies have provided a better overall understanding of the capabilities of mesenchymal stem cells (MSCs) and DPSCs, with recent evidence demonstrating that differentiation potential may depend, in part, on the tissue of origin used in MSC harvesting [6-8].

Studies done on DPSC differentiation have demonstrated the potential to form many tissue types, including neural, osteogenic, and vascular precursors [9-11]. Much progress has been made towards the in vitro and in vivo differentiation of DPSC towards specific cell lineages [12,13]. In fact, some evidence now suggests that individual growth factors, such as vascular

endothelial growth factor (VEGF), may be sufficient to induce partial differentiation of DPSC – although this may be more dependent upon specific biomarkers or DPSC characteristics [14-16].

It has been demonstrated that DPSCs can be stimulated using VEGF through the canonical Wnt-β-catenin pathway into differentiating into blood vessels that resembled embryonic vasculogenesis, revealing the importance of this growth factor (VEGF) in angiogenesis as well as its potential for regenerative vasculogenesis [11,12,16]. However, the majority of studies to date have examined how the family of VEGF ligands act on specific tyrosine kinase receptors to create intracellular responses in differentiated vascular endothelial cells, while the cellular responses to, and intracellular effects of, VEGF on various lineages of multipotent DPSCs remain relatively unknown [17].

Differentiation potential and stem-ness may be linked with specific intracellular MSC biomarkers such as the expression of Sox-2, Oct-4 and NANOG, which have been found to be highly associated with the pluripotency of cells, including DPSC [18-20]. The presence or absence of these biomarkers in cultured DPSCs may determine the ability of the isolates to differentiate and self-replicate [21-23]. Based upon this understanding, the primary objective of this study was to evaluate the effects of VEGF on several DPSC isolates and to further evaluate the expression of specific biomarkers that may indicate pluripotency, as well as differentiation.

Methodology

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".

Study Design

This retrospective study involved the analysis of DPSCs previously isolated from clinical patients, recruited at random the UNLV-SDM pediatric clinic. Inclusion criteria included adult patients or pediatric patients aged seven (7) or older with their parents or guardian's permission who agreed to participate and were scheduled for a tooth extraction of health (vital) intact teeth prior to the initiation of orthodontic treatment. Pediatric assent and Parental permission to consent for voluntary participation were obtained at the time of study enrollment. Exclusion criteria included any patient, parent or child that was not a patient of record at UNLV-SDM, any patient or guardian who declined to participate and any patients having teeth extracted due to injury (fracture), infection or other disease.

DPSC Collection (initial)

In brief, the overwhelming majority of patients who agreed to participate were scheduled for tooth extractions of third molars. Once extracted, each tooth was sectioned at the cemento-enamel junction (CEJ) to allow extraction of the dental pulp with an endodontic broach for transfer into a sterile microcentrifuge tube containing 1X phosphate-buffered saline (PBS).

Samples were stored on ice until transfer to a biomedical laboratory for processing and screening. To prevent research bias and prevent any patient identifying information from being disclosed, a randomly generated, non-duplicated number was assigned to each sample and concurrent patient demographic information collected. No patient-specific identifying information was subsequently available to any research team member.

Culture and Propagation

Briefly, cells were cultured and propagated for ten passages to determine the rate of growth and doubling time (DT). Passage (or split) for each DPSC isolate was 1:2 and confluence determined with trypan blue and BioRad TC20 automated cell counter (Hercules, CA), using the manufacturer recommended protocol. Data collected included total and live cell number and the resulting percentage of viable cells for analysis. Doubling time (DT) was categorized as rapid or rDT (~2 days), intermediate or iDT (4-6 days), and slow or sDT (10-12 days).

Experimental protocol

To determine any effects on DPSCs, the cells were plated into 96-well tissue culture treated plates at a concentration of 1.2 x 10^4 cells/mL. Negative (non-treated) control cells were compared with cells treated with vascular endothelial growth factor (VEGF) from ThermoFisher Scientific (PCH9394) at a concentration of 10 ng/mL. Eight replicates were performed in each experiment for all DPSC isolates, which were repeated for a total of three experimental trials (n=24).

RNA isolation

To assess any changes to differentiation, total RNA was isolated from each isolate using the Total RNA isolation reagent (TRIR) from Molecular Research Center (Cincinnati, OH) using the protocol recommended by the manufacturer. RNA was subsequently screened for quality and quantity using ratio measurements of absorbance at 260 and 280 nm (A260/A280 ratio).

Polymerase chain reaction (PCR)

Screening for changes to mRNA expression in each DPSC isolated was accomplished using the ABgene Reverse-iT One-Step RT-PCR protocol and reagent kit with specifications that included an initial reverse transcription at 47C for 30 minutes, followed by 30 cycles of denaturation at 95C for 10 minutes, annealing for 30 seconds at the appropriate temperature for each primer set, and final extension at 60C for one minute. Primers synthesize from Eurofins MWG Operon (Huntsville, AL) were:

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); Annealing temperature 67C

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

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

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

Alkaline phosphatase (ALP); Annealing temperature: 72C

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

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

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

Dentin sialophosphoprotein (DSPP); Annealing temperature: 68C

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

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

Optimal temperature T(opt): Lower temperature – 5C = 62C

Statistical analysis

Basic proliferation and viability information regarding the DPSC isolated were compiled and presented using simple descriptive statistics (counts and percentages). Comparisons of change to viability or proliferation were calculated and compared using two-tailed t-tests, which are appropriate for parametric data analysis. Due to the potential for Type I error, all analyses were subsequently confirmed using analysis of variance or multiple (ANOVA).

Results

To determine any effects on DPSC phenotypes, vascular endothelial growth factor (VEGF) was administered in 96-well assays (Figure 1). These results demonstrated that the majority of DPSC isolates were not significantly affected by VEGF administration, $p>0.05$. However, two DPSC isolates (dpsc-3882, dpsc-5653) had significant measurable decreases in proliferation under VEGF administration, $p=0.038$ and $p=0.041$ respectively. In addition, dpsc-3882 and dpsc-5653 were both categorized as having rapid doubling times or rDT.

