Influence of Mechanical Compression on Stem Cell Property in Human PDL Cells

Panita Panchamanon

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Oral Health Sciences
Prince of Songkla University
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Author Miss Panita Panchamanon

Major Program Oral Health Sciences

Major Advisor

.....

(Assoc. Prof. Dr. Chidchanok Leethanakul)

Co-advisor

.....

(Prof. Dr. Prasit Pavasant)

Examining Committee :

.....Chairperson

(Assoc. Prof. Dr. Nirada Dhanesuan)

.....Committee

(Assoc. Prof. Dr. Chidchanok Leethanakul)

.....Committee

(Prof. Dr. Prasit Pavasant)

.....Committee

(Asst. Prof. Dr. Suwanna Jitpakdeebodintra)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy degree in Oral health sciences in 2016

.....

(Assoc.Prof.Dr. Damrongsak Faroongsarng)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

.....Signature
(Assoc. Prof. Dr. Chidchanok Leethanakul)
Major Advisor

.....Signature
(Miss Panita Panchamanon)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

.....Signature
(Miss Panita Panchamanon)
Candidate

ชื่อวิทยานิพนธ์ อิทธิพลของแรงเชิงกลต่อความเป็นเซลล์ต้นกำเนิดของเซลล์เนื้อเยื่อเอ็นยึดปริทันต์

ผู้เขียน นางสาวพนิตา ปัญจมานนท์

สาขาวิชา วิทยาศาสตร์สุขภาพช่องปาก

ปีการศึกษา 2560

บทคัดย่อ

เอ็นยึดปริทันต์เป็นอวัยวะที่เกี่ยวข้องกับการรับแรง จากการบดเคี้ยวและแรงในการจัดฟัน โดยเอ็นยึดปริทันต์ประกอบด้วยกลุ่มเซลล์หลากหลายชนิด รวมถึงเซลล์ต้นกำเนิดซึ่งมีบทบาทในการรักษาภาวะสมดุลภายในของเอ็นยึดปริทันต์ หลักฐานเชิงประจักษ์นำเสนอแนวคิดเกี่ยวกับแรงเชิงกลต่อการเปลี่ยนแปลงสภาวะของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ (Mesenchymal stem cells) แต่อิทธิพลของการกระตุ้นเชิงกลต่อการเปลี่ยนแปลงคุณสมบัติของเซลล์ต้นกำเนิดในเอ็นยึดปริทันต์นั้นยังไม่เป็นที่แน่ชัด วัตถุประสงค์ เพื่อศึกษาการเปลี่ยนแปลงคุณสมบัติความเป็นเซลล์ต้นกำเนิดในเซลล์เอ็นยึดปริทันต์ภายใต้อิทธิพลของแรงกด วิธีวิจัย เซลล์เนื้อเยื่อเอ็นปริทันต์มนุษย์จากฟันกรามซี่ที่ 3 ทำการทดสอบเพื่อยืนยันคุณลักษณะสำคัญของเซลล์ต้นกำเนิดโดยดูการแสดงออกเฉพาะบนผิวเซลล์ต่อ CD73, CD90, CD105 และความสามารถในการเจริญพัฒนาไปเป็นเซลล์กระดูก (Osteoblast) และเซลล์ไขมัน (Adipocyte) ได้ การศึกษาครั้งนี้ใช้เซลล์ใน passage ที่ 3-5 โดยให้แบ่งกลุ่มและให้แรงกดแก่เซลล์ด้วยน้ำหนักที่แตกต่างกัน (กลุ่มควบคุม, 0.5 ก/ซม^2, 1.0 ก/ซม^2, 1.5ก/ซม^2, 2.0 ก/ซม^2) เป็นเวลา 24 ชั่วโมง จากนั้นทำการทดสอบโดยวิธี MTT assay, CFU-F assay, flow cytometry เพื่อดูการแสดงเฉพาะบนผิวเซลล์ต่อ STRO-1, CD73, CD90 และ CD105 และวัดปริมาณการแสดงออกของยีน (gene)ที่เกี่ยวข้องกับการเปลี่ยนแปลงของคุณสมบัติของเซลล์ต้นกำเนิด รวมถึงเพอริออสติน (periositin) ซึ่งเป็นเมทริกซ์เซลลูลาร์โปรตีนที่ตอบสนองต่อแรง ด้วยวิธีควอนทิเททีฟเรียลไทม์พีซีอาร์ (quantitative real-time PCR) ผลการศึกษา เพอริออสตินตอบสนองต่อแรงขนาดต่างๆแบบไบเฟสิก (biphasic effect) โดยมีการแสดงออกสูงสุดของเพอริออสตินเมื่อให้แรงขนาด 1.0 ก/ซม^2 และลดลงเมื่อได้รับแรงเพิ่มขึ้น ยีนทีจีเอฟเบต้า (TGF-β) นานอก (Nanog) ออคโฟ (Oct4) และออสเทอริกซ์ (Osx) ก็ให้ผลในทำนองเดียวกัน CFU-F assay ซึ่งแสดงถึงความสามารถในการแบ่งตัวทดแทนตัวเองได้ (self renwewal) พบการเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อให้แรง 1.0 ก/ซม^2 แต่ลดลงเมื่อให้แรง 0.5 ก/ซม^2 ไม่พบความแตกต่างระหว่างกลุ่มของการแสดงออกเฉพาะบนผิวเซลล์ทั้งหมด นอกจากนี้ยังพบความสัมพันธ์ระหว่างการแสดงออกของเพอริออสตินและนานอกภายใต้แรงกด

สรุปผลการศึกษา แรงกดมีผลต่อการเปลี่ยนแปลงสภาวะของเซลล์ต้นกำเนิดทั้งความสามารถในการแบ่งทดแทนตัวเองและความสามารถในการพัฒนาเจริญไปเป็นเซลล์กระดูกรวมถึงการแสดงออกของยีนเพอริออสตินโดยขึ้นกับขนาดของแรง การแสดงออกของเพอริออสตินอันเนื่องมาจากแรงกดมีความสอดคล้องกับการเปลี่ยนแปลงของความสามารถในการแบ่งตัวทดแทน ซึ่งเป็นไปได้ว่าเพอริออสตินมีบทบาทในการรักษาคุณสมบัติของเซลล์ต้นกำเนิดในเอ็นยึดปริทันต์ ทั้งนี้เพอริออสตินมีหน้าที่เชื่อมโยงกับการส่งสัญญาณของ Wnt และ PI3K/Akt ผ่านทางอินทิกริน (intergrin) ซึ่งทำหน้าที่ควบคุมคุณสมบัติของเซลล์ต้นกำเนิดในหลายๆระบบ

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ABSTRACT

Periodontal ligament (PDL) is a mechanical responsive tissue that subject to mastication and orthodontic force. PDL contains heterogenous cell population including residual stem cells, that play roles in maintaining PDL homeostasis. Evidences suggested that mechanical stimuli could modulate stem cell fate, however, the role of mechanical stimulation in stem cells properties of PDL stem cells is still unclear. Objective: To investigate the stem property alteration in human PDL cells under the influence of compressive force. Material and Methods: PDL stem cells were isolated from healthy periodontal tissue obtained from third molar extracted teeth. The characteristics of PDL stem cells were confirmed by the expression of CD73, CD90 and CD105 as well as the ability to differentiate into osteoblast-like cells. The 3rd - 5th passage of human PDL cells were used in the study. Cells were subjected to varied magnitude of static compressive force for 24 hours (control, 0.5g/cm^2, 1.0g/cm^2, 1.5g/cm^2 and 2.0g/cm^2). MTT assay, CFU-F assay, flow cytometry for immunophenotyping (STRO-1, CD73, CD90, CD105) and qPCR for gene expression were used to determine the stem property. The expression of periostin, a forced-responsive matricellular protein, was also examined. Result: A biphasic dose response of periostin expression was observed. Periostin was expressed maximum at 1.0g/cm^2 compression then declined. Nanog, Oct4, stemness regulatory gene, and osx, osteogenic differentiation marker, were expressed in consistent pattern with periostin expression. A correlation coefficient analysis also showed strong relationship between periostin and Nanog expression. CFU-F assay was used to represent the self renewal capacity. The results showed that CFU-F

increased at 1.0g/cm^2 and decreased at 0.5g/cm^2. All cell surface markers were expressed in all samples but no difference was found between groups. Conclusion: The results showed compressive force could effects stem cell fate in differential manner. The changes in periostin expression was associated with the self-renewal capacity changes. Increased periostin due to compressive force might play a role in stem properties in PDL, since periostin function involved Wnt signaling and PI3K/Akt signaling via integrin that regulate stemness in many stem cell systems.

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LIST OF ABBREVIATIONS AND SYMBOLS

PDL	= Periodontal Ligament
MTT	= The 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide = induced Pluripotent Stem cells
CFU-F	= Colony forming unit - fibroblast
g/cm2	= Gram per square centimetre
PI3/Akt	= Phosphoinositide 3-kinase/Protein kinase B
iPSCs	= induced pluripotent stem cells
ECM	= Extracellular matrix
PDLSCs	= Periodontal ligament stem cells
ESCs	= Embryonic stem cells
MSC	= Mesenchymal stem cell
MMP	= Matrix metalloprotienase
IL-13	= Interleukin 13
IL-14	= Interleukin 14
TGF-β	= Transforming growth factor beta
mRNA	= messenger Ribonucleic acid
FAK	= Focal adhesion kinase
BMSCs	= Bone marrow-derived mesenchymal stromal cells
hPDLC	= human periodontal ligament cell
VEGF	= Vascular endothelial growth factor
TSP	= Thrombospondin
TNF	= Tumor necrosis factor
LPS	= Lipopolysaccharide
hMSC	= human Mesenchymal stem cell
PBS	= Phosphate-buffered saline
cm2	= square centimeter
%	= Percentage
FBS	= Fetal bovine serum
℃	= Degree Celsius
CO_2	= Carbon dioxide
DMEM	= Dulbecco's modified essential medium
U/ml	= Unit per microliter

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

µg/ml	= microgram per microliter
h	= hour
qPCR	= Quantitative real-time Polymerase chain reaction
ml	= milliliter
mg/ml	= Microgram per milliliter
nm	= nanometer
FITC	= Fluorescein isothiocyanate
PE	= Phycoerythrin
APC	= Allophycocyanin
IgG	= Immunoglobulin G
IgM	= Immunoglobulin M
µg	= microgram
RNA	= Ribonucleic acid
µl	= microliter
DEPC-treated water	= Diethyl pyrocarbonate-treated water
mM	= Millimolar
µM	= Micromolar
min	= minute
s	= second
SCXA	= Scleraxis A
POSTN	= Periostin
Runx2	= Runt-related transcription factor 2
Osx	= Osterix
PPARɣ	= Peroxisome proliferator-activated receptor gamma
PCR	= Polymerase chain reaction
bp	= base pair
CT	= Control
LIF	= Leukemia inhibiting factor
FGF	= Fibrblast growth factor
HIF-1a	= hypoxia-inducible factor (HIF)-1alpha

CHAPTER 1

INTRODUCTION

Background and Rationale

The use of regenerative medicine and tissue engineering are growing continuously in treating obstinate disease. it also covers various functional tissues to repair and substitute tissues or organs that loss of function due to age, disease, trauma, or congenital defect. . Stem cells are widely used in cell-based therapy, regenerative medicine and tissue engineering due to their capacity for multiple lineages differentiation and self-renewing.[1-5] Stem cells have been broad classified in three groups, ground on the source. Embryonic stem cell are derived from embryonic sources and culture the fertilization of preimplantation blastocysts and are referred to as pluripotent. Pluripotent stem cells also can be evoked directly from adult stem cell by genetic reprogramming and are known as induced pluripotent stem cells (iPSCs). Although iPSCs inspired to accomplished cell therapy without immune rejection, It is concerned about safety in the issue of generating teratoma.[6] Actually, adult tissue which have high cell turnover rate, remains adult stem cell in for maintaining that tissue. These multipotent stem cell can undergo to a number of series of lineage. So adult stem cells could be an alternative. However a basic consequence still be necessary to be clarified to achieve an accurate control over stem cells behavior, in term of self-renewing and differentiation, especially, a more extensive knowledge about the interaction between stem cells and microenvironment (growth factor, cell to cell contacts and cell to extracellular matrix interaction), which is currently lacking.

