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INFLUENCE OF OXYGEN ON THE RESPONSE OF HUMAN PERIODONTAL LIGAMENT CELLS TO MECHANICAL STRESS

Miss Jittima Pumklin

A Dissertation Submitted in Partial Fulfillment of the Requirements
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จิตติมา พุ่มกลิ่น : อิทธิพลของออกซิเจนต่อการตอบสนองของเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ต่อแรงกดเชิงกล (INFLUENCE OF OXYGEN ON THE RESPONSE OF HUMAN PERIODONTAL LIGAMENT CELLS TO MECHANICAL STRESS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ทญ. ดร.กนกพร พะลัง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ทพ. ดร.ประสิทธิ์ ภวสันต์, หน้า.

แรงกดเชิงกล เช่น แรงจากการบดเคี้ยวที่ผิดปกติ มีการศึกษาทั้งในห้องปฏิบัติการและการศึกษาในสัตว์ทดลองพบว่าส่งเสริมต่อการแสดงออกของอินซูลินไลท์โกรทแฟคเตอร์และออสทีโอพอนทินในเนื้อเยื่อปริทันต์ อินซูลินไลท์โกรทแฟคเตอร์มีบทบาทหลากหลายในเซลล์ทั้งการอยู่รอดของเซลล์ การแบ่งตัว รวมทั้งการแปรสภาพของเซลล์ ในขณะที่ออสทีโอพอนทินเป็นหนึ่งในตัวบ่งชี้ของการเกิดการแปรสภาพของเซลล์เป็นเซลล์สร้างกระดูกและยังมีบทบาทสำคัญระหว่างกระบวนการก่อรูปกระดูก นอกจากนี้ยังพบว่าแรงจากการบดเคี้ยวที่ผิดปกติและภาวะพร่องออกซิเจนถือเป็นปัจจัยสำคัญที่สนับสนุนต่อการคงอยู่ของโรคปริทันต์อักเสบ และส่งเสริมให้เกิดการทำลายอวัยวะปริทันต์ อย่างไรก็ตามกลไกของแรงและภาวะพร่องออกซิเจนต่อการทำลายเนื้อเยื่อปริทันต์ยังไม่เป็นที่แน่ชัด ดังนั้น การศึกษานี้จึงสนใจถึงบทบาทของแรงกดเชิงกลที่มีผลต่อการแสดงออกของอินซูลินไลท์โกรทแฟคเตอร์และออสทีโอพอนทินในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ภายใต้สภาวะที่มีระดับออกซิเจนแตกต่างกัน

เซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ถูกกดด้วยแรงกดเชิงกลภายใต้สภาวะที่มีออกซิเจนและสภาวะเลียนแบบการพร่องออกซิเจนจากการใช้โคบอลท์คลอไรด์เป็นเวลา 24 ชั่วโมง ทำการตรวจวัดการแสดงออกของยีนส์ที่ต้องการศึกษาด้วยวิธีรีเวอร์สทรานสคริบชัน คอนเวนชันนอลและเรียลทาร์มโพลีเมอร์เรสเชนรีแอคชัน และตรวจวัดโปรตีนด้วยเทคนิคอีไลซา รวมทั้งใช้สารยับยั้งเพื่อศึกษาเส้นทางการส่งสัญญาณต่อการแสดงออกของยีนส์

ผลการศึกษาแสดงให้เห็นว่าแรงกดเชิงกลมีผลต่อการเพิ่มการแสดงออกของยีนส์อินซูลินไลท์โกรทแฟคเตอร์และออสทีโอพอนทินในเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ในสภาวะออกซิเจนปกติ ในขณะที่สภาวะพร่องออกซิเจนแรงกดเชิงกลยังมีผลส่งเสริมการแสดงออกของยีนส์ออสทีโอพอนทินเพิ่มมากขึ้น ซึ่งตรงข้ามกับยีนส์อินซูลินไลท์โกรทแฟคเตอร์ที่ถูกกดการแสดงออกเมื่อกระตุ้นด้วยแรงกดเชิงกลภายใต้สภาวะโคบอลท์คลอไรด์ นอกจากนี้เมื่อใช้สารยับยั้งต่อทรานส์ฟอร์มมิ่งโกรทแฟคเตอร์เบต้ารีเซบเตอร์วัน สามารถยับยั้งแรงกดเชิงกลต่อการกระตุ้นการแสดงออกของยีนส์อินซูลินไลท์โกรทแฟคเตอร์และออสทีโอพอนทินได้ในทั้งสภาวะที่มีออกซิเจนและพร่องออกซิเจนจากโคบอลท์คลอไรด์ นอกจากนี้ยังตรวจพบระดับโปรตีนของทรานส์ฟอร์มมิ่งโกรทแฟคเตอร์เบต้าวันเพิ่มมากขึ้นทั้งในกลุ่มที่ถูกแรงกดและแรงกดร่วมกับโคบอลท์คลอไรด์ สอดคล้องกับการกระตุ้นเซลล์ด้วยรีคอมบิแนนท์ทรานส์ฟอร์มมิ่งโกรทแฟคเตอร์เบต้าวัน สามารถเพิ่มการแสดงออกของยีนส์ทั้งสองได้ และเมื่อใช้ทรานส์ฟอร์มมิ่งโกรทแฟคเตอร์เบต้าวันกระตุ้นร่วมกับสภาวะที่มีโคบอลท์คลอไรด์ การตอบสนองของยีนส์ทั้งสองก็สอดคล้องกับผลจากแรงกดเชิงกลร่วมกับสภาวะที่มีโคบอลท์คลอไรด์เช่นกัน

โดยสรุป การศึกษานี้พบว่าแรงกดเชิงกลส่งเสริมให้มีการแสดงออกของยีนส์อินซูลินไลท์โกรทแฟคเตอร์และออสทีโอพอนทินในเนื้อเยื่อเพาะเลี้ยงเอ็นยึดปริทันต์ โดยมีกลไกการกระตุ้นผ่านทางทรานส์ฟอร์มมิ่งโกรทแฟคเตอร์เบต้าวัน และระดับของออกซิเจนมีผลต่อการแสดงออกของยีนส์ทั้งสองเมื่อถูกกระตุ้นด้วยแรงกดเชิงกล

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5276451532 : MAJOR ORAL BIOLOGY
KEYWORDS: HUMAN PERIODONTAL LIGAMENT CELLS / INTERMITTENT MECHANICAL STRESS / INSULIN LIKE GROWTH FACTOR-1 / OSTEOPONTIN / COBALT CHLORIDE / TRANSFORMING GROWTHA FACTOR-BETA1 / HYPOXIA

JITTIMA PUMKLIN: INFLUENCE OF OXYGEN ON THE RESPONSE OF HUMAN PERIODONTAL LIGAMENT CELLS TO MECHANICAL STRESS. ADVISOR: ASST. PROF. KANOKPORN BHALANG, Ph.D., CO-ADVISOR: PROF. PRASIT PAVASANT, Ph.D., pp.

Mechanical force, i.e. occlusal trauma was shown to promote insulin like growth factor-1 (IGF-1) and osteopontin (OPN) expression in periodontal ligament both in vitro and in vivo. IGF-1 plays a role in various cellular activity, including survival, proliferation, and differentiation while OPN is one of the intermediate protein for bone remodeling. Occlusal force and hypoxic condition are considered as the facilitating factors for periodontitis which is a worldwide disease resulting in the destruction of the periodontium. However, the mechanism by which force and hypoxia contributing to periodontal destruction is yet unclear. Thus, this study investigated the influence of the intermittent mechanical stress on *IGF-1* and *OPN* expression by human periodontal ligament cells (HPDLs) under normoxia and hypoxia.

The intermittent mechanical stress was applied to HPDLs with or without cobalt chloride ($CoCl_2$) for 24 hours. The gene expression was examined by conventional and real-time polymerase chain reaction. The protein expression was examined by ELISA assay. The signaling pathways regulating gene expression were investigated using chemical inhibitors.

The results showed that both *IGF-1* and *OPN* mRNA expression increased in the intermittent mechanical stress treated group and $CoCl_2$ synergistically enhanced the intermittent mechanical stress-induced *OPN* expression. In opposite to *IGF-1*, $CoCl_2$ attenuated the intermittent mechanical stress-induced *IGF-1* expression. The TGF-β receptor I inhibitor (SB431542) abolished *IGF-1* and *OPN* mRNA expression induced by intermittent mechanical stress with and without $CoCl_2$. Furthermore, the intermittent mechanical stress could induce TGF-β1 protein release in the presence and absence of $CoCl_2$. HPDLs treated with recombinant transforming growth factor-beta1 (rhTGF-β1) significantly upregulated both *IGF-1* and *OPN* mRNA levels. However, the combination of rhTGF-β1 and $CoCl_2$ significantly downregulated *IGF-1* expression while, this condition could upregulate *OPN* expression.

In conclusion, the results suggested that intermittent mechanical stress induced *IGF-1* and *OPN* expression in HPDLs through TGF-β1. The level of oxygen influenced to this phenomenon in HPDLs.

Field of Study: Oral Biology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

In oral cavity, mechanical stress was generated in many situations, such as mastication, functional/parafunctional habits, orthodontic treatment, and occlusal trauma. Occlusal force plays a pivotal role in the regulation of periodontium homeostasis (1-3). The mechanical force in the range of physiological condition involved in the maintaining of the periodontium system (4). However, the force exceeding physiological limitation could lead to pathological change, such as periodontal ligament (PDL) space widening, periodontium destruction, and alveolar bone resorption (5, 6). Periodontal ligament cells (PDLs) act as osteocytes in term of mechanosensor to receive and respond to mechanical stress. The mechanical forces also involve the fluid flow in periodontal tissue and PDLs perceived this stimulus (7). Several evidences demonstrated the effect of mechanical stress to cellular response, including PDLs (4, 8). I*n vitro* and *in vivo* study demonstrated that mechanical stress influenced PDLs behaviors. Moreover, the mechanical stimuli generated to PDLs is important for repairing the damage matrix, regulating alveolar bone remodeling as well as transferring signal to the surrounding cells and tissue (9). Understanding the PDLs behavior under physiologic and pathologic force will enhance the knowledge of biological response of PDLs in health and disease (10).

The mechanical force, especially traumatic occlusal force, is also considered as another factor related to periodontal diseases (11). The molecular mechanism(s), in which traumatic occlusal force enhances periodontal disease progression, is yet

unclear. Several clinical studies demonstrated that the occlusal force is a significant factor, influencing the progression of periodontitis (12-14). Previous studies showed the role of PDLs in responding to mechanical stress (7, 15, 16) such as increasing production of interleukin-1β (IL-1β) (17, 18), interleukin-6 (IL-6), interleukin-8 (IL-8) (18), insulin-like growth factor-1 (IGF-1) (19, 20), as well as osteopontin (OPN) (21). PDLs responded to static compressive stress by releasing ATP to induce IL-1β (22, 23), receptor activator of nuclear factor-κB ligand (RANKL)(24, 25), and osteopontin (21, 26). Further, increasing intracellular calcium (22, 27), changing actin filament organization (28), and upregulating of several cytokines or growth factor (19, 20, 29) were detected.

IGFs consist of several family members such as IGF-1 and IGF-2 (30). IGF-1 plays a role in various cellular activity, including survival, proliferation, and differentiation (31-37). IGF-1 is involved in several kinds of cells and tissues (30) while IGF-2 is important during prenatal development (32). IGF-1's role in bone homeostasis is well investigated. The osteogenic response under 4-point bending study was determined in WT mice but did not observe in the mice-deleted IGF-1 (38, 39). It has been illustrated that human PDLs (HPDLs) expressed the IGF-1 receptor, implying the ability to stimulate IGF-1 (40). Correspondingly to the study of IGF-1 distribution in both primary and permanent teeth, it was found that major of IGF-1 localized in the periodontium and suggested that PDL act as an IGF reservoir (32). Previous report showed that IGF-1 enhanced HPDLs survival by down-regulating pro-apoptotic molecules and inducing anti-apoptotic molecules (33). Moreover, IGF-1 was shown to promote proliferation, osteogenic differentiation in HPDLs and IGF-1 is a key mediator for wound healing process (35). In addition, dextran-co-gelatin

microspheres release local IGF-1 enhance regeneration of periodontium (41) and the combine effect between IGF-1 and other growth factors (GFs) increase bone formation and periodontal regeneration (42). It was noted that IGF-1 plays a key role in periodontium homeostasis. Although the relationship between mechanical stresses and upregulated IGF-1 release in PDLs were investigated (18, 19, 29), the molecular mechanism by which mechanical stress stimulates IGF-1 expression is yet unclear.

OPN is a multifunctional protein, participating in both physiological and pathological conditions. OPN is one of the intermediate markers for osteogenic differentiation and also plays a crucial role during bone remodeling (43-45). OPN is essential in regulating the attachment, adhesion including osteoblast and osteoclasts spreading on bone surface in the process of bone remodeling (43). The study of unloading using a tail suspension model in wild type and OPN knockout mice showed that unloading decreased bone formation and increased bone resorption in wild type mice. Nevertheless, these phenomenon did not occur in the OPN knockout mice. Thus, it was suggested that the function of OPN is closely related to bone remodeling under force application (46, 47). OPN expression was upregulated by various GFs, including transforming growth factor-β (TGF-β) (48, 49). Beside GFs, the OPN gene promotor contains Stress Response Element (SRE) for response to mechanical loading (50). In HPDLs, it has been shown that HPDLs upregulated OPN by static mechanical stress via ATP release (21, 26). Furthermore, it has been shown that OPN involved in inflammatory process. In this respect, OPN regulated the recruitment and migration of immune cells to inflamed site such as neutrophils, macrophages and T-cells d site (7, 48, 51). Clinically, the levels of OPN in gingival crevicular fluid (GCF) correlated with inflammation of periodontium (52-54). These data imply the interesting role of

OPN in mechanical stress related periodontal disease. Several techniques were employed to investigate the effect of mechanical stress *in vitro*, for example centrifugal force (3), weighted metal/glass cylinders (21), tensile strain (20), shear stress (55), and cyclic tensile stress (56, 57). However, the static mechanical stress may not directly relate to clinical situations since the periodontal tissues may exposure to the intermittent force during chewing cycle. Therefore, intermittent mechanical stress is represented the actual mastication better than other forms of mechanical stress.

Periodontitis is one of the major problems in oral health worldwide. This disease is a cause of periodontium destruction (58, 59). Generally, periodontitis is known as a chronic and progressive disease associated with low oxygen concentration in periodontal pocket (60-63). It has been demonstrated that the oxygen levels was lower that 2% in deep periodontal pockets (60). Further, the hypoxic condition was shown to promote apoptotic process and to enhance the *P. gingivalis* lipopolysaccharide-induced inflammatory mediator expression in PDL (64-66). Moreover, hypoxia directly plays an essential role in periodontium destruction via RANKL expression (67). Thus, the low oxygen concentration could be one of the crucial factors promoting periodontitis's progression.

The physiological force is one of the key factors in maintaining periodontium homeostasis (4). However, the knowledge regarding the influence of intermittent stress on PDL's behavior is yet lacking. Hence, the aims of this study were to examine the influence of intermittent mechanical stress on the *OPN* and *IGF-1* expression by HPDLs. Moreover, in pathological condition with low level of oxygen such as periodontitis, the physiological force may lead to tissue destruction (11, 13).

Thus, the combination of intermittent mechanical stress and hypoxia on *IGF-1* and *OPN* expression were also investigated. The intracellular signaling participating in these regulations was examined.

Research question

1. Do HPDLs respond to intermittent mechanical stress under *IGF-1* and *OPN* regulation?
2. Does oxygen influence *IGF-1* and *OPN* expression in HPDLs stimulated with intermittent mechanical stress?

Objectives and hypothesis

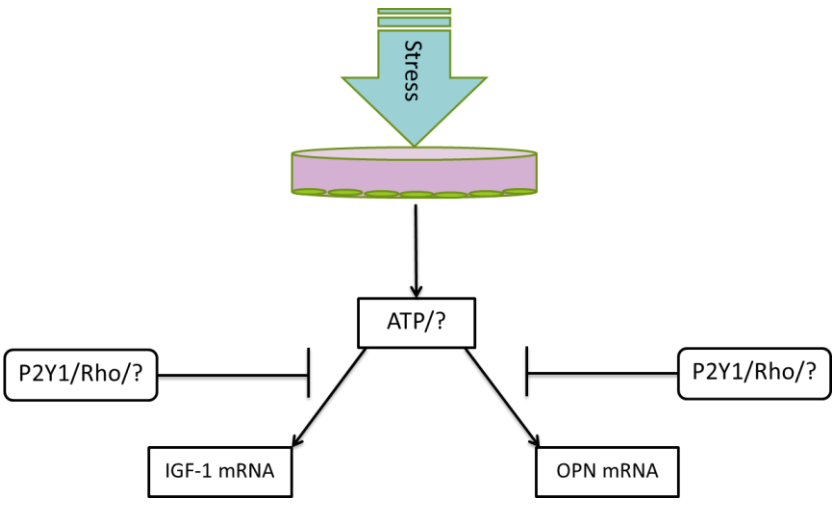
Objective 1.1: To investigate the role of intermittent compressive stress in regulating *IGF-1* and *OPN* expression in HPDLs.

Hypothesis: HPDLs stimulated by intermittent mechanical stress increase the mRNA level of *IGF-1* and *OPN* higher than the unstimulated group.

Objective 1.2: To investigate the intracellular signaling of intermittent mechanical stress in regulating *IGF-1* and *OPN* expression in HPDLs.

Hypothesis: Intermittent mechanical stress regulates *IGF-1* and *OPN* expression via the releasing of ATP.

Experimental design:



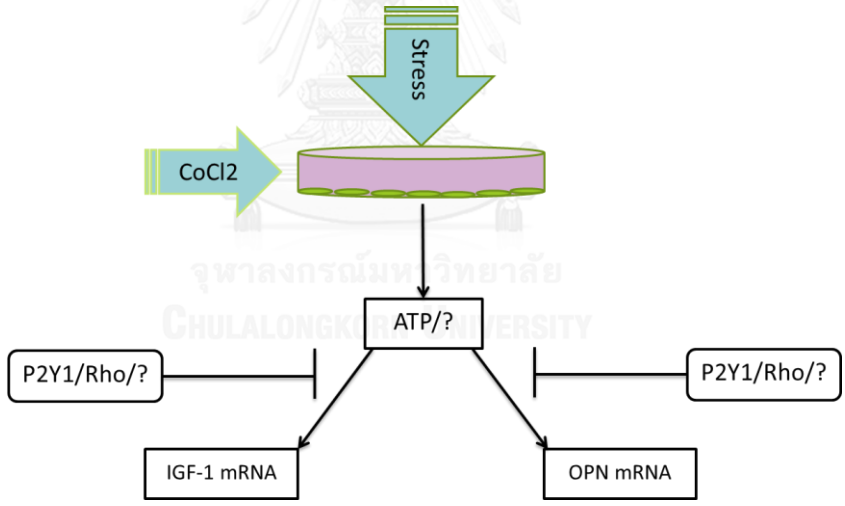
Objective 2.1: To investigate the influence of oxygen on *IGF-1* and *OPN* expression nder intermittent mechanical stress treated HPDLs.

Hypothesis: HPDLs activated by intermittent mechanical stress regulate *IGF-1* and *OPN* under chemical hypoxia is different from that in normoxia.

Objective 2.2: To compare the intracellular signaling of intermittent mechanical stress in regulating *IGF-1* and *OPN* under chemical hypoxia and normoxia in HPDLs.

Hypothesis: The intracellular signaling of intermittent mechanical stress regulates *IGF-1* and *OPN* expression in HPDLs in chemical hypoxia is different from that in normoxia.

Experimental design:



Expected benefit

The knowledge gained from this study will increase our understanding of the influence of oxygen on the response of human periodontal ligament cells (HPDLs) to intermittent stress. It will help us understand the biological response and the mechanism of HPDLs to intermittent mechanical stress under different level of oxygen.

Keywords

- Intermittent mechanical stress
- Hypoxia
- Cobalt chloride
- Human periodontal ligament cell
- Insulin-like growth factor-1
- Osteopontin
- Transforming growth factor-β1

Research design

Laboratory experimental research

CHAPTER II

REVIEW OF RELATED LITERATURE

The role of mechanical loading and biological response

Mechanical stimuli are important in cellular responses. Mechanotransduction system in the cell can translate the physiological signal to biochemical signal and finally cellular response. The immediate response of the cells to mechanical stimuli occur in seconds to minute and the biochemical signals occurs through several signaling mechanisms (68). In vitro and in vivo studies investigated the relation of mechanical loading and bone behavior (39, 69-71). However, the role of mechanical stimuli is not only influent bone homeostasis but also affect normal tissues and organ function in our body. In bone biology, the physiological force maintains the bone volume by regulating both bone resorption and bone formation. On the contrary, bone loss will be happen if the stimuli are absent. The cells that respond to mechanical stimuli in the bone are osteocyte which is the majority of cells in the bone. Osteocyte can connect between the cell and surrounding environment through long cell processes (69). Osteocyte activated by mechanical stimuli could release some molecules which are important in bone homeostasis such as endothelial/inducible Nitric Oxide Synthase (eNOS/iNOS) (72), Nitric Oxide (NO) (73), Prostaglandin E2 (PGE_2) (74), Connective tissue growth factor2 (CCN2) (75) including IGF-1(70) and OPN (50, 76). Pathological forces such as hypertension also generate the force to the cells, high pressure blood flows generate as like as sheer stress. The vascular endothelial cells can respond to this force by releasing inflammatory cytokine, growth factors, adhesion molecules and enzymes. All of these molecules are important to

cellular adaptation to the force (77, 78). As mentioned above, cells in our body can response to both of physiological force as well as pathological force.

Currently, it remains unclear how the mechanical stimuli can transmit the signal into biological response. However, in present knowledge, the influence of stressor effect to cell biological response can be summarize in fig. 2.1 (79, 80):

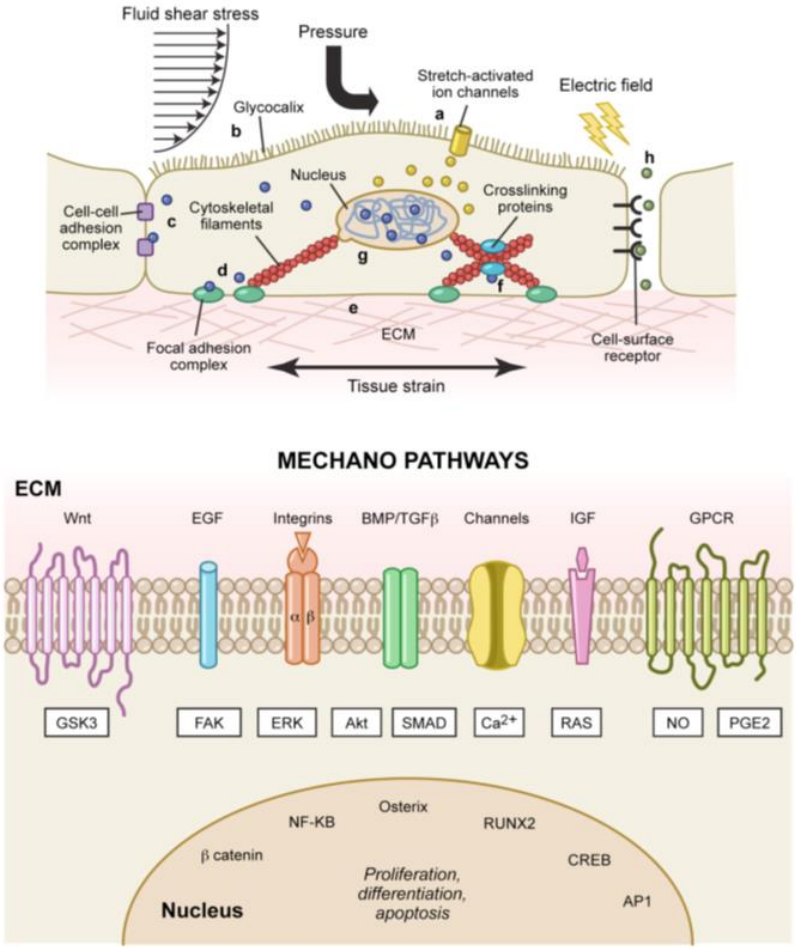


Figure 2.1 The current knowledge of cellular response to mechanical stimuli. (79).