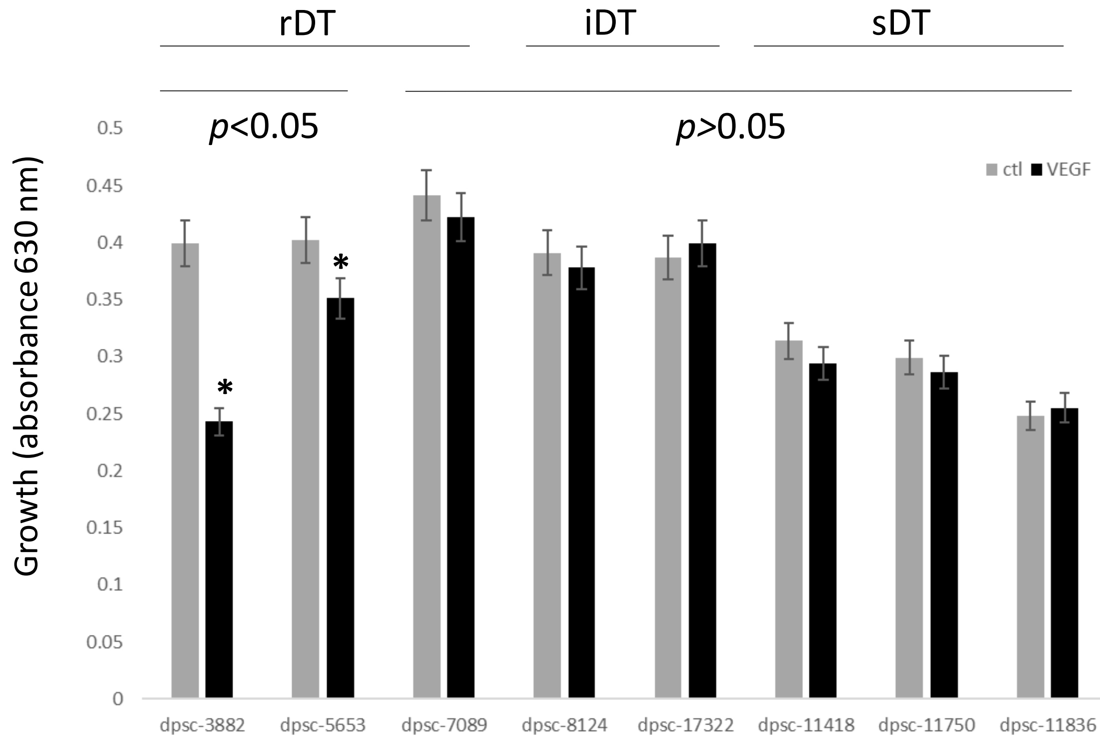

Figure 1. Effects of VEGF administration on DPSC growth. Administration of VEGF at 10 ng/mL had a significant effect on two rapidly dividing (rDT) DPSC isolates, dpsc-3882 and dpsc-5653 – which were significantly lower than the negative controls (p=0.038 and p=0.041, respectively). No other measurable effects were noted among the intermediate (iDT) or slow (sDT) DPSC isolates.

To evaluate if the observed changes in proliferation and cellular growth correlated with any changes to other DPSC phenotypes, cellular viability was also measured under VEGF administration (Figure 2). Although no significant changes to viability were noted among the iDT or sDT DPSC isolates under VEGF administration, all three of the rDT DPSC isolates demonstrated significant measurable increases to viability over the 72 hour time course, $p<0.05$.

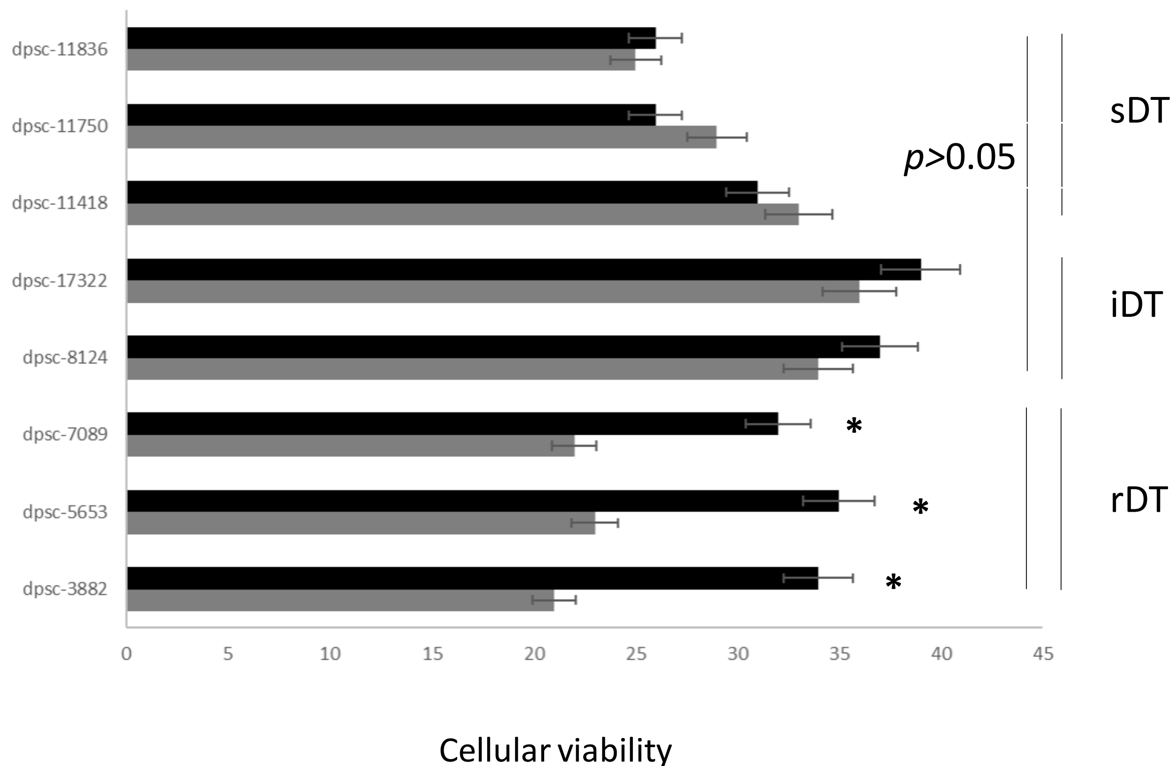

Figure 2. Effects of VEGF administration on DPSC viability. Administration of VEGF at 10 ng/mL had no significant effects on the intermediate (iDT) or slow (sDT) DPSC isolates, p>0.05. However, all three of the rapidly dividing or rDT DPSC isolates, dpsc-3882, dpsc-5653, dpsc-7089 exhibited increased viability (p=0.018, p=0.011, p=0.122, respectively).

To determine if any of the changes to cellular growth or viability induced by VEGF administration among the DPSC isolates were associated with changes to DPSC biomarkers for osteoblastic differentiation, RT-PCR screening of RNA was performed (Figure 3). In brief, primers specific for alkaline phosphatase (ALP) and dentin sialophosphoprotein (DSPP) were used to screen for mRNA expression of these biomarkers. These results demonstrated that VEGF administration was sufficient to induce mRNA expression of ALP in two DPSC isolates (dpsc-3882, dpsc-5653). In addition, VEGF administration was also sufficient to induce DSPP mRNA expression in one DPSC isolate (dpsc-3882).

