

DENTAL PULP STEM CELL DIFFERENTIATION POTENTIAL OF BMP-2 AND BMP-4

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Abstract

DENTAL PULP STEM CELL DIFFERENTIATION POTENTIAL OF BMP-2 AND BMP-4

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Introduction: Many studies are now evaluating the potential for dental pulp stem cells (DPSC) to assist with more complex and biotechnology applications, such as facilitating and promoting osseointegration following dental implants. However, the effects of factors that may control osseointegration and bone repair using DPSC including bone morphogenic proteins (BMP-2, BMP-4) are not yet well understood.

Objective: Based upon this lack of evidence, the primary goal of this project is to evaluate the potential effects of BMPs (alone or in combination with other growth factors) to induce factors associated with osteogenesis.

Methods: DPSC isolates from an existing repository (n = 13) were plated into 96-well experimental assays with the addition of BMP2, BMP-4 or a combination. Viability and growth assays were performed and RNA was collected and screened using quantitative polymerase chain reaction (qPCR).

Results: BMP-2 administration induced increased proliferation and viability among two rapidly dividing DPSC isolates, while administration of BMP-4 induced similar responses among different rapid and all the intermediate dividing DPSC isolates. The combination of BMP-2 and BMP-4 induced differential increases in growth and viability among a distinct subset of rapidly and slowly dividing DPSC isolates that did not respond to the isolated administration of BMP-2 or BMP-4 alone. In addition, the increased growth and proliferation among these distinct isolates was associated with increased expression of alkaline phosphatase (ALP).

Conclusions:

These results suggest that BMP-2 and BMP-4 (both alone and in combination) are sufficient to induce the production of the early bone biomarkers ALP within specific subsets of the DPSC isolates evaluated. Although these results represent a significant step towards our understanding of DPSC biology, further research will be needed to determine the additional factors and biomarkers that may facilitate osteogenic differentiation.

Key words: Dental Pulp Stem Cells (DPSC); BMP-2, BMP-4; Quantitative Polymerase Chain Reaction (qPCR); Alkaline Phosphatase (ALP)

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Chapter 1

Background and Significance

The ability to restore human organs and repair wounds has been an area of productive research in regenerative medicine [1-3]. Indeed, the potential of targeted regenerative therapy is monumental and could usher in a new age of stem cell-driven medicine [4,5]. The topic has become a major focus of research in all healthcare disciplines, and has been developing within dentistry due to the isolation and study of stem cells derived from oral cells and tissues [6,7].

Most of these regeneration efforts are focused on the regenerative properties of mesenchymal stem cells [8,9]. Most mesenchymal stem cells have not fully differentiated, which means they still have the potential to become different types of cells and tissues [10,11]. They retain the ability to proliferate extensively and may become the building blocks of many tissues and organ systems [12-14].

Mesenchymal stem cells can be found throughout the body, including bone marrow, blood, muscle, liver, brain, adipose tissue, skin, and the gastrointestinal tract [15,16]. One particularly accessible source of stem cells is in the pulp of human teeth, known as dental pulp stem cells (DPSCs) [17,18]. There are additional types of oral stem cells, such as gingival-derived mesenchymal stem cells (GMSC), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), and stem cells from the apical papilla (SCAP) that each possess significant differentiation potentials and the ability to regenerate specialized structures such as the dentin-pulp complex or periodontal ligament [19,20].

DPSCs have been the subject of much of scientific literature in this area due to their properties, such as their natural involvement in reparative dentinogenesis, a form of tooth repair following injury from various irritants such as dental attrition or caries [21,22]. In addition,

DPSCs have been shown to differentiate into odontoblast-like cells, a type of cell that assists in the aforementioned tooth repair and regeneration [23,24]. This has revealed that many of the cues that drive odontoblast differentiation among DPSC may share some overlapping pathways with osteoblast differentiation, which could lead to many new applications in the field of osseointegration and implant treatments or therapy [25,26].

The conditions required for DPSC cellular attachment, proliferation, differentiation and function towards osteoblastic differentiation involve specific growth factors, such as bone morphogenic proteins (BMP) [27,28]. In fact, previous work from this group has explored the role of BMP-2 and vascular endothelial growth factor (VEGF) on DPSC differentiation [29-31]. However, most models of bone development and repair suggest that a combination of BMPs, including BMP-2 and BMP-4 may be needed to stimulate stem cell differentiation into osteoblastic and osteoclastic lineages [32,33].

BMPs play a particularly fundamental role in the regulation of bone formation, maintenance, and repair with more than 20 different types divided into four distinct subfamilies [34,35]. BMP-2 and BMP-4 have the most important roles in osteoblast differentiation and cartilage regeneration and have been used clinically for therapy to treat bone defects, spinal fusion, osteoporosis, and root canal surgery [36,37]. Because these two growth factors may wield profound influence on DPSCs in their potential to form bone, understanding the interconnection between DPSCs and BMP-2/BMP-4 may help DPSC researchers closer to functional therapies involving tissue regeneration or osseointegration [38,39]. Based upon the lack of research in this area, the primary goal of this project is to evaluate the potential effects of BMP-2 and BMP-4 (alone and in combination) to induce factors associated with osteogenesis among DPSC.

Research Question

Based upon the lack of in-depth information regarding this type of bone biology, the first goal of this project will be to evaluate whether the sequential administration of BMP-2/BMP-4 is sufficient to induce chondrogenesis or osteogenesis among varying cell lines.

- A. Null hypothesis: BMP-2/BMP-4 administration is not sufficient to induce these effects among all cell lines.
- B. Alternative hypothesis: BMP-2/BMP-4 administration is sufficient to induce these effects among some cell lines but not others.

Approval

The protocol for this study was reviewed and approved by the Institutional Review Board (IRB) and Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas (UNLV) under #171612-1 “Retrospective Analysis of Dental Pulp Stem Cells (DPSC) from the UNLV School of Dental Medicine (SDM) Pediatric and Clinical Population” on February 21, 2021.

Research Design

Study approval

The protocol for this study was reviewed and approved by the Institutional Review Board (IRB) and Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas (UNLV) under #171612-1 “Retrospective Analysis of Dental Pulp Stem Cells (DPSC) from the UNLV School of Dental Medicine (SDM) Pediatric and Clinical Population” on

February 21, 2021. This was a retrospective study involving an existing biomedical repository of previously collected dental pulp stem cell (DPSC) isolates [29-31].

Original study protocol

The original study protocol for the establishment of the DPSC biorepository was also reviewed and approved by the IRB and OPRS at UNLV under OPRS#0907-3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp" on February 5, 2010. The inclusion criteria for the original study collection required voluntary patient participation and voluntary Informed consent from all adult patients over the age of 18 years. Patients younger than 18 were allowed to participate with voluntary Parental Permission from the parent or guardian and voluntary Pediatric Assent from each pediatric patient . Only patients of record were allowed to participate and the exclusion criteria included any patients or parents/guardians that declined to participate or were not scheduled for routine dental extraction.

Cell culture

This study utilized the existing biorepository DPSC isolates (n=18). All DPSC isolates were thawed briefly and centrifuged to remove the dimethyl sulfoxide (DMSO) used for cryopreservation, prior to resuspension in cell culture media. DPSC were cultured in Roswell Park Memorial Institute (RPMI) media with the addition of 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin antibiotic solution - all from Gibco (Waltham, MA). Cells were cultured in T25 cm³ tissue-culture treated flasks in a biosafety cabinet at 37°C, supplemented with 5% CO₂.