Earlier, the regulation of stem cell direction has been focus principally to genetic and molecular mediator, either *in vivo* or *in vitro*. But, It is now distinct that control of stem cell by extracellular matrix(ECM) has high possibility to happen through various biophysical mechanisms, such as ECM geometry, ECM stiffness, or mechanical signal transmitted from ECM to cell.[7] A better comprehesing in the role

and interplay of the mediators with traditional signalling cascade possibly lead to novel knowledge on the regulation of self-renewing and differentiation of stem cells. The intelligence to control cell behaviour, through physical and molecular interactions, may increase potentiality in tissue engineering from adult or embryonic stem cell.

The periodontal ligament has a rapid rate of turnover of PDL cell and extracellular component and also exist a population of stem cells. The periodontal ligament stem cells (PDLSCs) have capacity to differentiate into either fibroblast, cementoblast-like cells, osteoblast as well as adipocytes. Periodontal ligament stem cells seem closely-related mesenchymal stem cell populations. However, another publication proposed that pluripotent stem cells which are descended from the neural crest, might reside in the PDL. It is clearly seen that physical factor can have obvious effect on the overall behaviour of Mesenchymal stem cell (MSC) population but for the PDLSCs, that differ in origin, is still lack of studies to make an agreement.

The periodontal ligament is the soft fibrous connective tissue located between root of the tooth and bone that form alveolar socket. It has very high flexibility and adaptability to immediate changes from external loading and ability to preserve its width. This dense fibrous connective tissue is consisted of both collagenous and non-collagenous proteins along with growth factors, cytokines and matrix degradation enzymes, matrix metalloproteinases (MMPs). Collagen fibres are major component of periodontal ligament matrix, type I and III are the major type. Both contain cell-adhesive region and role the significant part in remodeling of periodontal tissue. Type III collagen provide structural support, and also function in contol cell proliferation, cell migration and related gene expression *via* integrin receptor. The non-collagenous part of the ECM versican, dextran sulphated biglycan, and decorin contribute to, which also includes glycoproteins such as fibronectin, vitronectin, thrombospondin, tenascin, periostin and osteopontin. Change in periodontal ligament ECM composition alters physical properties and transduction pathway that may lead stem cell fate. In orthodontic treatment, a distortion of the PDL matrix after force application lead to a modification in cell shape and cytoskeletal configuration, and simultaneously neuropeptide releasing from afferent nerve endings. A picture in the biomolecular level, these mechanical loading may

stimulate the release of prostaglandins, cytokines, growth factor and other mediators. All events purpose to keep PDL homeostasis but there is no study to show how the periodontal ligaments maintain their width. The balance of PDLSCs proliferation and differentiation is believed to be a key. Recent findings suggested that periostin is indispensable for the homeostasis of the periodontium and its remodeling following mechanical stress.[8] Perhaps, periostin may be crucial in forced controlling periodontal ligament stem cell behavior to maintain tissue structure.

Periostin is categorized as one of the matricellular proteins, which launched into the extracellular space but does not function as a structural protien. Matricellular proteins also can control direct cell fate, adhesion, motility and survival by serving communication between cell surface and the surrounding matrix. Matricellular protein also influence the assembly and turn-over of the ECM by their intensified expression at the site of tissue remodeling and wound healing.[9] Periostin has recently been implicated in fibrosis of respiratory organs caused by chronic pathological inflammation as a consequence of Th2-type immune responses. Periostin induces high levels of IL-4 or IL-13 in lung fibroblasts, recruitment of eosinophil, or TGF-β activation in epithelial cells airway lining and which is in turn related to bronchial asthma fibrosis. In immunohistochemical studies, periostin in PDL matrix was shown high levels of expression. Periostin is also engage with tenascin-C to assembly a complex ECM meshwork which includes type I collagen and fibronectin. When periostin were blocked out in mice, they were found dwarfed, narrower and smaller skulls and tiny forelimbs, ribs and cancellous bony trabeculae. PDL around incisors show widening, and abnormal remodelling and breaking down of alveolar bone.[10] The unstable structure of PDL shows abruptly progressive periodontitis-like disorder with a loss of the PDL's mechanical properties. In the process of fibrillogenesis, decreased collagen cross link was also found. Kii et al suggest that periostin is necessary for collagen matrix remodelling.[11] Without periostin, it seems to cause changing in many expression profile of non collagenous proteins.[12]

Without doubt, periostin and tenascin-C both matricellular protein have transcription factor that being controlled by cytoskeletal action, thus its expression are harmonizely induced by mechanical loading. In PDL, cyclic strain and TGF-β1 via FAK-dependent pathways was found the ability to regulate periostin

expression.[13] It was observed transiently reduced periostin expression in periodontal ligament of hypofunctional teeth[14], but periostin expression was raised on the compression side during orthodontic tooth movement.[15] In cancer, periostin is important in maintaining cancer stem cell, and inhibition of its function can interrupt metastasis.[16-19]

As describe above, the evidence showed that mechanical force related to periostin expression in periodontal ligament and alter periodontal ligament ECM properties. Periostin possibly roles both direct and indirect to alter ECM due to mechanical stimuli. Change in ECM also imply to stem cell fate whether proliferation or differentiation. There is now rarely report that demonstrated obviously effect of force to PDLSCs behavior and involvement in ECM composition. It is interesting to investigate the role of force, even the differential force, in periodontal ligament stem cell fate and possible mechanism which will enhance cell therapy. More over orthodontic clues that how the teeth move may also be elucidated.

Review of Literature

Tissue engineering and regenerative medicine that seek for repair or regenerate damage tissue through the combinations of cells, scaffolds, and soluble mediators.[20] This field is now rapidly developed and requires available cell sources that can provide desired function. Stem cells are crucial roles in development, tissue regeneration, and normal tissue remodelling, and they are also important for advancing the fields of regenerative medicine and tissue engineering because of their self-renewing capacity and pluripotency ability. Moreover, there are reported about the abated immunogenicity and immunosuppressive properties in many adult stem cells that give advantage for allogenic cell transplantation.

Stem cell type and sources

Stem cell means to a clonable, relatively undifferentiated progenitor cell which possess the two unique properties, dividing by maintaining their stemness and multi-lineage differentiation. A stem cell could either go extended self-renewing or decide differentiation through modulation of intracellular signals that were stimulated by varying factor.

Based on the cell obtaining source, stem cell are broadly classified into three category. Embryonic stem cells (ESCs) are original pluripotent progenitor cells obtained from primary mammalian embryo with their high capacity to extensively grow and differentiate into all cell type in the body. Even if ESCs have much more developmental efficiency, the usage of embryos to derive human ESCs lines provoke ethical mortal issue. The induced pluripotent stem cell (iPSC) was generated by genetical reprogramming somatic cell back to their potent state instead. But, genetic manipulations method to reprogram iPS cells might modulate their growth and developmental properties that probably restrict their use. The iPSC-based treatments also still are challenged about the safety because of tumorigenic properties.[6] Adult stem cells are multiple-lineage potent progenitor cells which could be obtained from various tissue including bone, bone marrow, blood vessels, skin and muscular tissue. Those tissues can be characterized by rapid turn over rate. Adult stem cells have responsibility for keeping tissue homeostasis. They have shown the ability to differentiate to other specific cell lineages over and above their tissue

origin. Even though proliferation and differentiation potential in these cell are limited, easily accessibility, immunocompatibility and no ethic dilemma are benefits over the ESCs

Mesenchymal stem cell (MSC) are member of adult stem cells that powerful to undergo differentiation into different specific cell lines. The term of MSCs now refer to a heterogenous mix of potent progenitors, which subclass populations can differentiate into mesoderm (osteoblasts, adipocytes, chondrocytes, tenocytes and visceral stromal cells), ectodermal (astrocytes, neurons) and endodermal (hepatocytes) originated cell.

Among various mesenchymal origin, Bone marrow stromal stem cells (BMSCs) were the first mesenchymal stem cell population that have been long and most studied. BMSCs were isolated from bone marrow for the first time and identified by their ability to form clonal colonies on plate at the low density . This characteristic is shown in other somatic stem cells that indicates their capacity to continue self-renewing. Even so, in vitro, BMSCs seem to have limitation in growing expansion.[21, 22] In vitro studies, BMSC has been shown ability to differentiating themselves into variety of cell types when cultured in particular conditions, including adipogenic, osteogenic, myeloid stromal, myogenic, and neurogenic lineages. Osteogenic, adipogenic, chondrogenic differentiation are the critical lineages to define multipotent MSCs.

BMSCs are heterogenous population, contain mixed phase of cell immaturity through differentiation pathways. Cell-surface markers are also used to defined the presumed MSCs. CD73, CD90, CD105, CD146, STRO-1, Nanog, Oct4, beta2 integrin positive are mostly reported as positive expression while CD14, CD34, CD45 and HLA-DR are expressed less.[23-29] The heterogeneity make it arduous to identify the certain phenotype template of a mesenchymal stem cell. To conform the identification of MSC and support emerging data among researchers in stem cell therapeutic uses, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC. “First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules.

Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro."[26]

Instead of using stem cell from bone marrow that cause pain, morbidity, and a few number of derived stem during harvesting, the newer alternative source for MSCs were suggested. MSCs derived from fat connective tissue could be gained by liposuction method and MSC-like populations from umbilical cord blood have been also popular to isolate and characterize. All MSCs from bone marrow, adipose tissue, and umbilical cord blood are same in morphological and immunophenotypical but not exact the same at all. Among those, proliferative potential of UCB- derived MSC show highest and the BMSC are lowest.[30]

Dental-tissues-derived mesenchymal stem cell-like populations are one of another isolated and characterized stem cells which reside in particularly tissues. The periodontium and dentine need to maintain some regenerative or reparative capacities. The residing multipotent progenitor cells are thought to be role in this critical tissue homeostasis and maintenance due to their differentiation capacity to go functional, specific cells. Nonetheless, when compared with BMSCs, dental derived stem cells seem to be narrower in their cellular potency as dental tissues have no process of remodelling like bone tissue do.[31] Moreover, dental mesenchyme is derived from 'ectomesenchyme' since it has been interacted with the neural crest.

