เบสิกไฟโบรบลาสโกรทแฟกเตอร์ควบคุมความสามารถในการแปรสภาพของเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันน้ำนมมนุษย์

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BASIC FIBROBLAST GROWTH FACTOR REGULATES THE DIFFERENTIATION ABILITY OF STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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นันทวรรณ เนาวโรจน์ : เบสิกไฟโบรบลาสโกรทแฟกเตอร์ควบคุมความสามารถในการแปรสภาพของเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันน้ำนมมนุษย์ (BASIC FIBROBLAST GROWTH FACTOR REGULATES THE DIFFERENTIATION ABILITY OF STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทพ. ดร. ธนภูมิ โอสถานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ทพ. ดร. ประสิทธิ์ ภวสันต์, 137 หน้า.

เบสิกไฟโบรบลาสโกรทแฟกเตอร์(บีเอฟจีเอฟ) มีบทบาทสำคัญในกระบวนการทางชีวภาพต่างๆของเซลล์ รวมถึงการเจริญเติบโต การมีชีวิตรอด การเคลื่อนย้าย และความสามารถในการแปรสภาพของเซลล์ต้นกำเนิด ในวิทยานิพนธ์นี้ที่มุ่งเน้นเฉพาะในเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันน้ำนมมนุษย์ จากการศึกษาพบว่า เซลล์ที่แยกจากเนื้อเยื่อในฟันน้ำนมมนุษย์มีลักษณะคล้ายเซลล์สร้างเส้นใย มีการแสดงออกของโปรตีนผิวเซลล์บ่งชี้ของเซลล์ต้นกำเนิดมีเซนไคม์ และมีความสามารถในการแปรสภาพไปเป็นเซลล์สร้างกระดูก เซลล์ไขมัน และเซลล์คล้ายเซลล์ประสาท ในส่วนแรกได้มีการทบทวนวรรณกรรมที่เกี่ยวข้องกับบทบาทของบีเอฟจีเอฟต่อเซลล์ต้นกำเนิดที่แยกได้จากเนื้อเยื่อของฟันน้ำนมมนุษย์ โดยหลักฐานแสดงให้เห็นว่า บทบาทของบีเอฟจีเอฟในการควบคุมเซลล์ต้นกำเนิดนั้นอาจขึ้นอยู่กับหลายปัจจัย อาทิ ปริมาณ ระยะเวลาในการกระตุ้น และชนิดของเซลล์ ในส่วนที่สองได้ทำการศึกษาผลของบีเอฟจีเอฟภายในเซลล์ต่อการเพิ่มจำนวน และการแปรสภาพไปเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดที่แยกได้จากเนื้อเยื่อในฟันน้ำนมมนุษย์ จากการศึกษาพบว่าการยับยั้งการแสดงออกของบีเอฟจีเอฟหรือการยับยั้งการทำงานของตัวรับไฟโบรบลาสโกรทแฟกเตอร์ ส่งผลให้เกิดการลดลงของจำนวนโคโลนีของเซลล์ แต่พบว่าเซลล์มีความสามารถในการแปรสภาพไปเป็นเซลล์สร้างกระดูกที่ดีขึ้น ในส่วนสุดท้ายเป็นการศึกษาถึงบทบาทของบีเอฟจีเอฟต่อการควบคุมความเป็นเซลล์ต้นกำเนิด ในเซลล์ต้นกำเนิดที่แยกได้จากเนื้อเยื่อในฟันน้ำนมมนุษย์ ผลการศึกษาพบว่า บีเอฟจีเอฟส่งเสริมการแสดงออกของยีนเร็กซ์1 ซึ่งเป็นยีนที่เกี่ยวข้องกับการบ่งชี้ภาวะพลูริโพเทน การกระตุ้นการแสดงออกของเร็กซ์1ด้วยบีเอฟจีเอฟนั้น เกิดผ่านตัวรับไฟโบรบลาสโกรทแฟกเตอร์และสัญญาณเอเคทีในเซลล์ นอกจากนั้นยังพบว่าอินเตอร์ลิวคิน6 มีบทบาทในกระบวนการที่บีเอฟจีเอฟส่งเสริมการแสดงออกของเร็กซ์1อีกด้วย โดยสรุปหลักฐานที่กล่าวข้างต้นสนับสนุนถึงบทบาทของบีเอฟจีเอฟ ในการควบคุมการคงสภาพของเซลล์ต้นกำเนิด และการควบคุมการแปรสภาพไปเป็นเซลล์สร้างกระดูกในเซลล์ต้นกำเนิดที่แยกจากเนื้อเยื่อในฟันน้ำนมมนุษย์

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

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NUNTHAWAN NOWWAROTE: BASIC FIBROBLAST GROWTH FACTOR REGULATES THE DIFFERENTIATION ABILITY OF STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH. ADVISOR: ASSOC. PROF. THANAPHUM OSATHANOON, D.D.S., Ph.D., CO-ADVISOR: PROF. PRASIT PAVASANT, D.D.S., Ph.D., 137 pp.

The basic fibroblast growth factor (bFGF) play a crucial role in various biological processes, including cell growth, survivor, migration and differentiation in several types of stem cells. In this dissertation was focusing particularly on stem cells from human exfoliated deciduous teeth (SHEDs). SHEDs exhibited fibroblast-like morphology and express mesenchymal stem cell surface markers. These cells were able to differentiate into various lineages, including osteogenic, adipogenic, and neurogenic lineages. First, the roles of bFGF in dental tissue-derived mesenchymal stem cells were reviewed. The evidences suggest that bFGF regulate stem cell behaviors may depend on several factors, including dose, exposure time and cells type. Second, roles of endogenous bFGF on cell proliferation and osteogenic differentiation were revealed. bFGF knockdown and chemical inhibition of fibroblast growth factor receptor led to the reduction of colony forming unit and the increase of osteogenic differentiation potency. Third, the effect of bFGF on stemness maintenance in SHEDs was examined. bFGF promoted a pluripotent marker, REX1, gene expression in SHEDs. The bFGF-induced REX1 expression was occurred via FGFR and Akt signaling pathway. In addition, the results demonstrated the participation of interleukin-6 in bFGF-induced REX1 expression. Together, these evidences support the influence of bFGF in the maintaining of stemness and controlling of differentiation ability toward osteogenic lineage in SHEDs.

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CHAPTER I
INTRODUCTION

BACKGROUND AND SIGNIFICANCE

1.1 Rationale

The basic fibroblast growth factor (bFGF) has different roles in stem cell biology, for example enhancing stemness maintenance and regulating stem cell differentiation (Kato and Gospodarowicz 1985, Tsutsumi, Shimazu et al. 2001). It has been shown that bFGF regulates self-renewal ability in several types of stem cells (Yeoh and de Haan 2007). In this regards, it is known that bFGF signalling has a crucial role in self-renewal capacity of human embryonic stem cells and human induced pluripotent stem cells (Amit, Carpenter et al. 2000). The addition of bFGF enhancing the expression of pluripotent markers, suggesting the stemness maintaining of human embryonic stem cells in pluripotent stage (Ludwig, Levenstein et al. 2006). This influence was noted even in the feeder-free culture condition (Ludwig, Levenstein et al. 2006). Previous studies from our group illustrated that the addition of exogenous bFGF stimulated colony-forming units and enhanced the mRNA expression of pluripotent stem cell markers in stem cells isolated from human exfoliated deciduous teeth (SHEDs) and human dental pulp stem cells (DPSCs) (Osathanon, Nowwarote et al. 2011, Sukarawan, Nowwarote et al. 2014). Moreover, the osteogenic differentiation ability was attenuated in the presence of exogenous bFGF in culture

medium. In this regard, the attenuation of alkaline phosphatase enzymatic activity and mineralization was noted in SHEDs, DPSCs, and periodontal ligament stem cells (PDLSCs) (Osathanon, Nowwarote et al. 2011, Osathanon, Nowwarote et al. 2013).

On the contrary, some publications suggested that bFGF promoted osteogenic differentiation (Bai, Li et al. 2013), (Yuan, Pan et al. 2013) . The discrepancy could be due to cell types, concentration, exposure times, and culture conditions. Thus, the specific study of the role of bFGF in particular cell type is indeed necessitated. Since SHEDs became the cell source of interest for mesenchymal stem cells due to the ease of access and great potency. These cells were able to differentiate into several cell lineages, including osteoblast, adipocyte, neuron, and insulin producing cell (Govindasamy, Ronald et al. 2011, Govitvattana, Osathanon et al. 2013). Therefore, the aim of this proposal is to investigate the influence of bFGF on SHEDs stem cell maintenance and differentiation.

1.2 Introduction

This dissertation composed of three parts. The first part described literature review regarding the role of bFGF in dental tissue-derived mesenchymal stem cells. The second part illustrated the influence of endogeneous role of bFGF on cell proliferation, colony forming unit, and osteogenic differentiation in SHEDs. Lastly, the intracellular mechanisms participating in bFGF induced pluripotent marker expression was examined.

First, to understand biological function of bFGF, A literature review regarding the role of bFGF in the behavior of stem cells was performed, focusing particularly on human dental tissue-derived mesenchymal stem cells (DMSCs). PubMed database was searched with assigned key words. Identified publications were evaluated for the inclusion criteria. Ensemble information illustrated that the expression of stem cell marker in DMSCs was enhanced by supplemented with bFGF. Nevertheless, the role of bFGF on osteogenic differentiation by DMSCs remained controvertible.

The second study regarding the role of endogeneous bFGF on SHEDs proliferation, colony forming unit, and osteogenic differentiation was investigated. Previous studies from our group performing in DMSCs illustrated that the addition of exogenous bFGF stimulated colony-forming units and enhanced the mRNA expression of pluripotent stem cell markers in SHEDs and human dental pulp stem cells (DPSCs). Moreover, the osteogenic differentiation ability was attenuated in the presence of exogenous bFGF in culture medium. In this regard, the attenuation of alkaline phosphatase enzymatic activity and mineralization was noted in SHEDs, DPSCs, and periodontal ligament stem cells (PDLSCs). The second part of this dissertation aimed to investigate the role of endogenous bFGF in SHEDs. Knockdown *bFGF* expression using shRNA against bFGF and chemical inhibitor for fibroblast growth factor receptor (FGFR) were employed. This study showed that the inhibition of bFGF signaling inhibited colony forming unit ability and promoted mineralization

by SHEDS. Thus, impeding bFGF signaling may be useful to enhance osteogenic differentiation of stem cells.

The third section of this dissertation demonstrated the intracellular mechanisms participating in bFGF induced pluripotent marker expression. From our previous studies showed that stem cell properties of SHEDs for instance stem cell marker expression and cellular differentiation were regulating by bFGF. However, the mechanism of bFGF regulated stem cell marker expression still unclear. The aim of this part was to investigate the regulation of stem cell marker expression by bFGF in SHEDs. The present study focused on REX1 expression, REX1 is a pluripotent stem cell marker and found in undifferentiated embryonic stem cells. Our results clearly demonstrated bFGF regulated stemness maintenance in SHEDs by stimulating REX1 expression through FGFR and Akt signaling pathway. Moreover, IL-6 was demonstrated to involve in bFGF-induced REX1 expression in SHEDs

1.3 Background and significance

1.3.1 Human exfoliated deciduous teeth (SHEDs)

Dental tissue-derived stem cells have been isolated and grown from pulp tissues of permanent human dental pulp, pulp tissues of primary teeth, periodontal ligament, and apical papilla (Huang, Gronthos et al. 2009). Stem cells isolated from human dental pulp tissues from permanent and primary teeth were similar in cell morphology (Shekar and Ranganathan 2012) but cells isolated from dental pulp

tissues of primary teeth show higher stem cell capacity than those isolated from permanent teeth (Wang, Sha et al. 2012).

SHEDs were able to differentiate into a variety of cell types, including neural cells, adipocytes, osteoblast and odontoblasts. In terms of surface mesenchymal stem cell marker, it has been shown to express STRO-1, CD146, SSEA4, CD90, CD73, CD 105, and lack of hematopoietic/endothelial markers (CD34, CD31) (Miura, Gronthos et al. 2003, Govitvattana, Osathanon et al. 2013). Moreover, pervious study in SHEDs showed that the isolation method may influence the SHEDs characteristics. In general, the utilisation of enzyme digestion or tissue-outgrowth resulted in the similar cell characters. Those cells expressed stem cell markers as well as containing multi potential differentiation ability, suggesting both methods are suitable to employ for stem cell isolation (Jeon, Song et al. 2014). However, it should be noted that cells isolated using enzymatic disaggregation exhibited stronger stem cell marker expression, but cells isolated form outgrowth method appeared to be prone for osteogenic differentiation (Jeon, Song et al. 2014). Thus, in the regard of bone regeneration, SHEDs from outgrowth isolation technique are more suitable for hard-tissue regeneration therapy in teeth (Jeon, Song et al. 2014).

SHEDs are simple and convenient source to obtain. Cells can be isolated during the childhood and stored in cell bank for future use. In addition, SHEDs has a potential to use as an autologous transplantation, resulting in the reduction of

potential immune reaction (Arora, Arora et al. 2009). In order to support this idea, the 2-year cryopreserved SHEDs were able to maintain stemness properties (Papaccio, Graziano et al. 2006). However, it should be noted that the utilisation of SHEDs in potential clinical treatment is not yet approved in any circumstance and the efficacy as well as safety should be carefully investigated and concerned.

1.3.2 Basic Fibroblast growth factor

Numerous studies have been shown the influence of bFGF on different biological functions, for example in embryonic development, disease progression, cell survival, cell differentiation, cell proliferation, vessel formation, muscle formation, healing, and regeneration. bFGF has different roles in stem cell biology, for example enhancing stemness maintenance and regulating stem cell differentiation. It has been shown that bFGF regulates self-renewal ability in several types of stem cells. In this regards, it is known that bFGF signalling has a crucial role in self-renewal capacity of human embryonic stem cells and human induced pluripotent stem cells. The detail can be found in chapter II.

1.3.3 Interleukin-6

Interleukin-6 (IL-6) is a member of the family of cytokines and acts as inflammatory cytokine that plays an active role in immunology, bone metabolism, reproduction, arthritis, neoplasia, and aging (Keller, Wanagat et al. 1996). IL-6 secrets by T-cell and macrophage to stimulate immune response also play role in attack

infection (van der Poll, Keogh et al. 1997). Furthermore, soluble IL-6 is secreted by osteoblast to stimulate osteoclast formation *in vitro* (Tamura, Udagawa et al. 1993, Roodman 2001) and *in vivo* (Ishimi, Miyaura et al. 1990). IL-6 has the effects on proliferation, differentiation, and maturation events depending on the nature of the target cells (Metcalf 1993).

1.3.4 Structure and signaling of Interleukin-6

IL-6 receptor, also known as CD126, has been shown in membrane bound and soluble form (Mullberg, Oberthur et al. 1994). IL-6 interacts with specific cell surface receptor including IL-6 receptor (IL-6R or gp80) and glycoprotein 130 (gp130), which forms one subunit of the type I cytokine receptor within the IL-6 receptor. This is necessary to transduce the biological signal into the target cells (Taga, Hibi et al. 1989, van der Poll, Keogh et al. 1997). The IL-6 structure reveals an interface rich in hydrophobic interactions that overlap the binding sites of IL-6 receptors (Gelinas, Davies et al. 2014).

A soluble form of the IL-6R (sIL-6R) can be generated by proteolytic cleavage of mIL-6R by the metalloproteinase TNFα-converting enzyme (TACE; ADAM17) and ADAM10 or alternatively spliced mRNA. IL-6 responses can be induced classically in cells expressing membrane bound form of IL-6R (mIL-6R) through a high-affinity tetrameric complex consisting of IL-6, IL-6R and two gp130 molecules (or a hexameric complex consisting of two IL-6, two mIL-6R and two gp130 molecules).

Alternatively, a process of trans-signalling a IL-6–sIL-6R (soluble IL-6R) complex directly binds to and signals through gp130 in cells lacking mIL-6R (Kopf, Bachmann et al. 2010).

The signaling occur when IL-6-IL-6R complex combined with double gp130, and then dimerizes and initiates signaling, these Janus kinases (JAKs) become activated and phosphorylate gp130 on five tyrosine residues, which stimulates the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-4, 5-bisphosphate3-kinase (PI3K) pathway. Phosphorylation of the other tyrosine residues leads to recruitment of STAT (signal transducer and activator of transcription) factors, which are phosphorylated, dimerize and travel to the nucleus where they stimulate transcription of target genes (Scheller, Garbers et al. 2014).

1.3.5 Biological function of Interleukin-6

IL-6 is a pleiotropic growth factor that has important roles in regulating hematopoiesis and the immune response, including inflammation. IL-6 signalling is a critical element of inflammation and repair in health and seems to support a central role in the pathogenesis of inflammatory diseases. IL-6 was originally identified as T cell-derived lymphokine that induces final maturation of B cells into antibody producing cells (Kishimoto 1989). IL-6 can be secreted by macrophage in response to specific microbial molecules (Li, Hsieh et al. 2009). IL-6 regulates human adipose tissue lipid metabolism and laptin production (Trujillo, Sullivan et al. 2004). IL-6

stimulates energy mobilization that leads to increased body temperature in muscle and fatty tissue. IL-6 stimulates osteoclast formation and function as well as regulation of RANK expression in preosteoclasts/osteoclasts (Palmqvist, Persson et al. 2002). Moreover, it has been reported that IL-6 promotes human epidermal keratinocyte proliferation and keratin cytoskeleton reorganization (Hernandez-Quintero, Kuri-Harcuch et al. 2006) Interestingly, IL-6 has crucial roles in wound healing, probably by regulating leukocyte infiltration, angiogenesis, and collagen accumulation (Lin, Kondo et al. 2003). Finally, IL-6 has been shown to interact with and affect various cells and organ systems, including the vascular endothelial cells, the endocrine system of the hypothalamic pituitary adrenal axis, and the neuropsychological system (Tanaka and Kishimoto 2014).

1.3.6 Interleukin-6 in stem cells

IL-6 originally identified in the immune system that is a multifunctional cytokine also found that IL-6 is important in stem cell maintenance, proliferation and differentiation in several tissues. Recent study illustrated that endogenous IL-6 binds to IL6Rs on cortical precursors to promote their self-renewal and maintain their numbers during embryogenesis (Gallagher, Norman et al. 2013) but the cytokine interleukin 6, which is not detectable at significant levels in embryonic stem cells, was induced with IL-6 after 4 day and this event can induced pluripotent stem cells at the onset of iPS reprogramming (Brady, Li et al. 2013). IL-6 highly expressed in the

bone marrow stromal cells (Ogasawara, Tsuji et al. 1996), maintains the proliferative and undifferentiated state of bone marrow-derived MSCs, an important parameter for the optimization of both in vitro and in vivo manipulation of MSCs (Pricola, Kuhn et al. 2009). IL-6 is a major cytokine in the central nervous system (Erta, Quintana et al. 2012). The stromal cells are a major source of IL-6 during repair (Tadokoro, Wang et al. 2014). Evidence strongly that targeting IL-6 signals may be useful as a cancer stem cell directed therapy (Wang, Lathia et al. 2009). Interestingly, IL-6 was found to play role in maintaining stemness of human exfoliated deciduous via REX-1 expression (Govitvattana, Osathanon et al. 2013).

1.3.7 Interukin-6 and basic fibroblast growth factor

IL-6 can be secreted by various types of cells, including lymphocytes, endothelial cells, fibroblasts and epithelial cells (Bartalena, Brogioni et al. 1995). IL-6 was considered as both pro- and anti-inflammatory molecule (Jones, Horiuchi et al. 2001). It has been shown that bFGF can stimulate IL-6 synthesis (Kozawa, Suzuki et al. 1997, Dankbar, Padro et al. 2000). Likewise, IL-6 stimulated bFGF expression was observed in the paracrine interaction between myeloma and marrow stromal cells (Bisping, Leo et al. 2003). However, the knowledge of interaction between and IL-6 in stemness maintenance in SHEDs is yet lacking. Interestingly only one report, IL-6 was found to play role in maintaining stemness of human exfoliated deciduous via REX-1 expression (Govitvattana, Osathanon et al. 2013).

1.4 RESEARCH OBJECTIVES

1. To investigate an influence of bFGF on stemness maintenance of SHEDs, in terms of cell proliferation, colony forming unit ability, and pluripotent marker expression *(REX1)*.
2. To investigate the effect of bFGF on osteogenic differentiation in SHEDs.

1.5 RESEARCH BOUNDARY

SHEDs used in this study were primary cells; they have a large variation of characteristics. This experiment was performed *in vitro*. Therefore, we could control the factors involved in the stemness maintenance and control differentiation capacity. The results from this study clarified function of bFGF on the stemness of SHEDs. However, the results might not represent what happens *in vivo*. Moreover, due to the heterogeneous population in isolated cells, the response might occur from subpopulation. Thus, the future investigation on specific population is indeed required. This experiment

1.6 BENEFICIAL OF THIS STUDY

This knowledge could be further applied for manipulate the culture of SHEDs in order to maintain stem cells. Further, the knowledge in the differentiation control could be beneficial for future osteogenic regenerative study.

CHAPTER II

REVIEW OF THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN DENTAL TISSUE-DERIVED MESENCHYMAL STEM CELLS

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Review of the role of basic fibroblast growth factor in dental tissue-derived mesenchymal stem cells

2.1 Abstract

Background: Basic fibroblast growth factor (bFGF) plays a crucial role in various biological processes, including cell growth, survival, migration, and differentiation. In stem cell biology, bFGF is employed to maintain stemness and regulate differentiation.

Objectives: To review the role of bFGF in the behavior of stem cells, focusing particularly on human dental tissue-derived mesenchymal stem cells (DMSCs).

Methods: The articles from January 1, 1990 to March 25, 2015 in the PubMed database were searched with assigned key words (dental stem cells and (bFGF or FGF2)). Titles and abstracts of the retrieved articles were evaluated to identify inclusion criteria.

Results: Sixty-five articles were identified from the PubMed database using the assigned keywords. Eighteen articles met the inclusion criteria including: (1) articles published in English, (2) articles describing the effects of endogenous and exogenous bFGF in cell culture and animal studies, and (3) the cell model used in the study was derived from dental-related tissues, and were employed as the main articles discussed in the present narrative review.

Conclusion: bFGF supplementation enhances stem cell marker expression in DMSCs. However, the role of bFGF on osteogenic differentiation by DMSCs remains controversial.

Keywords: Basic fibroblast growth factor, dental tissue-derived stem cells, differentiation, stemness

2.2 Abbreviations

Akt = protein kinase B

ALP = alkaline phosphatase

Ank = a 12-membrane spanning protein associated with progressive ankylosing mineralization

bFGF = basic fibroblast growth factor = fibroblast growth factor 2 (FGF2)

BMP = bone morphogenetic protein

Caspase = cysteine-aspartic acid protease

CDC2 = phosphorylated cell division cycle protein 2 homolog

CDK = cyclin-dependent kinase

DAG = diacylglycerol

DMSCs = dental tissue-derived mesenchymal stem cells

DPSCs = dental pulp stem cells

Dusp6 = dual specificity phosphatase 6

EMT = epithelial-to-mesenchymal transition

ERK = extracellular signal-regulated kinase = mitogenactivated protein kinase (MAPK)

ES cells = embryonic stem cells

FGF2 = fibroblast growth factor 2 = basic fibroblast growth factor (bFGF)

FGFR = fibroblast growth factor receptor

FRS2 = fibroblast growth factor receptor substrate 2

G2/M = end of G2/entry into mitosis

Gab1 = Grb2-associated-binding protein 1

GLUT = glucose transporter

GPDH = glycerol 3-phosphate dehydrogenase

Grb2 = growth factor receptor-bound protein 2

HDPCs = human dental pulp cells

HSPGs, heparin sulphate proteoglycans

iPS cells = induced pluripotent stem cells

IP3 = inositol-1,4,5-triphosphate

JAK = Janus kinase

JNK = c-Jun N-terminal kinases

MAPK = mitogen-activated protein kinase = extracellular signal-regulated kinase (ERK)

MEK = mitogen-activated protein kinase

MKP-3 = mitogen-activated protein kinase phosphatase-3

MSCs = mesenchymal stem cells

NF-kB = nuclear factor k-light-chain-enhancer of activated B cells

Oct4 = octamer-binding transcription factor 4

P = phosphorylation

p21 = cyclin-dependent kinase inhibitor

PC-1 = pyrophosphate-generating enzyme

PCP = noncanonical planar cell polarity

PDLSCs = periodontal ligament stem/progenitor cells

PI-3 kinase = phosphatidylinositol-4,5-bisphosphate 3-kinase

PIP2 = phosphatidyl-inositol-4,5-diphosphate

PKC = protein kinase C

PLGA = poly(lactic-co-glycolactic acid)

PLC = phospholipase C

PPARg2 = peroxisome proliferator-activated receptor-g2

Raf1 = RAF proto-oncogene serine/threonine-protein kinase

SCAPs = stem cells from the apical papilla

SH2 = Src homology 2

SHEDs = stem cells isolated from human exfoliated deciduous teeth

Shc = Src homology

SOS = Son of Sevenless

Spred = sprouty-related, EVH1 domain-containing protein

Spry = sprouty protein

SSEA-4 = stage-specific embryonic antigen 4

STAT1 = signal transducer and activator of transcription-1

XFLRT3 = (Xenopus) fibronectin leucine rich transmembrane protein 3

2.3 Introduction

Basic fibroblast growth factor (bFGF) or fibroblast growth factor 2 (FGF2), a member of the fibroblast growth factor family, regulates cell growth, differentiation, migration, and survival during development and regeneration (Ornitz and Itoh 2001, Thisse and Thisse 2005, Yun, Won et al. 2010). bFGF has various roles in stem cell biology including the maintenance of stemness and control of differentiation (Kato and Gospodarowicz 1985, Tsutsumi, Shimazu et al. 2001). To maintain stemness, bFGF regulates the self-renewing ability of several cell types (Yeoh and de Haan 2007). bFGF signaling plays a crucial role in the self-renewal capacity of human embryonic stem cells (ES) and human induced pluripotent stem cells (iPS) (Amit, Carpenter et al. 2000). Exogenous bFGF enhances the expression of pluripotent markers (Ludwig, Levenstein et al. 2006). We have shown that exogenous bFGF stimulates colony-forming units and enhances the mRNA expression of pluripotent stem cell markers in stem cells isolated from human exfoliated deciduous teeth (SHEDs) and human dental pulp stem cells (DPSCs) (Osathanon, Nowwarote et al. 2011, Sukarawan, Nowwarote et al. 2014). The role of bFGF in stem cell differentiation is controversial. We reported an inhibitory effect of bFGF on

osteogenic differentiation of MSCs (Osathanon, Nowwarote et al. 2011, Osathanon, Nowwarote et al. 2013, Sukarawan, Nowwarote et al. 2014), while others reported an inductive effect (Bai, Li et al. 2013, Yuan, Pan et al. 2013). Similarly, the effects of bFGF on adipogenic differentiation are controversial. These contradictory results may be a consequence of different cell types, concentrations, exposure times, and culture conditions.