Viability

Cell viability was assessed using the Trypan Blue exclusion assay and a TC20 automated Cell Counter from BioRad Technologies (Hercules, CA). Cell counts of live cells and total cells were enumerated according to the manufacturer protocol using Trypan Blue 0.4% solution from MP Biochemicals (Santa Ana, CA). DPSC isolates exhibiting good to excellent viability (50% - 95%) were selected for inclusion in this study (n=13).

Biomarker screening

RNA was isolated from each of the DPSC isolates using the phenol:chloroform extraction method. RNA concentration and quality were evaluated using the NanoDrop 2000 spectrophotometer from ThermoFisher Scientific (Fair Lawn, NJ). Absorbance readings at A230 nm, A260 nm, and A280 nm were assessed to determine RNA quality and quantity, which were all above the minimum standards for cDNA synthesis of 100 ng with A260:A280 ratios of 1.65 or greater. cDNA was generated from the RNA isolations using the One Step RT-PCR (cDNA synthesis) kit from New England Biolabs (NEB; Ipswich, MA). Screening for the presence of stem cell markers CD90 and CD105, as well as the absence of CD45 according to the International Society for Cellular Therapy (ISCT) criteria for stem cells, as previously described [40]. In addition, screening for other stem cell biomarkers Sox-2, Oct-4, and NANOG was confirmed.

qPCR screening

Molecular screening was then performed using qPCR reactions composed of the SYBR Green Master Mix from ThermoFisher Scientific. More specifically, each reaction contained ABsolute SYBR green (12.5 uL), nuclease-free water (7.5 uL), forward and primer (1.75 uL of each), and sample diluted to 1.0 ng/uL (1.5 uL). Setting for the qPCR reactions included enzyme activation

15 minutes at 95°C and 40 cycles consisting of 15 seconds denaturation at 95°C, annealing for 30 seconds at the primer pair-specific temperature, and final extension for 30 seconds at 72°C .

Positive control primers

GAPDH forward: 5'ATCTTCCAGGAGCGAGATCC-3'; 20 nt, 55% GC, Tm 66°C

GAPDH reverse: 5'ACCACTGACACGTTGGCAGT-3'; 20 nt, 55% GC, Tm 70°C

Beta-actin forward: 5'-GTGGGGTCCTGTGGTGTG-3'; 18 nt, 67% GC, Tm: 69 °C

Beta-actin reverse: 5'-GAAGGGGACAGGCAGTGA-3', 18 nt, 61% GC, Tm: 67 °C

ISCT control primer

CD90 forward: 5'-ATGAACCTGGCCATCAGCA-3'; 19 nt, 53% GC, Tm: 67 °C

CD90 reverse: 5'-GTGTGCTCAGGCACCCC-3'; 17 nt, 71% GC, Tm: 70 °C

CD105 forward: 5'-CCACTAGCCAGGTCTCGAAG-3'; 20 nt, 60% GC, Tm: 67 °C

CD105 reverse: 5'-GATGCAGGAAGACACTGCTG-3'; 20 nt, 55% GC, Tm: 66 °C

CD45 forward: 5'CATATTTATTTTGTCTTCTCCCA-3'; 24 nt, 33% GC, Tm: 60 °C

CD45 reverse: 5'-GAAAGTTTCCACGAACGG-3'; 18 nt, 50% GC, Tm: 61 °C

MSC biomarker primers

Sox-2 forward: 5'-ATGGGCTCTGTGGTCAAGTC-3'; 20 nt: 55% GC; Tm 67 °C

Sox-2 reverse: 5'-CCCTCCCAATTCCCTTGTAT-5'; 20 nt; 50% GC; Tm 64 °C

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

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

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

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

Differentiation primers

Alkaline phosphatase (ALP)

ALP forward: 5'-CACTGCGGACCATTCCCACGTCTT-3'; 24 nt, 58% GC, Tm: 74°C

ALP reverse: 5'-GCGCCTGGTAGTTGTTGTGAGCAT-3'; 24 nt, 54% GC, Tm: 72°C

Dentin sialophosphoprotein (DSPP)

DSPP forward: 5'-CAACCATAGAGAAAGCAAACGCG-3'; 23 nt, 48% GC, Tm: 67°C

DSPP reverse: 5'-TTTCTGTTGCCACTGCTGGGAC-3'; 22 nt, 55% GC, Tm: 70°C

Proliferation assays

All DPSC isolates meeting the viability standards for this study (n=13) were plated at 1.2×10^5 cells/mL in 96-well tissue culture treated flat bottom Corning Costar assay plates (Corning, NY) and allowed to proliferate in a biosafety level (BSL)-2 incubator at 37°C supplemented with 5% CO₂. Assays were performed using recombinant human Bone Morphogenic Protein (BMP) BMP-2 (#PHC7141) and BMP-4 (PHC9531) alone and in combination. Viability was assessed (as described above) and proliferation assays were then fixed at 24 hours (one day), 48 hours (two days) or 72 hours (three days) with 10% formalin and processed using Gentian Violet 1% w/v alcoholic solution from RICCA Chemical Company (Arlington, TX). 96-well assay plates were analyzed using a BioTek ELx808 microplate reader (Winooski, VT) at 630 nm and absorbance readings were exported into Microsoft Excel (Redmond, WA) for analysis.

Statistical analysis

Viability data from the initial thawing and cell culture, as well as from the end points of the proliferation assays were imported into Microsoft Excel (Redmond, WA) and differences between experimental conditions were measured using two-tailed Student's t-tests, which are appropriate for parametric analysis of continuous data. Any statistically significant differences were verified using Analysis of Variance (ANOVA) due to the possibility of error involved with analysis of multiple two-way t-tests. Significance levels were set at alpha (α) = 0.05.

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Chapter 2

Dental Pulp Stem Cell Differentiation Potential Of BMP-2 And BMP-4

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Abstract

Introduction: Many studies are now evaluating the potential for dental pulp stem cells (DPSC) to assist with more complex and biotechnology applications, such as facilitating and promoting osseointegration following dental implants. However, the effects of factors that may control osseointegration and bone repair using DPSC including bone morphogenic proteins (BMP-2, BMP-4) are not yet well understood. Based upon this lack of evidence, the primary goal of this project is to evaluate the potential effects of BMPs (alone or in combination with other growth factors) to induce factors associated with osteogenesis.

Methods: DPSC isolates from an existing repository (n=13) were plated into 96-well experimental assays with the addition of BMP-2, BMP-4 or a combination. Viability and growth assays were performed and RNA was collected and screened using quantitative polymerase chain reaction (qPCR).

Results: BMP-2 administration induced increased proliferation and viability among two rapidly dividing DPSC isolates, while administration of BMP-4 induced similar responses among different rapid and all the intermediate dividing DPSC isolates. The combination of BMP-2 and BMP-4 induced differential increases in growth and viability among a distinct subset of rapidly and slowly dividing DPSC isolates that did not respond to the isolated administration of BMP-2 or BMP-4 alone. In addition, the increased growth and proliferation among these distinct isolates was associated with increased expression of alkaline phosphatase (ALP).

Conclusions: These results suggest that BMP-2 and BMP-4 (both alone and in combination) are sufficient to induce the production of the early bone biomarkers ALP within specific subsets of the DPSC isolates evaluated. Although these results represent a significant step towards our understanding of DPSC biology, further research will be needed to determine the additional factors and biomarkers that may facilitate osteogenic differentiation.

Introduction

The ability to restore human organs and repair wounds has been an area of productive research in regenerative medicine [1-3]. Indeed, the potential of targeted regenerative therapy is monumental and could usher in a new age of stem cell-driven medicine [4,5]. The topic has become a major focus of research in all healthcare disciplines, and has been developing within dentistry due to the isolation and study of stem cells derived from oral cells and tissues [6,7].