Periodontal ligament and Periodontal ligament stem cell

Periodontal ligaments (PDLs) are a particularized connective tissue, consisting mainly of collagen fibers that connect a tooth to the alveolar bone. Periodontal ligament always expose to physical force, mastication, orthodontic force. The periodontal ligament function as supporter, protecter and receptor for mastication. Periodontal ligament has a rapid turn-over rate and also can quickly modify to uncertain regional condition. Periodontal ligament width are controlled precisely by unknown mechanism. Previous evidence has demonstrated that some residing cells are contained in PDL can go differentiation into either cementoblasts or osteoblasts.[32, 33] The progenitor remaining cell in PDL are suggested to function in maintaining tissue homeostasis and regeneration of PDL tissue.[34]

Periodontal ligament stem cells (PDLSCs) show a self-renewal capacity and present various cell surface markers same as BMSCs[35] PDLSCs likewise demonstrated the multiple potential to differentiate into several cell types, such as bone cells, fat cells, cartilage cell, and neuron cell, *in vitro*.[36] There is no specific tools to label periodontal ligament stem cells specifically. However, PDLSCs also present the MSC-associated cell surface markers STRO-1, CDs, and scleraxis which is expressed in PDLSCs higher than in BMSCs.[31] PDLSC was reported the ability to differentiate in to cells that can form colony and expand on biocompatible scaffold that proposed a handy and efficient autologous source of potent progenitors for bone tissue engineering.[37,38] Moreover, PDLSCs have an obvious ability to create both cementum-like and PDL-like tissue *in vivo*, supporting that PDLSCs possibly are member of a unique group of somatic stem cells. The induced PDLSCs while was transplanted into immunocompromised mice demonstrated the ability of tissue regeneration to form cementum/PDL-like structure, which characterized by a form of cementum-like mineralized tissues layer connected with PDL-like collagen fibers[35]. Huang et al. suggest that PDL possibly remain pluripotent progenitor cells, which migrate down from the neural crest.[39] Human PDL shows itself could be provide as an alternative resource for original progenitor to be applied in cell based therapies.

The pluripotent character and regenerative potential of periodontal ligament stem cells are depend on the lifetime of the donor. Increasing age reduce regenerative potential and pluripotent ability.[40] Recent study demonstrated the properties alteration of periodontal ligament cells during passages.[41] The differentiation potential to osteogenic of cultured cells during passage was decreased. In the later passage, the decrease of alkaline phosphatase activity was also present. However, For better understanding in the detailed of regulatory mechanism of osteogenesis and tendo/ligamentogenesis between passages, it still need more data to fulfil.

Recent research indicates the different isolation methods are able to be used effectively to isolated hPDLCs in a clinical environment.[42] The method that using to isolate hPDLSCs from PDL tissue can be both outgrowth or enzymatic dissociation. Outgrowth method was initiated by Somerman et al. to isolated PDL cell and has been widely used in many studies.[43] Enzymatic dissociation is an

another regular technique to gain single cell from primary tissues. With minimal exposure time of tissue to enzyme, this method was claimed a better preserving cell viability.

Stem cells, that found in adult tissue, are commonly present as a quiescent phase . To undergo self-renewal, It could be in two mechanism. To maintain a constant number of stem cell under physical condition, asymmetrical self-renewal is processed by each stem cell splits into one naive and another one functional cell. But in a condition of tissue injury or disease that loss of differentiated cell, a huge number of stem cells is required so stem cells go symmetric self-renewal, which stem cell divided into double stem cell, lead to a large volume of stem cell pool. How to control stem cell self-renewal is need to be clarified to further extend controlling stem cells response. Recent study show Wnt3a can promotes hPDLC expansion efficiently and still maintains the self-renewal capacity and osteogenic differentiation ability.[44]

Stem cell niche

A niche is a microenvironment that maintains stemness and directs stem cell fate. The niche microenvironment often takes the form of specialized cells that directly contact stem cells and secrete stem cell regulatory factors. Afterward Schofield's statement about this concept, other aspects of the stem cell environment were also extensively included and studied.[45] Significant factors of the niche cover stem cells and nearby cells direct interaction, secretory mediating factors, inflammation, scarring, extracellular matrix (ECM), physical factors such as shear stress , surface geometry and tissue elasticity, and other stress condition like hypoxia.[46] A complicated system of both physical and biochemical signals, from regional and systemic resource, in the niche direct stem cell to undergo expansion and specific differentiation.

According to the reciprocal interaction between cell and ECM causing various biochemical signaling, a strategy to enhance cell attachment, expansion, and differentiation for properly use in the natural biopolymers for scaffold materials has been developed. And the synthetic biomaterials have also been profited. A

functionalisation of the substrate surface is an attribute that has been demonstrated to control the stem cell activity.

Although the impact of biochemical factors, including soluble factors and ECM ligands has been extensively explored *in vitro*, the control of self-renewing and specific lineage determination of stem cells by these important factors is obscurely known and hard to apply in living organism. ECM components have a pivotal function in the regulated distribution signalling molecules. Moreover, the spatial and transient arrangement of ECM adhesive ligands *in vivo* is delicately adjusted and hard to accomplish through engineering procedure.

In addition, adapting techniques to control stem cell direction using biochemical cues, the evidences that loading factor is powerful enough to control stem cell direction *in vitro* are now increasing.[47-53] These cues can be detected and transduced through intracellular biochemical and functional responses by stem cells, this process is called mechanotransduction.

External loading and matrix dynamics are important in the controlling of stem cell direction. The image of the mechanotransduction procedure for stem cells perception and response to loadings and modulating in matrix mechanical properties in molecularly detail has yet to be clarified. The load equilibrium transfer through the mechanical of ECM-integrin-cytoskeleton that can accompany integrin-mediated adhesion signaling to regulate downstream integrated stem cell response. Focal adhesion (FA) is the sites where ECM connects mechanically to the actin cytoskeleton via integrin, and the biophysical signals was perceived. Integrin can be activated by different external force, such as mechanical strain, fluid shear stress or substrates with various stiffness, stimulating on cells that promote accumulation of signaling proteins to intensify FAs and to transmit biochemical signals into the cell. These mechanotransduction pathways set positive-feedback loops, when integrin binding stimulates actomyosin cytoskeleton contractility, consequently strengthen FAs. Therefore, the changing of cytoskeleton contractility inside the cell is correspondingly consequence to the strengthen of adhesion and the matrix stiffness and regulates stem cells behavior.[54]

Studies on effects from outside loadings including mechanical strain, fluid shear stress and compression on cell responsibility have been extensively worked and published for cardiovascular tissues, skeletal muscles, and adult stem cells such as hMSCs. Evidence involved to effects of mechanical force on the controlling stem cell direction *in vitro* has started to gather recently. Study using cyclic equibiaxial strain in the conditioned medium for mouse embryonic fibroblast demonstrated hESC differentiation was decreases and self-renewal was enhanced without selecting against survival of differentiated or undifferentiated cells.[55] Late study further exhibited that the TGF-β/activin/Nodal signaling pathway is a significant player, under mechanical strain, in suppressing hESC differentiation. In undifferentiated hESCs, TGF-β1, activin A, and Nodal transcription can be induced by mechanical stain and then result in upregulation of Smad2/3 phosphorylation. Moreover, data also showed the TGF-β superfamily activation of Smad2/3 was necessary for suppression of spontaneous differentiation of hESCs under mechanical stress, which farther suggested that mechanical strain possibly induce signaling both autocrine or paracrine in hESCs through TGF-β superfamily ligands.[56]

Due to the fact that sheer stress from blood flow influences the change and remodelling of blood vessel sized,[57] lead to various studies demonstrated the precise controlled shear stress was ability to use to induce mouse embryonic stem cells (mESCs) to differentiate into the line of endothelial cell and line of hematopoietic cells, In spite of the molecular mechanisms under these mechanical response of mESCs have not yet been investigated adequately. Chowdhury et al. used functionalized magnetic bead to demonstrate that local cyclic stress can induce spreading of mESCs through integrin-mediated adhesions with a concurrent decrease Oct3/4 gene expression.[58]

The recent publish suggest the concept “force isotropy” that explain how to encourage MSCs self-renewal and pluripotency maintaining.[59] Stem cell commitment can also be induced in a synergistic manner by applying biophysical factors; Exactly, cell have their own cytoskeletal tension due to adhesive forces from surrounding extracellular loads, which can modulate and control their fate. MSCs can manifest diverse phenotypes including adipose tissue, bone, cartilage, muscle and fibrous tissue. Fibrous tissues, such as tendon, could be obtained by anisotropic

traction forces exerted by cells and through the superimposition of anisotropic extracellular loading, such as mechanical stretching. However, this hypothesis still have to be proved in all cel type.

Extracellular matrix and stem cell

Extracellular matrix is a progressional and complicated environment which is individualized the biophysical, mechanical and biochemical characters for each tissue that also can control cell reaction. Stem cells have a essential responsibility for maintaining and regenerating of tissues and they are usually placed in a limited microenvironment, defined as niche.

ECM is pivotal influencer in stem cell niche, as it roles directly or indirectly to alter stem cells maintaining, expansion, self-renewing and differentiation. Various ECM molecules involve in regulatory functions of varied kinds of stem cells, and depend on its molecular composition the ECM and finely adjusted for giving the most suitable niche for stem cells in the various tissues. Recent data continues to increase on the effect of the extracellular matrix (ECM) on stem cell fate through physical interactions with cells, for example the controlling of cell geometry, ECM geometry/ topography at the nanoscale, ECM mechanical properties, and the transduction of mechanical or other biophysical factors to the cell.[7]

Stem cell and ECM Interactions can be mediated directly by integrins and other cell receptors. Although the variety of expected receptors relates in ECM–stem cell communication, a growing amount of studies indicated that integrins are considerable receptors related in ECM–stem cell interactions and in the binding, migration and recruitment of stem cells.[60-62] Integrins is a big family of heterodimeric transmembrane receptors that link the external cellular environment to the internal cell cytoskeleton, for regulating cell migration, proliferation, survival and differentiation. Several type of integrins are related to the direct adhesion to abundant of ECM compositions or to other cell surface adhesion molecules and receptors. Integrins can stimulate downstream signaling via focal adhesion kinase (FAK) and phosphoinositide 3-kinase (PI3K), then controlling the self-renewing and proliferation in many several stem cells.[63-65]

Growing evidence in ECM biology focus on biophysical characters of the ECM as a key factor of stem cell response. Cells controls their cytoskeleton tension, creating intracellular tension that are expressed to outside by focal adhesion sites. The focal adhesion complexes, which consist of integrins, adaptors and signaling proteins, anatomically connect to the actomyosin cytoskeleton with the ECM. The focal adhesion complexes, that coordinating with cytoskeleton, nuclear matrix, nuclear envelope and chromatin, become a complex mechanosensing machinery that decides how cells response to ECM generated force. The cellular response to mechanical factors could be defined as mechanotransduction. Mechanotransduction pathway caused variety of downstream mediating the biological effects of ECM elasticity, cell geometry and cytoskeletal organization.

In vitro study show the relativeness of ECM and its biomechanical properties for neural stem cell behavior that there are stiffness gradients in the hippocampus.[66] Furthermore, while glial scars or brain tumors present, including in aging, the behavior of neuron, neuronal stem cells and glioblastomal call change concurrently with mechanical properties change together. Human MSCs grew on the hydrogel, which have an tissue stiffness same as bone marrow, enhance their ability for self-renewing and maintaining multipotency, compared to MSCs on stiffer substrates. The substrate stiffness show the powerful regulation on the osteogenic differentiation in rat MSCs as well as the ECM macromolecules pre-adsorbed onto the biomaterial.[67] Even though amount of data focusing at substrate stiffness as a key factor in controlling stem cell direction, a new study proposed that cells can perceive ECM tethering, more than the overall stiffness of the material where they are located. The stem cells responding to the stiffness of the collagen fibers themselves indicate the new important function of ECM mechanical characters on the stem cell behavior within the niche and tissue.