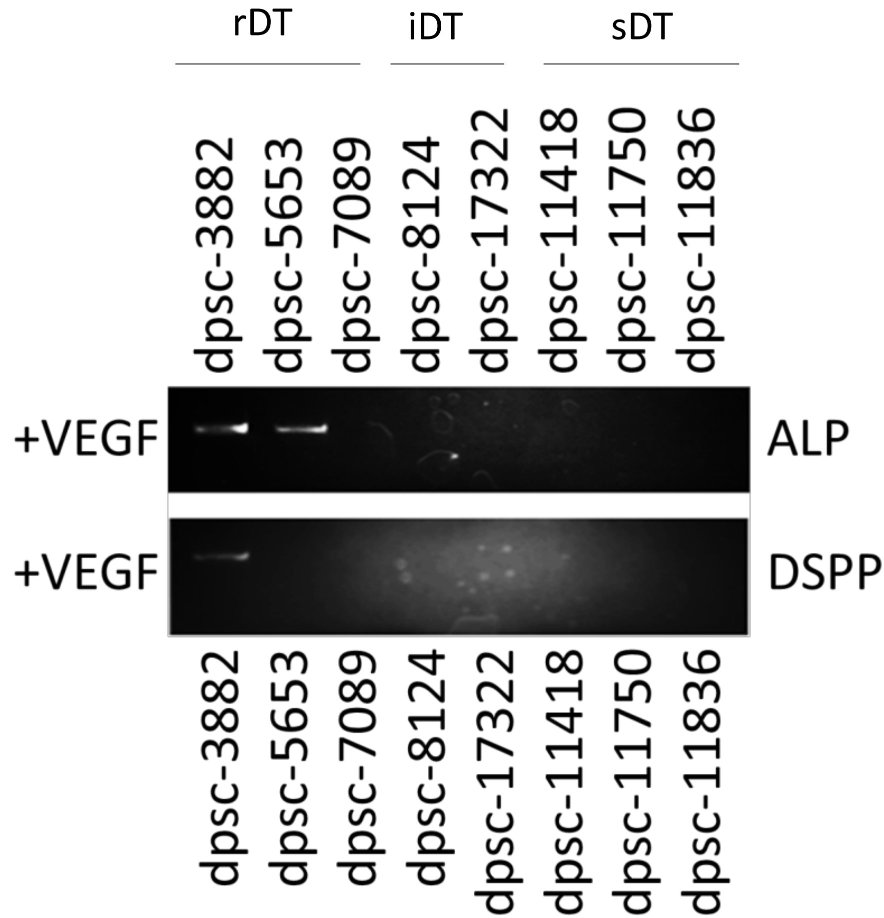

Figure 3. DPSC osteogenic mRNA biomarker induction following VEGF treatment. VEGF administration (10 ng/mL) was sufficient to induce mRNA expression of alkaline phosphatase (ALP) in two rDT DPSC isolates (dpsc-3882, dpsc-5653) and dentin sialophosphoprotein (DSPP) mRNA expression in dpsc3882. Expression of these mRNA biomarkers were not evident in any of the other DPSC isolates.

Finally, an evaluation of the MSC biomarkers for each DPSC isolate was performed to determine if there were any associations with VEGF responsiveness (Figure 4). This analysis revealed that MSC biomarkers Sox-2, Oct-4 and NANOG were differentially expressed by the DPSC isolates (Fig 4A). For example, the rDT DPSC isolates each had a distinct expression profile (dps-3882: Oct-4, NANOG; dpsc-5653: Sox-2, NANOG; dpsc-7089: Sox-2, Oct-4,

NANOG). In contrast, none of the iDT DPSC isolates expressed Oct-4, while none of sDT expressed either Sox-2 or Oct-4.

When combined with the results of VEGF assay, these data demonstrated that only the rDT DPSC isolates that expressed a combination of NANOG with either Oct-4 or Sox-2 (but not both) were responsive to VEGF administration (Fig. 4B). More specifically, the rDT DPSC isolate expressing a combination of Oct-4 and NANOG exhibited the most robust VEGF response, producing both ALP and DSPP (dpsc-3882). The rDT DPSC isolate expressing the combination of Sox-2 and NANOG exhibited some response to VEGF, producing ALP but not DSPP (dpsc-5653). However, the rDT isolate that expressed all three MSC biomarkers (Sox-2, Oct-4, NANOG) did not exhibit an osteogenic response to VEGF administration – similar to the negative response of the iDT DPSC isolates (Sox-2, NANOG) and sDT DPSC isolates (NANOG only).

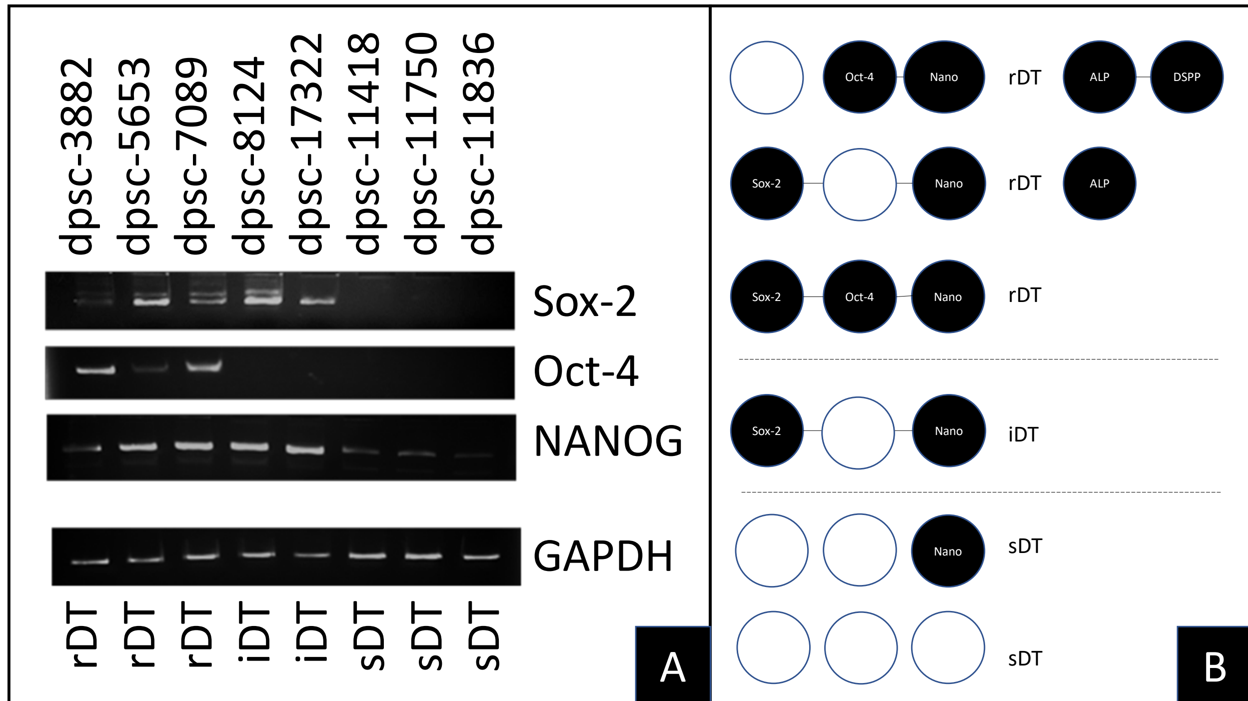

Figure 4. MSC mRNA biomarker analysis of DPSC isolates. Osteogenic VEGF response was observed in DPSC isolates expressing NANOG in combination with either Oct-4 (dpsc-3882) or Sox-2 (dpsc-5653) but not both (dpsc-7089). No osteogenic VEGF response was noted among the iDT (Sox-2, NANOG) or sDT (NANOG) DPSC isolates.