Most of these regeneration efforts are focused on the regenerative properties of mesenchymal stem cells [8,9]. Most mesenchymal stem cells have not fully differentiated, which means they still have the potential to become different types of cells and tissues [10,11]. They retain the

ability to proliferate extensively and may become the building blocks of many tissues and organ systems [12-14].

Mesenchymal stem cells can be found throughout the body, including bone marrow, blood, muscle, liver, brain, adipose tissue, skin, and the gastrointestinal tract [15,16]. One particularly accessible source of stem cells is in the pulp of human teeth, known as dental pulp stem cells (DPSCs) [17,18]. There are additional types of oral stem cells, such as gingival-derived mesenchymal stem cells (GMSC), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), and stem cells from the apical papilla (SCAP) that each possess significant differentiation potentials and the ability to regenerate specialized structures such as the dentin-pulp complex or periodontal ligament [19,20].

DPSCs have been the subject of much of scientific literature in this area due to their properties, such as their natural involvement in reparative dentinogenesis, a form of tooth repair following injury from various irritants such as dental attrition or caries [21,22]. In addition, DPSCs have been shown to differentiate into odontoblast-like cells, a type of cell that assists in the aforementioned tooth repair and regeneration [23,24]. This has revealed that many of the cues that drive odontoblast differentiation among DPSC may share some overlapping pathways with osteoblast differentiation, which could lead to many new applications in the field of osseointegration and implant treatments or therapy [25,26].

The conditions required for DPSC cellular attachment, proliferation, differentiation and function towards osteoblastic differentiation involve specific growth factors, such as bone morphogenic proteins (BMP) [27,28]. In fact, previous work from this group has explored the role of BMP-2 and vascular endothelial growth factor (VEGF) on DPSC differentiation [29-31]. However, most models of bone development and repair suggest that a combination of BMPs,

including BMP-2 and BMP-4 may be needed to stimulate stem cell differentiation into osteoblastic and osteoclastic lineages [32,33].

BMPs play a particularly fundamental role in the regulation of bone formation, maintenance, and repair with more than 20 different types divided into four distinct subfamilies [34,35].

BMP-2 and BMP-4 have the most important roles in osteoblast differentiation and cartilage regeneration, and have been used clinically for therapy to treat bone defects, spinal fusion, osteoporosis, and root canal surgery [36,37]. Because these two growth factors may wield profound influence on DPSCs in their potential to form bone, understanding the interconnection between DPSCs and BMP-2/BMP-4 may help DPSC researchers closer to functional therapies involving tissue regeneration or osseointegration [38,39]. Based upon the lack of research in this area, the primary goal of this project is to evaluate the potential effects of BMP-2 and BMP-4 (alone and in combination) to induce factors associated with osteogenesis among DPSC.

Methodology

Study approval

The protocol for this study was reviewed and approved by the Institutional Review Board (IRB) and Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas (UNLV) under #171612-1 “Retrospective Analysis of Dental Pulp Stem Cells (DPSC) from the UNLV School of Dental Medicine (SDM) Pediatric and Clinical Population” on February 21, 2021. This was a retrospective study involving an existing biomedical repository of previously collected dental pulp stem cell (DPSC) isolates [29-31].

Original study protocol

The original study protocol for the establishment of the DPSC biorepository was also reviewed and approved by the IRB and OPRS at UNLV under OPRS#0907-3148 "Isolation of Non-Embryonic Stem Cells from Dental Pulp" on February 5, 2010. The inclusion criteria for the original study collection required voluntary patient participation and voluntary Informed consent from all adult patients over the age of 18 years. Patients younger than 18 were allowed to participate with voluntary Parental Permission from the parent or guardian and voluntary Pediatric Assent from each pediatric patient . Only patients of record were allowed to participate and the exclusion criteria included any patients or parents/guardians that declined to participate or were not scheduled for routine dental extraction.

Cell culture

This study utilized the existing biorepository DPSC isolates (n=18). All DPSC isolates were thawed briefly and centrifuged to remove the dimethyl sulfoxide (DMSO) used for cryopreservation, prior to resuspension in cell culture media. DPSC were cultured in Roswell Park Memorial Institute (RPMI) media with the addition of 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin antibiotic solution - all from Gibco (Waltham, MA). Cells were cultured in T25 cm³ tissue-culture treated flasks in a biosafety cabinet at 37°C, supplemented with 5% CO₂.

Viability

Cell viability was assessed using the Trypan Blue exclusion assay and a TC20 automated Cell Counter from BioRad Technologies (Hercules, CA). Cell counts of live cells and total cells were enumerated according to the manufacturer protocol using Trypan Blue 0.4% solution from

MP Biochemicals (Santa Ana, CA). DPSC isolates exhibiting good to excellent viability (50% - 95%) were selected for inclusion in this study (n=13).

Biomarker screening

RNA was isolated from each of the DPSC isolates using the phenol:chloroform extraction method. RNA concentration and quality were evaluated using the NanoDrop 2000 spectrophotometer from ThermoFisher Scientific (Fair Lawn, NJ). Absorbance readings at A230 nm, A260 nm, and A280 nm were assessed to determine RNA quality and quantity, which were all above the minimum standards for cDNA synthesis of 100 ng with A260:A280 ratios of 1.65 or greater. cDNA was generated from the RNA isolations using the One Step RT-PCR (cDNA synthesis) kit from New England Biolabs (NEB; Ipswich, MA). Screening for the presence of stem cell markers CD90 and CD105, as well as the absence of CD45 according to the International Society for Cellular Therapy (ISCT) criteria for stem cells, as previously described [40]. In addition, screening for other stem cell biomarkers Sox-2, Oct-4, and NANOG was confirmed.

qPCR screening

Molecular screening was then performed using qPCR reactions composed of the SYBR Green Master Mix from ThermoFisher Scientific. More specifically, each reaction contained ABsolute SYBR green (12.5 uL), nuclease-free water (7.5 uL), forward and primer (1.75 uL of each), and sample diluted to 1.0 ng/uL (1.5 uL). Setting for the qPCR reactions included enzyme activation 15 minutes at 95°C and 40 cycles consisting of 15 seconds denaturation at 95°C, annealing for 30 seconds at the primer pair-specific temperature, and final extension for 30 seconds at 72°C .

Positive control primers

GAPDH forward: 5'-ATCTTCCAGGAGCGAGATCC-3'; 20 nt, 55% GC, Tm 66°C

GAPDH reverse: 5'-ACCACTGACACGTTGGCAGT-3'; 20 nt, 55% GC, Tm 70°C

Beta-actin forward: 5'-GTGGGGTCCTGTGGTGTG-3'; 18 nt, 67% GC, Tm: 69 °C

Beta-actin reverse: 5'-GAAGGGGACAGGCAGTGA-3', 18 nt, 61% GC, Tm: 67 °C

ISCT control primer

CD90 forward: 5'-ATGAACCTGGCCATCAGCA-3'; 19 nt, 53% GC, Tm: 67 °C

CD90 reverse: 5'-GTGTGCTCAGGCACCCC-3'; 17 nt, 71% GC, Tm: 70 °C

CD105 forward: 5'-CCACTAGCCAGGTCTCGAAG-3'; 20 nt, 60% GC, Tm: 67 °C

CD105 reverse: 5'-GATGCAGGAAGACACTGCTG-3'; 20 nt, 55% GC, Tm: 66 °C

CD45 forward: 5'-CATATTTATTTTGTCTTCTCCCA-3'; 24 nt, 33% GC, Tm: 60 °C

CD45 reverse: 5'-GAAAGTTTCCACGAACGG-3'; 18 nt, 50% GC, Tm: 61 °C

MSC biomarker primers

Sox-2 forward: 5'-ATGGGCTCTGTGGTCAAGTC-3'; 20 nt: 55% GC; Tm 67 °C

Sox-2 reverse: 5'-CCCTCCCAATTCCCTTGTAT-5'; 20 nt; 50% GC; Tm 64 °C

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

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

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

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

Differentiation primers

Alkaline phosphatase (ALP)