Matricellular protein

In the mid 1990s, Paul Bornstein proposed a new category of proteins, which are strong mediators in interactions between cell and matrix as called matricellular proteins. Matricellular proteins are launched to the outside of cell that do not exhibit primary structural functions but modulate cell function by interacting with cell-surface receptors, bioactive molecules, and matrix components like

collagens. Somehow, these matricellular proteins also turn against cell adhesion when become soluble molecules and induced rearrangement of FA and actin stress fibers, in termed intermediate adhesion.[68]

At first, matricellular proteins were identified only as transient, rather than fundamental composition of the ECM. They are take part into the ECM of remodeling tissues during development, wound healing, and in response to injury and stress, especially in fibrotic ECM, scar and tumor- associated stroma. Various matricellular proteins also be seen in body fluids and can adhere cells as soluble ligands. Matricellular proteins can bind to many soluble growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and (latent) transforming growth factor-b (TGF-β) and can regulate the actions of these factors through appearance to their receptors, by receptor binding blockage, or by induction of conformational alterations to modulate activity. Remarkably, two different matricellular proteins, Thrombospondin1(TSP-1) and tenascin-X can convert latent TGF-β to its biologically active form, indirectly regulation ECM expression.[69, 70] Matricellular proteins can directly or indirectly modify growth factor receptor signaling[69, 71-77]

The accurate controlling of matricellular protein expression has been recognized. The controlling of matricellular proteins is complicated. The regulation of expression of specific matricellular proteins was widely characterized through characterization of promoter regions and their interactions with transcription factors. Matricellular proteins can be regulated by microRNA as well as other epigenetic mechanisms (DNA methylation, histone acetylation/deacetylation).[78-83] Moreover, matricellular proteins can be auto-regulated through their receptors. There are reports of increasing TSP-1 expression under fibrotic condition due to cell contractility, shear forces, or mechanical stretch,[84-86] proposing probable expansion of TSP-1 in fibrotic tissues. Mechanical strain also promoted SPARC (secreted protein acidic and rich in cysteine) expression by podocytes, with involving to glomerulosclerosis.[87] By contrast, when force was increased, osteopontin was reduced by microgravity.[88] The regulation of matricellular proteins by mechanical stimuli seem likely to control stem cell differentiation in tissue specific niches and has significant meanings for tissue engineering.

Periostin

Periostin is a 90 kDa secreted ECM protein that belongs to the fasciclin family. It is constitute of an amino-terminal in the EMILIN family (EMI domain), a tandem repeat of 4 fas I domains (RD1-4), a carboxyl-terminal region and a heparin binding site at its C-terminal end.[89] It has been named Periostin, because its localization in the periodontal ligament and periosteum.[90] It presents in collagen-rich connective tissues, including the endocardial cushions, cardiac valves, periosteum and PDL.[12] Periostin is highly expressed in the fibroblasts in PDL and its role in collagen fibrillogenesis has been widely discussed.[14, 91, 92] Interestingly, periostin may have close relationship to mechanical stress, because periodontal tissues, heart valves, tendons and periosteum are all tissue related to loading in specific ways. Periostin also plays directly with extracellular matrix proteins such as type I collagen fibronectin and tenascin C via its internal EMI and FAS I domains. These findings suggest that periostin is a player among other extracellular matrix proteins of tissues subjected to mechanical force.

Periostin is a secreted protein that play role as both a cell attachment protein and an autocrine or paracrine factor where signals through $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin, the cell adhesion molecules, thereby regulating cell adhesion and mobility. Periostin roles in heart development, the normal physiological functions, but also roles in pathophysiological conditions, such as vascular disease, wound repair, osteogenesis, and tumorigenesis though.[93-95] Periostin potently increase survival of colon cancer cells via the Akt/protein kinase B pathway, resulting in promoting metastasis.[16] It has been reported that the interaction of periostin and integrin cause activation of the PI-3K/AKT and FAK-mediated signaling pathways and lead to the increased cell survival, angiogenesis, invasion, metastasis, and importantly, epithelial-mesenchymal transition of carcinoma cells.[93] In addition, periostin is particularly expressed in tissues involved to mechanical load, suggesting a possible function of periostin in the structure and integrity of connective tissues maintenance. In cardiac model, periostin was secreted from cardiac fibroblast, and the expression was increased after heart failure and in an overload hypertrophy condition.[96]

In PDL, Periostin is connected to type I collagen to regulates fibrillogenesis, which in turn to the controlling of biomechanical properties of fibrous

matrix around the tooth. Periostin expression is upregulated in the periodontal ligament upon mechanical stimulation and is necessary for maintaining the PDL integrity and function while serving occlusal load bearing. In vitro study also show the increasing periostin expression under the tensile force application to PDL cells. Conversely, Periostin mRNA expression show decreasing following without masticatory loading *in vivo*.[14] Postn-deficient mice ($Postn^{-/-}$) present severe impairment of incisor eruption, because of an inability to digest collagen fibers in the critical shear zone of the periodontal ligament.[11] Thus the enamel and dentin of the incisors is consequently compressed and disorganized.

The transiently decrease of periostin expression was observed in periodontal ligament tissue of hypofunctional teeth, while the compression side of orthodontically treated teeth increased periostin expression was presented.[15] Concordantly with mouse model, without mechanical loading, the PDL was found degradation of fiber system coincide with a decreased of periostin and connective tissue growth factor.[14] Mechanical stress increase periostin via Wnt5a expression. Wnt5a enhanced PDL-related gene expression and collagen production through TGFb1-mediated upregulation of periostin expression.[97] And enhancing the proliferation and migration of PDL cells, but suppressing osteoblastic differentiation of PDL cells also were found. Hypoxia can induce periostin expression, and periostin stimulation increases VEGF or MMP-2 mRNA expression via the avb3 integrin and ERK signalling pathways.[98] In vivo study show that periostin expression in human PDL fibroblasts could be decreased by Inflammatory mediators (TNF-a) and bacterial virulence factors (P. gingivalis LPS). In periodontitis, hPDLSCs proliferation potential was increased but osteogenic differentiation capacity was decreased.

Recent study suggest that periostin in the PDL plays important roles not only in the collagen fibrillogenesis, but also in the migration of hMSCs.[92] The co-culture indicate that periostin produced by hPDLFs promotes hMSC migration. Padial-Molina et al. also found that modulates hPDL proliferation, migration and PI3K/AKT/mTOR pathway.[99] Even though, preceding evidences suggest that mechanical stress alter periostin expression and raising periostin promotes stem cell proliferation, the effect of mechanical stress stimulated periostin on stemness capacity is still lacking.

Objective

To demonstrate the alteration of amount of human periodontal ligament stem cells, self-renewal capacity and amount of periostin expression due to static compressive mechanical stimulation

Hypothesis

We assumed that the amount of human periodontal ligament stem cell and their self-renewal capacity do not change due to each varied compressive force. The periostin expression also do not change.

Significance of This Study

This study could elucidate the effects of compressive force on the alteration of stem cell fate and the modification of periostin expression in hPDL cells. This probably expands the knowledge in stem cell clues to research and develop for stem cell application. And It could further explains the possible mechanism in maintaining the PDL during orthodontic tooth movement.

CHAPTER 2

MATERIALS AND METHODS

Isolation and Cultivation of hPDL Cells

This research was approved by the Institutional Ethics Committee Board of the Prince of Songkla University (EC5807-26-P-LR). Human PDL cells were prepared from third molar, approval having been granted by the Prince of Songkla University Ethics Committee to harvest the teeth with the consent of the donor and/or parent. The healthy donor age during 18-25 years, were included(n = 4) .The Teeth will be washed with phosphate-buffered saline (PBS) and the PDL attached to the middle third of the root removed with a scalpel. Base on explant method, the PDL tissues were minced into a smallest size as possible and plated onto 3.5 cm^2 single culture dishes and cultured for a week in Dulbecco's minimal essential medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 1% penicillin-streptomycin (10,000 U/mL), 1% Fungizone (250µg/mL) at 37°C in an atmosphere of 95% relative humidity and 5% CO_2. Confluent cultures were lifted with 0.25% trypsin-EDTA (Gibco) and passaged through a progression (25, 75 cm2) of tissue culture flasks (corning) until 2^{nd} passage. Cell preparations were established from each individual donor.

Human PDL cells were identified by spindle-shaped cell morphology, the scleraxis and periostin, specific marker gene expression, which are expressed in hPDL cells. To confirm stem cells characteristic, all samples in 2^{nd} passage were performed multi potential differentiation ability both adipogenic and osteogenic differentiation and immunophenyping of STRO-1, CD73, CD90 and CD105. For osteogenic differentiation, cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in osteogenic differentiation medium (Lonza Inc, Walkersville, MD, USA) according to manufacturer recommendation. After 21 days, cells were fixed in 10 % formalin and stained with 2 % Alizarin Red (Sigma Aldrich) to determine mineralization under phase contrast microscopy (Nikon Eclipse Ti-S; Nikon Instruments Inc., Melville, NY, USA). To confirm the adipogenic potential, hPDL cells

were incubated in supplemented DMEM growth medium until reached confluency. Then, adipogenic induction medium and adipogenic maintenance (Lonza) were replace according to manufacturer protocol. The cells were fixed with 10 % formalin (Sigma Aldrich) and stained with 0.5 % Oil Red O (Sigma Aldrich) in methanol (Sigma Aldrich). Red lipid droplet was identified under phase contrast microscope to indicate the adipogenic differentiation (Nikon Eclipse Ti-S).

Experimental design

Human PDL cells at the third and fourth passage were seeded into five wells in six-well plates with each well containing $3x10^5$ cells and cultivated for a night to allow cells adhere. All cells were starved with serum-free DMEM with 1% penicillin-streptomycin (10,000 U/mL), 1% Fungizone (250µg/mL) for 24h. The experiment was divided into five groups. The control group received no intervention for 24 hrs. Another four experimental groups were exert a compressive force 0.5 g/ cm^2, 1 g/cm^2, 1.5 g/cm^2 and 2 g/cm^2 each group using a 50ml-plastic tube cap with weighed coins as described above. The compressive force was then applied for 24 h. (Figure 1)

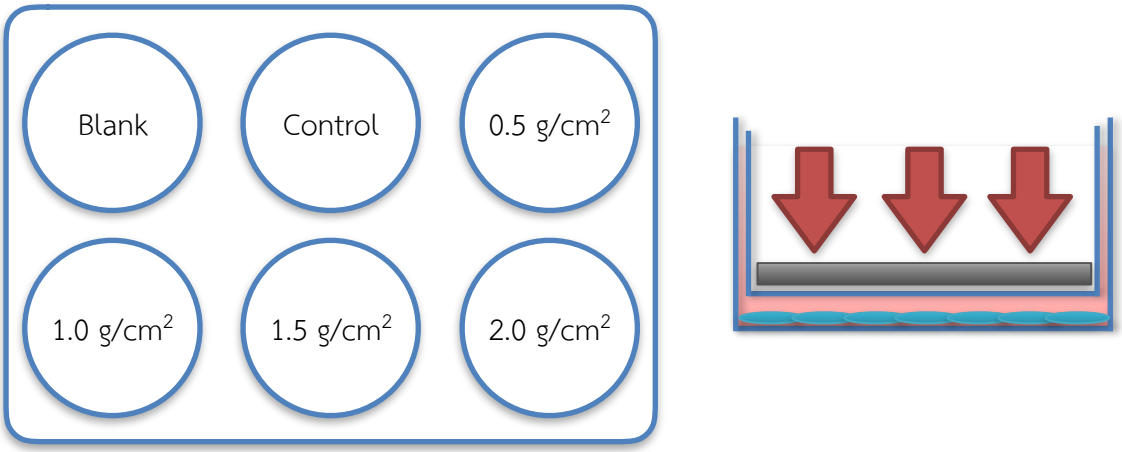

Figure 1 Experimental model (A) Illustration of experimental design in each 6-wells plate and (B) Method of static compressive mechanical stimuli application in each well. The total weight was varied by 0.5 g/cm^2, 1 g/cm^2, 1.5 g/cm^2 and 2 g/cm^2

Afterward, all cells were harvested follow the protocols. Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide

(MTT) assay. CFU-F assay was performed to represent the self renewal capacity. Flow cytometry was used for detemining immunophenotyping of stem cell (STRO-1, CD73, CD90, CD105) The expression of interesting genes were quantified using quantitative real-time polymerase chain reaction (qPCR). Each sample was done in triplicate.

MTT assay

MTT assay was performed to investigate cytotoxicity and cell viability. After 24hr of experiment, the medium was replaced with 1 mL MTT solution (5 mg/ml; (Sigma-Aldrich) of RPMI cell culture medium (Gibco BRL)) and incubated and light-protected for 3 hr at 37C with 5% CO2. After discarding the MTT solution, the purple formazan precipitate was dissolved in DMSO 1 ml. The optical density of precipitate was measure at an absorbance of 570 nm using microplate spectrophotometer (Multiskan™ GO; Thermo Scientific™ ,USA). Percentage of cell viability was calculated relative to the control

CFU-F assay

Cells in each wells were trypsinized with 0.25% Trypsin EDTA (Gibco), counted, then seed to six-well plates in low density (100 cells/well) after completed the experiment. Cells were cultured with supplemented DMEM for 21 days, the growth medium were change every 3 days. Then cells were fixed in 10% formalin and stained with 0.1% Toluidine blue (Sigma-Aldrich, USA) in 1% paraformaldehyde in PBS. Aggregates of >20 Toluidine blue staining cells were counted as a positive CFU-F. Then CFU-F efficiency was calculated, then compare with the control group.

Cell Surface Staining and Flow cytometry for immunophenotyping

Cells were trypsinized with 0.25% Trypsin EDTA (Gibco). Each group of experiment, cells were aliquoted into 1x105 cells/tube, then each of them were fixed in 10% formaldehyde and stored in FAC buffer (1% FBS, 0.1%Sodium azide in PBS). The following monoclonal antibodies (mouse anti-human) were used for flow cytometric immunophenotyping : CD90 FITC(Fluorescein isothiocyanate)(Abcam, Cambridge, MA, USA), CD105 PE (phycoerythrin)(BD biosciences, San Jose, CA, USA) CD73 (Abcam) with secondary conjugated FITC STRO-1 (Merck Millipore Darmstadt,

Germany) with secondary conjugated APC (Allophycocyanin), Isotype IgG (Biolegend, San Diego, CA, USA) Cells were analysed using FACSCalibur. Ten thousand events were acquired and analysed using the CellQuestTM program (BD Biosciences).

Total RNA isolation and qPCR

Total RNA was isolated from cultured cells using innuPREP DNA/RNA mini kits (Analytic-Jena, Konrad-Zuse-Strasse 1, Jena, Germany) according to the manufacturer's protocol and stored RNA at -80. The RNA concentration and purity were assessed using a spectrophotometer at 260nm and 280 nm. The cDNA were synthesized using a SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Aliquots containing amounts 1 µg of total RNA were mixed with 1 µl of 50 µM Oligo(dT)20, 1 µl of 10 mM dNTP mix and added diethyl pyrocarbonate-treated water (DEPC-treated water) to total volume 10 µl. Pipetting the sample to ensure thorough mixing. Then, the sample was incubated at 65°C for 5 min, placed on ice for at least 1 min. After that, Added 10 µl cDNA Synthesis Mix (the mixing of 2 µl of 10xRT Buffer, 4 µl of 25 mM MgCl2, 2 µl of 0.1 M DTT, 1 µl of RNaseOUTtm (40 U/µl), 1 µl of SuperScript® III RT (200 U/µl)). Then incubated at 50°C for 50 min, terminated reactions at 85°C for 5 min and followed by chilled on ice. After that, added 1 µl of RNase H and incubated for 20 min at 37°C. Then, the sample was used as template for qPCR.

Real-time PCR analyses were performed on a Rotor-Gene® Q (Qiagen, Qiagen Str. 1, Hilden, Germany) using SensiFASTTM SYBR No-ROX Kit (Bioline Inc, Taunton, MA, USA) (containing SYBR® Green I dye, dNTPs, stabilizers and enhancers). 1 µl of cDNA template was used as template for qPCR of all genes. Aliquots containing amounts cDNA template were mixed with 10 µl of 2x SensiFAST SYBR No ROX Mix, 0.8 µl of 5 µM forward primer, 0.8 µl of 5 µM reverse primer and added DEPC-treated water to total volume 20 µl. The primers for SCXA, POSTN, NANONG, OCT4, RUNX2, TGFB1, OSX, PPAR γ are listed in Table 1. The polymerase activation started the PCR at 95°C for 2 min, then denaturing at 95°C for 5s, following by annealing at a temperature optimized for each primer pair (Table 1) for 20s and an extension at 72°C for 20s for 35 cycles. The fluorescence data were analysed using Rotor-Gene Q software version 2.0.2 (Build 3) to determine Ct values. Ct values of

interested gene were calculated in relation to GAPDH Ct values that served as an internal control. The internal control gene was validated that its expression was unaffected by the experimental treatment. The levels of gene expression were calculated by the 2-ΔΔCt method. Changes in experimental groups were expressed as fold changes relative to the control. The efficiency for each specific primer was calculated base on the SYBR Green fluorescence curves and the standard dilution series by Rotor-Gene Q software. To ensure the presence of single amplification products, melting curves analysis was performed following PCR amplification to indicate the presence of a peak at the proper melting temperature for each gene sequence. In addition, 1.5% agarose gel electrophoresis of the PCR amplification products for each PCR reaction were performed.

Table 1 Primer sequences for qPCR

Target gene	Primer 5'-3'	Product size (BP)	Annealing temperature(°C)
GAPDH	**Forward :** GCACCGTCAAGGCTGAGAAC	142	60
	Reverse : ATGGTGGTGAAGACGCCAGT		
POSTN	**Forward :** TGTTGCCCTGGTTATATGAG	180	60
	Reverse : ACTCGGTGCAAAGTAAGTGA		
SCXA	**Forward :** AGACCGCACCAACAGCGTGAAC	127	62
	Reverse : CAGGTGCGAGATGTAGCTGGAG		
NANOG	**Forward :** GCTGGTTGCCTCATGTTATTATG	345	62
	Reverse : CCATGGAGGAAGGAAGAGGAGAG		
OCT-4	**Forward :** AGACCCAGCAGCCTCAAAATC	182	62
	Reverse : GCAACCTGGAGAATTTGTTCCT		
TGF-β	**Forward :** GGATAGCAACTATTGCTTCAGCTCC	130	62
	Reverse : AGGCTCCAAATGTAGGGGCAGGGCC		
RUNX2	**Forward :** CAGATGGGACTGTGGTTACTGT	169	62
	Reverse : GTGAAGACGGTTATGGTCAAGG		
OSX	**Forward :** ACCTACCCATCTGACTTTGCT	125	62
	Reverse : CCACTATTTCCCACTGCCTTG		
PPARγ	**Forward :** GCAAACCCCTATTCCATGCTG	167	62
	Reverse : CACGGAGCTGATCCCAAAGT		

Statistical Analysis

All statistical analyses were performed by commercially available software (SPSS Statistics 17; SPSS Inc., Chicago, IL). Values are expressed as average and standard deviations. Mean was obtained from the results of the 3 samples for each group. Shapiro-Wilk test was performed to determine the data set if it is distributed normally. Homogenity of Variance was also performed to confirm the normal of data set. In normal distribution data set, means between group were compared using oneway-ANOVA. Independent t-test was used to compared between control and another compression group. Kruskul Wallis was used to compared the medians between groups for nonparametric data . Man whitney-U test was used to compare the median between interesting groups. Spearman correlation was used to determine correlation between POSTN expression and the stem properties. Significance was defined as $p < 0.05$

CHAPTER 3

RESULT

Characterisation of hPDL cells

To confirm the originate of cell samples, the characterization of hPDL cells was performed, included stem cell characters. Spindle-shaped cells from PDL tissue were performed under inverted light microscope. Scleraxis A (SCXA), tendon specific transcription factor which well-known highly express in hPDL cell, was expressed distinctly in all samples. Each sample was shown ability in osteogenic differentiation and adipogenic differentiation. Self-renewal capacity were performed in CFU-F assay. All samples expressed STRO-1, CD73, CD90 and CD105, the stem cell surface marker, which can verify some stem cell properties. (Figure 2)

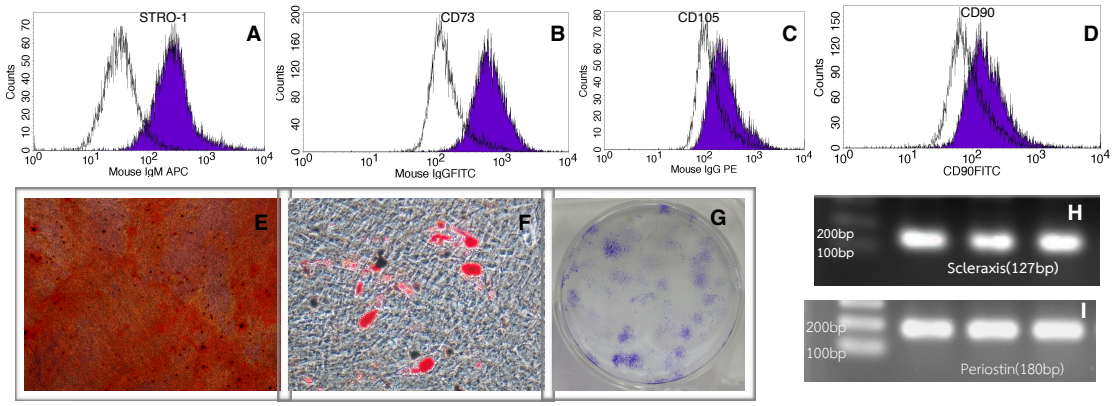

Figure 2 MSC immunophenoyping, osteogenic differentiation, adipogenic differentiation, CFU-F assay and SCXA gene expression analysis (A-D) STRO-1, CD73, CD9105, CD90 immunophenotype of stem cell surface marker were shown in surface marker analysis of hPDL cells with isotype control by flow cytometry (E) After hPDL cells were induction for osteogenic differentiation, calcium deposition was determined by Alizarin Red S staining. (F) Lipid droplet was stained with Oil Red O in adipogenic differentiation (G)Toluidine blue staining of hPDL cells in CFU-F assay, were performed to reflect self-renewal capacity. (H,I) qPCR product of scleraxis (SCXA) and periostin (POSTN) ,specific PDL marker, gene expression of samples were performed on electrophoresis gel.

