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ต่อการแปรสภาพของเซลล์เอ็นยึดปริทันต์ของมนุษย์เป็นเซลล์คล้ายเซลล์สร้างกระดูก

นางสาวจีรนันท์ มโนกวินโชค

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THE EFFECT OF PROSTAGLANDIN E2 ON DIFFERENTIATION OF HUMAN PERIODONTAL LIGAMENT CELLS TOWARD OSTEOBLAST-LIKE CELLS

Miss Jeeranan Manokawinchoke

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Oral Biology
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จีรนันท์ มโนกวินโชค : ผลของพรอสตาแกลนดิน อี2
ต่อการแปรสภาพของเซลล์เอ็นยึดปริทันต์ของมนุษย์เป็นเซลล์คล้ายเซลล์สร้างกระดูก.
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พรอสตาแกลนดิน อี2 มีบทบาทสำคัญต่อทั้งกระบวนการสร้าง และการสลายกระดูก การศึกษานี้มีจุดประสงค์เพื่อศึกษาผลของพรอสตาแกลนดิน อี2 ต่อการแปรสภาพเป็นเซลล์สร้างเนื้อเยื่อแข็งของเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ของมนุษย์ โดยการเลี้ยงเซลล์ในอาหารเลี้ยงเซลล์ ที่มีสารกระตุ้นกระบวนการแปรสภาพเป็นเซลล์สร้างเนื้อเยื่อแข็ง และกระตุ้นด้วย พรอสตาแกลนดิน อี2 ที่ความเข้มข้น 0.001-1 ไมโครโมลาร์ จากนั้นตรวจการตกตะกอนแร่ธาตุด้วยการย้อมสีอะลิซาริน เรด เอส, ตรวจระดับการแสดงออกของอาร์เอ็นเอนำรหัสด้วยเทคนิค เรียลทามโพลีเมอเรสเชนรีแอคชั่น ผลการศึกษาในครั้งนี้แสดงให้เห็นว่า พรอสตาแกลนดิน อี2 สามารถยับยั้งกระบวนการแปรสภาพเป็นเซลล์คล้ายเซลล์สร้างกระดูกของเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ได้ โดยให้ผลแปรผันตามความเข้มข้น ผลการตรวจระดับอาร์เอ็นเอนำรหัส พบว่าพรอสตาแกลนดิน อี2 เพิ่มการแสดงออกของรังซ์2 แต่ไม่มีผลต่อระดับการแสดงออกของออสติโอแคลซิน และเพิ่มการแสดงออกของทวิสต์1 ซึ่งเป็นตัวยับยั้งการทำงานของรังซ์2 ด้วย นอกจากนี้ การตรวจระดับโปรตีนด้วยเทคนิคเวสต์เทิร์น บลอท ยังพบว่าพรอสตาแกลนดิน อี2 เพิ่มการแสดงออกของรังซ์2 และทวิสต์1 ในระดับโปรตีนด้วย เมื่อศึกษากลไกการถ่ายทอดสัญญาณที่เกิดขึ้น โดยการใช้สารกระตุ้นและสารยับยั้งต่อรีเซบเตอร์ของพรอสตาแกลนดิน อี2 ชนิดต่างๆ พบว่า พรอสตาแกลนดิน อี2 ควบคุมการเปลี่ยนแปลงระดับการแสดงออกของรังซ์2 และทวิสต์1 ผ่านอีพี2 รีเซบเตอร์ และพีเคเอ โดยสรุป การศึกษาครั้งนี้แสดงให้เห็นว่าพรอสตาแกลนดิน อี2 ยับยั้งกระบวนการแปรสภาพเป็นเซลล์คล้ายเซลล์สร้างกระดูก ของเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์ ผ่านการควบคุมการเปลี่ยนแปลงระดับการแสดงออกของรังซ์2 และทวิสต์1 ซึ่งแสดงถึงบทบาทของพรอสตาแกลนดิน อี2 ในการควบคุมสมดุลย์บริเวณเนื้อเยื่อเอ็นยึดปริทันต์

