Role of p38 MAPK in Odontogenic Differentiation of Dental Pulp Stem Cells in Hypoxia

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LIST OF ABBREVIATIONS

AAE	American Association of Endodontists
α-MEM	Alpha Modification of Eagle's Medium
cDNA	Complimentary DNA
CEJ	Cementoenamel Junction
DAPI	4′,6-diamidino-2-phenylindole
DMP1	Dentin Matrix Protein 1
DNA	Deoxyribonucleic Acid
DPSC	Dental Pulp Stem Cell
dsDNase	Double-strand Specific DNase
DSPP	Dentin Sialophosphoprotein
FBS	Fetal Bovine Serum
IgG	Immunoglobulin G
MAPK	Mitogen-Activated Protein Kinase
MAP2K	Mitogen-Activated Protein Kinase-Kinase
mRNA	Messenger Ribonucleic Acid
PBS	Phosphate-Buffered Saline
RUNX2	Runt-Related Transcription Factor 2
SD	Standard Deviation

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SUMMARY

The multipotent nature of dental pulp stem cells (DPSCs) promises regenerative endodontic potential. A specific microenvironment controls the differentiation capability of DPSCs. Oxygen is known as a crucial regulator in the microenvironment. Certain dentin matrix proteins may help to promote the p38 mitogen activated protein kinase (MAPK) pathway. Understanding this molecular signaling mechanism could aid in obtaining predictable clinical outcomes. The aim of this study is to examine the role of p38 MAPK under oxygen-deprived conditions to reveal its effect on odontogenic DPSC differentiation.

Human DPSCs were isolated from healthy molars and cultured in basic and odontogenic media. The experimental group was treated with SB203580, a p38 inhibitor, under normoxic and hypoxic conditions. Immunocytochemistry and quantitative PCR analysis for various differentiation markers were performed.

Our PCR data demonstrate that p38 MAPK inhibition in normoxic conditions results in a significant upregulation of odontogenic genes such as DMP-1, DSPP, RUNX2, and OSX. Under hypoxia, this effect was reversed. These results were also supported by DSPP immunocytochemistry. When p38 MAPK was inhibited, the DSPP expression under hypoxia was significantly weaker as compared to normoxia.

Our results indicate that p38 MAPK constitutes a positive regulator of the odontogenic DPSC differentiation under hypoxia, demonstrating its essential role in designing for successful pulp-dentin complex regeneration engineering strategies.

1 INTRODUCTION

1.1 Background

In recent years within the regeneration field of dentistry, much attention has been given to stem cell biology as stem cells demonstrate remarkable translational capacity of regenerating dentin and damaged tooth structure (Cordeiro et al., 2008; Iohara et al., 2011). Dental pulp stem cells (DPSCs) can be acquired with relative ease compared to other types of stem cells such as embryonic stem cells and induced pluripotent stem cells. Several studies, including the present study, have shown that human DPSCs can successfully differentiate into odontoblast or odontoblast-like cells under specific culture conditions (Boyle et al., 2014; Jones et al., 2015). These differentiated cells were able to produce dentin.

Changes in the microenvironment have been shown to affect the phenotype of stem cells. The extracellular environment such as the extracellular matrix, secreted factors, and direct cell-to-cell contact have both positive and negative roles in stem cell differentiation and cell fate determination (Ra'em and Cohen, 2012; Gattazzo et al., 2014). Oxygen is one of the vital factors that has a variety of functions in stem cell proliferation and differentiation as well as in the production of growth factors that promote angiogenesis (Lennon et al., 2001; Koay and Athanasiou, 2008; Prasad et al., 2009; Abdollahi et al., 2011). The natural oxygen level in the body has significantly lower oxygen levels compared to lab *in vitro* oxygen levels. In the lab, 20% oxygen is routinely used, compared to 3% within tissues of the body (Csete, 2005). Thus, a careful consideration of microenvironmental oxygen levels for stem cell engineering strategies

needs to be addressed.