Discussion

Research that has evaluated DPSC differentiation has demonstrated the potential to form many cell types, including neural, osteogenic, and vascular precursors using cytokines and growth factors, such as vascular endothelial growth factor [11,12]. However, the phenotypic and cellular effects of VEGF on various lineages of multipotent DPSCs remains relatively unknown, therefore this study sought to evaluate these effects on several DPSC isolates with distinct markers of pluripotency[14-16]. The results of this study demonstrated that VEGF has distinct and specific effects on DPSC phenotypes, although these were not observed uniformly among all DPSC isolates.

For example, cellular growth and viability were markedly affected by VEGF only among the rapidly growing (rDT) DPSC isolates, which mirrors similar findings of VEGF effects on rapidly dividing MSC from other tissues [24-26]. In addition, VEGF appears to induce osteogenic biomarker expression in a subset of rDT DPSC isolates, a finding that appears to support observations of VEGF osteogenic effects in other MSCs [27-29]. To understand these observations more thoroughly, an analysis of MSC biomarker expression and the associations with osteogenic marker induction may be necessary [23,30,31].

New evidence has suggested that MSC biomarker expression in DPSCs may determine, in part, their differentiation potential and responsiveness to external stimuli [32-34]. The results of this study support these findings, with observations that rapidly dividing DPSC isolates expressing NANOG in combination with either Sox-2 or Oct-4 were responsive to VEGF administration. This research may also provide a potential explanation for the observation that DPSC expression of Oct-4, Sox-2 and NANOG were not responsive to VEGF administration, noting that “stemness” and pluripotency are correlated with MSC biomarker expression and that DPSC expression of more MSC biomarkers may indicate more than one stimulus or induction factor may be needed to facilitate differentiation [35,36].

Conclusions

The results of these data suggest that VEGF administration may be sufficient to induce partial differentiation of DPSC isolates, although this may be dependent upon the MSC biomarker expression of the DPSCs. In addition, the phenotypic changes to these DPSC isolates (decreased growth, increased viability) support these observations and may preliminary data to further research into the potential for osteogenic differential of DPSC. This may contribute to the overall, long-term goals of DPSC use for tissue regeneration and bioengineering.

Acknowledgements

Dr. Kingsley and Dr. Forgues would like to thank the Department of Advanced Education in Orthodontics and Dentofacial Orthopedics at the University of Nevada, Las Vegas, School of Dental Medicine for research funds to complete this pilot study.

Ethics

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

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Chapter 3

Effects of Vascular Endothelial Growth Factor (VEGF) alone and in combination on rapidly dividing Dental Pulp Stem Cells (DPSC)

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Abstract

Background: Dental pulp stem cells (DPSC) are known to possess many of the properties of pluripotent stem cells. Many researchers have focused their efforts to refine the range of growth factors that influence and modulate DPSC growth and differentiation. More specifically, some evidence has emerged that demonstrated Vascular Endothelial Growth Factor (VEGF) may be sufficient to enhance odontogenic and angiogenic potential of some DPSC isolates. Based upon this information, the primary objective of this study was to evaluate whether VEGF administration or concomitant VEGF and Bone Morphogenic Protein (BMP) administration induced any measurable effects on DPSCs.

Methods: This study used six (n=6) previously isolated and characterized DPSCs, cells, which were sorted into rapid doubling time (rDT ~1-2 days, n=3) and intermediate doubling time (iDT ~4-6 days, n=3). Each DPSC isolate was plated into 96-well assay plates for three days using media with and without VEGF, BMP-2 alone and in combination. Cellular growth and viability were measured for comparison.

Results: Administration of VEGF reduced growth in two of the rDT DPSC isolates (dpsc-3882, dpsc-5653), with no effect on the iDT DPSC isolates (dpsc-8124, dpsc-9894, dpsc-17322). A corresponding increase in cellular viability was noted among all the rDT DPSC isolates along with corresponding changes to cellular morphology, with no effect on the iDT DPSCs. BMP-2 exhibited no effects on either rDT or iDT DPSC isolates. The combination of VEGF and BMP-2 in combination had similar effects to the administration of VEGF in isolation.

Discussion: These data provide significant preliminary results that clearly demonstrate significant and pronounced effects of VEGF on at least one subset of rapidly dividing DPSC isolates. These effects include changes to cellular viability and growth, which are supported by clear changes to cellular adhesion and morphology. However, these data strongly suggest more reseach is needed to determine the underlying pathways triggered by VEGF administration in the cells and the pathophysiologic mechanisms that determine the responsiveness of these DPSC isolates but not others.

Key words: Dental Pulp Stem Cells (DSPC), Vascular Endothelial Growth Factor (VEGF).

Background

Dental pulp stem cells (DPSC) are known to possess many of the properties of pluripotent stem cells [1,2]. Recent studies have demonstrated that DPSCs may have therapeutic potential as multipotent stem cells with the capacity for reprogramming and bioengineering applications [3,4]. However, the ability to control and transform DPSC into specific lineages with precision and accuracy remains an elusive and motivating goal [5-7].

Many researchers have focused their efforts to refine the range of growth factors that influence and modulate DPSC growth and differentiation [8]. For example, some studies have demonstrated specific effects of fibroblast growth factor (FGF) as a transforming growth factor on DPSCs to facilitate odontoblast differentiation and dentin formation [9,10]. Others have focused attention on vascular endothelial growth factor (VEGF), which may play a critical role in DPSC regeneration, differentiation and pluripotency [11,12].

More specifically, some evidence has emerged that demonstrated VEGF may be sufficient to enhance odontogenic and angiogenic potential of some DPSC isolates [13,14]. In fact, research from this group recently demonstrated VEGF may have the potential to induce osteogenic phenotypes in some subsets of DPSC – although these effects were not uniform or consistent and were mainly restricted to the most rapidly dividing DPSCs [15]. Alternatively, one recent study demonstrated that VEGF administration or temporal "priming" of DPSC with VEGF may enhance their odontogenic and osteogenic differentiation potential in combination with other growth factors, such as bone morphogenic protein (BMP) [16].

Based upon this information, the primary objective of this study was to evaluate whether VEGF administration or concomitant VEGF and BMP administration induced any measurable effects on DPSCs.

Material and Methods

Protocol approval

This study was approved through the Office for the Protection of Research Subjects (OPRS) and the Institutional Review Board (IRB) at the University of Nevada, Las Vegas (UNLV) under protocol OPRS#763012-1 "Retrospective analytsis of dental pulp stem cells (DPSC) from the UNLV School of Dental Medicine (SDM) pediatric and clinical population.

The DPSC isolates were originally collected and obtained under OPRS#0907-3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp". Each DPSC isolate was given a unique identifying number to prevent bias and disallow any patient identifying information from being disclosed.