ALP forward: 5'-CACTGCGGACCATTTCCCACGTCTT-3'; 24 nt, 58% GC, Tm: 74°C

ALP reverse: 5'-GCGCCTGGTAGTTGTTGTGAGCAT-3'; 24 nt, 54% GC, Tm: 72°C

Dentin sialophosphoprotein (DSPP)

DSPP forward: 5'-CAACCATAGAGAAAGCAAACGCG-3'; 23 nt, 48% GC, Tm: 67°C

DSPP reverse: 5'-TTTCTGTTGCCACTGCTGGGAC-3'; 22 nt, 55% GC, Tm: 70°C

Proliferation assays

All DPSC isolates meeting the viability standards for this study (n=13) were plated at 1.2×10^5 cells/mL in 96-well tissue culture treated flat bottom Corning Costar assay plates (Corning, NY) and allowed to proliferate in a biosafety level (BSL)-2 incubator at 37°C supplemented with 5% CO₂. Assays were performed using recombinant human Bone Morphogenic Protein (BMP) BMP-2 (#PHC7141) and BMP-4 (PHC9531) alone and in combination. Viability was assessed (as described above) and proliferation assays were then fixed at 24 hours (one day), 48 hours (two days) or 72 hours (three days) with 10% formalin and processed using Gentian Violet 1% w/v alcoholic solution from RICCA Chemical Company (Arlington, TX). 96-well assays plates were analyzed using a BioTek ELx808 microplate reader (Winooski, VT) at 630 nm and absorbance readings were exported into Microsoft Excel (Redmond, WA) for analysis.

Statistical analysis

Viability data from the initial thawing and cell culture, as well as from the end points of the proliferation assays were imported into Microsoft Excel (Redmond, WA) and differences between experimental conditions were measured using two-tailed Student's t-tests, which are appropriate for parametric analysis of continuous data. Any statistically significant differences were verified using Analysis of Variance (ANOVA) due to the possibility of error involved with analysis of multiple two-way t-tests. Significance levels were set at alpha (α) = 0.05.

Results

Each of the DPSC isolates were placed into culture and doubling time (DT) was evaluated (Figure 1). These data confirmed that six of the DPSC isolates exhibited rapid doubling times (rDT) between 1.9 and 2.6 days, including dpSC-9765 (2.1 days), dpSC-7089 (dpSC-7089), dpSC-3924 (2.1 days), dpSC-5423 (2.2 days), dpSC-5653 (2.3 days), and dpSC-3882 (2.6 days). The average doubling time for the rDT isolates was 2.2 days. Some of the DPSC isolates exhibited an intermediate doubling time or iDT roughly twice as long as the rDT DPSCs, which ranged between 4.2 and 5.5 days. These included dpSC-8124 (4.2 days), dpSC-5243 (5.1 days), and dpSC-8604 (5.5 days). The doubling time for iDT DPSC isolates averaged approximately 4.9 days. Finally, four DPSC isolates exhibited significantly longer or slow doubling times (sDT), such as dpSC-11750 (10.4 days), dpSC-11418 (10.6 days), dpSC-17322 (11.2 days), and dpSC-11836 (12.9 days), which averaged 11.3 days.

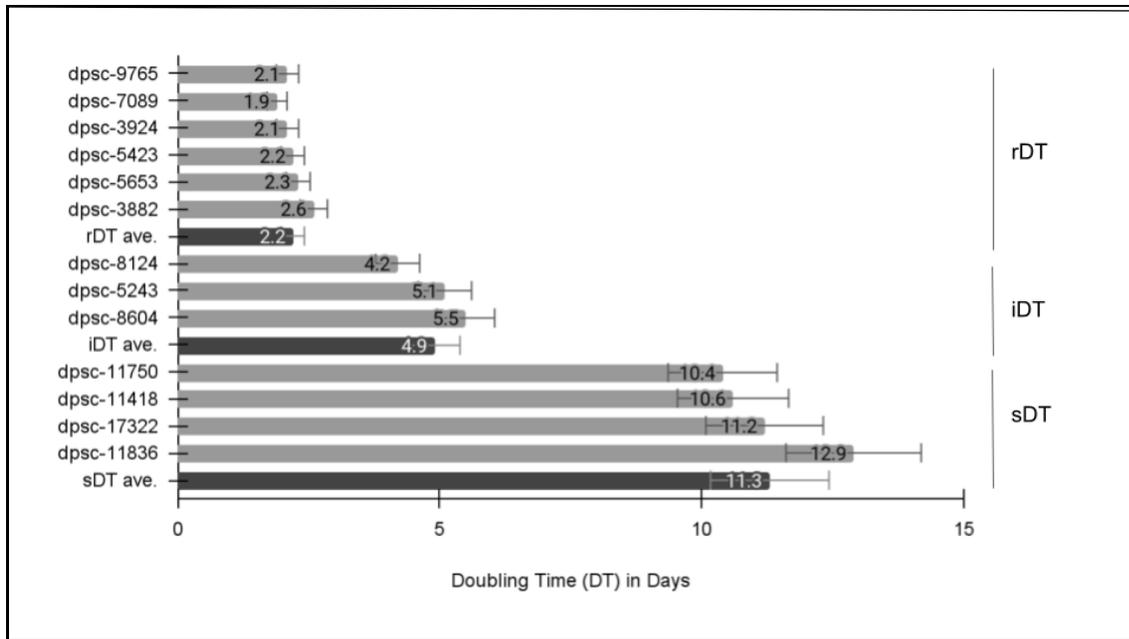


Figure 1. Baseline growth of the DPSC isolates. Six DPSC isolates exhibited rapid doubling times (rDT) between 1.9 and 2.6 days, averaging 2.2 days. Three DPSC isolates exhibited an intermediate doubling time or iDT between 4.2 and 5.5 days, averaging 4.9 days. Four DPSC isolates exhibited slow doubling times (sDT) between 10.4 and 12.9 days, averaging 11.3 days.

To evaluate the effect of these growth factors on DPSCs, BMP-2 was administered to the available DPSC isolates in three-day proliferation assays (Figure 2). These data demonstrated that the rapid and intermediate doubling time (rDT, iDT) isolates exhibited positive growth responses to BMP-2 compared with baseline (control) that ranged between 2.4% and 12.4%. Two DPSC isolates exhibited statistically significant responses, including dpsc-3924 (12.0%) and dpsc-3882 (12.4%), $p=0.011$. However, responses among the slow doubling time (sDT) isolates exhibited negative growth responses ranging between -1.3% to -7.9%.