สาขาวิชา	ชีววิทยาช่องปาก	ลายมือชื่อนิสิต .....
ปีการศึกษา	2556	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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JEERANAN MANOKAWINCHOKE: THE EFFECT OF PROSTAGLANDIN E2 ON DIFFERENTIATION OF HUMAN PERIODONTAL LIGAMENT CELLS TOWARD OSTEOBLAST-LIKE CELLS. ADVISOR: PROF. PRASIT PAVASANT, Ph.D., 58 pp.

Prostaglandin E2 (PGE_2) has been shown to be able to influence both bone formation and resorption. The purpose of this study was to investigate the effect of PGE_2 on osteogenic differentiation of human periodontal ligament (HPDL) cells. HPDL cells were cultured with 0.001-1 μM PGE_2 in osteogenic medium. In vitro mineral deposition was determined by Alizarin Red S staining, while gene expression profile was determined by real-time polymerase chain reaction. The results indicated that PGE_2 inhibited in vitro mineral deposition by HPDL cells in a dose dependent manner. PCR analyses showed that PGE_2 upregulated the expression of RUNX2 but had no effect on osteocalcin expression. Upregulation of TWIST1, the functional antagonist of RUNX2 was also observed. In addition, PGE_2 induced RUNX2 and TWIST1 protein synthesis was also detected by Western blot analysis. By using a chemical activator of EP receptors as well as siRNA against an EP receptor, it was shown that PGE_2 regulated both RUNX2 and TWIST1 via the EP2 receptor. The role of PKA in the inductive effect of PGE_2 was also demonstrated. In conclusion, the results of this study revealed that PGE_2 modulated the osteogenic differentiation of HPDL cells via regulating the expressison of RUNX2 and TWIST1. The results suggest a possible role of PGE_2 in regulation of the homeostasis of periodontal ligament tissue.

Field of Study:	Oral Biology	Student's Signature .....
Academic Year:	2013	Advisor's Signature .....

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CHAPTER I
INTRODUCTION

Periodontal ligament (PDL) is a connective tissue that connects between cementum and alveolar bone. It consists of heterogeneous cell population, collagenous and non-collagenous matrix constituents (Lekic and McCulloch, 1996). PDL anchors the tooth to the inner wall of the alveolar socket and plays a crucial role in providing support, protection and sensory input to the masticatory system (Beertsen et al., 1997). PDL also plays an important role in orthodontic tooth movement and participates in alveolar bone remodeling (Kang et al., 2010). Human periodontal ligament cells (HPDL), resided within the PDL tissue, have been shown to play important roles in all functions of PDL tissue, including alveolar bone remodeling. These cells are responsible for homeostasis of the periodontal tissue (Fukushima et al., 2005; Kanzaki et al., 2002) and play critical roles in the regeneration of periodontal tissues damaged by periodontitis (Kim et al., 2013).

Prostaglandin E2 (PGE_2) is a potent lipid mediator that functions in regulating a broad range of physiological activities in our body such as cardiovascular, endocrine, gastrointestinal, neural, pulmonary, reproductive, and visual systems (Akaogi et al., 2006; Kalinski, 2012). The actions of PGE_2 are mediated by activating cell surface receptor. There are at least four subtypes of cell surface receptor for prostaglandin E, E prostanoid (EP) 1-4, which are expressed in a tissue- and cell-specific manner (Breyer et al., 2001). PGE_2 has been described to involve in several biological phenomena. It can regulate inflammatory process in several tissues including synovial joint and its biosynthesis can be enhanced by several proinflammatory

cytokines such as IL-1β, TNF-α, and trauma (Martel-Pelletier et al., 2003). In addition, PGE_2 also plays a role on cell proliferation (Huang et al., 2007), differentiation (Kolodsick et al., 2003), and function of epithelial, mesenchymal, and immune cells (Aronoff et al., 2004; Huang et al., 2007; White et al., 2005). In bone, PGE_2 has been reported to influence behavior of bone cells resulting in the regulation of both bone formation and resorption (Coetzee et al., 2009; Hagino et al., 2005; Hu et al., 2004; Minamizaki et al., 2009).