Mechanical loading in oral cavity

The physiological function in oral cavity is mastication, deglutition and speech which are closely association with mechanical force. Pathological force can also be found in oral cavity, for example bruxism, parafunctional habit, trauma from occlusion and orthodontic treatment. Several cell types in oral cavity can respond to the mechanical forces. A study investigated the relationship between vertical facial type and maximum occlusal force. The maximum occlusal force measured from primary first molar is 524.5±153.0 N, 389.7±162.8 N and 272.6±149.1 N in brachyfacial, mesofacial and dolichofacial type, respectively (81). From this evidence, the vertical facial type is related to occlusal force. However, many factors have to be considered that can affect occlusal force such as sex, age and body shape (82, 83). The occlusal force of the boy is higher than the girl with the same age. In addition, the occlusal force will be increased when one gets older (83).

Occlusal trauma or trauma from occlusion is an occlusal force as a result of injury of periodontal attachment apparatus (14, 84). Trauma from occlusion can be classified to two types. First, excessive occlusal force applies to healthy teeth which have a normal supporting structure, this is primary trauma from occlusion. Second, normal occlusal force or excessive occlusal force applied and harmful to pathologic teeth, this is secondary trauma from occlusion (14, 85). Previous results from our group found that by applying compressive force to human dental pulp cells (HDPCs), representing inflammation situation or restorative procedures, HDPCs increased IL-6 expression via P2Y6 (86). Moreover, the human exfoliated deciduous teeth (SHED), in same technique loading they showed that compressive stress regulated IL-6 to activate Rex-1 expression via ATP-P2Y1. This result suggests the role of compressive

stress in SHEDs relate to maintain stemness properties (87, 88). In contrast to other study developed a novel technique to mimic the biting force. HDPCs activated by this technique showed increase HDPCs osteogenesis to promote bone formation (89). Taken together, the cellular response to mechanical stimuli is different depend on mechanical stress technique, cell type, duration of stimuli or amount of force.

Periodontium consist of 4 part including cementum, gingiva, alveolar bone and periodontal ligament which is a specialize connective tissue cover the tooth's root and connect between cementum into alveolar bone. The periodontal ligament tissue shows highly vascular innervation. Thus, it has highly turnover rate of the cellular and extracellular component to the physiologic and pathologic mechanical stimuli. Periodontal ligament consist of both cell and extracellular component. Various kinds of cell can be found in periodontal tissue including endothelial cells, fibroblasts, and epithelial cell rests of Malassez, cementoblasts and also osteoblast. Among them, the numerous of cell in healthy periodontal ligament is a fibroblasts. PDL-fibroblast plays a key role and is often referred to as PDL cells (PDLCs). Moreover, the periodontal ligament is a mechanoresponsive of the tooth and shows adaptation to the mechanical loading (4, 7, 90).

Several in vitro studies showed that the mechanical loading play an essential role in periodontium homeostasis (8, 16, 91). Similar to other cell type, PDLs also shows various responses to the mechanical stimuli. Previous studies in our groups, plastic cylinder containing metal coins was used to generate compressive forces. The results showed that HPDLs respond to compressive stress by releasing ATP to activate Rho kinase via P2Y1 and finally induce OPN expression (21, 26).

Nevertheless, releasing of ATP from compressive stress in HPDLs not only activate OPN expression but also effect to RANKL expression through prostaglandin E2 (PGE2) (24) and ATP could upregulate IL-1β via P2X7 receptor (22, 23). However, the previous study investigated that intermittent force mimic orthodontic treatment could activate RANKL expression via IL-1β (17). The summarized of static compressive stress to HPDLs propose in Figure 2.2.

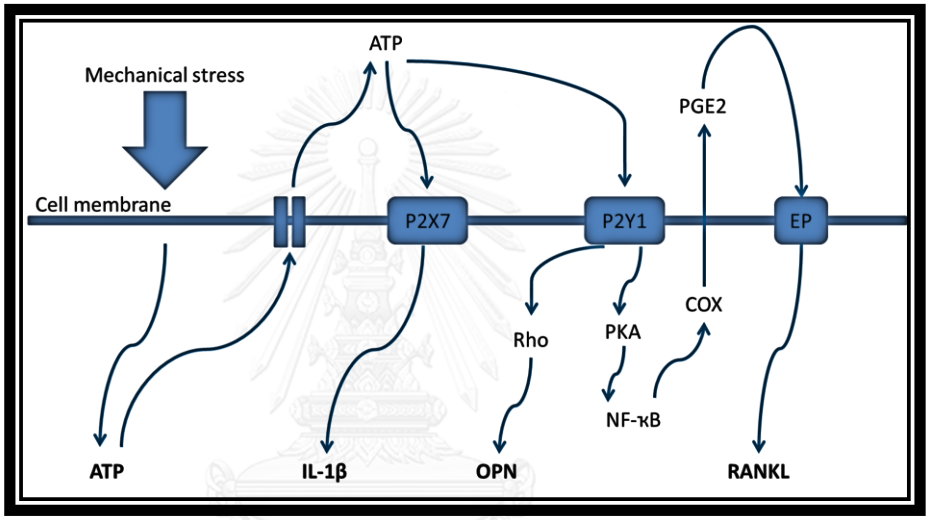


Figure 2.2 The diagram demonstrated the proposed signaling mechanism of the static compressive stress regulated HPDLs's behavior.

The influences of occlusal force in vivo models were investigated. Hypo-occlusal stimuli was generated in rat model, the data indicated that hypo-occlusal stimuli decreased PDLs proliferation by downregulate the expression of IGF-1 and IGF-1R. The recovery occlusal stimuli could reverse this effect in rat PDLs (29). Hyper-occlusal stimuli were used to study in mouse model represent the clinical trauma from occlusion. The results showed that hyper-occlusal force promoted osteoclastogenesis (92, 93) and upregulated both OPN and RANKL (92). Moreover,

the alveolar bone deposition was decrease in hyper-occlusal group when compared with control group (92).

The majority of studies mechanotransduction signaling on PDLs not only focused to regulate small signaling molecule and gene expression but also effect to behavior of PDLs and surrounding tissues. Studies of the effects of mechanical stress on the osteogenic differentiation of PDLs are widely investigated. Cyclic tensile stress to PDLs showed upregulated of osteogenic differentiation via ERK1/2/Elk1 MAPK pathway (16, 56, 91). However, some in vitro (94) and in vivo (95) evidence showed different result. Compressive stress activated PDLs upregulate ephrin-A2 expression which plays an essential role in bone homeostasis. Ephrin-A2 interacts with their receptor on osteoblast or PDLs and down-regulate Runx2/ALP which are osteogenic marker. The suggestion of this study is down-regulation of osteogenic differentiation by osteoblast or PDLs and promotes the bone resorption at the pressure site in orthodontic force (94). In addition to in vivo studies, they showed that constant force decreased Runx2 expression and PCNA-positive cells in rat model (95). Furthermore, cyclic stain can induce early apoptosis by upregulate caspase-3 via caspase-9 pathway in PDLs (96, 97). As described above, to understand of the PDL's response under physiologic and pathologic force might enhance the understanding of the PDL's behavior in health and disease. Thus, Fill et al. review and summarize the factor that influence the response of PDLs to mechanical stress is shown in Table 2.1 (10).

As described above, occlusal trauma is not applying only healthy teeth but also apply in the pathological teeth such as periodontitis. The relationship between trauma from occlusion and progression of periodontitis is not fully understood.

However, the main principle of periodontitis treatment is both eliminate the dental plaque to reduce the bacteria and following control the occlusal factors (84). The scientific evidence showed that heavy occlusal force increased tooth mobility by alveolar bone resorption and this effect is reversible if eliminate the occlusal force (14). Similar to the combination of inflammation and occlusal trauma (IO) in rat models showed that IO group increased osteoclast number, attachment loss and distribution of the area of immune-complex formation when compared to the control (13). Therefore, control the occlusal factor is an essential procedure for periodontitis treatment. The occlusal reduction in periodontitis teeth combined with eliminate the dental plaque showed decreasing of tooth mobility and eliminate the alveolar bone loss compared with the one without reduction (11).

Table 2.1 The influence factors affecting the periodontal ligament (10).

Factor	**Specifics**
Geometric configuration of the periodontium	N/A
Size and shape of tooth root	Bicuspid, canine, molar, and so forth
Region of the PDL	Regional differences and thickness
Physiological	Age, ethnicity, race, gender, and genetics
Environment	Dental and overall physical health, diet
Type of loading	Loading frequency, strain rate, loading velocity, and load direction
Material mechanics	Nonlinearities, compression/shear coupling, and intrinsic viscoelasticity

The model of mechanical loading for periodontal ligament

General physical function in oral cavity is mastication. During chewing, the intermittent force applies to the teeth and generates force into periodontium especially PDLs. The intermittent force or cyclic stain is necessary for periodontium homeostasis. However, some situation in PDLs such as orthodontic treatment did not generate intermittent mechanical loading but static compression is more represent. Therefore, several loading technique were used to study in PDLs. At present, the classification of mechanical loading system in PDLs divided to 2D culture system (18, 21, 26, 91) and 3D culture system (9, 98, 99).

2 dimensional (2D) cultured system

1. Cyclic tensile stain system

This is the most widely method to use in PDLs and has the commercial system such as Flexercell apparatus (100, 101). The principle of cyclic tensile stain is reforming the surface that cell attachment. The PDLs were cultured in silicone membrane that attach to the loading post and the silicone membrane were stretched by vacuum pressure. This system represents to cyclic tensile stain and can set the frequency of stretching. For PDLs, the frequencies of cyclic tensile stain start from 0.2 to 0.5 Hz, is commonly investigated (9) (Fig 2.3).

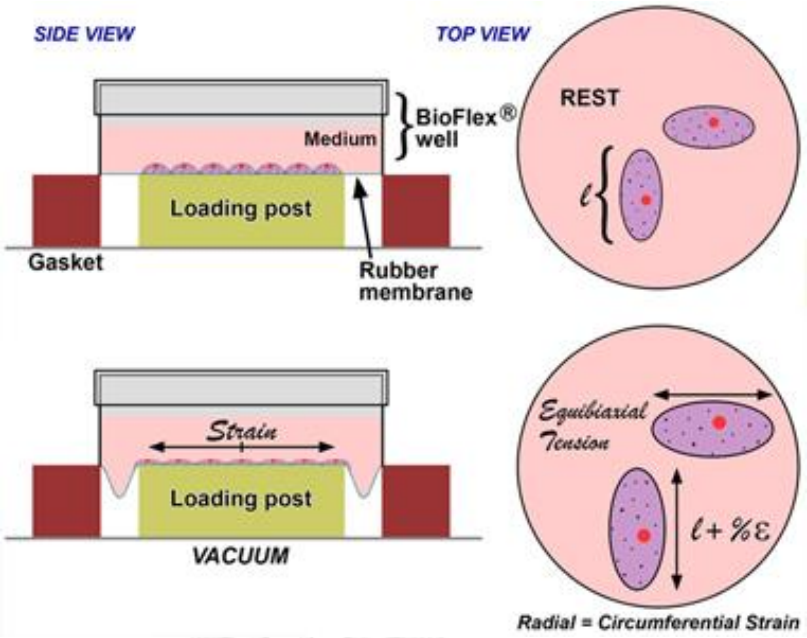


Figure 2.3 Flexercell compression systems for 2D monolayer cell culture

(http://www.flexcellint.com/applications1.htm)

2. Compressive weight loading system

This method can generate static compressive stress and unidirectional to monolayer cell culture. Generally, this method used to represent pressure site in orthodontic treatment. Principle of compressive weight loading apparatus is very simple. The glass cylinder or metal coins were required to put over the cultured cells. The number of glass cylinder or coins use for adjust the amount of compressive force. The normally amount of force in PDLs is estimate 0.5–5 g/cm^2. The most commonly used is 2 g/cm^2 (9). In addition, this technique is very simplest and easier method. Furthermore, it does not require a special tool for generate the stress (Fig 2.4) (18, 21, 26).

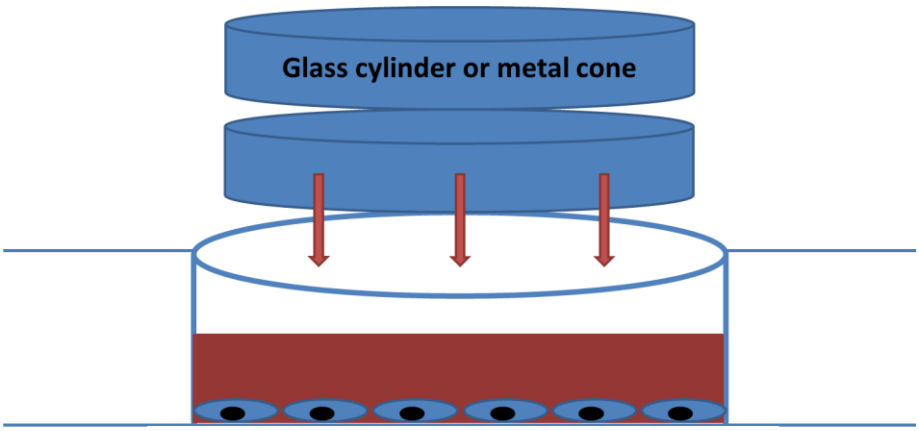


Figure 2.4 The compressive weight loading method.

3. Centrifugal force system

Centrifugal force is one of the static compressive force as same as compressive weight loading method. This method uses the special device to generate the force which is laboratory centrifuge. The range of centrifugal forces applied to PDLs is start from 10-50 g/cm^2 for represent pressure site of orthodontic forces. Although, this method is compressive force but it is not static force like weight loading method because the centrifuge is faster to move than weight loading system and the force that apply to the cell is like a shear stress (Fig 2.5) (9, 94, 102).

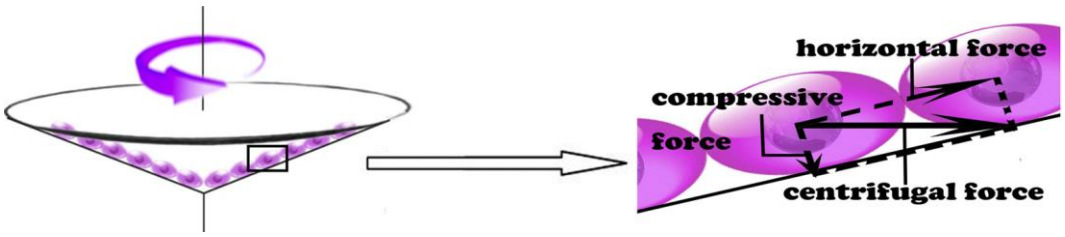


Figure 2.5 The centrifugation method (9).

4. Fluid sheer stress system

The cells were cultured in the environment that medium fluid flow. This system use to represent a blood flow in capillary, fluid flow in canaliculi of bone cells, fluid flow in dental pulp including PDLs when mastication (69, 77, 78, 103). Previous evidence demonstrated that PDLs responded to fluid flow by releasing of PGE2, NO and decreased alkaline phosphatase activity (104). Figure 2.6 present the device for fluid sheer stress system.

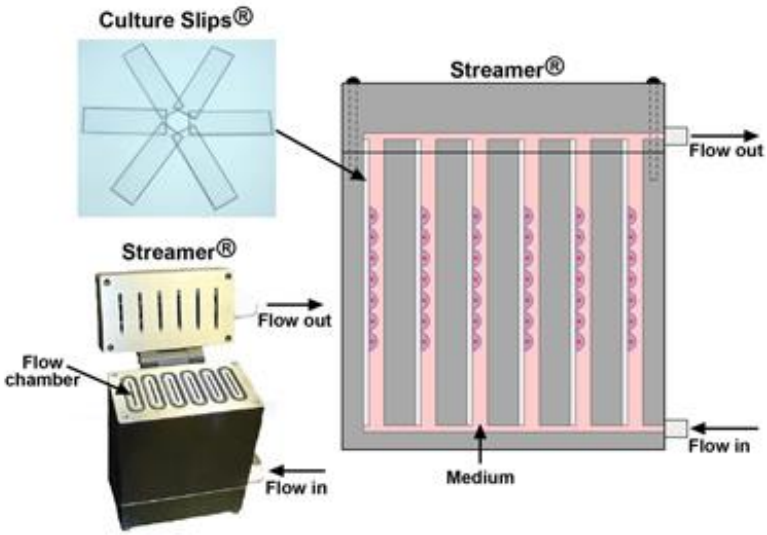


Figure 2.6 Fluid shear applied to cells in Streamer® device.
(http://www.flexcellint.com/applications1.htm)

5. Vibration system

PDLs were cultured in culture plate or container that recommend. Vibrator is used to generate the force to the cultured cells and adjust the amplitude or frequency to match with clinical situation (9, 105, 106). For PDLs, the range of amplitude and frequency is 0.3 g and 10–180 Hz, respectively. The effect of vibration method in

PDLs was investigated. The data showed that vibration-stimulated PDLs decreased proliferation. In contrast, it could increase markers of osteogenic differentiation in a frequency-dependent manner (106). The vibration system is showed in figure 2.7.

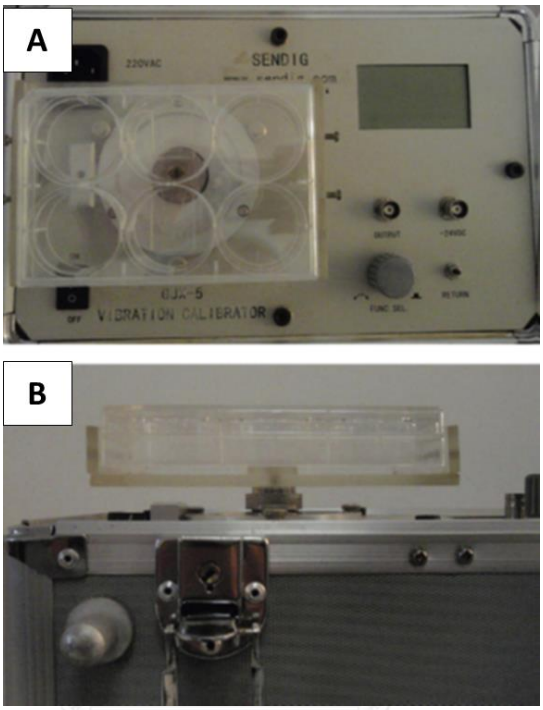


Figure 2.7 The GJX-5 vibration machine which use in PDLs vibration method. (A) Frontal view (B) Lateral view (106).

6. The hydraulic cylinder loading system

Mastication or orthodontic treatment creates a hydrostatic pressure that transmits to the periodontium. This machine principle is the hydraulic cylinder force directly to the cell medium. The type of force, amount of force or duration can set by software program. Cells were cultured in 6-well plate that the well fit to the cylinder plastic tube and the plate transfer to place at the platform of the machine. After setting the program, the hydraulic pumping part will be load to the cell medium and generate

the force. This system can generate two type of force; static force and intermittent force (Fig 3.1).

As describe above, to understand the principle of the mechanical loading system is very important to choose a suitable model for the specific research purpose. The appropriate indication of each system concluded in table 2.2 (9).

Table 2.2 The summarize of mechanical loading system (Adapted from Yang et. al., 2015) (9).

Loading system	**Characteristic**	**Simulation**	
		Masticatory force	Orthodontic force
Cyclic tensile stain system	Cyclic tension or static tension or compression	Suitable	Barely suitable
Compressive weight loading system	Static unidirectional compressive stress	Barely suitable	Suitable
Centrifugal force system	Static compressive stress	Barely suitable	Nearly suitable
Fluid sheer stress system	Discontinuous fluid shear strain	Barely suitable	Barely suitable
Vibration system	Vibration with adjustable frequency	Barely suitable	Barely suitable
The hydraulic cylinder loading system	Static or intermittent compressive stress	Suitable	Suitable

3 Dimensional (3D) cultured system

Presently, the in vitro researches about the effect of mechanical stress to PDLs were performed using 2D cultured system. However, 2D cultured system has a limitation to interpretation in the real situation. Therefore, the 3D cultured systems were developed to fill this gap. In 2D cultured system, the cells were cultured in monolayer. In opposite to 3D cultured system, the cells were plated in the scaffold. As

described above, the position of the cell may affect the mechanoresponse of the cell (9).

1. Cell in Scaffolds used for 3-D culture system

Mainly of extracellular matrix component in periodontal tissue is collagen type I (90). From this reason, the collagen type I is the most commonly used for scaffold in PDLs (2, 27, 78, 98). In addition, numerous type of scaffolds were developed to use in 3D cultured system. They mentioned that elastic modulus of PLGA is much higher than collagen and closely to natural PDLs (107, 108). Moreover, some study developed the new in vitro model and expected that it represent as same as the real mastication in human (89).

The effect of 2D cultured and 3D cultured systems were investigated in PDLs response (Fig. 2.8). The cDNA microarray was analyzed and found that mechanical force application in 3D cultured system regulated the 553 gene expression compare to 191 gene expression in 2D cultured system (109). Thus, it can conclude that mechanoresponse in PDLs depend on the position of the cells. Moreover, the responses of PDLs in 3D culture mechanical stress system were identified by microarray. They showed that 3 dimensional PDLs culture were stimulated by static force upregulated 85 genes and downregulated 23 genes expression. In addition, the genes were related in several cellular response including cell communication, cell signaling, cell cycle, mechanoresponse (27, 110).

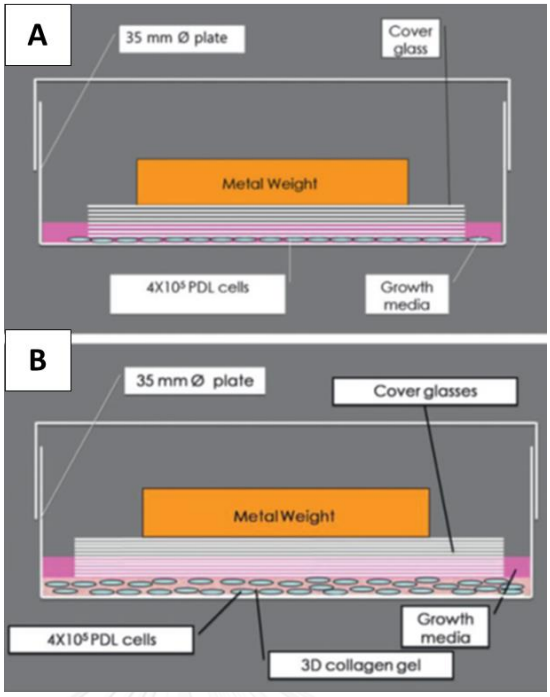


Figure 2.8 The 2D cultured system and 3D cultured system (109).

Periodontitis and hypoxic condition

Evolution of organisms has been needed to adapt to changes of environment especially the air concentrations. The ratio of earth's atmosphere was composed of CO_2 : N_2 : O_2 is 0.03:79:21(111, 112). For inspired air, the oxygen partial pressure (pO_2) is around 20% Oxygen level or 160 mmHg and the oxygen level decrease in the lung (Fig. 2.9). The pO_2 in the alveolar capillaries is around 104 mmHg. The ratio of oxygen level is depends on the type of organ. Moreover, the oxygen level in tissue is diffusion distance approximately 100-200 µm. The tissue which is so far from blood vessels estimate 100 µm is almost zero (111).