DMSCs have been introduced as a stem cell source because of their accessibility and availability. MSCs can be isolated from various dental-related tissues, including dental pulp, periodontal ligaments, apical papilla, and dental follicles (Egusa, Sonoyama et al. 2012). The isolated cells exhibit the stem cell characteristics, including the expression of mesenchymal stem cell markers and multipotential differentiation ability (Egusa, Sonoyama et al. 2012, Machado, Fernandes et al. 2012, Sedgley and Botero 2012). Although these cells share common characters, the DMSCs from different sources exhibit dissimilar characteristics and potency (Eleuterio, Trubiani et al. 2013, Kanafi, Ramesh et al. 2013, Hakki, Kayis et al. 2014, Sawangmake, Nowwarote et al. 2014). Various studies have examined the effect of bFGF on the behavior of these DMSCs. The results are varied. In the present article, the influence of bFGF on DMSCs is reviewed and discussed in terms of both stemness maintenance and cell differentiation.

2.4 Methods

The articles from January 1, 1990 to March 25, 2015 in PubMed database were searched using keywords. The keywords used in the search were (“1990/01/01”[Publication Date]:”2015/03/ 25"[Publication Date]) AND (dental stem cells and (bFGF or FGF2)). The title and abstract of retrieved articles were evaluated for inclusion in the review. The inclusion criteria were as follows: (1) articles published in English, (2) articles describing the effects of endogenous and exogenous bFGF in cell culture and animal studies, and (3) the cell model used in the study was derived from dental-related tissues.

2.5 Results

Sixty-five articles were identified from the PubMed database using the assigned keywords. Eighteen articles met with the inclusion criteria and were employed as the main articles discussed in the present narrative review.

2.5.1 bFGF and receptors

bFGF is a b-sheet protein that consists of 140 amino acids (Okada-Ban, Thiery et al. 2000). It contains two receptor binding sites, locating at residues 13–30 and 106–129 (Baird, Schubert et al. 1988, Yayon, Aviezer et al. 1993, Woodbury and Ikezu 2014). These residues bind to fibroblast growth factor receptors (FGFRs) on the cell surface (Powers, McLeskey et al. 2000). FGFRs consist of four subtypes: FGFR1, FGFR2, FGFR3, and FGFR4 (Kan, Wang et al. 1993, Hatch 2010). The preferential binding

ability of bFGF to its receptors may lead to a differential cell response (Powers, McLeskey et al. 2000). The levels and types of receptor expression are crucial factors regulating bFGF signaling. FGFR expression levels are altered during cell proliferation or differentiation. For example, actively proliferating cells express higher FGFR than the confluent cells, implying an influence of bFGF on cell proliferation (Wada, Gelfman et al. 2001). Moreover, FGFR levels are shown to increase or decrease during cell differentiation depending on cell type (Olwin and Hauschka 1988, Moscatelli 1994). The binding of bFGF to its receptors results in the activation of tyrosine kinase (Bikfalvi, Klein et al. 1997) and, subsequently, leads to initiation of various intracellular signaling, including phospholipase C (PLC)-g, protein kinase C (PKC), Ras (small GTPase)-mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3- kinase (PI-3 kinase)/protein kinase B (AKT), signal transducer and activator of transcription-1 (STAT1)/ cyclin-dependent kinase inhibitor (p21), Src homology (Shc), and Src pathways (Su, Du et al. 2008, Yang, Xia et al. 2008). Further, bFGF can bind heparin and bFGF signaling can be enhanced in the presence of heparin sulfate proteoglycan (Powers, McLeskey et al. 2000, Murakami 2011). Heparin sulphate promotes the stability of receptor dimerization and prevents aggregation of bFGF (Harada, Kettunen et al. 1999, Powers, McLeskey et al. 2000, Murakami 2011, Chen, Gulbranson et al. 2012). A diagram summarizing the intracellular signaling induced by bFGF is shown as Figure 1.

2.5.2 bFGF and stemness maintenance

bFGF has crucial role in maintaining the stemness properties of human embryonic stem (ES) and induced pluripotent stem (iPS) cells. In general, bFGF is used as a supplemental growth factor in the culture medium of these cells to maintain them in an undifferentiated state (Communications, Journal of the American Dental et al. 2005, Xu, Rosler et al. 2005, Park and Hong 2014). bFGF supplementation is able to maintain pluripotent marker expression in long-term culture of human ES cells (Villegas, Canham et al. 2010). Further, human ES cells cultured in bFGF supplemented serum-free culture medium are able to proliferate and maintain an undifferentiated state (Amit, Carpenter et al. 2000). Correspondingly, human ES cells can be maintained in an undifferentiated state in co-culture with bFGF expressing human feeder cells (Xu, Rosler et al. 2005, Park, Choi et al. 2010, Xi, Wang et al. 2010, Park, Kim et al. 2011). However, the effect of bFGF on several characteristics of human ES and iPS cells is dose- and cell line-dependent (Quang, Marquez et al. 2014).

bFGF is required to maintain the expression of the stemness markers octamer-binding transcription factor 4 (Oct4) and the transcription factor, Nanog, by human ES cells (Rosenblatt-Velin, Lepore et al. 2005, Yu, Pan et al. 2011, Kong, Tu et al. 2013). Mechanistically, bFGF maintains human ES cells in an undifferentiated state via the extracellular signal-regulated kinase (ERK)1/ 2-c-Fos/c-Jun signaling pathway

(Kang, Kim et al. 2005, Park, Choi et al. 2010). bFGF directly regulates Nanaog expression via the ERK– mitogen-activated protein kinase (MEK) pathway (Yu, Pan et al. 2011, Chen, Gulbranson et al. 2012). Supplementation with ERK inhibitor suppresses bFGF-induced Nanog expression in human ES cells (Yu, Pan et al. 2011, Chen, Gulbranson et al. 2012). Moreover, bFGF represses bone morphogenetic protein (BMP) signaling in human ES cells, resulting in attenuation of differentiation and promotion of self-renewal (Xu, Rosler et al. 2005). A combination treatment of bFGF and noggin (a BMP antagonist) can sustain the undifferentiated state of human ES cells in a feeder free culture (Wang, Zhang et al. 2005). Further, bFGF inhibits human iPS and ES cell apoptosis by the inhibition of activation of cysteine-aspartic acid protease (Caspase)-3 through the ERK/serine/ threonine-specific Akt signaling pathway, indirectly preventing differentiation (Wang, Lin et al. 2009).

bFGF is involved in the self-renewal and maintenance of the multipotential differentiation ability of mesenchymal stem cells (MSCs) (Kato and Gospodarowicz 1985, Tsutsumi, Shimazu et al. 2001). Exogenous bFGF supplementation or endogenous bFGF overexpression enhances proliferation of human MSC (Go, Takenaka et al. 2007, Zhang, Wang et al. 2013). Addition of exogenous bFGF does not alter the multipotential differentiative ability of these stem cells (Zhang, Wang et al. 2013). CyclinD1, cyclinD3, cyclin-dependent kinase (CDK)-4, and phosphorylated cell division cycle protein 2 homolog (CDC2) protein expression are dramatically upregulated in bFGF-treated human MSCs, resulting in enhancement of proliferation

(Ramasamy, Tong et al. 2012). Moreover, bFGF promoted the mRNA expression of pluripotent stem cell markers in MSC isolated from various tissues (Osathanon, Nowwarote et al. 2011, Ramasamy, Tong et al. 2012, Sukarawan, Nowwarote et al. 2014). Together, these results suggest an important role for bFGF in controlling the stemness of both human pluripotent and multipotent stem cells.

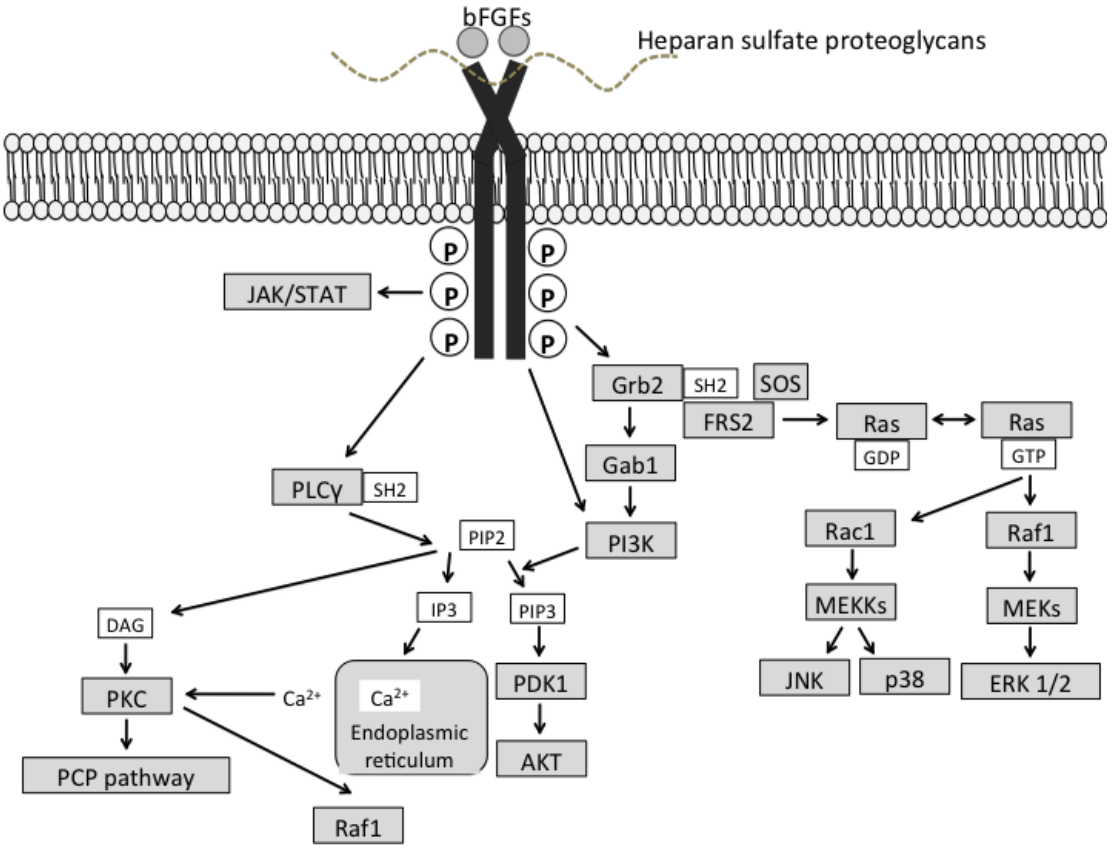

Figure 1 bFGF intracellular signaling. Activation by receptor autophosphorylation triggers diverse signaling cascades, including the Ras/ mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5- bisphosphate 3-kinase (PI-3 kinase)/protein kinase B (Akt), phospholipase C (PLC)-g/Ca2 and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways. Phosphorylation of the docking protein,

fibroblast growth factor receptor substrate 2 (FRS2) is followed by growth factor receptor-bound protein 2 (Grb2) activation, which in turns activates either the Ras/MAPK cascade via Son of Sevenless (SOS), or the PI-3 kinase/Akt pathway via Grb2-associated-binding protein 1 (Gab1). PI-3 kinase can also be activated directly by tyrosine phosphorylation or alternatively by Ras1. The other main transduction pathway involves PLC. The Src homology 2 (SH2) domain of the PLC interacts directly with the receptor leading to the hydrolysis of phosphatidyl-inositol-4,5-diphosphate (PIP2) to inositol-1,4,5-triphophate (IP-3) and diacylglycerol (DAG). Inositol-1,4,5-triphosphate (IP-3) releases Ca2+ from the endoplasmic reticulum (ER), while DAG activates protein kinase C (PKC) that in turn can activate the noncanonical planar cell polarity (PCP) pathway and RAF proto-oncogene serine/threonine-protein kinase (Raf1). Feedback inhibitors such as dual specificity phosphatase 6 (Dusp6)/mitogen-activated protein kinase phosphatase-3 (MKP-3), sprouty protein (Spry), FRS2a, sprouty-related, EVH1 domain-containing protein (Spred), and Sef involved in signal attenuation, and enhancers such as fibronectin leucine rich transmembrane protein 3 (XFLRT3) can also contribute to the overall levels of bFGF signaling. HSPGs, heparin sulphate proteoglycans; EMT, epithelial-to-mesenchymal transition; P, phosphorylation (modified from (Villegas, Canham et al. 2010) 2010 Wiley-Liss, Inc. with permission for reuse.

2.5.3 bFGF and cell differentiation

Addition of exogenous bFGF effects stem cell differentiation toward various lineages, including osteogenic, adipogenic, chondrogenic, myogenic, and neurogenic lineages. The present review focuses briefly on the osteogenic, adipogenic, and neurogenic lineages.

2.5.3.1 bFGF and osteogenic differentiation

The influence of bFGF on osteogenic differentiation is controversial. bFGF both positively and negatively regulates osteogenic differentiation. Fgf2-knockout mice exhibit reduced bone mass and osteogenic differentiation (Montero, Okada et al. 2000). Correspondingly, bFGF promotes cell proliferation and enhanced osteogenic treatment upregulates alkaline phosphatase (ALP) activity, osteocalcin mRNA expression, calcium deposition, and bone nodule formation in vitro (Sakaguchi, Janick et al. 1997, Pri-Chen, Pitaru et al. 1998). Moreover, bFGF and BMP-2 in combination synergistically induce osteogenic potency in rat bone marrow MSCs (Hanada, Dennis et al. 1997). The delivery of bFGF using various approaches (i.e. coral scaffold and collagen hydrogel) results in the promotion of osteogenic differentiation in human bone marrow MSCs (Park, Kim et al. 2006, Zheng, Su et al. 2011, Oh, Lee et al. 2012). However, the selective response of the osteogenic marker gene to bFGF, and the specific time point in which bFGF could promote osteogenic differentiation, suggested treatment with bFGF enhanced osteopontin, but decreased type I collagen

expression (Tanaka, Ogasa et al. 1999). Moreover, bFGF exposure at an early stage of differentiation promotes osteogenic differentiation, while inhibiting it in later stages (Qian, Jiayuan et al. 2014).

Besides the positive regulation of osteogenic differentiation by bFGF, some contradictory reports are noted (Osathanon, Nowwarote et al. 2011, Li, Qu et al. 2012, Osathanon, Nowwarote et al. 2013, Qian, Jiayuan et al. 2014). A combination of BMP-2 and bFGF inhibits the inductive effect of BMP-2 in a rat femur defect model of bone formation (Wang, Zou et al. 2013). Further, supplementation with bFGF alone could strongly attenuate osteogenic differentiation in MSC derived from various tissue sources (Lai, Krishnappa et al. 2011, Osathanon, Nowwarote et al. 2013). bFGF might alter a specific process during osteogenic differentiation. For example, exogenous bFGF reduces the ALP activity in osteogenic medium, but does not affect mineralization (Rose, Fitzsimmons et al. 2013). In mouse preosteoblasts, bFGF inhibits mineralization, possibly via the upregulation of pyrophosphate-generating enzyme (PC-1) and Ank, a 12-membrane spanning protein associated with progressive ankylosing mineralization (e.g. chondrocalcinosis and craniometaphyseal dysplasia), and downregulation of tissue nonspecific ALP. This bFGF-induced gene expression alteration results in the accumulation of a potent inhibitor of mineralization, pyrophosphate, leading to attenuation of mineralization (Hatch, Nociti et al. 2005, Hatch, Li et al. 2009). The intracellular mechanism(s) require further investigation to clarify the role of bFGF in osteogenic differentiation.

2.5.3.2 bFGF and adipogenic differentiation

In bFGF knockout mice, an increase of bone marrow fat accumulation assessing by osmium tetroxide labeling and Oil Red O staining was noted in vivo (Xiao, Sobue et al. 2010). Further, addition of exogenous bFGF can decrease intracellular lipid accumulation in bone marrow-derived cells in bFGF knockout mice (Xiao, Sobue et al. 2010).

Correspondingly, bFGF overexpression in human MSC results in a slight decrease of adipogenic marker expression, and attenuates intracellular lipid accumulation of cells cultured in an adipogenic induction medium (Fierro, Kalomoiris et al. 2011). Together, these results imply a suppressive effect of bFGF on adipogenic differentiation. Further, bFGF suppressed adipogenic differentiation via extracellular signal-regulated kinase 1 and 2 (ERK1/2) activation (Inoue, Imamura et al. 2009).

By contrast, bFGF enhances adipocyte differentiation in human embryonic stem cell-derived MSCs (Song, Li et al. 2014). Upregulation of peroxisome proliferator- activated receptor-g2 (PPARg2) by MSC is observed upon bFGF supplementation in an adipogenic induction medium (Neubauer, Fischbach et al. 2004). Moreover, bFGF binding to heparinized decellularized adipose tissues promotes adipose tissue formation in vivo (Lu, Li et al. 2014). In addition, bFGF supplementation upon adipogenic differentiation of human adipose-derive stem cells can enhance the upregulation of PPAR g2 and glucose transporter (GLUT) type 4

mRNA expression, lipid accumulation, and glycerol 3-phosphate dehydrogenase (GPDH) activity in vitro (Kakudo, Shimotsuma et al. 2007). An adipogenic enhancing effect was shown in a 3D culture system employing a poly (lactic-co-glycolactic acid) (PLGA) scaffold. Adipogenesis of bone marrow MSCs is enhanced upon bFGF supplementation (Neubauer, Hacker et al. 2005). The apparently contradictory effects of bFGF on adipogenic differentiation are noted to be similar to those on osteogenic differentiation (Xiao, Sobue et al. 2010). Thus, further investigation is required to determine the role of bFGF in adipogenic differentiation in specific cell types. bFGF and neurogenic differentiation

bFGF promotes neuronal differentiation. bFGF induces cell division and neuronal differentiation by chromaffin cells, olfactory neuroblastoma cells, amniotic epithelial cells, and spinal cord neurons (Stemple, Mahanthappa et al. 1988, Dai and Peng 1995, Niknejad, Peirovi et al. 2010). In MSCs, bFGF and neurotrophin 3 supplementation promoted neuronal differentiation (Guan, Xu et al. 2014). Further, bFGF promotes the mRNA expression of neuronal markers in various cells, including bone marrow derived MSCs, muscle-derived stem cells, DPSCs, and adipose stem cells (Jang, Cho et al. 2010, Hu, Wang et al. 2013, Kang, Kwon et al. 2013, Nakano, Edamura et al. 2014). For example, the addition of bFGF alone in neurobasal medium was sufficient to enhance neuronal differentiation of dental pulp stem cells (DPSCs), as determined by the expression of b3-tubulin (Osathanon, Nowwarote et al. 2011). Moreover, bFGF is indispensable for Schwann cell induction from bone

marrow MSCs and this process is regulated via the MAPK/ERK signaling pathway (Zhu, Yang et al. 2014).

2.5.3.3 bFGF and other cell differentiation

Beside the lineages discussed above, there are several reports of the influence of bFGF on cell differentiation potency toward other specific cell lineages i.e. epithelial, chondrogenic, and myogenic lineages. Exemplified by bFGF treatment of lens- epithelial cells promotes cell proliferation and lens- fiber differentiation (McAvoy and Chamberlain 1989). bFGF enhances mature cardiomyocyte differentiation from cardiac precursor cells and mouse embryonic stem cells (Rosenblatt-Velin, Lepore et al. 2005, Khezri, Valojerdi et al. 2007). Further, bFGF priming or bFGF immobilized on biomaterials can promote genotypic and phenotypic changes of MSCs toward fibroblasts (Subramony, Su et al. 2014, Duan, Hockaday et al. 2015).

2.5.4 bFGF in DMSCs

bFGF enhances stemness in many types of DMSCs. The bFGF enhances the expression of embryonic stem cell markers (Oct4, Rex1, and Nanog) in DPSCs (Osathanon, Nowwarote et al. 2011). Moreover, an increase in the number of cells recognized by the STRO monoclonal antibody (STRO-1+ cells) is observed in bFGF treated DPSCs and human periodontal ligament stem/ progenitor cells (PDLSCs) (Morito, Kida et al. 2009, Hidaka, Nagasawa et al. 2012). Supplementation of medium

in both short- and long-term cultures with bFGF leads to an increase in mRNA expression of pluripotent markers in SHEDs (Sukarawan, Nowwarote et al. 2014). Similarly, bFGF enhances stem cell marker expression in stem cells from the apical papilla (SCAPs) (Wu, Huang et al. 2012). An increase of colony forming unit ability is observed when SHEDs from normal and inflamed pulp tissues are supplemented with exogenous bFGF [(Kim, Park et al. 2014, Sukarawan, Nowwarote et al. 2014). Exogenous bFGF does not influence the proliferative ability of SHEDs (Li, Qu et al. 2012, Sukarawan, Nowwarote et al. 2014). By contrast, bFGF enhanced the proliferation of DPSCs, PDLSCs, and SCAPs (He, Yu et al. 2008, Morito, Kida et al. 2009, Lee, Um et al. 2012, Wu, Huang et al. 2012, Kono, Maeda et al. 2013). End of G2/entry into mitosis (G2/M) is upregulated when PDLSCs are treated with bFGF (Kono, Maeda et al. 2013). bFGF induces proliferation of human dental pulp cells (HDPCs) and tends to enhance stem cell surface marker proteins STRO-1 and stage-specific embryonic antigen 4 (SSEA-4) (Kim, Min et al. 2010).

Osteogenic differentiation is attenuated in the presence of exogenous bFGF in osteogenic culture medium. Attenuation of ALP enzymatic activity, osteogenic marker expression, and mineralization is noted in SHEDs, DPSCs, SCAPs, and PDLSCs (Murakami, Takayama et al. 2003, Osathanon, Nowwarote et al. 2011, Lee, Um et al. 2012, Wu, Huang et al. 2012, Osathanon, Nowwarote et al. 2013, Qian, Jiayuan et al. 2014). Correspondingly, FGFR inhibitor supplementation promotes ALP activity and mineralization by SHEDs in vitro (Nowwarote, Pavasant et al. 2015). bFGF possibly

inhibits the Wnt/-catenin signal transduction pathway, which has been shown in SHEDs (Li, Qu et al. 2012). Transplantation with PDLSCs and bFGF results in a decrease of bone formation in mice (Lee, Um et al. 2012). By contrast, exogenous bFGF enhances ALP activity, mineralization, and odontoblastic marker gene expression in primary HDPCs (Kim, Min et al. 2010). bFGF treatment of HDPCs induces chemokine mRNA expression via MAPKs (ERK1/2, p38, c-Jun N-terminal kinases (JNK)), nuclear factor k-light-chain-enhancer of activated B (NF-kB) cells, and PKC pathways (Kim, Min et al. 2010). bFGF pretreatment for 1 week before osteogenic induction enhances osteogenic differentiation ability in vitro and in vivo (Qian, Jiayuan et al. 2014). BMP-2 and bFGF promote the formation of new bone (Wang, Sha et al. 2012). Gelatin carriers releasing human recombinant bFGF induce periodontal regeneration in artificially created furcation class II bone defects in beagle dogs (Murakami, Takayama et al. 2003) and primates (Takayama, Murakami et al. 2001). The effects of bFGF in DMSCs remain controversial. A summary of the in vitro and in vivo effects of bFGF in dental-derived stem/progenitor cells is shown in Tables 1 and 2.