DPSC culture

All DPSC isolates were originally cultured for a minimum of ten (10) passages to ascertain the rate of growth or doubling time (DT). The average time between passaging for each DPSC isolate was characterized as either rapid doubling time (rDT) between 0-2 days and intermediate doubling time (iDT) between 4-6 days. All cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium with the addition of 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptocmycin from Fisher Scientific. Cells were maintained in 25 cm^2 tissue culture flasks at 5% CO_2 in humidified tissue culture chambers.

Experimental growth factors

The previous study from this group determined that only rapdily dividing or rDT DPSC isolates responded to VEGF administration, therefore - to determine any effects on DPSC isolates, rDT DPSC isolates were plated into 96-well assay plates for three days using media with and without additional growth factors. An additional set of experiments with intermediate or iDT DPSC isolates was used for comparison. Vascular Endothelial Growth Factor (VEGF) obtained from Fisher Scientific (PCH9394) was used at an experimental concentration of 10 ng/mL and Bone Morphogenic Protein (BMP-2) from Fisher Scientific (RP-8638) at a similar

concentration of 10 ng/mL. Cells were either plated without any growth factor as a negative control (CTL), with VEGF, BMP-2 or both VEGF and BMP-2 concomitantly.

Proliferation and Viability

Cells were grown for three days in each experimental condition (n=24 wells/plate) and each experiment was replicated in triplicate. Experimental assays were fixed with 10% buffered formalin for 24 hours. Following fixation, cells were stained with Gentian Violet and absorbance (growth) measured using a BioTek 808x 96-well plate reader. Higher absorbance readings correspond with higher growth measurements and larger cell numbers, as previously described [17,18]. Viability was measured using the Trypan Blue exclusion assay and a BioRad TC20 automated cell counter, as previously described [19,20]. Cells were imaged at 20X using an AxioVert inverted microscope from Ziess.

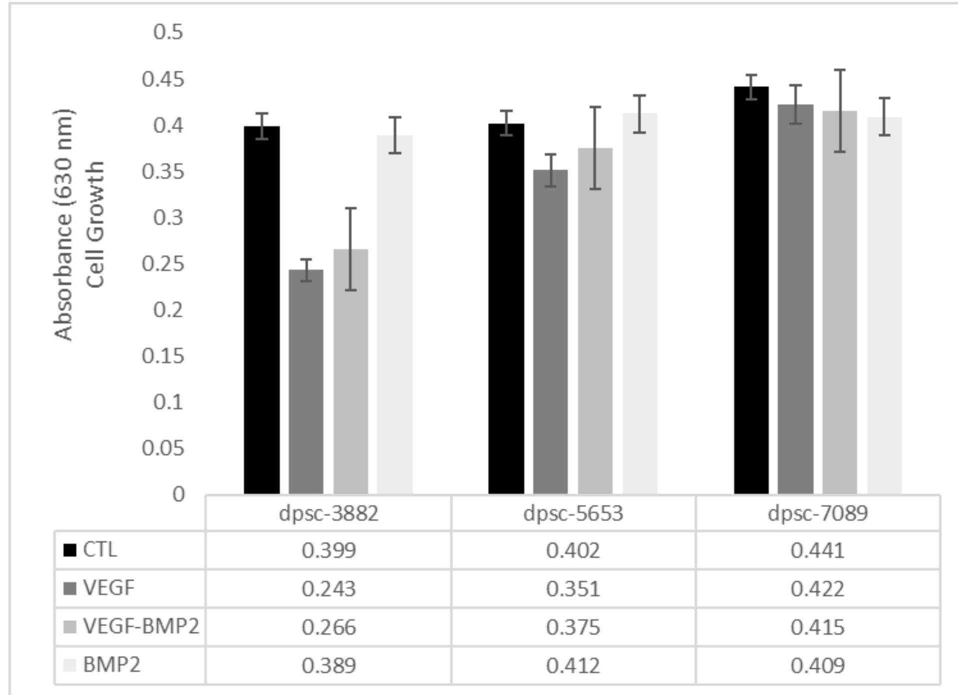
Statistical analysis

As continuous (parametric) data measurements were made using absorbance readings at 630 nm, differences between experimental and control treatments were evaluated using two-tailed t-tests. An alpha level of $\alpha=0.05$ was used to determine statistical significance.

Results

The results of the initial experimental assay using rapidly dividing or rDT DPSC isolates demonstrated that two DPSC isolates responded to VEGF administration (Figure 1). More specifically, VEGF administration induced significant, measurable decreases in cellular growth among dpsc-3882 (-39.1%, p=0.018) and dpsc-5653 (-12.6%, p=0.039) over three days compared with the negative control – with no measurable differences observed among dpsc-7089

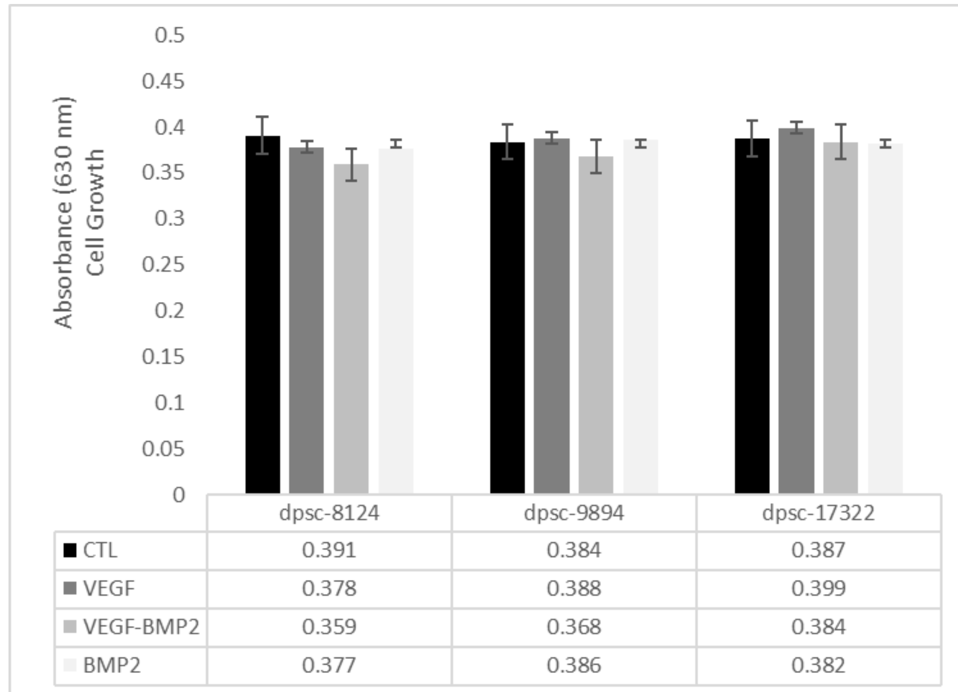
(-4.3%, p=0.852). In contrast, administration of BMP-2 did not significantly alter cellular growth among any of the rDT DPSC isolates, including dpsc-3882 (-2.5%, p=0.948), dpsc-5653 (+2.4%, p=0.823), and dpsc-7089 (-7.2%, p=0.742). However, concomitant administration of VEGF in combination with BMP-2 appeared to have a more modest effect on rDT DPSC isolates, reducing growth in dpsc-3882 (-33.3%, p=0.0241), dpsc-5653 (-6.7%, p=0.592), and dpsc-7089 (-5.8%, p=0.691).