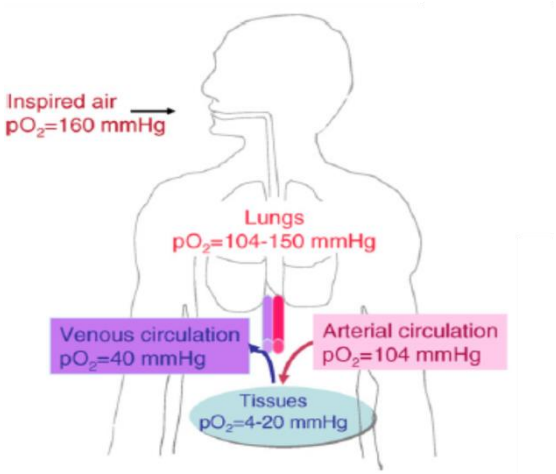


Figure 2.9 Physiological oxygen partial pressures (pO_2) in the body (Adapted from Brahimi and Pouyssegur, 2007) (111).

Oxygen is an essential element required to sustain life. Level of oxygen in organisms depends on both acute and chronic adaptation (113). In healthy tissues, the pO_2 is estimated to be 20-70 mmHg or 2.5-9% oxygen and drops to lower level (<1% oxygen) in wounds and infectious sites (114-116).

The oxygen levels in tissues are measured by oxygen demand and oxygen supply. Low levels of oxygen (hypoxia) occur in several situations such as development and pathological conditions. Such conditions, including cancer, ischemic disorder, diabetes, atherosclerosis and inflammatory diseases, present a tissue hypoxia or anoxia (complete lack of oxygen) (111, 114).

Inflammation is a defensive response after cellular injury, which may be caused by microbes, physical agents, chemicals, necrotic tissue and immune response. There are two causes of hypoxia in inflamed tissue (Fig. 2.10). Firstly, in inflammatory disease will be increase metabolic activity due to penetrating

inflammatory cells resulting in elevated oxygen demand (117). Secondly, the problem in blood supply lead to poor perfusion and cause vasculopathy which decrease oxygen supply of the inflammatory tissue (118, 119).

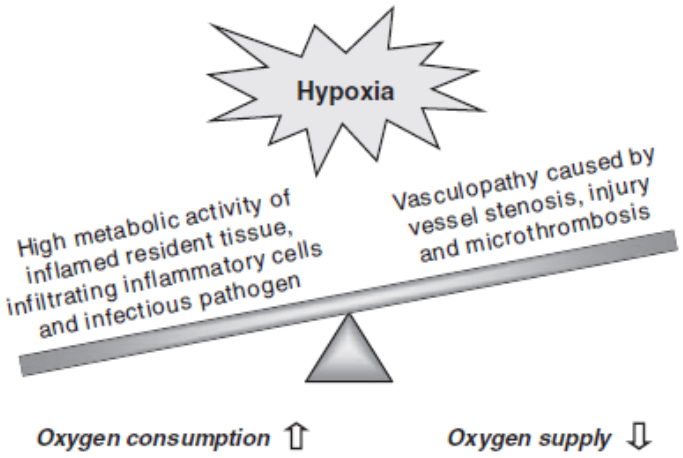


Figure 2.10 The model represent the hypoxic condition can be occur in inflammation site (115).

In inflammatory areas, oxygen drop to slight than 10 mmHg while in vitro experiments, the level of oxygen was set to 0.5%-5% by adjust the gas volume mixture (114). Many evidences showed the effect of oxygen and inflammation such as Wilson et al. (120) reported that hyperbaric oxygen could decreases inflammatory pain in animal model. In addition, Motohira et al. (121) investigated that hypoxia and reoxygenation stimulate many cytokines such as IL-6, IL-1β and PGE_2 in HPDLs. In case of periodontitis, low level of oxygen was presented in periodontal pocket. The range of oxygen level is estimate 5-27 mmHg depend on the pocket depth (61). All of these studies showed closely relation between oxygen and inflammation. The influence of hypoxia on development of inflammation depends on molecular pathways regulated by hypoxia-inducible factor or HIF.

Hypoxia-inducible factor

Cellular adaptations to hypoxia are directly results from hypoxia-inducible factor or HIF. HIF is a transcription factor which is composed of 2 subunits; HIF-α which is an oxygen-sensitive subunit and HIF-β or the aryl hydrocarbon receptor nuclear translocator or ARNT which is a constitutively-expressed subunit. HIF subunits are the basic helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family of transcription factors (114, 122, 123).

Currently, the three isoforms of HIF-α (HIF-1α, HIF-2α and HIF-3α) have been identified. Firstly, HIF-1α is identified in human and mouse tissues. It has been reported relate in physiological responses to hypoxic condition (124). Secondly, HIF-2α which shares the amino acid sequence with HIF-1α to 48%. HIF-2α is prominently expressed in endothelium, lung, and carotid organs (125, 126). Thirdly, HIF-3α was lately discovered, expresses in several tissues. Moreover, HIF-3α spicing form can interact with the HIF-1α and interfere its binding to DNA. Therefore, HIF-3α acts as a dominant-negative regulator of HIF-1(Fig. 2.11) (122).

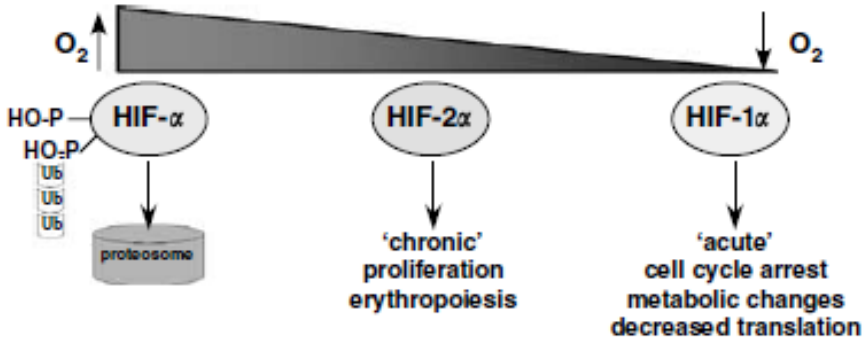


Figure 2.11 The level of oxygen to regulate HIF-α under different level of oxygen (126).

Domain structure of HIF (Fig. 2.12)

1. HIF-1α and HIF-1β are the basic helix-loop-helix–Per-ARNT-Sim (bHLH–PAS) protein family. This region is important for DNA-binding of transcription factors (127).
2. The Oxygen-Dependent Degradation Domain (ODDD) was hydroxylated and regulated stability of HIF-α (128).
3. The transactivation domains consisting N-terminal (N-TAD) and C-terminal (C-TAD). HIF-1α and HIF-2α also contain C-TAD and N-TAD except HIF-1β contains only one TAD. Two transactivation domains are essential for the transcription activity of HIF complex (122).

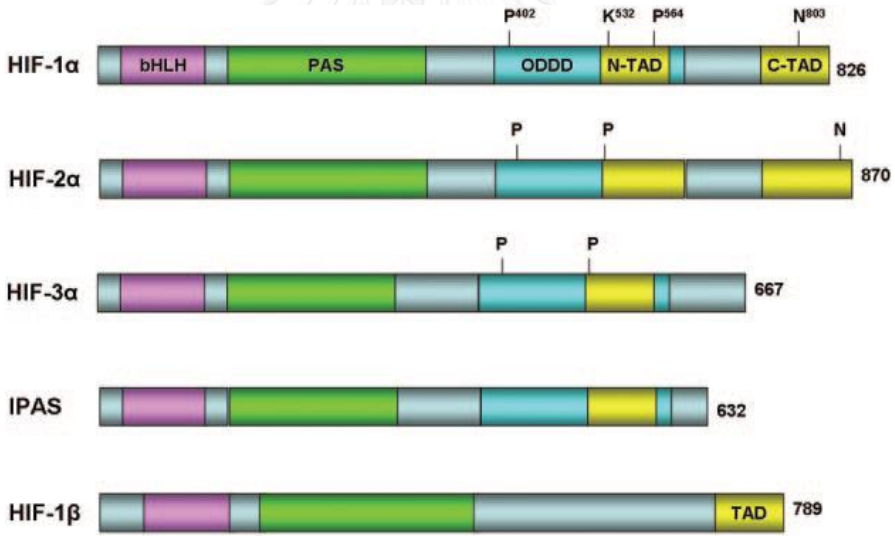


Figure 2.12 The domain structure of HIF family in human (122).

HIF-α cannot detect in the normoxia but it can detect under hypoxia. In contrast, HIF-β is constitutively not regulating by oxygen level. In nucleus, the HIF complex requires a co-activator named p300/CBP to bind into an active complex and finally, this complex will be bind to a hypoxia-response element (HRE) (129) of

target genes. Over 100 genes are activated or suppressed by HIF such as metabolism, angiogenesis, differentiation, survival and cell death. However, we can detect the HIF-α stabilize in cell culture at 5% of oxygen (40 mmHg) (111, 114).

The HIF-1 transcription factor is an essential regulator of cell to response under hypoxia. In the presence of oxygen, HIF-α subunits are hydroxylated by two enzymes (prolyl hydroxylase domains (PHDs) and factor inhibiting HIF (FIH)). The hydroxylation by PHDs promotes von-Hippel–Lindau (pVHL)-dependent proteolysis and HIF-α subunits are destroyed. In addition, hydroxylation of an asparaginyl residue by FIH, prevents binding of the co-activator p300 and results in the HIF-α inactive to transcriptional activity. In low or absence of oxygen, the HIF-α is stabilized, which permits HIF-α translocate to the nucleus and activate the target genes (Fig. 2.13) (125, 130). In human consist of 3 PHD isoforms as follows: PHD1, PHD2 and PHD3. The PHDs hydroxylate at proline 402 and 546 which are proline residues in ODDD of HIF-1α. These enzymes use co-factors to hydroxylation into HIF-α. The co-factors are 2-oxoglutarate (2-OG) (a-ketoglutarate), Fe (II) and ascorbate. It was reasoning why the iron chelators can stabilize HIF-α.

Moreover, PHD1/2/3 RNA interference was used to examine the important role of these enzymes to stabilize HIF-α under normoxia. The results showed that only PHD2 is important to stabilize HIF-1α in normoxia. It is meaning that PHD1 and PHD3 are not necessary in oxygen sensor. HIF-α accumulation in cytoplasm follows nuclear translocation, which is bound to hypoxia response element, referred to a recognition DNA sequence, 5′-RCGTG-3′, of the target genes such as angiogenesis, erythropoietin (EPO), apoptosis (Fig. 2.14) (131).

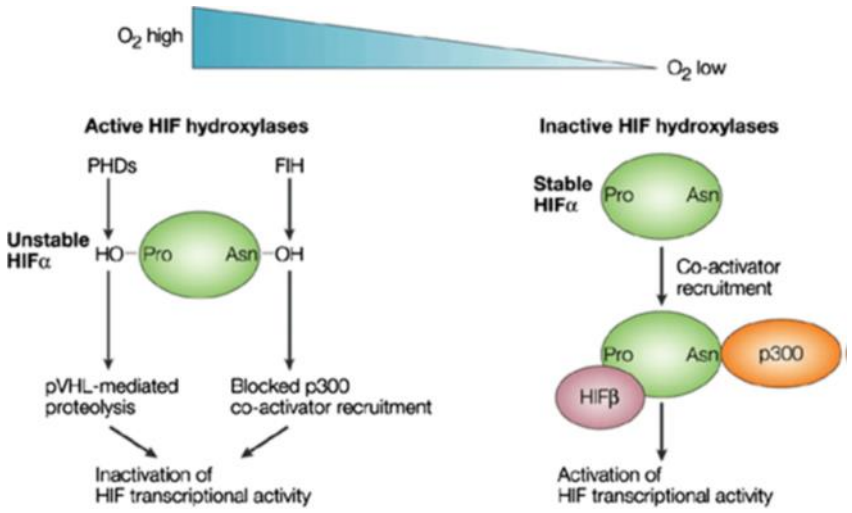


Figure 2.13 The diagram shows the hydroxylation of HIF-α by PHD and FIH (130).

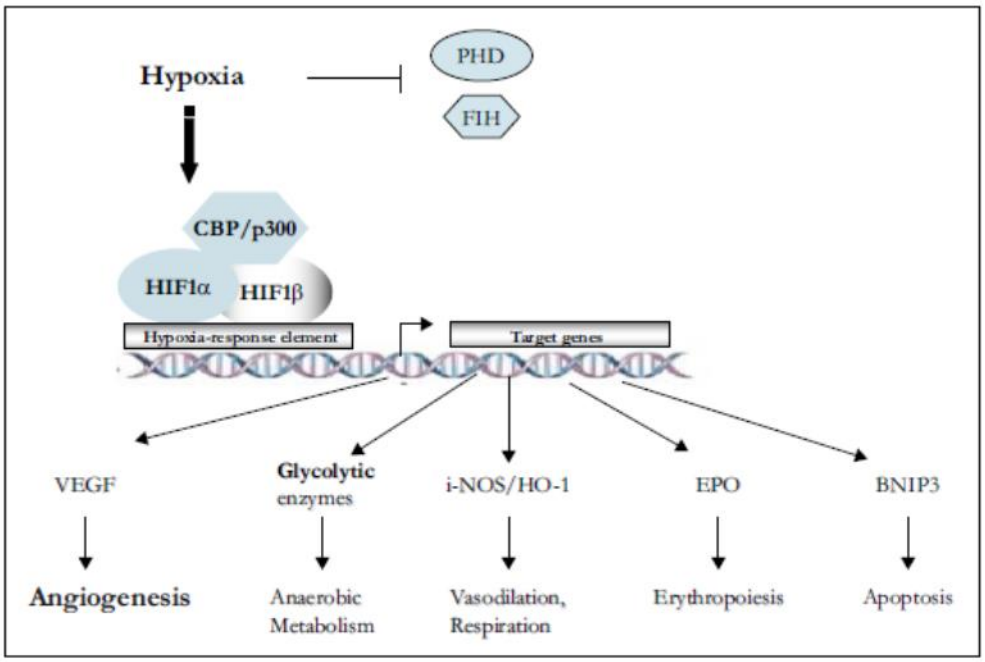


Figure 2.14 The influence of hypoxia to regulate the target gene (131) .

As describe above, HIF-α consists two TAD. The C-TAD is in HIF-α C-terminal part and N-TAD is in its N-terminal part. The C-TAD is hydroxylated by FIH and leading to its inactivity. Normally, PHDs and FIH have been work at different time. The PHDs are more sensitive to drop of oxygen level more than FIH.

At starting to drop of oxygen, the PHDs will be inactive before FIH. If the oxygen level drops to cutoff, the PHD and FIH will be inactive and some target genes required both N/C-TAD will be activated. However, we can detect some genes require the activity of one TAD. (Fig. 2.15) (131).

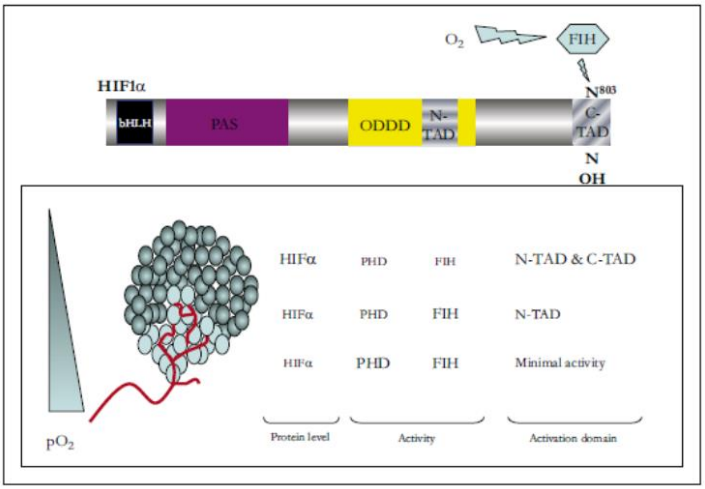


Figure 2.15 The activity of PHD and FIH under hypoxic condition. HIF-1α contains two transcription activation domains in the C-terminal part of the protein, referred to as the N-TAD and C-TAD. In different low level of oxygen will be activated different genes depend on gene requiring only N-TAD or both of them. (Adapted from Brahimi-Horn and Pouyssegur (2006)) (131).

HIF protein can detect in various cell type and to be stabilized in a hypoxic condition which is less than 5% oxygen or 40 mmHg in 2-4 h. However, HIF protein will be rapidly degraded within 20 min after oxygen return to 21% oxygen or 160 mmHg.

Cobalt chloride

As mentioned above, several studies investigated relationship between hypoxia and their interestingly target. However, the limitation of experimental design,

some model such as our study model cannot use physical hypoxia. Present, chemical hypoxia which is an easier technique was used to mimic hypoxic condition. Cobalt chloride ($CoCl_2$) is a popular agent that commonly uses to mimic hypoxia in several studies (132-137) and showed that it can stabilize HIF-1α in HPDLs (133). The main mechanism of $CoCl_2$ was proposed in 2 pathways. First, the evidence showed that $CoCl_2$ decreased intracellular ascorbate which is a co-factor of prolyl hydroxylase (PHD), a key enzyme in the oxygen sensing pathway (Fig. 2.16A) (138). Second, recently study showed that $CoCl_2$ binds directly to the iron part of a HIF-specific hydroxylase which is important enzyme to hydroxylate HIF-α. Thus, the function of $CoCl_2$ is prevent the degradation of HIF-α by block the interaction of HIF-α and VHL (Fig. 2.16B) (139).

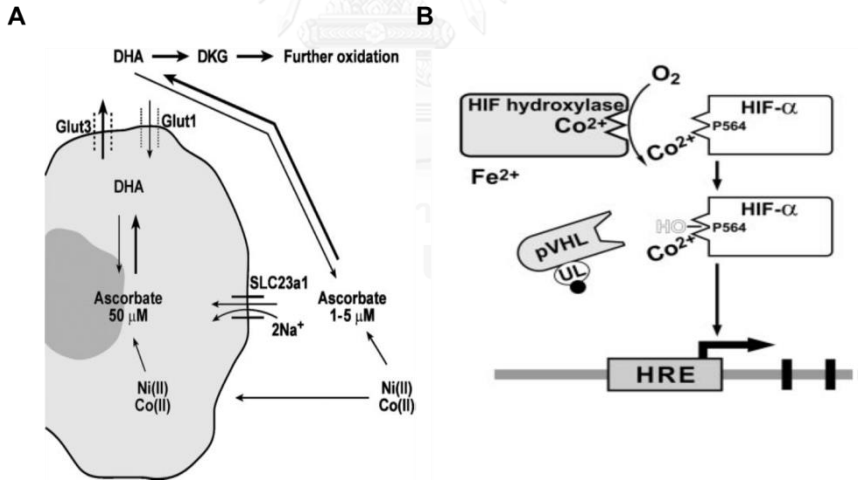


Figure 2.16 The propose mechanism of $CoCl_2$ to stabilize HIF-α. (138, 139).

Recently study investigated the effect of many type of PHD inhibitor including $CoCl_2$ in human gingival fibroblast and HPDLs. They demonstrated that $CoCl_2$ affect to cell viability, protein synthesis and also cell proliferation in a dose dependent manner (137). It is meaning that the concentration of $CoCl_2$ to use in

experiment is important. Moreover, $CoCl_2$ was used to study in osteogenic differentiation of HPDLs. The study proposed that $CoCl_2$ inhibited osteogenic differentiation by decreasing osteogenic marker gene; Runx2, ALP and OCN. Moreover, $CoCl_2$ suppressed alkaline phosphatase activity and upregulated stem cell marker in HPDLs (133). In addition, rat renal tubular NRK52E cells were treated with $CoCl_2$ and showed to increase osteopontin (OPN) expression via Akt/PKB/p38 pathway (140). A lot of study comparison between physical hypoxia and $CoCl_2$, various kind of cell such as mouse peritoneal macrophages, splenocytes or mouse Lewis lung carcinoma (LLC) cells were incubated in both hypoxia or $CoCl_2$ and they found that $CoCl_2$ stabilizes HIF-1α to create hypoxic-like conditions but the detail mechanism to promote some pathway is different depend on targeting genes and cell types (132, 141, 142). Thus, the interpretation of the results should be done with caution and implied for hypoxic mimic condition.

Transforming growth factor-beta (TGF-β)

In 1983, TGF-β was firstly discovered form moloney sarcoma virus-transformed cells (MSV-transformed 3T3 cell line) cultured medium. First time, it called sarcoma growth factor (SGF). After purified the crude of SGF, they found 2 form of SGF which they call types TGF-α or epidermal growth factor (EGF) and TGF-β (143). TGF-α is a ligand for EGF receptor (EGFR), while TGF-β binds to own its receptors which call TGF-β receptor (TβR) (144).

TGF-βs are a multifunctional of growth factor family. Currently, the isoform of TGF-β were identified to 3 forms; TGF-β1, TGF-β2 and TGF-β3 in mammals. All isoform of this protein share the 72-79% similar sequence of amino acid while arise

from different genes and chromosome (145, 146). TGF-βs is an extracellular protein that can express in many cell types including HPDLs. TGF-β1 is known to regulate a broad range of cellular biological processes such as cell differentiation&migration, extracellular matrix (ECM) protein production and wound healing (147-151). Moreover, TGF-β1 is an isoform that most studies.

Secretion of latent complex of TGF-βs

The previous study investigated that TGF-βs did not detect in culture medium. However, they used the acid to activate TGF-βs and TGF-βs were detected. As described from this study, the evidence suggests that cell can release the TGF-βs to culture medium in the latent form (146). Present knowledge, several studies demonstrated that TGF-βs is secreted to a high-molecular-mass protein complex target to the extracellular matrix (ECM). This complex is called large latent complex (LLC) which consists of 3 proteins; the mature TGF-β, the latency-associated protein (LAP) (152), and the latent TGF-β binding protein (LTBP) (Fig 2.17A). The small latent complex (SLC) consists of only 2 proteins; the mature TGF-β and LAP (Fig. 2.17B). After activation, the LAP will be cleaved from the complex and TGF-β will be released and activated (146). Latent complexes of TGF-β in the ECM are essential to prepare tissues with available storage of TGF-β. The TGF-β activation can rapidly release to active form (25 kD) and activate the signals (147).

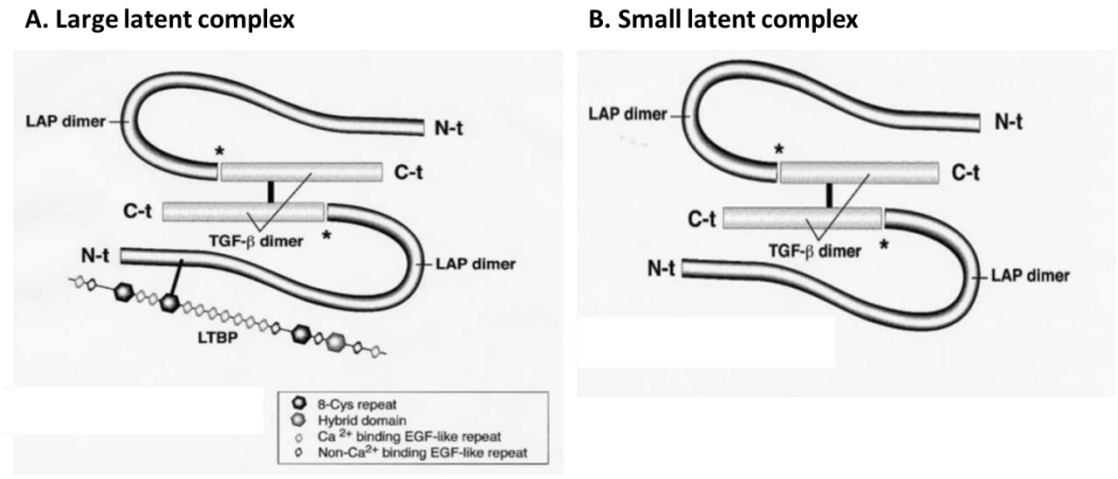


Figure 2.17 The illustration representation of the large (A) and small (B) latent complexes (146).