Table 1 *In vitro* effects of basic fibroblast growth factor (bFGF)

Cell types	In vivo results	References
DPSCs	(+) cell migration	(Suzuki, Lee et al. 2011)
	(+) cell proliferation	(Morito, Kida et al. 2009) (Lee, Kim et al. 2015) (He, Yu et al. 2008)
	(+) colony forming unit	(Osathanon, Nowwarote et al. 2011)
	(+) matrix deposition and cell viability	(Yang, Zhang et al. 2015)
	(+) stem cell marker expression (STRO-1, *OCT4*, *NANOG*, *REX1*)	(Morito, Kida et al. 2009) (Osathanon, Nowwarote et al. 2011)
	(+) osteoblast differentiation (6 day or 2 weeks bFGF priming)	(Qian, Jiayuan et al. 2014) (Lee, Kim et al. 2015)
	(+) neurogenic differentiation	(Sasaki, Aoki et al. 2008) (Osathanon, Nowwarote et al. 2011)

Table 1 *In vitro* effects of basic fibroblast growth factor (bFGF) (Continues)

Cell types	In vivo results	References
PDLSCs	(+) cell proliferation	(Kono, Maeda et al. 2013) (Lee, Um et al. 2012). (Lee, Kim et al. 2015) (Takeuchi, Hayashi et al. 2015)
	(-) c-kit expression	(Suphanantachat, Iwata et al. 2014)
	(-) osteoblast differentiation	(Lee, Um et al. 2012). (Osathanon, Nowwarote et al. 2013).
SHEDs	(+) colony forming unit	(Sukarawan, Nowwarote et al. 2014). (Nowwarote, Pavasant et al. 2015). (Osathanon, Nowwarote et al. 2013).
	(+) stem cell marker expression (*OCT4*, *NANOG*, *REX1*)	(Sukarawan, Nowwarote et al. 2014).

Table 1 *In vitro* effects of basic fibroblast growth factor (bFGF) (Continues)

Cell types	In vivo results	References
SHEDs	(-) osteoblast differentiation	(Nowwarote, Pavasant et al. 2015). (Osathanon, Nowwarote et al. 2013)
SCAPs	(+) cell proliferation and colony forming unit (+) stem cell marker expression (*OCT4*, *NANOG*, *REX1*, *SOX2*, STRO-1) (-) osteoblast differentiation	(Wu, Huang et al. 2012)
Dental pulp cells	(+) cell migration and cell proliferation (-) osteoblast differentiation	(Takeuchi, Hayashi et al. 2015)
Periodontal ligament cells	(+) cell migration and cell proliferation	(Takeuchi, Hayashi et al. 2015)
	(-) osteogenic differentiation	(Dangaria, Ito et al. 2009)
Dental follicle cells	(-) osteogenic differentiation	(Dangaria, Ito et al. 2009)

DPSCs = dental pulp stem cells; periodontal ligament stem/progenitor cells (PDLSCs)

SHEDs = stem cells isolated from human exfoliated deciduous teeth; stem cells from the apical papilla (SCAPs) octamer-binding transcription factor 4 (Oct4)

Table 2 *In vivo* investigation.

Cell types	In vivo results	References
PDLSCs	(-) bone formation in subcutaneous implantation	(Lee, Um et al. 2012)
DPSCs	(-) bone formation (1 week bFGF priming) (+) bone formation (2 week bFGF priming) (+) revascularization and cell migration in ectopic tooth slice transplantation model	(Qian, Jiayuan et al. 2014) (Yang, Zhang et al. 2015)
Primary dental pulp cells from deciduous teeth	(+) wound healing in murine full-thickness skin defect model	(Nishino, Ebisawa et al. 2011)
(In vivo delivery without cell incorporation)	(+) revascularization, recellulization, and odontoblastic differentiation in ectopic tooth transplantation model	(Takeuchi, Hayashi et al. 2015) (Suzuki, Lee et al. 2011)

DPSCs = dental pulp stem cells; PDLSCs = periodontal ligament stem/progenitor cells; SHEDs human exfoliated deciduous teeth.

2.5.5 Preclinical study of the use of bFGF in dentistry

bFGF-loaded hydrogel enhances revascularization and pulp-like tissue regeneration in human endodontic treated teeth implanted subcutaneously in mice

(Suzuki, Lee et al. 2011). bFGF-releasing scaffolds promoted robust dentin formation in a rat model of molar defect (Kikuchi, Kitamura et al. 2007). Controlled bFGF release results in localized dentin formation in the defect area (Ishimatsu, Kitamura et al. 2009). The dose administered is a critical factor for the dentin formation. A low dose (0.05 mg/ml) fails to promote dentin regeneration, while a high dose (5 mg/ml) results in scattered and incomplete dentin formation (Ishimatsu, Kitamura et al. 2009). Correspondingly, bFGF (dose 30 ng) did not promote dentin bridge formation, but instead fibrous formation with some inflammation (Hu, Zhang et al. 1998).

bFGF promotes periodontal tissue regeneration in canine periodontal defects (Murakami, Takayama et al. 2003, Shirakata, Taniyama et al. 2010, Saito, Saito et al. 2013). This regeneration may be the result of the proliferative effect of bFGF on periodontal ligament cells as demonstrated in vitro (Dereka, Markopoulou et al. 2006). A positive effect of bFGF is observed in a canine model of alveolar bone regeneration (Kinoshita, Matsuo et al. 2008, Matsumoto, Hoshino et al. 2012).

2.6 Conclusion

The regulation of stem cells behaviors by bFGF may depend on several factors, including dose, exposure time, and cell type. The influence of bFGF on differentiation is controversial. Careful investigations of bFGF function in specified cell types in specific settings are necessary to understand the complex regulation of dental MSC behaviors by bFGF.

2.7 Acknowledgments

This article was supported by the Ratchadapiseksomphot Endowment Fund of Chulalongkorn University, Faculty of Dentistry Research Funding and the Research Chair Grant 2012, the National Science and Technology Development Agency (NSTDA). Figure 1 is 2010 Wiley-Liss, Inc. with permission for reuse.

2.8 Conflict of interest statement

The authors have no conflicts of interest to declare.

CHAPTER III

ROLE OF ENDOGENEOUS BASIC FIBROBLAST GROWTH FACTOR IN STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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Role of endogenous basic fibroblast growth factor in stem cells isolated from human exfoliated deciduous teeth

3.1 Abstract

Objective: This study aimed to investigate the role of endogenous basic fibroblast growth factor (bFGF) in stem cells isolated from human exfoliated deciduous teeth.

Methods: Cells were isolated from dental pulp tissues of human exfoliated deciduous teeth. The expression of stem cell markers was determined using conventional semi-quantitative polymerase chain reaction (PCR) and flow cytometry. The multipotential differentiation ability was also examined. The lentiviral shRNA or fibroblast growth factor receptor (FGFR) inhibitor was employed to inhibit bFGF mRNA expression and signal transduction, respectively. The colony formation ability was determined by low-density cell seeding protocol. The mRNA expression was evaluated using real-time quantitative PCR. The osteogenic differentiation was examined using alkaline phosphatase enzymatic activity assay and alizarin red staining.

Results: The results demonstrated that the cells isolated from human exfoliated deciduous teeth (SHEDs) exhibited stem cell characteristics, regarding marker expression and multipotential differentiation ability (osteogenic, adipogenic, and neurogenic lineage). The sh-bFGF transduced SHEDs had lower colony forming unit and higher mineralization than those of the control. Similarly, the decrease of colony

number and the increase of mineral deposition were noted upon exposing cells to FGFR chemical inhibitor.

Conclusion: These results imply that the endogenous bFGF may participate in the colony formation and osteogenic differentiation ability. In addition, the inhibition of bFGF signalling may be useful to enhance osteogenic differentiation of stem cells.

3.2 Introduction

Basic fibroblast growth factor (bFGF) has the influences on various types of cells and organs in regard of cell growth, differentiation, migration, and survival regulation (Yun, Won et al. 2010). It has also shown that bFGF enhances stemness maintenance as deter- mined by self-renewal capacity and maintaining of differentiation potency in several stem cell types (Kato and Gospodarowicz 1985, Tsutsumi, Shimazu et al. 2001, Yeoh and de Haan 2007). In this respect, the bFGF signalling has a crucial role in self-renewal capacity of human embryonic stem cells and human induced pluripotent stem cells (Amit, Carpenter et al. 2000). The bFGF supplementation in culture medium is necessary to maintain those cells in pluripotent stage (Ludwig, Levenstein et al. 2006).

Dental pulp tissues from human exfoliated deciduous teeth (SHEDs) contained multipotent stem cells, which expressed several mesenchymal stem cell markers i.e. STRO-1, CD73, CD90, CD105, CD146, CD166 and SSEA4 (Miura, Gronthos et al. 2003, Wang, Wang et al. 2010, Yamaza, Kentaro et al. 2010, Silva Fde, Ramos et al.

2014). SHEDs exhibited high colony forming unit and proliferative ability compared to bone marrow-derived mesenchymal stem cells (BMMSCs) (Yamaza, Kentaro et al. 2010). Further, it has been shown that SHEDs were able to differentiate into several lineages, for example osteogenic, adipogenic, and neurogenic lineage (Miura, Gronthos et al. 2003, Wang, Wang et al. 2010, Yamaza, Kentaro et al. 2010, Silva Fde, Ramos et al. 2014).

Previous study from our group illustrated that exogenous bFGF stimulated colony-forming units and enhanced expression of pluripotent stem cell markers in SHEDs and human dental pulp stem cells (DPSCs) (Osathanon, Nowwarote et al. 2011, Sukarawan, Nowwarote et al. 2014). Moreover, the osteogenic differentiation ability was attenuated in the presence of exogenous bFGF in culture medium. In this regard, the attenuation of alkaline phosphatase enzymatic activity and mineralization was noted in SHEDs, DPSC, and periodontal ligament stem cells (PDLSCs) (Osathanon, Nowwarote et al. 2011, Osathanon, Nowwarote et al. 2013). Therefore, in the present study, we aimed to investigate the influence of endogenous bFGF on SHEDs' behaviours regarding cell proliferation, alkaline phosphatase expression, and mineralization.

3.3 Materials and methods

3.3.1 Cell culture

The protocol to obtain cells was approved by Human Ethic Committee, Faculty of Dentistry, Chulalongkorn University. The inclusion criteria for teeth collection were the deciduous teeth from 6 to 12 year-old patients without caries, periodontal disease and periapical lesion. The teeth were shedding or scheduling in the treatment plan, which the extraction was indicated i.e. prolong retention teeth. Pulp tissues from human exfoliated deciduous teeth were carefully removed and minced into small pieces. Cell explants were cultured in normal growth medium contain 10% FBS (Hyclone, USA), 2 mM L-glutamine (Gibco, USA), 100 U/ml penicillin (Gibco, USA), 100 mg/ml streptomycin (Gibco, USA), 5 mg/ml ampho- tericin B (Gibco, USA) in 100% humidity, 37 8C and 5% carbon dioxide. Culture medium was changed every 48h. After reaching confluence, the cells were subcultured at 1:3 ratio and cells at passage 4–10 were used in the experiments.

For differentiation experiment, the protocol was performed according to previous reports (Osathanon, Nowwarote et al. 2011, Osathanon, Subbalekha et al. 2012, Osathanon, Ritprajak et al. 2013). Briefly, cells were maintained in osteogenic medium (growth medium supplemented with 50 mg/ml ascorbic acid, 10 mM beta-glycerophosphate and 100 nM dexamethasone), adipogenic medium (growth medium containing 0.1 mg/ml insulin, 1 mM dexamethasone, 1 mM IBMX and 0.2

mM indomethacin), or neurogenic medium (neurobasal medium containing 2% B27, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 5 mg/ml amphotericin B, 20 ng/ml bFGF, and 20 ng/ml EGF). Cells cultured in normal growth medium were employed as the control. For neurogenic differentiation, the petri dishes were employed for creating floating condition.

In some experiment, the inhibitor of fibroblast growth factor receptor (FGFR) (SU5402; Calbiochem, USA) was added in the culture medium at the concentration of 20 mM.

3.3.2 Lentiviral shRNA transduction

Lentiviral shRNA particle against bFGF (sc-39446-v; Santa Cruz Biotechnology, USA). The control lentiviral particle (sc-108080; Santa Cruz Biotechnology, USA) was employed as the control. Transduction protocol was performed according to the manufacturing protocol. Briefly, cells were seeded in 60 mm plate culture and replaced with 3 ml Lentivirus transduction solution (contained growth medium, 10 mg polybrene (Sigma, USA) and 1 106 virus particles) for 17 h. Subsequently, the medium was changed to normal growth medium. The selection of cells expressing shRNA transgene was performed using 1 mg/ml puromycin in normal growth medium.

3.3.3 Flow cytometry

Primary antibodies were FITC-conjugated anti-human CD44 mAb (BD Biosciences Pharmingen, USA), PerCP-CyTM5.5- conjugated anti-CD90 antibody (BD Biosciences Pharmingen, USA), PE-conjugated anti-human CD105 mAb (BD Biosciences Pharmingen, USA), PerCP-conjugated anti-CD45 antibody (BD Biosciences Pharmingen, USA), purified anti-human CD73 mAb (Abcam, USA), or purified anti-human STRO-1 mAb (Merck Millipore, USA). For CD73 and STRO-1, the biotinylated- goat anti-mouse IgG or biotinylated-goat anti-mouse IgM was used as the secondary antibody, following by FITC-conjugated or APC-streptavidin, respectively. Stained cells were analyzed on a FACSCalibur using the CellQuest software (BD Bioscience, USA). The presented values were the mean fluorescence intensity (MFI).

3.3.4 Colony forming unit assay

To evaluate colony forming unit ability, the low-density cell seeding technique was employed. Cells (500 cells) were seeded into 60-mm-diameter culture dishes and maintained in the normal growth medium. In some experiments, FGFR inhibitor was added in the culture medium. After 14 days, cells were fixed with 10% formalin for 10 min, washed twice with PBS and stained with coomassie blue (Sigma, USA).

3.3.5 Cell proliferation assay

MTT assay is a metabolic assay, in which the viable cells can reduce a tetrazolium compound to a formazan product. This assay was employed in the present study to indirectly illustrate the viable cells at different time point. In other words, the cell proliferation can be indirectly assumed if the number of viable cells increases corresponding to time. Cells were seeded at density of 12,500 cells per wells in 24-well-plate. The MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed at day 1, 3, and 7. Cells were treated with 1 mg/ml MTT solution (USB Corporation, USA) for 15 min at 37 8C. Formazan crystals were solubilized using DMSO and glycine buffer. Subsequently, the absorbance was examined by microplate reader at 540 mm.

3.3.6 Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton1-X100, and performed unspecific blocking with 10% horse serum in PBS for 1 h. The anti-b3-Tubulin (Promega, USA) was prepared at 1:100 dilutions and further incubated with the samples for 18 h at room temperature. After washed with PBS, the cell was incubated with biotinylated-goat anti-mouse antibody (Invitrogen, USA) at 1:500 dilutions for 45 min. The detection was performed using streptavidin-FITC (Sigma, USA) system. Cells were also counterstained with DAPI. The fluorescence was

evaluated by fluorescence microscope (Apotome). The secondary antibody control (omitting of primary antibody) was employed as the negative control.

3.3.7 Polymerase chain reaction (PCR) analysis

Total cellular RNA was extracted using Trizol reagent. RNA sample (1 mg) was converted to cDNA used RT kit (Improm-IITM Reverse Transcription system; Promega, USA). The cDNA amplification in semi-quantitative polymerase chain reaction (PCR) was performed used Taq polymerase (Invitrogen, USA). The PCR product was analyzed by 1.8% agarose gel electrophoresis and stained with ethidium bromide staining.

For real-time quantitative polymerase chain reaction, the amplified cDNA was detected using SYBR green detection system (FastStart Essential DNA Green Master; Roche Diagnostic, USA) on MiniOpticon real-time PCR system (Bio-Rad, USA). The value of gene expression was normalized to GAPDH expression. The primer sequences were shown in Table 3.

Table 3 Primer sequences

Gene	**Sequences** (F; forward, R; reverse)		**Reference**
GAPDH	F	5’ TGAAGGTCGGAGTCAACGGAT 3’	NM002046.4
	R	5’ TCACACCCATGACGAACATGG 3’	
GAPDH (real-time)	F	5’ CACTGCCAACGTGTCAGTGGTG 3’	NM002046.4
	R	5’ GTAGCCCAGGATGCCCTTGAG 3’	
RUNX2	F	5’ CCCCACGACAACCGCACCAT 3’	NM001278478.1
	R	5’ CACTCCGGCCCACAAATC 3’	
ALP	F	5’ CGAGATACAAGCACTCCCACTTC 3’	NM000478.3
	R	5’ CTGTTCAGCTCGTACTGCATGTC 3’	
OCN	F	5’ ATGAGAGCCCTCACACTCCTC 3’	NM199173.2
	R	5’ GCCGTAGAAGCGCCGATAGGC 3’	
LPL	F	5’ GAGATTTCTCTGTATGGCACC 3’	NM000237.2
	R	5’ CTGCAAATGAGACACTTTCTC 3’	
PPARγ2	F	5’ GCTGTTATGGGTGAAACTCTG 3’	NM015869.4
	R	5’ ATAAGGTGGAGATGCAGGCTC 3’	
NMD	F	5’ CACTGATAACTCGCCGTCCT 3’	NM002045.3
	R	5’ CTCTTCAGCTTGGCTGCTCT 3’	
β3-TUBULIN	F	5’ GGCCTCTTCTCACAAGTACG 3’	NM006086.3
	R	5’ CCACTCTGACCAAAGATGAAA 3’	

Table 3 Primer sequences (Continue)

Gene	**Sequences** (F; forward, R; reverse)		**Reference**
CD105	F	5’ CATCACCTTTGGTGCCTTCC 3’	NM000118.2
	R	5’ CTATGCCATGCTGCTGGTGGA 3’	
CD73	F	5’ ACACTTGGCCAGTAAAATAGGG 3’	NM001204813.1
	R	5’ ATTGCAAAGTGGTTCAAAGTCA 3’	
CD44	F	5’ ACAAGTTTTGGTGGCACGCA 3’	NM001202557.1
	R	5’ CAATCTTCTTCAGGTGGAGC 3’	
CD90	F	5’ CACTCTCACACCAATGCGGG 3’	NM006288.3
	R	5’ CGTTAGGCTGGTCACCTTCT 3’	
C-Myc	F	5’ AAGACTCCAGCGCCTTCTCTC 3’	NM002467.4
	R	5’ GTTTTCCAACTCCGGGATCTG 3’	
C-Fos	F	5’ ATGAGCCTTCCTCTGACTCG 3’	NM005252.3
	R	5’ ACGCACAGATAAGGTCCTCC 3’	
bFGF	F	5’ GGCTTCTTCCTGCGCATCCAC 3’	NM002006.4
	R	5’ GGTAACGGTTAGCACACACTCCTT 3’	

3.3.8 Alkaline phosphatase activity assay

The cells were extracted in alkaline lysis buffer. Further, the cell lyses were incubated with the substrate solution containing 2 mg/ml of p-nitrophenol phosphate (Invitrogen, USA), 0.1 M 2-amino-2-methyl-1-propanol and 2 mM MgCl2 for 30 min at 37 8C. Subsequently, NaOH (50 mM) was added to stop the reaction. The absorbance of product was measured using microplate reader at 410 nm. Total protein was deter- mined using BCA assay (Thermo Scientific, USA). The value of enzyme activity was normalized to total protein.

3.3.9 Alizarin red S staining

Cells were fixed with cold methanol for 10 min. After washed by deionized water, the cells were stained with 1% alizarin red S solution (Sigma, USA) at room temperature. Each of the stain specimens was washed by deionized water and air-dried. The quantification was determined by eluting staining in 10 mM sodium phosphate. The absorbance of product was measured used microplate reader at 570 nm.

3.3.10 Oil red O staining

Cells were fixed in 10% formalin for 30 min. After washed by PBS, the specimens were incubated with 60% isopropanol for 5 min

and further stained with oil red O solution for 5 min at room temperature and subsequently washed with deionized water.

3.3.11 Enzyme-linked immunosorbent assay (ELISA)

The amount of bFGF protein in culture medium was determined by ELISA methods according to manuals of ELISA kits protocol (R&D Systems, USA). The absorbance was measured using microplate reader at 450 nm.

3.3.12 Statistical analyses

The data was presented as means standard deviations. Statistical significance was assessed by independent two samples t test. The differences at $p < 0.05$ was

considered as a statistical significant difference. At least three biological replicates were employed in each experiment.

3.4 Results

3.4.1 SHEDs characterization

The isolated cells expressed stem cell markers, including CD44, CD73, CD90, CD105, and STRO-1, in both mRNA and protein levels (Fig. 2A and B). CD45+ cells were rare in the isolated cell population (Fig. 2A). Further, the multipotential differentiation ability was determined. Upon maintained cells in osteogenic differentiation medium for 14 days, the mineral deposition and mRNA expression of osteoblast markers (RUNX2, ALP, and OCN) were increased (Fig. 2C). For adipogenic differentiation, cells were maintained in induction medium for 16 days. The intracellular lipid droplets were noted as examined by oil red O staining (Fig. 2D). In addition, the upregulation of adipogenic marker gene (LPL and PPARg) was observed (Fig. 2D). Lastly, cells were examined the neurogenic differentiation capacity using neruosphere formation assay. Cells were cultured in the neuroinduction medium for 7 days. The formation of neurosphere was noted and those spheres expressed b3-Tubulin as identified by immunocytochemistry staining (Fig. 2E). The increase mRNA expression of b3-Tubulin and neuromodulin (NMD) was also observed (Fig. 2E). Together, the results imply the stem cell characteristics of the cells isolated from remaining pulp tissues of human exfoliated deciduous teeth.

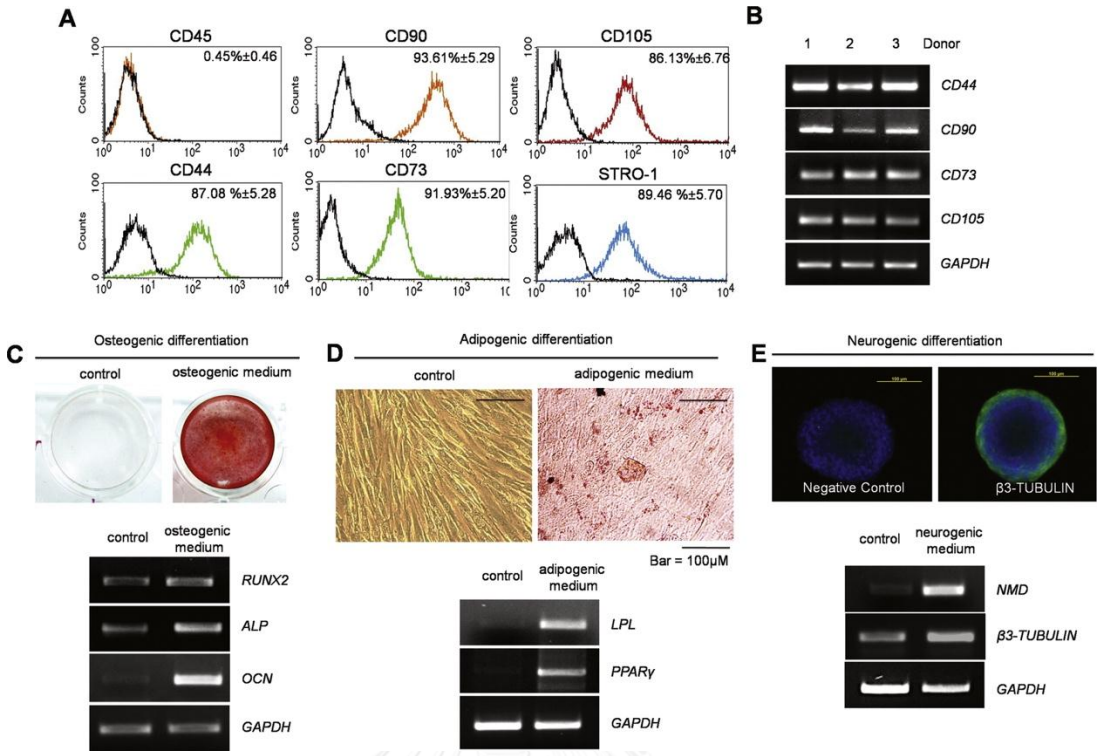

Figure 2 Stem cell characteristics. The expression of stem cells markers was evaluated using flow cytometry and conventional semi-quantitative PCR (A and B). The osteogenic, adipogenic, and neurogenic differentiation were evaluated at day 14, 16, and 7 after induction, respectively (C–E). The mineral deposition and intracellular lipid accumulation was determined using alizarin red and oil red O staining, respectively (C and D). The b3-Tubulin protein expression was evaluated by immunocytochemistry staining (E). The expression of differentiation marker was examined using conventional semi-quantitative PCR.