Mitogen-activated protein kinases (MAPKs) are an evolutionarily conserved family of enzymes. They are serine/threonine kinases that connect cell-surface receptors to critical regulatory targets within the cell and are essential mediators of various physiological functions in normal as well as disease conditions (Cuenda and Rousseau, 2007). Broadly, the p38 MAPK cascade, which is one of three major MAPK pathways, has been implicated in cellular differentiation, cell migration, inflammation, and even cancer. More specifically, it has been implicated in tooth development and enamel production (Greenblatt et al., 2015) as well as in periodontal tissue remodeling during orthodontic tooth movement (Jiang and Tang, 2018). p38 MAPK also appears to be involved in dentin production regulation. Lastly, dentin matrix proteins promoted bone marrow stromal cell differentiation through p38 MAPK signaling (Yu et al., 2014).

At the molecular level, growth factors, inflammatory cytokines, and many environmental stresses can activate p38 MAPK. Once activated, p38 MAPK affects downstream targets including other protein kinases, cytosolic substrates, transcription factors, and chromatin remodelers (Cuadrado et al., 2010). More specifically, most stimuli trigger the canonical pathway. This pathway involves the phosphorylation of p38 MAPK at a threonine and tyrosine residue on the activation loop by MAPK-kinase (MAP2K). Alternate pathways of p38 MAPK activation also exist (Cuadrado et al., 2010).

1.2 Specific Aims

The role of p38 MAPK in the differentiation of DPSCs under hypoxia is unknown. Our aim is to validate the role of p38 MAPK in p38 MAPK-mediated odontogenic DPSC differentiation, especially in the context of oxygen-deprived conditions. The results from the present study will contribute to the pulp-dentin complex regeneration engineering strategies for dentin repair in a damaged pulp.

1.3 Hypothesis

H_0: p38 MAPK inhibition has no effect on odontogenic DPSC differentiation.

H_1: p38 MAPK inhibition will effect odontogenic DPSC differentiation as measured by odontogenic lineage gene markers and proteins.

2 REVIEW OF THE LITERATURE

2.1 Regenerative Endodontic Therapy

Traditionally, immature necrotic teeth with apical periodontitis have been treated with apexification procedures using either long-term calcium hydroxide treatment or the MTA barrier method. A major disadvantage of apexification is that there is no potential for further development of the immature root end in terms of dentin thickness as well as root length. Additionally, long-term treatment with calcium hydroxide may predispose the already fragile immature root to fracture (Andreasen et al., 2002).

In 2001, Iwaya et. al were the first group to study revascularization of immature necrotic teeth with sinus tract. Their study found that this procedure was able to successfully treat clinical signs and symptoms and apical periodontitis, as well as encourage further dentin thickening and root end closure. Then in 2004, Banchs and Trope proposed a clinical protocol that is similar to the current AAE guidelines for revascularization based on observations of revascularization of reimplanted teeth, disinfection of the necrotic canal space, and induction of a blood clot into the canal. Their study also found successful results. Today, regenerative endodontic therapy is recommended as an alternative treatment to the traditional apexification procedures.

2.2 The Microenvironment

The triad of tissue engineering includes stem cells, a physical scaffold, and signaling molecules (Hargreaves et. al, 2008). But even with an appropriate source of stem cells, the differentiation lineage may not be appropriate due to an altered microenvironment. Once a canal becomes infected, the microenvironment is changed; and once the canal is disinfected with antimicrobials, the microenvironment is also changed. One example of this is that in the presence of LPS, stem cells from the apical papilla introduced into root canal segments were found to shift towards osteogenic phenotype as opposed to the odontogenic phenotype (Vishwanat et al., 2017). Many other authors have found that if the microenvironment is altered, stem cell fate will also be altered (Wagers, 2012; Scadden 2006). Thus, careful control of the microenvironment is vital to the success of regeneration strategies. The present study focuses the molecular signaling of stem cells to differentiate into the appropriate lineage.

2.3 p38 MAPK

As mentioned previously, the MAPKs are enzymes that regulate various physiologic functions when activated by affecting downstream targets including other enzymes, DNA transcription, and more. The MAPK pathway has been implicated in odontoblast differentiation during primary development as well as tertiary dentinogenesis. Odontoblast stimulation was associated with increased p38 MAPK activation during tertiary dentin formation via phosphorylation of p38 MAPK. Additionally, p38 MAPK nuclear translocation was increased, transporting it to where it serves as a transcription factor (Simon et. al, 2010). In contrast, the p38 MAPK pathway

has been found to maintain DPSC quiescence and that its inhibition stimulates DPSCs to exit the G_0 phase of the cell cycle for mitotic division to begin (Vandomme et al., 2014). Finally, the first *in vivo* study linking p38 MAPK to tooth morphogenesis found that in mice with p38 MAPK deletion, there was defective enamel secretion and lack of dental cusps (Greenblatt et al., 2015).