Process of activation TGF-βs latent form to active form and signaling pathway

Normally, the TGF-βs is secreted in large latent complex (LLC) from the cells and attached by covalent bond in ECM and fibrillin and LLCis activated later. Previous studies showed that non-physiological events can activate latent-TGF-βs including low pH, heating to 100°C or irradiation (144, 147). In addition, several physiological events were reported activate latent TGF-βs to active form such as plasmin (153), thrombospondin-1 (TSP-1)(154), matrix metalloproteinase-9 (MMP-9) (155), calpain (156), αvβ6 integrin (157), and αvβ8 integrin (Fig. 2.18) (158). The summaries of activation method showed in table 2.3. The process of activation requires two steps. The first step is the release of SLC of TGF-βs from ECM and fibrillin by cleaves LTBPs at the specific sites by proteases. The releasing of SLC will be bind to mannose-6-phosphate/insulin-like growth factor-II receptor on the cell surface. Then, the second step start by plasmin released from plasminogen on the cell surface or other proteases release active TGF-β. The principle of this step is released

the mature TGF-βs from the LAP by another proteolytic events or mechanical events such as mechanical stress (Fig. 2.18) (144, 157). LTBPs are a most important role in proteolysis mediated TGF-β activation. The excessive free of LTBP effect to activate active form of TGF-β (147). After activation, the free active from of TGF-β is able to activate with their receptors and signal transduction is started.

Table 2.3 The summarize of activation method of latent TGF-βs (147).

Activation method	Reference
Physicochemical	
Acidic cellular microenvironment	Jullien et al., 1989
Extremes of pH	Brown et al., 1990
Γ-irradiation	Barcellos-Hoff, 1993
Reactive oxygen species	Barcellos-Hoff and Dix, 1996
Enzymatic and by non-specified protein interactions	
Proteases	
Plasmin, cathepsin G	Lyons et al., 1988, 1990
Calpain	Abe et al., 1998
Kato III cells (unidentified protease)	Horimoto et al., 1995
MMP-9 and MMP-2	Yu and Stamenkovic, 2000
Cell co-cultivation (u-PA)	Sato and Rifkin, 1989
Glycosidases	Miyazono and Heldin, 1989
Thrombospondin-mediated	Schultz-Cherry and Murphy-Ullrich, 1993
Integrin αvβ6-mediated	Munger et al., 1999
Drug-induced	
Antiestrogens	Knabbe et al., 1987
Retinoids	Glick et al., 1989
Vitamin D3 derivatives	Koli and Keski-Oja, 1993
Glucocorticoids	Boulanger et al., 1995; Oursler et al., 1993

Currently, the three types of TGF-β receptors were identified. TGF-β receptor type I (TβRI) and type II (TβRII) are transmembrane serine/threonine kinase receptors with the single transmembrane domain. TGF-β receptor type III (TβRIII) is a co-receptor. Seven TβRIs, also named as activin-like receptor kinases (ALKs), ALK1–7. Five TβRIIs (TGFβR2, BMPR2, ACVR2, ACVR2B and AMHR2) and two TβRIIIs (betaglycan and endoglin) have been identified (144). The active form of TGF-β1

binds to the TβRII first and recruits TβRI form the heterodimer complex. TβRII is a part of active serine/threonine kinase due to the capacity to bind to TGF-β1, while TβRI is only activated by transphosphorylation of glycine/serine-rich domain of TβRI by TβRII after complex formation. TβRIII play a modulator of TGF-β1 by bind to TβRI-TβRII complex (159).

The intracellular signaling of TGF-β1 is mediated by Smad signaling pathway. TGF-β1 binds to the TβRII and then form heterodimer complex with TβRI and later that TβRI kinase activated Smad2 and Smad3 phosphorylation in the cytoplasm. After that Smad4 (Co-Smad) will be form the complex with phosphorylated Smad2 and Smad3 and the complex will be translocate into the nucleus and binds to transcription factors and regulates the target gene. In contrast, Smad7 which is act as an inhibitor of phosphorylated Smad2/3 by binding TβRI (144). The all process of TGF-β1 was activated and regulated the intracellular signaling shows in figure 2.19A. In addition to Smad pathway, TGF-β1 can activate Smad-independent pathway such as mitogen-activated protein kinase, PI3K kinase and Rho kinase proteins (Fig. 2.19B) (160).

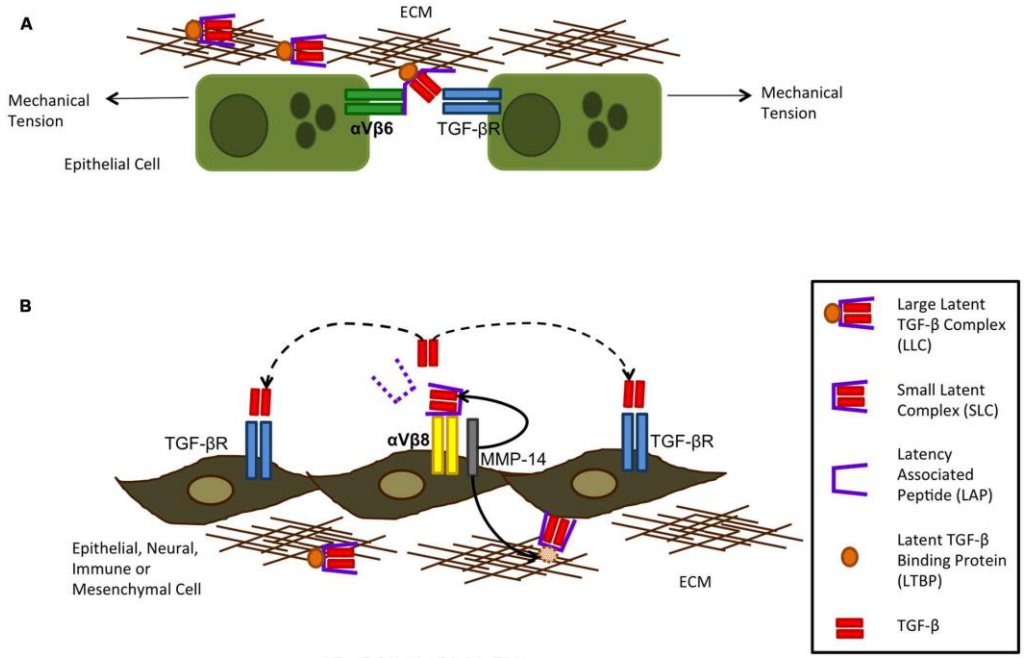


Figure 2.18 Integrin activation of TGF-β can occur via αVβ6 (A) and αVβ8 (B)(157).

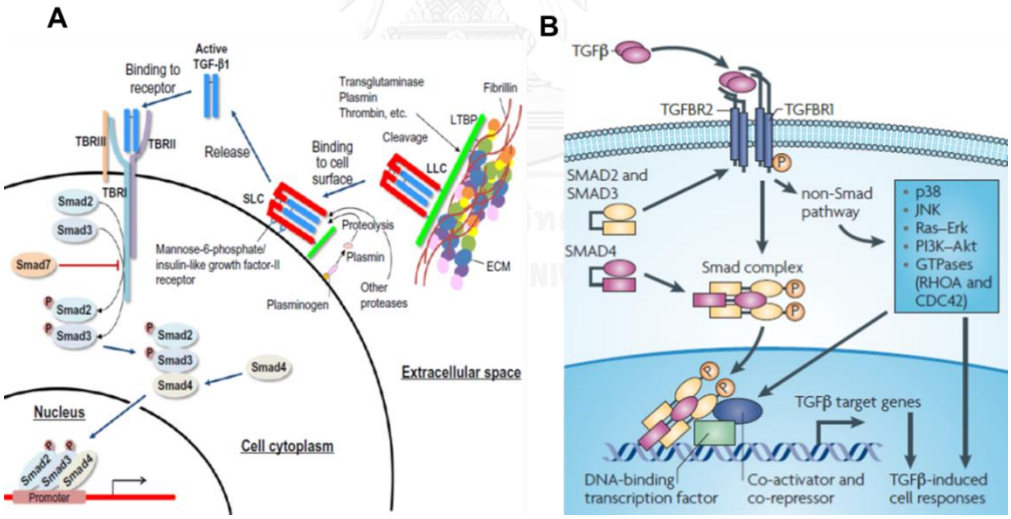


Figure 2.19 The activation process (A) and signaling pathway of TGF-β1 (B) (144, 161).

The function of TGF-β1 in periodontal ligament cells

TGF-β1 is well known expression in various cell types including normal cells as same as tumor cells (144). TGF-β1 plays a role in broad spectrum of cellular biological activity in the cells such as cell proliferation, migration, differentiation, apoptosis, and ECM protein production. Moreover, TGF-β1 was reported to involve in many developing process including embryogenesis, angiogenesis, wound healing and bone formation, and also diseases (144, 148, 150, 162). TGF-β1 knockout mice showed prenatal lethality by primary defects on vasculature in yolk sac and haematopoietic system. Thus, they suggested that haematopoietic or endothelial cell proliferation were decreased in loss of TGF-β1 function (163). In addition, the loss of the TGF-β1 gene showed effect to diffuse and mortal inflammation. After treatment with anti-inflammatory agents and/or immune suppressive agents showed extent the survival of mice-deleted TGF-β1 (164). Moreover, TGF-β1 knockout mice were used to also study the role of TGF-β1 after postnatal development. The data indicated that longitudinal bone growth and bone mass decreased after loss of TGF-β1 function (165). All of together, we can conclude that TGF-β1 plays a key role in multiple process and tissue.

TGF-β1 was also demonstrated several function in periodontal ligament cells. TGF-β1 increased the proliferation and regulated cytoskeletal rearrangement in HPDLs (166, 167). Moreover, recombinant TGF-β1 promoted differentiation in HPDLs by increasing ALP activity (168). The role of TGF-β1 in PDLs differentiation was investigated. The single dose of TGF-β1 activated differentiation in HPDLs, human mesenchymal stem cell (hMSC) and MC3T3 cells by increasing ALP activity, the expression of IGF-1, Runx2 and ALP while the prolong treatment of TGF-β1

showed inhibit the differentiation through suppression of IGF-1 signaling pathway (162).

In case of orthodontic treatment, the remodeling of periodontium response to mechanical force and relate to several cytokines. The tension side and pressure side in orthodontic teeth were examined. The evidence showed that both tension side and pressure side express several cytokine much more than the control such as type I collagen, IL-10, TNF-α, MMP-1, osteocalcin (OCN), osteoprotegerin (OPG), RANKL, tissue inhibitor of metalloproteinase-1 (TIMP-1) including TGF-β. Additional, at compression side upregulated the expression of RANKL, MMP-1 and TNF-α when compared with the tension side (169).

TGF-β1 acts as a pro- and anti-inflammatory effect. The inflammatory cell from HPDL-apical lesion expresses several inflammatory cytokines such as IL-1, IL-6 and IL-8. In addition, the treatment with TGF-β1 can suppress the production of inflammatory cytokines in this cell (170). Although IL-10 was important in downregulation of inflammatory responses in inflamed PDL tissue, this study showed that the anti-inflammatory effect of TGF-β1 was stronger than IL-10 (170). This result indicated that TGF-β1 is important to anti-inflammatory effect in HPDLs. In contrast to periodontitis, TGF-β1 was detected in serum, saliva as well as gingival crevicular fluid (GCF) in periodontitis's patient compared to the control. The TGF-β1 was suggested as a marker for periodontal disease progression (171).

Insulin-like growth factor (IGF)

The insulin-like growth factor (IGFs) family which is endocrine, paracrine and autocrine growth factors which are controlling both pre- and post-natal development.

In present, IGFs family composes their three ligands (insulin, IGF-I, and IGF-II), three surface receptors (the insulin receptor (IR), the IGF-I receptor (IGF-IR), and the mannose-6-phosphate IGF-II receptor (M6P/IGF-IIR), including at least six IGF-binding proteins (IGFBPs) which is protein that bind to IGFs and control the biological processes. However, the mechanisms of IGFBPs are not well understood (Fig. 2.20) (172). Several evidences interested to study IGFs family because IGFs is involved in physiological condition as well as pathological condition (173). Various cell types can express IGFs and represent biologically activity which affects the proliferation, chemotaxis, differentiation, matrix synthesis and cell survival (30, 32, 35, 172). IGF-I is part of the growth hormone and small size of protein with molecular weight 7.7 kDa, while IGF-II plays an essential role mainly during pre-natal development with molecular weight 7.5 kDa (30, 32).

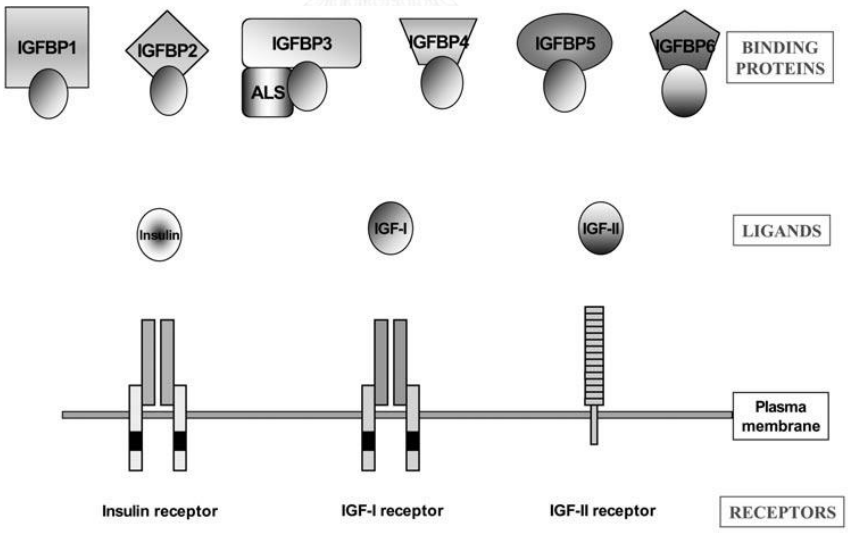


Figure 2.20 The IGFs family. The IGFs family including three membrane receptor, three ligands and six binding protein (172).

IGF-1 signaling pathway

IGFs ligands bind to their receptors and later stimulate intracellular signaling. The tyrosine kinase is activated after IGFs binding and plays a crucial role in signal transduction of MAPK or PI3K pathway (20).

In case of IGF-1 activation, the IGF-1 binds to tyrosine kinase IGF1R and leading Tyr residues were phosphorylated, followed activates downstream substrates. The IGF1R mediates the most of IGFs action and express nearly ubiquitously (40). The downstream of IGF-1 is insulin receptor substrate (IRS) and Shc were activated by tyrosine phosphorylations. Four isomers of IRS were found (IRS1-4). The roles of each IRS have the different function depend on cell types. IRS1 and IRS2 have been studied in bone. Since, osteoblasts express both IRS1 and 2, while chondrocyte express only IRS1 and osteoclasts also express IRS2. Currently, no evidences showed expression of IRS3 and IRS4 in bone cells or not. Finally, IRS1 bind to Shc/Grb2 and then activates PI3K and the extracellular signal-regulated kinase (174) mitogen-activated protein kinase (MAPK) (Fig. 2.21) (175).

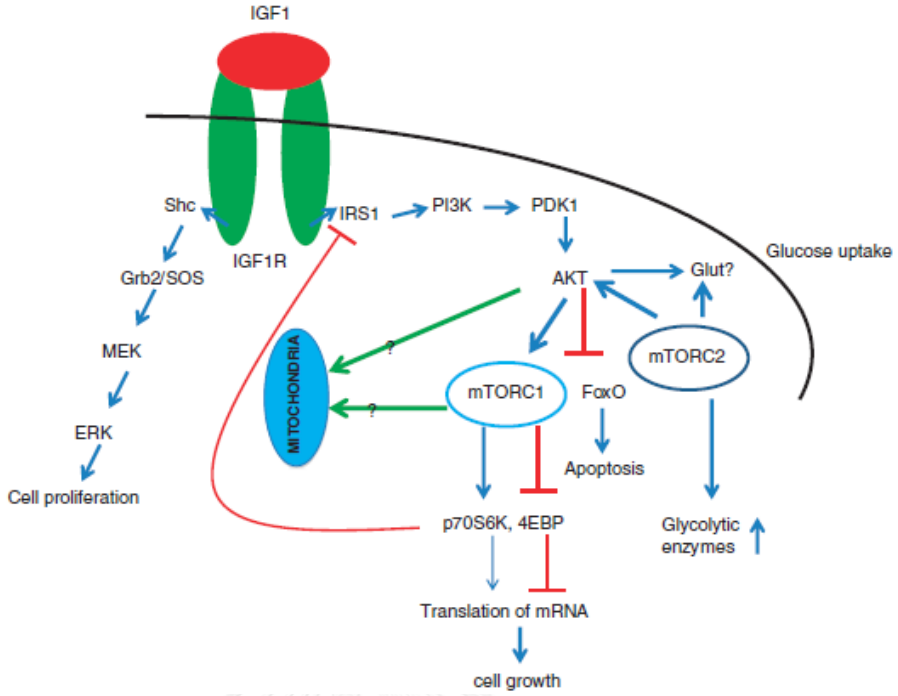


Figure 2.21 This figure shows the IGF-1 signaling pathway (175).

The role of IGF-1 in periodontal ligament cells

The role of IGF-1 in bone homeostasis is well investigated. The osteogenic response under 4-point bending was determined in wild type mice but did not observe in IGF-1condition knockout mice (38, 39). In addition to role of IGF-1 in bone, recently, the study from Werner et al concluded the role of IGFs family in oral biology including PDL homeostasis and pathological conditions (172). IGFs were detected in human cementum, PDL and dental pulp by immunohistochemical staining. IGF-1 could express in cementum, PDL and dental pulp, while IGF1R could detect in PDL-fibroblast and odontoblast (Table 2.4) (40). Form this evidence, they suggested that HPDLs plays a role for IGFs reservoir and the HPDL fibroblasts respond to the IGFs in a paracrine manner.

Table 2.4 IGFs family expressed in human cementum, HPDL and dental pulp investigated by immunohistochemical (40).

	Cementum			PDL		Pulp		Dentin/pred.
	AEFC	CIFC	Insertion	ECM	Fibroc.	ECM[a]	Odonto.	
IGF-I	(+)	Ø	++	++	Ø	++	Ø	Ø
IGF-II	++	Ø	++	+/(+)	Ø	Ø	Ø	Ø
IGF1R	Ø	Ø	Ø	Ø	+	Ø	(+)	Ø
IGFBP-1	Ø	Ø	+	+/Ø	Ø	+	(+)	(+)
IGFBP-2	(+)	Ø/(+)	+	(+)	Ø	Ø	Ø	Ø
IGFBP-3	(+)	Ø	+	Ø/(+)	Ø	++	(+)	+
IGFBP-4	Ø	Ø	Ø	(+)	Ø	Ø	Ø	Ø
IGFBP-5	(+)	Ø	++	(+)	Ø	Ø	Ø	Ø
IGFBP-6	Ø	Ø	Ø	+/++	Ø	+	Ø	(+)

AEFC, acellular extrinsic fiber cementum; CIFC, cellular intrinsic fiber cementum; insertion, insertion zone (of Sharpey's fibers); PDL, periodontal ligament; ECM, extracellular matrix, fibroc., fibrocytes; odonto., odontoblasts; pred., predentin.
Ø, no immunoreactivity; (+), weak immunoreactivity; +, moderate immunoreactivity; ++, strong immunoreactivity.
[a] Immunoreactivity in fibrotic areas; ECM around pulp denticles was reactive for IGF-I, IGFBP-1, -3, -5 and -6.

Other main function of IGF-1 in HPDL was reported. IGF-1 involve in cell survival and for HPDLs, IGF-1 enhanced cell survival in HPDLs compared with gingival fibroblast (GF) by reducing the apoptotic cells and DNA fragmentation. The action of IGF-1 in cell survival is upregulated Bcl-2 family which is the anti-apoptotic molecules, while is downregulated Bax, Bad, Bid, and Bak which are the pro-apoptotic molecules in HPDLs (33).

IGF-1 is important in proliferation, migration, differentiation, induce the DNA synthesis as well as promote wound healing in HPDLs (35, 176-178). Previous study in beagle dog model with natural periodontal disease and monkey with ligature-induced attachment loss showed that after 1 month treatment with PDGF/IGF-I, the attachment formation was upregulated in both beagle dog and monkey when compared to the control. Thus, IGF-1 plays an essential role to promote regeneration both hard and soft tissue of peiodontium (179, 180). IGF-1 is well known a potent mitogenic protein which can promote the osteogenic differentiation in HPDLs.

Recently, the study investigated that pathway that relate in IGF-1 induced osteogenic differentiation in HPDLs. They demonstrated that administration of IGF-1 to HPDLs in vitro enhanced ALP activity, mineralization and upregulation the gene and protein expression of RUNX2, OSX and OCN which is an osteogenic marker. Moreover, they found JNK and ERK were activated after IGF-1 treated in HPDLs. In vivo study, IGF-1 administration to the treated implants promoted tissue mineralization and expression of RUNX2, OSX, and OCN more than the control group (35).

In term of mechanical loading, the study generated hypo-occlusal stimuli in rat model. They demonstrated that rat periodontal tissue upregulated the expression of IGF-1 and IGF-1R when occlusal stimuli. In addition, the pattern of IGF-1 and IGF-1R is shown the same trend with cell proliferation. The hypo-occlusal stimuli affect to expression of both IGF-1 and IGF-1R including decreasing the proliferation in PDLs (29). Corresponding to other study in rat model, they applied the force to upper first molar tooth for represent the orthodontic force and detected the IGF-1, IGF-1R and IRS1. This data showed that IGF-I, IGF-IR and IRS1 could detect in periodontal tissues in both the control teeth and loading-teeth. In the loading teeth, the expression of IGF-1, IGF-1R and IRS1 strongly increase on the tensile side and decrease on the pressure side (19). In HPDLs is shown the similar result with rat PDLs. Cyclic tensile strain was used to apply the HPDLs for represent the chewing loading. HPDLs increased the gene and protein expression of IGF-1, IGFBP1 while, decreased the expression of IGFBP3 and IGFBP5. Although, other IGFs were detected, no significant different were found between experimental group and control group (20). As described above, we suggested that mechanical loading is closely related to regulate the IGFs family in PDLs.

Osteopontin (OPN)

Osteopontin (OPN) also known as bone sialoprotein I (BSP-1) or secreted phosphoprotein 1 (SPP1) which is one of the secreted phosphoglycoprotein. It is a non-collagenous protein and was identified from the bone's extracellular matrix. The weight of OPN is around 34 kDa. Several cell types can detect the OPN expression such as osteoclasts, osteoblasts, chondrocytes, and periodontal ligament cells (48). OPN is also expressed in various tissue such as bone, kidney, blood vessels including epithelial cells (181). In vivo, OPN was upregulated in some conditions such as sepsis, tumor metastasis while, in vitro several cytokines such as IL-12, TGF-β, EGF or PDGF can modulate OPN production (181).

The OPN is a multifunctional protein that involved in the regulation of both physiological and pathological condition (48). Some evidences showed OPN plays a key role in pro-inflammatory cytokine and inflammatory regulation (45, 52-54, 182). OPN is known to act as chemoattractant in monocytes and/or macrophages. OPN knockout mice with polymicrobial sepsis increased the survival rate when compared to the wild type. It suggested that the loss of OPN function protects the host from an excessive inflammatory response and increase mortality (45, 182).