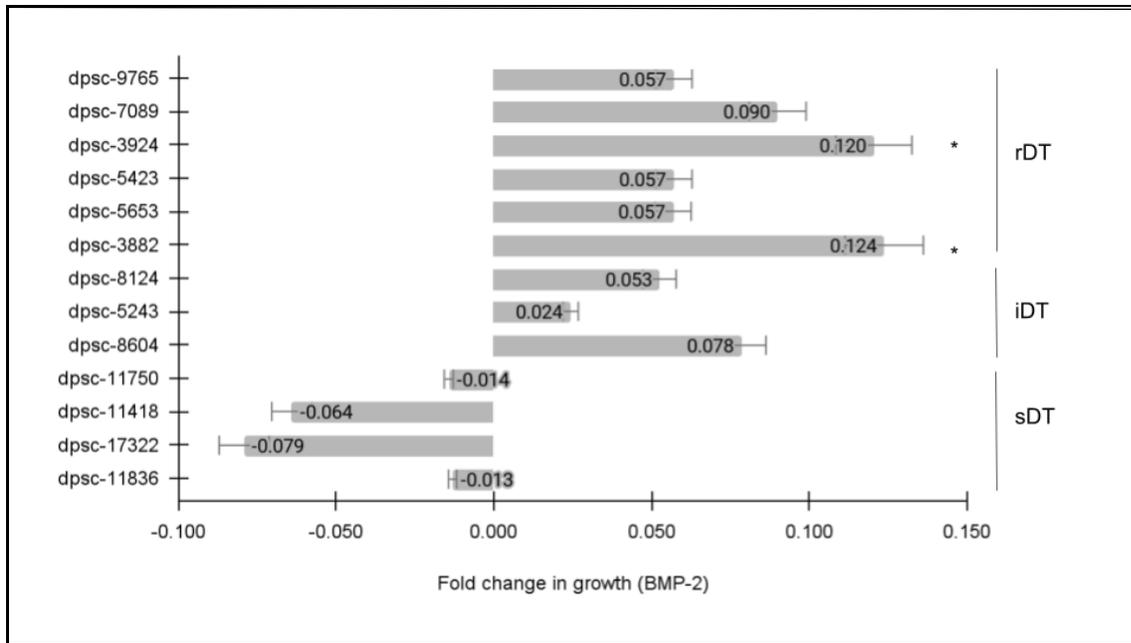


Figure 2. BMP-2 growth assay with DPSC isolates. Rapid and intermediate doubling time (rDT, iDT) isolates exhibited BMP-2 positive growth responses to BMP-2 between 2.4% and 12.4%, including dpsc-3924 (12.0%) and dpsc-3882 (12.4%), $p=0.011$. Slow doubling time (sDT) isolates exhibited negative growth responses between -1.3% to -7.9%.

To evaluate the effect of BMP-4, this growth factor was administered to the DPSC isolates in three-day proliferation assays (Figure 3). These results demonstrated that both rapid and intermediate doubling time (rDT, iDT) isolates exhibited positive growth responses to BMP-4, ranging from 4.5% to 25.8%. Two rDT DPSC isolates exhibited statistically significant increases to BMP-4, including dpsc-5653 (22.4%) and dpsc-3882(17.5%), $p=0.0067$. In addition, all three iDT DPSC isolates also exhibited strong positive growth responses to BMP-4, including dpsc-8124 (24.1%), dpsc-5243 (25.8%) and dpsc-8604 (12.9%), $p=0.011$. In contrast,

responses among the slow doubling time (sDT) isolates exhibited negative growth responses ranging between -1.1% to - 4.5%.

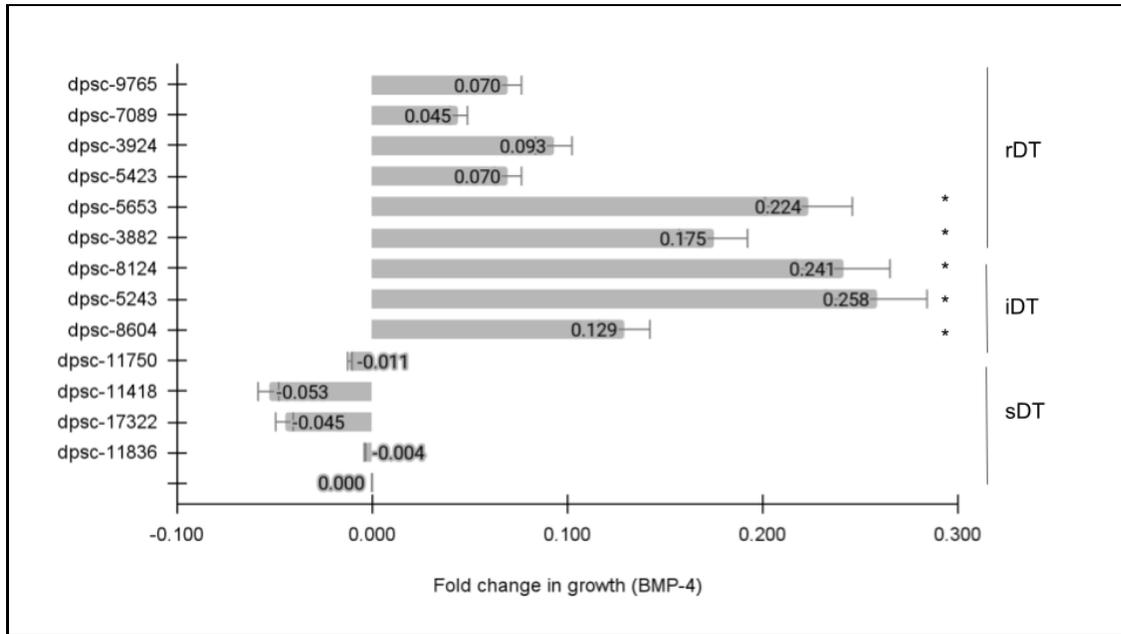


Figure 3. BMP-4 growth assay with DPSC isolates. Rapid and intermediate doubling time (rDT, iDT) isolates exhibited positive growth responses to BMP-4 between 4.5% to 25.8%. Significant responses were observed with two rDT isolates dpsc-5653 (22.4%) and dpsc-3882(17.5%), $p=0.0067$ and all three iDT DPSC isolates dpsc-8124 (24.1%), dpsc-5243 (25.8%) and dpsc-8604 (12.9%). Slow doubling time (sDT) isolates exhibited negative growth responses between -1.1% to - 4.5%.

To evaluate the effect of BMP-2 and BMP-4 in combination, growth assays were completed with each of the DPSC isolates over three days (Figure 4). These results of this experiment demonstrated that only three of the rapid doubling time (rDT) isolates exhibited positive growth responses to the combination of BMP-2 and BMP-4, including dpSC-9765 (19.2%), dpSC-7089 (12.0%) and dpSC-5423 (19.2%), $p=0.017$. Although none of the iDT isolates exhibited significant changes in growth, two of the slow (sDT) isolates exhibited statistically significant increases to the coadministration of BMP-2 BMP-4, including dpSC-11750 (19.3%) and dpSC-11418 (18.1%), $p=0.036$.