Our previous results showed that HPDL cells, upon activation with mechanical stress (Wongkhantee et al., 2007) or adenosine triphosphate (ATP) (Luckprom et al., 2010), increased PGE_2 production and secretion. Subsequently, increase PGE_2 could up-regulate the expression of receptor activator of nuclear factor kappa B ligand (RANKL), a key modulator of osteoclastogenesis (Boyle et al., 2003). The PGE_2-induced RANKL expression has been proposed to participate in periodontal remodeling and inflammation by regulation the release of inflammatory cytokines that play roles in osteoclastogenesis (Kanzaki et al., 2002; Luckprom et al., 2010). Although the role of PGE_2 in inflammatory process and osteoclast function has been demonstrated, the involvement of PGE_2 in osteogenic differentiation of human periodontal ligament cells is still unknown.

RESEARCH OBJECTIVE

To investigate the effect of PGE_2 in human periodontal ligament (HPDL) cells, especially the effects on osteogenic differentiation.

RESEARCH HYPOTHESIS

1. PGE_2 inhibits proliferation.
2. PGE_2 inhibits osteogenic differentiation .
3. PGE_2 suppressed the expressions of osteoblastogenesis related gene marker.
4. The function of PGE_2 on osteogenic differentiation in HPDL cells occurs via E prostanoid receptors

EXPECTED BENEFIT

This study will provide the basic knowledge regarding the mechanism of PGE_2 on the regulation of osteogenic differentiation. The knowledge will be beneficial for the clinical application in the future for modulating the healing and regeneration of periodontium by prostaglandin signaling pathway.

CHAPTER II
REVIEW OF RELATED LITERATURE

Prostaglandins (PGs)

Prostaglandins (PGs) are a group of lipid mediators that are produced from arachidonic acid in various tissues under physiologic and pathologic condition to maintain tissue homeostasis. Action of PGs occur through the group of cell surface receptor, prostaglandin receptors. At least five classes (ten receptors) of prostaglandin receptors have been reported. The expression of these receptors varies depending on cell types, indicating a wide variety of effects of prostaglandin actions in various cell types such as aggregation or disaggregation of platelets (Chulada et al., 2000; Gupta et al., 2003; Rao et al., 1980; Stamler et al., 1989), regulation of inflammatory mediation (Fattahi and Mirshafiey, 2012; Zhong et al., 2012), control cell growth (Jiang and Dingledine, 2013) and regulation of bone resorption (Singh et al., 2012).

Prostaglandin synthesis

Prostaglandins are synthesized from phospholipid on the plasma membrane. Arachidonic acid was synthesized from membrane phospholipid by the action of phospholipase A2. Later, by the action of one of the two cyclo-oxygenase isoforms (COX-1 or COX-2), arachidonic acid is converted into PGH_2. Subsequently, PGH_2 is further converted into a series of different PGs, such as prostaglandin E2 (PGE_2),

prostaglandin D2 (PGD_2), prostaglandin F2 alpha (PGF_{2a}), prostaglandin I2 (PGI_2) or prostacyclin, depend on the function of specific PG synthases (e.g., PGE synthase) located in a particular cell or tissue (see Fig.2.1) (Funk, 2001).

As described earlier, PGs act in an autocrine or paracrine manner via specific cell surface, seven-transmembrane domain, G protein–coupled receptors. There are five classes of cell surface PG receptors corresponding to the main PG species termed E prostanoid (EP), D prostanoid (DP), F prostanoid (FP), I prostanoid (IP), and thromboxane prostanoid (TP) (Hull et al., 2004) as shown in figure 2.1.