OPN is well known to involve in bone remodeling (Fig. 2.22). Osteoblasts which the bone cells play an essential role in matrix formation and osteoclasts are mainly control the resorption throughout its life span. Normally in the differentiation stage of osteoblast, the adhesion process of pre-osteoblasts attach to the bone surface is very important points. In this step, the pre-osteoblasts will become mature to osteoblasts and OPN is a one protein that act at this step (43). The role of OPN about

bone formation was reported in OPN-deficient mice. This study investigated calvarial suture closure under mechanical stress in wild type mice compared with OPN deficient mice. They found that OPN could detected in the osteoblast and osteocyte in new bone formation while, the OPN deficient mice decreased the new bone formation compared to the wild type (76). Although, OPN is involved in bone formation, the bone resorption is reported to relate with OPN. Osteoclast adhesion is an important action for destruction the bone. The adhesion of osteoclast allows them to attach and interact with bone matrix and release the essential molecules to resorb the bone. OPN–integrin αvβ3 binding plays an important to regulation the osteoclast activity (43). The study of unloading using a tail suspension model in wild type and OPN knockout mice showed that unloading increased bone resorption and decreased bone formation in wild type mice. However, these effects did not occur in the OPN knockout mice. Thus, they suggested that OPN plays a key role in bone remodeling under mechanical loading (46, 47).

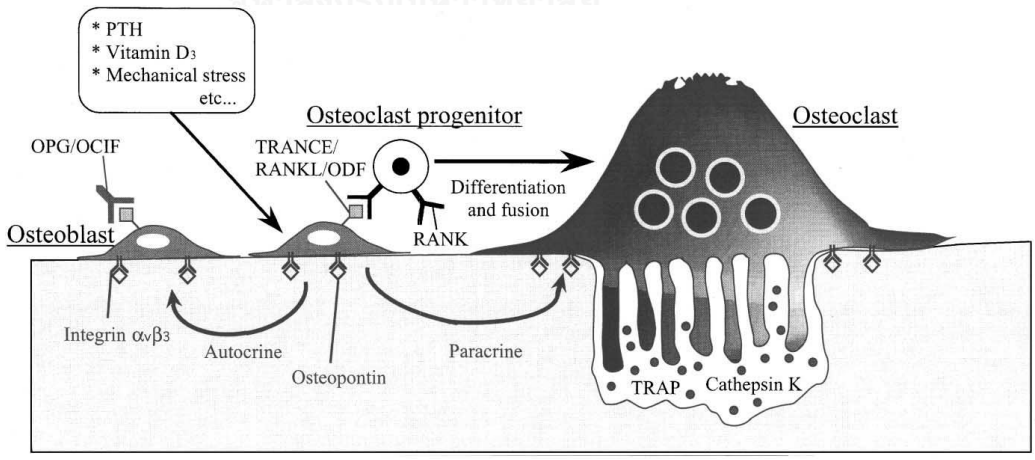


Figure 2.22 The role of OPN in bone remodeling (43).

OPN signaling pathway

The Arg-Gly-Asp (RGD) motif of OPN is an essential part for binding to integrin receptors. The αvβ3 integrin can be bind with OPN to activate the signaling events and primarily responsible for cell adhesion and migratory properties of OPN (48). After binding of OPN and αvβ3 integrin, the focal adhesion kinase (FAK), paxillin, tensin, and Src were phosphorylated and numerous signaling were activated such as proliferation, apoptosis, phagocytosis and cytoskeletal organization. Moreover, OPN binds with αvβ3 can also activate the intracellular Ca^{2+} by stimulating Ca^{2+} releasing from intracellular compartments and regulating extracellular Ca^{2+} influx via calmodulin-dependent, Ca^{2+}-ATPase (48).

The binding of OPN and each kind of integrin depend on the OPN RGD motif. Recently studies reported that OPN interacts with αvβ1, αvβ3 and αvβ5 integrins in cancer cells (183). In addition to integrin, OPN can bind to cell surface receptor; CD44 which is the main receptor for hyaluronate. CD44 has been studies in various kinds of cells including HPDLs. Earlier study demonstrated that knockdown CD44 affected to decrease proliferation and mineralization in HPDLs (184). After binding between CD44 and OPN, the cellular response is similar to those of OPN bind with integrins (Fig. 2.23) (48).

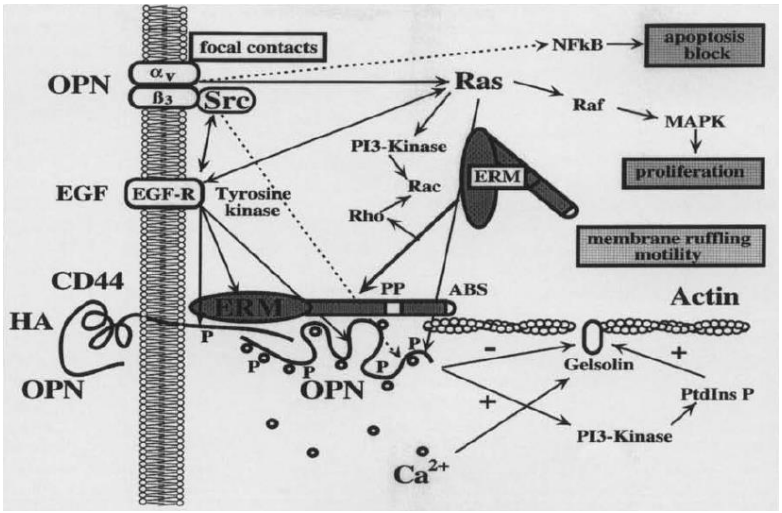


Figure 2.23 The summary of osteopontin signaling pathway. OPN are binding through the αvβ3 integrin or CD44, which affect to several cellular processes (48).

The role of OPN in periodontal ligament cells

The study about important of OPN in tooth root development in mice demonstrated that OPN is expressed at the root surface during cementogenesis and is also strongly expressed at the PDL region. In contrast to odontoblast, the OPN expression is lightly detected (185). Furthermore, the role of OPN in bone remodeling was reported. Previous report indicated that osteocyte responded to mechanical stress. The osteocyte is expressed the OPN gene and is increased the number of osteoclasts 17-fold compared the unloading group (44). Corresponding to another study used the OPN knockout mice model to determine the role of OPN in mechanical stress response. This study showed that $TRAP^{+}$ cells in OPN knockout mice is less detected when compare to wild type mice while, the collagen type I is not different between knockout and wild type group. Moreover, after orthodontic application force showed activation of OPN promotor. All together, these evidences concluded that OPN is an important molecule for bone remodeling under mechanical force application (50).

In addition to loading teeth, the role of OPN in unloading teeth model was also investigated. The study demonstrated that OPN is not related in continuous eruption of un-opposed molar. In contrast to tooth drifting, OPN is required in bone remodeling. They found that OPN expression and the number of osteoclast increased at bone surface in drift tooth while, $OPN^{-/-}$ mice showed diminish osteoclast number. Furthermore, the OPN treated cells induced RANKL expression via PI3K and MEK/ERK pathway (186). The role of OPN in bone remodeling summarized in figure 2.24.

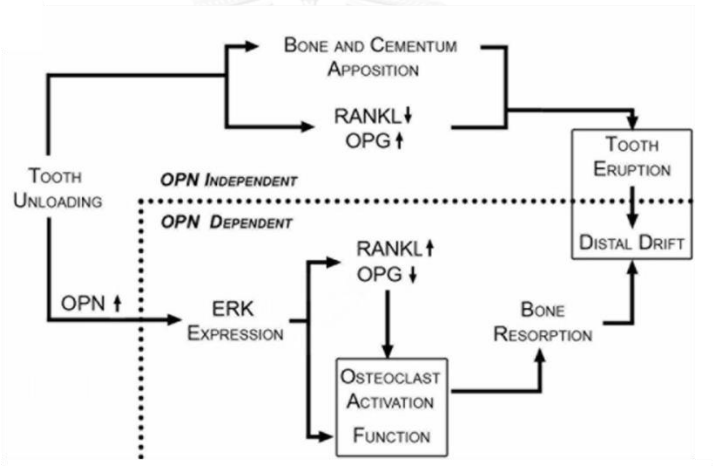


Figure 2.24 Proposed the role of OPN in unloading tooth and loading tooth (186).

In hyperocclusion mouse model represent to traumatic occlusion resulted induced RANKL, TRAP staining and also OPN expression. All together may support increasing osteoclasts activation and migration (92). The role of OPN in bone is more extensively study while, the role of OPN in HPDLs is still not clear. Previous studies in our group demonstrated that compressive stress induced OPN expression in HPDLs

via Rho/P2Y1/ATP pathway. They also indicated that compressive stress is upregulated RANKL expression in HPDLs (21, 26).

Moreover, the OPN acts as an inflammatory marker in periodontal disease. The OPN level in plasma and GCF were examined in both healthy patient, gingivitis patient and periodontitis patient. The data showed that OPN level in periodontitis group is highest in both plasma and GCF. After treatment, the level of OPN is significantly decreased. Thus, OPN is a biomarker for periodontal disease progression (53, 54).

CHAPTER III

RESERCH METHODOLOGY

Material

All cell culture media were purchased from Gibco BRL (BRL, Carlsbad, CA, USA). Tissue culture dishes and plastic tubes were purchased from Corning (Corning, NY, USA). Exogenous ATP, cyclohexamine, genistein, TGF-β receptor inhibitor (SB431542), src inhibitor, MRS2179 (P2Y1 inhibitor) and recombinant human TGF-β1 (rhTGF-β1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rho inhibitor was purchased from Calbiochem (Biosciences, San Diego, CA, USA). Cobalt chloride ($CoCl_2$) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Modular incubator chamber was purchased from Billups-Rothenberg, Inc. (PO Box 977 Del Mar, CA) and SPD-201/O_2 Gas detector was purchased from Protronics intertrade (Bangkok, Thailand).

Cell culture

HPDL cells were explanted from healthy periodontal ligament tissue of non-carious, freshly extracted third molars, or extracted for orthodontic treatment at Faculty of Dentistry, Chulalongkorn University. This protocol was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, the study code: HREC-DCU 2013-066. Briefly, the periodontal tissues were scraped from the middle of the teeth's root and placed in 35 mm tissue culture dish to allow cell migration in standard culture media (Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 100

units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. The cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2. After confluence, cells were detached with 0.25% trypsin-EDTA and sub-cultured at a ratio of 1:3 in 60-mm-tissue culture dishes and considered as passage 1. The experiments were conducted using cells from passage 3-7 and cells from at least 3 donors were utilized in each experiment.

Normoxic and hypoxic conditions

Hypoxic condition could generate by 2 systems; physiologic hypoxia and chemical hypoxia. The physiologic hypoxia was created in an airtight chamber de-oxygenated with the constant infusion of a hypoxic gas mixture (37°C, 5% CO_2, 5% H_2 and balanced N_2). The oxygen content was monitored with an oxygen analyzer (SPD-201/O_2 Gas detector). Similarly, normoxic group was maintained in a standard incubator (37°C, 5% CO_2, 21%O_2, balanced N_2).

The chemical hypoxia, $CoCl_2$ (a hypoxia mimetic agent) was used to imitate hypoxic conditions. $CoCl_2$ powder was dissolved in autoclaved deionized water to obtain the stock concentration of 300 mM. The HPDLs were incubated with $CoCl_2$ at 150 or 300 µM. The control groups were cultured in the absence of $CoCl_2$.

To examine the influence of oxygen level on OPN or IGF-1 expression, $1.5x10^5$ cells per well of each cell lines were seeded in 12-well culture plate. Oxygen deprivation were carried out in an incubator with <1%O_2 and 21%O_2 conditions for 24 h.

Cell viability

HPDLs were seeded at a density of $3x10^5$ per well in 6-well plates for apply the force and 24-well plates at density $5x10^4$ cells per well for treated with $CoCl_2$. Subsequently cells were starved with serum-free media 4 h before treatment. At 24 h, HPDLs were incubated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide solution for 30 min. Formazan crystals were solubilized in a DMSO/glycine buffer solution (0.1M glycine/ 0.1M sodium chloride pH10). The solution was further measured for an absorbance at 570 nm in a microplate reader (Elx800, Biotek, USA). The data were normalized to the control. All measurements were done in triplicate.

Intermittent mechanical stress application

The mechanical loading apparatus was manufactured by the Faculty of Engineering, Thai-Nichi Institute of Technology. The machine principle is the hydraulic cylinder force directly to the cell medium in 6-well plate (Fig. 1). This machine can be load to two types of stress, static stress and intermittent stress.

HPDLs were seeded in 6-well plates at a density of $3x10^5$ cells per well overnight. Subsequently, cells were starved with serum-free media 4 h before placing in the apparatus. Compressive force generator V2.5 software was used to set times, stress type and force. Intermittent mechanical stress was set to cycle (1/3 Hertz and force amount $1g/cm^2$).

In some experiments, $CoCl_2$ (150-300 μM), exogenous ATP (10 μM), Rho inhibitor (1.27 nM), MRS2179 (5μM), cyclohexamine (10 μM), genistein (92.5 μM), Monensin (100 μM), SB431542 (10 μM), rhTGF-β1 (1-10 ng/ml), or TGF-β1 antibody (5 μg/ml) was added in the culture condition.

Cell stimulation and signaling inhibition

HPDLs were seeded in 6-well plates, at a density of $3x10^5$ cells per well. The cells were starved with serum-free media for 4 h before adding 150 μM of $CoCl_2$ with or without each kind of inhibitor for 30 min prior to apply force. The concentration of inhibitors employed in the present study was 10 μM for cyclohexamide, 92.5 μM for genistein, 10 μM for SB431542, 0.26 μM for Src inhibitor, 5 μM for MRS2179 (P2Y1 inhibitor) and 1.27 nM for Rho inhibitor. In some experiments, HPDLs were treated with 10 μM of exogenous ATP or 1-10 ng/ml of rhTGF-β1. After 24 h, RNA was extracted for real-time polymerase chain reaction (real-time PCR) analysis. The culture medium was kept at-80ºC for ELISA assay. Non-stressed HPDLs served as the control group.

Reverse transcription-polymerase chain reaction (RT-PCR)

After the treatment, whole cellular RNA was extracted with Trizol reagent according to the manufacturer's instructions (Molecular research Center, Cincinnati, Ohio, USA). RNA was quantified using a nanodrop2000 spectromonometer (Thermo Scientific, Wilmington, DE, USA). One microgram of each RNA sample was converted to cDNA by Improm-IITM (Promega, Madison, WI, USA). Subsequent to reverse transcription, real-time PCR were performed. The primer oligonucleotide sequences used were based on reported genes sequences in Genbank and are shown in Table1.

The gene expression was detected by RT-PCR. A semi-quantitative polymerase-chain reaction (PCR) were performed in DNA thermal cyclers (Biometra, Gottingen, Germany) using Tag polymerase (Taq polymerase enzyme kit (Invitrogen)). One μl of cDNA were amplified with a PCR volume of 25 μl. The

mixture was heated initially at 95°C for 2 min and then followed by 23cycles of 18S ribosome, 28 cycle for TGF-β1 and 32 cycles for IGF-1 and OPN with denaturation at 94°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 1.30 min. The amplified DNA were then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining (Sigma-Aldrich St. Louis, MO, USA). The signal was capture by Gel Doc (Biogenomed, Bangkok, Thailand). The band density was determined using Bio1D software (Biogenomed, Bangkok, Thailand).

Real-time polymerase chain reaction (real-time PCR)

For quantitative real-time PCR, the reaction was performed in a LightCycler instrument (Roche Diagnostics) with the LightCycler® 480SYBR Green I Master kit according to the manufacturer's specifications. The mixture was heated initially at 95°C for 10 min and then followed by 40 cycles with denaturation at 94°C for 10s, annealing at 60°C for 10s, and extension at 72°C for 10s. Gene expression was normalized to the 18S ribosome expression. RelQuant software (Roche Diagnostics) was used to determine relative gene expression. The results are shown as fold change values relative to the control group.

Enzyme-linked immunosorbent assay (ELISA)

The protein was extracted from cells using radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with protease inhibitors. The culture medium was also collected for evaluated the secreted protein. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). The concentration of TGF-β1 was determined using ELISA assay according to the manufacturing protocol (Quantikines immunoassay, R&D Systems, USA). The final product was evaluated the absorbance at 450 nm using microplate reader (BioTek, ELx800, USA).

Luciferin-Luciferase bioluminescence assay

Culture medium was collected for extracellular ATP measurement by ENLITEN® kit, the ATP assay system Bioluminescence Detection kit (Promega,Madison, WI, USA). Briefly, the Enliten Luciferase/Luciferin (L/L) medium was mixed with the sample at 1:1 ratio (PACKARD, Promega,Madison, WI, USA). The light signal was immediately measured by Hybrid multi-Mode Microplate Reader (Synergy™H1, BioTek). A standard curve was generated using a serially diluted ATP standard.

Immunofluorescence staining

HPDLs were seeded on a cover slip and the cover slips were put in 6-well plate at a density of 1 x 10^5 cells/ well. The cells were starved with serum-free media 4 h before adding 150 μM of $CoCl_2$ 30 min prior to apply force. Cold methanol was used to fix the HPDLs for 10 min. The primary antibody to TGF-β1 (25 μg/ml, R & D Systems Inc.) was used to incubate HPDLs. overnight followed by secondary antibody goat anti-mouse IgG (Life technologies) for 40 min. Cells were incubated with DAPI for 15 min followed by streptavidin-fluorescein isothiocyanate for 40 min in the dark. PBS was used to wash the cover three times and mounted for microscopy with Prolong® Gold antifade reagent (Invitrogen). The Fluorescence microscope (Axiovert 40 CFL, Carl Zeiss, Gottingen, Germany) was used to investigate the HPDLs and using Axiocam MRc5 and the AxioVs40v4.7.2.0 software to take a picture.

Nuclei isolation

HPDLs were seeded in 6-well plates at a density of 3x10^5 per well for apply the force. At indication time, the nuclei were isolated by nuclei EZ prep nuclei

isolation kit following to the manufacturing protocol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the media were rinse out and washed the cells with PBS on ice. Harvest and lyse the cell by nuclei EZ lysis. Transfer the entire cell lysate to a separate 1.5 ml centrifuge tube, vortex, and set on ice for 5 min. Collect the nuclei by centrifugation at 500x g for 5 min at 4 °C. Aspirate the clear supernatant from each tube and set the nuclei pellet on ice. Resuspend and wash nuclei in 1 ml of ice cold Nuclei EZ lysis buffer as follows. Vortex nuclei pellet, mix well and set on ice for 5 minutes. Collect washed nuclei by centrifugation at 500x g for 5 min at 4 °C. Aspirate the clear supernatant and set the nuclei pellet on ice. Resuspend each nuclei pellet in 200 µl of ice cold Nuclei EZ storage buffer, vortex pellet and kept at -80°C before use.

Statistical analyses

The data were informed as mean ± standard deviation. The student t-test was used for two-group comparisons and one-way analysis of variance (176) followed by Turkey's *post hoc* test (SPSS, Chicago, IL, USA). The p value less than 0.05 was considered as statistically significant.

Table 3.1 Primer sequence for polymerase chain reaction

Primer	**Forward (5' - 3')**	**Reverse (5' - 3')**
IGF-1 (NM000618.3)	CAT GCC TGC TCA GAA GGG TA	GCC TCT GAT CCT TGA GGT GA
OPN (NM000582.2)	AGT ACC CTG ATG CTA CAG ACG	CAA CCA GCA TAT CTT CAT GGC TG
TGF-β1 (NM000660.5)	GCT AAT GGT GGA AAC CCA CA	AGT GAA CCC GTT GAT GTC CA
18S (NR003286.2)	GTG ATG CCC TTA GAT GTC C	CCA TCC AAT CGG TAG TAG C
qOPN(NM001251830.1)	AGG AGG AGG CAG AGC ACA	CTG GTA TGG CAC AGG TGA TG
qTGF-β1 (NM000660.5)	GGA TAC CAA CTA TTG CTT CAG CT	AGG CTC CAA ATG TAG GGG CAG GG
q18S (NR003286.2)	GGC GTC CCC CAA CTT CTT A	GGG CAT CAC AGA CCT GTT ATT

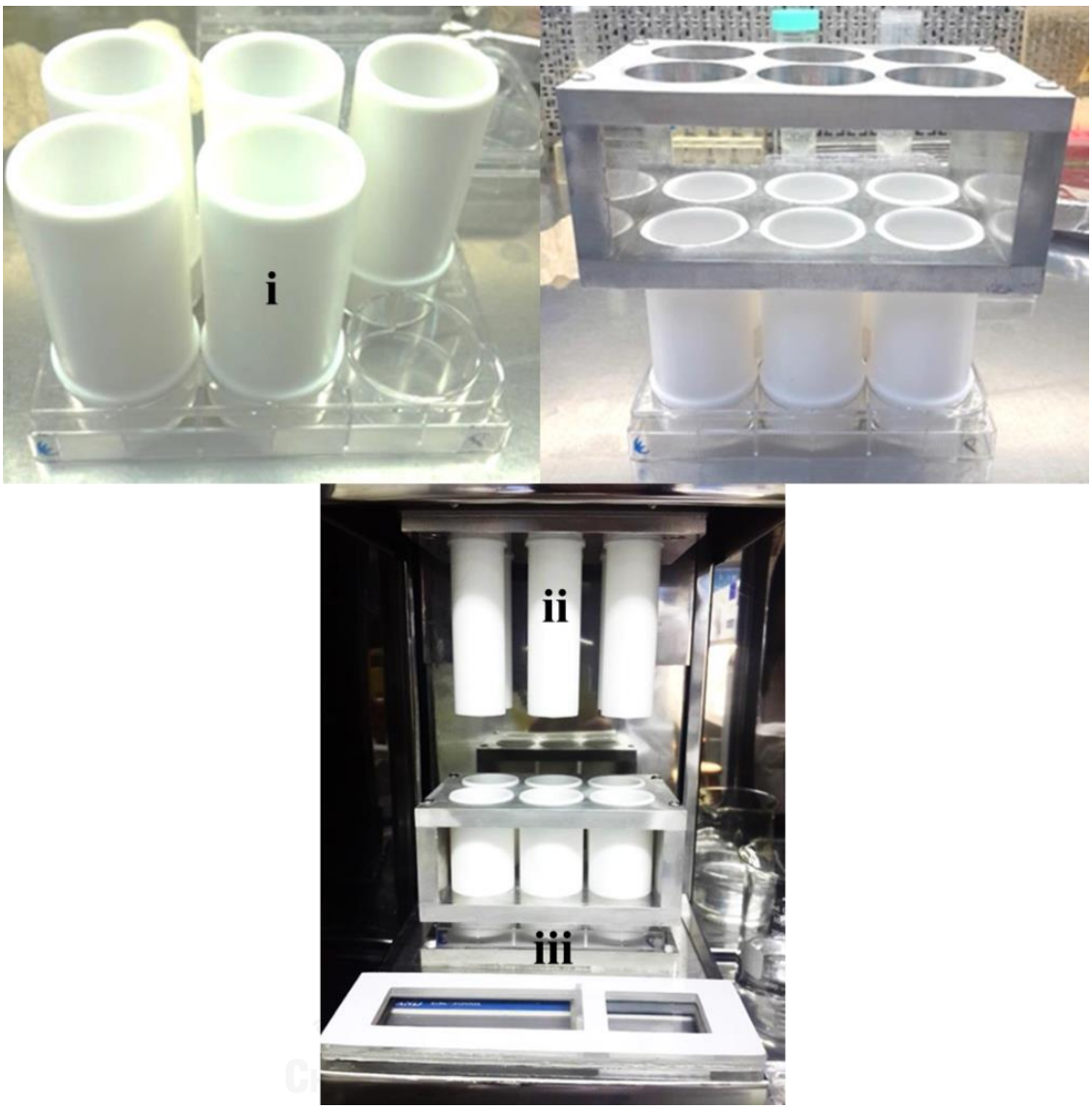


Figure 3.1 The machine compartment. (i) The removable plastic tube (ii) A hydraulic cylinder connecting to pumping part and (iii) The platform.