3.4.2 Influence of endogeneous bFGF on SHED's behaviours

To evaluate the requirement of endogenous bFGF in the regulation of stemness maintenance and osteogenic differentiation, the inhibition of endogeneous bFGF mRNA expression was performed using lentiviral shRNA. The marked reduction of bFGF mRNA expression was confirmed by both conventional semi-quantitative PCR and real-time PCR (Fig. 3A and B). In addition, the significant decrease of bFGF protein levels was noted using ELISA (Fig. 3C). Together, these results indicated the efficiency of the inhibition of bFGF expression in SHEDs. Further, cell proliferation was determined using MTT assay at day 1, 3 and 7 (Fig. 4A). The percentage of cell number was comparable between the sh-bFGF and the control. No significant difference on C-Fos and C-Myc was observed (Fig. 4B). Interestingly, the lower colony formation was noted in the sh-bFGF groups compared to the control (Fig. 4C). Upon culture cells in osteogenic condition, the ALP mRNA levels were slightly increased but no significant difference was noted (Fig. 4D). Though, the similar ALP enzymatic activity was observed. The significant difference was found at day 14 (Fig. 4E). For mineral deposition, the sh-bFGF groups exhibited higher calcium deposition compared to the control. The significance was noted at day 14 (Fig. 4F).

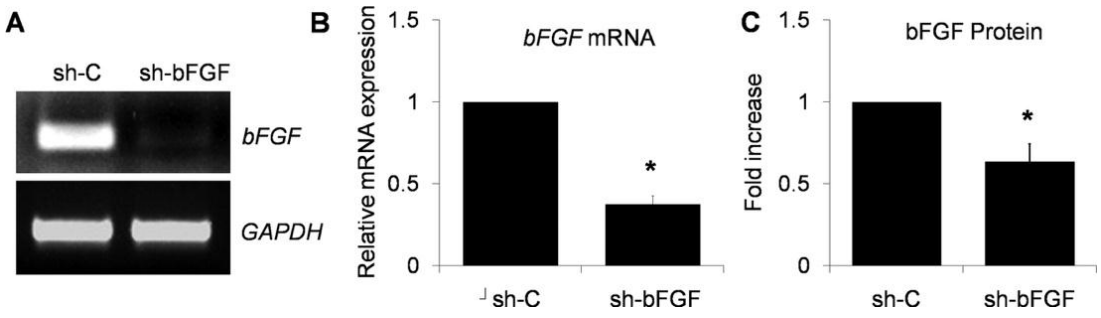

Figure 3 Transduction efficiency of sh-bFGF in stem cells isolated from human exfoliated deciduous teeth (SHEDs). SHEDs were transduced with a non-targeting control shRNA (sh-C) or shRNA targeting bFGF (sh-bFGF). bFGF mRNA expression was measured by conventional semi-quantitative PCR (A) and real-time quantitative PCR (B). The reduction of bFGF protein expression was determined by ELISA (C). The asterisks indicated the statistical significant difference compared to the control condition.

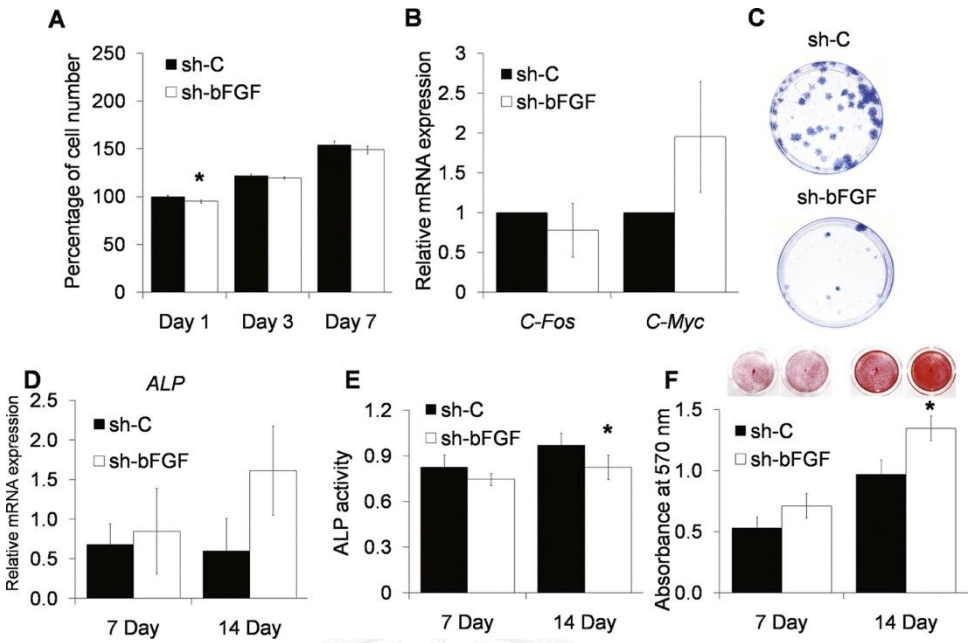

Figure 4 Influences of sh-bFGF transduction on SHEDs' behaviours. Graph illustrated the percentage of cell number as determined by MTT assay at day 1, 3, and 7 (A). The mRNA expression of proliferation related gene (C-Fos and C-Myc) was determined by real-time quantitative PCR (B). Colony formation was evaluated by coomassie blue staining at day 14 (C). Upon maintained cells in osteogenic medium for 7 and 14 days, the alkaline phosphatase (ALP) mRNA expression and enzymatic activity were examined (D and E). The mineral deposition was evaluated using alizarin red staining (F). The asterisks indicated the statistical significant difference compared to the control.

3.4.3 Effect of fibroblast growth factor receptor (FGFR) inhibition on SHEDs' behaviours

Chemical inhibition of FGFR was performed as an alternative approach to attenuate endogeneous bFGF signalling. The FGFR inhibitor (SU5402) was added in the condition medium. The results showed that the cell number was significant decreased in the condition supplemented with SU5402 (Fig. 5A). Correspondingly, the C-Fos and C-Myc mRNA expression was decreased compared to the control. The significant reduction was noted for C-Fos mRNA expression (Fig. 5B). In addition, the marked decreased of colony forming unit was noted in SU5402 supplemented condition (Fig. 5C). The trend of increased ALP mRNA expression, ALP enzymatic activity, and mineral deposition was noted in the presence of FGFR inhibitor at both 7 and 14 days (Fig. 5D–F).

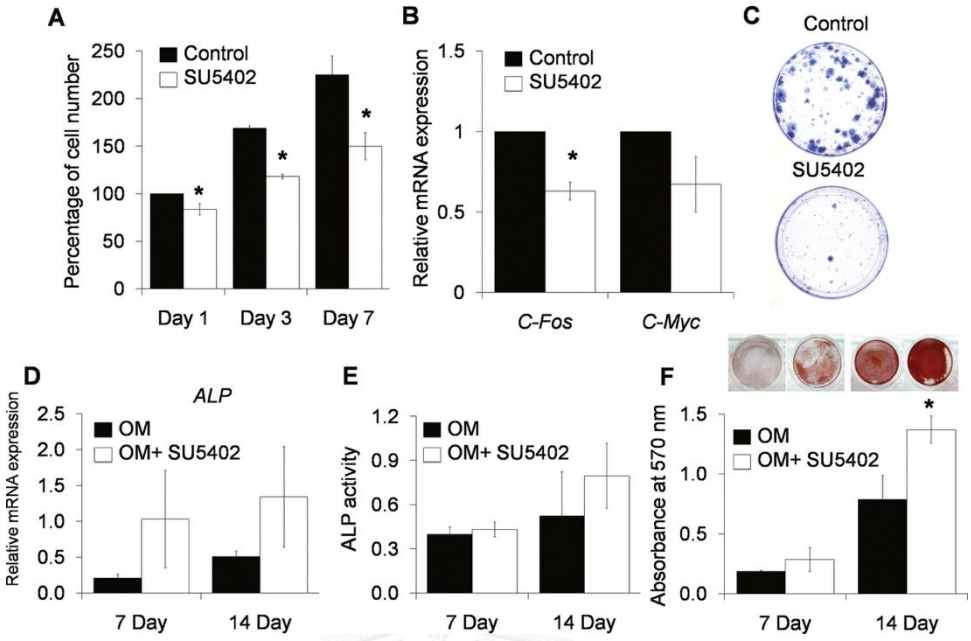

Figure 5 Influences of fibroblast growth factor receptor (FGFR) inhibitor on SHEDs' behaviours. Cells were maintained in the culture medium with or without the FGFR inhibitor (SU5402). Graph illustrated the percentage of cell number as determined by MTT assay at day 1, 3, and 7 (A). The mRNA expression of proliferation related gene (C-Fos and C-Myc) was determined by real-time quantitative PCR (B). The colony forming unit ability was evaluated at day 14 using coomasie blue staining (C). Further, cells were maintained in osteogenic induction medium for 7 and 14 days, the alkaline phosphatase (ALP) mRNA expression and enzymatic activity were examined (D and E). The mineral deposition was evaluated using alizarin red staining (F). The asterisks indicated the statistical significant difference compared to the control

3.5 Discussion

In the present study, we described the role of endogenous bFGF on cell proliferation and osteogenic differentiation of SHEDs. The attenuation of bFGF expression by shRNA did not exhibit the robust difference in proliferation and ALP expression. Though, the significant difference was noted for the colony formation and mineral deposition at day 14. It was hypothesized that the incomplete reduction of bFGF mRNA expression might result in the presence of bFGF protein, which can still bind to the receptor to initiate the intracellular signalling. Thus, the chemical inhibition of bFGF signalling was employed as an alternative approach. FGFR inhibitor (SU5402) interferes the binding of bFGF and its cell surface receptor, resulting in the blocking of further intracellular signal transduction. The FGFR inhibition significantly initiated the decrease of cell proliferation and colony formation. Under osteogenic differentiation, the inhibition of bFGF signalling resulted in the increase of ALP mRNA expression and enzymatic activity as well as mineral deposition. Therefore, these results suggest the role of endogenous bFGF in the control of SHEDs stemness maintenance and differentiation.

Correspondingly, it has been reported that the FGFR inhibition resulted in the differentiation enhancement and proliferation reduction in murine dental epithelial stem cell (Chang, Wang et al. 2013). However, it was noted in human adipose mesenchymal stem cells that FGFR inhibition reduced cell proliferation, colony

forming unit, and adipogenic differentiation ability, but did not influence the osteogenic differentiation (Zaragosi, Ailhaud et al. 2006, Rider, Dombrowski et al. 2008).

Our previous study illustrated that the addition of exogenous bFGF resulted in the enhancement of colony formation in SHEDs. Correspondingly, bFGF enhanced colony forming unit, cell proliferation, and cell migration by stem cells from inflamed dental pulp tissue from human deciduous teeth (inflamed SHEDs) (Kim, Park et al. 2014). Moreover, the similar effect of bFGF was noted in other stem cell types i.e. human stem cells from apical papilla (SCAPs) and megakaryocyte progenitor cell proliferation (Bruno, Cooper et al. 1993, Wu, Huang et al. 2012). In the present study, we demonstrated the reduction of cell proliferation and colony forming unit upon exposing SHEDs to SU5402, implying the role of bFGF signalling in stem cell proliferation. Correspondingly, it has previously shown that bFGF knockout human prostatic stromal cells had the decrease of cell proliferation (Wang, Cheng et al. 2011).

C-myc directly regulates cell cycle via control G1/s phase transition (Schmidt 1999). The c-myc protein has crucial function in ribosomal DNA transcription as well as RNA polymerase activation (Arabi, Wu et al. 2005) . In addition, c-myc has been shown to regulate cell proliferation in various cell types and its dysregulation results in tumor formation (Gordan, Thompson et al. 2007, Stoelzle, Schwarb et al. 2009, Jeong, Ahn et al. 2010, Cavalheiro, Matos-Rodrigues et al. 2014). C-fos was also

shown to regulate cell proliferation. The c-fos overexpression resulted in the enhanced promotor activity of cyclin A, a positive S phase regulator (Sylvester, Chen et al. 1998). The c-fos silencing led to the attenuation of cell proliferation (Zhang, Li et al. 2006). Further, it has been shown that bFGF regulated cell cycle via modulating the expression of c-myc and c-fos. The bFGF stimulated c-myc gene expression and stabilized c-myc protein (Lepique, Forti et al. 2000). In addition, c-fos could be activated by bFGF, resulting in the increase of cyclin D mRNA expression (Adepoju, Micali et al. 2014). Concur with the present study, FGFR inhibition led to the reduction of C-fos and C-Myc mRNA expression. Together, these data confirm the role of bFGF in maintaining stem cells population by promoting cell proliferation and colony formation.

The addition of exogenous bFGF resulted in the inhibition of osteoblast differentiation in various types of stem cells, including SHEDs, inflamed SHEDs, DPSCs, PDLSCs, and SCAPs (Miura, Gronthos et al. 2003, Osathanon, Nowwarote et al. 2011, Lee, Um et al. 2012, Wu, Huang et al. 2012, Osathanon, Nowwarote et al. 2013, Kim, Park et al. 2014). Particularly, exogenous bFGF suppressed ALP mRNA expression and enzymatic activity as well as mineral deposition in SHEDs (Osathanon, Nowwarote et al. 2013). Consistent with the present study, the increased trends of ALP mRNA expression and enzymatic activity as well as mineral deposition were noted upon treated SHEDs with FGFR inhibitor. The possible mechanism is that bFGF inhibited WNT/b-catenin pathway, leading to the attenuation of osteogenic differentiation

(Govitvattana, Osathanon et al. 2013). However, some publications reported that bFGF enhanced osteogenic differentiation (Pitaru, Kotev-Emeth et al. 1993, Sakaguchi, Janick et al. 1997). Therefore, the mechanism(s) of bFGF in osteogenic differentiation of mesenchymal stem cells requires further investigation.

In summary, the present study demonstrated the influence of endogenous bFGF on stem cell properties of SHEDs as well as their osteogenic differentiation ability. This knowledge could be further applied for the culture condition to maintain stemness of the SHEDs. In addition, the results for the present study could be beneficial for enhancing osteogenic differentiation of SHEDs, which could be subsequently utilized in the future osteogenic regenerative study.

3.6 Funding

This study was supported by the 2012 Research Chair Grant, Thailand National Science and Technology Development Agency (NSTDA).

3.7 Competing interests

All authors declare no conflict of interest.

3.8 Ethical approval statement

The protocol to obtain cells was approved by Human Ethic Committee, Faculty of Dentistry, Chulalongkorn University (No. 062/2012).

3.9 Acknowledgements

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CHAPTER IV

BASIC FIBROBLAST GROWTH FACTOR REGULATES REX1 EXPRESSION VIA IL-6 IN STEM CELLS ISOLATED FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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Basic fibroblast growth factor regulates REX1 expression via IL-6 in stem cells isolated from human exfoliated deciduous teeth

4.1 Abstract

Basic fibroblast growth factor (bFGF) regulates pluripotent marker expression and cellular differentiation in various cell types. However, the mechanism by which bFGF regulates *REX1* expression in stem cells isolated from human exfoliated deciduous teeth (SHEDs) remains unclear. The aim of the present study was to investigate the regulation of *REX1* expression by bFGF in SHEDs. SHEDs were isolated and characterized. Their mRNA and protein expression levels were determined using real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. In some experiments, chemical inhibitors were added to the culture medium to impede specific signaling pathways. Cells isolated from human exfoliated deciduous tooth dental pulp tissue expressed mesenchymal stem cell surface markers (CD44, CD73, CD90, and CD105). These cells differentiated into osteogenic and adipogenic lineages when appropriately induced. Treating SHEDs with bFGF induced *REX1* mRNA expression and this effect was attenuated by pretreatment with FGFR or Akt inhibitors. Cycloheximide pretreatment also inhibited the bFGF-induced *REX1* expression, implying the involvement of intermediate molecule(s). Further, the addition of an IL-6 neutralizing antibody attenuated the bFGF-induced *REX1* expression by SHEDs. In conclusion, bFGF enhanced *REX1* expression by SHEDs via

the FGFR and Akt signaling pathways. Moreover, IL-6 participated in the bFGF-induced *REX1* expression in SHEDs.

4.2 Introduction

Basic fibroblast growth factor (bFGF) regulates cell behavior and organogenesis in both development and regeneration (Thisse and Thisse 2005, Yun, Won et al. 2010). Numerous reports have shown that bFGF enhanced stemness maintenance, preserving stem cell multi-differentiation ability (Tsutsumi, Shimazu et al. 2001, Yeoh and de Haan 2007, Sukarawan, Nowwarote et al. 2014, Nowwarote, Pavasant et al. 2015). bFGF increases pluripotent stem cell marker gene expression, including *NANOG*, *OCT4*, and *REX1* by human stem cells from the apical papilla (SCAPs), human dental pulp (DPSCs), and human exfoliated deciduous teeth (SHEDs) (Osathanon, Nowwarote et al. 2011, Wu, Huang et al. 2012, Sukarawan, Nowwarote et al. 2014). Exogenous bFGF increases, while bFGF knockdown decreases, SHED colony forming unit ability (Sukarawan, Nowwarote et al. 2014, Nowwarote, Pavasant et al. 2015). In addition, bFGF treatment promotes human embryonic stem cell (hES) derivation (Wang, Xu et al. 2012). Together, these investigations imply an important role for bFGF in maintaining human stem cells' stemness.

REX1, a zinc finger transcription factor, is typically used as a pluripotent stem cell marker (Scotland, Chen et al. 2009, Son, Choi et al. 2013). REX1 is important in maintaining pluripotency in human pluripotent stem cells, with REX1 depletion

resulting in reduced stemness maintenance (Son, Choi et al. 2013). REX1 knockdown in hES resulted in lower pluripotent marker expression (OCT4, NANOG, and TRA-1-60) and higher lineage specific marker expression (NCAM, NKX2.5, and GATA4) (Son, Choi et al. 2013). Similarly, REX1 knockdown in human cord blood stem cells leads to significantly enhanced neurogenic differentiation (Langroudi, Forouzandeh et al. 2013). Further, adding REX1 to conventional reprograming factors improves the reprogramming efficiency of human fetal fibroblasts (Son, Choi et al. 2013). These results indicate the requirement of REX1 in the maintenance of human pluripotent stem cells.

Our previous study demonstrated that bFGF supplementation in both short-term and long-term culture enhanced *REX1* expression in SHEDs (Sukarawan, Nowwarote et al. 2014). In addition, previous work demonstrated that REX1 expression could be regulated by interleukin 6 (IL-6) in SHEDs (Govitvattana, Osathanon et al. 2013). Although, it has been reported that bFGF promoted IL-6 expression in various cells (Okamura, Sato et al. 1991, Kozawa, Suzuki et al. 1997, Kozawa, Tokuda et al. 1999, Bisping, Leo et al. 2003, Andoh, Bamba et al. 2004). The effect of bFGF on IL-6 expression is not yet identified in SHEDs. Moreover, the interaction/crosstalk between bFGF and IL-6 and intracellular mechanism controlling REX1 expression in SHEDs remains unclear. The present study investigated the intracellular signaling and the possible involvement of intermediate molecules in the bFGF-induced *REX1* expression in SHEDs.

4.3 Materials and methods

4.3.1 Cell isolation and culture

Cells were obtained from healthy exfoliated deciduous teeth of children 6–12 years old. The cell isolation protocol was approved by the Human Research Ethics Committee, Faculty of Dentistry, Chulalongkorn University (Study code: HREC-DCU 2015-007). The pulp tissues were removed and cut into small pieces. Cell explants were cultured in growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) (Hyclone, USA), 2mM L-glutamine (Gibco, USA), 100 unit/ml penicillin (Gibco, USA), 100 ug/ml streptomycin (Gibco, USA), and 5 ug/ml amphotericin B (Gibco, USA). The cells were maintained at 37°C in a humidified 5% carbon dioxide atmosphere. The culture medium was changed every 48 hours. When confluent, the cells were trypsinized with 0.25% trypsin-EDTA and subcultured at a 1:3 ratio and cells from passages 3–5 were used in the experiments.

The multilineage differentiation protocols were performed according to previous reports (Osathanon, Nowwarote et al. 2011, Osathanon, Ritprajak et al. 2013, Osathanon, Chuenjitkuntaworn et al. 2014). Briefly, cells were cultured in differentiation medium (Osteogenic induction medium consisting of growth medium supplemented with 50 mg/ml ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate or adipogenic induction medium consisting of growth medium

supplemented with 0.1 mg/ml insulin, 1 μM dexamethasone, 1 mM IBMX, and 0.2 mM indomethacin). Osteogenic and adipogenic marker gene expression was determined using real-time quantitative polymerase chain reaction (PCR). Alizarin red S and Oil Red O staining were used to evaluate mineral deposition and intracellular lipid accumulation, respectively. Cells cultured in growth medium served as the control.

The following concentrations of cytokines and chemical inhibitors were used in the present study: 5 or 10 ng/ml bFGF (Invitrogen, USA), 1 or 10 ng/ml IL-6 (R&D Systems Inc., USA), 20 μM FGFR inhibitor (SU5402; Calbiochem, USA), 10 μM MEK inhibitor (Calbiochem, USA), 10μM Akt inhibitor II (Calbiochem, USA), 2 μg/ml IL-6 neutralizing antibody (Anti-IL-6; R&D Systems Inc., USA), and 1 μg/mL cycloheximide (ta; Sigma, USA).

4.3.2 Flow cytometry

Cells were trypsinized and washed with phosphate buffered saline (PBS) containing 1% FBS. The cells were incubated for 15 min at 4°C in PBS containing 1% FBS with the primary antibodies at a 1:25 dilution. The antibodies used in the present study were FITC-conjugated anti-human CD44 mAb (BD Biosciences Pharmingen, USA), PerCP-CyTM5.5-conjugated anti-CD90 antibody (BD Biosciences Pharmingen, USA), PE-conjugated anti-human CD105 mAb (BD Biosciences Pharmingen, USA), PerCP-conjugated anti-CD45 antibody (BD Biosciences Pharmingen, USA), purified

anti-human CD73 mAb (Abcam, USA). For CD73, biotinylated-goat anti-mouse IgG was used as the secondary antibody, followed by FITC-conjugated or APC-streptavidin. The stained cells were analyzed on a FACSCalibur instrument using CellQuest software (BD Bioscience, USA). The presented values are the mean fluorescence intensity (MFI).

4.3.3 PCR analysis

Total RNA was extracted using RiboEx™ Total RNA isolation solution (GeneALL, Korea). RNA (1 ug) was converted to cDNA using an RT kit (Improm-II™ Reverse Transcription system; Promega, USA). For real-time quantitative PCR, the amplified cDNA was detected using the SYBR green detection kit (FastStart Essential DNA Green Master; Roche Diagnostic, USA) on a MiniOpticon real-time PCR system (Bio-Rad, USA). The gene expression value was normalized to 18S expression. The primer sequences are shown in Table 4.

Table 4 Primer sequences for real-time quantitative polymerase chain reaction

Gene	Accession numbers		Primer sequences
IL-6	NM000600.3	(Forward)	5’ ATGCAATAACCACCCCTGAC 3’
		(Reverse)	5’ AAAGCTGCGCAGAATGAGAT 3’
REX-1	NM174900.4	(Forward)	5’ TGGGAAAGCGTTCGTTGAGA 3’
		(Reverse)	5’ CACCCTTCAAAAGTGCACCG 3’
ALP	NM000478.4	(Forward)	5’ GACCTCCTCGGAAGACACTC 3’
		(Reverse)	5’ TGAAGGGCTTCTTGTCTGTG 3’
RUNX-2	NM001024630.3	(Forward)	5’ ATGATGACACTGCCACCTCTG 3’
		(Reverse)	5’ GGCTGGATAGTGCATTCGTG 3’
LPL	NM000237.2	(Forward)	5’ GAGATTTCTCTGTATGGCACC 3'
		(Reverse)	5' CTGCAAATGAGACACTTTCTC 3'
18S	NR003286.2	(Forward)	5’ GGCGTCCCCCAACTTCTTA3’
		(Reverse)	5’ GGGCATCACAGACCTGTTATT 3’

4.3.4 Alizarin Red S Staining

Cells were fixed with ice-cold methanol for 10 min and washed with deionized water. Mineral deposition was stained by a 1% Alizarin red S solution (Sigma, USA) for 3 min at room temperature. The staining was quantified by eluting

the stain in 10% cetylpyridium chloride monohydrate. The absorbance was measured at 570 nm.