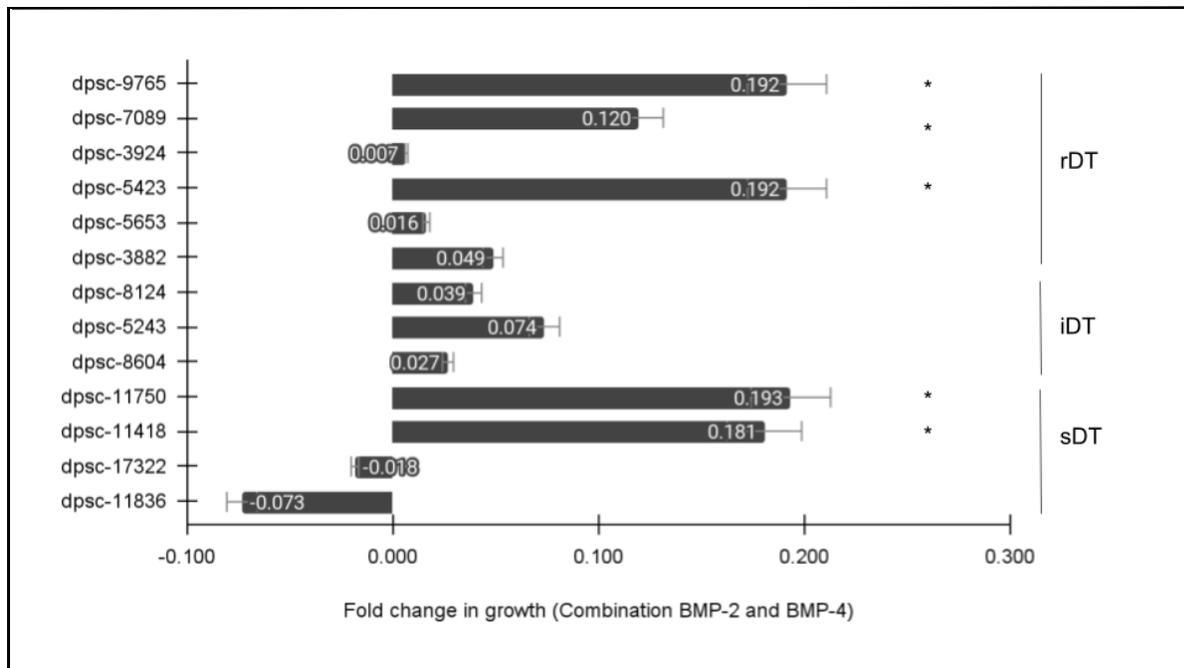


Figure 4. BMP-2 and BMP-4 combination growth assays with DPSCs. Three rapid doubling time (rDT) isolates exhibited positive growth, including dpsec-9765 (19.2%), dpsec-7089 (12.0%) and dpsec-5423 (19.2%), $p=0.017$ along with two of the slow (sDT) isolates, dpsec-11750 (19.3%) and dpsec-11418 (18.1%), $p=0.036$.

To evaluate the effect of BMP administration on other DPSC phenotypes, viability under each experimental condition was examined (Figure 5). These data demonstrated that no significant changes in viability were observed with any DPSC isolate under BMP-2 administration. In addition no significant changes were observed with DPSC isolate viability under BMP-4 administration. However, three rDT DPSC isolates exhibited significant increases in viability under combination (BMP-2, BMP-4) experimental treatment conditions, including dpsec-9765 (19.8%), dpsec-7089 (13.4%), and dpsec-5423 (11.3%), $p=0.48$. In addition, two of the sDT DPSC

isolates also exhibited increased viability under combination BMP treatment, including dpSC-11750 (19.8%) and dpSC-11418 (23.2%), $p=0.025$.

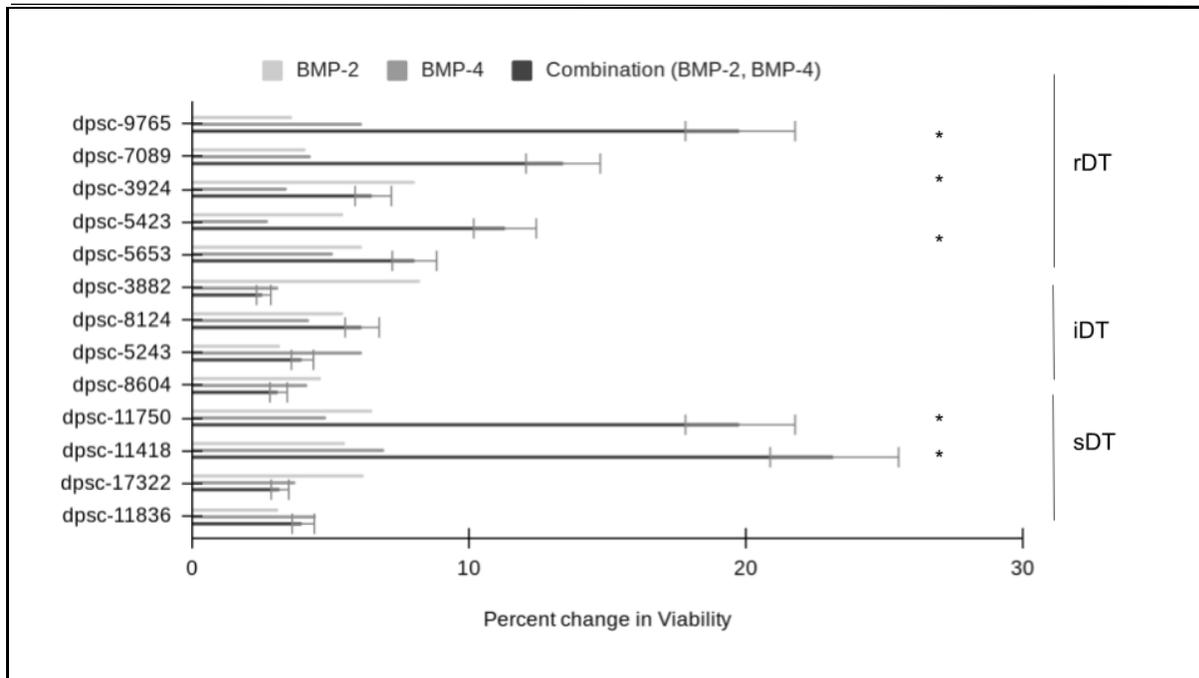


Figure 5. Evaluation of DPSC isolate viability under BMP administration. Three rapid doubling time (rDT) isolates exhibited increased viability under combination treatment, including dpSC-9765 (19.8%), dpSC-7089 (13.4%) and dpSC-5423 (11.3), $p=0.048$ along with two of the slow (sDT) isolates, dpSC-11750 (19.8%) and dpSC-11418 (23.2%), $p=0.025$.

To evaluate the intracellular mechanisms responsible for these observations, RNA was isolated and converted to cDNA for screening and analysis (Table 1). The data demonstrated that the concentration of RNA isolated from the rDT DPSC isolates (506 ng/uL) was not significantly different from that of the iDT (521 ng/uL) or sDT DPSC isolates (500 ng/uL), $p=0.867$. In addition, the RNA quality as measured by the absorbance ratio at A260 and A280 nm was similar among the rDT (1.79), iDT (1.78) and sDT (1.82) DPSC isolates, $p=0.962$.

The RNA was subsequently converted into cDNA to allow for qPCR screening and analysis. These data demonstrated that the average concentrations of cDNA from rDT (1586 ng/uL), iDT (1574 ng/uL) and sDT (1523 ng/uL) DPSC isolates was also not significantly different, $p=0.299$. In addition, the average purity of cDNA measured by the A260:A280 ratio was also very similar between the rDT (1.83), iDT (1.84) and sDT (1.87) DPSC isolates, $p=0.354$.

Table 1. RNA and cDNA analysis of DPSC isolates.

| DPSC isolates | RNA concentration [ng/uL] | RNA quality A260:A80 ratio | cDNA concentration [ng/uL] | cDNA purity A260:A280 ratio |
|----------------------|--|-----------------------------------|---|------------------------------------|
| rDT isolates | 506 +/- 39.3 ng/uL Range: 477 - 547 | 1.79 Range:1.74 - 1.89 | 1586 +/- 115 ng/uL Range: 1558 - 1626 ng/uL | 1.83 Range: 1.79-1.91 |
| iDT isolates | 521 +/- 32 ng/uL Range: 462 - 549 | 1.78 Range:1.76 - 1.83 | 1574 +/- 109 ng/uL Range:1455 - 1621 ng/uL | 1.84 Range: 1.86-1.92 |
| sDT isolates | 500 +/- 40.5 ng/uL Range: 458 - 522 | 1.82 Range:1.77 - 1.94 | 1523 +/- 80 ng/uL Range: 1451 -1621 ng/uL | 1.87 Range: 1.81-1.93 |

Screening the cDNA from each of the DPSC isolates revealed expression of the positive controls for the positive controls from the metabolic pathway (Glyceraldehyde 3-phosphate dehydrogenase or GAPDH) and the cytoskeletal structural control (beta actin) among all the DPSC isolates (Figure 6). In addition, these data also confirmed the presence and expression of International Society for Cellular Therapy (ISCT) positive control stem cell biomarkers CD73,

CD90 and CD105. Finally, no expression of the ISCT negative control marker CD45 was found among any of the DPSC isolates.

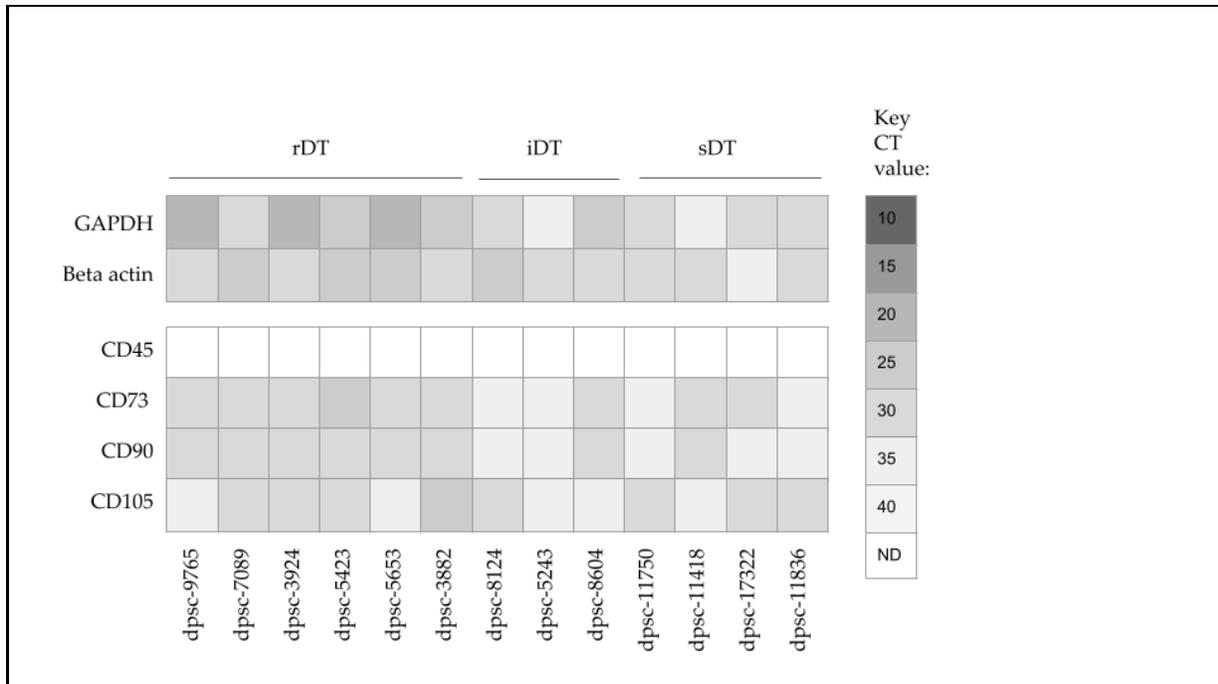


Figure 6. qPCR screening of the DPSC isolates. Expression of the positive controls (Glyceraldehyde 3-phosphate dehydrogenase or GAPDH) and beta actin were among all DPSC isolates. Expression of International Society for Cellular Therapy (ISCT) positive control stem cell biomarkers CD73, CD90 and CD105 was observed among all DPSC isolates, with no observed expression of the ISCT negative control marker CD45.

To evaluate the effects of BMP administration on DPSC isolates, qPCR screening for MSC and bone biomarkers was performed (Figure 7). These data demonstrated expression of MSC biomarkers Oct-4, Sox-2 and NANOG among all DPSC isolates evaluated. However, expression of these MSC biomarkers was relatively high among the rDT DPSC isolates. In addition, Sox-2 was highly expressed among both rDT and iDT DPSC isolates, while Oct-4 was highly expressed among the rDT and two of the sDT DPSC isolates (dpSC-11750 and dpSC-11418).

Evaluation of the bone differentiation biomarker alkaline phosphatase (ALP) and tooth differentiation biomarker dentin sialophosphoprotein (DSPP) revealed that BMP-2 induced expression of ALP among three of the rDT DPSC isolates (dpSC-7089, dpSC-3924, and dpSC-3882), which corresponded with their proliferative responses to BMP-2. Furthermore, administration of BMP-4 induced expression of ALP among two of the rDT DPSC isolates (dpSC-5653, dpSC-3882) and all three iDT DPSC isolates (dpSC-8124, dpSC-5243, dpSC-8604) but none of the other rDT or sDT DPSC isolates. Finally, the combination of BMP-2 and BMP-4 administration induced ALP expression among all of the rDT DPSC isolates, as well as two of the sDT DPSC isolates (dpSC-11750 and dpSC-11418) but not among any of the iDT or other sDT DPSC isolates. Expression of DSPP was observed only within the one rDT DPSC isolate, dpSC-5423.

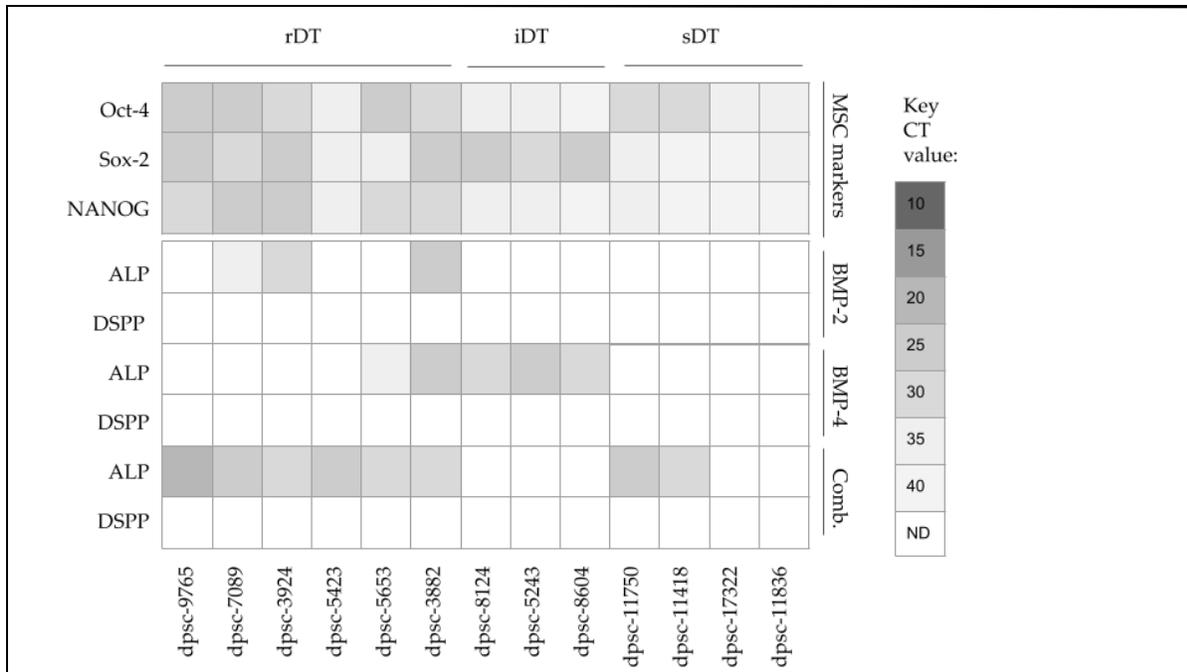


Figure 7. qPCR screening for MSC and differentiation biomarkers. Expression of MSC biomarkers Oct-4, Sox-2 and NANOG was observed among all DPSC isolates. Bone differentiation biomarker alkaline phosphatase (ALP) was induced by BMP-2 among three rDT DPSC isolates (dp1-7089, dp2-3924, and dp3-3882). BMP-4 induced expression of ALP among two of the rDT DPSC isolates (dp4-5653, dp3-3882) and all three iDT DPSC isolates (dp5-8124, dp6-5243, dp7-8604). Combination BMP-2 and BMP-4 administration induced ALP expression among all of the rDT DPSC isolates and two sDT DPSC isolates (dp8-11750 and dp9-11418). Expression of DSPP was observed only with the rDT DPSC isolate, dp1-5423.

Discussion

The goal of this study was to evaluate the potential for BMP administration to induce phenotypic changes among an existing repository of DPSC isolates. These data clearly demonstrated that the administration of BMP-2 and BMP-4 alone and in combination was sufficient to induce changes to some, but not all, DPSC isolates evaluated. These differential responses in both proliferation and viability were associated with changes in DPSC expression of the early bone differentiation biomarker alkaline phosphatase or ALP.

This study is among a growing number of studies that have demonstrated that BMP-2 administration may be sufficient to induce some phenotype changes among DPSCs, including ALP expression, that may suggest the potential for this growth factor to induce some aspects of bone and osteogenic differentiation [41,42]. As previously noted, some initial studies have suggested that DPSC may respond positively to BMP administration in vitro, although these were mainly restricted to evaluation of the effects of BMP-2 [27-31, 43]. In fact, only one previous study to date has evaluated the effects of both BMP-2 and BMP-4 on DPSC, which suggest that these findings require additional research in this area to confirm these observations and the potential use of BMP-4 to induce osteogenic responses among DPSC isolates [44].

This may be among the first studies to evaluate not only the phenotypic characteristics of DPSC and their responsiveness to BMP administration, but also to analyze the expression of MSC biomarkers and the association with DPSC responsiveness. Although previous work from this group demonstrated that DPSC responsiveness to BMP-2 was associated with reduced expression of Sox-2, that study analyzed only six DPSC isolates [31]. The current study greatly expands the range of DPSCs evaluated, including many rDT, iDT and sDT DPSC isolates. In

fact, these current results demonstrated that expression of Sox-2 appears to be associated with DPSC responsiveness to BMP-4 administration among the rDT and iDT DPSC isolates, while Oct-4 expression appears to correlate with BMP combination treatment among only the sDT DPSC isolates.

These results correspond with other studies that suggest some growth factors, such as bFGF and EGF may be sufficient to induce early stages of directed development including neural differentiation [45]. In addition, recent studies have also suggested that other forms of mechanical induction, including the use of extracellular matrix (ECM) may also be used to induce DPSC differentiation responses in vitro [46]. However, these data may represent the most comprehensive evaluation of DPSC responsiveness to BMP administration to date.

Analysis of these results should also come with awareness of the limitations posed by this specific study design. For example, this study included only DPSC available from an existing biorepository, which includes the possibility that long-term cryopreservation may have influenced the biomarkers expressed and responsiveness observed among these DPSCs [47,48]. In addition, this study was restricted to DPSCs within this biorepository, which suggests that additional studies may be needed to confirm these observations among other DPSCs from other repositories. Finally, at least one other study has suggested that growth factors may be needed in combination with ECM-directed stimulation to induce appropriate differentiation responses among DPSC - which may be the focus of future research in this area [49].

Conclusions

These results suggest that BMP-2 and BMP-4 (both alone and in combination) are sufficient to induce the production of the early bone biomarkers ALP within specific subsets of the DPSC isolates evaluated. Although these results represent a significant step towards our understanding of DPSC biology, further research will be needed to determine the additional factors and biomarkers that may facilitate osteogenic differentiation.

Consent

Not applicable. This study does not involve human subjects.

Ethical Approval

Not applicable. This study does not involve human subjects.

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Author Contributions

KK was responsible for the overall project design. DL, HL, and DF were responsible for data generation. KK was responsible for analysis and the writing of this manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest to report.

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Chapter 3

Summary and Conclusions:

The goal of this study was to evaluate the potential for BMP administration to induce phenotypic changes among an existing repository of DPSC isolates. These data clearly demonstrated that the administration of BMP-2 and BMP-4 alone and in combination was sufficient to induce changes to some, but not all, DPSC isolates evaluated. These differential responses in both proliferation and viability were associated with changes in DPSC expression of the early bone differentiation biomarker alkaline phosphatase or ALP.

This study is among a growing number of studies that have demonstrated that BMP-2 administration may be sufficient to induce some phenotype changes among DPSCs, including ALP expression, that may suggest the potential for this growth factor to induce some aspects of bone and osteogenic differentiation [41,42]. As previously noted, some initial studies have suggested that DPSC may respond positively to BMP administration in vitro, although these were mainly restricted to evaluation of the effects of BMP-2 [27-31, 43]. In fact, only one previous study to date has evaluated the effects of both BMP-2 and BMP-4 on DPSC, which suggest that these findings require additional research in this area to confirm these observations and the potential use of BMP-4 to induce osteogenic responses among DPSC isolates [44].

This may be among the first studies to evaluate not only the phenotypic characteristics of DPSC and their responsiveness to BMP administration, but also to analyze the expression of MSC biomarkers and the association with DPSC responsiveness. Although previous work from this group demonstrated that DPSC responsiveness to BMP-2 was associated with reduced expression of Sox-2, that study analyzed only six DPSC isolates [31]. The current study greatly

expands the range of DPSCs evaluated, including many rDT, iDT and sDT DPSC isolates. In fact, these current results demonstrated that expression of Sox-2 appears to be associated with DPSC responsiveness to BMP-4 administration among the rDT and iDT DPSC isolates, while Oct-4 expression appears to correlate with BMP combination treatment among only the sDT DPSC isolates.

These results correspond with other studies that suggest some growth factors, such as bFGF and EGF may be sufficient to induce early stages of directed development including neural differentiation [45], In addition, recent studies have also suggested that other forms of mechanical induction, including the use of extracellular matrix (ECM) may also be used to induce DPSC differentiation responses in vitro [46]. However, these data may represent the most comprehensive evaluation of DPSC responsiveness to BMP administration to date.

Based upon the lack of in-depth information regarding this type of bone biology, the first goal of this project will be to evaluate whether the sequential administration of BMP-2/BMP-4 is sufficient to induce chondrogenesis or osteogenesis among varying cell lines.

- A. Null hypothesis: BMP-2/BMP-4 administration is not sufficient to induce these effects among all cell lines. REJECT
- B. Alternative hypothesis: BMP-2/BMP-4 administration is sufficient to induce these effects among some cell lines but not others. ACCEPT

Limitations and Recommendations:

Analysis of these results should also come with awareness of the limitations posed by this specific study design. For example, this study included only DPSC available from an existing biorepository, which includes the possibility that long-term cryopreservation may have influenced the biomarkers expressed and responsiveness observed among these DPSCs [47,48]. In addition, this study was restricted to DPSCs within this biorepository, which suggests that additional studies may be needed to confirm these observations among other DPSCs from other repositories. Finally, at least one other study has suggested that growth factors may be needed in combination with ECM-directed stimulation to induce appropriate differentiation responses among DPSC - which may be the focus of future research in this area [49].

Appendix A

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