Effect of compressive mechanical compression on cell viability

To determine the the effect of compressive force on cell viability, grossly cell morphology was observed concordantly with performing the MTT assay. Obviously cell death in every groups were not seen under light microscope.(Figure 3A-E) Determination of cell viability by MTT assay, did not show statistical significant differences between each groups. (Figure 3F)

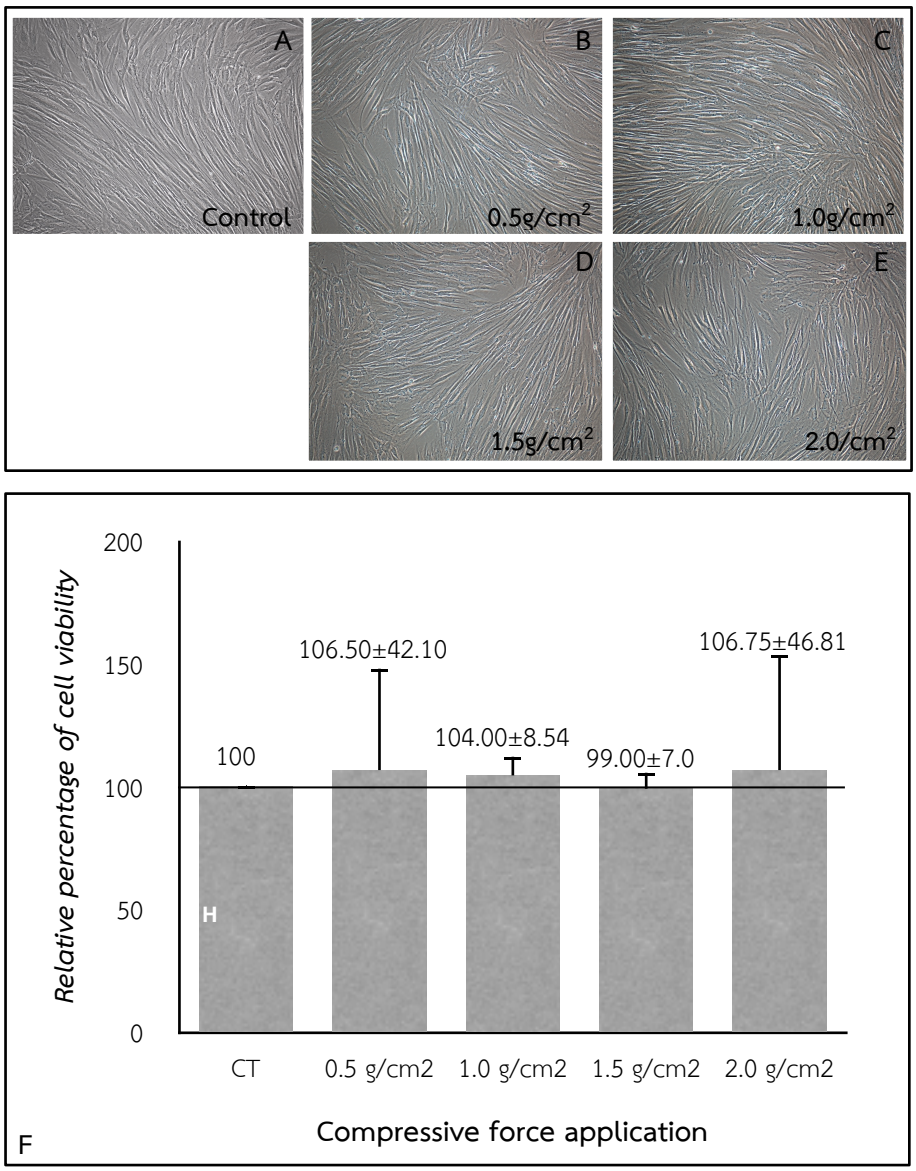

Figure 3 Cell viability under 24 hours compressive mechanical application (A-E) Spindle-shaped hPDL cells in each compressive mechanical stimuli (control, 0.5 g/cm^2, 1.0 g/cm^2, 1.5 g/cm^2 and 2.0 g/cm^2) after 24 hours were observed under 10x inverted light microscope, (F) Percentage of cell viability, measured by MTT assay, were analysed comparatively with the control group using Kruskal Wallis. Mean±SD were shown.

POSTN expression in hPDL respond to compressive mechanical stimuli in biphasic pattern

To evaluate the alteration of periostin due to force responding, periostin (POSTN) expression was determine using real-time PCR. Periostin expression responding to compressive force was performed in biphasic pattern, which gradually increased depend on increasing force magnitude then declined. POSTN was increasingly highest expressed at 1.0g/cm2 compressive force(1.59±0.16), and significantly increased compared with other groups. While, at 2.0g/cm2 POSTN was significantly decreased. (Figure 4)

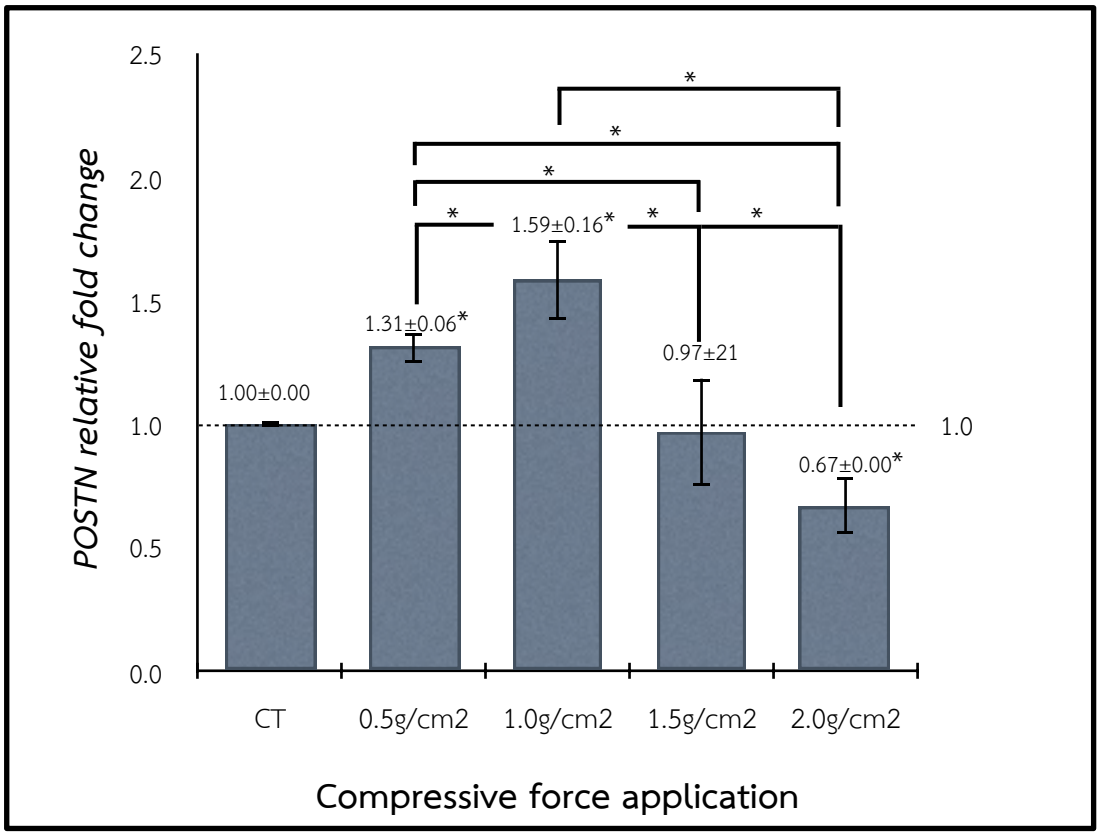

Figure 4 POSNT Gene expression responded to varied compressive fore : Relative expression values of POSTN were shown in Mean±SD. The fold change expressions were calculated comparing with control group according to ΔΔCT- method, GAPDH was used as reference gene. (*significantly difference $p<0.05$, Man-Whitney U test)

Effect of various magnitudes of compression force on stemness of hPDL cells

To evaluate influence of force on stemness, stem cell surface marker expression and CFU-F efficiency were compared between experiment groups. CFU-F efficiency was used to represent self-renewal capacity, that was calculated from performing CFU-F assay. Mann-Whitney U test was used to compared difference of CFU-F efficiency between groups. Percentage of relative CFU-F efficiency was highest increasingly expressed in 1.0 g/cm^2 compression group (126.53±26.94), and significantly increased compared with control. The significant decreased CFU-F efficiency was found in 0.5 g/cm2 compression group (79.95±12.96) compared with other groups. (Figure 5A)

Mesenchymal stem surface markers, CD73, CD90, CD105 and including STRO-1, were labeled with fluorescence and determined using flow cytometry. Percentage of CD90 in 1.0 g/cm2 compression group was expressed increasingly highest (184.32±112.45). However percentage of all stem cell surface marker expressions (STRO-1, CD73, CD90 and CD105) between groups were not found significant difference.

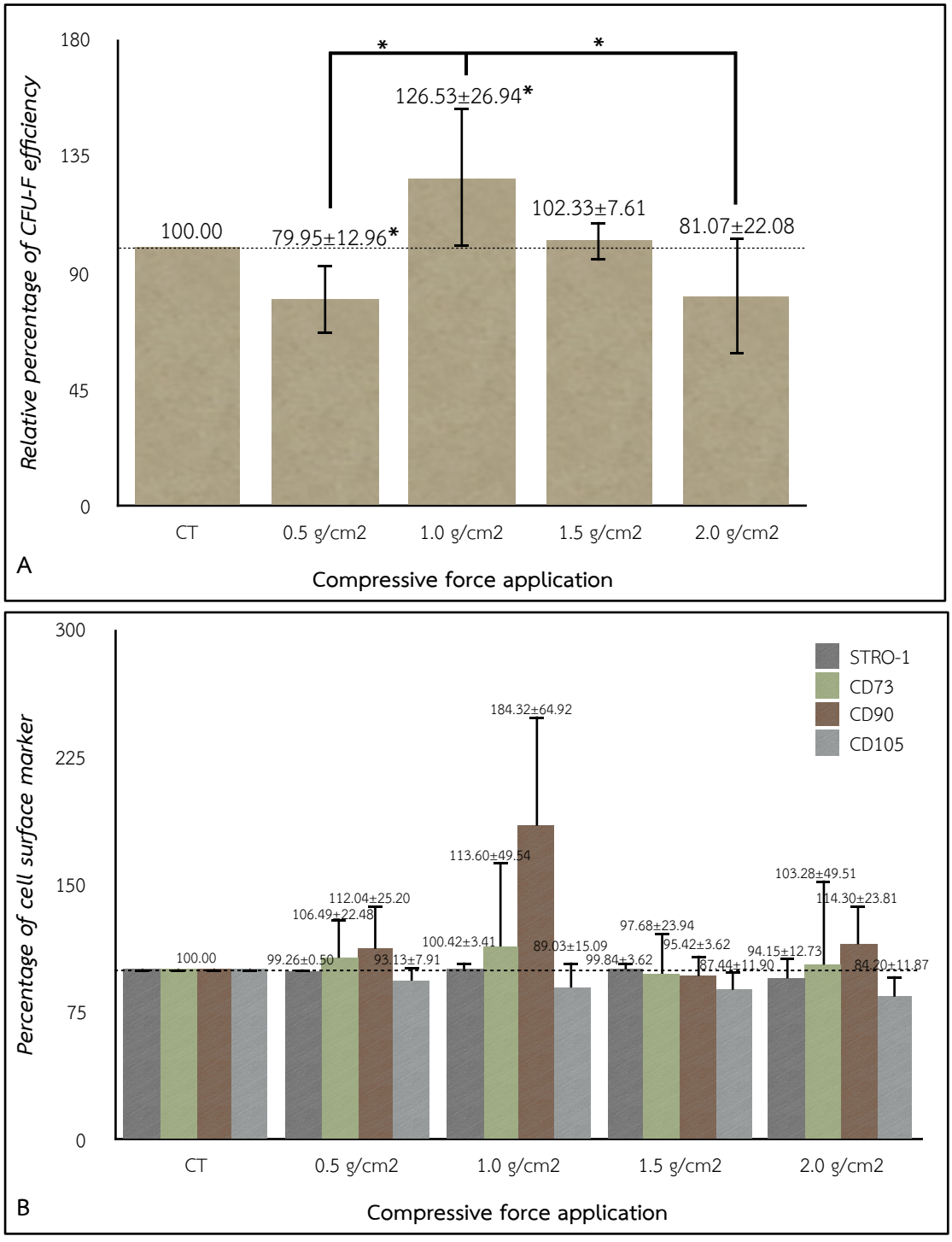

Figure 5 Self-renewal capacity and MSC cell surface markers changes in experiment groups : (A) CFU-F assay of hPDL cells in each experiment group was performed and percentage of CFU-F efficiency relatively compared with control group. (B) STRO-1 APC fluorescence labeled, CD73 FITC, CD90 FITC and CD105 PE cell surface markers in each groups were performed by flow cytometry and comparatively shown with control group. (Mean±SD, *significantly difference, $p < 0.05$, Man-Whitney U test)