4.3.5 Oil Red O Staining

Cells were fixed with 10% formalin for 30 minutes and washed with PBS. Subsequently, cells were incubated with 60% isopropanol for 5 minutes and further stained with Oil Red O solution at room temperature for 5 minutes and washed with deionized water.

4.3.6 Enzyme-linked immunosorbent assay (ELISA)

The amount of IL-6 protein in the culture medium was determined by ELISA per the ELISA kit protocol (R&D Systems Inc., USA). The absorbance was measured using a microplate reader at 450 nm.

4.3.7 Immunofluorescence staining

Cells were fixed in cold methanol, and performed unspecific blocking with 10% horse serum in PBS for 1 h. Primary antibody, REX-1 (R&D System, USA) was prepared dilutions 1:250 and then, incubated with the samples for 3 h at 37 $^{\circ}$C. After washed with PBS, the cell was incubated with biotinylated-secondary antibody (Abcam, USA) dilutions 1:500 for 45 min. The detection was performed using streptavidin-FITC (Sigma, USA). Cell nuclei were counterstained with DAPI. The fluorescence was evaluated by fluorescence microscope (Apotome). The secondary antibody control (omitting of primary antibody) was employed as the negative

control.

4.3.8 Statistical analysis

Cells from 5 donors were employed in the present study. The data is presented as box and whisker plots. Statistical significance was assessed using the Mann-Whitney U test for independent two-group comparison. For three or more group comparison, the Kruskal-Wallis test, followed by pairwise comparison was employed. Statistical analyses were performed using IBM SPSS Statistics for Mac, Version 22 (Armonk, NY, USA). Differences at $p<0.05$ were considered statistically significant.

4.4 Results

4.4.1 Stem cell characterization

The isolated cells expressed the mesenchymal stem cell markers CD44 (94.27%±4.77), CD73 (97.69%±2.38), CD90 (94.51%±4.07), and CD105 (87.33%±7.46) (Fig. 6A-D), while exhibiting negative staining for the hematopoietic marker CD45 (1.46%±0.46) (Fig. 6E). When culturing the cells in induction medium, the cells differentiated into osteogenic and adipogenic lineages. A significant up-regulation in *ALP* and *RUNX2* mRNA expression was observed after 7 days of osteogenic induction (Fig. 6F and G). At day 14, a significant increase in mineral deposition was found (Fig. 6H). For adipogenic differentiation, the cells were maintained in adipogenic induction medium for 16 days. Expression of the adipogenic marker gene *LPL* was significantly

increased (Fig. 6I), corresponding with increased accumulation of intracellular lipid accumulation as evaluated by Oil Red O staining (Fig. 6J). These results demonstrate that the cells isolated from pulp tissues of human exfoliated deciduous teeth exhibit mesenchymal stem cell characteristics.

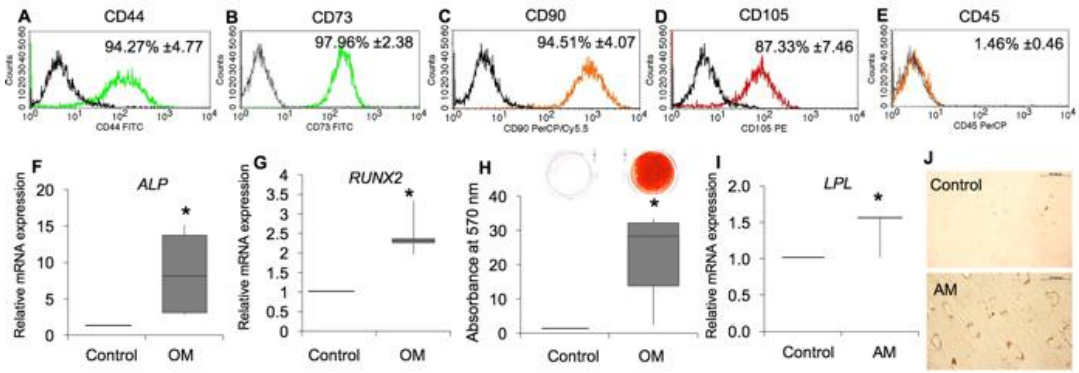

Figure 6 Characterization of the mesenchymal stem cells isolated from human exfoliated deciduous teeth (SHEDs). Mesenchymal stem cell surface marker levels (positive; CD44, CD73, CD90, and CD105 and negative; CD45) as determined by flow cytometry (A-E). Multilineage differentiation, osteogenic differentiation is shown by osteogenic marker gene expression at day 7 (*ALP* and *RUNX2*) by real-time quantitative polymerase chain reaction (F and G) and mineralization using alizarin red staining at day 14 (H). After adipogenic induction for 8 days, cells expressed the adipogenic marker gene *LPL* as analyzed by real-time quantitative polymerase chain reaction (I) and intracellular lipid droplets accumulation are present at 16 days as shown by oil red O staining (J). The asterisks indicate a significant difference compared with the control ($p<0.05$).

4.4.2 bFGF enhanced *REX1* mRNA expression in SHEDs

Cells were treated with 5 or 10 ng/ml bFGF for 24 h in serum free culture medium. The results showed that bFGF induced *REX1* mRNA expression in SHEDs (Fig. 7A). A significant increase in *REX1* expression was observed when SHEDS were treated with 10 ng/ml bFGF. In our time-course study, the cells were treated with 10ng/ml bFGF for 6, 12, and 24 h. A significant up-regulation in *REX1* mRNA level was demonstrated at 24 hours after bFGF treatment (Fig. 7B).

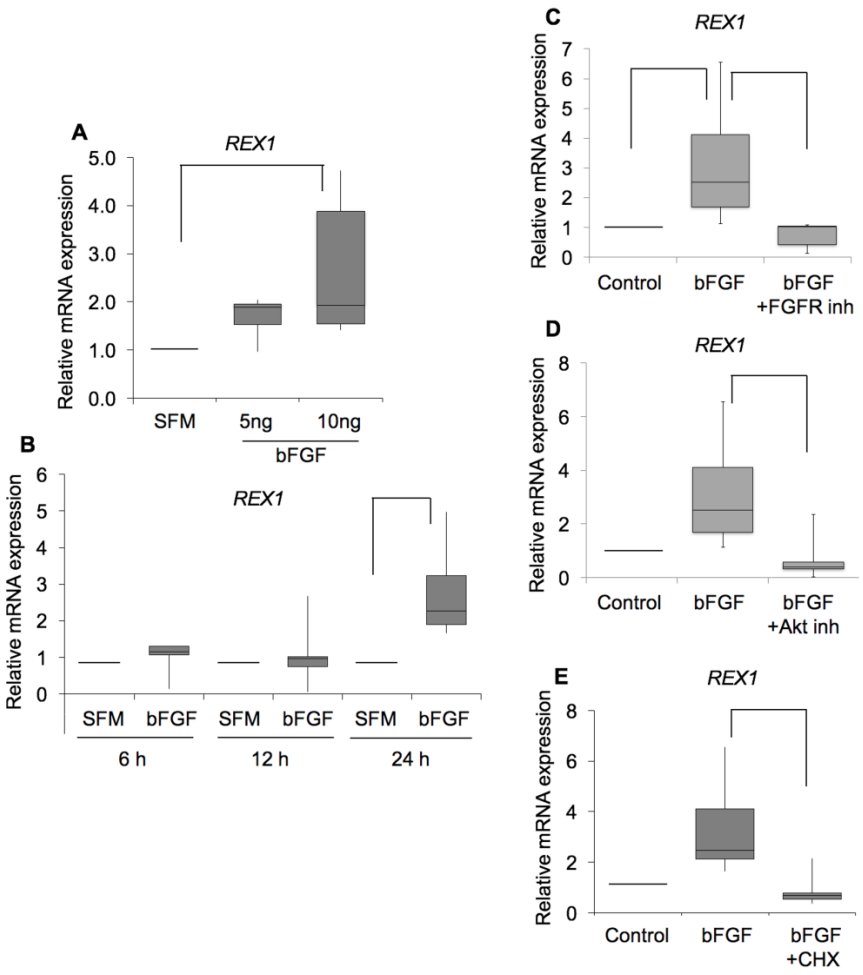

Figure 7 Effect of exogenous basic fibroblast growth factor on *REX-1* mRNA expressions in SHEDs. Exogenous bFGF dose-dependently induced *REX-1*mRNA expression in SHEDs as evaluated using real-time quantitative PCR (A) with a significant increase at 10ng/ml. Treatment with 10ng/ml bFGF from 6–24 h increased *REX-1* mRNA expression with a significant increase at 24 h (B). The bFGF-induced *REX-1* mRNA expression is inhibited by fibroblast growth factor receptor (FGFR inh) (C), Akt pathway (Akt inh) (D) and protein synthesis inhibitors (Cycloheximide; CHX) (E). The bars indicate a significant difference ($p<0.05$).

4.4.3 bFGF induced *REX1* mRNA expression via the FGFR and Akt pathways

SHEDs were pretreated with either an FGFR or Akt inhibitor for 30 min prior to bFGF treatment and then maintained in culture medium for 24 h. Pretreatment with the FGFR or Akt inhibitor significantly attenuated the bFGF-induced *REX1* expression (Fig. 7C and D). To determine whether bFGF-induced *REX1* expression required the translation of intermediate molecule(s), cycloheximide, a protein synthesis inhibitor, was used. SHEDs were pretreated with cycloheximide for 30 min before bFGF treatment. The results indicated that the bFGF-induced *REX1* mRNA level was significantly decreased by cycloheximide pretreatment (Fig. 7E), indicating the involvement of intermediate molecule(s). The REX-1 protein expression was evaluated using immunocytochemistry staining (Fig. 8). Similar results of REX-1 protein expression was observed as compared to mRNA expression pattern. In this regard, bFGF induced REX-1 protein expression (Fig. 8B). This effect was attenuated by pretreatment with FGFR inhibitor, Akt inhibitior, or cycloheximide (Fig. 8C-E).

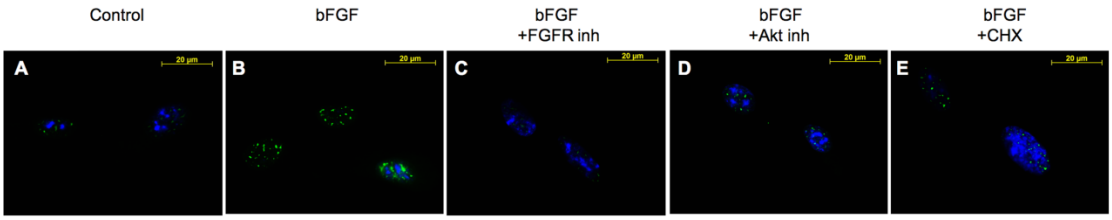

Figure 8 Effect of exogenous basic fibroblast growth factor on REX-1 protein expressions in SHEDs**.** Cells were treated with bFGF (10ng/ml) for 24 h and REX-1 protein expression was evaluated using immunocytochemistry. bFGF induced REX-1 expression (B) as compared with the control (A). FGFR inhibitor (FGFR inh)(C), Akt inhibitor (Akt inh) (D), or cycloheximide (CHX) (E) attenuated bFGF-induced REX-1 protein expression in SHEDs.

4.4.4 bFGF enhanced REX1 expression through IL-6

Previous work showed that IL-6 regulated *REX1* mRNA expression in SHEDs (Govitvattana, Osathanon et al. 2013). The present study confirmed that IL-6 treatment enhanced *REX1* expression (Fig. 9). A significant increase in *REX1* mRNA was observed when cells were exposed to 10 ng/ml IL-6. The upregulation of REX-1 protein expression was observed after IL-6 treatment (Fig. 9B and 9C). This result indicates the participation of IL-6 in the regulation of *REX1* expression in SHEDs.

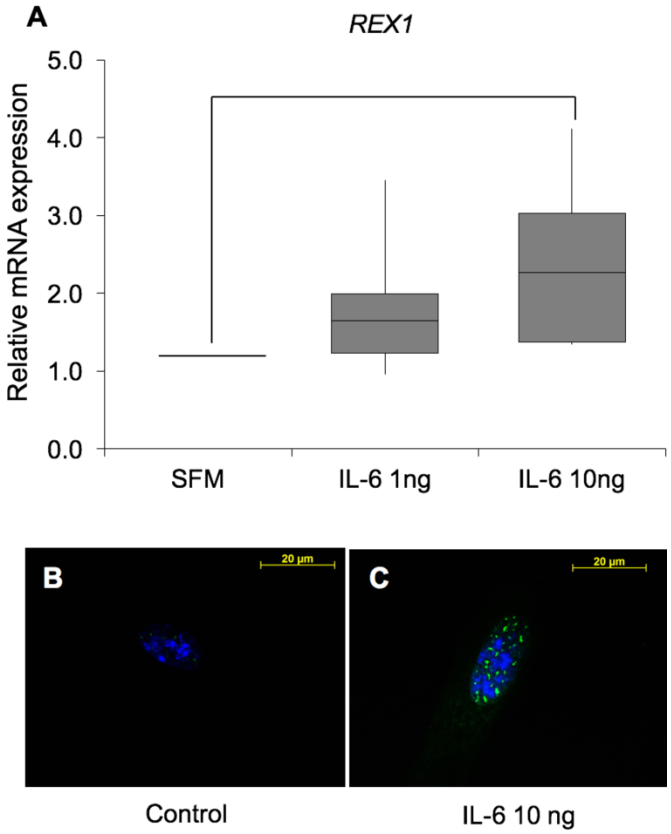

Figure 9 Effect of exogenous IL-6 on REX-1 expression in SHEDs. Exogenous IL-6 dose dependently induced *REX-1* mRNA expression in SHEDs with a significant increase at 10 ng/ml, as evaluated using real-time quantitative PCR. Cells were treated with IL-6 for 24 h and and REX-1 protein expression was evaluated using immunocytochemistry (B and C). The bars indicate a significant difference compared with the control ($p<0.05$).

bFGF treatment enhanced IL-6 expression at both the mRNA and protein levels (Fig. 10A-B). A significant increase in IL-6 mRNA expression was noted when the SHEDs were treated with 10 ng/ml bFGF. Further, in the time course experiment,

bFGF significantly induced *IL-6* mRNA expression at 24 h (Fig. 10C). However, a significant up-regulation of IL-6 protein expression was observed as early as 12 h after bFGF treatment (Fig. 10D). In contrast, IL-6 treatment did not influence *bFGF* expression levels in SHEDs (Supplement Fig. 1).

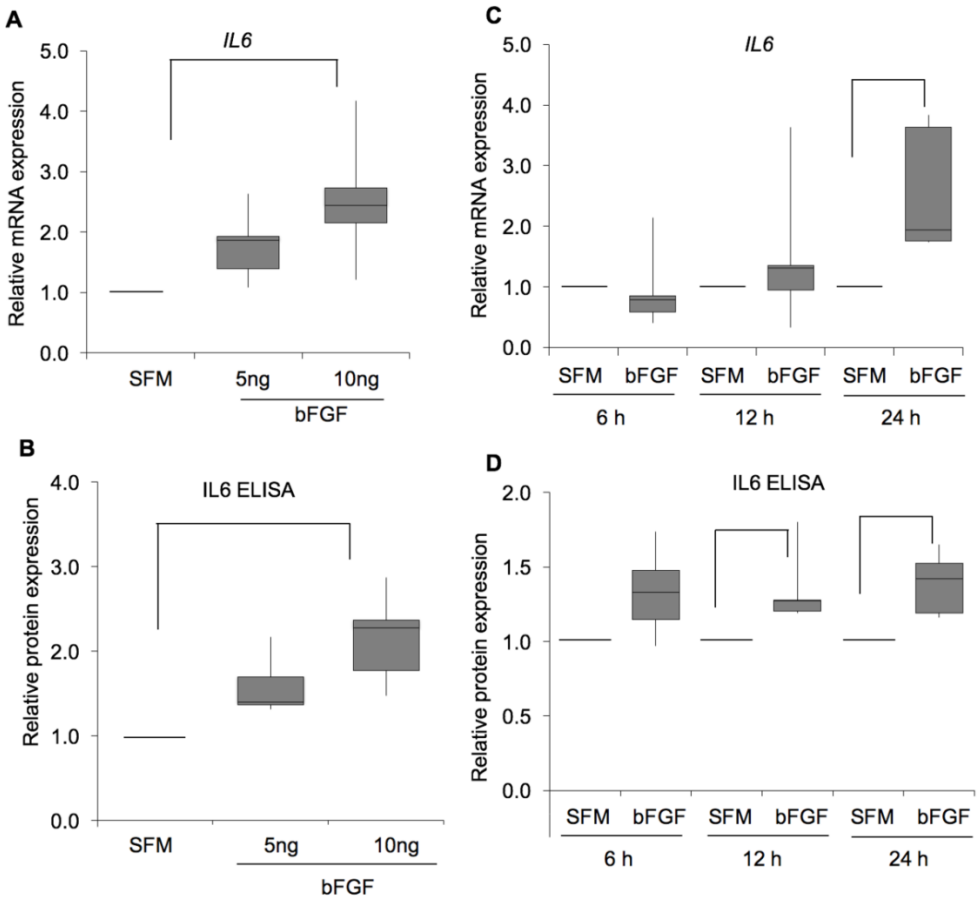

Figure 10 Effect of basic fibroblast growth factor on *IL-6* mRNA expression and IL-6 protein level in SHEDs. Exogenous bFGF dose dependently induced *IL-6* mRNA expression in SHEDs, with a significant increase at 10 ng/ml, as evaluated using real-time quantitative polymerase chain reaction (A). SHEDs were maintained in serum free medium with exogenous bFGF. bFGF dose-dependently increased IL-6 protein levels with a significant increase at 10 ng/ml as shown by ELISA (B). Treatment with 10 ng/ml bFGF from 6–24 h significantly increased mRNA expression at 24 h (C) and significantly increased protein levels at 12 and 24 h (D). The bars indicate a significant difference compared with the control ($p<0.05$).

We further investigated whether bFGF-induced *IL-6* expression utilizes similar signaling pathways to those of bFGF-induced *REX1* expression. SHEDs were pretreated with an FGFR, MEK, or Akt inhibitor for 30 min prior to bFGF treatment. The results demonstrated that the FGFR, MEK, and Akt inhibitors attenuated the bFGF-enhanced *IL-6* expression (Fig. 11A-C). However, cycloheximide failed to inhibit the effect of bFGF on IL-6 expression (Fig. 11D).

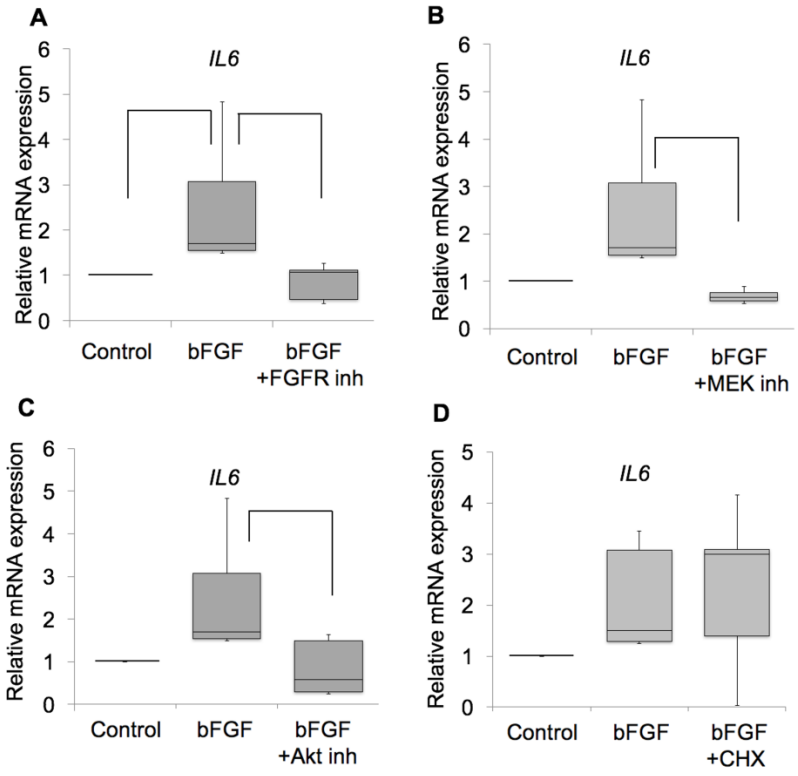

Figure 11 Effect of fibroblast growth factor receptor inhibition and intracellular signaling inhibition on bFGF-induced *IL-6* mRNA expression in SHEDs. Exogenous bFGF significantly induced *IL-6* mRNA expression (A), whereas the inhibition of FGFR (B), MEK, and AKT (C) significantly decreased *IL-6* mRNA expression. Cycloheximide, however, did not inhibit IL-6 mRNA expression (D). The bars indicate a significant difference compared ($p<0.05$).

To determine the involvement of IL-6 in bFGF-induced *REX1* expression, cells were pretreated with an anti-IL-6 neutralizing antibody. When neutralizing IL-6, bFGF-induced *REX1* mRNA expression was significantly attenuated (Fig. 12). Correspondingly, neutralizing IL-6 antibody attenuated bFGF-induced REX-1 protein

expression (Fig. 12B). Taken together, these results indicate that IL-6 participates in the bFGF-induced *REX1* expression in SHEDs.

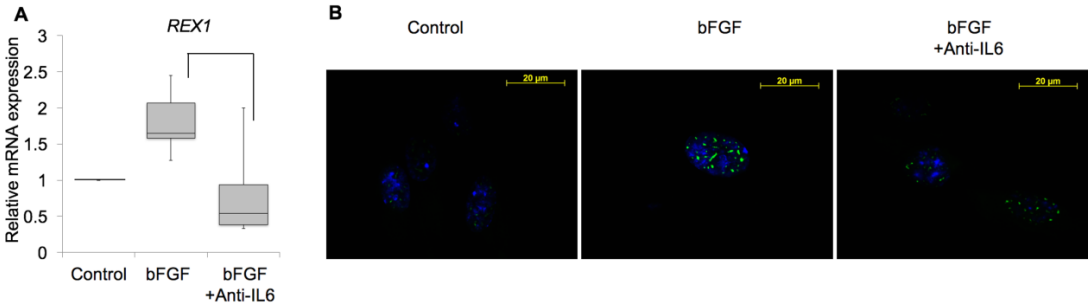

Figure 12 Involvement of IL-6 on bFGF-induced REX-1 expression. REX-1 mRNA expression when SHEDs were pretreated with an anti-IL-6 neutralizing antibody prior to bFGF treatment (A). Cells were treated with IL-6 (10ng/mL) for 24 h with or without anti-IL-6 neutralizing antibody pretreatment and REX-1 protein expression was determined using immunocytochemistry staining (B). The bars indicate a significant difference ($p<0.05$).

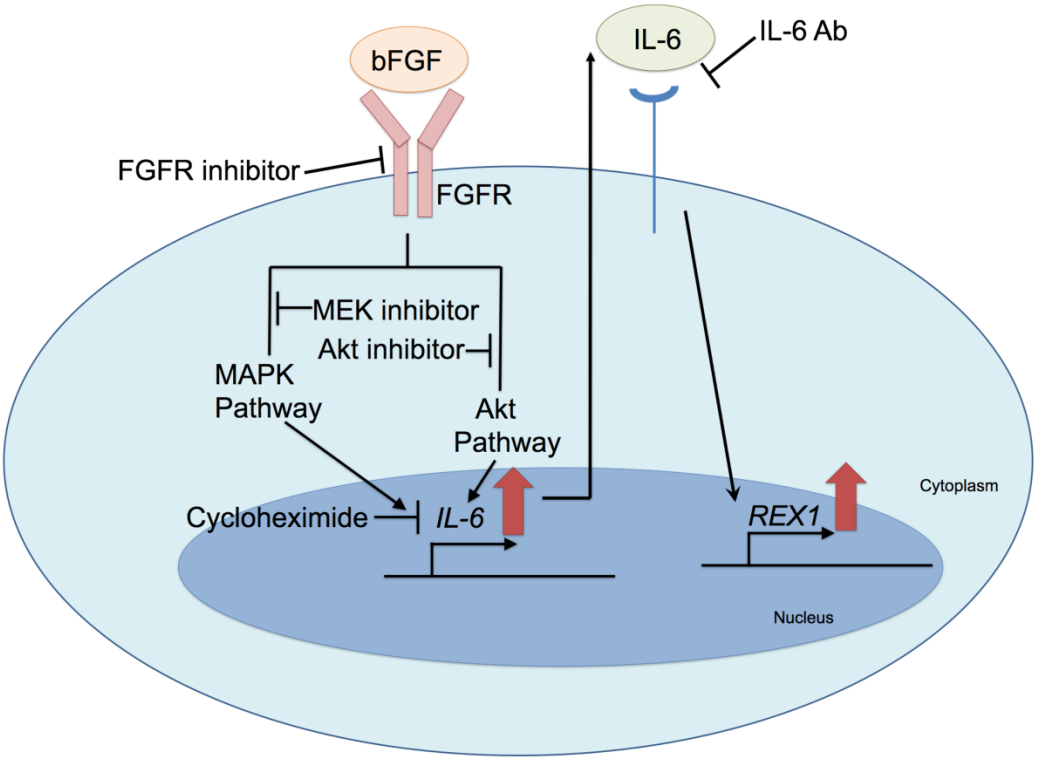

Figure 13 Schematic model of the mechanism of the bFGF-nduced REX1 expression in SHEDS. bFGF signals through the FGF receptor, activating the MAPK and AKT intracellular signaling pathways as shown by the use of signaling inhibitors, and inducing the expression and secretion of IL-6. Extracellular IL-6 then stimulates the expression of REX1 as demonstrated by the use of the IL-6 neutralizing antibody. Cycloheximide inhibition of IL-6 abrogates the bFGF-induced REX1 expression, indicating its intermediate role in REX1 expression.

4.5 Discussion

The present study investigated the signaling mechanisms whereby bFGF stimulated the expression of *REX1* in SHEDs. Our results demonstrated that bFGF induced *REX1* expression via the FGFR and Akt signaling pathways. In addition, the regulation of IL-6 by bFGF participates in the bFGF-induced *REX1* expression in SHEDs.

The isolated cells exhibited mesenchymal stem cell surface markers and differentiated into osteogenic and adipogenic lineages when appropriately induced. Thus, our results confirm the stem cell properties of cells isolated from the dental pulp tissue of human exfoliated deciduous teeth. These findings are consistent with those of previous studies (Govitvattana, Osathanon et al. 2013, Osathanon, Nowwarote et al. 2013, Nowwarote, Pavasant et al. 2015).

REX-1 is a pluripotent stem cell marker expressed in undifferentiated cells (Son, Choi et al. 2013). Serial passage of human amnionic mesenchymal stem cells exhibited decreased *REX-1* mRNA expression (Fatimah, Tan et al. 2013). In addition, decreased *REX-1* expression was observed in SHEDS at passage 10 compared with those at passage 5 (Sukarawan, Nowwarote et al. 2014). In our study, we demonstrated that bFGF enhanced *REX-1* mRNA expression in SHEDs. Our result corresponds with that of a previous study where bFGF promoted *REX1* expression in SCAPs (Wu, Huang et al. 2012). Long-term bFGF treatment upregulated *REX1* expression in SHEDs at passage 10 (Sukarawan, Nowwarote et al. 2014). These results

imply that bFGF regulates *REX1* expression and may participate in stemness maintenance in SHEDs.

bFGF utilizes various intracellular pathways to regulate cell responses, e.g. phosphatidylinositol-4,5-bisphosphate 3- kinase (PI-3 kinase)/protein kinase B (Akt), JAK/STAT, Ras (small GTPase)-mitogen-activated protein kinase, and Phospholipase C-gamma (PLCγ) (Nowwarote, Sawangmake et al. 2015). For example, bFGF regulated mesenchymal stem cell migration via the Akt pathway (Schmidt, Ladage et al. 2006). Furthermore, bFGF induced neural progenitor cell proliferation via PI3K/GSK3 signaling (Jin, Hu et al. 2005) and promoted bone marrow mesenchymal stem cell proliferation through the Akt and ERK1/2 pathways (Choi, Kim et al. 2008). In dental tissue-derived mesenchymal stem cells, previous studies demonstrated that bFGF enhanced neurogenic differentiation in human dental pulp cells via the PLCγ signaling pathway and inhibited osteogenic differentiation in SHEDs via the ERK1/2 signaling pathway (Osathanon, Nowwarote et al. 2011, Li, Qu et al. 2012). In the present study, we demonstrated that bFGF regulated *REX1* expression via the FGFR and Akt signaling pathways, implying that bFGF controls cell lineage by stimulating multiple pathways. Therefore, specific intracellular signaling initiation is crucial in bFGF regulated cell responses.

The present study illustrated that bFGF increased *REX1* expression via upregulating IL-6 at both the mRNA and protein levels. However, IL-6 treatment did

not affect *bFGF* expression levels in SHEDs. Cycloheximide pretreatment resulted in the attenuation of the bFGF-induced *REX1* expression, indicating the involvement of intermediate molecule(s). A previous study reported that IL-6 treatment increased *REX1* expression in SHEDs (Govitvattana, Osathanon et al. 2013). Interestingly, the results in the present study demonstrated that bFGF regulated IL-6 expression in SHEDs via the FGFR and Akt signaling pathways similar to that of the bFGF regulated *REX1* expression. Further, IL-6 neutralization led to reduced bFGF-induced *REX1* expression, suggesting the participation of IL-6 in the regulation of *REX1* by bFGF. These results indicate that bFGF stimulates IL-6 secretion, which induces *REX1* expression. Correspondingly, IL-6 treatment also induces *REX1* expression in SHEDs. Consequently, IL-6 may play a role as an intermediate molecule to maintain SHED stemness. A schematic model of our proposed pathway is shown in Figure 13.

Previous publications reported that bFGF induced IL-6 protein synthesis in murine pre-osteoblast cells (MC3T3-E1), human omental microvascular endothelial cells, human pancreatic periacinar myofibroblast cells, and human bone marrow stromal cells (Okamura, Sato et al. 1991, Kozawa, Suzuki et al. 1997, Kozawa, Tokuda et al. 1999, Bisping, Leo et al. 2003, Andoh, Bamba et al. 2004). bFGF-induced IL-6 expression is hypothesized to involve in acute inflammation process, tumor progression, and bone metabolism. The present study indicates the involvement of bFGF-induced IL-6 in the regulation of stemness maintenance partly via the upregulation of REX1 in SHEDs.

In MC3T3-E1 cells, bFGF promoted nuclear factor interleukin-6 (NFIL-6) production and cycloheximide failed to inhibit the bFGF-induced NFIL-6 and IL-6 expression (Hurley, Abreu et al. 1996). Corresponding with this study, we found that the effect of bFGF on IL-6 expression in SHEDs was not inhibited by cycloheximide treatment. These results imply that bFGF directly regulates IL-6 expression.

A previous study demonstrated that bFGF induced IL-6 expression by regulating intracellular Ca^{2+} mobilization in MC3T3-E1 cells (Kozawa, Suzuki et al. 1997). Moreover, the use of a MEK or p38 MEP kinase inhibitor attenuated the bFGF-induced IL-6 expression in MC3T3-E1 cells and human pancreatic periacinar myofibroblast cells (Kozawa, Tokuda et al. 1999, Andoh, Bamba et al. 2004, Tokuda, Takai et al. 2008). Similarly, in the present study, FGFR and MEK inhibitors abolished the inductive effect of bFGF on IL-6 expression in SHEDs, confirming these previous findings. In addition, the present study reveals another potential intracellular pathway, which regulates IL-6 expression after bFGF treatment in SHEDs. We demonstrated the involvement of the Akt signaling pathway, with the Akt inhibitor attenuating the bFGF-mediated IL-6 expression in SHEDs.

Several reports illustrated that IL-6 enhanced bFGF expression in various cell types, such as basal cell carcinoma and myeloma cells (Bisping, Leo et al. 2003, Jee, Chu et al. 2004). IL-6 promoted bFGF expression via the JAK and PI3K signaling pathways in basal cell carcinoma cells (Jee, Chu et al. 2004). However, our study

demonstrated that IL-6 did not influence bFGF expression in SHEDs. Therefore, this result implies that bFGF is upstream to IL-6 and a positive feedback loop that synergistically enhances IL-6 expression may not occur in SHEDs.

IL-6 is generally known as a cytokine that regulates inflammatory responses. However, other studies indicate that IL-6 is also an important molecule in stemness maintenance and cell differentiation (Pricola, Kuhn et al. 2009, Gallagher, Norman et al. 2013, Govitvattana, Osathanon et al. 2013, Yoon, Kim et al. 2014, Govitvattana, Osathanon et al. 2015). It has been shown that IL-6 supplementation together with soluble IL-6 receptor enhanced the osteogenic differentiation of periodontal ligament cells and adipose derived stem cells (Iwasaki, Komaki et al. 2008, Huh and Lee 2013). Further, IL-6 induces lineage commitment and loss of stemness maintenance via the decrease of SOX2 expression in BM-MSCs (Yoon, Kim et al. 2014). On the contrary, IL-6 inhibited stem cell differentiation. IL-6 attenuated the chondrogenic differentiation of bone marrow derived mesenchymal stem cells (BM-MSCs) (Wei, Shen et al. 2013). In SHEDs, Previous reports demonstrated that IL-6 induced REX-1 expression via the JAK and ATP-P2Y1 signaling pathways (Govitvattana, Osathanon et al. 2013, Govitvattana, Osathanon et al. 2015). In the present study, we found that bFGF induced REX-1 expression partly via IL-6, suggesting that IL-6 plays a role in stemness maintenance in SHEDs. Similarly, previous report demonstrated that IL-6 promoted cell proliferation and inhibited adipogenic as well as chondrogenic differentiation in BM-MSCs (Pricola, Kuhn et al. 2009). Though, osteogenic

differentiation by BM-MSCs did not alter by the addition of IL-6 (Pricola, Kuhn et al. 2009). Therefore, the influence of IL-6 on SHED differentiation potency should be further investigated.

In summary, bFGF regulates stemness maintenance in SHEDs by enhancing *REX-1* mRNA expression via the FGFR and Akt signaling pathways. Moreover, IL-6 is also involved in the bFGF-induced *REX1* expression. Results from the present study reveal the mechanism regulating stemness maintenance in SHEDs and could be used to apply for an *in vitro* maintaining of stem cells.

4.6 Acknowledgements

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4.7 Disclosure Statement

All authors declare no conflict of interest.

CHAPTER V

SUMMARY

5.1 SUMMARY

As described in Chapter 2, bFGF regulate stem cell behaviours, depending on several factors, including dose, exposure time and cells type. Therefore, a careful investigation of bFGF function in specified cell types in specific settings are necessary to understand the complex regulation of dental MSC behaviors by bFGF. Our experiments finding are to gain sufficiency basic knowledge in stem cell properties in SHEDs by bFGF. The inhibition of endogenous bFGF signaling led to the decrease of colony forming unit ability and the increase of mineralization by SHEDs. These results corresponded with previous reports demonstrating the influence of exogenous bFGF on SHEDs proliferation and differentiation. Our present study also demonstrated that bFGF may participate in stemness maintenance in SHEDs, partly via the upregulation of REX1 expression. The results showed that exogenous bFGF enhanced REX1 expression via FGFR and Akt signalling pathway. Moreover, exogenous IL-6 also participates in bFGF-induced *REX1* expression in SHEDs .

Due to ethical problem, dental tissue-derived stem cells are a good source of stem cells for use in regenerative medicine. However, a cell amplification process is indeed required to secure a comfortable cell number for suitable potential. Taken from our results, these evidences support the influence of bFGF in the maintaining of

stemness. In addition, the regulation of differentiation, particularly osteogenic differentiation, could be controlled by bFGF. All knowledge could be used to apply for an *in vitro* maintaining during amplification process. Moreover, control differentiation of stem cell for used in the future regenerative medicine.

5.2 POTENTIAL PITFALLS

This study was performed in human primary cell must have been approved protocol by ethical committee, faculty of dentistry, Chulalongkorn University. The experiment was performed in the laboratory-controlled parameters, there for the result not represent in the physiological of human body. Even though method to isolate cell, tooth location, cell passage, medium culture, genetic in each patient, sex, had affect to SHEDs.

Role of endogenous bFGF were investigated both genetic manipulation and chemical inhibition on stemness maintenance determined by following stem cell marker gene expression, cell cell proliferation and differentiation potency. Thus, effect of endogenous bFGF was depend on time and method to use in this study and effect of exogenous bFGF depend on dose and frequent.

5.3 FUTURE STUDY

The future investigation on specific population is indeed required. This knowledge could be further applied for the culture condition to maintain stemness of the SHEDs. In addition, the results for the present study could be beneficial for

enhancing osteogenic differentiation of SHEDs, which could be subsequently utilized in the future osteogenic regenerative study.

Beside role of bFGF in stem cells *in vitro*, various potential application of bFGF in regenerative medicine could be further investigated. For example, bFGF-priming biomaterials could be examined for the enhancement of tissue and organ healing *in vivo.*

REFERENCES

CHAPTER I

Bai, Y., P. Li, G. Yin, Z. Huang, X. Liao, X. Chen and Y. Yao (2013). "BMP-2, VEGF and bFGF synergistically promote the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells." Biotechnol Lett **35**(3): 301-308.

Bartalena, L., S. Brogioni, L. Grasso and E. Martino (1995). "Interleukin-6 and the thyroid." Eur J Endocrinol **132**(4): 386-393.

Bisping, G., R. Leo, D. Wenning, B. Dankbar, T. Padro, M. Kropff, C. Scheffold, M. Kroger, R. M. Mesters, W. E. Berdel and J. Kienast (2003). "Paracrine interactions of basic fibroblast growth factor and interleukin-6 in multiple myeloma." Blood **101**(7): 2775-2783.

Brady, J. J., M. Li, S. Suthram, H. Jiang, W. H. Wong and H. M. Blau (2013). "Early role for IL-6 signalling during generation of induced pluripotent stem cells revealed by heterokaryon RNA-Seq." Nat Cell Biol **15**(10): 1244-1252.

Dankbar, B., T. Padro, R. Leo, B. Feldmann, M. Kropff, R. M. Mesters, H. Serve, W. E. Berdel and J. Kienast (2000). "Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma." Blood **95**(8): 2630-2636.

Erta, M., A. Quintana and J. Hidalgo (2012). "Interleukin-6, a major cytokine in the central nervous system." Int J Biol Sci **8**(9): 1254-1266.

Gallagher, D., A. A. Norman, C. L. Woodard, G. Yang, A. Gauthier-Fisher, M. Fujitani, J. P. Vessey, G. I. Cancino, N. Sachewsky, K. Woltjen, M. P. Fatt, C. M. Morshead, D. R. Kaplan and F. D. Miller (2013). "Transient maternal IL-6 mediates long-lasting changes in neural stem cell pools by deregulating an endogenous self-renewal pathway." Cell Stem Cell **13**(5): 564-576.

Gelinas, A. D., D. R. Davies, T. E. Edwards, J. C. Rohloff, J. D. Carter, C. Zhang, S. Gupta, Y. Ishikawa, M. Hirota, Y. Nakaishi, T. C. Jarvis and N. Janjic (2014). "Crystal structure of interleukin-6 in complex with a modified nucleic acid ligand." J Biol Chem **289**(12): 8720-8734.

Govindasamy, V., V. S. Ronald, A. N. Abdullah, K. R. Nathan, Z. A. Ab Aziz, M. Abdullah, S. Musa, N. H. Kasim and R. R. Bhonde (2011). "Differentiation of dental pulp stem cells into islet-like aggregates." J Dent Res **90**(5): 646-652.

Govitvattana, N., T. Osathanon, S. Taebunpakul and P. Pavasant (2013). "IL-6 regulated stress-induced Rex-1 expression in stem cells from human exfoliated deciduous teeth." Oral Dis **19**(7): 673-682.

Hernandez-Quintero, M., W. Kuri-Harcuch, A. Gonzalez Robles and F. Castro-Munozledo (2006). "Interleukin-6 promotes human epidermal keratinocyte

proliferation and keratin cytoskeleton reorganization in culture." Cell Tissue Res **325**(1): 77-90.

Ishimi, Y., C. Miyaura, C. H. Jin, T. Akatsu, E. Abe, Y. Nakamura, A. Yamaguchi, S. Yoshiki, T. Matsuda, T. Hirano and et al. (1990). "IL-6 is produced by osteoblasts and induces bone resorption." J Immunol **145**(10): 3297-3303.

Jones, S. A., S. Horiuchi, N. Topley, N. Yamamoto and G. M. Fuller (2001). "The soluble interleukin 6 receptor: mechanisms of production and implications in disease." FASEB J **15**(1): 43-58.

Keller, E. T., J. Wanagat and W. B. Ershler (1996). "Molecular and cellular biology of interleukin-6 and its receptor." Front Biosci **1**: d340-357.

Kishimoto, T. (1989). "The biology of interleukin-6." Blood **74**(1): 1-10.

Kopf, M., M. F. Bachmann and B. J. Marsland (2010). "Averting inflammation by targeting the cytokine environment." Nat Rev Drug Discov **9**(9): 703-718.

Kozawa, O., A. Suzuki and T. Uematsu (1997). "Basic fibroblast growth factor induces interleukin-6 synthesis in osteoblasts: autoregulation by protein kinase C." Cell Signal **9**(6): 463-468.

Li, Y. Y., L. L. Hsieh, R. P. Tang, S. K. Liao and K. Y. Yeh (2009). "Interleukin-6 (IL-6) released by macrophages induces IL-6 secretion in the human colon cancer HT-29 cell line." Hum Immunol **70**(3): 151-158.

Lin, Z. Q., T. Kondo, Y. Ishida, T. Takayasu and N. Mukaida (2003). "Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice." J Leukoc Biol **73**(6): 713-721.

Metcalf, D. (1993). "Hematopoietic regulators: redundancy or subtlety?" Blood **82**(12): 3515-3523.

Mullberg, J., W. Oberthur, F. Lottspeich, E. Mehl, E. Dittrich, L. Graeve, P. C. Heinrich and S. Rose-John (1994). "The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site." J Immunol **152**(10): 4958-4968.

Ogasawara, H., T. Tsuji, D. Hirano, Y. Aoki, M. Nakamura and H. Kodama (1996). "Induction of IL-6 production by bone marrow stromal cells on the adhesion of IL-6-dependent hematopoietic cells." J Cell Physiol **169**(1): 209-216.

Osathanon, T., N. Nowwarote and P. Pavasant (2011). "Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLCgamma signaling pathway." J Cell Biochem **112**(7): 1807-1816.

Palmqvist, P., E. Persson, H. H. Conaway and U. H. Lerner (2002). "IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae." J Immunol **169**(6): 3353-3362.

Pricola, K. L., N. Z. Kuhn, H. Haleem-Smith, Y. Song and R. S. Tuan (2009). "Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism." J Cell Biochem **108**(3): 577-588.

Roodman, G. D. (2001). "Biology of osteoclast activation in cancer." J Clin Oncol **19**(15): 3562-3571.

Scheller, J., C. Garbers and S. Rose-John (2014). "Interleukin-6: from basic biology to selective blockade of pro-inflammatory activities." Semin Immunol **26**(1): 2-12.

Sukarawan, W., N. Nowwarote, P. Kerdpon, P. Pavasant and T. Osathanon (2014). "Effect of basic fibroblast growth factor on pluripotent marker expression and colony forming unit capacity of stem cells isolated from human exfoliated deciduous teeth." Odontology **102**(2): 160-166.

Tadokoro, T., Y. Wang, L. S. Barak, Y. Bai, S. H. Randell and B. L. Hogan (2014). "IL-6/STAT3 promotes regeneration of airway ciliated cells from basal stem cells." Proc Natl Acad Sci U S A **111**(35): E3641-3649.

Taga, T., M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano and T. Kishimoto (1989). "Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130." Cell **58**(3): 573-581.

Tamura, T., N. Udagawa, N. Takahashi, C. Miyaura, S. Tanaka, Y. Yamada, Y. Koishihara, Y. Ohsugi, K. Kumaki, T. Taga and et al. (1993). "Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6." Proc Natl Acad Sci U S A **90**(24): 11924-11928.

Tanaka, T. and T. Kishimoto (2014). "The biology and medical implications of interleukin-6." Cancer Immunol Res **2**(4): 288-294.

Trujillo, M. E., S. Sullivan, I. Harten, S. H. Schneider, A. S. Greenberg and S. K. Fried (2004). "Interleukin-6 regulates human adipose tissue lipid metabolism and leptin production in vitro." J Clin Endocrinol Metab **89**(11): 5577-5582.

van der Poll, T., C. V. Keogh, X. Guirao, W. A. Buurman, M. Kopf and S. F. Lowry (1997). "Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia." J Infect Dis **176**(2): 439-444.

Wang, H., J. D. Lathia, Q. Wu, J. Wang, Z. Li, J. M. Heddleston, C. E. Eyler, J. Elderbroom, J. Gallagher, J. Schuschu, J. MacSwords, Y. Cao, R. E. McLendon, X. F. Wang, A. B. Hjelmeland and J. N. Rich (2009). "Targeting interleukin 6 signaling suppresses glioma stem cell survival and tumor growth." Stem Cells **27**(10): 2393-2404.

Yuan, S., Q. Pan, C. J. Fu and Z. Bi (2013). "Effect of growth factors (BMP-4/7 & bFGF) on proliferation & osteogenic differentiation of bone marrow stromal cells." Indian J Med Res **138**: 104-110.

CHAPTER II

Amit, M., M. K. Carpenter, M. S. Inokuma, C. P. Chiu, C. P. Harris, M. A. Waknitz, J. Itskovitz-Eldor and J. A. Thomson (2000). "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture." Dev Biol 227(2): 271-278.

Bai, Y., P. Li, G. Yin, Z. Huang, X. Liao, X. Chen and Y. Yao (2013). "BMP-2, VEGF and bFGF synergistically promote the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells." Biotechnol Lett 35(3): 301-308.

Baird, A., D. Schubert, N. Ling and R. Guillemin (1988). "Receptor- and heparin-binding domains of basic fibroblast growth factor." Proc Natl Acad Sci U S A 85(7): 2324-2328.

Bikfalvi, A., S. Klein, G. Pintucci and D. B. Rifkin (1997). "Biological roles of fibroblast growth factor-2." Endocr Rev 18(1): 26-45.

Chen, G., D. R. Gulbranson, P. Yu, Z. Hou and J. A. Thomson (2012). "Thermal stability of fibroblast growth factor protein is a determinant factor in regulating self-renewal, differentiation, and reprogramming in human pluripotent stem cells." Stem Cells 30(4): 623-630.

Communications, A. D. A. D. o., A. Journal of the American Dental and A. D. A. C. o. S. Affairs (2005). "For the dental patient. Tooth eruption: The primary teeth." J Am Dent Assoc 136(11): 1619.

Dai, Z. and H. B. Peng (1995). "Presynaptic differentiation induced in cultured neurons by local application of basic fibroblast growth factor." J Neurosci 15(8): 5466-5475.

Dangaria, S. J., Y. Ito, C. Walker, R. Druzinsky, X. Luan and T. G. Diekwisch (2009). "Extracellular matrix-mediated differentiation of periodontal progenitor cells." Differentiation 78(2-3): 79-90.

Dereka, X. E., C. E. Markopoulou, A. Mamalis, E. Pepelassi and I. A. Vrotsos (2006). "Time- and dose-dependent mitogenic effect of basic fibroblast growth factor combined with different bone graft materials: an in vitro study." Clin Oral Implants Res 17(5): 554-559.

Duan, B., L. A. Hockaday, S. Das, C. Y. Xu and J. T. Butcher (2015). "Comparison of mesenchymal stem cell source differentiation towards human pediatric aortic valve interstitial cells within 3D engineered matrices." Tissue Eng Part C Methods.

Egusa, H., W. Sonoyama, M. Nishimura, I. Atsuta and K. Akiyama (2012). "Stem cells in dentistry--part I: stem cell sources." J Prosthodont Res 56(3): 151-165.

Eleuterio, E., O. Trubiani, M. Sulpizio, F. Di Giuseppe, L. Pierdomenico, M. Marchisio, R. Giancola, G. Giammaria, S. Miscia, S. Caputi, C. Di Ilio and S. Angelucci (2013). "Proteome of human stem cells from periodontal ligament and dental pulp." PLoS One 8(8): e71101.

Fierro, F. A., S. Kalomoiris, C. S. Sondergaard and J. A. Nolta (2011). "Effects on proliferation and differentiation of multipotent bone marrow stromal cells engineered to express growth factors for combined cell and gene therapy." Stem Cells 29(11): 1727-1737.

Go, M. J., C. Takenaka and H. Ohgushi (2007). "Effect of forced expression of basic fibroblast growth factor in human bone marrow-derived mesenchymal stromal cells." J Biochem 142(6): 741-748.

Guan, M., Y. Xu, W. Wang and S. Lin (2014). "Differentiation into neurons of rat bone marrow-derived mesenchymal stem cells." Eur Cytokine Netw.

Hakki, S. S., S. A. Kayis, E. E. Hakki, S. B. Bozkurt, G. Duruksu, Z. S. Unal, G. Turac and E. Karaoz (2014). "Comparison of MSCs Isolated From Pulp and Periodontal Ligament." J Periodontol: 1-17.

Hanada, K., J. E. Dennis and A. I. Caplan (1997). "Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells." J Bone Miner Res 12(10): 1606-1614.

Harada, H., P. Kettunen, H. S. Jung, T. Mustonen, Y. A. Wang and I. Thesleff (1999). "Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling." J Cell Biol 147(1): 105-120.

Hatch, N. E. (2010). "FGF signaling in craniofacial biological control and pathological craniofacial development." Crit Rev Eukaryot Gene Expr 20(4): 295-311.

Hatch, N. E., Y. Li and R. T. Franceschi (2009). "FGF2 stimulation of the pyrophosphate-generating enzyme, PC-1, in pre-osteoblast cells is mediated by RUNX2." J Bone Miner Res 24(4): 652-662.

Hatch, N. E., F. Nociti, E. Swanson, M. Bothwell and M. Somerman (2005). "FGF2 alters expression of the pyrophosphate/phosphate regulating proteins, PC-1, ANK and TNAP, in the calvarial osteoblastic cell line, MC3T3E1(C4)." Connect Tissue Res 46(4-5): 184-192.

He, H., J. Yu, Y. Liu, S. Lu, H. Liu, J. Shi and Y. Jin (2008). "Effects of FGF2 and TGFbeta1 on the differentiation of human dental pulp stem cells in vitro." Cell Biol Int 32(7): 827-834.

Hidaka, T., T. Nagasawa, K. Shirai, T. Kado and Y. Furuichi (2012). "FGF-2 induces proliferation of human periodontal ligament cells and maintains differentiation potentials of STRO-1(+)/CD146(+) periodontal ligament cells." Arch Oral Biol 57(6): 830-840.

Hu, C. C., C. Zhang, Q. Qian and N. B. Tatum (1998). "Reparative dentin formation in rat molars after direct pulp capping with growth factors." J Endod 24(11): 744-751.

Hu, F., X. Wang, G. Liang, L. Lv, Y. Zhu, B. Sun and Z. Xiao (2013). "Effects of epidermal growth factor and basic fibroblast growth factor on the

proliferation and osteogenic and neural differentiation of adipose-derived stem cells." Cell Reprogram 15(3): 224-232.

Inoue, S., M. Imamura and Y. Tabata (2009). "Adipogenic differentiation of adipo-stromal cells incubated with basic fibroblast growth factor in solution and coated form." J Biomater Sci Polym Ed 20(4): 483-494.

Ishimatsu, H., C. Kitamura, T. Morotomi, Y. Tabata, T. Nishihara, K. K. Chen and M. Terashita (2009). "Formation of dentinal bridge on surface of regenerated dental pulp in dentin defects by controlled release of fibroblast growth factor-2 from gelatin hydrogels." J Endod 35(6): 858-865.

Jang, S., H. H. Cho, Y. B. Cho, J. S. Park and H. S. Jeong (2010). "Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin." BMC Cell Biol 11: 25.

Kakudo, N., A. Shimotsuma and K. Kusumoto (2007). "Fibroblast growth factor-2 stimulates adipogenic differentiation of human adipose-derived stem cells." Biochem Biophys Res Commun 359(2): 239-244.

Kan, M., F. Wang, J. Xu, J. W. Crabb, J. Hou and W. L. McKeehan (1993). "An essential heparin-binding domain in the fibroblast growth factor receptor kinase." Science 259(5103): 1918-1921.

Kanafi, M. M., A. Ramesh, P. K. Gupta and R. R. Bhonde (2013). "Influence of hypoxia, high glucose, and low serum on the growth kinetics of mesenchymal stem cells from deciduous and permanent teeth." Cells Tissues Organs 198(3): 198-208.

Kang, H. B., J. S. Kim, H. J. Kwon, K. H. Nam, H. S. Youn, D. E. Sok and Y. Lee (2005). "Basic fibroblast growth factor activates ERK and induces c-fos in human embryonic stem cell line MizhES1." Stem Cells Dev 14(4): 395-401.

Kang, M. L., J. S. Kwon and M. S. Kim (2013). "Induction of neuronal differentiation of rat muscle-derived stem cells in vitro using basic fibroblast growth factor and ethosuximide." Int J Mol Sci 14(4): 6614-6623.

Kato, Y. and D. Gospodarowicz (1985). "Sulfated proteoglycan synthesis by confluent cultures of rabbit costal chondrocytes grown in the presence of fibroblast growth factor." J Cell Biol 100(2): 477-485.

Khezri, S., M. R. Valojerdi, H. Sepehri and H. Baharvand (2007). "Effect of basic fibroblast growth factor on cardiomyocyte differentiation from mouse embryonic stem cells." Saudi Med J 28(2): 181-186.

Kikuchi, N., C. Kitamura, T. Morotomi, Y. Inuyama, H. Ishimatsu, Y. Tabata, T. Nishihara and M. Terashita (2007). "Formation of dentin-like particles in dentin defects

above exposed pulp by controlled release of fibroblast growth factor 2 from gelatin hydrogels." J Endod 33(10): 1198-1202.

Kim, J., J. C. Park, S. H. Kim, G. I. Im, B. S. Kim, J. B. Lee, E. Y. Choi, J. S. Song, K. S. Cho and C. S. Kim (2014). "Treatment of FGF-2 on stem cells from inflamed dental pulp tissue from human deciduous teeth." Oral Dis 20(2): 191-204.

Kim, Y. S., K. S. Min, D. H. Jeong, J. H. Jang, H. W. Kim and E. C. Kim (2010). "Effects of fibroblast growth factor-2 on the expression and regulation of chemokines in human dental pulp cells." J Endod 36(11): 1824-1830.

Kinoshita, Y., M. Matsuo, K. Todoki, S. Ozono, S. Fukuoka, H. Tsuzuki, M. Nakamura, K. Tomihata, T. Shimamoto and Y. Ikada (2008). "Alveolar bone regeneration using absorbable poly(L-lactide-co-epsilon-caprolactone)/beta-tricalcium phosphate membrane and gelatin sponge incorporating basic fibroblast growth factor." Int J Oral Maxillofac Surg 37(3): 275-281.

Kong, Y. P., C. H. Tu, P. J. Donovan and A. F. Yee (2013). "Expression of Oct4 in human embryonic stem cells is dependent on nanotopographical configuration." Acta Biomater 9(5): 6369-6380.

Kono, K., H. Maeda, S. Fujii, A. Tomokiyo, N. Yamamoto, N. Wada, S. Monnouchi, Y. Teramatsu, S. Hamano, K. Koori and A. Akamine (2013). "Exposure to transforming growth factor-beta1 after basic fibroblast growth factor

promotes the fibroblastic differentiation of human periodontal ligament stem/progenitor cell lines." Cell Tissue Res 352(2): 249-263.

Lai, W. T., V. Krishnappa and D. G. Phinney (2011). "Fibroblast growth factor 2 (Fgf2) inhibits differentiation of mesenchymal stem cells by inducing Twist2 and Spry4, blocking extracellular regulated kinase activation, and altering Fgf receptor expression levels." Stem Cells 29(7): 1102-1111.

Lee, J. H., S. Um, J. H. Jang and B. M. Seo (2012). "Effects of VEGF and FGF-2 on proliferation and differentiation of human periodontal ligament stem cells." Cell Tissue Res 348(3): 475-484.

Lee, T. H., W. T. Kim, C. J. Ryu and Y. J. Jang (2015). "Optimization of treatment with recombinant FGF-2 for proliferation and differentiation of human dental stem cells, mesenchymal stem cells, and osteoblasts." Biochem Cell Biol: 1-8.

Li, B., C. Qu, C. Chen, Y. Liu, K. Akiyama, R. Yang, F. Chen, Y. Zhao and S. Shi (2012). "Basic fibroblast growth factor inhibits osteogenic differentiation of stem cells from human exfoliated deciduous teeth through ERK signaling." Oral Dis 18(3): 285-292.

Lu, Q., M. Li, Y. Zou and T. Cao (2014). "Delivery of basic fibroblast growth factors from heparinized decellularized adipose tissue stimulates potent de novo adipogenesis." J Control Release 174: 43-50.

Ludwig, T. E., M. E. Levenstein, J. M. Jones, W. T. Berggren, E. R. Mitchen, J. L. Frane, L. J. Crandall, C. A. Daigh, K. R. Conard, M. S. Piekarczyk, R. A. Llanas and J. A. Thomson (2006). "Derivation of human embryonic stem cells in defined conditions." Nat Biotechnol 24(2): 185-187.

Machado, E., M. H. Fernandes and S. Gomes Pde (2012). "Dental stem cells for craniofacial tissue engineering." Oral Surg Oral Med Oral Pathol Oral Radiol 113(6): 728-733.

Matsumoto, G., J. Hoshino, Y. Kinoshita, Y. Sugita, K. Kubo, H. Maeda, Y. Ikada and Y. Kinoshita (2012). "Alveolar bone regeneration using poly-(lactic acid-co-glycolic acid-co-epsilon-caprolactone) porous membrane with collagen sponge containing basic fibroblast growth factor: an experimental study in the dog." J Biomater Appl 27(4): 485-493.

McAvoy, J. W. and C. G. Chamberlain (1989). "Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration." Development 107(2): 221-228.

Montero, A., Y. Okada, M. Tomita, M. Ito, H. Tsurukami, T. Nakamura, T. Doetschman, J. D. Coffin and M. M. Hurley (2000). "Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation." J Clin Invest 105(8): 1085-1093.

Morito, A., Y. Kida, K. Suzuki, K. Inoue, N. Kuroda, K. Gomi, T. Arai and T. Sato (2009). "Effects of basic fibroblast growth factor on the development of the stem cell properties of human dental pulp cells." Arch Histol Cytol 72(1): 51-64.

Moscatelli, D. (1994). "Autocrine downregulation of fibroblast growth factor receptors in F9 teratocarcinoma cells." J Cell Physiol 160(3): 555-562.

Murakami, S. (2011). "Periodontal tissue regeneration by signaling molecule(s): what role does basic fibroblast growth factor (FGF-2) have in periodontal therapy?" Periodontol 2000 56(1): 188-208.

Murakami, S., S. Takayama, M. Kitamura, Y. Shimabukuro, K. Yanagi, K. Ikezawa, T. Saho, T. Nozaki and H. Okada (2003). "Recombinant human basic fibroblast growth factor (bFGF) stimulates periodontal regeneration in class II furcation defects created in beagle dogs." J Periodontal Res 38(1): 97-103.

Nakano, R., K. Edamura, T. Nakayama, K. Teshima, K. Asano, T. Narita, K. Okabayashi and H. Sugiya (2014). "Differentiation of canine bone marrow stromal cells

into voltage- and glutamate-responsive neuron-like cells by basic fibroblast growth factor." J Vet Med Sci.

Neubauer, M., C. Fischbach, P. Bauer-Kreisel, E. Lieb, M. Hacker, J. Tessmar, M. B. Schulz, A. Goepferich and T. Blunk (2004). "Basic fibroblast growth factor enhances PPARgamma ligand-induced adipogenesis of mesenchymal stem cells." FEBS Lett 577(1-2): 277-283.

Neubauer, M., M. Hacker, P. Bauer-Kreisel, B. Weiser, C. Fischbach, M. B. Schulz, A. Goepferich and T. Blunk (2005). "Adipose tissue engineering based on mesenchymal stem cells and basic fibroblast growth factor in vitro." Tissue Eng 11(11-12): 1840-1851.

Niknejad, H., H. Peirovi, A. Ahmadiani, J. Ghanavi and M. Jorjani (2010). "Differentiation factors that influence neuronal markers expression in vitro from human amniotic epithelial cells." Eur Cell Mater 19: 22-29.

Nishino, Y., K. Ebisawa, Y. Yamada, K. Okabe, Y. Kamei and M. Ueda (2011). "Human deciduous teeth dental pulp cells with basic fibroblast growth factor enhance wound healing of skin defect." J Craniofac Surg 22(2): 438-442.

Nowwarote, N., P. Pavasant and T. Osathanon (2015). "Role of endogenous basic fibroblast growth factor in stem cells isolated from human exfoliated deciduous teeth." Arch Oral Biol 60(3): 408-415.

Oh, S. A., H. Y. Lee, J. H. Lee, T. H. Kim, J. H. Jang, H. W. Kim and I. Wall (2012). "Collagen three-dimensional hydrogel matrix carrying basic fibroblast growth factor for the cultivation of mesenchymal stem cells and osteogenic differentiation." Tissue Eng Part A 18(9-10): 1087-1100.

Okada-Ban, M., J. P. Thiery and J. Jouanneau (2000). "Fibroblast growth factor-2." Int J Biochem Cell Biol 32(3): 263-267.

Olwin, B. B. and S. D. Hauschka (1988). "Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture." J Cell Biol 107(2): 761-769.

Ornitz, D. M. and N. Itoh (2001). "Fibroblast growth factors." Genome Biol 2(3): REVIEWS3005.

Osathanon, T., N. Nowwarote, J. Manokawinchoke and P. Pavasant (2013). "bFGF and JAGGED1 regulate alkaline phosphatase expression and mineralization in dental tissue-derived mesenchymal stem cells." J Cell Biochem 114(11): 2551-2561.

Osathanon, T., N. Nowwarote and P. Pavasant (2011). "Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLCgamma signaling pathway." J Cell Biochem 112(7): 1807-1816.

Park, J. H. and J. Hong (2014). "Continuous release of bFGF from multilayer nanofilm to maintain undifferentiated human iPS cell cultures." Integr Biol (Camb) 6(12): 1196-1200.

Park, M. S., S. S. Kim, S. W. Cho, C. Y. Choi and B. S. Kim (2006). "Enhancement of the osteogenic efficacy of osteoblast transplantation by the sustained delivery of basic fibroblast growth factor." J Biomed Mater Res B Appl Biomater 79(2): 353-359.

Park, Y., I. Y. Choi, S. J. Lee, S. R. Lee, H. J. Sung, J. H. Kim, Y. D. Yoo, D. H. Geum, S. H. Kim and B. S. Kim (2010). "Undifferentiated propagation of the human embryonic stem cell lines, H1 and HSF6, on human placenta-derived feeder cells without basic fibroblast growth factor supplementation." Stem Cells Dev 19(11): 1713-1722.

Park, Y., J. H. Kim, S. J. Lee, I. Y. Choi, S. J. Park, S. R. Lee, H. J. Sung, Y. D. Yoo, D. H. Geum, C. W. Choi, S. H. Kim and B. S. Kim (2011). "Human feeder cells can support the undifferentiated growth of human and mouse embryonic stem cells using their own basic fibroblast growth factors." Stem Cells Dev 20(11): 1901-1910.

Powers, C. J., S. W. McLeskey and A. Wellstein (2000). "Fibroblast growth factors, their receptors and signaling." Endocr Relat Cancer 7(3): 165-197.

Pri-Chen, S., S. Pitaru, F. Lokiec and N. Savion (1998). "Basic fibroblast growth factor enhances the growth and expression of the osteogenic phenotype of dexamethasone-treated human bone marrow-derived bone-like cells in culture." Bone 23(2): 111-117.

Qian, J., W. Jiayuan, J. Wenkai, W. Peina, Z. Ansheng, S. Shukai, Z. Shafei, L. Jun and N. Longxing (2014). "Basic fibroblastic growth factor affects the osteogenic differentiation of dental pulp stem cells in a treatment-dependent manner." Int Endod J.

Quang, T., M. Marquez, G. Blanco and Y. Zhao (2014). "Dosage and cell line dependent inhibitory effect of bFGF supplement in human pluripotent stem cell culture on inactivated human mesenchymal stem cells." PLoS One 9(1): e86031.

Ramasamy, R., C. K. Tong, W. K. Yip, S. Vellasamy, B. C. Tan and H. F. Seow (2012). "Basic fibroblast growth factor modulates cell cycle of human umbilical cord-derived mesenchymal stem cells." Cell Prolif 45(2): 132-139.

Rose, L. C., R. Fitzsimmons, P. Lee, R. Krawetz, D. E. Rancourt and H. Uludag (2013). "Effect of basic fibroblast growth factor in mouse embryonic stem cell culture and osteogenic differentiation." J Tissue Eng Regen Med 7(5): 371-382.

Rosenblatt-Velin, N., M. G. Lepore, C. Cartoni, F. Beermann and T. Pedrazzini (2005). "FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes." J Clin Invest 115(7): 1724-1733.

Saito, A., E. Saito, Y. Kuboki, M. Kimura, T. Nakajima, F. Yuge, T. Kato, Y. Honma, T. Takahashi and N. Ohata (2013). "Periodontal regeneration following application of basic fibroblast growth factor-2 in combination with beta tricalcium phosphate in class III furcation defects in dogs." Dent Mater J 32(2): 256-262.

Sakaguchi, D. S., L. M. Janick and T. A. Reh (1997). "Basic fibroblast growth factor (FGF-2) induced transdifferentiation of retinal pigment epithelium: generation of retinal neurons and glia." Dev Dyn 209(4): 387-398.

Sasaki, R., S. Aoki, M. Yamato, H. Uchiyama, K. Wada, T. Okano and H. Ogiuchi (2008). "Neurosphere generation from dental pulp of adult rat incisor." Eur J Neurosci 27(3): 538-548.

Sawangmake, C., N. Nowwarote, P. Pavasant, P. Chansiripornchai and T. Osathanon (2014). "A feasibility study of an in vitro differentiation potential toward insulin-producing cells by dental tissue-derived mesenchymal stem cells." Biochem Biophys Res Commun 452(3): 581-587.

Sedgley, C. M. and T. M. Botero (2012). "Dental stem cells and their sources." Dent Clin North Am 56(3): 549-561.

Shirakata, Y., K. Taniyama, T. Yoshimoto, M. Miyamoto, N. Takeuchi, T. Matsuyama and K. Noguchi (2010). "Regenerative effect of basic fibroblast growth factor on periodontal healing in two-wall intrabony defects in dogs." J Clin Periodontol 37(4): 374-381.

Song, X., Y. Li, X. Chen, G. Yin, Q. Huang, Y. Chen, G. Xu and L. Wang (2014). "bFGF promotes adipocyte differentiation in human mesenchymal stem cells derived from embryonic stem cells." Genet Mol Biol 37(1): 127-134.

Stemple, D. L., N. K. Mahanthappa and D. J. Anderson (1988). "Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: a sequence of events in sympathetic development." Neuron 1(6): 517-525.

Su, N., X. Du and L. Chen (2008). "FGF signaling: its role in bone development and human skeleton diseases." Front Biosci 13: 2842-2865.

Subramony, S. D., A. Su, K. Yeager and H. H. Lu (2014). "Combined effects of chemical priming and mechanical stimulation on mesenchymal stem cell differentiation on nanofiber scaffolds." J Biomech 47(9): 2189-2196.

Sukarawan, W., N. Nowwarote, P. Kerdpon, P. Pavasant and T. Osathanon (2014). "Effect of basic fibroblast growth factor on pluripotent marker expression and colony forming unit capacity of stem cells isolated from human exfoliated deciduous teeth." Odontology 102(2): 160-166.

Suphanantachat, S., T. Iwata, J. Ishihara, M. Yamato, T. Okano and Y. Izumi (2014). "A role for c-Kit in the maintenance of undifferentiated human mesenchymal stromal cells." Biomaterials 35(11): 3618-3626.

Suzuki, T., C. H. Lee, M. Chen, W. Zhao, S. Y. Fu, J. J. Qi, G. Chotkowski, S. B. Eisig, A. Wong and J. J. Mao (2011). "Induced migration of dental pulp stem cells for in vivo pulp regeneration." J Dent Res 90(8): 1013-1018.

Takayama, S., S. Murakami, Y. Shimabukuro, M. Kitamura and H. Okada (2001). "Periodontal regeneration by FGF-2 (bFGF) in primate models." J Dent Res 80(12): 2075-2079.

Takeuchi, N., Y. Hayashi, M. Murakami, F. J. Alvarez, H. Horibe, K. Iohara, K. Nakata, H. Nakamura and M. Nakashima (2015). "Similar in vitro effects and pulp regeneration in ectopic tooth transplantation by basic fibroblast growth factor and granulocyte-colony stimulating factor." Oral Dis 21(1): 113-122.

Tanaka, H., H. Ogasa, J. Barnes and C. T. Liang (1999). "Actions of bFGF on mitogenic activity and lineage expression in rat osteoprogenitor cells: effect of age." Mol Cell Endocrinol 150(1-2): 1-10.

Thisse, B. and C. Thisse (2005). "Functions and regulations of fibroblast growth factor signaling during embryonic development." Dev Biol 287(2): 390-402.

Tsutsumi, S., A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi and Y. Kato (2001). "Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF." Biochem Biophys Res Commun 288(2): 413-419.

Villegas, S. N., M. Canham and J. M. Brickman (2010). "FGF signalling as a mediator of lineage transitions--evidence from embryonic stem cell differentiation." J Cell Biochem 110(1): 10-20.

Wada, M., C. M. Gelfman, H. Matsunaga, M. Alizadeh, L. Morse, J. T. Handa and L. M. Hjelmeland (2001). "Density-dependent expression of FGF-2 in response to oxidative stress in RPE cells in vitro." Curr Eye Res 23(3): 226-231.

Wang, G., H. Zhang, Y. Zhao, J. Li, J. Cai, P. Wang, S. Meng, J. Feng, C. Miao, M. Ding, D. Li and H. Deng (2005). "Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers." Biochem Biophys Res Commun 330(3): 934-942.

Wang, H., Q. Zou, O. C. Boerman, A. W. Nijhuis, J. A. Jansen, Y. Li and S. C. Leeuwenburgh (2013). "Combined delivery of BMP-2 and bFGF from nanostructured colloidal gelatin gels and its effect on bone regeneration in vivo." J Control Release 166(2): 172-181.

Wang, X., G. Lin, K. Martins-Taylor, H. Zeng and R. H. Xu (2009). "Inhibition of caspase-mediated anoikis is critical for basic fibroblast growth factor-sustained culture of human pluripotent stem cells." J Biol Chem 284(49): 34054-34064.

Wang, X., X. J. Sha, G. H. Li, F. S. Yang, K. Ji, L. Y. Wen, S. Y. Liu, L. Chen, Y. Ding and K. Xuan (2012). "Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells." Arch Oral Biol 57(9): 1231-1240.

Woodbury, M. E. and T. Ikezu (2014). "Fibroblast growth factor-2 signaling in neurogenesis and neurodegeneration." J Neuroimmune Pharmacol 9(2): 92-101.

Wu, J., G. T. Huang, W. He, P. Wang, Z. Tong, Q. Jia, L. Dong, Z. Niu and L. Ni (2012). "Basic fibroblast growth factor enhances stemness of human stem cells from the apical papilla." J Endod 38(5): 614-622.

Xi, J., Y. Wang, P. Zhang, L. He, X. Nan, W. Yue and X. Pei (2010). "Human fetal liver stromal cells that overexpress bFGF support growth and maintenance of human embryonic stem cells." PLoS One 5(12): e14457.

Xiao, L., T. Sobue, A. Esliger, M. S. Kronenberg, J. D. Coffin, T. Doetschman and M. M. Hurley (2010). "Disruption of the Fgf2 gene activates the adipogenic and suppresses the osteogenic program in mesenchymal marrow stromal stem cells." Bone 47(2): 360-370.

Xu, C., E. Rosler, J. Jiang, J. S. Lebkowski, J. D. Gold, C. O'Sullivan, K. Delavan-Boorsma, M. Mok, A. Bronstein and M. K. Carpenter (2005). "Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium." Stem Cells 23(3): 315-323.

Yang, H., Y. Xia, S. Q. Lu, T. W. Soong and Z. W. Feng (2008). "Basic fibroblast growth factor-induced neuronal differentiation of mouse bone marrow stromal cells requires FGFR-1, MAPK/ERK, and transcription factor AP-1." J Biol Chem 283(9): 5287-5295.

Yang, J. W., Y. F. Zhang, Z. Y. Sun, G. T. Song and Z. Chen (2015). "Dental pulp tissue engineering with bFGF-incorporated silk fibroin scaffolds." J Biomater Appl.

Yayon, A., D. Aviezer, M. Safran, J. L. Gross, Y. Heldman, S. Cabilly, D. Givol and E. Katchalski-Katzir (1993). "Isolation of peptides that inhibit binding of basic

fibroblast growth factor to its receptor from a random phage-epitope library." Proc Natl Acad Sci U S A 90(22): 10643-10647.

Yeoh, J. S. and G. de Haan (2007). "Fibroblast growth factors as regulators of stem cell self-renewal and aging." Mech Ageing Dev 128(1): 17-24.