CHAPTER IV

RESULTS

Intermittent mechanical stress induced *IGF-1* and *OPN* expression in HPDLs at 24 h

We began by investigate the effect of intermittent mechanical stress to HPDLs viability and morphology using a microscope at 100X magnification. HPDLs morphology was similar in all the groups (Fig. 4.1A) and mechanical stress did not affect the HPDLs viability (Fig. 4.1B). Next, HPDLs were stimulated with intermittent mechanical stress (1 g/cm^2) at 2h, 4h, 8h and 24 h. After stress application, the *IGF-1* and *OPN* mRNA expression were analyzed by RT-PCR and real-time PCR. The results showed that *IGF-1* and *OPN* expression statistical significantly increased at 24 h (Fig. 4.1C&D). Thus, HPDLs could respond to intermittent mechanical stress by upregulate both *IGF-1* and *OPN* expression at 24 h.

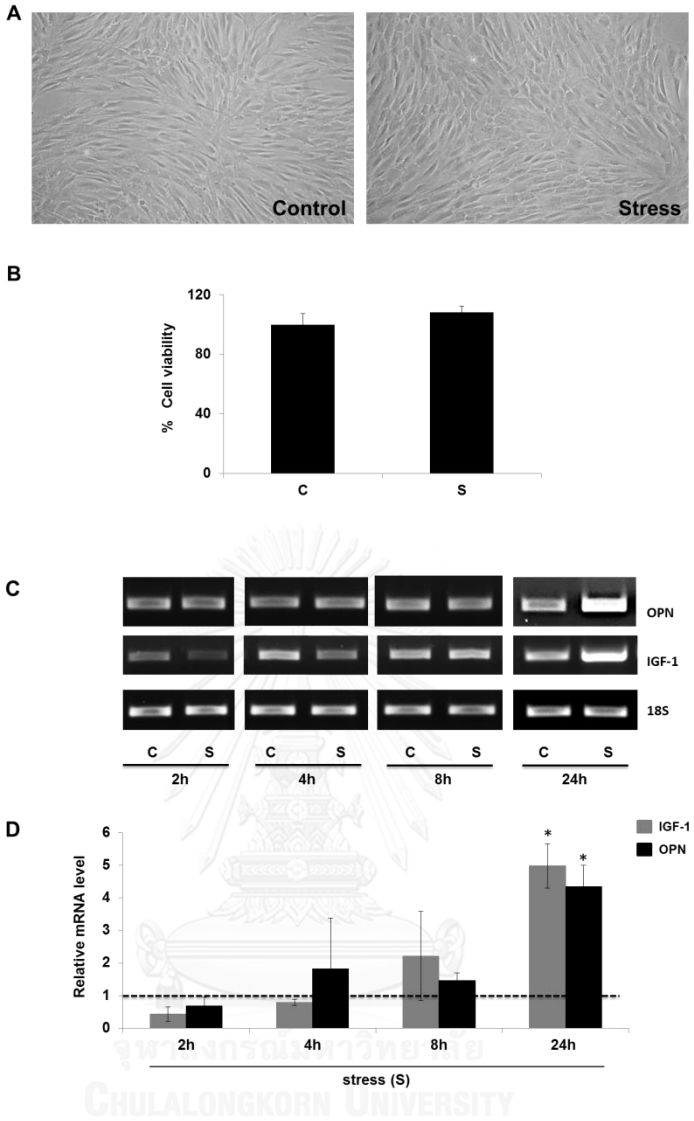


Figure 4.1 Effect of intermittent mechanical stress on *IGF-1* and *OPN* expression. HPDLs were treated with intermittent mechanical stress for 24 h. (A) The picture showed morphology of HPDLs (100X, original magnification). (B) The cell viability was examined using MTT assay. The *IGF-1* and *OPN* expression of the intermittent mechanical stress-treated HPDLs at 2h, 4h, 8h, and 24 h using RT-PCR (C) and real-time PCR (D). The dot line represented the expression levels of the control. The asterisks indicated statistical significance. (C; the control condition, S; the intermittent mechanical stress treatment condition)

Intermittent mechanical stress increased releasing of exogenous ATP

Previously, our group reported that the static compressive stress induced the ATP release to promote *OPN* and *IL-1β* expression by HPDLs (22, 26). In the present study, we further evaluated whether the intermittent mechanical stress altered ATP release and relate to *IGF-1* and *OPN* expression or not. At 24 h, the intermittent mechanical stress significantly enhanced ATP release by HPDLs (Fig. 4.2A). Further, we investigate the role of exogenous ATP on *IGF-1* and *OPN* expression. After HPDLs were exposed to exogenous ATP for 24 h, the increase of *OPN* mRNA levels was significantly noted (Fig. 4.2B&C). However, exogenous ATP did not effect to *IGF-1* expression in HPDLs. Together, the results suggest that intermittent mechanical stress-induced ATP release might be promote *OPN* expression in HPDLs.

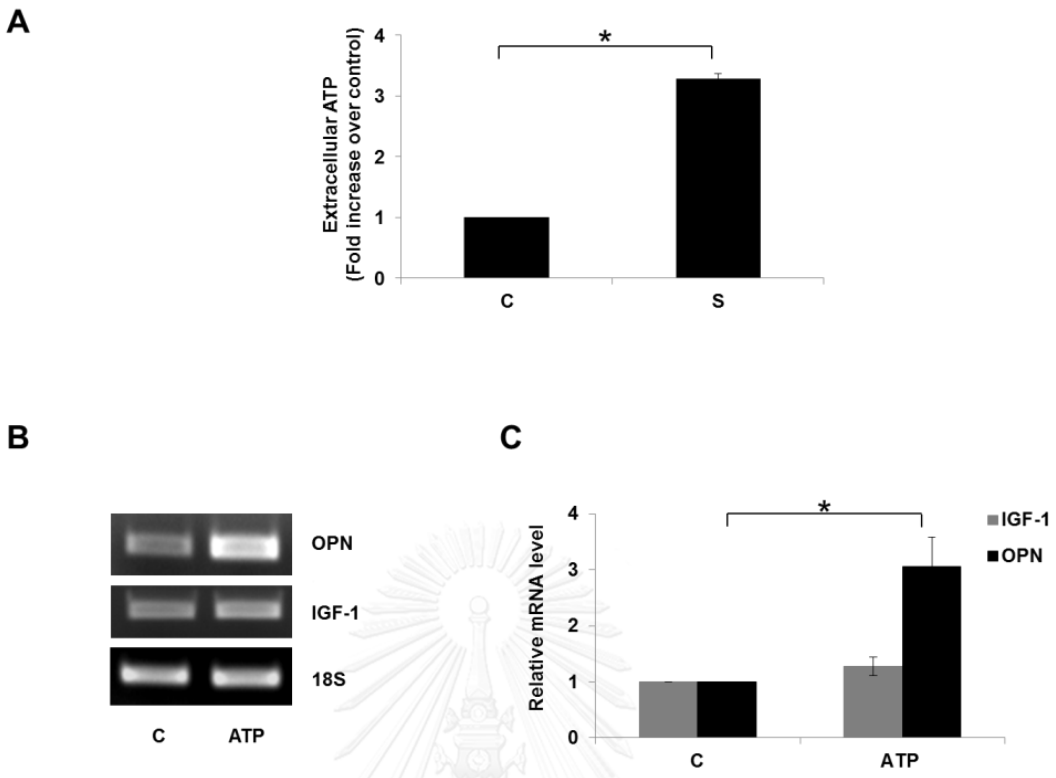


Figure 4.2 Effect of intermittent mechanical stress on ATP release. (A) The extracellular ATP was measured after treating cells with intermittent mechanical stress for 24 h using Luciferin-Luciferase bioluminescence assay. The gene expression was evaluated using RT-PCR (B) and confirmed by real-time PCR (C) upon treating cells with exogenous ATP (10 μM) for 24 h. The asterisks indicated statistical significance. (C; the control condition, S; the intermittent mechanical stress treatment condition)

Intermittent mechanical stress upregulated *OPN* expression via different pathway to static compressive stress

In addition, previously our group reported that static compressive force promoted *OPN* expression by HPDLs via ATP/P2Y1/Rho kinase signaling pathway (21, 26). However, in this study, Rho inhibitor or P2Y1 inhibitor (MRS2179) could not inhibit intermittent mechanical stress-induced *OPN* expression (Fig. 4.3A-D). Therefore, these results imply that the mechanisms of intermittent mechanical stress-induced *OPN* expression in HPDLs may different to those of the static mechanical stress.

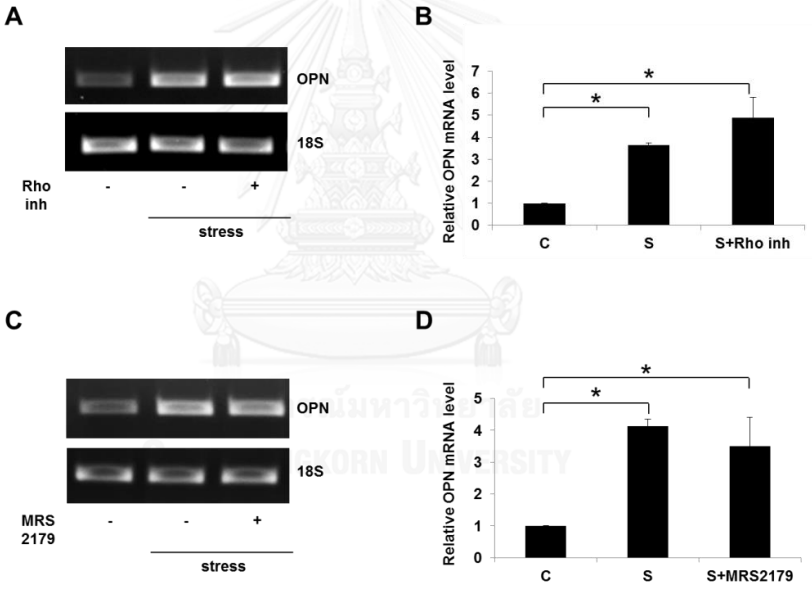


Figure 4.3 The effect of Rho inhibitor and MRS 2179. Rho inhibitor (1.27 nM) and MRS2179 (P2Y1 inhibitor; 5 μM) did not abolish the effect of intermittent mechanical stress on *OPN* expression. The *OPN* mRNA levels were evaluated using RT-PCR (A, C) and real-time PCR (B, D) upon treating cells in various conditions with the supplementation of Rho inhibitor ; A&B or MRS2179; C&D. The asterisks

indicated statistical significance. (C; the control condition, S; the intermittent mechanical stress treatment condition)

Intermittent mechanical stress required intermediate protein to induce *IGF-1* and *OPN* expression

Previous our data indicated intracellular signaling between static and intermittent compressive stress is different. Therefore, we start to use cyclohexamide, a chemical inhibitor of protein translation, was pretreated prior to apply the intermittent mechanical stress (Fig. 4.4A-B). Cyclohexamide completely inhibited the effect of intermittent stress-induced *IGF-1* and *OPN* expression by HPDLs. Furthermore, genistein is an autophosphorylation inhibitor of tyrosine kinases (187) was used to further identify the mechanism *IGF-1* and *OPN* expression (Fig. 4.4C-D). Corresponding to the effect of cyclohexamide, genistein abolished the intermittent mechanical stress-induced *IGF-1* and *OPN* expression by HPDLs.

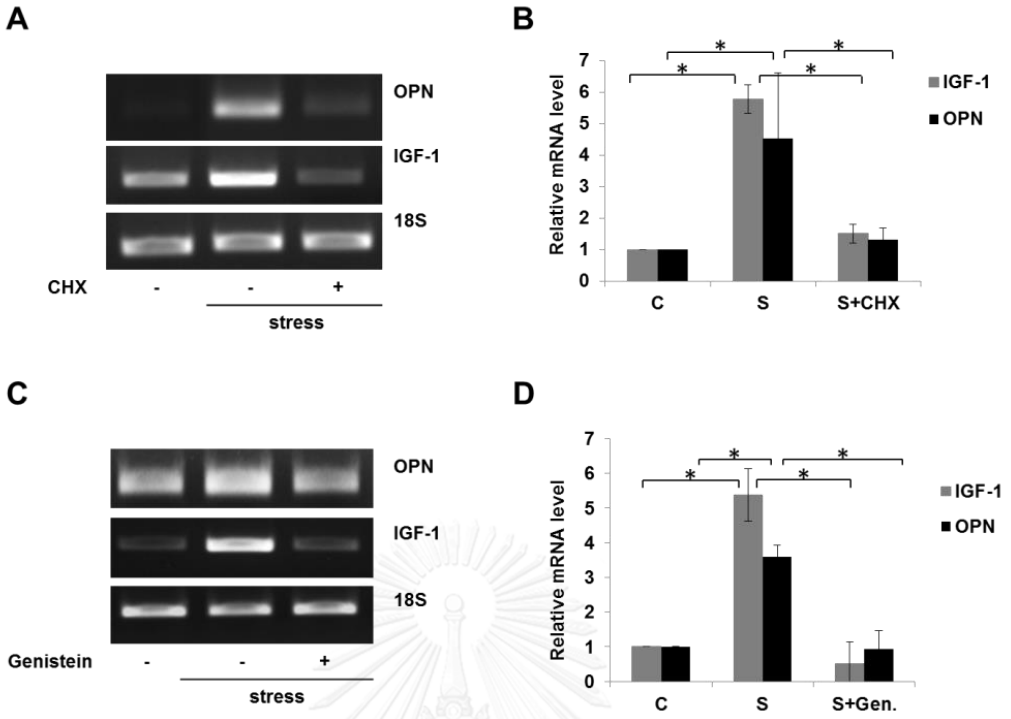


Figure 4.4 Intermittent mechanical stress required the intermediate protein to induce *IGF-1* and *OPN* expression. (A&B) Cyclohexamide (10μM), (C&D) genistein (92.5 μM) were pre-treated 30 min prior to apply the intermittent mechanical stress for 24 h. The mRNA expression was determined by RT-PCR (A&C) and real-time PCR (B&D). Asterisks indicated statistically significant. (C; the control condition, S; the intermittent mechanical stress treatment condition, CHX; cyclohexamide, GEN; genistein)

Participation of TGF-β1 in the intermittent mechanical stress-upregulated *IGF-1* and *OPN* gene expression

Having demonstrated that the intermittent mechanical stress-induced *IGF-1* and *OPN* mRNA expression was inhibited by genistein. Further, a TGF-β receptor I inhibitor (SB431542) was employed to clarify mechanism (Fig. 4.5A-B) because TGF-β1 has been shown to modulate the expression of IGF-1 and OPN in several cell types (49, 162, 188, 189) and intermittent compressive force could upregulate TGF-β1 expression by HPDLs (190). The result illustrated that SB431542 completely suppressed the intermittent mechanical stress-induced *IGF-1* and *OPN* expression by HPDLs. To confirm the role of TGF-β1 in this phenomenon, the neutralizing antibody against TGF-β1 (TGF-β1Ab) was used to block the binding of TGF-β1 and its receptors. Correspondingly with SB431542 treatment, the neutralizing antibody against TGF-β1 reduced the *IGF-1* and *OPN* transcription under intermittent mechanical stress stimulation (Fig. 4.5C-D). To further determine the intermittent mechanical stress induced *IGF-1* and *OPN* expression via TGF-β1. Recombinant TGF-β1 (rhTGF-β1) was used to stimulate HPDLs for 24 h. Our data indicated that rhTGF-β1 could activate both *IGF-1* and *OPN* expression in HPDLs (Fig. 4.6A-B). Taken together, we suggested that intermittent mechanical stress induced *IGF-1* and *OPN* expression in HPDLs via TGF-β1. Finally, to confirm the intermittent mechanical stress induced *IGF-1* and *OPN* expression through TGF-β1 protein secretion, we collected the cell culture medium from intermittent mechanical stress-treated group (CMS) as well as the control group (CMC) and transferred to another set of unstimulated HPDLs for 24 h. Surprisingly, both of *IGF-1*and *OPN* expression

in those cells incubated with CMS-treated group and CMC-treated group did not different (Fig. 4.6C-D).

Thus, we further measured the TGF-β1 protein levels in both condition medium and found that TGF-β1 protein levels in CMS did not different to CMC (Fig. 4.6E). However, the whole cell lysate from intermittent mechanical stress-treated group expressed significantly higher TGF-β1 protein levels than the control group (Fig. 4.6E). These evidences may imply that intermittent mechanical stress-induced TGF-β1 protein to activate *IGF-1* and *OPN* expression in HPDLs.

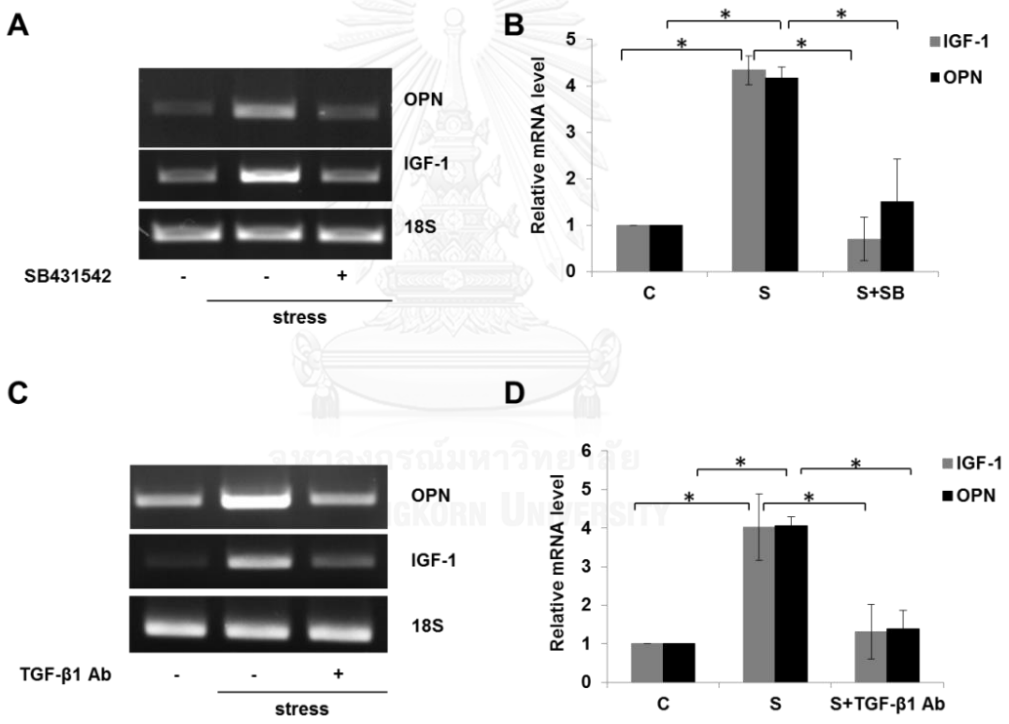


Figure 4.5 TGF-β1 related to intermittent mechanical stress-induced *IGF-1* and *OPN* expression. (A&B) SB431542 (TGF-β receptor I inhibitor; 10 μM) or (C&D) TGF-β1 neutralizing antibody (5 μg/ml) was used to pre-treat HPDLs 30 min before applying the intermittent mechanical stress for 24 h. The gene expression was measured by RT-PCR (A&C) and real-time PCR (B&D). (C; the control condition, S; the intermittent

mechanical stress treatment condition, SB; SB431542, TGF-β1 Ab; TGF-β1 neutralizing antibody)

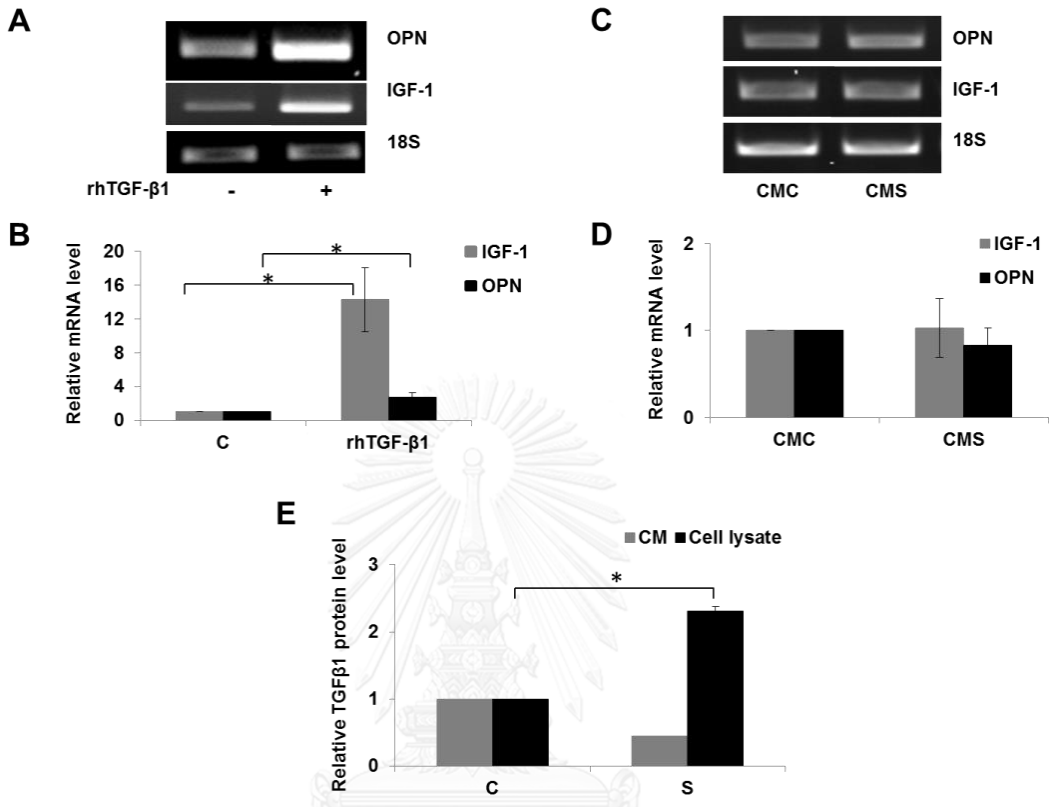


Figure 4.6 Participation of TGF-β1 in the intermittent mechanical stress-regulated gene expression. (A&B) *IGF-1* and *OPN* mRNA levels was examined upon HPDLs were treated with rhTGF-β1 (10 ng/ml) for 24 h. (C&D) HPDLs were treated with cell culture medium from intermittent mechanical stress-treated group (CMS) or untreated group (CMC) 24 h. The gene expressions were determined by RT-PCR (A&C) and real-time PCR (B&D). (E) The TGF-β1 protein in condition medium and whole cell lysate was measured by ELISA assay. Asterisks indicated statistically significant. (C; the control condition, S; the intermittent mechanical stress treatment condition, rhTGF-β1; recombinant TGF-β1)

Hypoxic-mimic condition involved to intermittent mechanical stress-regulated *IGF-1* and *OPN* expression in HPDLs

Hypoxic condition was mimicked using the $CoCl_2$ supplementation at 150-300 μM. We began by investigating the toxicity of $CoCl_2$ and stress plus $CoCl_2$ to HPDLs at 24 h by MTT assay (Fig. 4.7A-B). There was no significant difference in cell viability in all groups. Next, we investigated the combined effect of $CoCl_2$ and intermittent mechanical stress to HPDLs morphology using a microscope at 100X magnification. The morphology of HPDLs was similar in all the groups (Fig. 4.7C).