Expression of mRNA stem cell marker and differentiation marker alteration

To explore effect of force on stemness deeply, determination mRNA expression of Nanog and Oct4 , important stem cell markers essential in self-renewal maintaining, was also performed. One way-ANOVA showed significant difference of Nanog and Oct4 expression between groups. At 1.0 g/cm^2 compressive force, Nanog and Oct4 expression were increased significantly, while Oct4 was decreased In 2.0 g/cm$^{2.}$. Both are compared with control group by LSD post hoc test. (Figure 5A, 5B)

TGF-β, Runx2, Osx and PPARɣ were evaluated as stem cell differentiation marker. PPARɣ, adipose cell differentiation marker, had highly expression in 1.0 g/cm^2 compression group, but there was not found statistically significant difference. (Figure 5D) TGF-β was compared using Man-whitney U test. The significant increasingly difference were found at 0.5 and 1.0 g/cm^2 compressive force when compared with control.(Figure 5C) Runx2 and Osx expression were performed in similar pattern. There was no significant difference of Runx2 expression, while Osx expression was significantly increased at 1.0 g/cm^2 compressive force when compared with control.(Figure 5E,5F)

Correlation between periostin expression and stem properties alteration in hPDL cells due to varied compressive force

A Spearman's correlation was run to determine the relationship between Magnitude of force, periostin and stem cell properties ;CFU-F efficiency, STRO-1, CD73, CD90, CD105, Nanog, Oct4, TGF-β, PPARɣ, Runx2 and Osx expression. There was a very strong, positive monotonic correlation between periostin expression and Nanog expression (=0.842, $p < .000$), strong correlation between periostin expression and TGF-β expression (=0.668, $p < .05$) and Osx expression(=0.679, $p < .05$). And there was a moderate correlation between periostin expression and Oct4 expression (=0.581 , $p < 0.05$) While another remaining factors showed no significant correlation between each other.

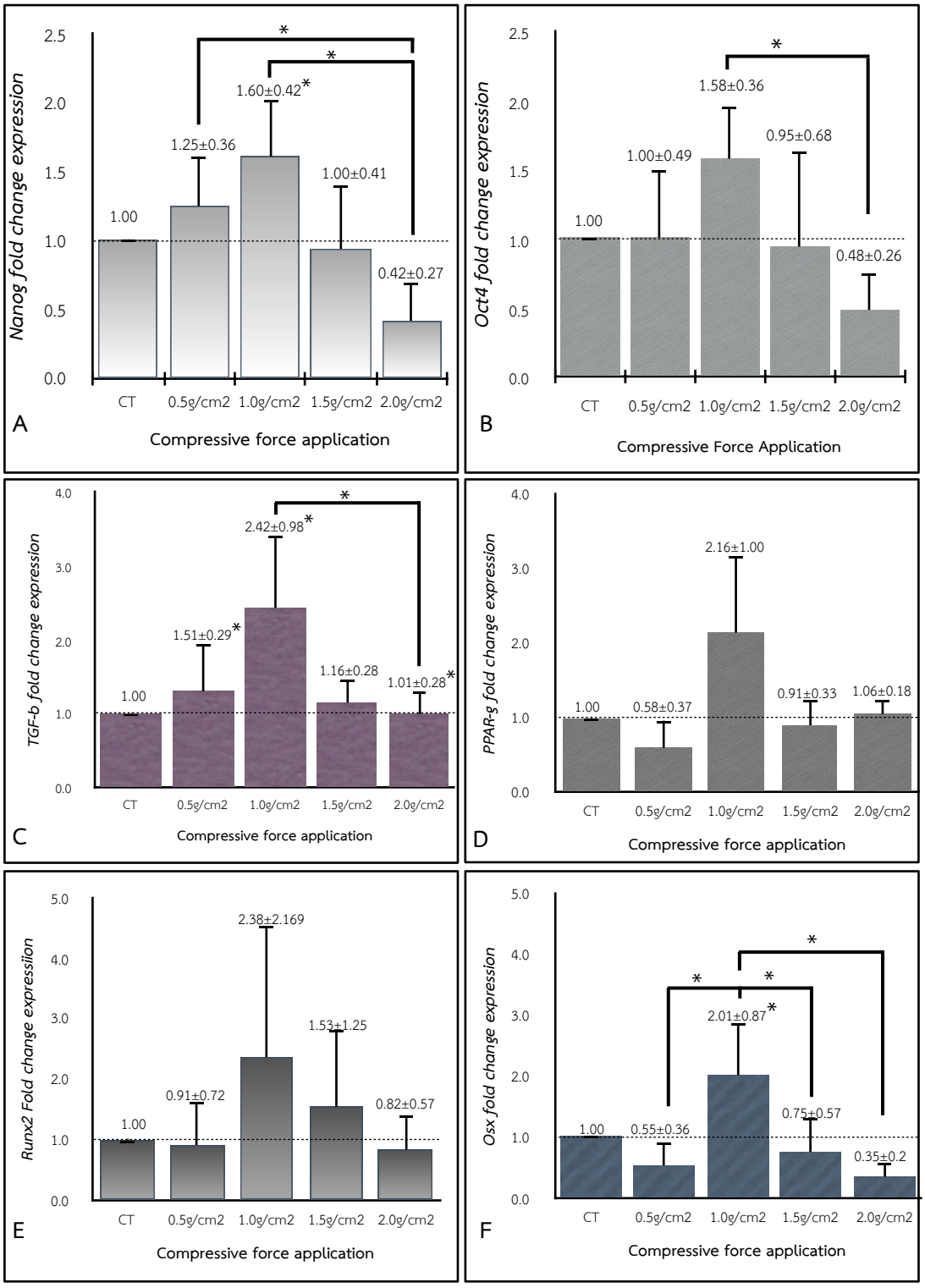

Figure 6 Nanog, Oct4, TGF-β, PPARɣ, Runx2 and Osx relative fold change expression : (A) Nanog expression (B) Oct4 expression were compared mean using ANOVA and LSD post hoc test. (C) TGF-β expression significant difference was determined using Kruskall Wallis and Man-Whithey U test (D) PPARɣ expression and (E) Runx2 were not found significant difference between group using Kruskal Wallis,and (F) Osx expression (*significantly difference $p<0.05$)

CHAPTER 4

DISCUSSION

In addition to the biomechanical factor, mechanical signals are becoming recognizing as altering cell fate factor. Orthodontic tooth movement is another interesting mechanical model for cellular response. In orthodontic tooth movement, force application induces cellular dynamic changes in the PDL and alveolar bone, which is remodeled. The sequence of cellular changes that occurs in these tissues has been extensively studied.[100-103] Many studies reported the role of mechanical compression in promoting periodontal ligament-induced osteroclastogenesis that leads to bone resorption activity.[104-106] Even if there was extensively numerous studies about orthodontic tooth movement, why the teeth can move while PDL width was maintained is still interesting clues. PDL contains some residual stem cells, that is know well as a key regulator in homeostasis and regenerative. PDL stem cell probably have a crucial role in PDL remodeling in orthodontic tooth movement. Most studies in effect of mechanical stimuli on HPDLSC used cyclic compression model to mimic force load from mastication.[8, 13, 107] Moreover stem cells need their niche, the microenvironment, to control their fate. The ECM change was reported the involvement in alteration stem properties.[59] Relationship between ECM and HPDLSC fate in previous studies was little bit mention. This study is the first time to investigate influence of static compression, various magnitude on stem properties in PDL cells and relate to ECM change.

Periostin is one of the local contributing factors in bone and periodontal tissue remodeling following mechanical stress during experimental tooth movement[15]. Periostin is very closely related to the mechanical sensor.[91] In vitro PDL fibroblasts respond to mechanical strain to regulate periostin expression. Periostin expression was shown to increase in area of compression and decrease in area of tension.[15] In this study showed the responding of periostin expression to static compression in biphasic pattern. Low magnitude of compressive force enhance periostin expression, while high magnitude of compressive force decreased periostin expression. Maximum increasing periostin expression was shown at 1.0 g/cm^2 and the decreasing was shown at 2.0 g/cm^2(Figure 4). The highest response of periostin

expression was reported at 2.0 g/cm^2, which is magnitude of force that was widely used for compression force model in in vitro studies. Because it was reported that the production of cytokine and expression of mRNAs coding for osteoclastogenic molecules reaches a peak at this level of compression force. That was conflict with this study. However, the vary in number of seeding cell, culture condition and compressive force application models between laboratory probably could alter the results. Even in clinical orthodontics, there is still no evidence-based for recommended magnitude of force for the optimal efficiency tooth movement.

The previous study suggested that periostin response to mechanical loading was controlled by TGF-β in the PDL fibroblasts, potentially through the activation of Latent TGF-β.[107] In this study, the TGF-β and periostin mRNA was expressed in the same pattern, with strong correlation (Figure 4,5C). That is concordant with the prior evidences.

Periostin is key factor for maintaining tissue integrity under biomechanical, bacterial and inflammatory challenges.[108] Periostin can regulate collagen I fibrillogenesis. Periostin deletion induces a reduction in collagen fiber diameter that result in a decreased tissue modulus of elastic.[12] In vitro, periostin expression is specifically reduced in human PDL fibroblast by the effects of inflammatory cytokines and bacteria by products. The reduction of periostin expression in heavier force experiment in this study might indicate the excessive compression force can cause overabundantly inflammation and damage the PDL tissue.

Stem cell fate, either self-renewing or differentiation, depends upon various microenvironmental cues including soluble growth factors, extracellular matrix, and mechanical forces. Self-renewal capacity is the ability to simultaneously replenish the stem cell pool that allow them to sustain tissue development and maintenance. Self-renewal capacity in this study demonstrated decreasing when apply 0.5 g/cm^2, the lowest compressive force, then increasing at 1.0 g/cm^2 compressive force. And there was no difference in another heavier force groups compared with control. These suggested that mechanical stimuli is important in maintaining stemness. Insufficient force probably causes lossing of stem cells that leads to inability to maintain tissue homeostasis. And there might be some range of

force that promotes tissue remodeling. These results assert that force possibly controls the stem cell fate.

In addition, at 1.0 g/cm2, self-renewal capacity was increased conformingly with increasing periostin expression. Studies on cardiac and cancer cells have extensively reported that periostin can bind through it FAS-I domain to the intergrin $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_6\beta_4$ and enhance cell proliferation and survival, migration and metastasis.[16, 92, 109] Thereby, Increasing periostin expression probably affects the self-renewal capacity in hPDL cell via integrin receptor.

Moreover, in this experimental design, not only the compressive mechanical stimuli were set up, but also unintentionally created the hypoxia stress condition due to very small air-contacted surface. The study demonstrated effect of hypoxia on hPDL cells that results in hypoxic cell death with apoptosis and autophagy, due to activation of the HIF-1a pathway and hypoxia was also capable of suppressing the proliferation and migration ability, changing the morphology of hPDLCs.[110, 111] The reduction of O_2 show decreasing the growth rate of hPDL cell and alteration on morphology of hPDLC. In cancer cell study, periostin can promotes invasiveness and resistance to hypoxia induced cell death.[18] Another study suggested that POSTN decreases HIF-1a accumulation in hPDL under hypoxic conditions, possibly by inhibiting TGF-β/SMAD2 signaling to regulate apoptosis.[112] In the other hand, the recent study suggest that hypoxia enhanced PDLSC clone formation and proliferation by activating the p38/MAPK and ERK/MAPK signaling pathways.[113] But in this study, there was no difference of cell viability between group to indicate the obvious cell death. However, to elucidate this question, HIF-1a might be observed and evaluate if it is involved in CFU-F efficiency alteration. That clue might cause a weak correlation between the periostin expression and CFU-F efficiency.

Downstream effectors of periostin binding to integrin include FAK, Rho/PI3 kinase, and Akt/PkB signaling pathways, which induce migration, proliferation and matrix formation. PI3K/Akt signaling regulates stemness in many stem cell system. [114] PI3K/Akt signaling plays a pivotal role in maintaining pluripotency via transcriptional factor Nanog. Nanog is an essential transcriptional factor for the maintenance of ES cell self renewal. In this studies, the expression of Nanog and

periostin have a very strong correlation. It could possibly reveal that periostin maintains stemness of hPDL cells via integrin through PI3K/Akt signaling. Adipogenic stem cell (ADSC) study has shown that cells transfected with periostin exhibited a higher proliferation rate compared with the untreated negative control ADSCs. Studies have suggested canonical Wnt signaling keeps stem cells in a self-renewing and undifferentiated state.[115, 116] Periostin could be up-regulated by TGF-β or even was raised straightforwardly from mechanical effect. To eliminate the suspicion, both TGF-β and periostin should be blocked out. Perhaps, compressive mechanical stimuli change the architecture of matrix and cell-matrix interaction directly. Then the integrin should be investigated for more

Oct4 is considered to be an important stem cell marker and essential transcription factor during human embryogenesis. Several studies suggested a role for Oct4 in sustaining self-renewal capacity of adult somatic stem cells. Some study indicated that TGF-β initiates the expression of pluripotent transcription factor Oct4 but the mechanism still unknown.[117] This study showed that Oct4 expression was increasing high and conformed with CFU-F efficiency, TGF-β and periostin expression. The correlation between Oct4 expression The under- or overexpression of Oct4 can lead to differentiation in specific tissue. No correlation was found between Oct4 and differentiation marker gene expression in this study. The decrease and increase of Oct4 expression might not reach the threshold to stimulate the differentiation.

Transducing periostin can also achieved by to Notch receptor that affect osteoblast differentiation and play important roles in anti-cell death function under mechanical stress.[118, 119] Study in aotic valve also demonstrated that periostin deletion can causes derepression of the osteogenic potential mesenchymal cells via Notch1 signaling.[120] However, other studies showed that Notch signaling could also promote osteogenic differentiation through cross-talk with BMP2 signaling. RUNX2 plays an essential role upstream of osteoblastic differentiation in osteogenic specification. Osterix (Osx) is an osteoblast-specific transcription factor required for the differentiation of pre-osteoblasts into functional osteoblasts. Osx acts downstream of Runx2. This study, Runx2 and Osx expression, was raised highest in 1.0 g/cm^2 compression group but only Osx was significantly different. As the previous study demonstrated the expression pattern of Osx after force loading as time-dependent manner while Runx2 showed rapid decreased after initial increasing.[121]

This could reveal that the compressive force induced periostin expression involved in stem cell osteogenic differentiation.

Both self-renewal capacity and differentiation were enhanced in the same magnitude of compressive force, which could mean the well regulation of stem cells was performed responding to specific range of stimuli to maintain their homeostasis. Heavy force is intolerable to keep the stem properties, and might lead to the tissue destruction. And fluffy compression is also inadequate to maintain tissue integrity thogh

In pilot studies observed the stem cell surface markers expression in hPDL between from force and non-force applied teeth and found it was altered. However, in this study, there was no difference of stem cell surface markers expression between group. hPDLs from forced applied teeth were unknown type of force and accurate magnitude. Possibly, the difference of force application became in the difference results.

HPDLSC studies usually isolated stem cell for the experiment. Even after obtaining the clinical characteristic of HPDLSCs, the cultured HPDLSCs can be induced to differentiate under experimental cell culture conditions in the laboratory that might result in vary report.[122-124]

Although the results of the present study do not provide conclusive insight as to the cause-and-effect relationships between mechanical compression and self-renewal capacity and differentiation on hPDL cell, we tentatively propose that there is some range of optimum magnitude of mechanical stimuli, represented by an increased periostin expression in compression model that might alter microenvironment and affect stem cell self renewal capacity and osteogenic differentiation, possibly *via* integrin. And excessive force should be avoid to not damage the tissue. Further studies are needed to understand more fully the role of mechanical compression on stemness maintaining and association with ECM.

CHAPTER 5

CONCLUSION

This study demonstrate that static compressive mechanical stimuli influences stem cell fate both self-renewal capacity through preservation of colony forming early progenitor cells and Nanog and Oc4 expression and osteogenic differentiation in hPDL cells in differential manner. Periostin expression, matricellular protein, responding to compressive force was shown change due to magnitude of compression force in biphasic pattern The changes in periostin expression was associated with the self-renewal capacity changes and osteogenic differentiation potential. Increased periostin due to compressive force might play a role in stem properties in hPDL, since periostin function involved Wnt signaling and PI3K/Akt signaling via integrin that regulate stemness in many stem cell systems.

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APPENDICES

Table 2. Test of normality and homogeneity of variance of Variables

Varable	Test of Normality		Homogeneity of Variance
	Komogorov-Smirnov sig.	Shapiro-Wilk sig.	sig
POSTN	0.200	0.636	0.001
NANOG	*0.200*	*0.699*	*0.112*
OCT4	*0.200*	*0.577*	*0.066*
RUNX2	0.003	0.007	0.007
TGF-β	0.030	0.008	0.066
OSX	*0.183*	*0.201*	*0.051*
PPAR-g	0.008	0.003	0.011
CFU-F efficiency	0.042	0.035	
MTT assay	0.004	0.008	
STRO-1	0.000	0.000	0.012
CD73	*0.710*	*0.216*	*0.114*
CD90	0.000	0.000	0.006
CD105	*0.015*	*0.750*	*0.127*

Table 3. Test of normality and homogeneity of variance of Variables

Varable	Test Statistic	
	ANOVA sig.	Kruskal Wallis test sig.
POSTN		0.002
NANOG	*0.016*	
OCT4	*0.037*	
RUNX2		0.606
TGF-β		0.021
OSX	*0.018*	
PPAR-g		0.086
CFU-F efficiency		0.033
MTT assay		0.777
STRO-1		0.496
CD73	*0.966*	
CD90		0.627
CD105	*0.413*	

ที่ ศธ 0521.1.03/1004

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
15 ถนนกาญจนวณิชย์
อ.หาดใหญ่ จ.สงขลา 90110

หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง อิทธิพลของแรงเชิงกลต่อความเป็นเซลล์ต้นกำเนิดของเซลล์เนื้อเยื่อเอ็นยึดปริทันต์

รหัสโครงการ EC5807-26-P-HR

หัวหน้าโครงการ รองศาสตราจารย์ ดร.ทพญ.ชิดชนก ลีธนะกุล

ผู้ร่วมโครงการวิจัย ทันตแพทย์หญิงพนิตา ปัญจมานนท์

สังกัดหน่วยงาน ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Research Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

ในคราวประชุมครั้งที่ 8/2558 **เมื่อวันที่** 20 สิงหาคม 2558

ให้ไว้ ณ วันที่ 16 กันยายน 2559

(รองศาสตราจารย์ ดร.ทพญ.ศรีสุรางค์ สุทธปรียาศรี)
ประธานคณะกรรมการจริยธรรมในการวิจัย

หมายเหตุ :- ออกแทนหนังสือรับรองฉบับที่ ศธ 0521.03/1021 ลงวันที่ 7 กันยายน 2558 เนื่องจากคณะกรรมการสอบโครงร่างวิทยานิพนธ์มีมติให้เปลี่ยนแปลงชื่อโครงการวิจัย และระเบียบวิธีวิจัย

RESEARCH ETHICS COMMITTEE (REC)
BUILDING 1 5TH FLOOR ROOM 504
TEL. 66-74-287533, 66-74-287504
FAX. 66-74-287533

FACULTY OF DENTISTRY
PRINCE OF SONGKLA UNIVERSITY
HADYAI, SONGKHLA 90112, THAILAND
TEL. 66-74-212914, 66-74-429871, 66-74-287500
FAX. 66-74-429871, 66-74-212922

Documentary Proof of Ethical Clearance
Research Ethics Committee (REC)
Faculty of Dentistry, Prince of Songkla University

The Project Entitled Influence of Mechanical Compression on Stem Cell Properties in Human PDL Cell

REC Project No. : EC5807-26-P-HR

Principal Investigator : Assoc. Prof. Dr. Chidchanok Leethanakul

Co-Principal Investigator : Miss Panita Panchamanon

Approved by Research Ethics Committee (REC), Faculty of Dentistry, Prince of Songkla University.

This is to certify that REC is in full Compliance with International Guidelines for Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Date of Approval : **16 SEPTEMBER 2016** **No. of Approval** : MOE 0521.1.03/ **1004**

(Assoc. Prof. Dr. Srisurang Suttapreyasri)
Chairman of Research Ethics Committee

บันทึกข้อความ

ส่วนงาน กลุ่มงานบริการวิชาการ ศูนย์คอมพิวเตอร์ โทร. 2109, 2116
ที่ มอ 150/152 **วันที่** 24 มกราคม 2560
เรื่อง อนุญาตใช้ซอฟต์แวร์โปรแกรม SPSS

เรียน หัวหน้าภาควิชาทันตกรรมป้องกัน

ตามหนังสือที่ มอ 670/50 ลงวันที่ 18 มกราคม 2560 ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ ได้ขอความอนุเคราะห์ให้ ทพญ.พนิตา ปัญจมานนท์ นักศึกษาหลักสูตรปรัชญาดุษฎีบัณฑิต สาขาวิชาวิทยาศาสตร์สุขภาพช่องปาก ใช้ซอฟต์แวร์ลิขสิทธิ์โปรแกรมประมวลผลข้อมูลสำเร็จรูป SPSS ของศูนย์คอมพิวเตอร์ มหาวิทยาลัยสงขลานครินทร์ ในการทำวิจัย เรื่อง Influence of mechanical compression on stem cell property in human PDL cell นั้น

ศูนย์คอมพิวเตอร์ ยินดีให้ความอนุเคราะห์การใช้ซอฟต์แวร์ดังกล่าว โดยโปรแกรมที่จัดซื้อชื่อ SPSS Statistics Bass 17.0 for Windows EDU S/N 5065845 จัดซื้อเมื่อวันที่ 23 ธันวาคม 2551 จากบริษัท อีบิซิเน็ต จำกัด ที่อยู่ 24/128 ซอยลาดพร้าว 21 แยก 3 แขวงจอมพล เขตจตุจักร กรุงเทพฯ ผลิตจาก SPSS inc. Chicago USA

จึงเรียนมาเพื่อโปรดทราบ

(รองศาสตราจารย์ ดร.มนตรี กาญจนะเดชะ)
ผู้อำนวยการศูนย์คอมพิวเตอร์

F-AD06 REV-0

VITAE

Name Miss Panita Panchamanon

Student ID 5610830009

Educational Attainment

Degree	Name of Institution	Year of Graduation
DDS.	Prince of Songkla University	2010

Scholarship Awards during Enrolment

Graduate School Research Scholarship, Prince of Songkla University 2013-2016

Faculty of Dentistry, Prince of Songkla University 2013-2016

Work – Position and Address

Dentist, Professional level, Kapho Hospital, Karubi, Kapho, Pattani 90230

E-mail : indpang@gmail.com

List of Publication and Proceeding

Panchamamon P, Leethanakul C editors Mechanical Stress Induces Human Periodontal Ligament Stem Cells Proliferation. Multidisciplinary Approach in Dentistry 2016; 2016 Dec 15-17, Centara Grand at Central World, Bangkok; 2016

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