Yu, P., G. Pan, J. Yu and J. A. Thomson (2011). "FGF2 sustains NANOG and switches the outcome of BMP4-induced human embryonic stem cell differentiation." Cell Stem Cell 8(3): 326-334.

Yuan, S., Q. Pan, C. J. Fu and Z. Bi (2013). "Effect of growth factors (BMP-4/7 & bFGF) on proliferation & osteogenic differentiation of bone marrow stromal cells." Indian J Med Res 138: 104-110.

Yun, Y. R., J. E. Won, E. Jeon, S. Lee, W. Kang, H. Jo, J. H. Jang, U. S. Shin and H. W. Kim (2010). "Fibroblast growth factors: biology, function, and application for tissue regeneration." J Tissue Eng 2010: 218142.

Zhang, X., Y. Wang, Y. Gao, X. Liu, T. Bai, M. Li, L. Li, G. Chi, H. Xu, F. Liu, J. Y. Liu and Y. Li (2013). "Maintenance of high proliferation and multipotent potential of human hair follicle-derived mesenchymal stem cells by growth factors." Int J Mol Med 31(4): 913-921.

Zheng, Y. H., K. Su, Y. T. Jian, S. J. Kuang and Z. G. Zhang (2011). "Basic fibroblast growth factor enhances osteogenic and chondrogenic differentiation of

human bone marrow mesenchymal stem cells in coral scaffold constructs." J Tissue Eng Regen Med 5(7): 540-550.

Zhu, H., A. Yang, J. Du, D. Li, M. Liu, F. Ding, X. Gu and Y. Liu (2014). "Basic fibroblast growth factor is a key factor that induces bone marrow mesenchymal stem cells towards cells with Schwann cell phenotype." Neurosci Lett 559: 82-87.

CHAPTER III

Adepoju, A., N. Micali, K. Ogawa, D. J. Hoeppner and R. D. McKay (2014). "FGF2 and insulin signaling converge to regulate cyclin D expression in multipotent neural stem cells." Stem Cells 32(3): 770-778.

Amit, M., M. K. Carpenter, M. S. Inokuma, C. P. Chiu, C. P. Harris, M. A. Waknitz, J. Itskovitz-Eldor and J. A. Thomson (2000). "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture." Dev Biol **227**(2): 271-278.

Arabi, A., S. Wu, K. Ridderstrale, H. Bierhoff, C. Shiue, K. Fatyol, S. Fahlen, P. Hydbring, O. Soderberg, I. Grummt, L. G. Larsson and A. P. Wright (2005). "c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription." Nat Cell Biol **7**(3): 303-310.

Bruno, E., R. J. Cooper, E. L. Wilson, J. L. Gabrilove and R. Hoffman (1993). "Basic fibroblast growth factor promotes the proliferation of human megakaryocyte progenitor cells." Blood **82**(2): 430-435.

Cavalheiro, G. R., G. E. Matos-Rodrigues, A. L. Gomes, P. M. Rodrigues and R. A. Martins (2014). "c-Myc regulates cell proliferation during lens development." PLoS One **9**(2): e87182.

Chang, J. Y., C. Wang, J. Liu, Y. Huang, C. Jin, C. Yang, B. Hai, F. Liu, R. N. D'Souza, W. L. McKeehan and F. Wang (2013). "Fibroblast growth factor signaling is essential for self-renewal of dental epithelial stem cells." J Biol Chem **288**(40): 28952-28961.

Gordan, J. D., C. B. Thompson and M. C. Simon (2007). "HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation." Cancer Cell **12**(2): 108-113.

Govitvattana, N., T. Osathanon, S. Taebunpakul and P. Pavasant (2013). "IL-6 regulated stress-induced Rex-1 expression in stem cells from human exfoliated deciduous teeth." Oral Dis **19**(7): 673-682.

Jeong, K. C., K. O. Ahn and C. H. Yang (2010). "Small-molecule inhibitors of c-Myc transcriptional factor suppress proliferation and induce apoptosis of

promyelocytic leukemia cell via cell cycle arrest." Mol Biosyst **6**(8): 1503-1509.

Kato, Y. and D. Gospodarowicz (1985). "Sulfated proteoglycan synthesis by confluent cultures of rabbit costal chondrocytes grown in the presence of fibroblast growth factor." J Cell Biol **100**(2): 477-485.

Kim, J., J. C. Park, S. H. Kim, G. I. Im, B. S. Kim, J. B. Lee, E. Y. Choi, J. S. Song, K. S. Cho and C. S. Kim (2014). "Treatment of FGF-2 on stem cells from inflamed dental pulp tissue from human deciduous teeth." Oral Dis **20**(2): 191-204.

Lee, J. H., S. Um, J. H. Jang and B. M. Seo (2012). "Effects of VEGF and FGF-2 on proliferation and differentiation of human periodontal ligament stem cells." Cell Tissue Res **348**(3): 475-484.

Lepique, A. P., F. L. Forti, M. S. Moraes and H. A. Armelin (2000). "Signal transduction in G0/G1-arrested mouse Y1 adrenocortical cells stimulated by ACTH and FGF2." Endocr Res **26**(4): 825-832.

Ludwig, T. E., M. E. Levenstein, J. M. Jones, W. T. Berggren, E. R. Mitchen, J. L. Frane, L. J. Crandall, C. A. Daigh, K. R. Conard, M. S. Piekarczyk, R. A. Llanas and J. A. Thomson (2006). "Derivation of human embryonic stem cells in defined conditions." Nat Biotechnol **24**(2): 185-187.

Miura, M., S. Gronthos, M. Zhao, B. Lu, L. W. Fisher, P. G. Robey and S. Shi (2003). "SHED: Stem cells from human exfoliated deciduous teeth." Proceedings of the National Academy of Sciences **100**(10): 5807-5812.

Osathanon, T., N. Nowwarote, J. Manokawinchoke and P. Pavasant (2013). "bFGF and JAGGED1 regulate alkaline phosphatase expression and mineralization in dental tissue-derived mesenchymal stem cells." J Cell Biochem **114**(11): 2551-2561.

Osathanon, T., N. Nowwarote and P. Pavasant (2011). "Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLCgamma signaling pathway." J Cell Biochem **112**(7): 1807-1816.

Osathanon, T., P. Ritprajak, N. Nowwarote, J. Manokawinchoke, C. Giachelli and P. Pavasant (2013). "Surface-bound orientated Jagged-1 enhances osteogenic differentiation of human periodontal ligament-derived mesenchymal stem cells." J Biomed Mater Res A **101**(2): 358-367.

Osathanon, T., K. Subbalekha, P. Sastravaha and P. Pavasant (2012). "Notch signalling inhibits the adipogenic differentiation of single-cell-derived mesenchymal stem cell clones isolated from human adipose tissue." Cell Biol Int **36**(12): 1161-1170.

Pitaru, S., S. Kotev-Emeth, D. Noff, S. Kaffuler and N. Savion (1993). "Effect of basic fibroblast growth factor on the growth and differentiation of adult stromal bone marrow cells: enhanced development of mineralized bone-like tissue in culture." J Bone Miner Res **8**(8): 919-929.

Rider, D. A., C. Dombrowski, A. A. Sawyer, G. H. Ng, D. Leong, D. W. Hutmacher, V. Nurcombe and S. M. Cool (2008). "Autocrine fibroblast growth factor 2 increases the multipotentiality of human adipose-derived mesenchymal stem cells." Stem Cells **26**(6): 1598-1608.

Sakaguchi, D. S., L. M. Janick and T. A. Reh (1997). "Basic fibroblast growth factor (FGF-2) induced transdifferentiation of retinal pigment epithelium: generation of retinal neurons and glia." Dev Dyn **209**(4): 387-398.

Schmidt, E. V. (1999). "The role of c-myc in cellular growth control." Oncogene **18**(19): 2988-2996.

Silva Fde, S., R. N. Ramos, D. C. de Almeida, E. J. Bassi, R. P. Gonzales, S. P. Miyagi, C. P. Maranduba, O. A. Sant'Anna, M. M. Marques, J. A. Barbuto, N. O. Camara and C. M. da Costa Maranduba (2014). "Mesenchymal stem cells derived from human exfoliated deciduous teeth (SHEDs) induce immune modulatory profile in monocyte-derived dendritic cells." PLoS One **9**(5): e98050.

Stoelzle, T., P. Schwarb, A. Trumpp and N. E. Hynes (2009). "c-Myc affects mRNA translation, cell proliferation and progenitor cell function in the mammary gland." BMC Biol **7**: 63.

Sukarawan, W., N. Nowwarote, P. Kerdpon, P. Pavasant and T. Osathanon (2014). "Effect of basic fibroblast growth factor on pluripotent marker expression and colony forming unit capacity of stem cells isolated from human exfoliated deciduous teeth." Odontology **102**(2): 160-166.

Sylvester, A. M., D. Chen, K. Krasinski and V. Andres (1998). "Role of c-fos and E2F in the induction of cyclin A transcription and vascular smooth muscle cell proliferation." J Clin Invest **101**(5): 940-948.

Tsutsumi, S., A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi and Y. Kato (2001). "Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF." Biochem Biophys Res Commun **288**(2): 413-419.

Wang, J., X. Wang, Z. Sun, X. Wang, H. Yang, S. Shi and S. Wang (2010). "Stem cells from human-exfoliated deciduous teeth can differentiate into dopaminergic neuron-like cells." Stem Cells Dev **19**(9): 1375-1383.

Wang, K., L. Cheng, Y. Liang, D. Liu, K. Li and P. Wang (2011). "Adenovirus-mediated delivery of shRNA against bFGF mRNA suppresses growth of cultured human primary prostatic stromal cells." Mol Biol Rep **38**(2): 971-976.

Wu, J., G. T. Huang, W. He, P. Wang, Z. Tong, Q. Jia, L. Dong, Z. Niu and L. Ni (2012). "Basic fibroblast growth factor enhances stemness of human stem cells from the apical papilla." J Endod **38**(5): 614-622.

Yamaza, T., A. Kentaro, C. Chen, Y. Liu, Y. Shi, S. Gronthos, S. Wang and S. Shi (2010). "Immunomodulatory properties of stem cells from human exfoliated deciduous teeth." Stem Cell Res Ther **1**(1): 5.

Yeoh, J. S. and G. de Haan (2007). "Fibroblast growth factors as regulators of stem cell self-renewal and aging." Mech Ageing Dev **128**(1): 17-24.

Yun, Y. R., J. E. Won, E. Jeon, S. Lee, W. Kang, H. Jo, J. H. Jang, U. S. Shin and H. W. Kim (2010). "Fibroblast growth factors: biology, function, and application for tissue regeneration." J Tissue Eng **2010**: 218142.

Zaragosi, L. E., G. Ailhaud and C. Dani (2006). "Autocrine fibroblast growth factor 2 signaling is critical for self-renewal of human multipotent adipose-derived stem cells." Stem Cells **24**(11): 2412-2419.

Zhang, L., X. Li, M. Zhao, P. He, W. Yu, J. Dong, G. Liu, C. Li and X. Shi (2006). "Antisense oligonucleotide targeting c-fos mRNA limits retinal pigment

epithelial cell proliferation: a key step in the progression of proliferative vitreoretinopathy." Exp Eye Res **83**(6): 1405-1411.

CHAPTER IV

Andoh A, Bamba S, Fujino S, Inatomi O, Zhang Z, Kim S, Takayanagi A, Shimizu N, Fujiyama Y. 2004. Fibroblast growth factor-2 stimulates interleukin-6 secretion in human pancreatic periacinar myofibroblasts. Pancreas 29:278-83.

Bisping G, Leo R, Wenning D, Dankbar B, Padro T, Kropff M, Scheffold C, Kroger M, Mesters RM, Berdel WE, Kienast J. 2003. Paracrine interactions of basic fibroblast growth factor and interleukin-6 in multiple myeloma. Blood 101:2775-83.

Choi SC, Kim SJ, Choi JH, Park CY, Shim WJ, Lim DS. 2008. Fibroblast growth factor-2 and -4 promote the proliferation of bone marrow mesenchymal stem cells by the activation of the PI3K-Akt and ERK1/2 signaling pathways. Stem Cells Dev 17:725-36.

Fatimah SS, Tan GC, Chua K, Fariha MM, Tan AE, Hayati AR. 2013. Stemness and angiogenic gene expression changes of serial-passage human amnion mesenchymal cells. Microvasc Res 86:21-9.

Gallagher D, Norman AA, Woodard CL, Yang G, Gauthier-Fisher A, Fujitani M, Vessey JP, Cancino GI, Sachewsky N, Woltjen K, Fatt MP, Morshead CM, Kaplan DR, Miller FD. 2013. Transient maternal IL-6 mediates long-lasting changes in neural stem cell pools by deregulating an endogenous self-renewal pathway. Cell Stem Cell 13:564-76.

Govitvattana N, Osathanon T, Taebunpakul S, Pavasant P. 2013. IL-6 regulated stress-induced Rex-1 expression in stem cells from human exfoliated deciduous teeth. Oral Dis 19:673-82.

Govitvattana N, Osathanon T, Toemthong T, Pavasant P. 2015. IL-6 regulates stress-induced REX-1 expression via ATP-P2Y1 signalling in stem cells isolated from human exfoliated deciduous teeth. Arch Oral Biol 60:160-6.

Huh JE, Lee SY. 2013. IL-6 is produced by adipose-derived stromal cells and promotes osteogenesis. Biochim Biophys Acta 1833:2608-16.

Hurley MM, Abreu C, Marcello K, Kawaguchi H, Lorenzo J, Kalinowski J, Ray A, Gronowicz G. 1996. Regulation of NFIL-6 and IL-6 expression by basic fibroblast growth factor in osteoblasts. J Bone Miner Res 11:760-7.

Iwasaki K, Komaki M, Mimori K, Leon E, Izumi Y, Ishikawa I. 2008. IL-6 induces osteoblastic differentiation of periodontal ligament cells. J Dent Res 87:937-42.

Jee SH, Chu CY, Chiu HC, Huang YL, Tsai WL, Liao YH, Kuo ML. 2004. Interleukin-6 induced basic fibroblast growth factor-dependent angiogenesis in basal cell carcinoma cell line via JAK/STAT3 and PI3-kinase/Akt pathways. J Invest Dermatol 123:1169-75.

Jin L, Hu X, Feng L. 2005. NT3 inhibits FGF2-induced neural progenitor cell proliferation via the PI3K/GSK3 pathway. J Neurochem 93:1251-61.

Kozawa O, Suzuki A, Uematsu T. 1997. Basic fibroblast growth factor induces interleukin-6 synthesis in osteoblasts: autoregulation by protein kinase C. Cell Signal 9:463-8.

Kozawa O, Tokuda H, Matsuno H, Uematsu T. 1999. Involvement of p38 mitogen-activated protein kinase in basic fibroblast growth factor-induced interleukin-6 synthesis in osteoblasts. J Cell Biochem 74:479-85.

Langroudi L, Forouzandeh M, Soleimani M, Atashi A, Golestaneh AF. 2013. Induction of differentiation by down-regulation of Nanog and Rex-1 in cord blood derived unrestricted somatic stem cells. Mol Biol Rep 40:4429-37.

Li B, Qu C, Chen C, Liu Y, Akiyama K, Yang R, Chen F, Zhao Y, Shi S. 2012. Basic fibroblast growth factor inhibits osteogenic differentiation of stem cells from human exfoliated deciduous teeth through ERK signaling. Oral Dis 18:285-92.

Nowwarote N, Pavasant P, Osathanon T. 2015a. Role of endogenous basic fibroblast growth factor in stem cells isolated from human exfoliated deciduous teeth. Arch Oral Biol 60:408-15.

Nowwarote N, Sawangmake C, Pavasant P, Osathanon T. 2015b. Review of the role of basic fibroblast growth factor in dental tissue-derived mesenchymal stem cells. Asian Biomedicine 9:271-283.

Okamura K, Sato Y, Matsuda T, Hamanaka R, Ono M, Kohno K, Kuwano M. 1991. Endogenous basic fibroblast growth factor-dependent induction of collagenase and interleukin-6 in tumor necrosis factor-treated human microvascular endothelial cells. J Biol Chem 266:19162-5.

Osathanon T, Chuenjitkuntaworn B, Nowwarote N, Supaphol P, Sastravaha P, Subbalekha K, Pavasant P. 2014. The responses of human adipose-derived mesenchymal stem cells on polycaprolactone-based scaffolds: an in vitro study. Tissue Engineering and Regenerative Medicine 11:239-246.

Osathanon T, Nowwarote N, Manokawinchoke J, Pavasant P. 2013a. bFGF and JAGGED1 regulate alkaline phosphatase expression and mineralization in dental tissue-derived mesenchymal stem cells. J Cell Biochem 114:2551-61.

Osathanon T, Nowwarote N, Pavasant P. 2011. Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp

stem cells through a FGFR and PLCgamma signaling pathway. J Cell Biochem 112:1807-16.

Osathanon T, Ritprajak P, Nowwarote N, Manokawinchoke J, Giachelli C, Pavasant P. 2013b. Surface-bound orientated Jagged-1 enhances osteogenic differentiation of human periodontal ligament-derived mesenchymal stem cells. J Biomed Mater Res A 101:358-67.

Pricola KL, Kuhn NZ, Haleem-Smith H, Song Y, Tuan RS. 2009. Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. J Cell Biochem 108:577-88.

Schmidt A, Ladage D, Schinkothe T, Klausmann U, Ulrichs C, Klinz FJ, Brixius K, Arnhold S, Desai B, Mehlhorn U, Schwinger RH, Staib P, Addicks K, Bloch W. 2006. Basic fibroblast growth factor controls migration in human mesenchymal stem cells. Stem Cells 24:1750-8.

Scotland KB, Chen S, Sylvester R, Gudas LJ. 2009. Analysis of Rex1 (zfp42) function in embryonic stem cell differentiation. Dev Dyn 238:1863-77.

Son MY, Choi H, Han YM, Cho YS. 2013. Unveiling the critical role of REX1 in the regulation of human stem cell pluripotency. Stem Cells 31:2374-87.

Sukarawan W, Nowwarote N, Kerdpon P, Pavasant P, Osathanon T. 2014. Effect of basic fibroblast growth factor on pluripotent marker expression and colony

forming unit capacity of stem cells isolated from human exfoliated deciduous teeth. Odontology 102:160-6.

Thisse B, Thisse C. 2005. Functions and regulations of fibroblast growth factor signaling during embryonic development. Dev Biol 287:390-402.

Tokuda H, Takai S, Hanai Y, Matsushima-Nishiwaki R, Yamauchi J, Harada A, Hosoi T, Ohta T, Kozawa O. 2008. (-)-Epigallocatechin gallate inhibits basic fibroblast growth factor-stimulated interleukin-6 synthesis in osteoblasts. Horm Metab Res 40:674-8.

Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, Takagishi K, Kato Y. 2001. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem Biophys Res Commun 288:413-9.

Wang Y, Xu C, Wang H, Liu J, Hui S, Li N, Liu F, Li J. 2012. Efficient derivation of human embryonic stem cell lines from discarded embryos through increases in the concentration of basic fibroblast growth factor. Hum Cell 25:16-23.

Wei H, Shen G, Deng X, Lou D, Sun B, Wu H, Long L, Ding T, Zhao J. 2013. The role of IL-6 in bone marrow (BM)-derived mesenchymal stem cells (MSCs) proliferation and chondrogenesis. Cell Tissue Bank 14:699-706.

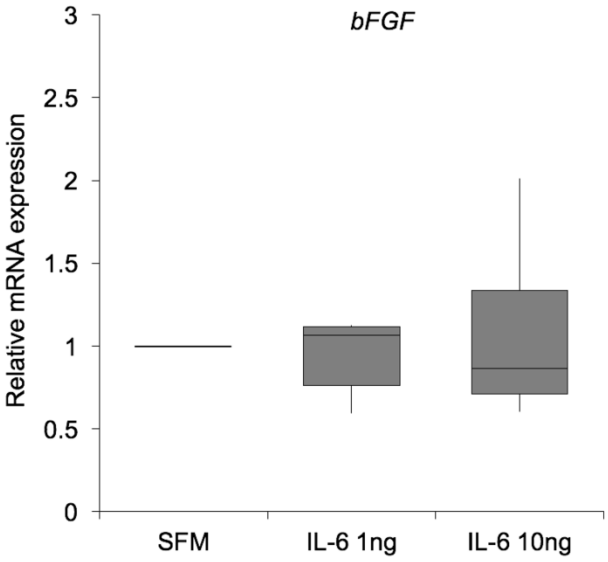
Wu J, Huang GT, He W, Wang P, Tong Z, Jia Q, Dong L, Niu Z, Ni L. 2012. Basic fibroblast growth factor enhances stemness of human stem cells from the apical papilla. J Endod 38:614-22.

Yeoh JS, de Haan G. 2007. Fibroblast growth factors as regulators of stem cell self-renewal and aging. Mech Ageing Dev 128:17-24.

Yoon DS, Kim YH, Lee S, Lee KM, Park KH, Jang Y, Lee JW. 2014. Interleukin-6 induces the lineage commitment of bone marrow-derived mesenchymal multipotent cells through down-regulation of Sox2 by osteogenic transcription factors. FASEB J 28:3273-86.

Yun YR, Won JE, Jeon E, Lee S, Kang W, Jo H, Jang JH, Shin US, Kim HW. 2010. Fibroblast growth factors: biology, function, and application for tissue regeneration. J Tissue Eng 2010:218142.

APPENDICES



Supplementary Figure 1 Effect of IL-6 on *bFGF* mRNA expression in SHEDs.

VITA

Miss Nunthawan Nowwarote was born on 14th May 1982 in Yasothon province, Thailand. She graduated with B.Sc. (Bachelor of Science in animal science) from Rajamangala University of Technology Tawan-Ok in 1999 – 2003 and M.Sc. (Master degree of science in physiology of animal) from Kasetsart University, Master of Science in 2004 – 2006. She had worked at mineralize tissue research unit at faculty of dentistry, Chulalongkorn University for 6 years. She got Poster Award: 6th Hiroshima Conference on Education and Science in Dentistry 2015 21-30 October 2015 at Hiroshima Japan by Oral presentation and Poster presentation in title “Basic fibroblast growth factor induced interleukin 6 expression by stem cell isolated from human exfoliated deciduous teeth”. Her academic publications are as follows:

1. Nowwarote, N., W. Sukarawan, P. Pavasant and T. Osathanon (2016). "Basic Fibroblast Growth Factor Regulates REX1 Expression Via IL-6 In Stem Cells Isolated from Human Exfoliated Deciduous Teeth." J Cell Biochem.

2. Nowwarote,N.,P.PavasantandT.Osathanon(2015)."Roleofendogenousbasic fibroblast growth factor in stem cells isolated from human exfoliated deciduous teeth." Arch Oral Biol 60(3): 408-415.

3. Nowwarote,N.,C.Sawangmake,P.PavasantandT.Osathanon(2015)."Review of the role of basic fibroblast growth factor in dental tissue-derived mesenchymal stem cells." Asian Biomedicine 9(3): 271-283.

4. Nowwarote, N., T. Osathanon, P. Jitjaturunt, S. Manopattanasoontorn and P. Pavasant (2013). "Asiaticoside induces type I collagen synthesis and osteogenic differentiation in human periodontal ligament cells." Phytother Res 27(3): 457- 462.

5. Chuenjitkuntaworn, B., T. Osathanon, N. Nowwarote, P. Supaphol and P. Pavasant (2016). "The efficacy of polycaprolactone/hydroxyapatite scaffold in combination with mesenchymal stem cells for bone tissue engineering." J Biomed Mater Res A 104(1): 264-271.

6. Osathanon,T.,N.NowwaroteandP.Pavasant(2016)."Expressionandinfluence of Notch signaling in oral squamous cell carcinoma." J Oral Sci 58(2): 283-294.

7. Osathanon, T., C. Sawangmake, N. Ruangchainicom, P. Wutikornwipak, P. Kantukiti, N. Nowwarote and P. Pavasant (2016). "Surface properties and early murine pre-osteoblastic cell responses of phosphoric acid modified titanium surface." J Oral Biol Craniofac Res 6(1): 2-9.

8. Osathanon,T.,N.Nowwarote,P.PavasantandW.Sukarawan(2015)."Influence of Jagged1 on apoptosis-related gene expression: a microarray database analysis." Genes & Genomics 37(10): 837-843.

9. Osathanon,T.,B.Chuenjitkuntaworn,N.Nowwarote,P.Supaphol,P.Sastravaha, K. Subbalekha and P. Pavasant (2014). "The responses of human adipose- derived mesenchymal stem cells on polycaprolactone-based scaffolds: an in vitro study." Tissue Engineering and Regenerative Medicine 11(3): 239-246.

10. Osathanon, T., C. Sawangmake, N. Nowwarote and P. Pavasant (2014). "Neurogenic differentiation of human dental pulp stem cells using different induction protocols." Oral Dis 20(4): 352-358.