The role of hypoxic-mimic condition to intermittent mechanical stress was observed by RT-PCR and confirmed by real-time PCR. HPDLs were incubated with $CoCl_2$ 30 min before intermittent mechanical stress application for 24 h. The results showed that $CoCl_2$ did not significantly effect to *IGF-1* and *OPN* expression in normal culture (Fig. 4.7D-F). However, $CoCl_2$ significantly inhibited *IGF-1* expression upon the intermittent mechanical stress treatment in a $CoCl_2$ dose dependent manner. In opposite to *OPN* expression, we found that $CoCl_2$ enhanced intermittent mechanical stress induced *OPN* expression in a $CoCl_2$ dose dependent manner too. These data indicated that the effect of intermittent mechanical stress regulated *IGF-1* and *OPN* expression in HPDLs under normoxia is different to hypoxia. Therefore, next part we first investigated influence of chemical hypoxia to intermittent mechanical stress regulated *IGF-1* expression and following to *OPN* expression.

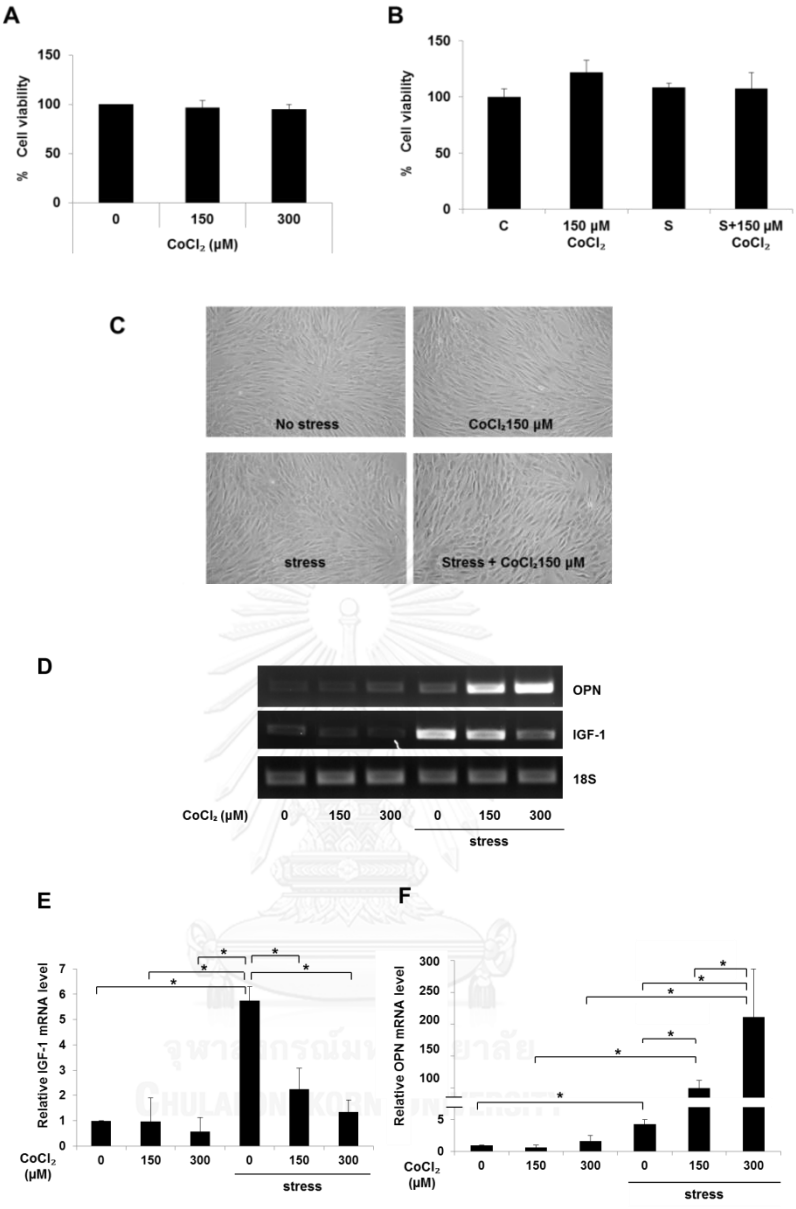


Figure 4.7 Effect of hypoxic-mimic condition to intermittent mechanical stress regulated *IGF-1* and *OPN* expression. HPDLs viability upon exposure to $CoCl_2$ or combined with intermittent mechanical stress was evaluated using MTT assay (A-B) and microscopic examination (C; 100X, original magnification) at 24 h. HPDLs were treated in each condition for 24 h. The mRNA expressions were evaluated by RT-PCR (D) and real-time PCR (E&F). Asterisks indicated statistically significant.

Hypoxic-mimic condition attenuated intermittent mechanical stress induced *IGF-1* expression

Previous results showed $CoCl_2$ attenuated intermittent mechanical stress-induced *IGF-1* expression by HPDLs. To further examine, HPDLs were pretreated with $CoCl_2$ 30 min prior stimulate with 2 ng/ml of rhTGF-β1. We found that $CoCl_2$ also inhibited the rhTGF-β1 induced *IGF-1* expression (Fig. 4.8A-B). In addition to rhTGF-β1-treated HPDLs under physical hypoxia (0.5% O_2) also inhibited the IGF-1 expression but no statistical significant (Fig. 4.8C). Thus, we hypothesized that $CoCl_2$ may affect to release of TGF-β1 protein from intermittent mechanical stress. To evaluate this hypothesize, whole cell lysate of stimulated-HPDLs under hypoxic-mimic condition were measured by ELISA. Surprisingly, the supplementation of $CoCl_2$ led to the slightly increase of TGF-β1 protein expression. Though, no statistical significance was observed. However, TGF-β1 protein level stimulated by intermittent mechanical stress under hypoxic-mimic condition was more increased than stress application alone (Fig. 4.8D). Therefore, our hypothesis was rejected. We concluded that $CoCl_2$ did not affect to release of TGF-β1 protein expression but it affect the intracellular signaling of TGF-β1 in order to induce *IGF-1* expression in HPDLs.

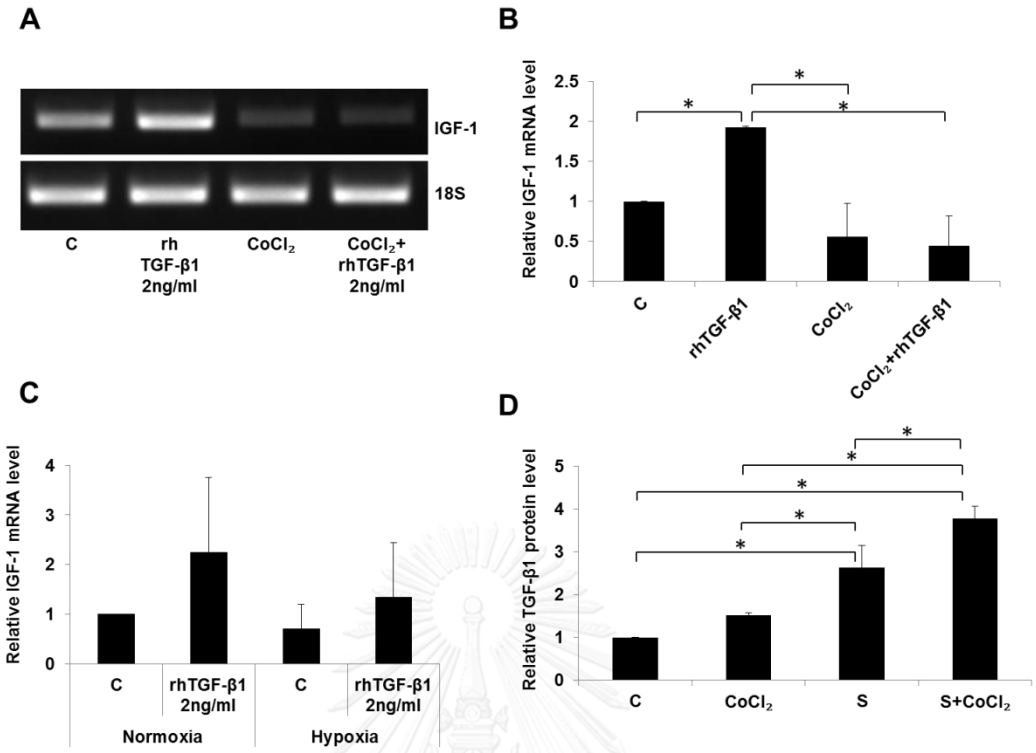


Figure 4.8 Hypoxic mimic condition arrested intermittent mechanical stress-induced *IGF-1* expression. HPDLs were cultured with rhTGF-β1 (2 ng/ml) with or without $CoCl_2$ (150 μM) for 24 h. *IGF-1* expression was measured using RT-PCR (A) and real-time PCR (B). HPDLs were treated with rhTGF-β1 (2ng/ml) under 21% and 0.5% oxygen. *IGF-1* expression was examined by real-time PCR (C).The TGF-β1 protein in whole cell lysate was measured by ELISA assay (D). Asterisks indicated statistically significant. (C; the control condition, S; the intermittent mechanical stress treatment condition, rhTGF-β1; recombinant TGF-β1)

Intermittent mechanical stress upregulated *OPN* expression under hypoxic mimic condition in the ATP independent manner

Similarly to our results in normoxia that we showed intermittent mechanical stress induced release of exogenous ATP to activate *OPN* expression. To determine this effect in chemical hypoxia, $CoCl_2$ was used to pretreat the HPDLs 30 min prior intermittent mechanical stress application. In the present study, we further evaluated whether $CoCl_2$ and the intermittent mechanical stress altered ATP release (Fig. 4.9A). At 24 h, the results demonstrated that $CoCl_2$ treatment led to the slightly increase of ATP release. However, no statistical significance was noted. The intermittent mechanical stress significantly enhanced ATP release by HPDLs. The combination treatment of $CoCl_2$ and the intermittent mechanical stress did not have additional effect on ATP release as compared to those treated with intermittent mechanical stress alone.

Further, we investigated the role of exogenous ATP on *OPN* expression. After HPDLs were exposed to exogenous ATP for 24 h, the increase of *OPN* mRNA levels was significantly noted (Fig. 4.9B). Moreover, the addition of $CoCl_2$ in combination with exogenous ATP did not enhance *OPN* expression compared to the control. Together, the results suggest that intermittent mechanical stress-induced ATP release might be promoted *OPN* expression in normoxia but not in the hypoxia.

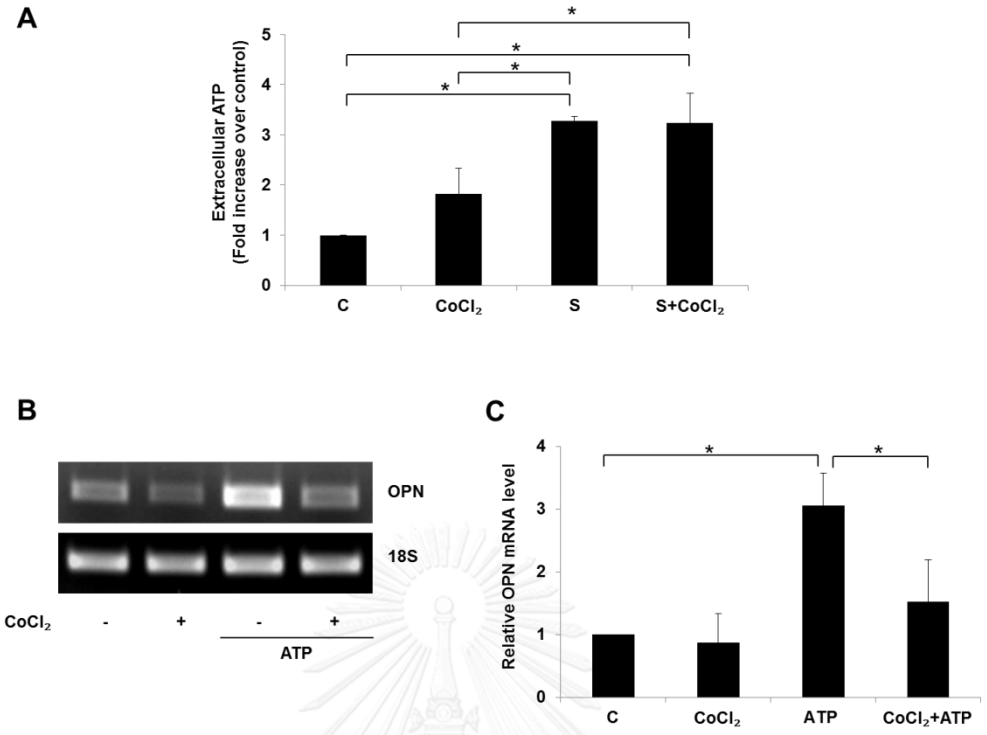


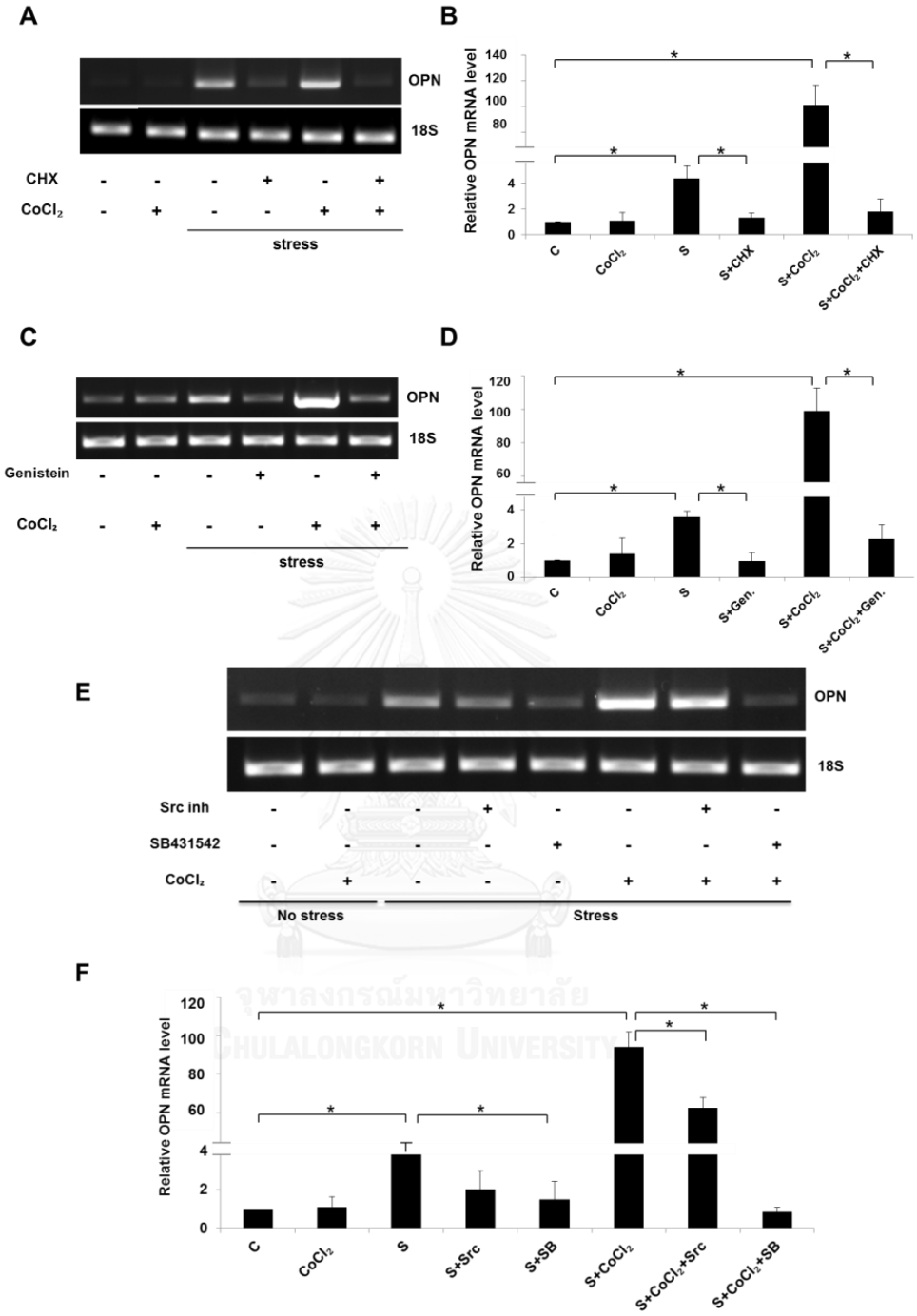
Figure 4.9 Hypoxic mimic condition abolished exogenous ATP-induced *OPN* expression. (A)The extracellular ATP was measured after treating HPDLs with intermittent mechanical stress with or without $CoCl_2$ for 24 h using Luciferin-Luciferase bioluminescence assay. HPDLs were cultured with exogenous ATP (10μM) with or without $CoCl_2$ (150 μM) for 24 h. *OPN* expression was measured using RT-PCR (B) and real-time PCR (C). Asterisks indicated statistically significant. (C; the control condition, S; the intermittent mechanical stress treatment condition)

Intermittent mechanical stress increased *OPN* expression under hypoxic-mimic condition via TGF-β1 pathway

Correspondingly to normoxia, we firstly investigated intermittent mechanical stress-induced *OPN* expression in hypoxia require intermediate protein or not. Cyclohexamide was used again by adding with $CoCl_2$ 30 min prior to intermittent mechanical stress application for 24 h (Fig. 4.10A-B). The data indicated that intermittent mechanical stress induced *OPN* expression under hypoxic mimic condition through intermediate protein. Following to normoxia, HPDLs were pretreated with genistein and $CoCl_2$ together 30 min before apply the stress for 24 h. Corresponding to the effect of cyclohexamide, genistein could completely inhibit *OPN* expression-induced by intermittent mechanical stress under chemical hypoxia (Fig. 4.10C-D). Next, we used SB431542, which is a TGF-β1 receptor inhibitor (a tyrosine kinase receptor inhibitor) and also an inhibitor of src, which is a family of non-receptor tyrosine kinases for clarify mechanism. The *OPN* mRNA expression was analyzed by RT-PCR and real-time PCR (Fig. 4.10E-F). We observed that SB431542 completely suppressed *OPN* expression induced by intermittent mechanical stress not only normoxia but also hypoxia while the src inhibitor partially decreased *OPN* expression in both conditions.

To confirm this phenomenon, rhTGF-β1 was used to treat HPDLs with or without $CoCl_2$ for 24 h. The data showed consistency with previously our results, the addition of exogeneous rhTGF-β1 plus $CoCl_2$ resulted in the upregulation of *OPN* mRNA levels at 24 h (Fig. 4.10G-H). However, the $CoCl_2$ did not have the additional effect on rhTGF-β1-induced *OPN* expression. In contrast to physical hypoxia, the *OPN* expression was upregulated under hypoxic condition but no statistical significant

while, rhTGF-β1-treated HPDLs under hypoxia (0.5% O_2) showed increased level of *OPN* expression as same as under $CoCl_2$ condition (Fig. 4.10I). Thus, the data indicated that the intermittent mechanical stress promotes TGF-β1 release and further initiates TGF-β signaling pathway to induce *OPN* expression by HPDLs in both normoxic and hypoxic mimic condition.


A
OPN
18S
CHX
CoCl2
stress
B
Relative OPN mRNA level
C
CoCl2
S
S+CHX
S+CoCl2
S+CoCl2+CHX
C
Genistein
D
S+Gen.
S+CoCl2+Gen.
E
Src inh
SB431542
No stress
Stress
F
S+Src
S+SB
S+CoCl2+Src
S+CoCl2+SB

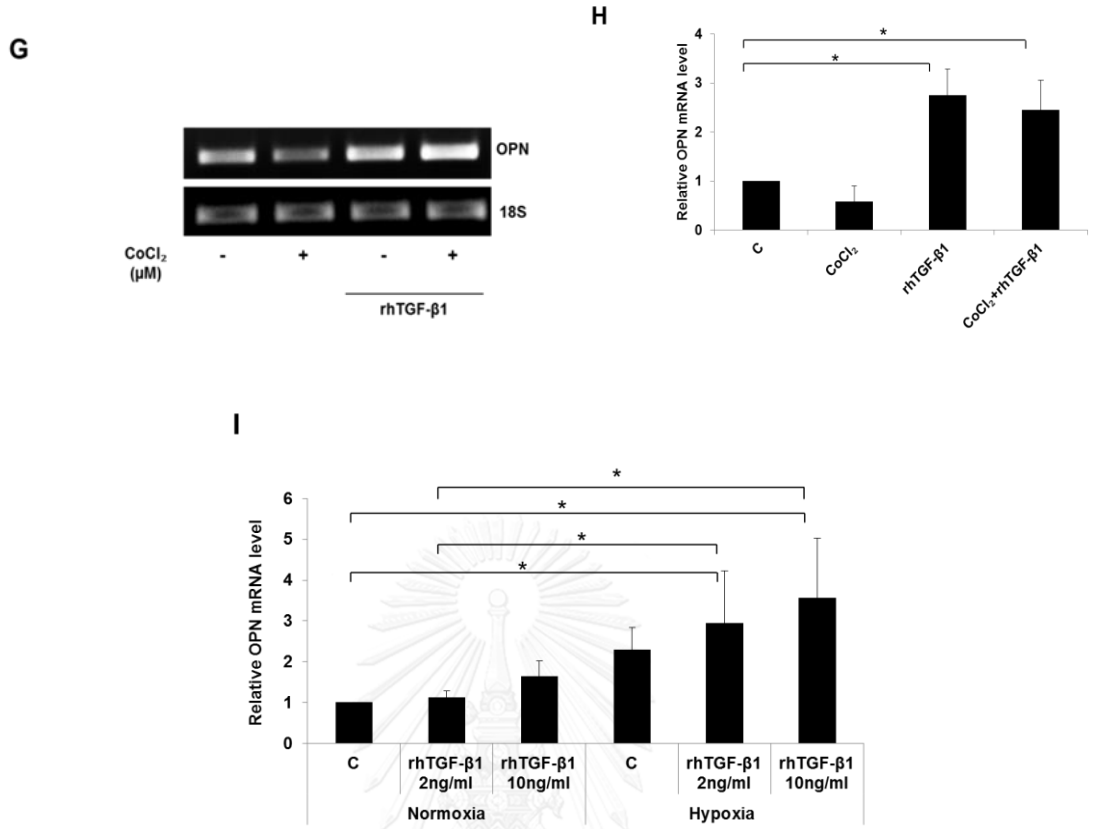


Figure 4.10 Hypoxic mimic condition enhanced intermittent mechanical stress-induced *OPN* expression via TGF-β1 pathway. HPDLs were incubated with cyclohexamide (10 μM; A&B), genistein (92.5 μM; C&D), src inhibitor (0.26 μM) and SB431542 (10μM; E&F) with or without $CoCl_2$ (150 μM) 30 min prior apply the intermittent mechanical stress for 24 h. (G&H) HPDLs were treated with rhTGF-β1 (2-10ng/ml) with or without $CoCl_2$ (150μM) for 24 h or under hypoxia (0.5% O_2). The mRNA expression was determined by RT-PCR (A, C, E, G) and real-time PCR (B, D, F, H, I). Asterisks indicated statistically significant. (C; the control condition, S; the intermittent mechanical stress treatment condition, CHX; cyclohexamide, GEN; genistein, SB; SB431542, rhTGF-β1; recombinant TGF-β1)

CHAPTER V

DISCUSSION AND CONCLUSION

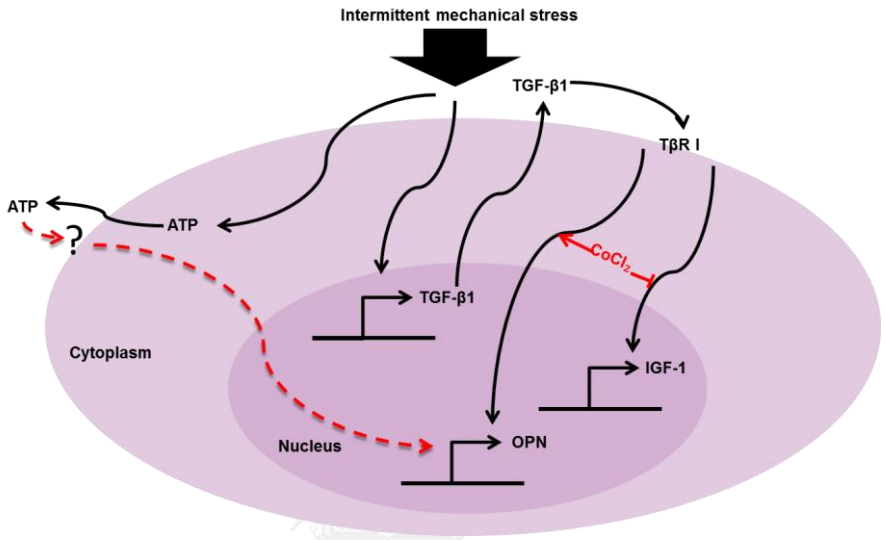


Figure 5.1 The diagram demonstrated the proposed signaling mechanism of the intermittent mechanical stress-induced *IGF-1* and *OPN* expression by HPDLs.