	dpsc-3882	dpsc-5653	dpsc-7089
CTL	0.399	0.402	0.441
VEGF	0.243	0.351	0.422
VEGF-BMP2	0.266	0.375	0.415
BMP2	0.389	0.412	0.409

Figure 1. Growth factor effects on rapidly dividing (rDT) DPSC isolates. Administration of VEGF reduced growth in dpsc-3882 and dpsc-5653 significantly (-39.1%, p=0.018; -12.6%, p=0.039) with no effect on dpsc-7089 (-4.3%, p=0.852). Administration of BMP-2 alone exhibited no significant effects on growth in rDT DPSC isolates, although VEGF-BMP-2 in combination had a modest effect on dpsc-3882 (-33.3%, p=0.0241).

The results of the subsequent experimental assay using intermediate dividing or iDT DPSC isolates demonstrated no significant or measurable effects on these DPSC isolates (Figure 2). More specifically, the administration of VEGF did not exhibit any significant effect on cellular growth in any of the iDT DPSC isolates, including dpsc-8124 (-3.3%, p=0.137), dpsc-9894 (+1.3%, p=0.539), and dpsc-17322 (+3.1%, p=0.165). The concomitant administration of VEGF and BMP-2 also had no significant effect on cellular growth in dpsc-8124 (-8.1%, p=0.0579), dpsc-9894 (-4.2%, p=0.441), and dpsc-17322 (-0.8%, p=0.887). These results were similar to the observations with the administration of BMP-2 on dpsc-8124 (-3.6%, p=0.189), dpsc-9894 (+0.5%, p=0.653), and dpsc-17322 (-1.3%, p=0.794).



	dpsc-8124	dpsc-9894	dpsc-17322
CTL	0.391	0.384	0.387
VEGF	0.378	0.388	0.399
VEGF-BMP2	0.359	0.368	0.384
BMP2	0.377	0.386	0.382

Figure 2. Growth factor effects on intermediate dividing (iDT) DPSC isolates. Administration of VEGF exhibited no significant effect on iDT DPSC growth: dpsc-8124 (-3.3%, p=0.137), dpsc-9894 (+1.3%, p=0.539), and dpsc-17322 (+3.1%, p=0.165) – similar to the effects of BMP-2: dpsc-8124 (-3.6%, p=0.189), dpsc-9894 (+0.5%, p=0.653), dpsc-17322 (-1.3%, p=0.794). Combined VEGF and BMP-2 also had no significant effects: dpsc-8124 (-8.1%, p=0.0579), dpsc-9894 (-4.2%, p=0.441), dpsc-17322 (-0.8%, p=0.887).

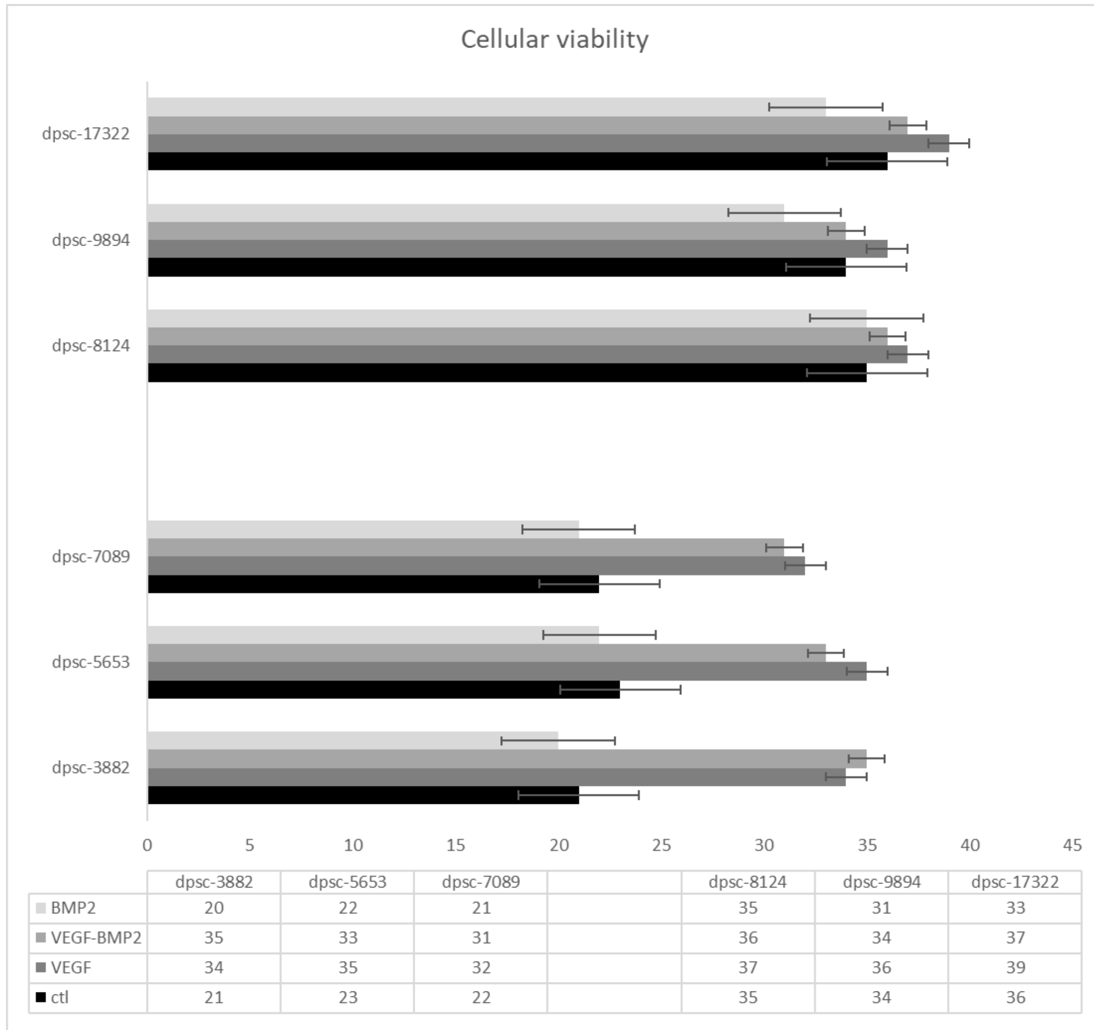
To evaluate whether any of the observations in cellular growth were associated with any changes to survival, cellular viability was evaluated under each control and experimental condition (Figure 3). These data demonstrated an overall higher viability for the iDT DPSC isolates at baseline: dpsc-8124 (34%), dpsc-9894 (33%), dpsc-17322 (36%) compared with the rDT DPSC isolates: dpsc-3882 (21%), dpsc-5653 (23%), dpsc-7089 (22%), which was statistically significant, p=0.0067.

The addition of VEGF significantly increased cellular viability among the rDT DPSC isolates, such as dpsc-3882 (35%, +61.9%), dpsc-5653 (35%, +52.1%), and dpsc-7089 (32%, +45.4%), p=0.0081. However, no corresponding increase in viability was observed in any iDT

DSPC isolate under , including dpsc-8124 (37%, +5.4%), dpsc-9894 (36%, +5.8%), and dpsc-17322 (39%, +8.3%), p=0.102.

However, the addition of BMP-2 did not significantly alter cellular viabilty among the rDT DPSC isolates, inclduing dpsc-3882 (20%, -4.7%), dpsc-5653 (22%, -4.3%), and dpsc-7089 (21%, -4.5%), p=0.287. Similarly, no effects were observed among the iDT DPSC isolates, such as dpsc-8124 (35%, no change), dpsc-9894 (31%, -8.8%), and dpsc-17322 (33%, -8.3%), p=0.221.

The concomitant administration of VEGF and BMP-2 exhibited some effects on rDT but not iDT cellular viability. More specifically, VEGF-BMP-2 significantly increased viability among the rDT DPSC isolates, dpsc-3882 (35%, +66.7%), dpsc-5653 (33%, +43.4%), and dpsc-7089 (31%, +40.9%), p=0.0036. In contrast, viability among the iDT DPSC isolates was not significantly changed under VEGF-BMP-2 administration, dpsc-8124 (36%, 2.8%), dpsc-9894 (34%, no change), dpsc-17322 (37%, +2.7%), p=0.566



	dpsc-3882	dpsc-5653	dpsc-7089		dpsc-8124	dpsc-9894	dpsc-17322
BMP2	20	22	21		35	31	33
VEGF-BMP2	35	33	31		36	34	37
VEGF	34	35	32		37	36	39
ctl	21	23	22		35	34	36

Figure 3. Growth factor effects on DPSC cellular viability. The addition of VEGF (alone or in combination) significantly increased viabilty among the rDT DPSC isolates (dpsc-3882, dpsc-5653, dpsc-8124) but had no effects on the iDT DPSC isolates (dpsc-8124, dpsc-9894, dpsc-17322). No changes in cellular viability were observed with the administration of BMP-2 in either the rDT or iDT DPSC isolates.

Microscopy was performed to more closely evaluate the effects of VEGF on growth and viability of the rDT DPSC isolates (Figure 4). This analysis revealed that the increases in cellular viability and decrease in growth were associated with significant changes to cellular morphology in both dpsc-3882 (Fig. 1A, Fig. 1D) and dpsc-5653 (Fig. 1B, Fig. 1E). It was also noted that

significant cell adhesion, cell spreading and increased cellular size was noted – although fewer overall numbers of cells were present. In addition, dpsc-7089 also contained a smaller proportion of cells with changes to adhesion, spreading and increased size (Fig. 1C, Fig. 1F). No changes to cell number, size or shape were noted among the iDT DPSC isolates (data not shown).

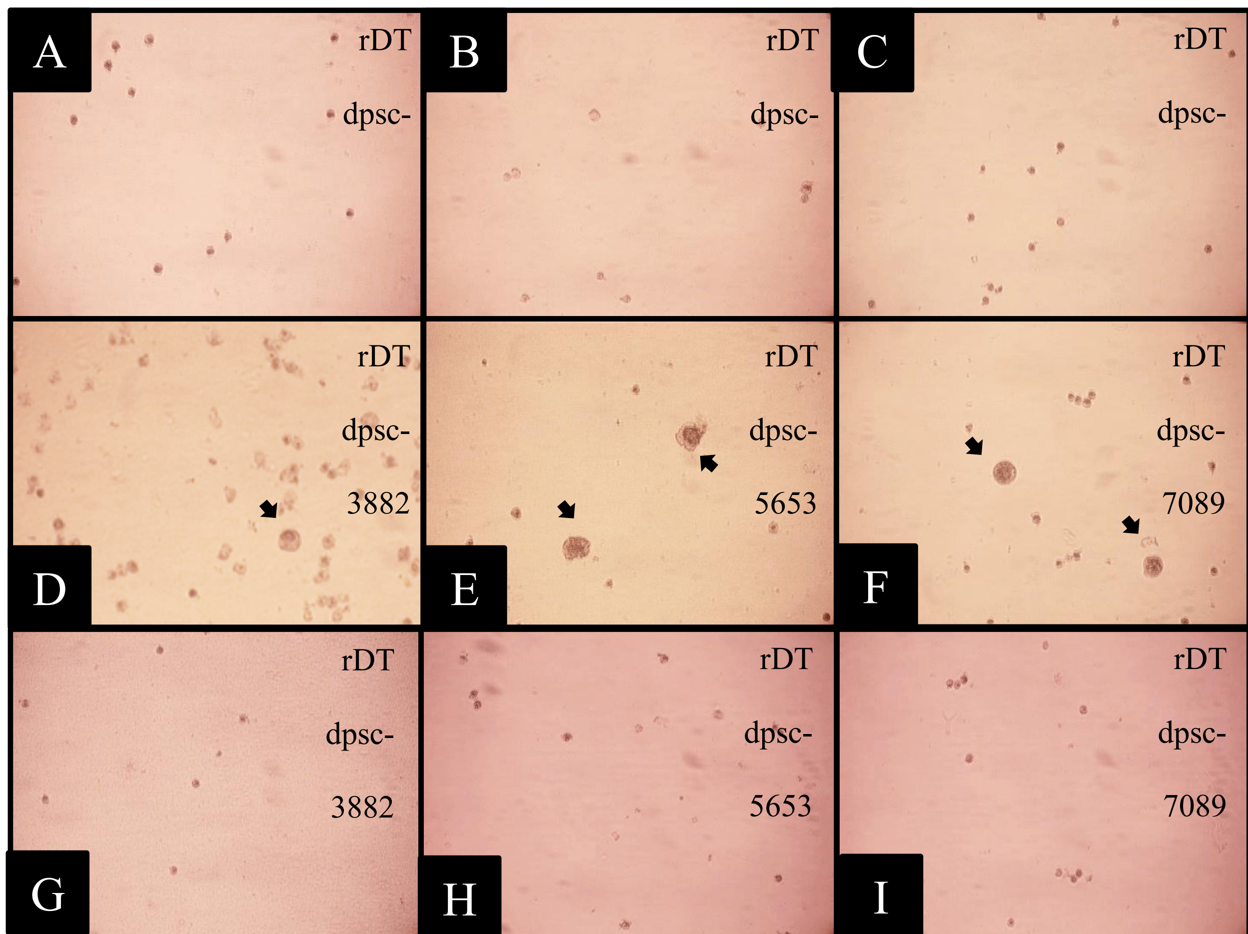


Figure 4. Cellular morphology of rDT DPSC isolates in control and experimental conditions. The cellular morphology of rDT dpsc-3882 (A), dpsc-5653 (B), and dpsc-7089 (C) was marked altered by the addition of VEGF (D-F). Although an overall decrease in cell number was observed in dpsc-3882 and dpsc-5653 (D,E), an overall increase in cell size, cellular adherence and cell spreading was observed. No changes were observed with BMP-2 (G-I).

Discussion

The main objective of this study was to evaluate whether VEGF administration or concomitant VEGF and BMP administration induced any measurable effects on DPSCs. These results demonstrated that administration of VEGF was sufficient to reduce growth in two of the rDT DPSC isolates, with no effect on any of the iDT DPSC isolates. Moreover, a corresponding increase in cellular viability was noted among all the rDT DPSC isolates along with corresponding changes to cellular morphology, with no effect on the iDT DPSCs. These results were in stark contrast to the effects of BMP-2, which exhibited no effects on either rDT or iDT DPSC isolates. In addition, the combination of VEGF and BMP-2 in combination had similar effects to the administration of VEGF in isolation, which may suggest VEGF may be primarily responsible for the observed changes to cellular phenotypes.

Although these data strongly suggest that VEGF may alter rDT cellular phenotypes, such as viability, proliferation and morphology, the limited scope of this study was not sufficient to allow for the elucidation of specific pathways responsible for these effects. The limited evidence that exists for MSC has recently suggested that VEGF may act on mesencymal stem cells through ERK- and FAK-dependent mechanisms that increase cellular migration – although there is no evidence to suggest these same pathways may be active among the rDT DPSC isolates [21,22].

The few studies that have explored VEGF in MSC or DPSC have been mainly restricted to studies of vasculogenic differentiation, an important topic for bioengineering and therapeutic applications [23, 24]. However, the potential for VEGF to induce other cellular phenotypes and differentiation into lineages other than angiogenic and endothelial lineages remains an exciting and potentially revolutionary application for stem cell therapy [25,26].

Conclusions

These data provide significant preliminary results that clearly demonstrate significant and pronounced effects of VEGF on at least one subset of rapidly dividing DPSC isolates. These effects include changes to cellular viability and growth, which are supported by clear changes to cellular adhesion and morphology. Taken together, these data strongly suggest more reseach is needed to determine the underlying pathways triggered by VEGF administration in the cells and the pathophysiologic mechanisms that determine the responsiveness of these DPSC isolates but not others.

Competing Interests

The authors declare that there are no conflicts of interest.

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Chapter 4: Summary and Conclusions

The two studies presented provide further insight into the effects of vascular endothelial growth factor (VEGF) on the characteristics of pluripotent dental pulp stem cells (DPSC). It was the hope that the presence of VEGF would induce differentiation in cultured DPSCs showing a change in their presenting characteristics and intracellular mRNA biomarker expression.

Chapter 2 of this paper delivered the results of a retrospective study that sought to determine if the presence of VEGF alone with DPSCs would have any effect on their characteristics. Eight cell lines were cultured for ten rounds to determine their doubling times. Doubling time (DT) was categorized as rapid or rDT (~2 days), intermediate or iDT (4-6 days), and slow or sDT (10-12 days). These categories were then used to help analyze the data collected. The results of this study revealed that two specific rDT isolates (dpsc-3882, dpsc-5653) showed a significant measurable change in their growth when VEGF was administered (p=0.038 and p=0.041). No significant changes in growth were seen with any of the iDT or sDT isolates. When evaluating viability, the same two rDT isolates showed a statistically significant change (p=0.018 and p=0.011). Primer screening specific for alkaline phosphatase (ALP) and dentin sialophosphoprotein (DSPP) revealed rDT isolate dpsc-3882 to be expressing both DSPP and ALP, and rDT isolate dpsc-5653 to be only expressing ALP mRNA. Lastly, this study found that only the rDT group expressed a combination of NANOG with either Oct-4 or Sox-2. These rDT cell lines were the cell lines that showed significant change in both the growth and viability tests.

Chapter 3 presented the study done on the comparison of DPSC characteristics when cultured with VEGF alone, bone morphogenic protein-2 (BMP-2) alone, and when cultured with a combination of both VEGF and BMP-2. The rDT and iDT DPSC isolates were used in this

study. Growth and viability were studied and measured for comparison. The results showed that the rDT isolates had significantly decreased growth when only VEGF was present, however, when BMP-2 and VEGF were administered in combination the effect on growth was more moderate. No significant affect was seen on growth when just BMP-2 was cultured with rDT DPSCs. Additionally, no significant change was seen on growth in any of the iDT samples. When evaluating viability, the addition of VEGF alone was seen to have a statistically significant effect on rDT isolates and no effect on iDT isolates. BMP-2 also did not alter the viability in either the rDT or iDT groups. The combination of VEGF and BMP-2 was seen to have the same significant effect on viability that VEGF alone did in the rDT group. No change was seen in the iDT combination group. Cellular microscopy from this study noted cell morphology changes in dpsc-3882 and dpsc-5653 isolates. Cell adhesions, cell spreading and an increased cellular size was noted.

Based on the findings in each study, both alternative hypotheses can be accepted.

1. Is there an effect on characteristics of the DPSC lines when cultured with VEGF?

    Alternative (H_A) hypothesis: There will be an effect on the characteristics of the DPSC isolates when cultured with VEGF

2. Is there an effect on characteristics of the DPSC lines when cultured with VEGF in combination with BMP-2?

    Alternative (H_A) hypothesis: There will be an effect on the characteristics of the DPSC isolates when cultured with VEGF in combination with BMP-2

Limitations and Recommendations

A significant limitation in the two studies presented is the number of DPSC cell lines available. Since DPSC collecting is only being done every couple of years, it has not been possible to maintain numerous cell lines. Funding for additional collection, as well as the purchase of standard cell lines, would help to expand the breadth of the study and allow for more standardized study. With the results from this study, different rDT cell lines would allow for follow-up studies to assist in determining the specific requirements for a reaction to VEGF.

A follow-up study on different concentrations of VEGF and BMP-2 would provide more insight into the relationship that the two cytokines have on DPSCs. It would ultimately help to determine whether there is a competitive or noncompetitive interaction that is affecting the characteristics of DPSCs.

Appendix A

UNLV Biomedical IRB - Administrative Review
Notice of Excluded Activity

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

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

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

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

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

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

Office of Research Integrity - Human Subjects
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Appendix B

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University of Nevada, Las Vegas

I, **Karl Kingsley,** holder of copyrighted material entitled **Effects of vascular endothelial growth factor (VEGF) on dental pulp stem cells (DPSC),** authored by **Cale Forgues,** and **Karl Kingsley** originally published in **Current Research in Medicine, March 2019 (4-19)**, hereby give permission for the author to use the above described material in total or in part for inclusion in a Master's thesis at the University of Nevada, Las Vegas.

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March 1, 2019

Signature | Date

Karl Kingsley, PhD, MPH | Professor

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Appendix C

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Signature	Date
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Thesis Title:

The Effects of Vascular Endothelial Growth Factor on the Characteristics of Pluripotent Dental Pulp Stem Cells

Thesis Examination Committee:

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