2.4 Oxygen and Stem Cells

It has been shown that oxygen levels can affect stem cell differentiation and behavior. Rat marrow-derived mesenchymal stem cells in low oxygen tension proliferated more in quantity and more rapidly. There were also more bone cells and elevated markers of osteogenesis (Lennon, 2001). Human embryonic stem cells in low oxygen tension had increased ability to produce type I and II collagen and had different CD markers present on their cell surfaces (Koay, 2008). Finally, hypoxia has been used to promote differentiation into various cell lines while also having an influence on the production of growth factors involved in angiogenesis (Abdollahi, 2011).

3 MATERIALS AND METHODS

3.1 Cell Cultures

DPSCs were isolated from healthy immature third molars by the explant outgrowth method as described by Karamzadeh et. al (informed patient consent and institutional review board approval of the protocol #20110129 used at the University of Illinois at Chicago). Teeth were collected and stored in basic media, which consisted of alpha modification of Eagle's medium (α-MEM), 10% FBS, penicillin, and streptomycin and transported to the laboratory at 4°C. The remainder of the preparation for stem cell isolation was completed under sterile conditions. Teeth were cleaned with Betadine then rinsed with phosphate-buffered saline (PBS) and held with sterile extraction forceps. Remaining tissue was cleaned off of the tooth surface with a scalpel. The tooth was split with a hammer and chisel at the CEJ to expose the pulp and the pulp tissue was removed from the tooth. The tissue was rinsed twice with PBS, minced into 1-2mm pieces, rinsed twice with PBS, and seeded into proliferation media, which consists of α-MEM, 10% FBS, L-ascorbic acid 2-phosphate, L-glutamine, penicillin, streptomycin, and amphotericin B.

Cells were then seeded into a 6 well plate at 5,000 cells per well and DPSCs were then cultured for 24 days and treated with a p38 MAPK antagonist, SB203580—first in basic media for 72 hours and subsequently for 21 days in odontogenic media, which consists of α-MEM, 10% FBS, L-ascorbic acid 2-phosphate, L-glutamine, penicillin, streptomycin, dexamethasone, β-glycerol phosphate, and monopotassium phosphate. The medium was changed every three days. This procedure was done under hypoxic conditions (3% oxygen) and repeated under normoxic conditions (20% oxygen) in a tri-

gas incubator. The DPSCs were cultured at 37°C and 5% CO_2. DPSCs between 2nd and 4th passages were used throughout the study.

3.2 Immunophenotyping

250,000 cells per tube were incubated with anti-human antibodies for 30 minutes. After incubation, PBS was added to the to each tube and centrifuged for five minutes. The pellet was re-suspended in PBS and CD markers including CD34, CD73, CD105, and CD29 were analyzed with flow cytometry analysis.

3.3 Mineralization Assay

Fourth passage DPSCs subject to their respective control or treatment were fixed with 10% paraformaldehyde for 15 minutes then washed three times with distilled water. 1mL of Alizarin red dye was added for 20 minutes then washed four times with deionized water with gentle rocking to remove any additional Alizarin red. 1mL of 10% acetic acid was added to prevent the cells from drying. The cells were then subject to visual inspection and imaging.

3.4 Quantitative PCR Analysis

Second passage DPSCs were cultured in 6 well plates at 5,000 cells per well concentration for both control and experimental groups. This was done in three separate well plates per group. Total mRNA was isolated with 1 ml TriZol (Invitrogen, Life Technologies) and analyzed using a Fisher Scientific NanoDrop 2000 for density and

260/280 readings used to standardize the samples to 10ng/μL with RNase-free water. Then the mRNA were converted to cDNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit with dsDNase using the manufacturer provided protocol in a BioRad T100 thermal cycler. The cDNA samples were then used for real time PCR and the expression of specific genes of interest were probed for by using Applied Biosystems Fast SYBR green reagent using 60 cycles based on the manufacturer provided protocol.