The physiological force is one of the important factors in maintaining periodontium homeostasis (4). However, in pathological condition (i.e. periodontal disease), the physiological force may lead to tissue destruction (11, 13). This study investigated the influence of intermittent mechanical stress on *IGF-1* and *OPN* mRNA expression in HPDLs under normoxic and hypoxic mimic condition to represent the clinical situation in which occlusal force facilitated periodontitis progression. Our data showed that intermittent mechanical stress alone could upregulate both *IGF-1* and *OPN* expression in HPDLs. Further, we found that the intermittent mechanical stress promoted *IGF-1* and *OPN* expression via TGF-β1 signaling pathway in normoxic condition.

Surprisingly, the hypoxic mimic condition using $CoCl_2$ synergistically enhanced intermittent mechanical stress-induced *OPN* expression. In contrast, the hypoxic mimic condition could attenuate the intermittent compressive stress-induced *IGF-1* expression, implying that occlusal force may not induced *IGF-1* expression in deep periodontal pocket, where it was considered as a hypoxic microenvironment. In opposite to *OPN* expression, we also found that the intermittent mechanical stress induced *OPN* expression through TGF-β1 signaling pathway in both normoxia and hypoxia. The propose signaling mechanism is illustrated in **Figure 5.1.**

IGF-1 plays an important role in bone growth and development (39, 191) and promotes cell proliferation and osteogenic differentiation in HPDLs (31, 35). In addition, the *in vitro* study demonstrated that IGF-1 is a growth factors that early response in mechanical stress (20) and play a key role in bone formation under mechanical loading (38, 39). In the *in vivo* orthodontic tooth movement model, the orthodontic force or occlusal stimuli significantly enhanced IGF-1 expression in

HPDLs (19). Correspondingly, our data showed that intermittent mechanical stress induced *IGF-1* expression in HPDLs. Although, currently evidences supported the influence of mechanical stress induced IGF-1, no evidences explore about the detail signaling mechanism of this action.

The role of OPN was reported in both physiological and pathological situation. It has been shown that OPN involves in adhesion, attachment, and spreading of osteoclasts to the bone surface during bone resorption (92, 186). Moreover, OPN is an important role in response to mechanical stress (46, 47, 50). The *in vivo* model of excessive force showed OPN expression at the area of the periodontal ligament destruction in rat (5). In addition, it was noted that the exposing of rat bone cells to mechanical stress resulted in the increase OPN expression (44). Static compressive stress showed the role of ATP to activate numerous signaling in HPDLs (21-25) including static compressive force promoted OPN expression by HPDLs via Rho kinase/ATP/P2Y1 signaling pathway (21, 26). Although, the intermittent mechanical stress could upregulate ATP by HPDLs, Rho inhibitor or P2Y1 inhibitor could not inhibit intermittent mechanical stress-induced *OPN* expression in both normoxic and hypoxic condition. Therefore, these results imply that the mechanisms of intermittent mechanical stress-induced *OPN* expression in HPDLs may different to those of the static mechanical stress. Furthermore, exogenous ATP did not activated *IGF-1* expression by HPDLs. Thus, we concluded that ATP releasing from intermittent mechanical stress could activate only *OPN* expression but did not relate in *IGF-1*.

To clarify the intracellular mechanism(s) of the intermittent mechanical stress induced *IGF-1* and *OPN* expression, cyclohexamide was used to investigate the intermediate protein. Our results found that the pretreatment with cyclohexamide

inhibited the intermittent mechanical stress-induced *IGF-1* and *OPN*, implying that the intermediate protein is required in this phenomenon. Previous studies demonstrated that *IGF-1* and *OPN* could be upregulated by several types of growth factors (48) including TGF-β (49, 162, 189). Although, serine/threonine kinase activity was implicated in TGF-β signaling, several evidences support involvement of tyrosine kinases as well (187, 192, 193). Thus, genistein which is a tyrosine kinase receptor inhibitor was used. The results showed that genistein also inhibited the intermittent mechanical stress-induced *IGF-1* and *OPN* expression. Therefore, we chose SB431542, which is a potent and specific inhibitor of the TGF-β receptor I (152) to clarify detail mechanism. Corresponding to genistein, SB431542 could completely inhibit the influence of the intermittent mechanical stress on *IGF-1* and *OPN* expression, suggesting the participation of TGF-β1 signaling.

The present study also reported that the intermittent mechanical stress induced TGF-β1 production. Corresponding with recently evidence, they observed intermittent mechanical stress induced TGF-β1-activated SOST and periostin by HPDLs (190). Moreover, the mechanical stretch, static & intermittent compressive force, and shear stress could enhance TGF-β1 expression in various cell types (55, 57, 190, 194). Thus, the present study was the first report demonstrated that the intermittent mechanical stress promoted *IGF-1*and *OPN* expression by HPDLs through TGF-β1 pathway.

The intermittent compressive stress enhanced the increase of TGF-β1 protein expression in cell lysate and the addition of rhTGF-β1 resulted in the upregulation of *IGF-1* and *OPN* expression similar to those treated with the intermittent mechanical stress. The relationship between TGF-β1 and OPN were investigated. Rat

immortalized renal proximal tubular cells (IRPTCs) were treated with TGF-β1 showed OPN upregulation (49). This phenomenon was observed in smooth muscle cells (189). Correspondingly, previously study demonstrated that the TGF-β1 treatment significantly increased IGF-1 expression in a dose and time dependent manner in human marrow stromal osteoblast precursor cells (188). It was also shown that single-dose administration of TGF-β1 promoted the osteogenic maker expression via the expression of IGF-1 since the knockdown of insulin receptor substrate 1 could attenuate the TGF-β1-induced osteogenic marker expression (162). However, it should be noted that the repeat-dose of TGF-β1 led to the inhibition of IGF-1 expression and subsequently caused the suppression of osteogenic differentiation in HPDLs, human mesenchymal stem cells, and murine preosteoblast (MC3T3-E1 cells) (162). Moreover, TGF-β inhibited migration in C2C12 skeletal muscle satellite cell and P19 embryonal carcinoma cell via decreasing IGF-1 (151). Collectively, several evidences indicated the closely relationship between TGF-β1 and IGF-1 in a positively or negatively regulator depend on cell types.

Several evidences showed influence of hypoxia on HPDL's behavior. For example, the hypoxia enhanced LPS induced inflammation (64, 195) and the hypoxia promoted TGF-β1 induced extracellular matrix synthesis (148).The effect of hypoxia in periodontal pocket is associated with virulent of periodontal disease such as the strain of anaerobic bacteria growth (61, 196), the increase of the host-inflammatory response (64, 195), and the enhancement of alveolar bone loss (121, 197). Therefore, both intermittent mechanical stress and hypoxia are contributing factors to periodontal disease progression, leading us to investigate the effect of combined those two factors on HPDLs. $CoCl_2$ was employed to mimic hypoxic condition in the culture system

due to the limitation of loading machine setting in a hypoxic chamber. Our group previously reported that $CoCl_2$ supplementation in culture medium induced the expression of a master transcription factor of the adaptive response to hypoxia, hypoxia-inducible factor-1alpha (HIF-1α) (133). It was demonstrated that HIF-1α was increased in periodontal pockets more than control group (63). Several publications were employed $CoCl_2$ in the study to mimic hypoxic condition in culture and illustrated the similar effects of $CoCl_2$ and hypoxia (132, 136, 198, 199). Though, it should be noted that the $CoCl_2$ supplementation might not fully represent the physical hypoxia (i.e. hypoxic chamber) as the main action is specific on the stabilization of HIF-1α (133, 139). It has been shown that hypoxic chamber and hypoxic mimic agent ($CoCl_2$) may utilize the different detailed regulatory pathway (200). Thus, the interpretation of the results in the present study should be done with caution and implied for hypoxic mimic condition. Thus, the results from the present study may not directly imply to those of physical hypoxia setting. Further experiment is required to fully investigate the role of hypoxia on the intermittent compressive stress-induced *IGF-1* or *OPN* expression in HPDLs.

In this study, artificial hypoxic agent, $CoCl_2$ abolished the intermittent mechanical stress-induced *IGF-1* expression in HPDLs. This phenomenon is consistent to physical hypoxia. This condition represents the clinical situation, where the physiological force was loaded on periodontitis's teeth. Therefore, this data assumed that hypoxia attenuated the intermittent mechanical stress-induced osteogenic differentiation through decrease *IGF-1* expression in HPDLs. Though, the further investigation is indeed required to claim this hypothesis. Recently, it was demonstrated that the cyclic tensile stress under hypoxic condition regulated

proliferation and osteogenic differentiation in HPDLs via MAPK pathway (56). Thus, this information could imply that the type, amount, and direction of force may play an important role in the HPDLs' response under hypoxic condition.

The present study showed that hypoxic mimic condition attenuated the intermittent mechanical stress-induced *IGF-1* expression in HPDLs. However, in unloading condition, $CoCl_2$ did not significantly affect *IGF-1* expression. Corresponding with previous studies, physical hypoxia and $CoCl_2$ attenuated *IGF-1* expression via the suppression of Runx2 and the induction of C/EBPδ in rat osteoblasts (201). Runx2 could bind to the upstream element in IGF-1 gene promotor and regulated *IGF-1* expression (201). Further, in systemic investigation, the serum IGF levels were decreased in acute respiratory distress patients, which were a hypoxia state (202). On contrary, it was shown that $CoCl_2$ decreased *IGF-1* expression in fish muscle (203). In addition, the IGF-1 expression was upregulated by hypoxia in HepG2 cells (204). Further, in the present study, we demonstrated that $CoCl_2$ inhibited rhTGF-β1-induced *IGF-1* expression in HPDLs. The previous study demonstrated that hypoxia inhibited TGF-β1-induced transformation in rabbit corneal keratocyte (205). Together, the further study to evaluate, the mechanism of hypoxic mimic condition on the inhibition of TGF-β1-induced *IGF-1* expression in HPDLs is necessitated.

Opposite to *IGF-1*, we demonstrated that $CoCl_2$ treatment alone did not influence *OPN* mRNA levels in HPDLs but physical hypoxia alone could induce *OPN* expression. As mention above, hypoxic chamber and hypoxic mimic agent (CoCl2) may utilize the different detailed regulatory pathway (200). Thus, the interpretation of

the results in the present study should be done with caution and implied for hypoxic mimic condition. On the contrary, $CoCl_2$ was previously shown to promoted *OPN* expression in rat tubular NRK52E cells via the p38MAPK signaling pathway (140). Moreover, we showed in the present study that the supplementation of $CoCl_2$ did not significantly increase the ATP release by HPDLs. Though, $CoCl_2$ was able to increase ATP production by human skin keloid fibroblasts, while it showed to decrease cellular ATP levels in extravillous trophoblast cell line (206, 207). These contradict results may imply the different regulation of $CoCl_2$ in different cell types. Interestingly, we also noted in the present study that $CoCl_2$ enhanced the intermittent compressive stress-induced *OPN* expression. Further, the $CoCl_2$ supplementation did not alter the intermittent mechanical stress-promoted ATP release by HPDLs and the exogenous ATP failed to enhance *OPN* expression in the presence of $CoCl_2$. Together, these data suggest that the intermittent mechanical stress induced OPN mRNA expression in normoxic or hypoxic mimic condition may be utilized different intracellular mechanism(s).

Normally, TGF-β signaling is activated and phosphorylate the R-Smad2/3 and Co-Smad (Smad4) will be bind to a complex before translocate to the nucleus. The co-activator such as CREB binding protein (CBP)/p300 will be recruited to regulate the target genes. This co-activator is not required for TGF-β1 only. CBP/p300 will be requiring for HIF-1α. Thus, it might be noted that HIF-1α competition for co-activator. Finally, the TGF-β response is altering (205). However, the cross-talk between HIF-1α and TGF-β is probably depending on the cell type. The HIF-1α and Smad complex showed synergistically activate erythropoietin gene (208), collagen (209) and OPN (210). Therefore, it might possible that *IGF-1* expression under

hypoxia in our result required the CBP/p300 to activation. However, this co-activator is not necessary for *OPN* expression. To understanding this phenomenon, the future study is required.

Subsequently, the present study also demonstrated that HPDLs treated with rhTGF-β1 could induce *OPN* expression in the presence or absence of the hypoxic mimic agent. However, the addition of $CoCl_2$ did not synergist the induction effect. Together, we concluded that the intermittent mechanical stress induced *OPN* mRNA expression via TGF-β1 signaling, corresponding with previous studies that shown the relation of TGF-β1 and OPN (149, 211).

In conclusion, our results demonstrated that intermittent mechanical stress increased *IGF-1* and *OPN* expression by HPDLs and the intermittent mechanical stress-induced *IGF-1* expression via TGF-β1 signaling pathway in HPDLs. Further, the hypoxic mimic agent could abolish this effect. In opposite to OPN expression, intermittent mechanical stress under both conditions could activate *OPN* expression via TGF-β1 pathway. Our study suggests that the intermittent mechanical stress plays an important role in the regulation of the periodontal ligament homeostasis by upregulation of *IGF-1* and *OPN* expression. However, the level of oxygen was an essential factor to HPDLs's response to intermittent mechanical stress. The complete intracellular network is required further investigation.

Future studies and preliminary data

- To investigate the relationship between ATP releasing and TGF-β1 signaling.
- To study the relationship between integrin and TGF-β1 activation under intermittent mechanical stress.
- To study the intracellular signaling of TGF-β1 regulate IGF-1 and OPN expression in both normoxic and hypoxic-mimic condition.

Intermittent mechanical stress could upregulate *IGF-1* and *OPN* expression in normoxia. However, under hypoxic mimic condition, TGF-β1 protein did not induce *IGF-I* expression-stimulated by intermittent mechanical stress which is opposite to *OPN* expression. Previous data, we found that $CoCl_2$ enhanced the TGF-β1 protein releasing-activated from intermittent mechanical stress. Taken together, we wondered if $CoCl_2$ has any effects in TGF-β1 induced *IGF-1* and *OPN* expression in HPDLs.

The first hypothesis is hypoxic-mimic condition affect to the localization of TGF-β1 protein and this phenomenon influenced to activate the gene expression.

To evaluate the location of TGF-β1 under each condition, HPDLs were stimulated with intermittent mechanical stress under normoxia and chemical hypoxia. Immunofluorescence staining was used to detect TGF-β1 protein and each group represents 3 serial sectional levels of the HPDLs. The figure showed that intermittent mechanical stress promoted TGF-β1 proteins translocate to the nucleus (Fig.5.2 J-L) while chemical hypoxia inhibited this phenomenon and promoted the TGF-β1 accumulated at the nuclear membrane (Fig. 5.2 G-I and M-O). From these results, we

assumed that *IGF-1* upregulation required TGF-β1 nuclear translocation, but this situation did not necessary for *OPN* expression in HPDLs.

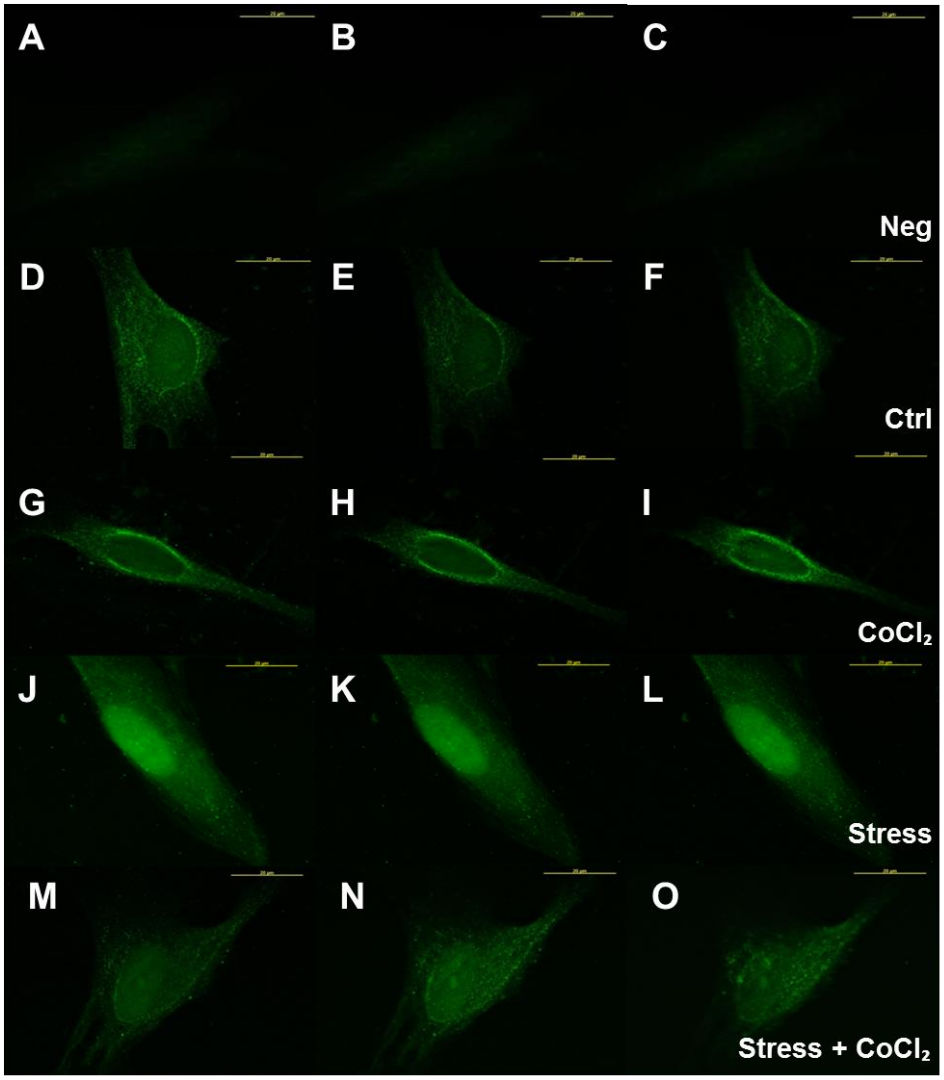


Figure 5.2 Hypoxic-mimic condition blocked intermittent mechanical stress-promoted TGF-β1 nuclear translocation. HPDLs were treated $CoCl_2$ with or without intermittent mechanical stress for 24 h. Immunofluorescence staining showed the positive staining of TGF-β1 (green). Each group represents 3 serial sectional levels of HPDLs. Scale bars = 20 μm

However, no evidence indicated TGF-β1 protein can move into the nucleus before. Thus, to confirm the intermittent mechanical stress promoted the translocation of TGF-β into the nucleus of HPDLs. After stimulated-HPDLs by intermittent mechanical stress with or without $CoCl_2$, HPDLs were isolated the nucleus using nuclear isolation kit and following measured the TGF-β1 protein by ELISA. The results indicated that the level of TGF-β1 protein in nucleus increased in intermittent mechanical stress compared with the control group (Fig. 5.3). However, the nuclear isolation kit might not separate the nuclear membrane from the nuclei extract. Thus, the data showed that the combination of intermittent mechanical stress and $CoCl_2$ showed the TGF-β1 protein was higher than intermittent mechanical stress alone.

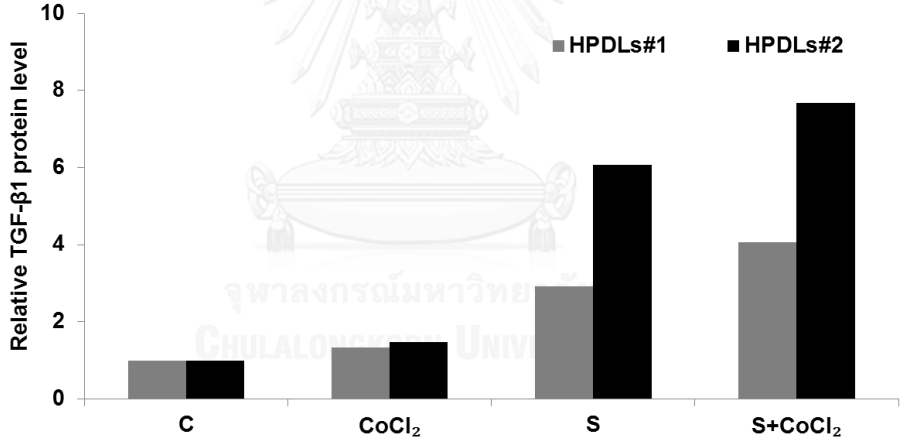


Figure 5.3 Intermittent mechanical stress with and without $CoCl_2$ promoted TGF-β1 nuclear translocation in HPDLs. The nuclei of HPDLs were isolated and the TGF-β1 protein level was analyzed by ELISA. The results showed from two cell lines. (C; the control condition, S; the intermittent mechanical stress treatment condition)

The interesting topics for investigation the first hypothesis include:

- To investigate the TGF-β1 protein nuclear translocation alone or with the receptor by co-immunoprecipitation (Co-IP) and double immunofluorescence straining with TβRII/I.
- To investigate the TGF-β1 protein location has an effect to their function by specific inhibitor such as brefeldin A(BFA); disrupt the structure and function of golgi apparatus, monensin; the protein transport inhibitor and accumulate the cytokine or protein in the golgi complex and endoplasmic reticulum and N-ethylmaleimide (NEM); the vestibular transport blocking.

The second hypothesis is TGF-β1 use the different pathway to activate *IGF-1* and *OPN* under hypoxic-mimic condition.

The interesting topics for investigation the second hypothesis include:

- To investigate the intracellular signaling molecule of TGF-β1 after intermittent mechanical stress stimulation in both normoxia and hypoxia for example the expression of TβRI, TβRII, smad or non-smad pathway using inhibitor, real-time PCR, western blot analysis and Co-IP.

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APPENDIX

VITA

Miss Jittima Pumklin was born on July 4, 1980 in Phitsanulok, Thailand. She graduated the degree of Doctor of Dental Surgery (D.D.S.) with second class honors from Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand in 2003. After graduation, she enrolled as a lecturer in Restorative department, Faculty of Dentistry, Naresuan University from April 2003-May 2004. She started her post-graduate program of Master of Sciences Program (M.Sc.) at Department of Occlusion, Chulalongkorn University, Bangkok, Thailand in 2004. She finished in this program in 2007 and returned to work at Naresuan University for 1 year before start to Degree of Doctor of Philosophy Program in Oral Biology at Faculty of Dentistry, Chulalongkorn University again in next year. The research component of this degree was performed at the Research Unit of Mineralized Tissue (RUMT), Faculty of Dentistry, Chulalongkorn University. At present, she returns to work in Restorative department, Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand.