Effect of cyclic tensile force on the expression of bone morphogenetic protein 9 and interleukin 6 in human periodontal ligament cells

Miss Yanee Tantilertanant

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Oral Biology
Common Course
Faculty of Dentistry
Chulalongkorn University
Academic Year 2018

ผลของแรงดึงแบบเป็นรอบต่อการแสดงออกของโบนมอร์โฟเจนเนติกโปรตีน 9 และอินเตอร์ลิวคิน 6 ในเซลล์เอ็นยึดปริทันต์มนุษย์

น.ส.ญาณี ตันติเลิศอนันต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาช่องปาก ไม่สังกัดภาควิชา/เทียบเท่า
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	Effect of cyclic tensile force on the expression of bone morphogenetic protein 9 and interleukin 6 in human periodontal ligament cells
By	Miss Yanee Tantilertanant
Field of Study	Oral Biology
Thesis Advisor	Associate Professor Dr. Neeracha Sanchavanakit

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the Faculty of Dentistry
(Assistant Professor Dr. SUCHIT POOLTHONG)

DISSERTATION COMMITTEE

..... Chairman
(Professor Dr. PRASIT PAVASANT)

..... Thesis Advisor
(Associate Professor Dr. Neeracha Sanchavanakit)

..... Examiner
(Assistant Professor Dr. Chalida Limjeerajarus)

..... Examiner
(Dr. Nuttha Klincumhom)

..... External Examiner
(Assistant Professor Dr. Weerachai Singhatanadgit)

ญาณี ตันติเลิศอนันต์ : ผลของแรงดึงแบบเป็นรอบต่อการแสดงออกของโบนมอร์โฟเจนเนติกโปรตีน 9 และอินเตอร์ลิวคิน 6 ในเซลล์เอ็นยึดปริทันต์มนุษย์. (
Effect of cyclic tensile force on the expression of bone morphogenetic protein 9 and interleukin 6 in human periodontal ligament cells) อ.ที่ปรึกษาหลัก : รศ. ทญ. ดร.นีรชา สารชวนะกิจ

เนื้อเยื่อเอ็นยึดปริทันต์เป็นเนื้อเยื่อพิเศษที่อยู่ระหว่างฟันและกระดูก ทำหน้าที่ในการช่วยรับแรง เซลล์เอ็นยึดปริทันต์เป็นเซลล์ที่สามารถรับรู้แรงได้ โดยในการกระตุ้นจากแรงในช่องปาก เซลล์เอ็นยึดปริทันต์สามารถสร้างสารต่างๆที่มีความจำเป็นต่อการรักษาสมดุลของเนื้อเยื่อไว้ได้ เซลล์เอ็นยึดปริทันต์ยังสามารถเปลี่ยนแปลงตัวเองไปเป็นเซลล์สร้างกระดูก และเซลล์สร้างเส้นใยคอลลาเจนได้ เซลล์เอ็นยึดปริทันต์รับแรงในช่องปากในลักษณะผลรวมของแรงกระทำ โดยมีแรงส่วนใหญ่อยู่ในรูปแรงดึงแบบเป็นรอบ แรงดึงแบบเป็นรอบมีบทบาทสำคัญในการกระตุ้นเซลล์เอ็นยึดปริทันต์ให้รักษาสมดุลของเนื้อเยื่อทั้งเนื้อเยื่อแข็งและเนื้อเยื่ออ่อน การศึกษานี้ได้แสดงให้เห็นถึงความสามารถของแรงดึงแบบเป็นรอบในการกระตุ้นการแสดงออกของยีนที่ช่วยเหนี่ยวนำการสร้างกระดูกเช่น โบนมอร์โฟเจนเนติกโปรตีน รวมไปถึงแสดงให้เห็นว่าแรงดังกล่าวสามารถเร่งการสะสมแร่ธาตุโดยเซลล์เอ็นยึดปริทันต์ ในกลุ่มโบนมอร์โฟเจนเนติกโปรตีนนี้ โบนมอร์โฟเจนเนติกโปรตีน 9 มีศักยภาพในการเหนี่ยวนำให้เกิดการสร้างกระดูกสูงที่สุด อย่างไรก็ตาม ผลของแรงดึงแบบเป็นรอบต่อการแสดงออกของยีนโบนมอร์ฟอจีนิกโปรตีน 9 และการสะสมแร่ธาตุนั้นยังไม่เป็นที่ทราบแน่ชัด การศึกษานี้ได้แสดงให้เห็นถึงการใช้แรงดึงแบบเป็นรอบอย่างต่อเนื่องเป็นเวลา 6 ชั่วโมงสามารถกระตุ้นการแสดงออกของยีนโบนมอร์ฟอจีนิกโปรตีน 9 และการสะสมแร่ธาตุในเซลล์เอ็นยึดปริทันต์ได้ โดยการกระตุ้นการแสดงออกของยีนนี้เกิดผ่านโมเลกุลเอทีพีและตัวรับชนิดพีทูวายวัน กลไกการกระตุ้นนี้เกิดผ่านการเพิ่มขึ้นของระดับแคลเซียมอิออนในเซลล์ และฟอสโฟไลเปส ซี นอกจากนี้ การใช้สารแอนติบอดี้ต่อโบนมอร์โฟเจนเนติกโปรตีน 9 ยังสามารถลดระดับการสะสมแร่ธาตุจากแรงดึงได้ด้วย แสดงให้เห็นว่าแรงดึงแบบเป็นรอบนี้สามารถกระตุ้นการสร้างสารที่เกี่ยวข้องกับการสร้างกระดูกได้ ซึ่งชี้นำให้เห็นว่าแรงดึงมีผลต่อการควบคุมสมดุลของเนื้อเยื่อแข็งในเนื้อเยื่อเอ็นยึดปริทันต์ นอกจากเนื้อเยื่อแข็งแล้ว การศึกษานี้ยังแสดงให้เห็นว่าแรงดึงแบบเป็นรอบสามารถกระตุ้นการสร้างไซโตไคน์ที่เกี่ยวข้องกับการอักเสบอย่างอินเตอร์ลิวคิน 6 เซลล์เอ็นยึดปริทันต์อาจต้องใช้สารเหล่านี้ในการปรับเปลี่ยนสมดุลของเนื้อเยื่ออ่อนเพื่อตอบสนองต่อแรงกระตุ้น ผลของสารอินเตอร์ลิวคิน 6 ต่อการแสดงออกของยีนของเอนไซม์เมทริกซ์เมตาโลโปรตีนเนสซึ่งเป็นเอนไซม์ที่จำเป็นต่อการปรับเปลี่ยนเนื้อเยื่อนั้นยังไม่เป็นที่ทราบแน่ชัด การศึกษานี้พบว่าแรงดึงแบบเป็นรอบสามารถทำให้เกิดการเพิ่มขึ้นของอินเตอร์ลิวคิน 6 และเอนไซม์เมทริกซ์เมตาโลโปรตีนเนสไปพร้อมๆกัน อินเตอร์ลิวคิน 6 สามารถเพิ่มการแสดงออกของยีนเมทริกซ์เมตาโลโปรตีนเนส 3 ได้ซึ่งพิสูจน์ด้วยการใช้สารแอนติบอดี้ต่ออินเตอร์ลิวคิน 6 และการใช้สารอินเตอร์ลิวคิน 6 ในการกระตุ้นเซลล์ แสดงให้เห็นว่าแรงดึงแบบเป็นรอบนี้มีความสำคัญต่อการปรับเปลี่ยนเนื้อเยื่ออ่อนในเนื้อเยื่อเอ็นยึดปริทันต์ด้วย

สาขาวิชา	ชีววิทยาช่องปาก	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5676051532 : MAJOR ORAL BIOLOGY

KEYWORD: periodontal ligament cells, cyclic tensile force, bone morphogenetic proteins 9, interleukin 6, matrix metaloproteinase 3

Yanee Tantilertanant :

Effect of cyclic tensile force on the expression of bone morphogenetic protein 9 and interleukin 6 in human periodontal ligament cells. Advisor: Assoc. Prof. Dr. Neeracha Sanchavanakit

Periodontium is a specialized tissue laying between tooth and surrounding bone. It functions as force cushion. In particular stimulation including mechanical force, periodontal ligament (PDL) cells which are mechanosensitive cells have the ability to secrete specific cytokines and proteins. PDL cells also have the potential to differentiate into osteoblast-like cells or fibroblast-like cells. PDL cells perceive combination of force in oral cavity in which major component is cyclic tensile force (CTF). CTF play an essential role in modulating PDL cells to maintain both hard and soft tissue integrity. Here, we demonstrated that CTF was able to stimulate the expression of osteoinductive genes including bone morphogenetic proteins (BMPs) by PDL cells. Among the BMPs, BMP9 is one of the most potent osteogenic BMPs. We showed that continuously applied CTF for only the first 6 hours stimulated the synthesis of BMP9 which enhanced the mineral deposition by 14 days. Stimulation of *BMP9* expression depended on ATP and $P2Y_1$ receptors by an increased level of intracellular Ca^{2+} through the phospholipase C (PLC) pathway. In addition, CTF also upregulated the expression of interleukin 6 (IL6) and MMPs. We demonstrated that CTF induced IL6 expression coincided with an increased matrix metaloproteinase 3 (MMP3) expression. A neutralizing IL6 antibody attenuated the CTF-increased MMP3 expression, whereas stimulating the cells with recombinant human IL6 increased MMP3 expression. Thus, CTF-induced IL6 increased the expression of MMP3. Collectively, our findings suggest an essential modulatory role of CTF in the homeostasis and regeneration of the periodontium.

Field of Study: Oral Biology Student's Signature .....

Academic Year: 2018 Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest appreciation to my thesis advisor, Associated Professor Neeracha Sanchavanakit for her noteworthy support and all advice not only in my research but also in coaching my life. I greatly thank for her generosity and patience she exerted on my work.

I would also like to show my intense gratefulness to Professor Dr. Vincent Everts for his practical suggestions, guidance, and energy he spent on my work. He is the great teacher and excellent role model. He draw my inspiration back to research life. He is worth to my sincere appreciation for his priceless relationship he has been given to me and my family.

I am notably thankful for all of my thesis committee: Professor Dr. Prasit Pavasant, Assistant Professor Dr. Weerachai Singhatanadgit, Assistant Professor Dr. Chalida Limjeerajarus, and Dr. Nuttha Klincumhom for reviewing my work and their valuable comments.

Moreover, I am also thankful to all my lab colleague for their suggestion and kind relationship during my working time in the Center of Excellence for Regenerative Dentistry, Faculty of Dentistry, Chulalongkorn University.

I truly appreciate Chulalongkorn University for being my warm-hearted home. Chulalongkorn University is my second home that raise me up, give me great experiences and opportunity to obtain the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship from Graduate School, Chulalongkorn University.

Special appreciation is expressed to Miss Mathaneeya Bunbanjerdsuk for her valuable cheer and friendship.

Finally, I considerably thank to my family for consistently helping me during my PhD. study. The last but by no means least, my husband, Mr. Thanapol Tangkriengkij, for invaluable his encouragement and hold my hand along the way.

Yanee Tantilertanant

TABLE OF CONTENTS

Lists of Tables

Page

Lists of Figures

Page

Chapter 1 Introduction

Background and rationale

Mechanical force normally occurs in the oral cavity during mastication, orthodontic treatment, parafunctional behavior and others. Such forces transmit from tooth to tooth-surrounding tissues which are cementum, periodontal ligament, and alveolar bone. Changes in force loading are important for normal functioning and homeostasis of these tissues. Occlusal hypofunction has been reported to cause irregular arrangement of the fibers and a decrease of periodontal space (Kaneko et al., 2001). Heavy occlusal force leads to an increase in the width and volume of periodontal space (Steigman et al., 1989). These observations suggest that normal loading force is essential for the homeostasis of periodontal tissue.

Periodontal ligament

The periodontal ligament (PDL) is the connective tissue that connects the tooth to its surrounding alveolar bone. The PDL is essential in transferring applied force from the tooth to the bone. Cells of the PDL are mechanosensitive and perceive force from various origin such as masticatory force and (orthodontic) tooth movement. The cells of the PDL consist of a mixed population of cell types including those which have multipotent mesenchymal stem cell properties that can differentiate in response to a variety of stimuli to either maintain homeostasis or to participate in modeling of the periodontal tissue (McCulloch et al., 2000; Seo et al.,

2004). The involvement of PDL cells is modulated by their responses to physiological occlusal forces which constitutively exist in the oral environment (Kawarizadeh et al., 2005; Saminathan et al., 2012; Yamashiro et al., 2007).

Mechanical force affects the PDL

Mode of action of mechanical force that persists in oral tissue like periodontal ligament tissue is roughly divided by mode of action into compressive, tensile, and shear force (Woda et al., 2006). Despite a different proportion on each area at the time, all three types of force exert together on the periodontal ligament and its neighboring tissues (Benazzi et al., 2016). Hence, the behavior and responses of the cells in the periodontium result from a combination of forces the cells perceive dynamically (Benazzi et al., 2016). Most studies of force in PDL cells are related to masticatory and orthodontic forces. Recently, an *in vivo* study (Kalajzic et al., 2014) on orthodontic force has been performed using a cyclic pattern since PDL cells sense force not only from orthodontic treatment but also force in conjunction with mastication. Previous studies have shown that cyclic tensile force (CTF) mimics the main profile of occlusal force (Fujihara et al., 2010; Matsuda et al., 1998). Moreover, CTF appears to occur also on the tension side during orthodontic tooth movement (Li et al., 2013a; Tang et al., 2012; Wescott et al., 2007). Different reports demonstrate a correlation between CTF during physiological occlusal force and PDL cellular activities in terms of osteogenic differentiation (Fujihara et al., 2010; Li et al., 2013a; Shen et al., 2014; Tang et al., 2012; Yang et al., 2006).

Mechanical force induces synthesis of signaling molecules

Periodontal ligament cells respond to force by sensing and transducing it into biological activity called mechanotransduction (Jaalouk and Lammerding, 2009). One of the mechanisms by which mechanical force drives cellular responses is through the release of signaling molecules, for examples, purine/pyrimidine such as ATP (Kariya et al., 2015; Wongkhantee et al., 2008). Since the purine/pyrimidine signaling molecules are promptly released after force sensing, they are considered to be one of the earliest signals in translating a mechanical stimulus into biological responses. Tension force was also able to induce ATP release which subsequently could promote osteogenesis by osteoblasts (Kariya et al., 2015; Sun et al., 2013). This promoting effect was proposed to be due to a stimulated expression of BMP2, 4 and 5 (Ayala-Pena et al., 2013).

Cyclic tensile force in a physiological range induced the expression of essential molecules and growth factors associated with bone formation such as RunX2 (Li et al., 2013a; Tang et al., 2012), OSX (Tang et al., 2012), ALP (Wescott et al., 2007), and BMP-2 and -6 (Suzuki et al., 2014; Wescott et al., 2007) in PDL cells. Following orthodontic treatment, an increased expression of osteogenic mediator-associated genes and proteins were found in the PDL cells located on the tension side (Shen et al., 2014). Perceiving a mechanical force also enhances the cell's capability to differentiate into cementoblasts and osteoblasts or to participate in modulating osteoclastogenesis (Fujihara et al., 2010; Kanzaki et al., 2002; King and

Hughes, 1999; Long et al., 2002; Yang et al., 2006). These events are sequentially controlled by the synthesis and release of cytokines, and growth and differentiation factors such as receptor activator of nuclear factor kappaB ligand (RANKL) , osteoprotegerin (OPG) and bone morphogenetic proteins (BMPs).

BMPs and the periodontal ligament

BMPs belong to the transforming growth factor (TGF)-β superfamily. Certain BMPs, e.g. BMP-2, -4, -6, -7, and -9, are classified as osteogenic factors which are able to induce osteoblast differentiation and lead to bone formation (Cheng et al., 2003; Kang et al., 2004). An increased expression of BMP-2 has been reported to be induced by cyclic tensile force and was mediated through COX2 (Suzuki et al., 2014).

Of the different osteogenic BMPs, BMP-9 has currently been suggested having the most potent osteogenic capability. The BMP-9-induced ectopic bone formation was not inhibited either by BMP-3 (Kang et al., 2004) or by noggin (Wang et al., 2013b), which can inhibit other osteogenic BMPs. Moreover, rhBMP-9 was recently reported to induce higher levels of alkaline phosphatase and *in vitro* calcification comparing to rhBMP-2 (Fujioka-Kobayashi et al., 2016a; Fujioka-Kobayashi et al., 2016b). Exogenous BMP-9 was shown to have a high potential to induce expression of osteogenic differentiation genes such as Runx2, OSX, ALP, Hey1 in MSCs and also PDL cells (Fuchigami et al., 2016; Sharff et al., 2009). Human PDL cells transfected with adenoviruses expressing BMP-9 can elevate the expression level of osteogenic

differentiation genes and promote mineralization through ERK and p38 signaling pathways (Ye et al., 2014). However, effect of cyclic tensile force on expression of BMP-9 in PDL cells and its biological function have not been elucidated.

Interleukins and the PDL

Physical loading also influences the regeneration and maintenance of the PDL integrity via cellular activity such as proliferation, migration, and extracellular matrix remodeling (McCulloch et al., 2000). PDL cells are embedded in an extracellular matrix which consists of both fibrillar and non-fibrillar proteins. Fibrillar collagens, major extracellular matrix components of the PDL have been shown to be reorganized and remodeled under the influence of mechanical loading (Kalajzic et al., 2014; Nakagawa et al., 1994). The remodeling of these matrix constituents is likely modulated by different cytokines such as interleukin 8 and -6. Physiologic CTF can increase the synthesis of these and other cytokines e.g. interleukin 8 (Maeda et al., 2007), interleukin 6 (Wada et al., 2017), TGF-β (Wada et al., 2017). At low expression level these inflammatory cytokines may be needed for tissue remodeling. Younger persons with a higher periodontal tissue metabolic activity have a higher IL6 expression level higher than adults (Grzibovskis et al., 2011). Moreover, in response to a mechanical force including masticatory and orthodontic forces, PDL cells also have the capacity to synthesize and release a number of proteolytic enzymes (e.g. MMP3).

Interleukin 6 (IL6) is a multi-functional cytokine that modulates in various organ systems a variety of physiological and pathological events such as cell proliferation, differentiation, cell survival, and inflammation. Next to immune cells, many cell types are reported to produce IL6; these include chondrocytes, osteoblasts, endothelial cells, muscle cells, fibroblasts, keratinocytes, and certain tumor cells (Hosokawa et al., 2014; Iwasaki et al., 2008; Kuhn et al., 2014; Tang et al., 2011). Regarding the periodontal tissue, increases in IL-6 levels were found in the compression zones of orthodontically moved teeth which coincided with attracted macrophages and stimulated osteoclastic activity (Teixeira et al., 2010). CTF was shown to increase the expression of IL6 by a human immortalized PDL cell line (Wada et al., 2017). Next to that, IL6 increases the expression of *Runx2* and *ALP,* thus promoting osteogenic differentiation of human PDL cells (Iwasaki et al., 2008). The cytokine also enhances cancer cell migration through an increased level of *MMP2* and *MMP9* (Kossakowska et al., 1999). Recently, IL6 has been shown to promote mesenchymal stem cell migration through an up-regulation of *MMP1* and *MMP3* (Casson et al., 2018).

MMPs and the PDL

Matrix metalloproteinases (MMPs) belong to a family of proteolytic enzymes collectively responsible for the physiological and pathological degradation of extracellular matrix proteins, including collagens, fibronectin, laminin and proteoglycans. Moreover, MMPs participate in proteolytic activation of several other

enzymes and growth factors/mediators thereby facilitating tissue remodeling and integrity. The MMP family is divided into 5 groups; collagenases (MMP1, 8, and 13), stromelysins (MMP3, 10, and 11), gelatinases (MMP2 and 9), and membrane-bound MMPs (MMP14, 15, 16, and 17), and some others (matrilysin-MMP7, macrophage elastase-MMP12, enamelysin-MMP20) depending on their substrate specificity (Jablonska-Trypuc et al., 2016; Nagase and Woessner, 1999).

The remodeling of collagen which is extremely fast in the PDL requires the orchestration of different types of proteases, among which the MMPs. MMPs play a central role in PDL remodeling both physiologically and pathologically due to their ability to cleave native triple-helical interstitial collagens. These enzymes are synthesized by a variety of cell types in the periodontium including PDL fibroblasts. The expression of MMPs is modulated by a wide variety of factors including interaction between cell to cell or cell to ECM, growth factors, cytokines, and hormones (Page-McCaw et al., 2007).

It is interesting to understand the cellular behavior of PDL under mechanical situation. The advantages of the studies presented in this thesis are not only to explain the general behavior and capability of the cells but the findings can also be used to guide the treatment that require some particular molecules induced by cells under mechanical circumstances.

Research question

Thus, this raises the question how human PDL cells respond to CTF and whether this response affects their biological function.

Research hypothesis

We hypothesize that CTF, which mimics the physiologic masticatory force, affects the remodeling capacity of human PDL cells.

Research Objective

This present study intended to investigate the response of human PDL cells to CTF that resembles the physiologic masticatory force

Specific aims

We divided the aims of the studies as follows:

1. To investigate the effect of CTF on the expression of osteogenic genes and the mineralization by human PDL cells

- ***Hypothesis***: CTF increases the expression of osteogenic genes and the mineralization by human PDL cells. If so, such osteogenic genes may be involved in the mineralization induced by PDL cells.

- ***Experimental design***

    i. *Expression of osteogenic genes*: Human PDL cells in fresh serum-free DMEM were subjected to CTF with an elongation strain of 10% and

the frequency of 60 rpm continuously for 2 and 6 hours. RNA and proteins were extracted and analyzed by quantitative real-time PCR (RT-PCR) and ELISA, respectively.

ii. *In vitro Mineralization*: Six-hour CTF-induced PDL cells were then cultured in the osteogenic medium (OM) for 12-14 days. To further investigate the role of osteogenic BMP9, cells were cultured with 0.2 $\mu g.mL^{-1}$ neutralizing anti-BMP9 monoclonal IgG antibody or a non-specific mouse IgG antibody as a control antibody. The addition of anti-BMP9-antibody in non-CTF loaded *in vitro* mineralization cultures (control cultures) was also performed. *In vitro* mineralization was then analyzed by Alizarin red S staining.

2. To investigate the effect of CTF on ATP release from human PDL cells and the involvement of ATP in the expression of osteogenic genes by human PDL cells.

- ***Hypothesis***: CTF induces ATP release which then increases the expression of osteogenic genes by human PDL cells.

- ***Experimental design***

i. The amount of extracellular ATP was measured using an ATP bioluminescence detection kit. culture medium was collected

during CTF loading of human PDL cells at 0, 10, 20, 30, 60, 120 and 360 minutes.

ii. Human PDL cells were cultured in the presence of 0, 0.1, 1 and 10 μM exogenous ATP for 6 hours. RNA and proteins were extracted and analyzed by quantitative real-time PCR (RT-PCR).

iii. Apyrase (2.5 U), an ecto-ATPase, was added to the cells thirty-minutes prior to a six-hour CTF loading. RNA and proteins were extracted and analyzed by quantitative real-time PCR (RT-PCR).

3. To clarify the pathway involved in the expression of CTF-increased osteogenic genes.

- ***Hypothesis***: CTF increases osteogenic genes through the involvement of ATP and its receptors.

- ***Experimental design***

i. Various inhibitors of signaling pathways were used to investigate involvement of CTF-increased osteogenic genes.

ii. Agonists of receptors suspected to participate, were used to identify the involvement in the upregulation of CTF-increased osteogenic genes.

4. To examine the effect of CTF on the expression of IL6 and enzymes by human PDL cells

   - ***Hypothesis***: CTF induces the expression of IL6, MMPs and TIMPs.

   - ***Experimental design***

     i. Cells in fresh serum-free DMEM were subjected to CTF with an elongation strain of 10% and the frequency of 60 rpm continuously for 2 and 6 hours. CTF-loaded cells were further incubated for 24 and 48 hours. RNA and proteins were extracted and analyzed by quantitative real-time PCR (RT-PCR) and ELISA, respectively.

5. To investigate the involvement of CTF-induced IL6 in the upregulation of MMPs

   - ***Hypothesis***: CTF-induced IL6 increases MMP expression

   - ***Experimental design***

     i. After finishing a six-hour CTF, cells were incubated with 0.07 $\mu g.mL^{-1}$ of a specific anti-IL6 mouse monoclonal antibody or with 0.07 $\mu g.mL^{-1}$ of a non-specific mouse IgG as a control for another 24 and 48 hours. RNA and culture media were collected and analyzed for gene expression and secreted proteins.

ii. Human PDL cells were treated with recombinant human IL6 at concentrations of 0.1, 1, 10 $ng.mL^{-1}$ or a control vehicle for 24 hours. RNA and culture media were collected and analyzed for gene expression and secreted proteins.

iii. Various inhibitors were used to pretreat human PDL cells prior to IL6 stimulation for 24 hours in order to clarify possible mechanisms of upregulation.

Chapter 2 Literature review

Mechanical force

Mechanical force is one of the most important environmental stimulators for cellular activity. Such forces are involved in almost all biological processes of cells such as proliferation, migration, differentiation, and even apoptosis. From development to maintenance, mechanical force influences the form and function of structures in our bodies. Hemodynamic pressure and shear force are necessary for cardiac development (Granados-Riveron and Brook, 2012). Shear and tensile force in blood vessels provided protective factors for prevention of blood vessel damage (Birukov et al., 2003) and regulative factors for vessel remodeling (Langille and O'Donnell, 1986; Rochier et al., 2011; Tuttle et al., 2001). In the musculoskeletal system, external mechanical force impacts the shape of trabecular bone (Huiskes et al., 2000). Bone resorption increases and bone formation decreases in microgravity or unloading conditions as found in bed rest condition or in cosmonauts (Vico et al., 2000; Zerwekh et al., 1998). Moreover, epithelial cells lining in the intestinal wall undergo cyclic strain stimulating cell proliferation (Basson et al., 1996).

Oral structures also experience mechanical force from mastication, speech, parafunctional habits, and orthodontic treatment. One of the mechanosensitive cell types in the oral cavity is the periodontal ligament (PDL) cell. This cell type is embedded in the extracellular matrix of the periodontal ligament. This connective

tissue connects the tooth to its surrounding alveolar bone. The PDL perceives the force from the tooth and transfers it to the bone acting as a force absorber. Periodontal ligament perceives mechanical force which is roughly divided by mode of action into compressive, tensile, and shear force. All three types of forces exert together on the periodontal ligament and its neighboring tissues (Benazzi et al. 2016). During chewing cycle, stress pattern changes noticeably. Hence, the behavior and responses of the cells in the ligament result from the combination of forces that the cells perceive dynamically (Benazzi et al., 2016). Cells sense mechanical force through their mechano-sensors which will activate or inhibit intracellular signaling cascades and finally induce both non-transcriptional and transcriptional changes (Huang et al., 2004; Jaalouk and Lammerding, 2009).

Mechanotransduction

Cells respond to mechanical forces and translate this into a biological signal which is subsequently translated into cellular responses by a process called mechanotransduction (Huang et al., 2004; Jaalouk and Lammerding, 2009). Cells perceive the change of their environment from mechanical stimuli through diverse cell surface receptors including integrins, stretch-activated ion channels, G protein coupled-receptors, and growth factor receptors.

Integrins

Almost all cell types are attached to components of the extracellular matrix. When the matrix is subjected to physical force, ECM proteins can be

non-stretched or stretched which transmit signals to the adherent cells through specific cell-adhesion molecules like integrins. This results in changes of cell binding properties and intracellular signaling (Jaalouk and Lammerding, 2009; Oberhauser et al., 2002). Integrins link the extracellular environment to intracellular protein complexes and mediate signals through the cytoskeleton. The primary function of integrins is binding the cell to extracellular matrix. They play an important role in mediating the mechanical signaling and to recruit cytoplasmic proteins in order to propagate mechanical forces to alter the cellular responses or activities (Miyamoto et al., 1995). Many signaling molecules recruitment to propagate intracellular signal cascades will turn on or inhibit gene expression in the nucleus (Ingber, 2006; Miyamoto et al., 1995). The induction signal induced by integrins also results in a change of transmembrane channels (Ingber, 2006)leading to a release of some molecules such as ATP.

Mechanosensitive ion channels

Stress-sensitive ion channels are plasma membrane bound channels that cross the membrane and play an essential role in transportation of ions like calcium from the exterior to the interior environment of the cell. Under the influence of mechanical forces, the ion channels have the capacity to change their conformation resulting to an open or closed state of the channel. These alterations may also be induced by elements of the

cytoskeleton that tug on the cytoplasmic portion of the channels. Consequently, they establish pores across cell membranes and reform their conformations leading to an altered rate of open and closed state of channels (Ghazi et al., 1998; Jaalouk and Lammerding, 2009). Ion influx then results in alterations of membrane potentials and intracellular ion concentration. It is followed by the release of intracellular messengers which may either initiate phosphorylation cascades resulting in changes of gene transcription or a rapid release of biological mediators (Mobasheri et al., 2005).

G protein coupled-receptors

G protein coupled receptors (GPCR) are receptors used by the cells to sense the mechanical signaling. The binding of an external signal molecule to a GPCR stimulates signaling pathways inside the cell that are involved in a large variety of processes. Moreover, some of the components can interact with mechanosensory channels in response to pain or inflammation (Geppetti et al., 2015; Veldhuis et al., 2015). GPCRs are also reported as mechanosensors that respond to fluid shear stress in neutrophils and in endothelial cells (Chachisvilis et al., 2006; Makino et al., 2006). Furthermore, one type of purine/pyrimidine receptors is GPCR known as P2Y receptors.

Growth factor receptors

This type of receptors has also been recognized as mechanosensing receptors. VEGFR2 has been reported as mechanosensor to shear stress in endothelial cells (Shay-Salit et al., 2002). Epidermal growth factor receptor (EGFR) can also be activated in a ligand-independent manner after being stimulated by ECM stiffness (Rosenthal, 2017).

Signaling mediators

After sensing force, one cellular response is the immediate release of some signaling molecules such as ATP (Luckprom et al., 2011; Romanello et al., 2001; Wongkhantee et al., 2008), UTP (Lazarowski and Boucher, 2001), PGE_2 (Luckprom et al., 2010), and nitric oxide (Bakker et al., 2001). In the present study, we focus on ATP as extracellular signaling molecule. Interestingly, ATP was reported a sudden release after mechanical loading in several cell types such as PDL cells and osteoblasts (Kariya et al., 2015; Luckprom et al., 2011; Takahara et al., 2014).

ATP (adenosine 5'-triphosphate)

ATP, one of nucleotides, belongs to the purine family. It is made of a nitrogenous base, a sugar and a chain of three phosphate groups bound to the sugar. ATP is well-known as the universal intracellular source of readily available chemical energy for all living cells. It is synthesized in mitochondria and found in the cytoplasm at a concentration of around 2-5 mM (Traut, 1994). Not only being the intracellular source of power, ATP is also functioning as extracellular ligands to

transfer signaling to the target cells. It is accepted to initiate and regulate many biological processes including cell proliferation (Coppi et al., 2007), differentiation (Atarashi et al., 2008), inflammation (Idzko et al., 2014), and apoptosis (Wen and Knowles, 2003). Unlike lytic release from cell death (Bodin and Burnstock, 2001), ATP can be non-lytically released through connexin hemichannels (Luckprom et al., 2011), anion channels (Liu et al., 2008; Sabirov and Okada, 2005) or pannexin channels (Lohman and Isakson, 2014; Woehrle et al., 2010).

Several cells can release ATP from intracellular storage into the extracellular environment when they undergo certain conditions such as mechanical loading in osteoblasts and periodontal ligament cells (Costessi et al., 2005; Ito et al., 2014; Kanzaki et al., 2006; Luckprom et al., 2011; Romanello et al., 2001; Wongkhantee et al., 2008), inflammation (Okada et al., 2013), and hypoxia (Bergfeld and Forrester, 1992).

Released ATP is utilized to amplify information by an autocrine network or to transfer the information to neighboring cells as a paracrine signaling (Lazarowski et al., 2011). Such ATP interact with purinoceptors expressed on target cell surfaces in order to regulate numerous biological processes. In PDL cells, ATP/$P2Y_1$ is involved in expression of osteopontin and RANKL (Luckprom et al., 2010; Wongkhantee et al., 2008), while ATP/$P2X_7$ is capable of increasing IL-1β expression (Kanjanamekanant et

al., 2013). ATP also induced BMP-2, -4, -5 expression in rat osteoblasts which probably is associated with $P2Y_2$ (Ayala-Pena et al., 2013).

Extracellular ATP is quickly catalyzed by a group of cell-surface or soluble extracellular enzymes named ecto-nucleotidase 5'-triphosphate diphosphohydrolase (NTPDase) family into ADP, AMP, nucleoside and phosphate (Lazarowski et al., 2003; Zimmermann, 1996).

ATP-Receptors

ATP exerts its signal as an extracellular ligand through specific receptors, the purinoceptors. Purinoceptors are categorized into two groups: P1 and P2. P1 receptors are preferentially activated by adenosine. P2 receptors, which consist of 2 distinct families, P2X and P2Y, respond to extracellular nucleotides like ATP, ADP, UTP, or UDP. P2 receptors are expressed on the cell surface of almost all cell types.

- ***P1 receptors*** are divided in to four distinct subtypes: A_1, A_{2A}, A_{2B} and A_3 receptors. They are members of the G protein-coupled receptor (GPCR) superfamily and bind preferentially to adenosine (Jacobson and Gao, 2006).

- ***P2X receptors*** are ionotropic receptors and membrane ion channels that open in response to binding of extracellular ATP. There are 7 isoforms ($P2X_{1-7}$) which are permeable to ions like Ca^{2+}, Na^{+}, K^{+}, and Cl^{-}. Once P2X receptors bind to ATP, the receptors will change their conformation and

generate a pore for ions to travel through the membranes (Syed and Kennedy, 2012). $P2X_1$ and $P2X_3$ receptors swiftly desensitize in the presence of ATP, whereas the $P2X_2$ receptor channel remains open for as long as ATP is bound to it (Koshimizu et al., 2000; Li et al., 2013b).

- ***P2Y receptors*** are metabotropic receptors. There are 8 isoforms of P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) which are all members of the G protein-coupled receptor (GPCR) superfamily. P2Y receptors can be classified into 4 groups. $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ are activated by adenine nucleotides. $P2Y_2$ and $P2Y_4$ respond to both adenine and uridine nucleotides. $P2Y_6$ is a receptor for uridine nucleotides. $P2Y_{14}$ is stimulated by UDP-sugar (Abbracchio et al., 2003). While ATP can fully activate $P2Y_2$ and $P2Y_{11}$, it can be a partial agonist of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors (Van Kolen and Slegers, 2006). The complexion of ligand and receptors subsequently activate phospholipase C (PLC) phosphoinositide hydrolysis and protein kinase C. $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ can also signal through a change of the level of adenylate cyclase (Novak, 2003).

Periodontal ligament (PDL)

Periodontal ligament is a part of the periodontium which consists of gingiva, cementum, periodontal ligament (PDL), and alveolar bone. It acts as mechanical force absorber and mediates the transmission of force from tooth to the alveolar

bone. PDL is a unique connective tissue consisting of a meshwork of collagenous fibrils that connect the cementum to the alveolar bone. The width of the PDL is approximately 0.15 to 0.38 mm (Lindhe and Karring, 1989). The ligament fibers possess nonlinear and viscoelastic properties respective to their location and direction (Cattaneo et al., 2009; Toms et al., 2002). For the cellular components the PDL consists of a mixed population of cell types (McCulloch et al., 2000). The PDL has the capacity to regenerate and remodel in order to maintain homeostasis and tissue integrity (Lekic and McCulloch, 1996). Apart from the PDL cells (fibroblasts), PDL contains nerve fibers, immune cells, blood vessels, osteoblasts and osteoclasts on the bony surface and cementoblasts on the cementum. The PDL also harbors a population of multipotent stem cells of the mesenchymal lineage (Silverio et al., 2010). They act as a reservoir of cells to differentiate into different cell types including those with dentinogenic and osteogenic potential (Choi et al., 2015). They also can differentiate into cementoblast-like cells, adipocytes, and collagen-producing cells (Seo et al., 2004). These findings indicate the high potential of PDL cells to repair and regenerate periodontal tissues.

PDL cells exposed to the physical force results in the regeneration and maintenance of tissue integrity due to their activities such as proliferation, migration, and extracellular matrix remodeling (Kawarizadeh et al., 2005; Kook et al., 2011; McCulloch et al., 2000; Saminathan et al., 2012). In response to various stimuli including mechanical force, PDL cells are able to synthesize and release particular

cytokines and enzymes (Kanzaki et al., 2002; Kook et al., 2011; Rosselli-Murai et al., 2013; Wada et al., 2017; Yamashiro et al., 2007) and also able to differentiate into other cell types such as cementoblasts or osteoblasts (King and Hughes, 1999; Li et al., 2013a; Yang et al., 2006).

Mechanical force in PDL environment

Sources of mechanical force in oral cavity are diverse such as physiologic occlusal force, orthodontic force, speech, and facial expression. Teeth and surrounding tissues are subjected to occlusal force every day, suggesting an essential role plaid by physiological occlusal force in homeostasis of the periodontium. Non-occluded teeth have an irregular arrangement of periodontal fibers and a narrowing periodontal space (Kaneko et al., 2001). Occlusal force also increases PDL cell proliferation to maintain PDL structure (Mine et al., 2005). Occlusal force is transmitted through the cementum and the PDL fiber and seems to indirectly load on alveolar bone cells. Interestingly, the finding from both *in vivo* and *in vitro* finite element analyses demonstrated that a large strain occurs in PDL fibers, while strain occurring in alveolar bone is too low to cause bone remodeling (Chen et al., 2014; Melsen, 2001; Toms et al., 2002). Physiologic occlusal force is essential for the proliferation of PDL cells.

Increasing evidence supports the role of PDL cells in tissue maintenance and alveolar bone remodeling by releasing regulatory proteins (Funakoshi et al., 2013; Ku et al., 2009; Li et al., 2013a; Maeda et al., 2007; Suzuki et al., 2014). These proteins may result in either tissue regeneration/healing or tissue destruction, depending on environmental stimulations that affect these regulatory proteins.

A tooth can move when it is subjected to force, either by orthodontic or masticatory force. Compression and tension force were reported to promote bone resorption and enhance bone formation, respectively, in order to maintain alveolar bone homeostasis. PDL also attained shear force through interstitial fluid within the tissue. The interstitial fluid will be distorted and squeezed within the space that surrounds the PDL cells, thus producing shear stress to the cells. Studies using finite element analysis (Cattaneo et al., 2005; Cattaneo et al., 2009; McCormack et al., 2014) revealed that mechanical force on periodontal tissue does not occur as a simple compression and tension, but it happens in combination.

Researchers tried to invent systems to simulate *in vivo* mastication; but none of those systems could mimic the real situation. This suggests that one type of force has to be studied at a time. As part of the occlusal force, tensile force occurs in almost all parts of the PDL ligament with the

exception of the most apical part (Benazzi et al., 2016). In the present study we will focus on the effect of cyclic tensile force which resembles masticatory force.

Tension force and PDL

Tension force can change both morphology (Bolcato-Bellemin et al., 2000) and the spatial arrangement of subjected cells (Li et al., 2013a; Liu et al., 2012; Wang et al., 2013a). Tension force influences the expression and production of proteins involved in a wide variety of cellular functions. Some cellular responses to tensile stress of PDL cells are essential for bone formation. Tensile force was reported to enhance the expression of many osteogenic genes such as Runx2, ALP, BMPs (Shen et al., 2014; Suzuki et al., 2014; Tang et al., 2012; Wescott et al., 2007). Cyclic tensile stress seems to promote not only osteoblast differentiation but also to suppress osteoclastogenesis as osteoblast increased osteoprotegerin (OPG) protein level concurrently with a decreased RANKL protein synthesis in a magnitude-dependent manner (Tang et al., 2006; Tsuji et al., 2004). However, another study reported the opposite. It showed that tensile force loaded on osteoblasts can induce osteoclastogenesis (Chen et al., 2015). Duration of force application also has an effect on osteogenic gene expression. Suzuki and colleagues reported that PDL cells subjected to cyclic tensile stress showed a higher expression of BMP2 as early as 3 hours of application, while

its expression decreased when PDL cells were stretched for 12 and 24 hours (Suzuki et al., 2014). These findings suggest the essential role of force in the balance of hard tissue remodeling.

Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs) are the largest subfamily of transforming growth factor beta (TGFβ) superfamily. BMPs were initially detected for their ability to induce ectopic bone formation and therefore named bone morphogenetic proteins (Urist, 1965). BMPs are involved in embryonic development and maintenance of tissue homeostasis in the adult stage. Currently, there are more than 20 BMPs identified which play significant roles in many organ systems such as musculoskeletal system, neural system, cardiovascular system (Bragdon et al., 2011). Based on their remarkably potential in the regulation of osteoblast differentiation and subsequent bone formation, BMP-2, -4, -6, -7, and -9 are classified as osteogenic BMPs (Bergeron et al., 2009; Cheng et al., 2003; Kang et al., 2009; Kang et al., 2004; Peng et al., 2003).

BMP-2, -6 and -9 were demonstrated to be the most potent proteins to induce the differentiation of mesenchymal stem cells into osteoblasts. They are also able to induce both early and late osteogenic markers as well as mineralization (Cheng et al., 2003; Kang et al., 2004). BMP-2, -4 and -7 are

very significant in the development. The knockout of these genes showed embryonic lethality in mice (Wang et al., 2014). Not only in development, BMPs also play important roles in maintenance of adult tissues. Overexpression of BMP-2 is probably involved in chondrocyte proliferation in osteoarthritis patient (Yeh et al., 2007).

BMPs are ligands for type I and II serine/threonine kinase receptors. Type I receptors consist of 5 subclasses: ALK1 (Acvrl1), ALK2 (ActRI), ALK3 (BRIa), ALK4 (ActRIb), and ALK6 (BRIb), whereas type II receptors are subdivided into 3 subclasses: BR II, ActRIIa, and ActRIIb (Bragdon et al., 2011). To drive the further signals requires binding of ligand to heterodimeric of type I receptors and heterodimeric of type II receptor.

After BMPs complex with their receptors, the signaling pathway can occur via Smad or non-Smad dependent pathways. For a Smad-dependent pathway, activated receptor will phosphorylate regulatory Smad which are either Smad-1, -5, or -8 regarding to the type of ligands. Common Smad (co-Smad), Smad-4, inhibitory Smad (I-Smad), and Smad-6/-7, are also activated. Smad will then translocate to nucleus and mediate target gene transcription. For non Smad-dependent pathway, BMPs can affect gene expression in nucleus through other pathways such as extracellular signal-regulated kinase (ERK), mitogen activated protein kinases (MAPKs), C-jun N-terminal kinase

(JNK), phosphoinositol-3 kinase (PI3K), protein kinase (PK) A, PKC and PKD (Bragdon et al., 2011).

In the oral cavity, a higher dose of BMP-2 was associated with ankylosis by inducing apoptosis and cytotoxicity in PDL cells (Muthukuru, 2013). Despite of being unable to change the width of the periodontal space, BMP-7 promoted proliferation of PDL cells (Rajshankar et al., 1998).

BMPs were reported to be up-regulated in some cells subjected to force. A certain amount of compressive force was reported to upregulate BMP-2, -4, -6, and -7 expression in human osteoblastic cells (Mitsui et al., 2006). Tension force was shown to upregulate BMP-2 and -4 in rats (Sato et al., 1999), while osteogenesis distraction in rabbits increased BMP-2, -4, and -7 (Rauch et al., 2000). In murine osteoblasts, a 4-point bending force was shown to upregulate BMP-2 and -4 and these factors subsequently supported bone formation (Wang et al., 2010). Not only in bone cells, tendon-derived stem cells were also shown to enhance BMP-2 expression after stretching (Rui et al., 2011). Moreover, shear force was demonstrated to increase BMP-2 and -4 expression by endothelial and smooth muscle cells (Csiszar et al., 2009). BMP-2, concurrent with occlusal force, is involved in the formation of cementum as shown in an *in vivo* model (King and Hughes, 1999). BMP-2 and -6 were reported to be upregulated in PDL cells loaded by tensile force

(Wescott et al., 2007); similar to results presented in 2014 (Suzuki et al., 2014). These findings indicate that mechanical force can induce BMP production in several cell types. Nonetheless, a possible relationship between mechanical force and BMP-9 has not been elucidated.

BMP-9

BMP-9, also known as GDF2 (Growth differentiation factor 2), was first reported in fetal mouse liver and was subsequently shown to be produced by human hepatocytes to promote their proliferation (Song et al., 1995). It is secreted in the circulation in two forms: 60% is in an active form and 40% is in an inactive form (Bidart et al., 2012). BMP-9 is associated with cell proliferation of hematopoietic progenitor cells. It has been shown to be involved in many biological processes including vascular quiescence, maturation of vessels, glucose homeostasis, maintenance of the basal forebrain neurons, and promotion of critical enzymes of lipid metabolism (Luther et al., 2011).

Studies both in primary murine mesenchymal progenitor cells (MPCs) and a MPC cell line described that retinoic acid induced BMP9 expression and subsequently enhanced BMP9-induced osteogenic markers and mineralization through BMPR/Smad signaling pathway (Zhang et al., 2010). Moreover, extra-pituitary growth hormone (GH) can induce BMP9 production which

potentiates osteogenesis in mouse MPCs (Huang et al., 2012). In addition, retinoic acid and GH acted synergistically with BMP9 to promote ectopic bone formation (Huang et al., 2012; Zhang et al., 2010).

BMP9 has an extraordinary potential in conducting osteogenesis (Kang et al., 2004; Peng et al., 2004). BMP9-induced Smad phosphorylation was not affected by noggin and BMP3, antagonists that inhibit other BMPs pathways (Bergeron et al., 2009; Kang et al., 2004; Wang et al., 2013b). Moreover, in contrast to other BMPs the functional BMP9 structure conserves a pro-domain. This pro-domain seems to stabilize and protect BMP9 instead of inhibiting its function (Brown et al., 2005; Li et al., 2016b). Furthermore**,** BMP9 has some cross-talk to other cytokines and synergistically augment their effects such as epidermal growth factor (EGF) (Liu et al., 2013), TGF-β (Li et al., 2015), Wnt3A (Zhang et al., 2015), and insulin-like growth factor 1 (IGF1) (Chen et al., 2016). BMP9-induced osteogenic signaling requires ALK1 and ALK2 (Brown et al., 2005; Townson et al., 2012).

BMP9 was introduced to PDL cells through either adenoviral transfection or by a synthetic recombinant agent. This resulted in an enhanced expression of *Runx2*, an increase of ALP activity and an enhancement of mineralization (Ye et al., 2014). Fuchigami et al. investigated the effects of recombinant human (rh) BMP-9 on osteoblastic differentiation of human PDL cells. rhBMP-9

potently promoted ALP activity, expression of Runx2, Id1, bone sialoprotein (BSP) and osteopontin (OPN). They also confirmed that noggin could not inhibit rhBMP9-induced ALP activity (Fuchigami et al., 2016). However, only exogenous BMP-9 treated human PDL cells were studied. The potential of human PDL cells in producing BMP-9 has not been investigated.

PDL responds to tension force in order to maintain soft tissue integrity. tensile force is implicated in extracellular matrix remodeling as it changes the expression of matrix metalloproteinases (MMPs) and their inhibitors, TIMPs (Ma et al., 2015; Tsuji et al., 2004). Tensile force can upregulate the expression of type I collagen which is the most abundant extracellular protein in the periodontal ligament (Kook et al., 2011)

Interleukin 6 (IL-6)

Interleukin 6 (IL-6) is a cytokine that is involved in both physiologic and pathologic processes. IL6 shows pleotropic properties on different cell types including proliferation, cell recruitment, regeneration, differentiation, and migration (Hosokawa et al., 2014; Iwasaki et al., 2008; Kuhn et al., 2014; Tang et al., 2011). IL6 has been synthesized not only by immune cells but also by other cell types including fibroblast, endothelial cells, neurons (März et al., 1998 ; Shirakura et al., 2018; Sundararaj et al., 2009). IL6 is also present in the periodontal ligament as shown by immunohistochemistry (Grzibovskis et al., 2011). The production of IL-6 has been induced by several factors including

cytokines and mechanical stress. The inflammatory cytokine interleukin 1β (IL-1β) was reported to increase IL6 secretion in human PDL cells (Hosokawa et al., 2014). Some studies have shown that static as well as cyclic tensile force induces the expression of IL6 by PDL cells (Jacobs et al., 2013; Wada et al., 2017). LPS toxin also increases the release of IL6 in human periodontal ligament cells(Jonsson et al., 2008).

The signaling of IL6 requires a receptor complex which consists of IL6 binding type I transmembrane glycoprotein; IL6-R, and a type I transmembrane signal transducer protein gp130 (Scheller et al., 2011; Scheller et al., 2014). PDL cells have been shown to express IL6R, and gp130 subunit (Hosokawa et al., 2014). After forming the complex, IL6-signal transduction occurs. This transduction includes the activation of JAK/STAT, PI3K, and MAPK signaling pathways (Scheller et al., 2011). IL6 signaling depends on two pathways: a classical pathway and a trans-signaling pathway In the classical signaling pathway, IL-6 binds to the membrane bound cell surface receptor before binding to gp130 and further stimulation of downstream signaling pathways (Scheller et al., 2011). However, transmembrane-IL6 receptor is dominantly expressed by some cell types such as hepatocytes, and a leukocyte subpopulation (Jones et al., 2001). Interestingly, gp130 is found in all cell types including periodontal cells. Cells can respond to a complex of

IL6 bound to a naturally occurring soluble form of the IL6-receptor (sIL6-R). Such a pathway is named trans-signaling (Scheller et al., 2011). The soluble form of the IL6-receptor remarkably expands the range of IL6 target cells. Intracellular signaling is subsequently triggered via activation of gp130-associated JAK/STAT signaling pathway. Additionally, IL6 also activates phosphorylation of MAP kinase pathway (Jones et al., 2001).

IL6 alters the production of many cytokines and enzymes in order to regulate cellular activities. For example, IL6 increased *Runx2* and *ALP* expression by human PDL cells causing osteoblastic differentiation (Iwasaki et al., 2008). IL6 is able to recruit and maintain survival of neutrophils and macrophages through upregulating CCL20 (Hosokawa et al., 2014). IL-6 showed its potential to enhance migration of cancer cells through an increase of *MMP-2*, *MMP-9*, and *TIMP-1* (Kossakowska et al., 1999). Recently, IL-6 was shown to influence cell migration of mesenchymal stem cells through upregulation of *MMP-1* and *MMP-3* (Casson et al., 2018). One of the possible functions of the cytokine is its role in bone remodeling. IL6 not only promotes osteoclastogenesis by RANKL induction in mouse osteoblasts (Udagawa et al., 1995), but also seems to play a role in osteoblast differentiation of both human osteoblastic cells (Nishimura et al., 1998) and human PDL cells (Iwasaki et al., 2008). IL6-deficient mice reveal a more extensive bone destruction at periapical area coinciding with high number of

osteoclast activity (Balto et al., 2001), suggesting IL6 in PDL tissue is important for bone protection. These findings suggest that the CTF-induced IL6 may control the balance of bone remodeling.

Moreover, IL6 increases expression of SOCs3 by human PDL cells and thereby negatively control a pro-inflammatory response of the cells (Fukushima et al., 2010). IL6 also increases CCL20 production in human PDL cells resulting in the recruitment of immune cells like Th17 to the tissue (Hosokawa et al., 2014). IL6 is able to enhance C-reactive protein production by human PDL fibroblasts contributing to the induction of Th1 and Th17 cytokines (Hernandez-Caldera et al., 2018). These findings together suggest a role of IL6 induced by CTF in an immunologic crosstalk to modulate the homeostasis of periodontium. IL6 affects not only PDL cells and osteoblasts but it also affects endothelial cells. IL6 enhances the expression of ICAM1 and VCAM1 by the endothelial cells (Watson et al., 1996) probably causing the recruitment of lymphocytes to PDL tissue. IL6 increases VEGF production by endothelial cells through Src-FAK signaling (Huang et al., 2016) which may promote angiogenesis to the tissue.

Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) belong to a family of proteolytic enzymes collectively responsible for the physiological and pathological degradation of extracellular matrix proteins, including collagens, fibronectin,

laminin and proteoglycans. MMPs, a group of zinc-dependent endopeptidases, are involved in the cellular responses to their microenvironment. These enzymes are responsible for the degradation of most, if not all, different extracellular matrix components thereby modulating tissue structure. They also facilitate several activities such as cell migration, activation of enzymes and growth factors, or even intracellular multifunctional proteins (Sternlicht and Werb, 2001). The orchestration of expression of MMPs and their activity is consequently crucial for a number of physiologic and pathologic processes (Sternlicht and Werb, 2001).

Currently, 24 human MMPs have been identified. The MMP family is divided into 5 groups; collagenases (MMP1, 8, 13), stromelysins (MMP3, 10, 11), gelatinases (MMP2 and 9), and membrane-bound MMPs (MMP14, 15, 16, and 17), and others (matrilysin-MMP7, macrophage elastase-MMP12, enamelysin- MMP20) depending on their substrate specificity (Jablonska-Trypuc et al., 2016; Nagase and Woessner, 1999). The expression of MMPs is mostly found in the connective tissue cells, mainly in fibroblasts. MMPs are also expressed in neutrophils, monocytes, macrophages and endothelial cells (Hrabec et al., 2007).

MMPs are synthesized as inactive pro-enzyme. The structure of MMPs is basically composed of an inhibitory pro-domain that controls the latency of enzyme, the catalytic domain, and the C-terminal hemopexin-like domain

which is involved in the recognition of MMP substrates (Franco et al., 2017). Several factors impact on the expression of MMPs including interaction between cell to cell or cell to ECM, growth factors, cytokines, and hormones (Page-McCaw et al., 2007). The activity of MMPs is strictly regulated through gene expression, proenzyme activation, and enzyme inhibition by endogenous inhibitors such as tissue inhibitors of MMPs (TIMPs) (Franco et al., 2017). Nowadays, four TIMPs are reported in human. They are broad-spectrum MMP inhibitors, however, they differ among their specificity for MMPs.

The expression of MMPs can be induced by many effectors such as growth factors, physical stress, cytokines, and chemical agents. Several studies demonstrated the effect of mechanical force on expression of *MMPs*. Tooth movement in rats up-regulated the expression of *MMP8* and *MMP13* (Takahashi et al., 2003), and CTF has been reported to up-regulate the expression of *MMP1* by human PDL cells (Kook et al., 2011). Cyclic mechanical stretching, at 10% strain, for 24 hours, increased *MMP2* expression by PDL cells (Chen et al., 2013). Not only mechanical stress, some cytokines, for example IL6, also affect the expression of MMPs. IL6 has been reported to induce *MMP9* expression in murine macrophages (Kothari et al., 2014). In cancer cells, IL6 increases MMP14 production through regulation of p53 protein. The expression of this MMP causes the destruction of the

extracellular matrix (Cathcart et al., 2016). Finally, IL6 can stimulate the expression of *MMP1* and *MMP3* in bone marrow-derived mesenchymal stem cells (Casson et al., 2018).

As other tissues, the PDL consists of the extracellular matrix which maintain mechanical and biochemical properties. Mechanical-loaded PDL cell demonstrated a changed expression of collagen (Kalajzic et al., 2014; Kook et al., 2009). The modulation of collagen requires the orchestration of different types of proteinase. MMPs are expressed mainly by fibroblasts in connective tissue. Several studies demonstrated the effect of mechanical force on expression of *MMPs*. Tooth movement in rats upregulated the expression of *MMP-8* and *MMP-13* (Takahashi et al., 2003). Moreover, CTF creates the alteration of cell to cell/ ECM interaction initiating changes in *MMP-1* expression (Kook et al., 2011).

MMP1, 8 and MMP14 (MT1-MMP) are categorized as collagenases. Their main substrate is collagen which form the majority of the ECM components in PDL tissue. Denatured collagen is subsequently degraded by MMP2, gelatinase, and MMP3 (Page-McCaw et al., 2007; Sternlicht and Werb, 2001). MMP3 and MMP14 can activate some other MMPs including MMP1 and MMP8 (He et al., 1989; Holopainen et al., 2003; Suzuki et al., 1990). MMP8 has been reported to be closely associated to periodontal disease (Butler and Overall, 2009; Franco et al., 2017).

MMP3, stromelysin 1, is synthesized as a prepro-enzyme and secreted as an inactive pro-MMP, similar to other MMPs (Nagase and Woessner, 1999). Among other substrates, MMP3 degrades collagen III, IV, and V, proteoglycans, fibronectin, elastin, laminin, and gelatin (Page-McCaw et al., 2007; Sternlicht et al., 1999) which are also components in the ECM of the PDL (Kaku and Yamauchi, 2014; van den Bos and Tonino, 1984). To regulate tissue architecture, MMP3 mediates a proteolytic cascade of ECM-degradation through activation of the proMMPs (e.g. MMP1, 8, 9, 13) (He et al., 1989; Knauper et al., 1996; Ogata et al., 1992; Suzuki et al., 1990). By mimicking the orthodontic force with a combination of mechanical force and LPS, an increase of MMP3 was found suggesting involvement of MMP3 in PDL remodeling in case of orthodontic tooth movement (Lisboa et al., 2013). High levels of MMP3 are also found in inflammation tissue like chronic periodontitis (Letra et al., 2012).

Chapter 3 Materials and Methods

Cell isolation and culture

The protocol for all experiments was reviewed and approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Bangkok. Primary human PDL cells were obtained from third molars that had been planned to be removed from patients in Faculty of Dentistry, Chulalongkorn University. All patients had already approved the informed consent. The periodontal ligament from the middle third of the root was scraped off and cut into pieces using blade no.15. The periodontal ligament tissue was placed in a 35-mm culture dish (Corning, New York, NY) and bathed in culture medium. All cells in this study were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 $U.mL^{-1}$ penicillin, 100 $\mu g.mL^{-1}$ streptomycin, and 5 $\mu g.mL^{-1}$ amphotericin B in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The medium and all supplements were from Gibco (BRL, Carlsbad, CA). Monolayer cells were sub-cultured into 1:3 ratio using 0.25% trypsin 0.1% EDTA and maintained in the same culture medium and condition. The medium was refreshed every 2 days before being sub-cultured or being used in the experiment. Cells from the third to the seventh passage were used.

Prior to starting the experiment, cells were seeded and cultured in 10% FBS supplemented DMEM for 24 hours and the culture medium was changed to serum-

free DMEM for overnight-starvation in all experiments in this study. Each experimental condition was analyzed at least in triplicate.

Silicone sheet preparation

Silicone sheets were prepared from Polydimethylsiloxane trimethylsiloxy(PDMS) (Silastic T-4, Dow Corning, GmbH, Germany) in dimension of 2.5 x 2 x 0.5 cm^3. After completed recommended setting time, the silicone sheet will be surface activated with dielectric barrier discharge (DBD) plasma energy and stabilized activated surface with glutaraldehyde cross-linking. The silicone sheets were then disinfected with UV light for 30 minutes per each side. The sterile silicone sheets were coated with 0.1% gelatin (Sigma Aldrich) dissolved in warm deionized water using orbital shaker at the room temperature and were kept overnight in a humidified atmosphere of 95% air, 5% CO_2 at 37°C incubator. After protein coating, all silicone sheets were dried and kept sterile. These treatments modify surface from hydrophobic into hydrophilic property in order to support cell adhesion.

A tension generating device

A tension generating device is a custom-made developed at the Faculty of Dentistry, Chulalongkorn University as shown in Figure 3.1. The device consists of chamber to contain the medium and handgrips to tightly hold the silicone membrane. This apparatus generates a uniformly uniaxial stretching on the silicone membrane. The range of frequency, duration and elongation were precisely controlled by a software program.

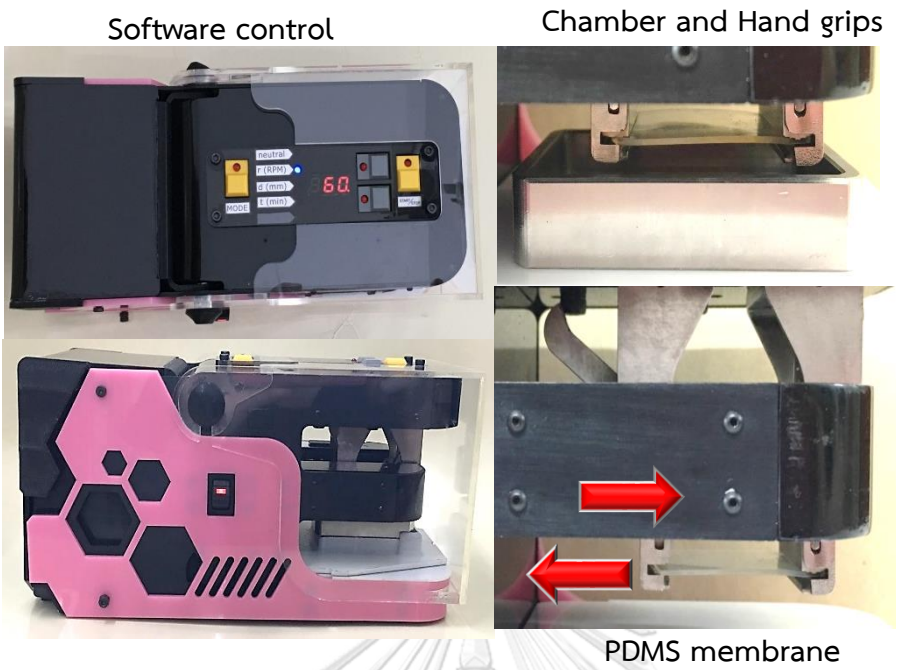

Figure 1 A tension-generating device

A uni-axial stretch device composed of software-controlled part that allows user to set three factors (frequency, duration, and elongation), two handgrips to tightly hold the silicone membrane, and chamber to contain culture medium. One handgrip steadily holds one side of membrane, meanwhile, another side pulls the membrane followed the selected parameters. The device utilizes direct clamping of membranes previously grown in non-loaded culture.

Application of cyclic tensile force (CTF)

The primary human PDL cells were seeded at a density of $2x10^5$ cells on 0.1% gelatin coated silicone membranes (Silastic T-4, Dow Corning, GmbH, Germany) and cultured in 10% FBS supplemented DMEM for 24 hours. The culture medium was changed to serum-free DMEM for overnight-starvation prior to the CTF. CTF was applied by a uni-axial stretch device developed at the Faculty of Dentistry, Chulalongkorn University This device generates a uniformly uniaxial stretching on the assembled silicone membrane by grasping both sides of membrane and stretching out followed the selected parameters. Thus, force was transferred to cells seeded on membrane directly. The range of frequency, duration and elongation were precisely controlled by a software program.

Cells in fresh serum-free DMEM were subjected to CTF with an elongation strain of 10% and the frequency of 60 rpm for desired timepoints in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Control cells were cultured under the same conditions by seeding on the gelatin-coated silicone membrane and placing in the stretch apparatus without applying mechanical loading.

Cell viability assay

The viability of cells was determined by an MTT assay. MTT 3(4,5-dimethylthiazol-2-yl-) 2,5-diphenyltetrazolium bromide (Sigma Aldrich) was reduced by mitochondrial dehydrogenase in viable human PDL cells to formazan, which was dissolved by 9:1 DMSO-glycine buffer solution. Then, the optical density was

measured by spectrophotometer at 550 nm. The amount of the formazan is proportional to the number of living cells. The viability of primary challenged with chemicals or CTF was determined by this assay before starting all experiments. Each experimental condition was analyzed in triplicate with cells obtained from at least three different donors. All parameters setting for CTF application (range of frequency, duration and elongation) were set within the range of unaffected viability. Also, all chemicals were used within a range of nontoxic concentrations.

In vitro mineralization assay

After CTF loading or chemical challenging, primary human PDL cells were cultured in osteogenic medium (OM): DMEM supplemented with 10% FBS, 50 μg.mL^{-1} ascorbic acid and 5 mM β-glycerophosphate (Sigma Aldrich) for 14–21 days. *In vitro* mineralization was analyzed as described previously (Techatharatip et al., 2018). Cells were fixed with cold methanol for 10 minutes. Afterwards, cells were washed with deionized water and stained for calcium precipitation by 1% Alizarin red S solution (Sigma Aldrich) for 5 minutes. Excess dye was removed by washing with deionized water. The mineral deposits were visualized by an inverted digital camera. Mineral deposition was quantified following extraction by using 10% cetylpyridinium chloride monohydrate (Sigma Aldrich). The absorbance was measured at 570 nm.

ATP measurement assay

The amount of extracellular ATP was measured using ATP assay system bioluminescence detection kit (ENLITEN®, Promega, WI). According to the manufacturer's instruction, 50 μL of culture medium was collected during CTF loading of human PDL cells at 0, 10, 20, 30, 60, 120, and 360 minutes. A 50 μL of Enliten Luciferase/Luciferin medium was added to the sample in the microplate. The signal was immediately measured by a hybrid multi-mode reader (Synergy H1; S/ N264252, BioTek, Winooski, VT). A calibration curve was generated for each luciferase assay by serial dilution of an ATP standard.

Inhibitor and peptides treatment

In the present study, chemical agents were used according to the objective of each experiment as followings:

The examination of role of ATP and its receptor

i. Prior to CTF loading, 2. 5 U/ mL apyrase (Sigma-Aldrich Chemicals, St. Louis, MO) or 15 μM suramin (Calbiochem, EMD Chemicals, San Diego, CA) or 5 μM MRS2179 [a specific $P2Y_1$ receptor antagonist (Sigma-Aldrich Chemicals, St. Louis, MO)] were used to thirty-minute pretreat human PDL cells seeded on gelatin-coated silicone membrane.

ii. The human PDL cells were seeded at a density of $1x10^5$ cells/well in 12-well plate (Corning, New York, NY) and were

overnight-starved with serum-free medium prior to treatment with different compounds which were ATP (Sigma-Aldrich) at concentration of 1, 10 and 100 μM, 0.3 and 0.5 nM MRS2365 [a $P2Y_1$ receptor agonist (Sigma-Aldrich)], 5 μM MRS2179 [a specific $P2Y_1$ receptor antagonist (Sigma-Aldrich)]

The investigation of signaling pathway(s)

iii. To investigate the involved pathway of CTF-stimulated BMP9, human PDL cells were seeded at a density of $1x10^5$ cells/well in 12-well plate for 24 hours before overnight starvation with serum-free medium. Then, they were treated with different inhibitors and compounds as followings: 2 μM U-73122 [a phospholipase C inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA)], 20 μM cAMPS-Rp [a competitive antagonist of cAMP-induced activation of PKA (Tocris Bioscience, Minneapolis, MN)], and 12.5 nM Thapsigargin (Sigma Aldrich).

iv. To investigate the associated pathway of IL6-induced MMP3, human PDL cells were seeded at a density of $1x10^5$ cells/well in 12-well plate for 24 hours before overnight starvation with serum-free medium. Then, they were treated with different inhibitors and compounds as followings: 15 nM JAK inhibitor I (Calbiochem, EMD Chemicals, San Diego, CA), 6 μM STAT3

inhibitor V (Calbiochem), 1.4 μM PI3K inhibitor (LY294002, Calbiochem), 40 nM JNK inhibitor II (SP600125, Calbiochem), 2.5 μM ERK1/2 inhibitor (PD98059, Calbiochem), and 35 nM p38 inhibitor (SB203580, Calbiochem). They were used to pretreat the cells for 30 minutes prior to a twenty-four-hour stimulation with 1 $ng.mL^{-1}$ recombinant human IL6 (R&D Systems Inc.).

Neutralizing antibody and recombinant compound

Neutralizing antibody to BMP9

- To investigate the role of BMP9 in CTF-induced mineralization, after six-hour continuously 10% CTF application, cells were further cultured in OM with 0.2 $\mu g. mL^{-1}$ neutralizing anti-BMP9 monoclonal IgG antibody (Catalog #MAB3209, R&D Systems Inc., Minneapolis, MN). or the same concentration of non-specific mouse IgG control antibody (Catalog #MAB002, R&D Systems Inc.) for 14 days.
- Human PDL cells were cultured and treated with 0.2 $\mu g. mL^{-1}$ specific anti-BMP9 mouse monoclonal antibody (Catalog #MAB3209, R&D Systems Inc., Minneapolis, MN). The control of the experiment was

cultured with 0. 2 μg. mL^{-1} non-specific mouse IgG (Catalog #MAB002, R&D Systems Inc.). The cells were cultured in osteogenic medium (OM): DMEM supplemented with 10% FBS, 50 μg.mL^{-1} ascorbic acid and 5 mM β-glycerophosphate (Sigma Aldrich) for 14–21 days.

Neutralizing antibody to IL6

To test whether endogenously produced IL6 modulated the expression of MMPs, after a six hour-CTF application, cells were incubated with 0.07 μg.mL^{-1} of a specific anti-IL6 mouse monoclonal antibody (Catalog #MAB2061-sp, R&D Systems Inc., Minneapolis, MN) or with 0.07 μg.mL^{-1} of a non-specific mouse IgG (Catalog #MAB002, R&D Systems Inc.) as a control for another 24 and 48 hours.

Recombinant human IL6

Primary human PDL cells between the third and sixth passage were seeded at a density of $1x10^5$ cells/well in 12-well plates, and stimulated with recombinant human IL6 (R&D Systems Inc.) at concentrations of 0.1, 1, 10 ng.mL^{-1} or a control vehicle for 24 hours.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

After the experiment was done, cells in all experiments that required mRNA analysis were added with 1 mL of Trizol Reagent (Ribo-ex, Molecular research Center, Cincinnati, OH) according to the manufacturer's instructions in order to extract RNA. One microgram of total RNA was used to generate complimentary DNA (cDNA) by reverse transcription reaction using AMV reverse transcriptase and oligo dT (Improm-II reverse transcription system, Promega, Madison, WI). Complementary DNA was amplified using Taq polymerase (Invitrogen, NY) in a DNA thermal cycler (Biometra, Gottingen, Germany). One microliter of cDNA was subjected to real-time PCR using SYBR Green I dye. PCR was performed in a LightCycler®96 (Roche Diagnostic Co., Indiana) with the Light Cycler 480 SYBR Green I Master kit (Roche Diagnostic Co., Indiana). The amplification profile consisted of 40 cycles of 95°C for 3 seconds, followed by 60°C for 20 seconds and subsequently 72°C for 10 seconds. All real-time PCR reactions were performed in triplicate, and the specificity of the PCR products was verified by melting curve analysis. Reaction product was quantified using RelQuant software (Roche Diagnostics Co., Indiana) with GAPDH as the reference gene. Primer sequences were shown in table 1.

Table 1 primer sequences

gene	sequence	
BMP2	F'	5’- GCGTGAAAAGAGAGACTG -3
	R'	5’- CCATTGAAAGAGCGTCCAC -3’
BMP6	F'	5’- TGCAGGAAGCATGAGCTG -3’
	R'	5'- CAGGGACTTGAGGAGGGTAGATC-3'
BMP9	F'	5'- CCTGGGCACAACAAGGAC-3'
	R'	5'- CCTTCCCTGGCAGTTGAG-3'
GAPDH	F'	5'- CAGTGCCAACGTGTCAGTGGTG-3'
	R'	5'- GTAGCCCAGGATGCCCTTGAG-3'
IL6	F'	5’- ATGCAATAACCACCCCTGAC -3’
	R'	5’- AAAGCTGCG CAGAATGAGAT-3’
IL6R	F'	5'- CAAGCCTCCCAGTGCAAGA-3
	R'	5’- CAATGGCAATGCAGAGGAGC-3’
MMP1	F'	5’- AGAGAGCAGCTTCAGTGACA -3’
	R'	5’- CTTGAGCTGCTTTTCCTCCG -3'
MMP2	F'	5’- TTGACGGTAAGGACGGACTC -3’
	R'	5’- ACTTGCAGTACTCCCCATCG -3’
MMP3	F'	5’- ATTCCATGGAGCCAGGCTTTC -3’
	R'	5’- CATTTGGGTCAAACTCCAACTGT -3’
MMP8	F'	5’- AACCACCACTTCCTCTCCTG -3’
	R'	5’- ATAGTGTGTGCCCTCCT -3’
MMP14	F'	5’- GGAGACAAGCATTGGGTGTT-3’
	R'	5’- GGTAGCCCGGTTCTACCTTC -3’
Noggin	F'	5'- GGAGGAAGTTACAGATGTGGCTG-3'
	R'	5'- CACTCGGAAATGATGGGGTATG-3'
$P2Y_1$	F'	5’- AAAACTAGCCCCCTGCAACT-3’
	R'	5’- GATCTGATGCCGGATGAACT-3’
TIMP1	F'	5’- ACTTCCACAGGTCCCACAAC-3’
	R'	5’- GCATTCCTCACGCCAACAG -3’
TIMP2	F'	5’- CGACATTTATGGCAACCCT-3’
	R'	5’- ATTCCTTCTTTCCTCCAACG-3’

Enzyme-linked immunosorbent assay (ELISA)

Level of human BMP9 protein in both conditioned media and cell lysate was analyzed. Conditioned media were collected after the experiment was done, and the cells were lysed in 200 µl lysis buffer (150 mM NaCl, 10 mM phosphate buffer pH 7.0, 1% NP-40, 0.1% sodium deoxycholate, 1 mM PMSF). Culture media and cell lysates were centrifuged at 15,000 x g for 5 minutes, and the supernatants were collected for analysis. For IL6 and MMP3, level of protein production was detected in conditioned media only. The protein concentration was measured by BCA Protein Assay® (Bio-Rad Laboratories, Hercules, CA). The amount of BMP9 was measured by ELISA kit (R&D Systems Inc.). Each sample was assayed in triplicate. The assays were performed as instructed by the ELISA manufacturer and the results were normalized to the amount of total protein.

Statistical analysis

All experiments were performed at least three times in independent experiments. The data were presented as mean ± standard deviation (s.d.). Two independent sample t-tests and ANOVA, followed by Tukey's test were performed using SPSS version 22 (Armonk, NY) software for statistical analysis. Differences at $p < 0.05$ were considered to be statistically significant.

Chapter 4 Results

Cell viability of human PDL cells after CTF application

Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm for 2 and 6 hours. This strain has been shown to represent physiological occlusal strain (Fujihara et al., 2010; Li et al., 2013a; Pini et al., 2002; Woda et al., 2006).

Analysis by an MTT assay revealed no significant effect of CTF on cell viability between CTF-loaded and non-CTF-loaded control as shown in Figure 2.

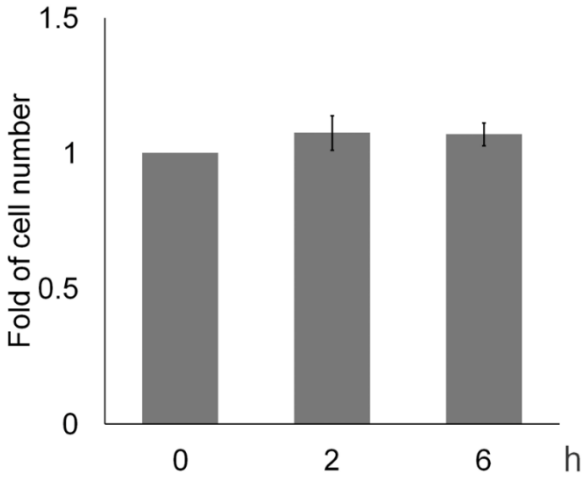

Figure 2 The viability of human PDL cells under CTF loading analyzed by MTT assay

Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm continuously for 2 and 6 hours in a serum-free condition. Bar graphs demonstrate the relative fold of cell number of the CTF-loaded groups compared to that of the non-CTF-loaded control. Data were presented as mean ± s.d. from 3 separate experiments.

CTF upregulates the expression of osteo-inductive genes by human PDL cells

Previous studies demonstrated that CTF upregulated *BMP2* and *BMP6* mRNA expression by human PDL cells (Suzuki et al., 2014; Wescott et al., 2007). In the present study, the expression of *BMP2* and *BMP6* mRNA was evaluated. The results showed significantly increased expression of these genes by CTF-loaded human PDL cells in a time dependent manner as shown in Figure 3A and B.

Since noggin is the extracellular inhibitor of most of BMPs, the expression of *noggin* was investigated. CTF also significantly increased *noggin* mRNA expression as shown in Figure 3C. This finding demonstrates that osteogenic inducers and inhibitors are concurrently stimulated by the same condition.

Because the BMP antagonist noggin blocks osteogenic BMPs except BMP9 (Wang et al., 2013b), the expression and synthesis of BMP9 were examined. The mRNA expression of *BMP9* significantly increased after 2 and 6 hours of CTF loading as shown in Figure 3D.

To investigate the synthesis of BMP9, after a six-hour CTF loading, the cells were further cultured for other 48 hours after which protein was collected from cell lysate and culture media for analysis of BMP9 protein by ELISA. The results demonstrated a significant increase of BMP9 in CTF-loaded human PDL cells compared to non-CTF-loaded control. Most of the BMP9 was found in the conditioned medium and appeared to be secreted as shown in Figure 3E.

Furthermore, the expression level of *BMP2, BMP6, and BMP9* comparing to *GAPDH* expression was also investigated. Among BMPs, the expression level of *BMP2* was the highest, followed by that of *BMP6* and *BMP9* as shown in Figure 3F.

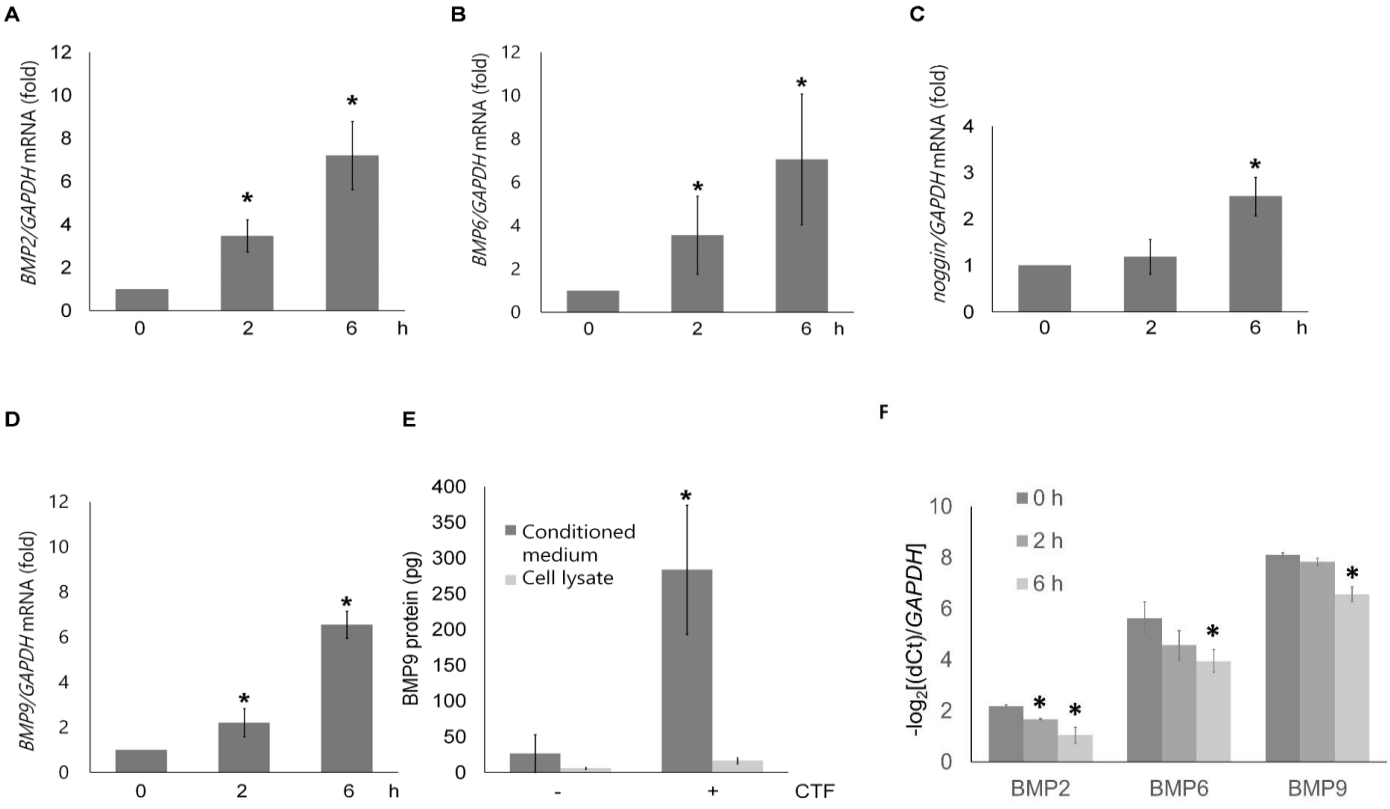

Figure 3 CTF up-regulates the expression of noggin and BMP9 by human PDL cells.

Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm continuously for 2 and 6 hours in serum-free medium. Then cells were extracted for analyses of *BMP2, BMP6, noggin* and *BMP9* mRNA expression by semi-quantitative RT-PCR. CTF significantly increased **(A)** *BMP2*, **(B)** *BMP6*, **(C)** *noggin* and **(D)** *BMP9* mRNA expression in a time-dependent manner. The relative mRNA expression levels of the different *BMPs* and *noggin* are presented as a fold-induction compared to non-CTF-loaded cells. **(E)** Cell lysates and conditioned media (CM) were collected after 48 hours and analyzed for BMP9 by ELISA. A significant increase of the level of BMP9 was found in both cell lysate and culture media compared to the non-CTF-loaded cells. The results were normalized to the amount of total

protein. **(F)** qRT-PCR data are demonstrated as the delta-Ct values normalized to GAPDH and delta-delta Ct to compare expression levels between different time points of applied CTF of BMP2, 6 and 9. A higher value represents a lower expression level. The results are expressed as means ± s.d. from 3 separate experiments. * $p <$ 0.05 vs. non-CTF controls.

CTF stimulates mineral deposition by human PDL cells

Human PDL cells have the capacity to express osteogenic genes such as *Runx2* and *ALP* after stimulation by CTF (Liu et al., 2012; Shen et al., 2014; Wescott et al., 2007). In the present study, the effect of CTF on *in vitro* mineralization by human PDL cells was further investigated. After a six-hour CTF loading, cells were further cultured in osteogenic medium for 14 days. The mineralization was assessed by Alizarin Red S staining. CTF increased the mineral deposition by human PDL cells compared to non-CTF-loaded OM condition, while no mineral deposition was found under growth medium condition, (GM) as shown in Figure 4A. Quantification of the alizarin staining demonstrated a three-fold increase of mineral deposition compared to the non-CTF-loaded control as shown in Figure 4B. Cells grown in a conventional growth medium demonstrated no mineral deposition at all.

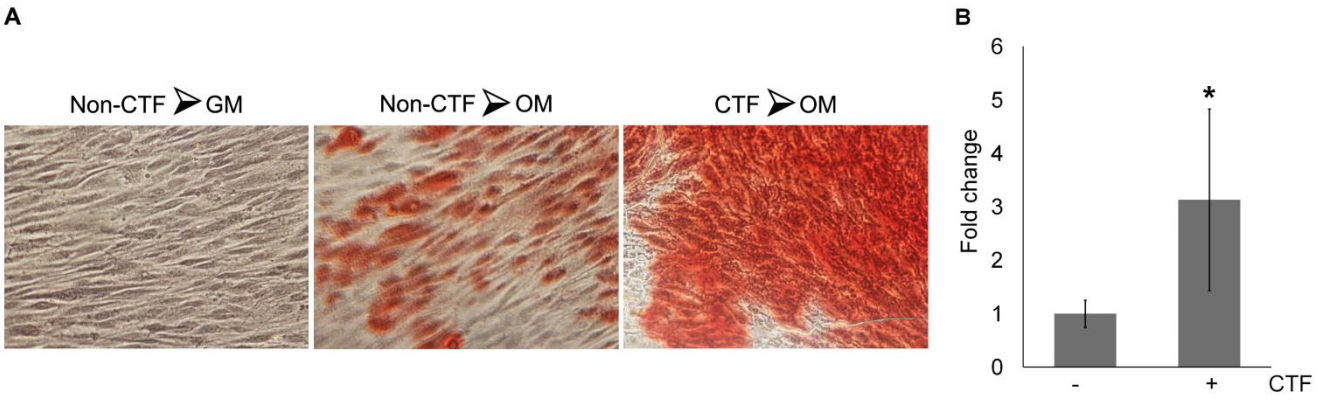

Figure 4 CTF stimulates in vitro mineral deposition by human PDL cells.

Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm continuously for 6 hours in serum-free medium. Cells were cultured further for 14 days in osteogenic medium (OM) consisting of DMEM supplemented with 10% FBS, 50 μg.mL^{-1} ascorbic acid and 5 mM β-glycerophosphate. **(A)** Alizarin red S staining was performed at day 14 for evaluation of mineral deposition. CTF strongly increased mineral deposition compared to non-CTF-loaded OM condition, while no mineral deposition was found under growth medium condition (GM). **(B)** Graphs demonstrate 3-fold increase of the Alizarin red S staining by CTF-loaded over non-CTF-loaded conditions, as quantified by eluting with 10% cetylpyridinium chloride monohydrate. Each assay was done in at least three independent experiments. The results are expressed as means ± s.d. from 3 separate experiments. * $p < 0.05$ vs. non-CTF control.

BMP9 participates in CTF-induced mineral deposition by human PDL cells

To subsequently investigate the participation of BMP9 in *in vitro* CTF-stimulated mineral deposition by human PDL cells. After a six-hour CTF loading, cells were further cultured in OM with 0.2 μg.mL^{-1} neutralizing anti-BMP9 monoclonal IgG antibody or the same concentration of non-specific mouse IgG control antibody for 14 days. The mineralization was evaluated by Alizarin Red S staining. CTF-increased mineral deposition by human PDL cells showed partial reduction in the presence of anti-BMP9 monoclonal IgG antibody when comparing to CTF-loaded condition with or without non-specific mouse IgG control antibody as shown in Figure 5A. Quantification of the alizarin staining demonstrated almost 45% decrease of mineral deposition compared to the non-specific mouse IgG control as shown in Figure 5B. The results suggest that BMP9 involved in CTF-increased mineral deposition by human PDL cells.

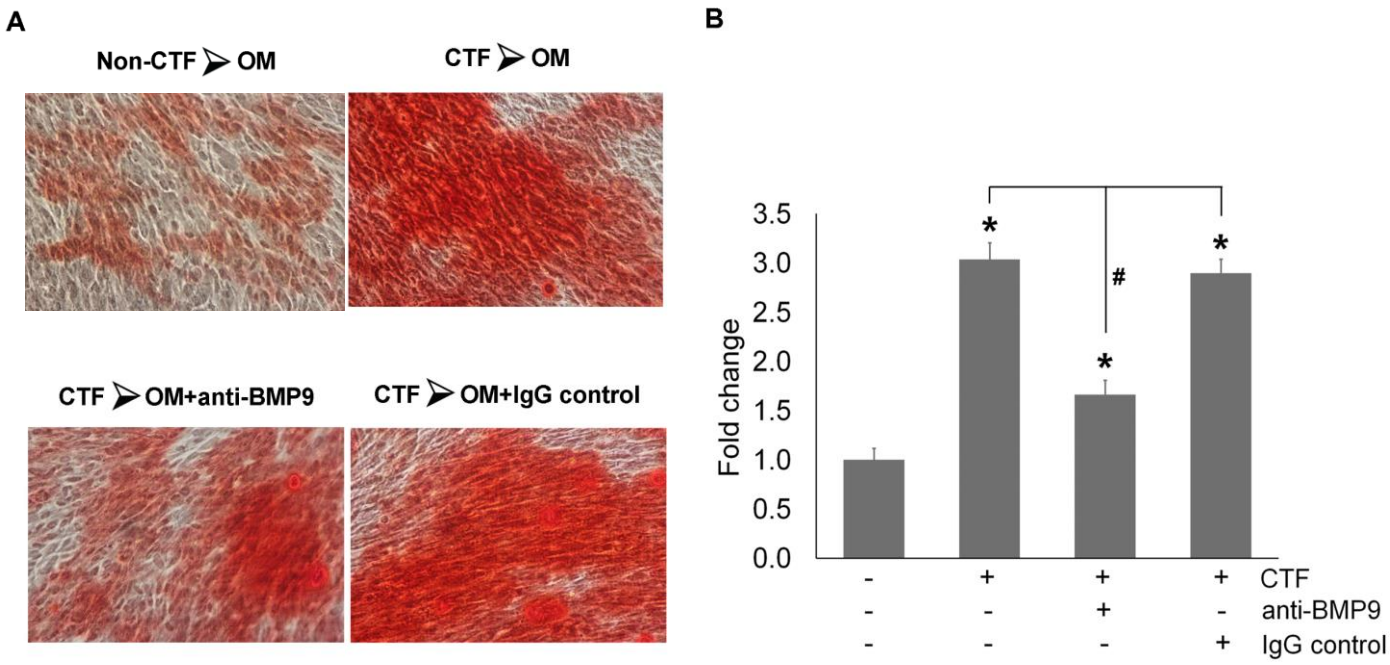

Figure 5 BMP9 participates in CTF-enhanced* in vitro *mineral deposition by human PDL cells.

Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm continuously for 6 hours in serum-free medium. Cells were cultured further for 14 days in osteogenic medium (OM) consisting of DMEM supplemented with 10% FBS, 50 $\mu g.mL^{-1}$ ascorbic acid and 5 mM β-glycerophosphate in the presence or absence of 0.2 $\mu g.mL^{-1}$ neutralizing anti-BMP9 monoclonal IgG antibody or 0.2 $\mu g.mL^{-1}$ non-specific mouse IgG control antibody. Cells cultured in an OM without CTF were included as an experimental control. **(A)** Cells were stained with Alizarin red S at day 14 to demonstrate mineralization. **(B)** Graphs demonstrate a fold change of the amount of staining. Each assay was done in at least three independent experiments. The results are expressed as means ± s.d. from 3 separate experiments. *$p < 0.05$ vs. control, # $p < 0.05$ between 2 experimental groups.

BMP9 participates in *in vitro* mineral deposition by human PDL cells

To investigate the effect of BMP9 in *in vitro* mineral deposition in non-CTF loaded condition, cells were cultured in an osteogenic medium with the presence of 0. 2 μg. mL^{-1} neutralizing anti-BMP9 monoclonal IgG antibody or the same concentration of non-specific mouse IgG control antibody. Alizarin Red S staining demonstrated a partial reduction of mineral deposition in the presence of anti-BMP9 monoclonal IgG antibody as shown in Figure 6A. The staining was quantitated and revealed a 41% decrease of mineral deposition in the presence of the anti-BMP9 antibody as shown in Figure 6B. These results indicate that endogenous BMP9 plays a crucial role in *in vitro* mineral deposition by human PDL cells.

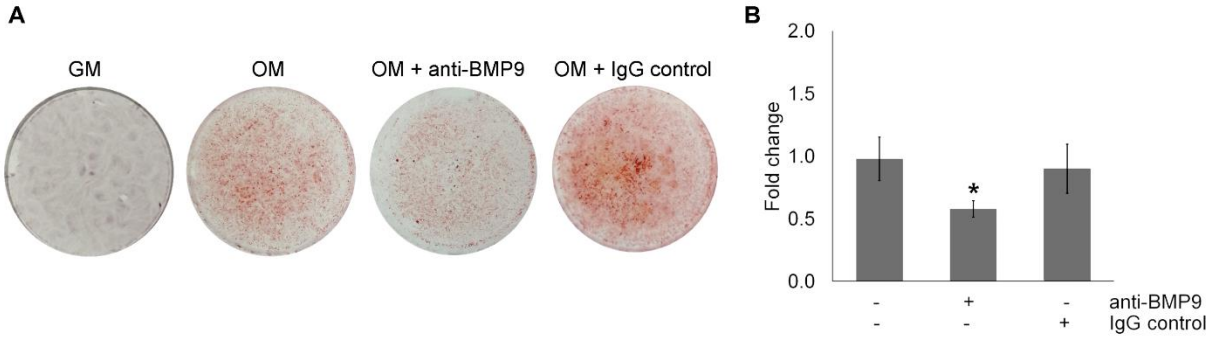

Figure 6 BMP9 participates in in vitro mineral deposition by human PDL cells.

Human PDL cells were cultured in DMEM supplemented with 10% FBS growth media (GM), OM, OM with 0.2 μg.mL^{-1} anti-BMP9 monoclonal IgG antibody, or OM with 0.2 μg.mL^{-1} non-specific mouse IgG for 21 days. **(A)** Cells were stained with Alizarin red S to demonstrate mineralization. **(B)** Graphs demonstrate a fold change of the amount of staining. Each assay was done in at least three independent experiments. The results are expressed as means ± s.d. from 3 separate experiments.* $p < 0.05$ vs. control, # $p < 0.05$ between 2 experimental groups.

CTF mediates ATP release by human PDL cells

Previous studies have shown that mechanical force either compressive, tensile or shear force could trigger the release of ATP (Kariya et al., 2015; Luckprom et al., 2011; Takahara et al., 2014). In the present study, the effect of CTF on the release of ATP was investigated. Human PDL cells were subjected to 10% CTF with a frequency of 60 rpm for 6 hours. During this period, after 10, 20, 30, 60, 120, and 360 minutes 50 μl of conditioned medium was collected for measuring the amount of ATP using an ATP luminescence assay. The results demonstrated an increase in the release of ATP under the influence of CTF. A calibration curve was generated by serial dilution of an ATP standard. ATP was released rapidly within 10 minutes after loading and then the level gradually decreased despite a continuous significantly higher amount than the control as shown in Figure 7.

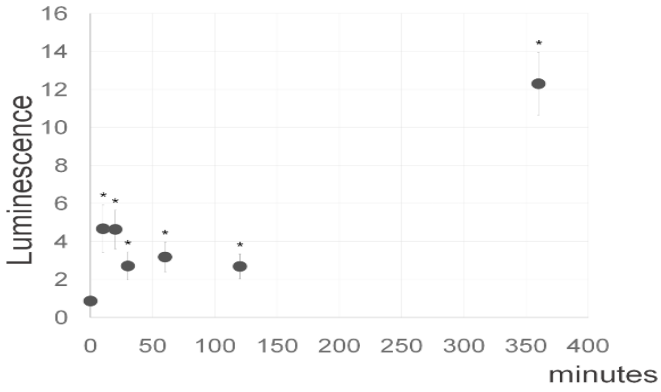

Figure 7 CTF mediates ATP release by human PDL cells.

Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm continuously for 2 hours in serum-free condition. The amount of extracellular ATP was measured using ATP assay system bioluminescence detection kit. 50 μL of culture medium was collected at 0, 10, 20, 30, 60, 120, and 360 minutes. A 50 μL of ENLITEN® Luciferase/Luciferin medium was added to the sample in the microplate. The signal was measured by a hybrid multi-mode reader. A calibration curve was generated by serial dilution of an ATP standard. The graph demonstrates the fold increase of ATP in the culture medium from CTF-loaded to those of the non-CTF-loaded control at each time point. The results demonstrated a rapid release of ATP within 10 minutes after CTF loading and the level was maintained higher than the control during the time course. The results are presented as mean ± s.d. from 3 separate experiments. * $p < 0.05$ vs. non-CTF control.

ATP stimulates BMP9 expression by human PDL cells

Since CTF stimulated the secretion of both BMP9 and ATP, ATP may be involved in the expression of *BMP9*. The presence of 2.5 $U.mL^{-1}$ apyrase**,** an ecto-ATPase, during CTF application was able to abolish the CTF-stimulated *BMP9* as shown in Figure 8A.

Thus, to confirm that extracellular ATP affected the expression of *BMP9*, human PDL cells were cultured in the presence of 0, 0.1, 1 and 10 μM ATP for 6 hours. The results indicated that the expression of *BMP9* mRNA was significantly stimulated by ATP in a dose-dependent manner as shown in Figure 8B. This finding demonstrates for the first time that extracellular ATP induces *BMP9* mRNA expression.

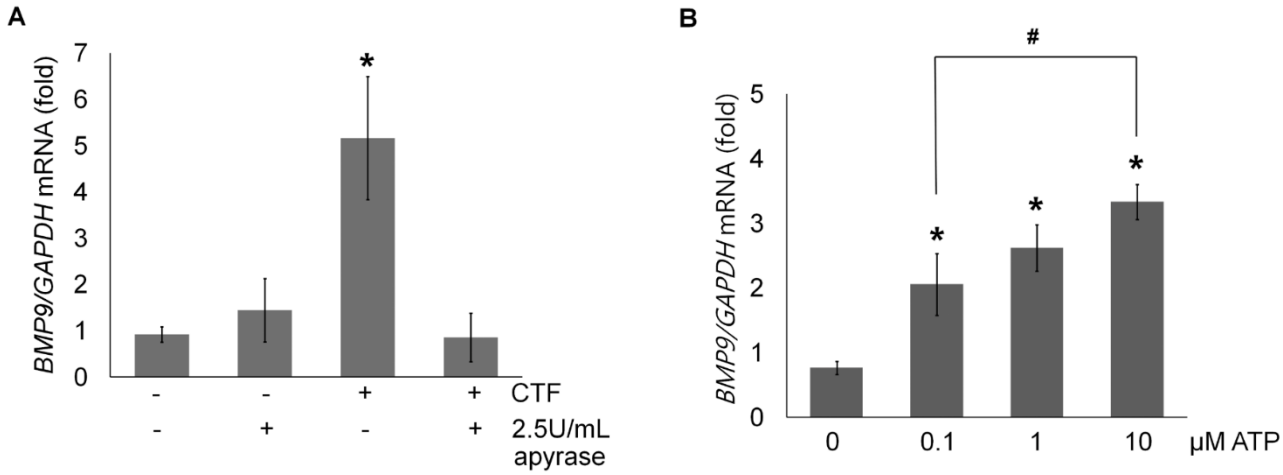

Figure 8 ATP stimulates BMP9 mRNA expression by human PDL cells.

(A) Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm continuously for 6 hours in serum-free condition. Cells were cultured with 2.5 $U.mL^{-1}$ apyrase to degrade extracellular ATP. The expression of *BMP9* was determined by RT-PCR demonstrating that apyrase abolished the CTF-stimulated *BMP9* expression. **(B)** Human PDL cells were cultured without or with 0.1, 1 and 10 μM ATP in serum-free medium for 6 hours. The expression of *BMP9* was significantly stimulated by ATP in a dose-dependent manner. The relative mRNA expression level of *BMP9/GAPDH* was presented as a fold-induction of the treated group compared to the control. The results are presented as means ± s.d. from 3 separate experiments. * $p < 0.05$ vs control, # $p < 0.05$ between 2 experimental groups.

CTF mediates ATP-induced BMP9 expression through the $P2Y_1$ receptor

The pathways involved in the CTF-mediated and ATP-induced *BMP9* expression was also identified. P2 purinoceptors have been known as a family of cell surface receptors that recognize extracellular nucleotides, therefore the possible involvement of different P2 receptors in the CTF-stimulated BMP9 expression was analyzed.

First, the effect of suramin, an inhibitor of heterodimeric G protein activation, was analyzed. Suramin acts as a broad-spectrum antagonist of P2Y purinergic receptors (Satrawaha et al., 2011). Human PDL cells were subjected to CTF in the presence or absence of 15 μM suramin. In the presence of suramin, the CTF-stimulated *BMP9* mRNA expression was completely abolished as shown in Figure 9A. These results indicate the involvement of P2Y receptors in CTF-stimulated *BMP9* expression. Next, MRS2179, a specific $P2Y_1$ receptor antagonist, was employed in an attempt to elucidate the subtype of P2Y receptor involved (Luckprom et al., 2010). Interestingly, similar to suramin, MRS2179 significantly inhibited the CTF-stimulated *BMP9* expression, suggesting a crucial role of $P2Y_1$ in the regulation of transcription as shown in Figure 9B.

To confirm the role of the $P2Y_1$ receptor in the expression of *BMP9*, different concentrations of MRS2365, a highly potent selective $P2Y_1$ receptor agonist (Lu et al., 2007), were added to non-CTF-loaded human PDL cells. Under these conditions

BMP9 expression significantly increased in a dose dependent manner as shown in Figure 9C. Finally, *$P2Y_1$* mRNA expression proved to be significantly increased in CTF-loaded human PDL cells as shown in Figure 9D.

Taken together, the data strongly suggest that CTF stimulates *BMP9* mRNA expression through the $P2Y_1$ receptor. The $P2Y_1$ receptor may be involved in ATP-upregulated *BMP9* expression by human PDL cells. Therefore, cells were stimulated with 10 μM ATP for 6 hours in the presence or absence of MRS2179. The results showed that ATP-induced *BMP9* expression was remarkably decreased by MRS2179 as shown in Figure 9E. The results indicate that ATP stimulates expression of *BMP9* via the $P2Y_1$ receptor.

Finally, the secondary signaling molecule(s) downstream to $P2Y_1$ receptor participated in upregulation of *BMP9* expression by human PDL cells was/were speculated. Since $P2Y_1$ receptor regularly triggers the release of intracellular calcium from the endoplasmic reticulum through the activation of phospholipase C (PLC) (Jiang et al., 2017), cells were added with 2 μM U-73122, a phospholipase C inhibitor (Luckprom et al., 2010) or were added with 12.5 nM thapsigargin, the drug that increase intracellular calcium level (Issaranggun Na Ayuthaya et al., 2017), before cells were stimulated with 0.5 nM MRS2365 for 6 hours. Comparing to the expression by human PDL cells stimulated with MRS2365 alone, *BMP9* expression significantly decreased in the presence of U-73122 while the expression significantly increased in the presence of thapsigargin, calcium agonist as shown in Figure 9F. The results

suggest that intracellular calcium is necessary in the upregulation of *BMP9* expression by $P2Y_1$ agonist-stimulated human PDL cells. Moreover, cAMP and PKA was reported as candidates downstream to $P2Y_1$ receptor (del Puerto et al., 2012; Shabbir and Burnstock, 2009). Adding 20 μM cAMPs-RP, a competitive antagonist of cAMP-induced activation of PKA, prior to the stimulation with MRS2365 was also performed. In the presence of cAMPs-RP, *BMP9* expression did not differ from the expression by human PDL cells

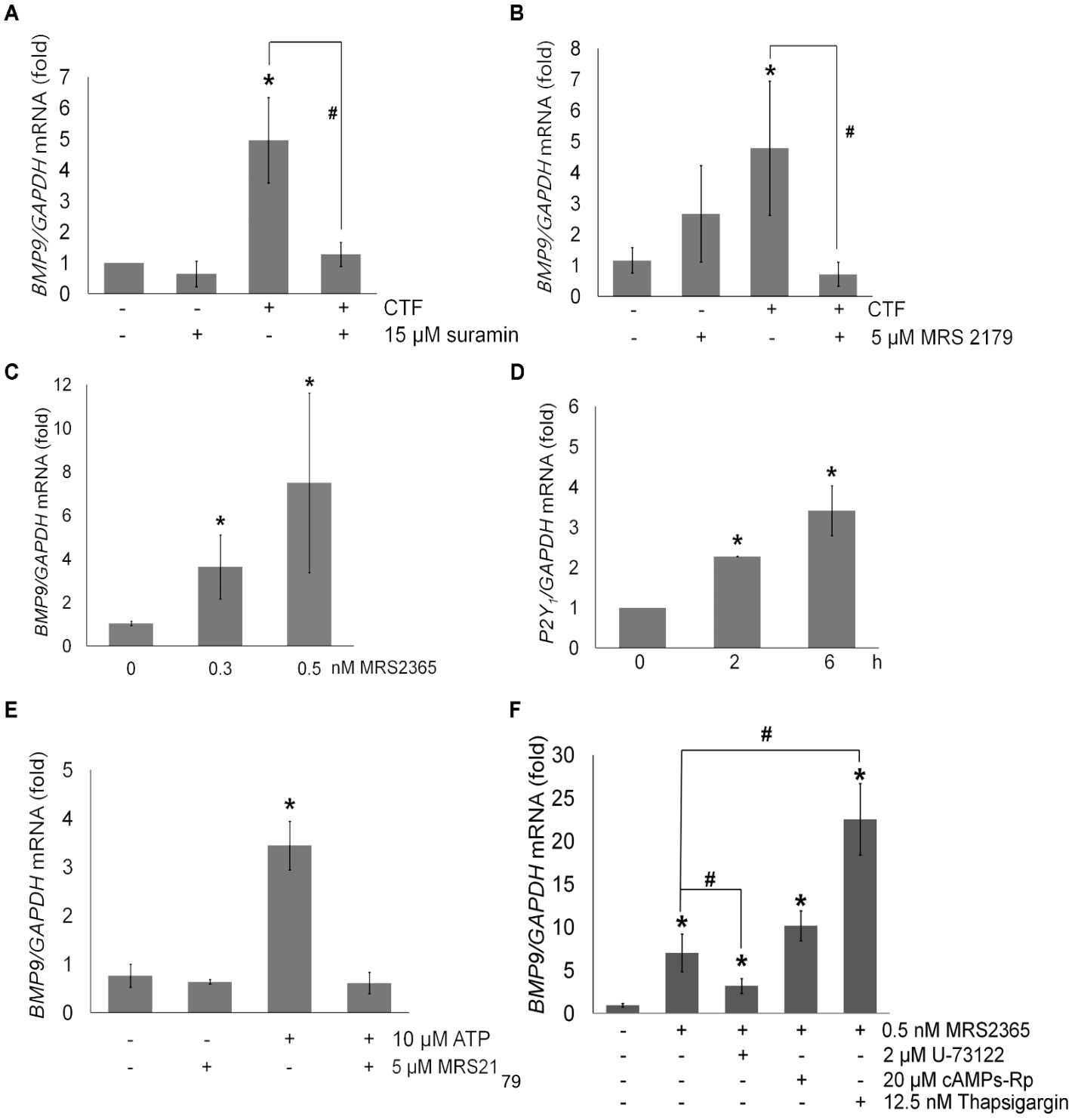
A
BMP9/GAPDH mRNA (fold)
0 1 2 3 4 5 6 7
*
#
- - + + CTF
- + - + 15 μM suramin
B
BMP9/GAPDH mRNA (fold)
0 1 2 3 4 5 6 7 8
*
#
- - + + CTF
- + - + 5 μM MRS 2179
C
BMP9/GAPDH mRNA (fold)
0 2 4 6 8 10 12
* *
0 0.3 0.5 nM MRS2365
D
P2Y1/GAPDH mRNA (fold)
0 1 2 3 4 5 6
* *
0 2 6 h
E
BMP9/GAPDH mRNA (fold)
0 1 2 3 4 5
*
- - + + 10 μM ATP
- + - + 5 μM MRS21
F
BMP9/GAPDH mRNA (fold)
0 5 10 15 20 25 30
# #
* * * *
- + + + + 0.5 nM MRS2365
- - + - - 2 μM U-73122
- - - + - 20 μM cAMPs-Rp
- - - - + 12.5 nM Thapsigargin

Figure 9 CTF-mediated ATP up-regulates BMP9 expression through $P2Y_1$ receptor.

Human PDL cells were subjected to 10% elongation strain of CTF at a frequency of 60 rpm continuously for 6 hours in serum-free condition. Culturing in the presence of **(A)** 15 μM suramin, a broad spectrum P2Y receptor antagonist, and by **(B)** 5 μM MRS2179, a specific $P2Y_1$ receptor antagonist abolished the CTF-stimulated *BMP9* expression. **(C)** MRS2365, a specific $P2Y_1$ agonist, stimulated expression of *BMP9* in a dose dependent manner. **(D)** The expression of *$P2Y_1$* mRNA was increased at 2 and 6 hours by CTF. **(E)** Addition of 5 μM MRS2179 reversed the 10 μM ATP stimulated *BMP9* expression. **(F)** Addition of 2 μM U-73122, a phospholipase C inhibitor, reduced 0.5 nM MRS2365-stimulated *BMP9* expression, whereas addition of 12.5 nM thapsigargin enhanced 0.5 nM MRS2365-stimulated *BMP9* expression. 20 μM cAMPs-RP, a competitive antagonist of cAMP-induced activation of PKA, had no significant effect on the expression *of BMP9* compared to those stimulated with 0.5 nM MRS2365 alone. The expression of *BMP9* was determined by RT-PCR. The relative mRNA expression level of *BMP9/GAPDH* was presented as a fold-induction compared to the control. The results are presented as means ± s.d. from 3 separate experiments. * $p < 0.05$ vs. control, # $p < 0.05$ between 2 experimental groups.

Collectively, a model of CTF-stimulated BMP9 expression by human PDL mediated by ATP via the $P2Y_1$ receptor was proposed as shown in Figure 10.

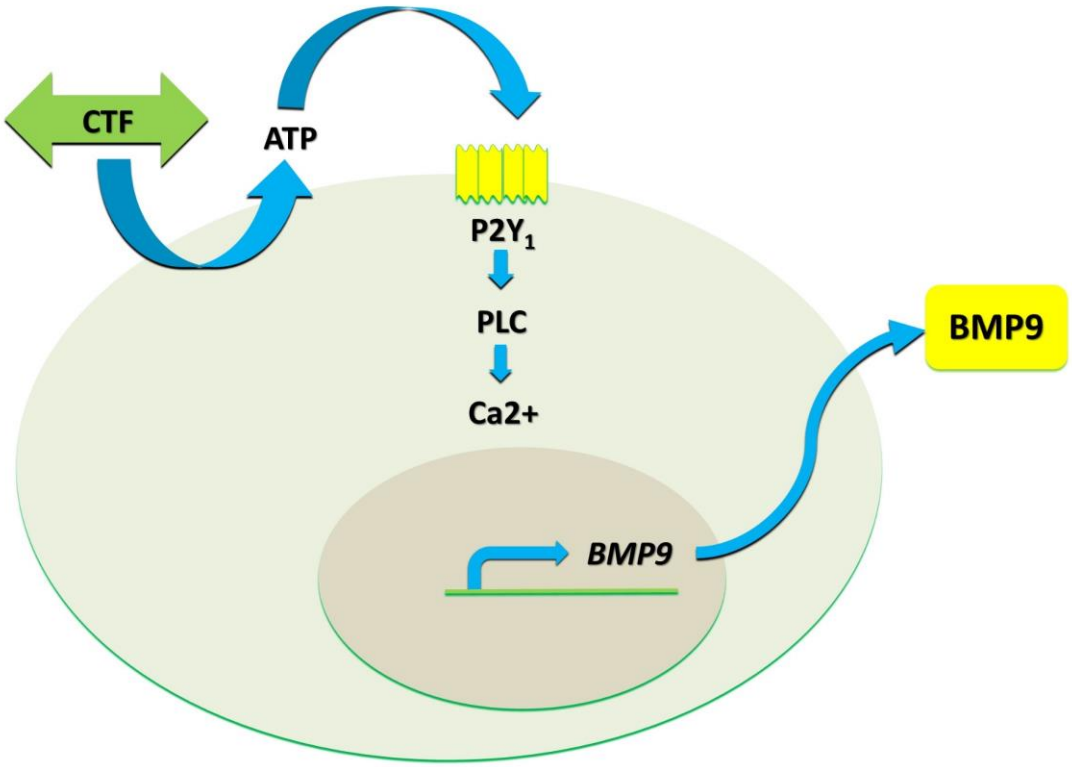

Figure 10 A proposed model for CTF stimulation of the expression of BMP9.

A proposed model demonstrates how CTF stimulates *BMP9* expression in human PDL cells. CTF mediates ATP release which subsequently induces a stimulated synthesis of BMP9 via $P2Y_1$ receptor. This activated receptor regulates the expression of *BMP9* by increasing the intracellular level of Ca^{2+} through the phospholipase C (PLC) pathway.

Addition to the effect of CTF on osteo-induction by human PDL cells, the investigation of effects of CTF on other aspects that associated with periodontal homeostasis was performed in this study.

CTF upregulates the expression of cytokine and enzymes by human PDL cells

The synthesis of IL6

Since CTF was reported to upregulate the expression of inflammatory cytokines such as *IL6*, the expression and synthesis of *IL6* were re-investigated by real-time PCR analysis. The results demonstrated that CTF applied for 2 and 6 hours significantly up-regulated the expression of *IL6* mRNA at both time points; being highest at 6 hours as shown in Figure 11A. Quantitative analysis of IL6 protein by ELISA demonstrated a significant increase of IL6 in the culture media by 24 hours as shown in Figure 11B. Cells grown under the same condition without CTF were used as a control and did not show any upregulation of the cytokine.

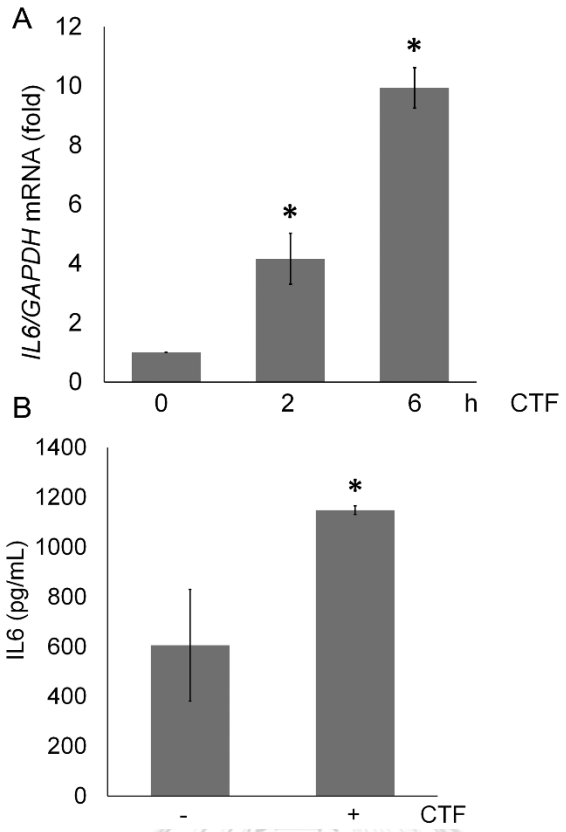

Figure 11 CTF up-regulates the synthesis of IL6 by human PDL cells.

Human PDL cells seeded on gelatin-coated silicone membranes were subjected to CTF at 10% elongation strain and 60 rpm of frequency continuously for 2 and 6 hours in serum-free medium. **(A)** Cells were processed for analysis of *IL6* mRNA expression by real time-qPCR. CTF significantly increased *IL6* mRNA expression in a time-dependent manner. The relative mRNA expression level of *IL6* are presented as a fold-change compared to non-CTF-loaded cells. **(B)** Cultured media were collected after a subsequent culture period of 24 hours and analyzed for IL6 protein by ELISA. A significant increase of the level of IL6 protein was found in CTF-loaded compared to the non CTF-loaded cells. The results were shown as means ± S.D. from 3 independent experiments. * $p < 0.05$ vs. non-CTF control.

The expression of MMPs and TIMPs by human PDL cells

Physiologic strain has been shown to influence extracellular matrix remodeling by modulating enzyme expression (Fujihara et al., 2010; Kalajzic et al., 2014; Kook et al., 2011). Therefore, we examined the expression levels of *MMP1*, *MMP2*, *MMP3*, *MMP8*, *MMP14, TIMP1 and TIMP2* proteins involved in PDL remodeling, after a continuous six-hour CTF by real-time PCR. We analyzed expression immediately after the six-hour CTF and after culturing for another 24 hours.

Immediately after load-withdrawal, the expression of most analyzed MMPs, except for *MMP8*, *TIMP1* and *TIMP2,* were significantly increased compared to non-loaded cells as shown in Figure 12A-G. Following a further twenty-four-hour culture period, the expression of *MMP1* (Fig. 12A) and *MMP3* (Fig. 12C) had increased even five-fold and six-fold, respectively. At this time point, the expression of *MMP2* (Fig. 12B) and *MMP14* (Fig. 12E) were reduced to control levels. No change was observed in the expression of *MMP8* (Fig. 12D), *TIMP1* (Fig. 12F) and *TIMP2* (Fig. 12G).

Since Kook and colleagues (Kook et al., 2011) have shown already that CTF up-regulated *MMP1* as well as the underlying mechanism that resulted in its increase, in the current study, we focused on MMP3. The level of MMP3 protein secreted in the medium after six-hour CTF-loading with an extended twenty-four-hour culture period was analyzed by ELISA. The results showed a

significant increase of MMP3 compared to the non-CTF loaded control as shown in Figure 12F.

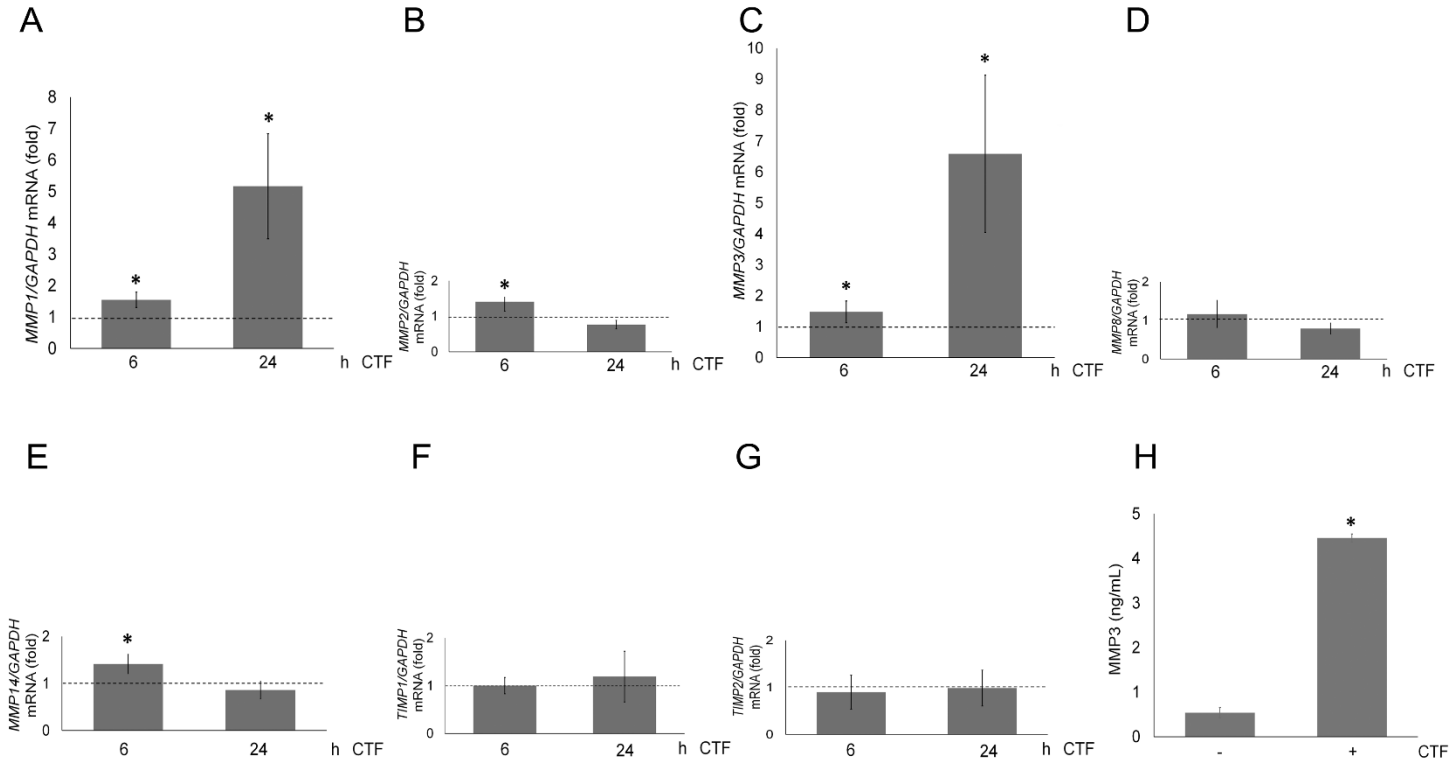

Figure 12 Effect of CTF on the expression of MMPs and TIMPs by human PDL cells

Human PDL cells seeded on gelatin-coated silicone membranes were subjected to CTF at 10% elongation strain and 60 rpm of frequency for 6 hours in serum-free medium. The expression of **(A)** *MMP1*, **(B)** *MMP2*, **(C)** *MMP3*, **(D)** *MMP8*, **(E)** *MMP14*, **(F)** *TIMP1* or **(G)** *TIMP2* was analyzed by real-time qPCR immediately after a 6 h CTF and after culture further for another 24 hours. The relative mRNA expression was presented as a fold-change compared to non-CTF-loaded cells (dotted-line). **(H)** Cultured media were collected after a subsequent culture period of 48 hours and analyzed for MMP3 by ELISA. A significant increase of the level of MMP3 protein was found in the CTF-loaded cells compared to the non CTF-loaded cells ($p < 0.05$). The

results were shown as means ± S.D. from 3 independent experiments. * $p < 0.05$ vs. non-CTF control. CTF=cyclic tensile force

CTF up-regulates the expression of MMP3 by human PDL cells through IL6

To assess whether IL6 was involved in the increased synthesis of MMP3, human PDL cells were loaded with six-hour CTF, after which 0.07 $\mu g. mL^{-1}$ IL6 antibody or 0.07 $\mu g. mL^{-1}$ non-specific IgG antibody was added. The cultures continued for another 24 and 48 hours followed by mRNA and protein analyses. The results demonstrated that the neutralizing antibody significantly decreased both the levels of *MMP3*-mRNA expression as shown in Figure 13A and synthesized MMP3 as shown in Figure 13B compared to the control group to which non-specific IgG was added ($p < 0.05$). The results indicate that IL6 plays a major role in CTF induced MMP3 synthesis.

The expression of IL6 receptor (*L6R*) was also investigated to assess whether the cells had the capacity to respond to IL6 in an autocrine manner. CTF was shown to up-regulate expression of *IL6R* as shown in Figure 13C.

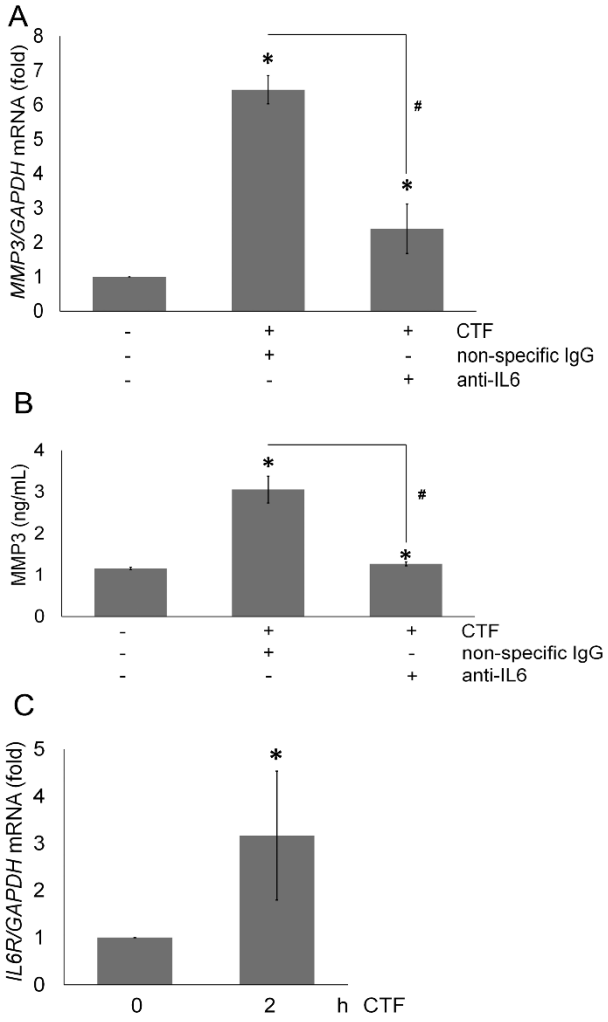

Figure 13 CTF up-regulates the expression of MMP3 in human PDL cells through IL6

Human PDL cells seeded on gelatin-coated silicone membranes were subjected to CTF at 10% elongation strain and 60 rpm of frequency continuously for 6 hours in serum-free medium. After terminating the CTF, 0.07 μg.mL^{-1} neutralizing anti-IL6 monoclonal IgG antibody or 0.07 μg.mL^{-1} non-specific mouse IgG control antibody was added and the cultures continued for 24 and 48 hours. Then expression of MMP3 was analysed by **(A)** qPCR and **(B)** ELISA. **(C)** the expression of IL6R after two-hour-loaded CTF was analysed by qPCR. The relative mRNA expression normalized to

GAPDH was presented as a fold-change compared to non-CTF-loaded cells. For protein analysis, the amount was normalized to the cell number. The results were shown as means ± S.D. from 3 independent experiments. * $p < 0.05$ vs. non-CTF control, # $p < 0.05$ between 2 experimental groups. CTF=cyclic tensile force

Cell viability of human PDL cells after adding of recombinant human IL6

Primary human PDL cells were challenged with recombinant IL6 at a concentration of 0, 0.1, 1 and 10 ng.mL^{-1} for 24 hours. The viability of cells under these conditions was tested by an MTT assay. Concentrations up to 10 ng.mL^{-1} of recombinant human IL6 had no significant effect on cell viability as shown in Figure 14.

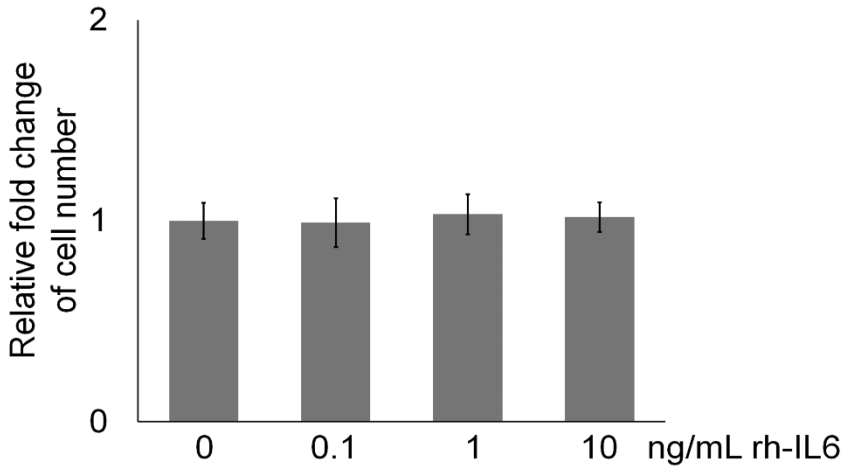

Figure 14 MTT analysis on the viability of IL6-treated human PDL cells.

Human PDL cells were cultured in the absence or presence of various concentrations of recombinant human IL6 in serum-free medium for 24 hours. Cell viability was examined using MTT assay. Bar graphs demonstrate the relative fold of cell number as mean ± S.D. compared to that of cells in a control group. Each condition was performed in triplicate, and data were analyzed from 3 different experiments.

IL6 up-regulates the expression of *MMP3* by human PDL cells

To confirm that IL6 could indeed stimulate expression of *MMP3* by human PDL cells, cells were treated with different concentrations of recombinant human IL6. These concentrations were within the range of released cytokine by CTF-stimulated human PDL cells (see Figure 11B). The results showed that 0.1, 1 and 10 $ng.mL^{-1}$ of recombinant human IL6 significantly up-regulated the mRNA expression as shown in Figure 15A and the concentration at 1 and 10 $ng.mL^{-1}$ significantly increased protein synthesis of MMP3 ($p < 0.05$) as shown in Figure 15B. Since the amount of IL6 released by the PDL cells was shown to be about 1 ng/ml (see Figure 11B), such concentration was chosen for subsequent experiments.

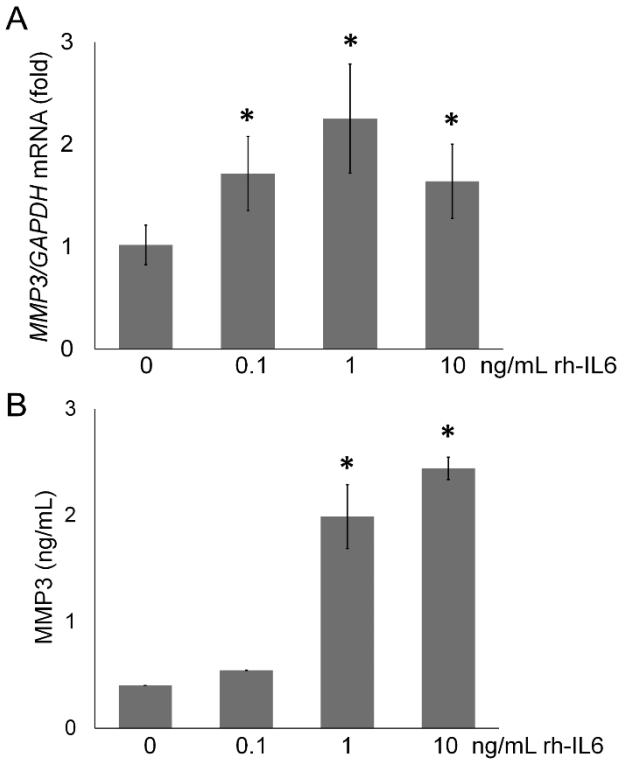

Figure 15 IL6 up-regulates the expression of MMP3 by human PDL cells

Human PDL cells were cultured in the absence or presence of 0.1, 1 and 10 ng.mL^{-1} recombinant human IL6 in serum-free media for 24 hours. The expression of MMP3 mRNA and protein were analyzed by **(A)** RT-qPCR and **(B)** ELISA, respectively. The relative expression of *MMP3* mRNA normalized by that of *GAPDH* was presented as a fold-change compared to non-treated controls. For protein analysis, the amount was normalized to the cell number. Graphs demonstrate means ± S.D. from 3 independent experiments. * $p < 0.05$ vs. control.

IL6 up-regulates MMP3 by human PDL cells via PI3K and MAPKs

Next, the possible regulatory intracellular pathways involved in the IL6 up-regulation of MMP3 expression was also analyzed. As the JAK/STAT3 pathway is a classical IL6 signaling downstream route for gene transcription regulation (Heinrich et al., 2003), JAK and STAT3 were blocked. Human PDL cells were pretreated for thirty-minutes with different concentrations (1-15nM) of a JAK inhibitor or with a STAT3 inhibitor V (1-6 μM). This was followed by an incubation with 1 ng/mL recombinant human IL6 for 24 hours. The qPCR results demonstrated that the inhibitors had no effect on IL6-induced expression of *MMP3* as shown in Figure 16. IL-6 has been shown also to activate the PI3K and MAPKs pathways (Hodes et al., 2016). Therefore, the possible role of PI3K, JNK, ERK and p38 MAPKs was examined using the PI3K inhibitor LY294002 (1.4 μM), the JNK inhibitor II SP600125 (40nM), the ERK1/2 inhibitor PD98059 (2.5 μM) and the p38 inhibitor SB203580 (35nM). The results showed that the PI3K and MAPKs inhibitors significantly reduced *MMP3* expression ($p < 0.05$) induced by IL6 stimulation as shown in Figure 16. These findings strongly suggest the involvement of the PI3K and MAPKs pathways in the IL6-modulated expression of *MMP3* by human PDL cells.

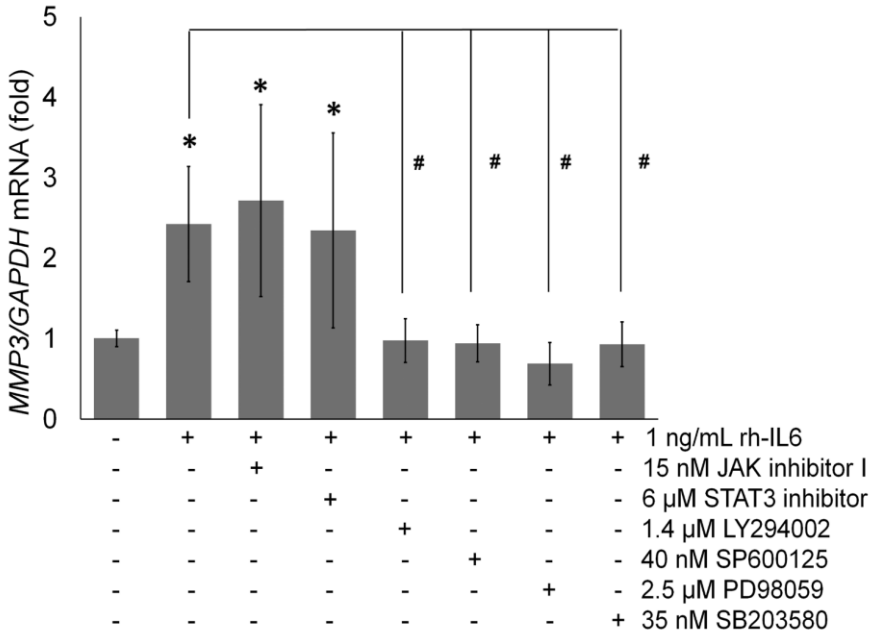

Figure 16 IL6 up-regulates MMP3 expression in human PDL cells via PI3K and MAPKs

Primary human PDL cells were seeded at a density of $1x10^5$ cells/well in 12-well plate. Cells were pretreated with inhibitors for different signaling molecules 30 minutes prior to an incubation with 1 $ng.mL^{-1}$ recombinant human IL6 for 24 hours. RNA was extracted for analysis by real-time qPCR. The inhibitors used in the present study were 15 nM JAK inhibitor I, 6 μM STAT3 inhibitor V, 1.4 μM PI3K inhibitor (LY294002), 40 nM JNK inhibitor II (SP600125), 2.5 μM ERK1/2 inhibitor (PD98059), and 35 nM p38 inhibitor (SB203580). The relative expression of *MMP3* mRNA normalized by that of *GAPDH* was presented as a fold-change compared to the non-treated control group. Graphs demonstrate means ± S.D. from 3 independent experiments. * $p < 0.05$ vs. control, # $p < 0.05$ between 2 experimental groups.

Collectively, we propose a model of CTF-upregulated MMP3 being mediated by CTF-increased IL6 as shown in Figure 17.

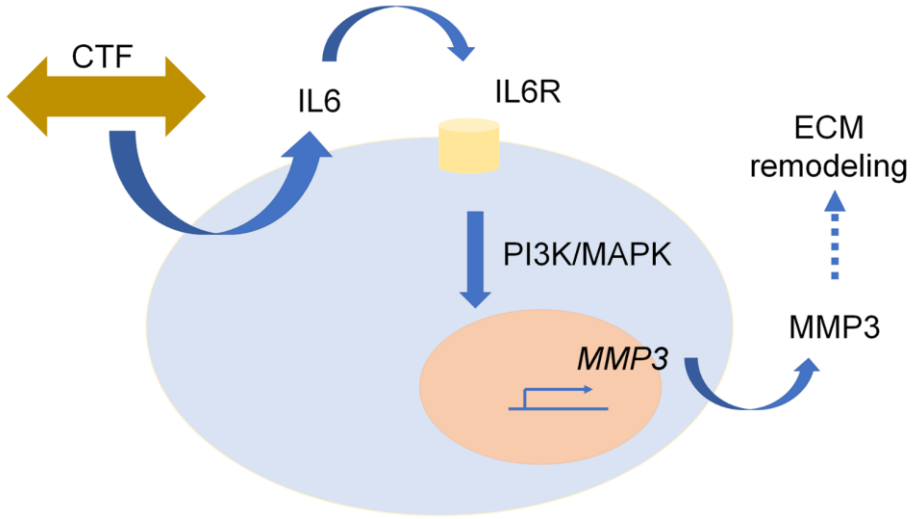

Figure 17 A proposed model of how CTF increases MMP3 via an up-regulation of IL6

A proposed model of how CTF increases MMP3 via an up-regulation of IL6 through MAP kinase and PI3K signaling pathways in human periodontal ligament cells, and its role in matrix remodeling.

Chapter 5 Discussion and Conclusion

This study aimed to clarify the behavioral response of human PDL cells to cyclic tensile force (CTF). The significant findings of our study are the CTF induced mineral deposition by human PDL cells and the concomitant stimulated expression of *BMP9* under these conditions. BMP9 proved to be crucial for the deposition of mineral. Moreover, we demonstrated that the force increased expression of *BMP9* depended on ATP and $P2Y_1$ receptors through the activation of phospholipase C and calcium signaling. Additionally, we demonstrated that CTF upregulated the synthesis of IL6 as well as the expression of *MMP1, 2, 3* and *14* by human PDL cells. A neutralizing IL6 antibody attenuated the production of MMP3 indicating for the first time that CTF-upregulated MMP3 expression depended on the activity of IL6. Finally, we have also shown that the PI3K and MAPK intracellular signaling pathways were essential for the IL6-mediated MMP3 upregulation.

The PDL is known as one of the most highly metabolically active connective tissues. It is characterized by a constant renewal of the extracellular matrix of which fibrillar collagen is the most abundant constituent. The metabolism of collagen in PDL has to be accurately controlled by a balance between synthesis and degradation. Mechanical loading caused by mastication or orthodontic tooth movement affects cell differentiation, remodeling of the extracellular matrix, and

the synthesis of inflammatory cytokines of PDL cells (Jacobs et al., 2013; Jacobs et al., 2014).

In this research study, cyclic tensile force was used in the range that mimic physiologic occlusal force of 10% CTF with a frequency of 60 rpm. The average frequency of a masticatory cycle is considered to be around 70 rpm (Pini et al., 2002) or between 0.5-3 Hz (Woda et al., 2006). A CTF at 10% has been taken to represent physiological occlusal strain (Fujihara et al., 2010; Li et al., 2013a; Matsuda et al., 1998).

Tensile force used in this study was uniaxial stretching. As cells randomly oriented themselves over the *in vitro* membrane and are thus not always in the direction of strain, they may experience less strain than that actually induced. Appear as found *in vivo* situation in periodontium as multidirectional (Hirashima et al., 2016). This suggests cell behavior varies according to the stretching direction. Our device can deliver a uniform strain at the central seeding area. Software-controlled handgrips allows precise control over grip-to-grip displacement and strain rate. This can imply that the tensile loading is sum of stretching force on membrane which eventually transfer to the seeded cells.

Substrate for tension generating device is made from polydimethylsiloxane trimethylsiloxy (PDMS) which is one type of silicone. The hydrophobic silicone membrane was surface modified using plasma surface activation, glutaraldehyde cross-linking, and collagen/gelatin coating to increase cell adhesion. In this research, gelatin coating was chosen. Cell can attach to gelatin as well as to collagen despite

of different integrin binding (Davidenko et al., 2016). Our result of cell viability indicated that cells can spread and attach on the gelatin coating membrane without any toxicity. Moreover, the result of *BMP2* upregulation was consistent with previous studies in which were used type I collagen coating membrane (Suzuki et al., 2014; Wescott et al., 2007). The upregulation of *IL6* was also in agreement with previous study using collagen coating (Wada et al., 2017). Our results suggested that in some circumstances, gelatin can also replace type I collagen in order to pre-coat surface for cell adherence.

Previous studies have shown that CTF increases the expression of osteogenic genes such as *Runx2*, *ALP*, and *osterix* by human PDL cells (Liu et al., 2012; Shen et al., 2014; Wescott et al., 2007), thus indicating that these cells obtain an osteoblast-like phenotype. Our finding showed that CTF also increases mineral deposition by these cells. So, it appears that tensile force stimulates the formation of bone. In line with our findings are studies which demonstrated that CTF enhanced the mineralization capacity of human bone marrow stem cells and osteoblasts (Jansen et al., 2010; Wang et al., 2016; Ye et al., 2012). Since PDL cells have been shown to be osteoblast-like cells, our findings suggest a role of these cells in bone formation under the influence of tensile force such as during tooth movement.

In the present study, CTF was found to stimulated the synthesis of BMP9 by human PDL cells. Even though its expression level was lower compared to that of BMP2 and 6, this BMP has a very high potency, up to 10-times higher than BMP2, to

induce osteogenic differentiation of different cell types including human PDL cells (Fuchigami et al., 2016; Fujioka-Kobayashi et al., 2016a; Wang et al., 2013b). A stimulating effect of CTF on the expression of other osteogenic BMPs was shown for *BMP2* and *6* (Suzuki et al., 2014; Wescott et al., 2007), but so far not for *BMP9*. It was of interest to note that we found a concomitant increase in the expression of *noggin*. Noggin is a well-known BMP inhibitor, in particular of BMP2 and BMP6. Noggin is, however, unable to inhibit the BMP9-dependent effect on osteogenesis (Wang et al., 2013b). This appears to suggest that the CTF-induced mineralization as found in the present study is mediated by BMP9.

Here, BMP9 was demonstrated that it played a role in *in vitro* mineral deposition in both the absence and the presence of CTF. The osteogenic BMPs including BMP9 are osteogenic inducers which promote osteogenic differentiation in mesenchymal stem cells (Cheng et al., 2003), thus their function is essentially required during an initiation phase of differentiation. CTF accelerated the capability of human PDL cells to form mineral deposition already by 14 days, instead of the period normally needed, 28 days. A neutralizing antibody to BMP9 decreased the mineral deposition by human PDL cells. This finding indicates that CTF-stimulated BMP9 by human PDL cells participates in *in vitro* mineral deposition.

As mentioned in the literature review, BMP9 is implicated in a wide variety of cellular activities. The CTF-stimulated BMP9 is possibly responsible to other functions in PDL cells or has paracrine effects to other cell types in the tissue. BMP9 can

stimulate the proliferation of primary bovine chondrocytes and extracellular matrix deposition resulting in chondrocyte hypertrophy and mineralization (Blunk et al., 2003). Not only chondrocytes, BMP9 also increase proliferation rate of HepG2 liver tumor cells and primary rat hepatocytes (Song et al., 1995). BMP9 has a potential in promoting proliferation and tube formation of mouse stem cell-derived endothelial cells (Suzuki et al., 2010). Using a competitive inhibitor to BMP9 receptors can impair the angiogenic response to VEGF by endothelial cells resulting in decrease of tumor growth (Cunha et al., 2010). These findings suggest the promoting effect on cellular proliferation in periodontium by BMP9. On the contrary, BMP9 was reported to has anti-angiogenic effect. BMP9 could attenuate the cell migration and growth rate of endothelial cells. It can activate the genes that play roles in vascular maturation, for example, Id1, Id2, Smad6/7, and endoglin (David et al., 2007). This may imply that BMP9 may function as a vascular maturation factor in the PDL tissue. Other function of BMP9 in periodontium except bone formation should be further investigated.

The ability of PDL cells to respond to BMP9 in an autocrine manner was also demonstrated. Human PDL cells express different BMP9 receptors including ALK1 and 2 of BMP receptor type I, the BMP receptor type II (BMPRII), ActR type IIA (ActRIIA), and ActR type IIB (ActRIIB), and the co-receptor endoglin (Fuchigami et al., 2016). Expression of *ALK1* and *ALK2* receptors is required for BMP9 induced osteogenic signaling (Luo et al., 2010; Townson et al., 2012), and previous studies showed that PDL cells are able to respond to BMP9 through these receptors (Fuchigami et al.,

2016). So, it seems that the basal expression of these receptors is sufficient for the cells to respond to BMP9.

Normally, BMPs are secreted in a latent form and require activation in the extracellular environment. BMP9 differs in this respect from other BMPs since its noncovalent-bound pro-domain is readily displaced when its functional domain contacts its receptor (Kienast et al., 2016). Consequently, latent and active BMP9 have equivalent biological activities (Brown et al., 2005; Li et al., 2016b). This strongly suggests that the secreted BMP9 can interact with its receptor.

Finally, the CTF-stimulated *BMP9* expression depended on ATP was also shown. CTF induced the release of ATP from human PDL cells. This finding is in line with data presented in previous studies which showed that the release of ATP could be enhanced by many cell types, including human PDL cells, if subjected to physical stresses such as shear, compression, hydrostatic pressure and plasma membrane stretch (Lazarowski et al., 2011). The released ATP acts as signaling molecule to induce *BMP9* expression. Proof for this statement was found by using apyrase, an enzyme that degrades extracellular ATP (Wongkhantee et al., 2008). Under these conditions, the CTF-induced *BMP9* expression was strongly inhibited. Moreover, the addition of ATP stimulated *BMP9* expression. ATP was previously reported to induce *BMP2, 4,* and *5* expression by rat osteoblasts (Ayala-Pena et al., 2013). So, it seems that ATP plays a role in the induction of different osteogenic BMPs. Some evidence indicates that ATP induces osteogenic differentiation by mesenchymal stem cells and

human osteoblasts (Kariya et al., 2015; Sun et al., 2013). Our data and those presented by others strongly suggest that this differentiation is mediated by BMPs, among which BMP9.

Exogenous ATP exerts its effects as a signaling molecule partly through P2 receptors, which can be categorized into P2X ($P2X_{1-7}$) and P2Y ($P2Y_{1,2,4,6,11-14}$) (Lazarowski et al., 2011). Our data suggest that $P2Y_1$ is the receptor responsible in the CTF-upregulated *BMP9* expression. By using MRS2179, a selective $P2Y_1$ receptor antagonist, *BMP9* expression stimulated by CTF was inhibited, while addition of MRS2365, a selective $P2Y_1$ receptor agonist, stimulated the expression. Moreover, only the expression of *$P2Y_1$*, and none of the other P2s, increased under the influence of CTF. Since the $P2Y_1$ receptor is known to be more selective to ADP (Houston et al., 2008; Palmer et al., 1998), we assume that ADP is generated from ATP due to the presence of ectoenzymes (Lazarowski et al., 2011). Collectively, we propose that the CTF-induced ATP-mediated increased *BMP9* expression occurs through $P2Y_1$ receptors. In support of this assumption is the finding that $P2Y_1$ receptors are involved in adipogenesis and proliferation of MSCs (Ciciarello et al., 2013). This suggests that $P2Y_1$ may play crucial roles in differentiation of the cells upon mechanical stimulation.

With respect to the intracellular signaling cascade we demonstrated that inhibition of PLC activity partially reduced the expression of *BMP9*, while an increase of intracellular Ca^{2+} enhanced. The partial attenuation of *BMP9* expression by the

PLC inhibitor indicates the presence of yet unknown other signal pathways. One of these pathways may be via modulating adenylyl cyclase (AC) which alters cAMP level by triggering the activation of protein kinase A (PKA) (Shabbir and Burnstock, 2009). Our results make it unlikely that cAMP is part of the signaling cascade. Thus, our study suggests that the $P2Y_1$ stimulated *BMP9* expression depends on an increased level of intracellular Ca^{2+} which is mediated by phospholipase C (PLC). This finding result can be concluded that ATP stimulates the expression of BMP9 by PDL cells via $P2Y_1$/PLC/ Ca^{2+}. However, ATP was previously reported to increase RANKL expression through pathway dependent on $P2Y_1$, cAMP dependent protein kinase, NF**κ**B, and COX (Luckprom et al., 2010). ATP also exerted its signal to inhibit mineral deposition in human osteoblast through binding with $P2X_7$ (Sindhavajiva et al., 2017). So, it is interestingly suggested the role of ATP in controlling balance of bone modeling. This facilitates the periodontal tissue in maintaining homeostasis.

CTF also increases the expression of some particular cytokines including inflammatory cytokines for example, IL6, IL8, or TNF-**α**. Some studies have shown that static as well as cyclic tensile force induces the expression of IL6 by PDL cells (Jacobs et al., 2013; Wada et al., 2017). These studies, however, employed periodontal cell lines whereas we analyzed the response of primary PDL cells. So, it appears that both cell lines as well as primary cells respond in a similar way to CTF by an increased production of IL6.

The actual presence of IL6 in the periodontal ligament has been shown by immunohistochemistry (Grzibovskis et al., 2011). These authors demonstrated different levels of IL6 in normal human PDL from different age groups. The expression of IL6 proved to be higher in tissue samples of younger persons than in those of an adult group. The higher expression coincided with higher metabolic activity of the periodontal tissue.

IL6 induces cell signaling through an IL6 receptor (IL6R) and a signal transducing subunit (gp130) (Scheller et al., 2014). PDL cells have been shown to express IL6R, and gp130 subunit (Hosokawa et al., 2014). In the present study we detected an increase of *IL6R* expression after two-hour CTF stimulation. These findings indicate that human PDL cells have the capacity to respond to CTF-induced IL6 in an autocrine manner.

The released IL6 from CTF-loaded human PDL cells probably affects several cell types in the periodontium both in a paracrine and autocrine manner. One of the possible functions of the cytokine is its role in bone remodeling. IL6 not only promotes osteoclastogenesis by RANKL induction in mouse osteoblasts (Udagawa et al., 1995), but also seems to play a role in osteoblast differentiation of both human osteoblasts (Nishimura et al., 1998) and human PDL cells (Iwasaki et al., 2008). IL6-deficient mice reveal more bone destruction in the periapical area; an area characterized by a high number of active osteoclasts (Balto et al., 2001). These

findings suggest that IL6 in PDL tissue is important for bone protection. The possible role of CTF-induced IL6 in control the balance of bone remodeling should be further studied.

IL6 increases also expression of SOCs3 by human PDL cells and thereby negatively controls a pro-inflammatory response of the cells (Fukushima et al., 2010). The cytokine stimulates CCL20 production in human PDL cells resulting in the recruitment of immune cells like Th17 cells to the tissue (Hosokawa et al., 2014). IL6 is able to enhance C-reactive protein production by human PDL fibroblasts contributing to the induction of Th1 and Th17 cytokines (Hernandez-Caldera et al., 2018). These findings together suggest a role of IL6 induced by CTF in an immunologic crosstalk to modulate the homeostasis of the periodontium. IL6 affects not only PDL cells and osteoblasts but it also affects endothelial cells. It enhances the expression of ICAM1 and VCAM1 by endothelial cells (Watson et al., 1996) probably causing the recruitment of lymphocytes to the PDL tissue. IL6 increases VEGF production by endothelial cells through Src-FAK signaling (Huang et al., 2016) which may promote angiogenesis. Collectively, the data strongly suggest a central role played by IL6 in the homeostasis of the periodontium.

One possible function of IL6 is to modulate the synthesis of proteolytic enzymes. IL6 has a potential to modulate various MMPs in many cell types. In mouse calvaria, IL6 also showed its ability to regulate various MMPs (MMP2, 3, 9, and 13) (Kusano et al., 1998). IL6 enhanced the migration of cancer cells through the

induction of MMP2, 9, and 13 (Kossakowska et al., 1999; Tang et al., 2011). Not only in cancer cells, IL6 modulated the upregulation of *MMP9* through the activation of Erk1/2 in macrophage (Kothari et al., 2014). Recently, IL6 was shown to induce cellular migration of mesenchymal stem cells through the enhancement of *MMP1* and *3* expression (Casson et al., 2018).

CTF also stimulates the expression of proteolytic enzymes. In this study, the six-hour CTF upregulates the expression of *MMP1*, *MMP2*, *MMP3*, and *MMP14.* Type I collagen, the main constituent of the ECM in the PDL, is degraded by MMP1 and MMP14, and denatured collagen is subsequently digested by MMP2 (Page-McCaw et al., 2007; Sternlicht and Werb, 2001). MMP3 and MMP14 can activate some other MMPs including MMP1 and MMP8 (He et al., 1989; Holopainen et al., 2003; Suzuki et al., 1990). Expression of MMP8, an enzyme previously reported to be associated with periodontal diseases (Butler and Overall, 2009; Franco et al., 2017), was found no to be changed under the influence of CTF. CTF did also not influence expression of *TIMP1* and/or *TIMP2*. This finding is in line with previous *in vitro* and *in vivo* studies which showed that mechanical force did not change the expression of *TIMPs* (Barthelemi et al., 2012; Carano and Siciliani, 1996; Redlich et al., 2001). Since the expression of TIMPs appears unaffected, this imbalance between MMPs and TIMPs would drive to the degradation mode of tissue remodeling. However, further studies are needed to elucidate the exact ratio of this disproportion.

Interestingly, the expression level of *MMP1* and *MMP3* proved to be increased also after 24 hours even though no more force was applied. In contrast herewith, the expression of *MMP2* and *MMP14* returned to the basal level. Our results on the expression of MMP1, are in line with data presented by Kook and colleagues who found that after force loading, PDL cells upregulated *MMP1* (Kook et al., 2011). Together, these findings suggest that MMP1 and MMP3 may play an important role in the turnover of the extracellular matrix of the PDL.

MMP3 is regulated by many factors, for example, cytokines, growth factors, and hormones. IL1α can increase the expression of *MMP3* by mouse osteoblasts in order to induce the bone resorption (Kusano et al., 1998). IL1β also upregulated the production of MMP3 by human synovial fibroblast (Sodin-Semrl et al., 2000). In some cancer cells, MMP3 was modulated by reactive oxygen species in order to induce transformation of cancer cells (Radisky et al., 2005). Fibroblast growth factor-2 (FGF-2) enhances the expression of MMP3 in endothelial cells via Erk signaling pathway (Derivaux et al., 2000). Additionally, gonadotropins and steroid hormones also induce the upregulation of MMP3 expression in granulosa cells (Zhu et al., 2014). These evidences suggest various factors modulate the MMP3 production indicating the wide variety cellular function of MMP3.

MMP3 was reported the upregulation in orthodontic patient (Capelli et al., 2011) and patient with chronic periodontitis (Letra et al., 2012). MMP3 was also found

increased in synovial fluid from patients who had knee injury (Lohmander et al., 1999). These suggested the involvement of MMP3 in both physiologic and pathologic situation. MMP3 can induce the FasL cleavage in osteosarcoma cell line inducing osteoclast apoptosis (Garcia et al., 2013). This suggested its other role in controlling balance of bone metabolism under mechanical loading. MMP3 cleaves the CTGF and VEGF complex in extracellular matrix resulting in the release of VEGF to promote the endothelial differentiation(Li et al., 2016a). Silencing MMP3 in mesenchymal stem cells derived both from adipose tissue and bone marrow showed lower blood flow recovery *in vivo* (Kim et al., 2007). These also suggest the role of MMP3 in endothelial differentiation and angiogenesis in the tissue in order to maintain homeostasis or facilitate tissue healing. Not only function as a secretory endopeptidase, MMP3 has also functioned intracellularly as a trans-regulator of some genes such as connective tissue growth factor (*CTGF*) detecting by nuclear translocation of MMP3 protein (Eguchi et al., 2008). This also suggested another role in tissue remodeling of MMP3 through modulation of connective tissue-mediated gene. Another sample of intracellular function of MMP3 is the reported about nuclear translocation of active MMP3 could increase apoptosis rate in ovary cells (Si-Tayeb et al., 2006).

In physiologic condition, MMP3 is found in serum at highest level of 1 $ng.mL^{-1}$ concentration which is found the declination corresponding to sample age (Thrailkill et al., 2005). High concentration of MMP3 (> 5 $ng.mL^{-1}$) was mostly found in

inflammatory or pathologic tissue such as cancer or periodontitis (Cymbaluk-Ploska et al., 2018; Reddy et al., 2012). MMP3 showed very low level of concentration in healthy (<1 $ng.mL^{-1}$) and after treatment of periodontitis (<5 $ng.mL^{-1}$) (Reddy et al., 2012). These suggested that in physiologic condition, only small amount of MMP3 is required. Our result of low level of MMP3 indicates the role of MMP3 in physiologic stimulation in tissue.

MMP2 and 14 expression were shown increased after continuous application of six-hour CTF. However, the up-regulatory effect had abated and the mRNA level had returned to the comparable level to non-CTF-loaded control. This indicates that MMP2 and 14 were directly upregulated by CTF. Our result was in agreement with previous finding that showed two-fold increase of *MMP2* expression after continuous stretching for 24 and 48 hours (Chen et al., 2013). Although, for MMP14, previous study showed unchanged expression by human PDL cells after twelve-hour radial stretching application (Bolcato-Bellemin et al., 2000), other study showed increase of MMP14 expression after mechanical force application for 1 and 4 days (Barthelemi et al., 2012). It is suggested that *MMP2, 14* expression required continuous mechanical stimulation. MMP2 participates in the expression of BSP and OC which are required at late stage of osteogenic differentiation (Barthelemi et al., 2012). MMP14 increased ALP expression in order to induced crystal deposition in osteoblast (Manduca et al., 2009). This suggests the role of MMP2 and 14 in promoting osteoblast differentiation during loaded by long-period mechanical force.

In conclusion, this study demonstrated that under the influence of cyclic tension force, human PDL cells secrete BMP9 and deposit mineral. BMP9 was shown to play an important role in the deposition of this mineral. BMP9 can be part of the mechanism of tensile force induced bone formation by human PDL cells. Moreover, BMP9 is involved in many biological processes including maturation of vessels (Luther et al., 2011). Furthermore, CTF also induces IL6 and further increases the expression of MMPs. We propose that under masticatory circumstances, PDL cells play a role in the maintenance of the homeostasis of the periodontal ligament tissue by mediating both hard and soft tissue remodeling.

Findings from this study may be used for future treatment. For example, using molecules like BMP9 or ATP in the treatment of bony defects or by coating implants to increase the osseointegration. Induction of an optimal mechanical force, thus induction of optimal levels of BMP9 or IL6 will result in proper healing of the periodontium in patients suffering from severe bone loss from periodontal disease. In case of patients who lost bone or require bone augmentation, an optimal mechanical force may help to obtain the bone back to normal.

Follow up studies can be the investigation of molecular mechanisms of CTF-increased noggin. This may clarify how CTF modulates the balance of BMPs and their inhibitors. Moreover, since there are other cell types in the periodontium, the paracrine effect of CTF-loaded cells should be investigated. For example, co-culture of CTF-loaded cells with other cell types such as osteoblasts or cementoblasts.

Other studies can focus on the question whether force can help healing of tissue by combining particular molecules like BMP9 or inflammatory cytokines *in vivo*. These studies will give an answer whether force can be applied to prevent or heal defects of tissues.

REFERENCES

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G. 2003. Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends in pharmacological sciences 24(2):52-55.

Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, Yagita H, Ishii N, Evans R, Honda K, Takeda K. 2008. ATP drives lamina propria T(H)17 cell differentiation. Nature 455(7214):808-812.

Ayala-Pena VB, Scolaro LA, Santillan GE. 2013. ATP and UTP stimulate bone morphogenetic protein-2,-4 and -5 gene expression and mineralization by rat primary osteoblasts involving PI3K/AKT pathway. Experimental cell research 319(13):2028-2036.

Bakker AD, Soejima K, Klein-Nulend J, Burger EH. 2001. The production of nitric oxide and prostaglandin E(2) by primary bone cells is shear stress dependent. Journal of biomechanics 34(5):671-677.

Balto K, Sasaki H, Stashenko P. 2001. Interleukin-6 deficiency increases inflammatory bone destruction. Infect Immun 69(2):744-750.

Barthelemi S, Robinet J, Garnotel R, Antonicelli F, Schittly E, Hornebeck W, Lorimier S. 2012. Mechanical forces-induced human osteoblasts differentiation involves MMP-2/MMP-13/MT1-MMP proteolytic cascade. J Cell Biochem 113(3):760-772.

Basson MD, Li GD, Hong F, Han O, Sumpio BE. 1996. Amplitude-dependent modulation of brush border enzymes and proliferation by cyclic strain in human intestinal Caco-2 monolayers. Journal of cellular physiology 168(2):476-488.

Benazzi S, Nguyen HN, Kullmer O, Kupczik K. 2016. Dynamic Modelling of Tooth Deformation Using Occlusal Kinematics and Finite Element Analysis. PloS one 11(3):e0152663.

Bergeron E, Senta H, Mailloux A, Park H, Lord E, Faucheux N. 2009. Murine preosteoblast differentiation induced by a peptide derived from bone

morphogenetic proteins-9. Tissue engineering Part A 15(11):3341-3349.

Bergfeld GR, Forrester T. 1992. Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovasc Res 26(1):40-47.

Bidart M, Ricard N, Levet S, Mallet C, David L, Subileau M, Tillet E, Feige J-J, Bailly S. 2012. BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain. Cell Mol Life Sci 69:313-324.

Birukov KG, Jacobson JR, Flores AA, Ye SQ, Birukova AA, Verin AD, Garcia JG. 2003. Magnitude-dependent regulation of pulmonary endothelial cell barrier function by cyclic stretch. American journal of physiology Lung cellular and molecular physiology 285(4):L785-797.

Blunk T, Sieminski AL, Appel B, Croft C, Courter DL, Chieh JJ, Goepferich A, Khurana JS, Gooch KJ. 2003. Bone morphogenetic protein 9: a potent modulator of cartilage development in vitro. Growth Factors 21(2):71-77.

Bodin P, Burnstock G. 2001. Purinergic signalling: ATP release. Neurochemical research 26(8-9):959-969.

Bolcato-Bellemin AL, Elkaim R, Abehsera A, Fausser JL, Haikel Y, Tenenbaum H. 2000. Expression of mRNAs encoding for alpha and beta integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts. Journal of dental research 79(9):1712-1716.

Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. 2011. Bone morphogenetic proteins: a critical review. Cellular signalling 23(4):609-620.

Brown MA, Zhao Q, Baker KA, Naik C, Chen C, Pukac L, Singh M, Tsareva T, Parice Y, Mahoney A, Roschke V, Sanyal I, Choe S. 2005. Crystal structure of BMP-9 and functional interactions with pro-region and receptors. The Journal of biological chemistry 280(26):25111-25118.

Butler GS, Overall CM. 2009. Updated biological roles for matrix metalloproteinases and new "intracellular" substrates revealed by degradomics. Biochemistry 48(46):10830-10845.

Capelli J, Jr., Kantarci A, Haffajee A, Teles RP, Fidel R, Jr., Figueredo CM. 2011. Matrix metalloproteinases and chemokines in the gingival crevicular fluid during orthodontic tooth movement. Eur J Orthod 33(6):705-711.

Carano A, Siciliani G. 1996. Effects of continuous and intermittent forces on human fibroblasts in vitro. Eur J Orthod 18(1):19-26.

Casson J, O'Kane S, Smith CA, Dalby MJ, Berry CC. 2018. Interleukin 6 Plays a Role in the Migration of Magnetically Levitated Mesenchymal Stem Cells Spheroids. Applied Sciences 8(412).

Cathcart JM, Banach A, Liu A, Chen J, Goligorsky M, Cao J. 2016. Interleukin-6 increases matrix metalloproteinase-14 (MMP-14) levels via down-regulation of p53 to drive cancer progression. Oncotarget 7(38):61107-61120.

Cattaneo PM, Dalstra M, Melsen B. 2005. The finite element method: a tool to study orthodontic tooth movement. Journal of dental research 84(5):428-433.

Cattaneo PM, Dalstra M, Melsen B. 2009. Strains in periodontal ligament and alveolar bone associated with orthodontic tooth movement analyzed by finite element. Orthodontics & craniofacial research 12(2):120-128.

Chachisvilis M, Zhang YL, Frangos JA. 2006. G protein-coupled receptors sense fluid shear stress in endothelial cells. Proceedings of the National Academy of Sciences of the United States of America 103(42):15463-15468.

Chen L, Zou X, Zhang R, Pi C, Wu N, Yin L, Deng Z. 2016. IGF1 potentiates BMP9-induced osteogenic differentiation in mesenchymal stem cells through the enhancement of BMP/Smad signaling. BMB reports 49(2):122-127

Chen YJ, Jeng JH, Chang HH, Huang MY, Tsai FF, Yao CC. 2013. Differential regulation of collagen, lysyl oxidase and MMP-2 in human periodontal ligament cells by low- and high-level mechanical stretching. J Periodontal Res 48(4):466-474.

Chen YJ, Shie MY, Hung CJ, Liu SL, Huang TH, Kao CT. 2015. Osteoblasts subjected to tensile force induce osteoclastic differentiation of murine macrophages in a coculture system. Journal of Dental Sciences 10(1):81-87.

Chen YJ, Shie MY, Hung CJ, Wu BC, Liu SL, Huang TH, Kao CT. 2014. Activation of focal adhesion kinase induces extracellular signal-regulated kinase-mediated osteogenesis in tensile force-subjected periodontal ligament fibroblasts but not in osteoblasts. Journal of bone and mineral metabolism 32(6):671-682.

Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC. 2003. Osteogenic activity of the

fourteen types of human bone morphogenetic proteins (BMPs). The Journal of bone and joint surgery American volume 85-A(8):1544-1552.

Choi JK, Hwang HI, Jang YJ. 2015. The efficiency of the in vitro osteo/dentinogenic differentiation of human dental pulp cells, periodontal ligament cells and gingival fibroblasts. International journal of molecular medicine 35(1):161-168.

Ciciarello M, Zini R, Rossi L, Salvestrini V, Ferrari D, Manfredini R, Lemoli RM. 2013. Extracellular purines promote the differentiation of human bone marrow-derived mesenchymal stem cells to the osteogenic and adipogenic lineages. Stem Cells Dev 22(7):1097-1111.

Coppi E, Pugliese AM, Urbani S, Melani A, Cerbai E, Mazzanti B, Bosi A, Saccardi R, Pedata F. 2007. ATP modulates cell proliferation and elicits two different electrophysiological responses in human mesenchymal stem cells. Stem cells 25(7):1840-1849.

Costessi A, Pines A, D'Andrea P, Romanello M, Damante G, Cesaratto L, Quadrifoglio F, Moro L, Tell G. 2005. Extracellular nucleotides activate Runx2 in the osteoblast-like HOBIT cell line: a possible molecular link between mechanical stress and osteoblasts' response. Bone 36(3):418-432.

Csiszar A, Lehoux S, Ungvari Z. 2009. Hemodynamic forces, vascular oxidative stress, and regulation of BMP-2/4 expression. Antioxidants & redox signaling 11(7):1683-1697.

Cunha SI, Pardali E, Thorikay M, Anderberg C, Hawinkels L, Goumans MJ, Seehra J, Heldin CH, ten Dijke P, Pietras K. 2010. Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. J Exp Med 207(1):85-100.

Cymbaluk-Ploska A, Chudecka-Glaz A, Pius-Sadowska E, Machalinski B, Menkiszak J, Sompolska-Rzechula A. 2018. Suitability assessment of baseline concentration of MMP3, TIMP3, HE4 and CA125 in the serum of patients with ovarian cancer. J Ovarian Res 11(1):1.

David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S. 2007. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109(5):1953-1961.

Davidenko N, Schuster CF, Bax DV, Farndale RW, Hamaia S, Best SM, Cameron RE. 2016. Evaluation of cell binding to collagen and gelatin: a study of the effect of 2D and 3D architecture and surface chemistry. J Mater Sci Mater Med 27(10):148.

del Puerto A, Diaz-Hernandez JI, Tapia M, Gomez-Villafuertes R, Benitez MJ, Zhang J, Miras-Portugal MT, Wandosell F, Diaz-Hernandez M, Garrido JJ. 2012. Adenylate cyclase 5 coordinates the action of ADP, P2Y1, P2Y13 and ATP-gated P2X7 receptors on axonal elongation. J Cell Sci 125(Pt 1):176-188.

Derivaux CC, Pintucci G, Yu P, Silver E, Seghezzi G, Grossi EA, Steinberg BM, Baumann FG, Colvin SB, Mignatti P, Galloway AC. 2000. Fibroblast growth factor-2 induces stromelysin-1 (MMP3) expression in endothelial cells by activating mitogen-activated protein kinases. Journal of American College of Surgeon 191(4):s37.

Eguchi T, Kubota S, Kawata K, Mukudai Y, Uehara J, Ohgawara T, Ibaragi S, Sasaki A, Kuboki T, Takigawa M. 2008. Novel transcription-factor-like function of human matrix metalloproteinase 3 regulating the CTGF/CCN2 gene. Mol Cell Biol 28(7):2391-2413.

Franco C, Patricia HR, Timo S, Claudia B, Marcela H. 2017. Matrix Metalloproteinases as Regulators of Periodontal Inflammation. Int J Mol Sci 18(2).

Fuchigami S, Nakamura T, Furue K, Sena K, Shinohara Y, Noguchi K. 2016. Recombinant human bone morphogenetic protein-9 potently induces osteogenic differentiation of human periodontal ligament fibroblasts. European journal of oral sciences 124(2):151-157.

Fujihara C, Yamada S, Ozaki N, Takeshita N, Kawaki H, Takano-Yamamoto T, Murakami S. 2010. Role of mechanical stress-induced glutamate signaling-associated molecules in cytodifferentiation of periodontal ligament cells. The Journal of biological chemistry 285(36):28286-28297.

Fujioka-Kobayashi M, Sawada K, Kobayashi E, Schaller B, Zhang Y, Miron RJ. 2016a. Osteogenic potential of rhBMP9 combined with a bovine-derived natural bone mineral scaffold compared to rhBMP2. Clinical oral implants research 00:1-7.

Fujioka-Kobayashi M, Sawada K, Kobayashi E, Schaller B, Zhang Y, Miron RJ. 2016b. Recombinant Human Bone Morphogenetic Protein 9 (rhBMP9) Induced Osteoblastic Behavior on a Collagen Membrane Compared With rhBMP2. J

Periodontol 87(6):e101-107.

Fukushima A, Kajiya H, Izumi T, Shigeyama C, Okabe K, Anan H. 2010. Pro-inflammatory cytokines induce suppressor of cytokine signaling-3 in human periodontal ligament cells. J Endod 36(6):1004-1008.

Funakoshi M, Yamaguchi M, M. A, S. F, K. K. 2013. Effect of Compression Force on Apoptosis in Human Periodontal Ligament Cells. Journal of Hard Tissue Biology 22(1):41-50.

Garcia AJ, Tom C, Guemes M, Polanco G, Mayorga ME, Wend K, Miranda-Carboni GA, Krum SA. 2013. ERalpha signaling regulates MMP3 expression to induce FasL cleavage and osteoclast apoptosis. J Bone Miner Res 28(2):283-290.

Geppetti P, Veldhuis NA, Lieu T, Bunnett NW. 2015. G Protein-Coupled Receptors: Dynamic Machines for Signaling Pain and Itch. Neuron 88(4):635-649.

Ghazi A, Berrier C, Ajouz B, Besnard M. 1998. Mechanosensitive ion channels and their mode of activation. Biochimie 80(5-6):357-362.

Granados-Riveron JT, Brook JD. 2012. The impact of mechanical forces in heart morphogenesis. Circ Cardiovasc Genet 5(1):132-142.

Grzibovskis M, Urtane I, Pilmane M. 2011. Specific signaling molecule expression in periodontal ligaments in different age groups: pilot study. Stomatologija 13(4):117-122.

He CS, Wilhelm SM, Pentland AP, Marmer BL, Grant GA, Eisen AZ, Goldberg GI. 1989. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc Natl Acad Sci U S A 86(8):2632-2636.

Hernandez-Caldera A, Vernal R, Paredes R, Veloso-Matta P, Astorga J, Hernandez M. 2018. Human periodontal ligament fibroblasts synthesize C-reactive protein and Th-related cytokines in response to interleukin (IL)-6 trans-signalling. Int Endod J 51(6):632-640.

Hirashima S, Ohta K, Kanazawa T, Okayama S, Togo A, Uchimura N, Kusukawa J, Nakamura KI. 2016. Three-dimensional ultrastructural analysis of cells in the periodontal ligament using focused ion beam/scanning electron microscope tomography. Sci Rep 6:39435.

Holopainen JM, Moilanen JA, Sorsa T, Kivela-Rajamaki M, Tervahartiala T, Vesaluoma

MH, Tervo TM. 2003. Activation of matrix metalloproteinase-8 by membrane type 1-MMP and their expression in human tears after photorefractive keratectomy. Invest Ophthalmol Vis Sci 44(6):2550-2556.

Hosokawa Y, Shindo S, Hosokawa I, Ozaki K, Matsuo T. 2014. IL-6 trans-signaling enhances CCL20 production from IL-1beta-stimulated human periodontal ligament cells. Inflammation 37(2):381-386.

Houston D, Costanzi S, Jacobson KA, Harden TK. 2008. Development of selective high affinity antagonists, agonists, and radioligands for the P2Y1 receptor. Comb Chem High Throughput Screen 11(6):410-419.

Hrabec E, Naduk J, Strek M, Hrabec Z. 2007. [Type IV collagenases (MMP-2 and MMP-9) and their substrates--intracellular proteins, hormones, cytokines, chemokines and their receptors]. Postepy Biochem 53(1):37-45.

Huang E, Zhu G, Jiang W, Yang K, Gao Y, Luo Q, Gao JL, Kim SH, Liu X, Li M, Shi Q, Hu N, Wang L, Liu H, Cui J, Zhang W, Li R, Chen X, Kong YH, Zhang J, Wang J, Shen J, Bi Y, Statz J, He BC, Luo J, Wang H, Xiong F, Luu HH, Haydon RC, Yang L, He TC. 2012. Growth hormone synergizes with BMP9 in osteogenic differentiation by activating the JAK/STAT/IGF1 pathway in murine multilineage cells. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 27(7):1566-1575.

Huang H, Kamm RD, Lee RT. 2004. Cell mechanics and mechanotransduction: pathways, probes, and physiology. American journal of physiology Cell physiology 287(1):C1-11.

Huang YH, Yang HY, Huang SW, Ou G, Hsu YF, Hsu MJ. 2016. Interleukin-6 Induces Vascular Endothelial Growth Factor-C Expression via Src-FAK-STAT3 Signaling in Lymphatic Endothelial Cells. PLoS One 11(7):e0158839.

Huiskes R, Ruimerman R, van Lenthe GH, Janssen JD. 2000. Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. Nature 405(6787):704-706.

Idzko M, Ferrari D, Eltzschig HK. 2014. Nucleotide signalling during inflammation. Nature 509(7500):310-317.

Ingber DE. 2006. Cellular mechanotransduction: putting all the pieces together again.

FASEB journal : official publication of the Federation of American Societies for Experimental Biology 20(7):811-827.

Issaranggun Na Ayuthaya B, Everts V, Pavasant P. 2017. Interleukin-12 Induces Receptor Activator of Nuclear Factor-Kappa B Ligand Expression by Human Periodontal Ligament Cells. J Periodontol 88(7):e109-e119.

Ito M, Arakawa T, Okayama M, Shitara A, Mizoguchi I, Takuma T. 2014. Gravity loading induces adenosine triphosphate release and phosphorylation of extracellular signal-regulated kinases in human periodontal ligament cells. Journal of investigative and clinical dentistry 5(4):266-274.

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(10):937-942.

Jaalouk DE, Lammerding J. 2009. Mechanotransduction gone awry. Nature reviews Molecular cell biology 10(1):63-73.

Jablonska-Trypuc A, Matejczyk M, Rosochacki S. 2016. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. J Enzyme Inhib Med Chem 31(sup1):177-183.

Jacobs C, Grimm S, Ziebart T, Walter C, Wehrbein H. 2013. Osteogenic differentiation of periodontal fibroblasts is dependent on the strength of mechanical strain. Arch Oral Biol 58(7):896-904.

Jacobs C, Walter C, Ziebart T, Grimm S, Meila D, Krieger E, Wehrbein H. 2014. Induction of IL-6 and MMP-8 in human periodontal fibroblasts by static tensile strain. Clin Oral Investig 18(3):901-908.

Jacobson KA, Gao ZG. 2006. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 5(3):247-264.

Jansen JH, Eijken M, Jahr H, Chiba H, Verhaar JA, van Leeuwen JP, Weinans H. 2010. Stretch-induced inhibition of Wnt/beta-catenin signaling in mineralizing osteoblasts. J Orthop Res 28(3):390-396.

Jiang LH, Mousawi F, Yang X, Roger S. 2017. ATP-induced Ca(2+)-signalling mechanisms in the regulation of mesenchymal stem cell migration. Cell Mol Life Sci 74(20):3697-3710.

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

Jonsson D, Nebel D, Bratthall G, Nilsson BO. 2008. LPS-induced MCP-1 and IL-6 production is not reversed by oestrogen in human periodontal ligament cells. Arch Oral Biol 53(9):896-902.

Kaku M, Yamauchi M. 2014. Mechano-regulation of collagen biosynthesis in periodontal ligament. J Prosthodont Res 58(4):193-207.

Kalajzic Z, Peluso EB, Utreja A, Dyment N, Nihara J, Xu M, Chen J, Uribe F, Wadhwa S. 2014. Effect of cyclical forces on the periodontal ligament and alveolar bone remodeling during orthodontic tooth movement. Angle Orthod 84(2):297-303.

Kaneko S, Ohashi K, Soma K, Yanagishita M. 2001. Occlusal hypofunction causes changes of proteoglycan content in the rat periodontal ligament. Journal of periodontal research 36(1):9-17.

Kang Q, Song WX, Luo Q, Tang N, Luo J, Luo X, Chen J, Bi Y, He BC, Park JK, Jiang W, Tang Y, Huang J, Su Y, Zhu GH, He Y, Yin H, Hu Z, Wang Y, Chen L, Zuo GW, Pan X, Shen J, Vokes T, Reid RR, Haydon RC, Luu HH, He TC. 2009. A comprehensive analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. Stem cells and development 18(4):545-559.

Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC. 2004. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene therapy 11(17):1312-1320.

Kanjanamekanant K, Luckprom P, Pavasant P. 2013. Mechanical stress-induced interleukin-1beta expression through adenosine triphosphate/P2X7 receptor activation in human periodontal ligament cells. Journal of periodontal research 48(2):169-176.

Kanzaki H, Chiba M, Sato A, Miyagawa A, Arai K, Nukatsuka S, Mitani H. 2006. Cyclical tensile force on periodontal ligament cells inhibits osteoclastogenesis through

OPG induction. Journal of dental research 85(5):457-462.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. 2002. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 17(2):210-220.

Kariya T, Tanabe N, Shionome C, Manaka S, Kawato T, Zhao N, Maeno M, Suzuki N, Shimizu N. 2015. Tension force-induced ATP promotes osteogenesis through P2X7 receptor in osteoblasts. Journal of cellular biochemistry 116(1):12-21.

Kawarizadeh A, Bourauel C, Gotz W, Jager A. 2005. Early responses of periodontal ligament cells to mechanical stimulus in vivo. J Dent Res 84(10):902-906.

Kienast Y, Jucknischke U, Scheiblich S, Thier M, de Wouters M, Haas A, Lehmann C, Brand V, Bernicke D, Honold K, Lorenz S. 2016. Rapid Activation of Bone Morphogenic Protein 9 by Receptor-mediated Displacement of Pro-domains. J Biol Chem 291(7):3395-3410.

Kim Y, Kim H, Cho H, Bae Y, Suh K, Jung J. 2007. Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. Cell Physiol Biochem 20(6):867-876.

King GN, Hughes FJ. 1999. Effects of occlusal loading on ankylosis, bone, and cementum formation during bone morphogenetic protein-2-stimulated periodontal regeneration in vivo. J Periodontol 70(10):1125-1135.

Knauper V, Lopez-Otin C, Smith B, Knight G, Murphy G. 1996. Biochemical characterization of human collagenase-3. J Biol Chem 271(3):1544-1550.

Kook SH, Hwang JM, Park JS, Kim EM, Heo JS, Jeon YM, Lee JC. 2009. Mechanical force induces type I collagen expression in human periodontal ligament fibroblasts through activation of ERK/JNK and AP-1. J Cell Biochem 106(6):1060-1067.

Kook SH, Jang YS, Lee JC. 2011. Involvement of JNK-AP-1 and ERK-NF-kappaB signaling in tension-stimulated expression of type I collagen and MMP-1 in human periodontal ligament fibroblasts. J Appl Physiol (1985) 111(6):1575-1583.

Koshimizu TA, Van Goor F, Tomic M, Wong AO, Tanoue A, Tsujimoto G, Stojilkovic SS. 2000. Characterization of calcium signaling by purinergic receptor-channels

expressed in excitable cells. Molecular pharmacology 58(5):936-945.

Kossakowska AE, Edwards DR, Prusinkiewicz C, Zhang MC, Guo D, Urbanski SJ, Grogan T, Marquez LA, Janowska-Wieczorek A. 1999. Interleukin-6 regulation of matrix metalloproteinase (MMP-2 and MMP-9) and tissue inhibitor of metalloproteinase (TIMP-1) expression in malignant non-Hodgkin's lymphomas. Blood 94(6):2080-2089.

Kothari P, Pestana R, Mesraoua R, Elchaki R, Khan KM, Dannenberg AJ, Falcone DJ. 2014. IL-6-mediated induction of matrix metalloproteinase-9 is modulated by JAK-dependent IL-10 expression in macrophages. J Immunol 192(1):349-357.

Ku SJ, Chang YI, Chae CH, Kim SG, Park YW, Jung YK, Choi JY. 2009. Static tensional forces increase osteogenic gene expression in three-dimensional periodontal ligament cell culture. BMB reports 42(7):427-432.

Kuhn KA, Manieri NA, Liu TC, Stappenbeck TS. 2014. IL-6 stimulates intestinal epithelial proliferation and repair after injury. PLoS One 9(12):e114195.

Kusano K, Miyaura C, Inada M, Tamura T, Ito A, Nagase H, Kamoi K, Suda T. 1998. Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 139(3):1338-1345.

Langille BL, O'Donnell F. 1986. Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. Science 231(4736):405-407.

Lazarowski ER, Boucher RC. 2001. UTP as an extracellular signaling molecule. News Physiol Sci 16:1-5.

Lazarowski ER, Boucher RC, Harden TK. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. Molecular pharmacology 64(4):785-795.

Lazarowski ER, Sesma JI, Seminario-Vidal L, Kreda SM. 2011. Molecular mechanisms of purine and pyrimidine nucleotide release. Adv Pharmacol 61:221-261.

Lekic P, McCulloch CA. 1996. Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. Anat Rec 245(2):327-341.

Letra A, Silva RM, Rylands RJ, Silveira EM, de Souza AP, Wendell SK, Garlet GP, Vieira AR.

2012. MMP3 and TIMP1 variants contribute to chronic periodontitis and may be implicated in disease progression. J Clin Periodontol 39(8):707-716.

Li C, Zhen G, Chai Y, Xie L, Crane JL, Farber E, Farber CR, Luo X, Gao P, Cao X, Wan M. 2016a. RhoA determines lineage fate of mesenchymal stem cells by modulating CTGF-VEGF complex in extracellular matrix. Nat Commun 7:11455.

Li L, Han M, Li S, Wang L, Xu Y. 2013a. Cyclic tensile stress during physiological occlusal force enhances osteogenic differentiation of human periodontal ligament cells via ERK1/2-Elk1 MAPK pathway. DNA and cell biology 32(9):488-497.

Li M, Silberberg SD, Swartz KJ. 2013b. Subtype-specific control of P2X receptor channel signaling by ATP and Mg2+. Proceedings of the National Academy of Sciences of the United States of America 110(36):E3455-3463.

Li R, Yan Z, Ye J, Huang H, Wang Z, Wei Q, Wang J, Zhao L, Lu S, Wang X, Tang S, Fan J, Zhang F, Zou Y, Song D, Liao J, Lu M, Liu F, Shi LL, Athiviraham A, Lee MJ, He TC, Zhang Z. 2016b. The Prodomain-Containing BMP9 Produced from a Stable Line Effectively Regulates the Differentiation of Mesenchymal Stem Cells. International journal of medical sciences 13(1):8-18.

Li XL, Liu YB, Ma EG, Shen WX, Li H, Zhang YN. 2015. Synergistic effect of BMP9 and TGF-beta in the proliferation and differentiation of osteoblasts. Genetics and molecular research : GMR 14(3):7605-7615.

Lindhe J, Karring T. 1989. The anatomy of the periodontium. In: Lindhe. J, editor. Textbook of Clinical Periodontology. 2nd ed. ed. Copenhagen: Munksgaard. p 19-69

Lisboa RA, Andrade MV, Cunha-Melo JR. 2013. Toll-like receptor activation and mechanical force stimulation promote the secretion of matrix metalloproteinases 1, 3 and 10 of human periodontal fibroblasts via p38, JNK and NF-kB. Arch Oral Biol 58(6):731-739.

Liu HT, Sabirov RZ, Okada Y. 2008. Oxygen-glucose deprivation induces ATP release via maxi-anion channels in astrocytes. Purinergic signalling 4(2):147-154.

Liu M, Dai J, Lin Y, Yang L, Dong H, Li Y, Ding Y, Duan Y. 2012. Effect of the cyclic stretch on the expression of osteogenesis genes in human periodontal ligament cells. Gene 491(2):187-193.

Liu X, Qin J, Luo Q, Bi Y, Zhu G, Jiang W, Kim SH, Lia M, Su Y, Nan G, Cui J, Zhang W, Li R, Chen X, Kong Y, Zhang J, Wang J, Rogers MR, Zhang H, Shui W, Zhao C, Wang N, Liang X, Wu N, He Y, Luu HH, Haydon RC, Shi LL, Li T, He T, Li M. 2013. Cross-talk between EGF and BMP9 signalling pathways regulates the osteogenic differentiation of mesenchymal stem cells. Journal of cellular and molecular medicine 17(9):1160-1172.

Lohman AW, Isakson BE. 2014. Differentiating connexin hemichannels and pannexin channels in cellular ATP release. FEBS Lett 588(8):1379-1388.

Lohmander LS, Ionescu M, Jugessur H, Poole AR. 1999. Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. Arthritis Rheum 42(3):534-544.

Long P, Liu F, Piesco NP, Kapur R, Agarwal S. 2002. Signaling by mechanical strain involves transcriptional regulation of proinflammatory genes in human periodontal ligament cells in vitro. Bone 30(4):547-552.

Lu W, Reigada D, Sevigny J, Mitchell CH. 2007. Stimulation of the P2Y1 receptor up-regulates nucleoside-triphosphate diphosphohydrolase-1 in human retinal pigment epithelial cells. The Journal of pharmacology and experimental therapeutics 323(1):157-164.

Luckprom P, Kanjanamekanant K, Pavasant P. 2011. Role of connexin43 hemichannels in mechanical stress-induced ATP release in human periodontal ligament cells. Journal of periodontal research 46(5):607-615.

Luckprom P, Wongkhantee S, Yongchaitrakul T, Pavasant P. 2010. Adenosine triphosphate stimulates RANKL expression through P2Y1 receptor-cyclo-oxygenase-dependent pathway in human periodontal ligament cells. Journal of periodontal research 45(3):404-411.

Luo J, Tang M, Huang J, He BC, Gao JL, Chen L, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Bi Y, Luo X, Jiang W, Su Y, Shen J, Kim SH, Huang E, Gao Y, Zhou JZ, Yang K, Luu HH, Pan X, Haydon RC, Deng ZL, He TC. 2010. TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. The Journal of biological chemistry 285(38):29588-29598.

Luther G, Wagner ER, Zhu G, Kang Q, Luo Q, Lamplot J, Bi Y, Luo X, Luo J, Teven C, Shi

Q, Kim SH, Gao JL, Huang E, Yang K, Rames R, Liu X, Li M, Hu N, Liu H, Su Y, Chen L, He BC, Zuo GW, Deng ZL, Reid RR, Luu HH, Haydon RC, He TC. 2011. BMP-9 induced osteogenic differentiation of mesenchymal stem cells: molecular mechanism and therapeutic potential. Curr Gene Ther 11(3):229-240.

Ma J, Zhao D, Wu Y, Xu C, Zhang F. 2015. Cyclic stretch induced gene expression of extracellular matrix and adhesion molecules in human periodontal ligament cells. Archives of oral biology 60(3):447-455.

Maeda A, Soejima K, Bandow K, Kuroe K, Kakimoto K, Miyawaki S, Okamoto A, Matsuguchi T. 2007. Force-induced IL-8 from periodontal ligament cells requires IL-1beta. Journal of dental research 86(7):629-634.

Makino A, Prossnitz ER, Bunemann M, Wang JM, Yao W, Schmid-Schonbein GW. 2006. G protein-coupled receptors serve as mechanosensors for fluid shear stress in neutrophils. American journal of physiology Cell physiology 290(6):C1633-1639.

Manduca P, Castagnino A, Lombardini D, Marchisio S, Soldano S, Ulivi V, Zanotti S, Garbi C, Ferrari N, Palmieri D. 2009. Role of MT1-MMP in the osteogenic differentiation. Bone 44(2):251-265.

März P, Cheng J, Gadient R, Patterson P, Stoyan T, Otten U, S. R-J. 1998 Sympathetic neurons can produce and respond to interleukin 6. Proc Natl Acad Sci U S A 95(6):3251-3256. .

Matsuda N, Yokoyama K, Takeshita S, Watanabe M. 1998. Role of epidermal growth factor and its receptor in mechanical stress-induced differentiation of human periodontal ligament cells in vitro. Archives of oral biology 43(12):987-997.

McCormack SW, Witzel U, Watson PJ, Fagan MJ, Groning F. 2014. The biomechanical function of periodontal ligament fibres in orthodontic tooth movement. PloS one 9(7):e102387.

McCulloch CA, Lekic P, McKee MD. 2000. Role of physical forces in regulating the form and function of the periodontal ligament. Periodontol 2000 24:56-72.

Melsen B. 2001. Tissue reaction to orthodontic tooth movement--a new paradigm. European journal of orthodontics 23(6):671-681.

Mine K, Kanno Z, Muramoto T, Soma K. 2005. Occlusal forces promote periodontal healing of transplanted teeth and prevent dentoalveolar ankylosis: an

experimental study in rats. The Angle orthodontist 75(4):637-644.

Mitsui N, Suzuki N, Maeno M, Yanagisawa M, Koyama Y, Otsuka K, Shimizu N. 2006. Optimal compressive force induces bone formation via increasing bone morphogenetic proteins production and decreasing their antagonists production by Saos-2 cells. Life sciences 78(23):2697-2706.

Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. The Journal of cell biology 131(3):791-805.

Mobasheri A, Barrett-Jolley R, Carter SD, Martin-Vasallo P, Schulze-Tanzil G, Shakibaei M. 2005. Functional Roles of Mechanosensitive Ion Channels, ss1 Integrins and Kinase Cascades in Chondrocyte Mechanotransduction. In: Kamkin A, Kiseleva I, editors. Mechanosensitivity in Cells and Tissues. Moscow.

Muthukuru M. 2013. Bone morphogenic protein-2 induces apoptosis and cytotoxicity in periodontal ligament cells. Journal of periodontology 84(6):829-838.

Nagase H, Woessner JF, Jr. 1999. Matrix metalloproteinases. J Biol Chem 274(31):21491-21494.

Nakagawa M, Kukita T, Nakasima A, Kurisu K. 1994. Expression of the type I collagen gene in rat periodontal ligament during tooth movement as revealed by in situ hybridization. Arch Oral Biol 39(4):289-294.

Nishimura R, Moriyama K, Yasukawa K, Mundy GR, Yoneda T. 1998. Combination of interleukin-6 and soluble interleukin-6 receptors induces differentiation and activation of JAK-STAT and MAP kinase pathways in MG-63 human osteoblastic cells. J Bone Miner Res 13(5):777-785.

Novak I. 2003. ATP as a signaling molecule: the exocrine focus. News Physiol Sci 18:12-17.

Oberhauser AF, Badilla-Fernandez C, Carrion-Vazquez M, Fernandez JM. 2002. The mechanical hierarchies of fibronectin observed with single-molecule AFM. Journal of molecular biology 319(2):433-447.

Ogata Y, Enghild JJ, Nagase H. 1992. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. J Biol Chem 267(6):3581-3584.

Okada SF, Ribeiro CM, Sesma JI, Seminario-Vidal L, Abdullah LH, van Heusden C, Lazarowski ER, Boucher RC. 2013. Inflammation promotes airway epithelial ATP release via calcium-dependent vesicular pathways. Am J Respir Cell Mol Biol 49(5):814-820.

Page-McCaw A, Ewald AJ, Werb Z. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8(3):221-233.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA, Harden TK. 1998. Agonist action of adenosine triphosphates at the human P2Y1 receptor. Mol Pharmacol 54(6):1118-1123.

Peng Y, Kang Q, Cheng H, Li X, Sun MH, Jiang W, Luu HH, Park JY, Haydon RC, He TC. 2003. Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. Journal of cellular biochemistry 90(6):1149-1165.

Peng Y, Kang Q, Luo Q, Jiang W, Si W, Liu BA, Luu HH, Park JK, Li X, Luo J, Montag AG, Haydon RC, He TC. 2004. Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. The Journal of biological chemistry 279(31):32941-32949.

Pini M, Wiskott HW, Scherrer SS, Botsis J, Belser UC. 2002. Mechanical characterization of bovine periodontal ligament. J Periodontal Res 37(4):237-244.

Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z, Bissell MJ. 2005. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature 436(7047):123-127.

Rajshankar D, McCulloch CA, Tenenbaum HC, Lekic PC. 1998. Osteogenic inhibition by rat periodontal ligament cells: modulation of bone morphogenic protein-7 activity in vivo. Cell Tissue Res 294(3):475-483.

Rauch F, Lauzier D, Croteau S, Travers R, Glorieux FH, Hamdy R. 2000. Temporal and spatial expression of bone morphogenetic protein-2, -4, and -7 during distraction osteogenesis in rabbits. Bone 27(3):453-459.

Reddy NR, Roopa D, Babu DS, Kumar PM, Raju CM, Kumar NS. 2012. Estimation of matrix metalloproteinase-3 levels in gingival crevicular fluid in periodontal disease,

health and after scaling and root planing. J Indian Soc Periodontol 16(4):549-552.

Redlich M, Reichenberg E, Harari D, Zaks B, Shoshan S, Palmon A. 2001. The effect of mechanical force on mRNA levels of collagenase, collagen type I, and tissue inhibitors of metalloproteinases in gingivae of dogs. J Dent Res 80(12):2080-2084.

Rochier A, Nixon A, Yamashita N, Abe R, Abe R, Madri JA, Sumpio BE. 2011. Laminar shear, but not orbital shear, has a synergistic effect with thrombin stimulation on tissue factor expression in human umbilical vein endothelial cells. Journal of vascular surgery 54(2):480-488.

Romanello M, Pani B, Bicego M, D'Andrea P. 2001. Mechanically induced ATP release from human osteoblastic cells. Biochemical and biophysical research communications 289(5):1275-1281.

Rosenthal CK. 2017. EGFR probes matrix stiffness. Nat Cell Biol 19(6):600.

Rosselli-Murai LK, Almeida LO, Zagni C, Galindo-Moreno P, Padial-Molina M, Volk SL, Murai MJ, Rios HF, Squarize CH, Castilho RM. 2013. Periostin responds to mechanical stress and tension by activating the MTOR signaling pathway. PLoS One 8(12):e83580.

Rui YF, Lui PP, Ni M, Chan LS, Lee YW, Chan KM. 2011. Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 29(3):390-396.

Sabirov RZ, Okada Y. 2005. ATP release via anion channels. Purinergic signalling 1(4):311-328.

Saminathan A, Vinoth KJ, Wescott DC, Pinkerton MN, Milne TJ, Cao T, Meikle MC. 2012. The effect of cyclic mechanical strain on the expression of adhesion-related genes by periodontal ligament cells in two-dimensional culture. J Periodontal Res 47(2):212-221.

Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N. 1999. Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. Journal of bone and mineral research : the official journal of the American

Society for Bone and Mineral Research 14(7):1084-1095.

Satrawaha S, Wongkhantee S, Pavasant P, Sumrejkanchanakij P. 2011. Pressure induces interleukin-6 expression via the P2Y6 receptor in human dental pulp cells. Archives of oral biology 56(11):1230-1237.

Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 1813(5):878-888.

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

Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364(9429):149-155.

Shabbir M, Burnstock G. 2009. Purinergic receptor-mediated effects of adenosine 5'-triphosphate in urological malignant diseases. Int J Urol 16(2):143-150.

Sharff KA, Song WX, Luo X, Tang N, Luo J, Chen J, Bi Y, He BC, Huang J, Li X, Jiang W, Zhu GH, Su Y, He Y, Shen J, Wang Y, Chen L, Zuo GW, Liu B, Pan X, Reid RR, Luu HH, Haydon RC, He TC. 2009. Hey1 basic helix-loop-helix protein plays an important role in mediating BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. The Journal of biological chemistry 284(1):649-659.

Shay-Salit A, Shushy M, Wolfovitz E, Yahav H, Breviario F, Dejana E, Resnick N. 2002. VEGF receptor 2 and the adherens junction as a mechanical transducer in vascular endothelial cells. Proceedings of the National Academy of Sciences of the United States of America 99(14):9462-9467.

Shen T, Qiu L, Chang H, Yang Y, Jian C, Xiong J, Zhou J, Dong S. 2014. Cyclic tension promotes osteogenic differentiation in human periodontal ligament stem cells. International journal of clinical and experimental pathology 7(11):7872-7880.

Shirakura K, Ishiba R, Kashio T, Sakai M, Fukushima Y, Yamamoto N, Manabe S, Shigesada N, Tanaka T, Hino N, Aird WC, Doi T, Okada Y. 2018. Endothelial Robo4 regulates IL-6 production by endothelial cells and monocytes via a crosstalk mechanism in inflammation. Biochem Biophys Res Commun

495(1):801-806.

Si-Tayeb K, Monvoisin A, Mazzocco C, Lepreux S, Decossas M, Cubel G, Taras D, Blanc JF, Robinson DR, Rosenbaum J. 2006. Matrix metalloproteinase 3 is present in the cell nucleus and is involved in apoptosis. Am J Pathol 169(4):1390-1401.

Silverio KG, Rodrigues TL, Coletta RD, Benevides L, Da Silva JS, Casati MZ, Sallum EA, Nociti FH, Jr. 2010. Mesenchymal stem cell properties of periodontal ligament cells from deciduous and permanent teeth. Journal of periodontology 81(8):1207-1215.

Sindhavajiva PR, Sastravaha P, Arksornnukit M, Pavasant P. 2017. Purinergic 2X7 receptor activation regulates WNT signaling in human mandibular-derived osteoblasts. Archives of oral biology 81:167-174.

Sodin-Semrl S, Taddeo B, Tseng D, Varga J, Fiore S. 2000. Lipoxin A4 inhibits IL-1 beta-induced IL-6, IL-8, and matrix metalloproteinase-3 production in human synovial fibroblasts and enhances synthesis of tissue inhibitors of metalloproteinases. J Immunol 164(5):2660-2666.

Song JJ, Celeste AJ, Kong FM, Jirtle RL, Rosen V, Thies RS. 1995. Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation. Endocrinology 136(10):4293-4297.

Steigman S, Michaeli Y, Yitzhaki M, Weinreb M. 1989. A three-dimensional evaluation of the effects of functional occlusal forces on the morphology of dental and periodontal tissues of the rat incisor. Journal of dental research 68(8):1269-1274.

Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z. 1999. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell 98(2):137-146.

Sternlicht MD, Werb Z. 2001. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17,:463-516.

Sun D, Junger WG, Yuan C, Zhang W, Bao Y, Qin D, Wang C, Tan L, Qi B, Zhu D, Zhang X, Yu T. 2013. Shockwaves induce osteogenic differentiation of human mesenchymal stem cells through ATP release and activation of P2X7 receptors. Stem Cells 31(6):1170-1180.

Sundararaj KP, Samuvel DJ, Li Y, Sanders JJ, Lopes-Virella MF, Huang Y. 2009.

Interleukin-6 released from fibroblasts is essential for up-regulation of matrix metalloproteinase-1 expression by U937 macrophages in coculture: cross-talking between fibroblasts and U937 macrophages exposed to high glucose. J Biol Chem 284(20):13714-13724.

Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H. 1990. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). Biochemistry 29(44):10261-10270.

Suzuki R, Nemoto E, Shimauchi H. 2014. Cyclic tensile force up-regulates BMP-2 expression through MAP kinase and COX-2/PGE2 signaling pathways in human periodontal ligament cells. Experimental cell research 323(1):232-241.

Suzuki Y, Ohga N, Morishita Y, Hida K, Miyazono K, Watabe T. 2010. BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. Journal of cell science 123(Pt 10):1684-1692.

Syed NH, Kennedy C. 2012. Pharmacology of P2X receptors. WIREs Membr Transp Signal 1:16-30.

Takahara N, Ito S, Furuya K, Naruse K, Aso H, Kondo M, Sokabe M, Hasegawa Y. 2014. Real-time imaging of ATP release induced by mechanical stretch in human airway smooth muscle cells. Am J Respir Cell Mol Biol 51(6):772-782.

Takahashi I, Nishimura M, Onodera K, Bae JW, Mitani H, Okazaki M, Sasano Y, Mitani H. 2003. Expression of MMP-8 and MMP-13 genes in the periodontal ligament during tooth movement in rats. J Dent Res 82(8):646-651.

Tang CH, Chen CF, Chen WM, Fong YC. 2011. IL-6 increases MMP-13 expression and motility in human chondrosarcoma cells. J Biol Chem 286(13):11056-11066.

Tang L, Lin Z, Li YM. 2006. Effects of different magnitudes of mechanical strain on Osteoblasts in vitro. Biochemical and biophysical research communications 344(1):122-128.

Tang N, Zhao Z, Zhang L, Yu Q, Li J, Xu Z, Li X. 2012. Up-regulated osteogenic transcription factors during early response of human periodontal ligament stem cells to cyclic tensile strain. Arch Med Sci 8(3):422-430.

Teixeira CC, Khoo E, Tran J, Chartres I, Liu Y, Thant LM, Khabensky I, Gart LP, Cisneros G, Alikhani M. 2010. Cytokine expression and accelerated tooth movement. J Dent

Res 89(10):1135-1141.

Thrailkill KM, Moreau CS, Cockrell G, Simpson P, Goel R, North P, Fowlkes JL, Bunn RC. 2005. Physiological matrix metalloproteinase concentrations in serum during childhood and adolescence, using Luminex Multiplex technology. Clin Chem Lab Med 43(12):1392-1399.

Toms SR, Lemons JE, Bartolucci AA, Eberhardt AW. 2002. Nonlinear stress-strain behavior of periodontal ligament under orthodontic loading. American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics 122(2):174-179.

Townson SA, Martinez-Hackert E, Greppi C, Lowden P, Sako D, Liu J, Ucran JA, Liharska K, Underwood KW, Seehra J, Kumar R, Grinberg AV. 2012. Specificity and structure of a high affinity activin receptor-like kinase 1 (ALK1) signaling complex. The Journal of biological chemistry 287(33):27313-27325.

Traut TW. 1994. Physiological concentrations of purines and pyrimidines. Molecular and cellular biochemistry 140(1):1-22.

Tsuji K, Uno K, Zhang GX, Tamura M. 2004. Periodontal ligament cells under intermittent tensile stress regulate mRNA expression of osteoprotegerin and tissue inhibitor of matrix metalloprotease-1 and -2. Journal of bone and mineral metabolism 22(2):94-103.

Tuttle JL, Nachreiner RD, Bhuller AS, Condict KW, Connors BA, Herring BP, Dalsing MC, Unthank JL. 2001. Shear level influences resistance artery remodeling: wall dimensions, cell density, and eNOS expression. American journal of physiology Heart and circulatory physiology 281(3):H1380-1389.

Udagawa N, Takahashi N, Katagiri T, Tamura T, Wada S, Findlay DM, Martin TJ, Hirota H, Taga T, Kishimoto T, Suda T. 1995. Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors. J Exp Med 182(5):1461-1468.

Urist MR. 1965. Bone: formation by autoinduction. Science 150(3698):893-899.

van den Bos T, Tonino GJ. 1984. Composition and metabolism of the extracellular matrix in the periodontal ligament of impeded and unimpeded rat incisors. Arch

Oral Biol 29(11):893-897.

Van Kolen K, Slegers H. 2006. Integration of P2Y receptor-activated signal transduction pathways in G protein-dependent signalling networks. Purinergic signalling 2(3):451-469.

Veldhuis NA, Poole DP, Grace M, McIntyre P, Bunnett NW. 2015. The G protein-coupled receptor-transient receptor potential channel axis: molecular insights for targeting disorders of sensation and inflammation. Pharmacological reviews 67(1):36-73.

Vico L, Collet P, Guignandon A, Lafage-Proust MH, Thomas T, Rehaillia M, Alexandre C. 2000. Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. Lancet 355(9215):1607-1611.

Wada S, Kanzaki H, Narimiya T, Nakamura Y. 2017. Novel device for application of continuous mechanical tensile strain to mammalian cells. Biol Open 6(4):518-524.

Wang J, Wang CD, Zhang N, Tong WX, Zhang YF, Shan SZ, Zhang XL, Li QF. 2016. Mechanical stimulation orchestrates the osteogenic differentiation of human bone marrow stromal cells by regulating HDAC1. Cell Death Dis 7:e2221.

Wang L, Pan J, Wang T, Song M, Chen W. 2013a. Pathological cyclic strain-induced apoptosis in human periodontal ligament cells through the RhoGDIalpha/caspase-3/PARP pathway. PloS one 8(10):e75973.

Wang L, Zhang X, Guo Y, Chen X, Li R, Liu L, Shi C, Guo C, Zhang Y. 2010. Involvement of BMPs/Smad signaling pathway in mechanical response in osteoblasts. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 26(6):1093-1102.

Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S, Idowu O, Li M, Shen C, Hu A, Haydon RC, Kang R, Mok J, Lee MJ, Luu HL, Shi LL. 2014. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes Dis 1(1):87-105.

Wang Y, Hong S, Li M, Zhang J, Bi Y, He Y, Liu X, Nan G, Su Y, Zhu G, Li R, Zhang W, Wang J, Zhang H, Kong Y, Shui W, Wu N, He Y, Chen X, Luu HH, Haydon RC, Shi LL, He TC, Qin J. 2013b. Noggin resistance contributes to the potent osteogenic

capability of BMP9 in mesenchymal stem cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 31(11):1796-1803.

Watson C, Whittaker S, Smith N, Vora AJ, Dumonde DC, Brown KA. 1996. IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes. Clin Exp Immunol 105(1):112-119.

Wen LT, Knowles AF. 2003. Extracellular ATP and adenosine induce cell apoptosis of human hepatoma Li-7A cells via the A3 adenosine receptor. British journal of pharmacology 140(6):1009-1018.

Wescott DC, Pinkerton MN, Gaffey BJ, Beggs KT, Milne TJ, Meikle MC. 2007. Osteogenic gene expression by human periodontal ligament cells under cyclic tension. Journal of dental research 86(12):1212-1216.

Woda A, Mishellany A, Peyron MA. 2006. The regulation of masticatory function and food bolus formation. J Oral Rehabil 33(11):840-849.

Woehrle T, Yip L, Elkhal A, Sumi Y, Chen Y, Yao Y, Insel PA, Junger WG. 2010. Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse. Blood 116(18):3475-3484.

Wongkhantee S, Yongchaitrakul T, Pavasant P. 2008. Mechanical stress induces osteopontin via ATP/P2Y1 in periodontal cells. Journal of dental research 87(6):564-568.

Yamashiro K, Myokai F, Hiratsuka K, Yamamoto T, Senoo K, Arai H, Nishimura F, Abiko Y, Takashiba S. 2007. Oligonucleotide array analysis of cyclic tension-responsive genes in human periodontal ligament fibroblasts. Int J Biochem Cell Biol 39(5):910-921.

Yang YQ, Li XT, Rabie AB, Fu MK, Zhang D. 2006. Human periodontal ligament cells express osteoblastic phenotypes under intermittent force loading in vitro. Front Biosci 11:776-781.

Ye G, Li C, Xiang X, Chen C, Zhang R, Yang X, Yu X, Wang J, Wang L, Shi Q, Weng Y. 2014. Bone morphogenetic protein-9 induces PDLSCs osteogenic differentiation through the ERK and p38 signal pathways. International journal of medical sciences 11(10):1065-1072.

Ye Y, Du Y, Guo F, Gong C, Yang K, Qin L. 2012. Comparative study of the osteogenic

differentiation capacity of human bone marrow- and human adipose-derived stem cells under cyclic tensile stretch using quantitative analysis. Int J Mol Med 30(6):1327-1334.

Yeh TT, Wu SS, Lee CH, Wen ZH, Lee HS, Yang Z, Nimni ME, Han B. 2007. The short-term therapeutic effect of recombinant human bone morphogenetic protein-2 on collagenase-induced lumbar facet joint osteoarthritis in rats. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 15(12):1357-1366.

Zerwekh JE, Ruml LA, Gottschalk F, Pak CY. 1998. The effects of twelve weeks of bed rest on bone histology, biochemical markers of bone turnover, and calcium homeostasis in eleven normal subjects. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 13(10):1594-1601.

Zhang H, Wang J, Deng F, Huang E, Yan Z, Wang Z, Deng Y, Zhang Q, Zhang Z, Ye J, Qiao M, Li R, Wang J, Wei Q, Zhou G, Luu HH, Haydon RC, He TC, Deng F. 2015. Canonical Wnt signaling acts synergistically on BMP9-induced osteo/odontoblastic differentiation of stem cells of dental apical papilla (SCAPs). Biomaterials 39:145-154.

Zhang W, Deng ZL, Chen L, Zuo GW, Luo Q, Shi Q, Zhang BQ, Wagner ER, Rastegar F, Kim SH, Jiang W, Shen J, Huang E, Gao Y, Gao JL, Zhou JZ, Luo J, Huang J, Luo X, Bi Y, Su Y, Yang K, Liu H, Luu HH, Haydon RC, He TC, He BC. 2010. Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. PloS one 5(7):e11917.

Zhu G, Kang L, Wei Q, Cui X, Wang S, Chen Y, Jiang Y. 2014. Expression and regulation of MMP1, MMP3, and MMP9 in the chicken ovary in response to gonadotropins, sex hormones, and TGFB1. Biol Reprod 90(3):57.

Zimmermann H. 1996. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Progress in neurobiology 49(6):589-618.

VITA

NAME	Yanee Tantilertanant
DATE OF BIRTH	17 December 1982
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	Faculty of Dentistry, Chulalongkorn University
HOME ADDRESS	86/38 Mantana Onnut-Wongwain 4 Village Kanchanapisek Rd., Dokmai Prawej Bangkok 10250, Thailand
PUBLICATION	Tantilertanant, Y, Niyompanich, J, Everts, V, Supaphol, P, Pavasant, P, Sanchavanakit, N. Cyclic tensile force stimulates BMP9 synthesis and in vitro mineralization by human periodontal ligament cells. J Cell Physiol. 2019; 234: 4528– 4539. https://doi.org/10.1002/jcp.27257