3.5 Immunocytochemistry

Cells were fixed, permeabilized, and saturated as previously described (Chmilewsky et al., 2016b). Then, cells were incubated for four hours with mouse anti-DMP1 (1:1000), rabbit anti-p38 MAPK (1:250) and mouse anti-DSPP (1:500) or their respective control isotypes. Finally, cells were incubated for 40 minutes with a mix of Alexa Fluor-594 anti-mouse IgG, Alexa Fluor-488 anti-rabbit IgG (2 μg/mL) and/or DAPI (1 μg/mL). The coverslips were sealed and photographs taken using a Leica DMI6000 B microscope. The integrated density of each condition was determined and statistically analyzed by using ImageJ 1.49v software.

3.6 Statistical Analysis

All experiments were repeated at least three times. Statistical significance was determined using the Student's *t* test to compare the different treatments and their respective controls. Data were expressed as means ±SD and considered significant for $P < 0.05$.

4 RESULTS

4.1 Confirmation of Odontogenic Differentiation

To demonstrate the *in vitro* role of p38 MAPK in DPSC odontogenic differentiation both in normal and oxygen-deprived conditions, isolated DPSCs were differentiated into odontoblast-like cells and treated with a p38 inhibitor using our modified differentiation protocol. The human DPSCs were acquired from molars and cultured using basic media and odontogenic media for 24 days. A general p38 MAPK inhibitor, SB203580, was used to treat the cells from day 1 to day 24 (Fig. 1). The homogeneous purity of DPSC population was checked and validated as in our previous reports (Boyle et al., 2014; Jones et al., 2015).

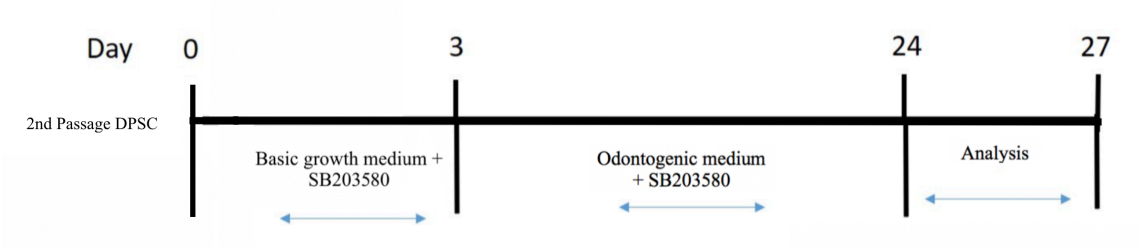

Figure 1

After full differentiation, the identity of the cells was analyzed with real-time PCR and immunocytochemistry by probing for several odontoblast lineage-specific markers. The differentiated cells demonstrated odontoblast-like characteristics as they expressed odontogenic cell markers such as dentin matrix protein 1 (DMP1) (Fig. 4), runt-related transcription factor 2 (RUNX2) (Fig. 4), and dentin sialophosphoprotein (DSPP) mRNA (Fig. 5) as well as protein (Fig. 3, Fig. 6). These are well-known odontoblast lineage-specific genes (Almushayt et al., 2006).

4.2 Alizarin Red Staining

Alizarin mineralization staining also confirmed a calcification (Fig. 2). Thus, our comprehensive odontogenic marker analysis demonstrate successful odontogenic differentiation of the DPSCs, both in normal and oxygen-deprived conditions.

Figure 2

4.3 Confirmation of p38 MAPK Expression

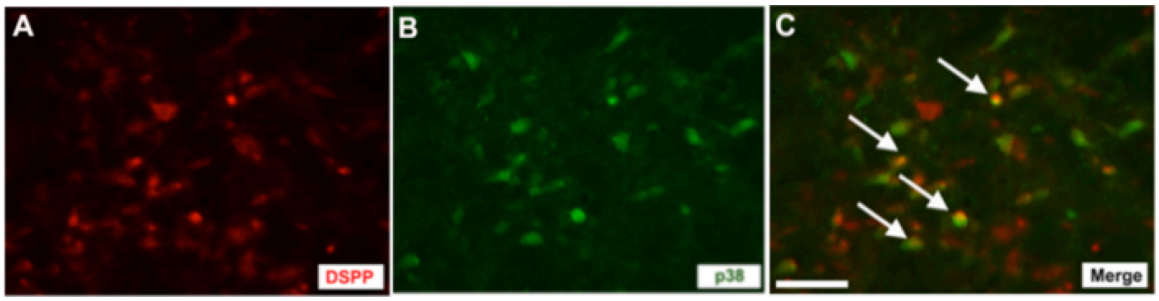

Figure 3

We next identified the DSPP and p38 MAPK expression in DPSCs-derived differentiated cells. Double fluorescence immunostaining with anti-DSPP and anti-p38 MAPK antibodies demonstrate that the differentiated cells expressed both DSPP and p38 MAPK, confirming a successful odontogenic differentiation as well as the presence of p38 MAPK expression (Fig. 3). In the merged image, the co-localization of both DSPP and p38 MAPK can be seen.

4.4 Quantitative PCR Analysis

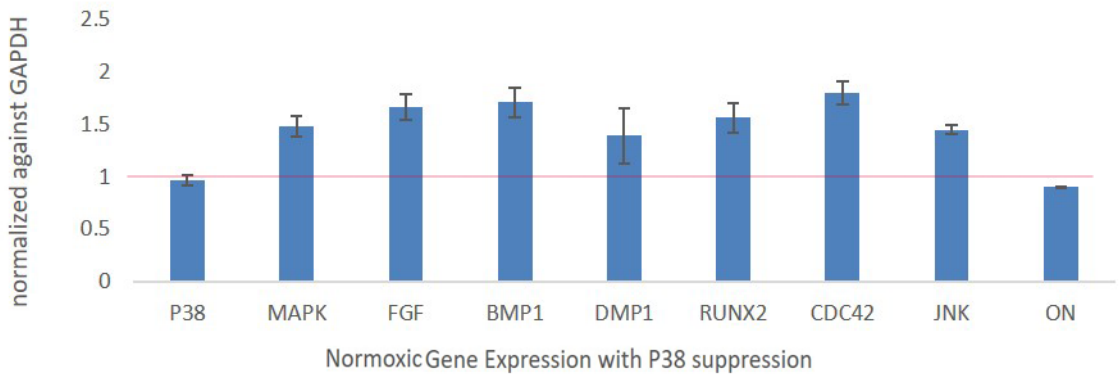

Figure 4

PCR gene expression analysis of p38 MAPK, all MAPK, fibroblast growth factor (FGF), bone morphogenetic protein 1 (BMP1), DMP1, RUNX2, cell division control protein 42 homolog (CDC42), c-Jun N-terminal kinase (JNK), and osteonectin (ON) in the normoxic SB203580 treatment group compared to control (represented as a value '1' of each respective gene) is shown in Figure 4. The data demonstrate a significant upregulation of odontoblast lineage genes such as DSPP, RUNX2 and DMP1 in the SB203580 treatment group compared to control (Fig. 4). The data is normalized against

GAPDH, a housekeeping gene involved in glycolysis. Thus, p38 MAPK inhibition results in a significant upregulation of odontogenic differentiation gene expression.

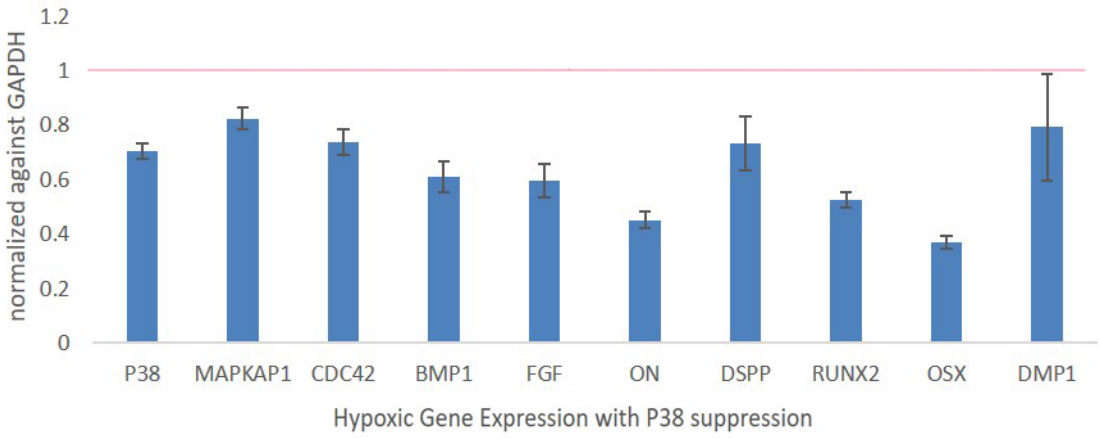

Figure 5

Under hypoxia and with p38 MAPK inhibition, the expression of odontoblast lineage genes, such as DSPP, RUNX2, and DMP1 is significantly decreased compared to control (without p38 MAPK inhibition) and normoxia groups (with p38 MAPK inhibition) (Fig. 5). Thus, p38 MAPK inhibition under hypoxia results in a significant reduction in odontogenic differentiation gene expression.

4.5 Immunocytochemistry

To further support the gene expression data, we next performed the DSPP protein immunostaining with DPSCs-differentiated cells. In normal cells, without hypoxia induction and SB203580 treatment, the DSPP expression is evenly present in the cytoplasm of the differentiated cells (Fig. 6A, D). However, the DSPP expression is

significantly upregulated in the normoxic SB203580 treatment group (Fig. 6B, E) suggesting p38 MAPK`s negative role in odontogenic DPSC differentiation. Unexpectedly, this effect was completely reversed in the oxygen-deprived condition where DSPP expression is significantly downregulated in the differentiated cells under hypoxia (Fig. 6C, F).

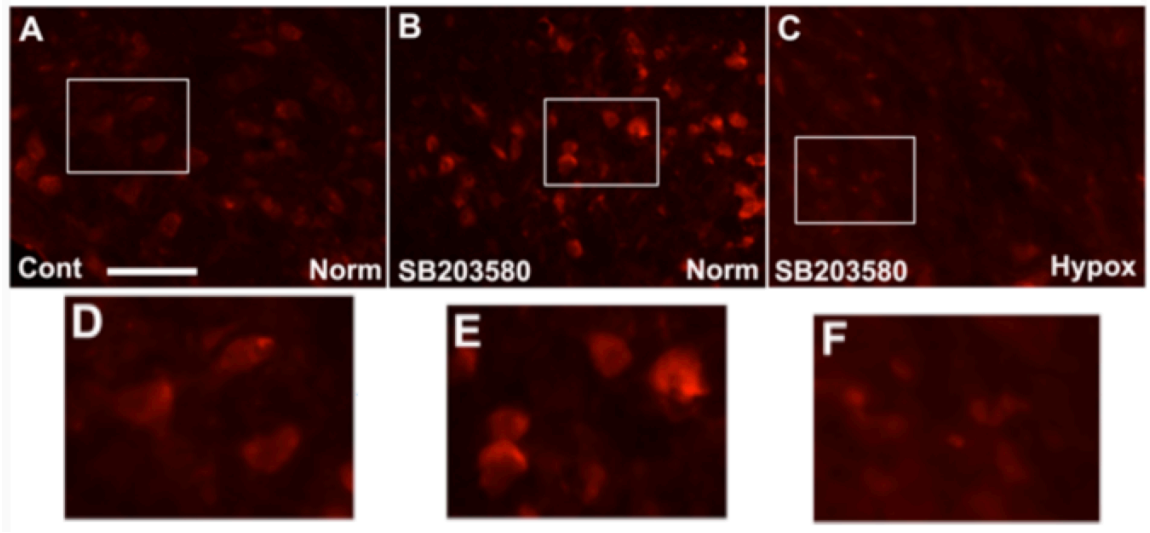

Figure 6

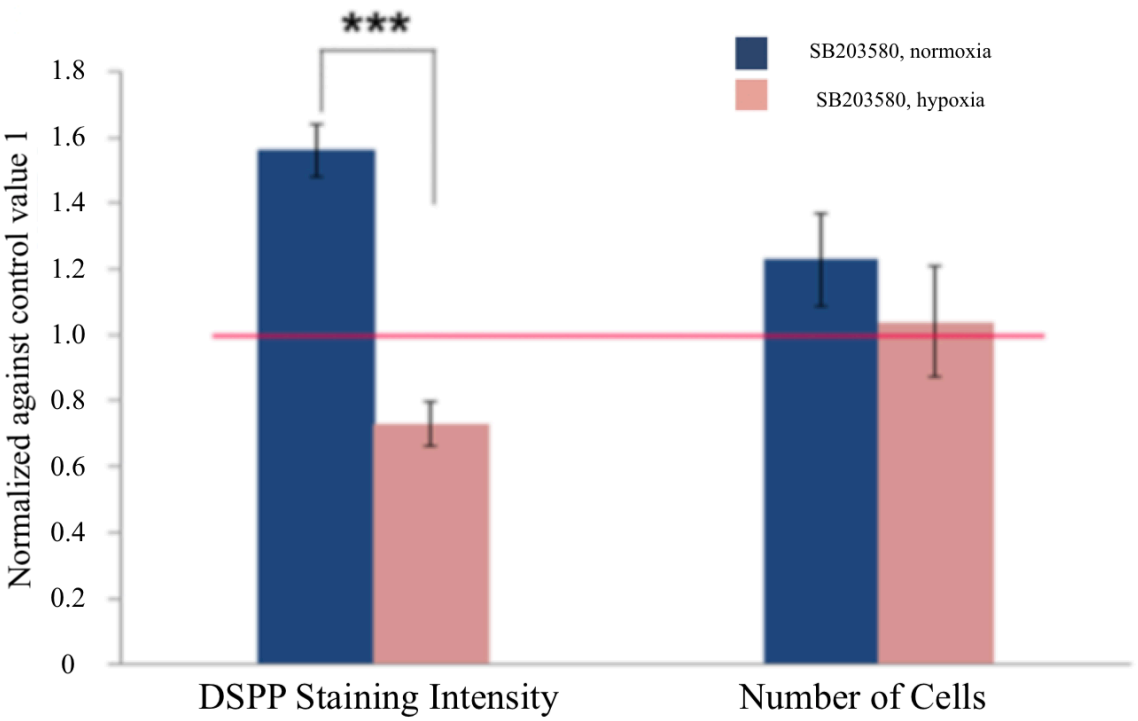

Figure 7

The DSPP expression staining intensity was quantified using ImageJ software. As shown in Fig. 7, the SB203580 treatment under hypoxia resulted in a significant downregulation ($p<0.005$) in DSPP expression of the differentiated cells (pink: 0.73 ± 0.06, N=5) compared to the SB203580 treatment under normoxia group (blue: 1.56 ± 0.08, N=4). Taken together, our data demonstrates that p38 MAPK has a negative role on odontogenic DPSC differentiation and this effect was reversed in an oxygen-deprived condition.

4.6 Proliferation Capacity

In order to rule out that the difference in odontogenic gene expression is due to increased or decreased cell number during DPSC differentiation, we next examined

proliferation capacity of the differentiated cells. The number of DSPP-positive cells was counted and analyzed in a fixed area of 1 X 1 mm^2 in the captured images. The number of the differentiated cells did not show a significant difference between the normoxic SB203580 treatment (1.23 ± 0.14, N=4) and hypoxic SB203580 treatment (1.04 ± 0.17, N=4, $p>0.05$) groups. Our analysis demonstrates that hypoxia induction has no significant effect on odontogenic proliferation of DPSCs.

5 DISCUSSION

5.1 Review

In this study, we first successfully differentiated our isolated DPSCs into odontoblast-like cells. Our results demonstrate that p38 MAPK constitutes a negative regulator of odontogenic DPSC differentiation as the p38 MAPK inhibition induces a significant upregulation of odontogenic lineage gene expression such as DMP1, RUNX2 and DSPP. Inhibition under hypoxia induction showed opposite results. Our results confirm that the physical changes in microenvironment, such as hypoxia, can play an important role in altering the differentiation properties of DPSCs.

5.2 The Role of p38 MAPK in Mesenchymal Stem Cell Differentiation

The role of p38 MAPK in several different types of stem cell differentiation and proliferation has been studied. However, the results in some of these studies regarding osteogenic and chondrogenic differentiation from mesenchymal stem cells (MSCs) have been controversial. Both positive and negative roles of p38 MAPK in bone tissue differentiation have been reported (Stanton and Beier, 2006; Bobick et al., 2010; Li et al., 2010; Kim and Im, 2010). It appears that the role of p38 MAPK in stem cell differentiation is complicated and many factors including the cell culture condition, timing, and dose of inhibitor treatment seem to play vital roles in MSC differentiation.

5.3 The Role of Oxygen in Stem Cell Differentiation

There have been a number of studies suggesting that oxygen plays a role in maintaining an undifferentiated status of stem cells as well as in stem cell differentiation

and proliferation. One study found that embryonic stem cells (ESCs) could maintain a longer period of undifferentiated status under low oxygen (Prasad et al., 2009). Another study found that human ESCs and mesenchymal stem cells derived cells showed more bone and cartilage differentiation in an oxygen-deprived condition (Lennon et al., 2001, Koay and Athanasiou, 2008). Surprisingly, our study demonstrates that the hypoxia induction oppositely regulates differentiation gene expression compared to the normoxic condition. This observation is consistent with our pilot study, which found that the immune complement fragment C5a plays a positive role in odontogenic differentiation of DPSCs in a normoxic environment. However, this effect was reversed in an oxygen-deprived condition. Treatment with a C5a receptor blocker induced a significant downregulation of odontogenic differentiation genes such as DMP1, ON, RUNX2, DSPP in normoxic condition, but under hypoxia, these genes' expression were significantly increased (Pasiewicz et al., unpublished observation). We do not understand the exact mechanism of this phenotype change. Further detailed molecular pathway analysis is needed.

5.4 The Role of p38 MAPK in DPSC Proliferation

Neither p38 MAPK inhibition nor hypoxia induction resulted in a meaningful change in DPSCs proliferation. It seems that several environmental factors including the differentiation stage, stem cell type, degree of hypoxia, and culture conditions are crucial factors in the differentiation phenotype determination.

6 CONCLUSION

In this study, we report that both the activity of p38 MAPK and oxygen levels are important regulators in the differentiation of DPSCs. Controlling the microenvironment in stem cell differentiation is a vital strategy for successful dentin regeneration. Our data provides an important basis for future DPSC-mediated pulp-dentin regeneration engineering.

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- Accepted into the Guaranteed Professional Program Admissions (GPPA) program for dentistry.

August 2004 – June 2008, Hinsdale Central High School

- Graduated in the top 5% of the class.

DENTAL EXPERIENCE

July 2015 – June 2016, General Practice Residency at Jesse Brown VA Hospital
- General Practice Resident at an ADA accredited one-year program.

June 2015 – Present, Associate at Hilary Cheng, D.D.S. Dental Office
- Practice as a general practitioner.

March 2012 – Present, Volunteer at Guaranteed Professional Program Admissions Pre-Clinic Night

June 2008 – Present, Volunteer at Tzu Chi Foundation Charity Health Fair

2004 – June 2015, Intern/Assistant at Hilary Cheng, D.D.S. Dental Office

May 2014 – May 2015, D4 Advanced Endodontic Track

May 2014 – May 2015, Honors Endodontics Seminar

February 2012 – May 2015, Volunteer at Illinois Center for Rehabilitation and Education (ICRE)

July 1-5, 2013, Externship at John H. Stroger, Jr. Hospital of Cook County Oral and Maxillofacial Surgery Department

July 2013, Volunteer at Bears Care

PROFESSIONAL ACTIVITIES

May 2015 – Present, American Association of Endodontists Member

August 2011 – Present, American Student Dental Association Member

- August 2013 – May 2014, Held Membership Chair position

July 2013 – Present, Appointed ambassador of Prince Philip Dental Hospital in Hong Kong concerning rotation program

November 2013 – Present, Delta Sigma Delta Rho Chapter Member

May 2014 – May 2015, Assistant Researcher in the lab of Dr. Satish Alapati

August 2011 – August 2012, Class Representative on Class Executive Board

AWARDS

- May 2015, Earned the American Association of Endodontists Student Achievement Award.
- April 2009, Earned the General Chemistry Award as nominated by my professor, Dr. Melita Balch, and as elected by the University of Illinois at Chicago Chemistry Department.
- November 2007, Earned the Sportsmanship Award as a part of the Varsity soccer team as elected by coaches and fellow teammates.