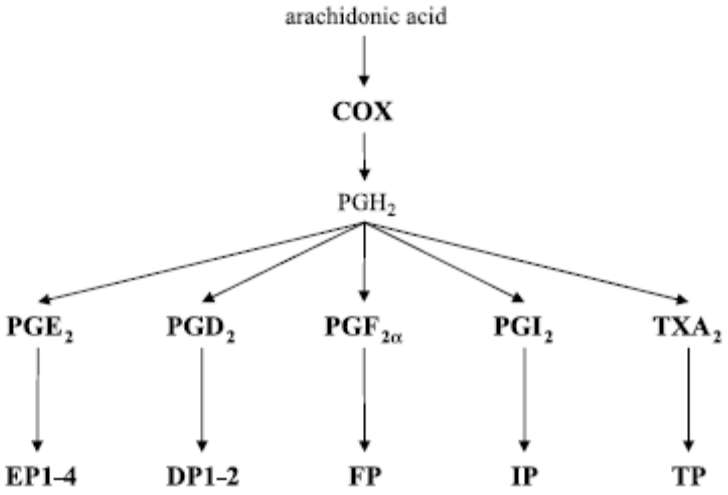

Figure 2.1 PGs synthesis pathway

(Funk, 2001)

Prostaglandin E2 (PGE_2)

Prostaglandin E subgroup comprises of Prostaglandin E1 (PGE_1) and Prostaglandin E2 (PGE_2). Prostaglandin E1 (PGE_1) has been known pharmaceutically as alprostadil (Cawello et al., 1995), a vasodilator drug used in the treatment of erectile dysfunction (Harding et al., 2002). Prostaglandin E2 (PGE_2) has been known in

medicine as dinoprostone and has been commonly used in labour to soften cervix and cause uterine contraction. PGE_2 also induces body temperature causing fever via its action in hypothalamus. In bone, PGE_2 can stimulate osteoblasts to induce the release of cytokines that initiate bone resorption (Kim et al., 2010).

Prostaglandin E2 (PGE_2) is one of the most ubiquitous and important endogenous lipid mediators. It exerts pleiotropic actions that vary depending on the tissue environment and cell type (Funk, 2001). PGE_2 is produced constitutively in almost all tissues. However, its synthesis can be increased in response to microbes, cytokines, hormones, and growth factors that induce the expression of cyclooxygenase (COX) and PGE synthase enzymes. There are at least three PGE synthase isoforms, cytosolic PGE synthase, microsomal PGE synthase-1 and PGE synthase-2, described. Moreover, three isoforms of COX have been reported (Funk, 2001).

Generally, it has been recognized that PGE_2 involved in the process of inflammation to cause fever, pain, and local tissue edema. Moreover, the function of PGE_2 also involves the mechanism of proliferation, function and differentiation of epithelial, mesenchymal and immune cells (Aronoff et al., 2004; Huang et al., 2007; Kolodsick et al., 2003; White et al., 2005). PGE_2 could also promote tumorigenesis (Greenhough et al., 2009). In bone, PGE_2 has been shown to exert the effect on both resorption and formation (Hagino et al., 2005). During mechanical stress-induced tooth movement, prostaglandins, particularly the E type, are produced mainly by periodontal ligament (PDL) cells (Kanzaki et al., 2002; Ren et al., 2002) and osteoblasts (Mitsui et al., 2005) to promote bone resorption through the stimulation

of osteoclast differentiation and function (Leiker et al., 1995; Yamasaki et al., 1980; Yamasaki et al., 1982; Yamasaki et al., 1984).

Cyclooxygenase (COX)

Cyclooxygenase (COX) or prostaglandin-endoperoxide synthase (PTGS) (EC 1.14.99.1) is an enzyme that is responsible for formation of prostanoids, a group of important mediators including prostaglandins, prostacyclin and thromboxane. An attempt to inhibit function of COX pharmacologically can attenuate the symptoms of inflammation and pain (Bello, 2012; Cleland et al., 2006). Non-steroidal anti-inflammatory drugs, such as aspirin and ibuprofen, exert their effects by inhibition of COX function. As described earlier, there are three isozymes of COX encoded by distinct gene products, a constitutive COX-1 and an inducible COX-2, which differ in their regulation of expression and tissue distribution. COX-3 is the alternative splice forms of COX-1, however, the function in mammals is still unclear. The expression of these enzymes is differentially regulated by relevant cytokines and growth factors (Vardeh et al., 2009; Yang et al., 2013).

Cyclooxygenase-1 (COX-1), also known as prostaglandin G/H synthase 1, prostaglandin-endoperoxide synthase 1 or prostaglandin H2 synthase 1, is an enzyme encoded by the *PTGS1* gene in human (Funk et al., 1991; Yokoyama and Tanabe, 1989). Cyclooxygenase-2 (COX-2) or Prostaglandin-endoperoxide synthase 2 is encoded by the *PTGS2* gene (Hla and Neilson, 1992). COX-1 is constitutively expressed in many tissues and is the predominant form in gastric mucosa and in the kidneys. Inhibition of COX-1 reduces the production of cytoprotective PGE_2 and PGI_2

in the stomach, which may cause gastric ulceration. COX-2 is generally an inducible gene, expressed only in induced-cells such as cells in the inflammatory tissues.

Prostaglandin E2 receptors

PGE_2 generates signal through specific cell surface receptors known as E-prostanoid (EP) receptors. EP receptor is divided into four subtypes, EP1-4. Each subtype of EP receptors differs in its signaling pathways and the sensitivity to selective agonists and antagonists (Narumiya et al., 1999). Action of EP2 and EP4 is known to activate adenylate cyclase, while EP1 activated phospholipase C, and EP3 is either lowering intracellular cyclic AMP levels or activating phospholipase C, depending on the alternatively spliced isoform (Breyer et al., 1998; Shamir et al., 2004; Tsuboi et al., 2002). Functions of each EP was shown in Figure 2.2.

Subtype	IC_{50}/K_d	Agonists[a]	Antagonists	Functional assay	Signaling
EP1[b]	1-20 nM	17-phenyl-trinor-PGE_2, iloprost sulprostone	SC19220 SC51089 AH6809	Contracts: guinea-pig ileum, gastric fundus trachea	IP3/DAG/PKC
EP2	20 nM	Butaprost AH13205	—	Relaxes: rabbit ear artery, guinea-pig ileum, trachea	Increased cAMP
EP3[b]	0.3-2 nM	MB28767 sulprostone misoprostol 11-deoxy-PGE_1	—	Contracts: chick ileum, inhibits gastric acid secretion	Decreased cAMP/G_i rho, and other pathways
EP4	2-11 nM	11-deoxy-PGE_1 MB28767(?) misoprostol	AH23848	Dilates: saphenous vein, jugular vein ductus arteriosus	Increased cAMP

Abbreviations are: EP, E-prostanoid; IP_3, inositol triphospate: PKC, protein kinase C.
[a] These agonists are only relatively selective and may activate other EP receptors at higher concentrations
[b] splice variants exists that may have alternate signaling and functional effects

Figure 2.2 EP receptor signaling pathways

(Breyer et al., 1998)

Among these receptors, EP2 and 4, whose function involve the regulation of cAMP, have been shown to play roles in osteoblast function and differentiation (Alander and Raisz, 2006; Minamizaki et al., 2009; Paralkar et al., 2003).

Prostaglandin E2 and bone remodeling

Prostaglandin E2 (PGE_2) has bimodal functions. PGE_2 has been shown to be able to promote bone formation during bone remodeling process (Mitsui et al., 2005). However, the prominent function of PGE_2 has been reported as a potent stimulator of bone resorption (Yamasaki et al., 1980; Yamasaki et al., 1982).

During bone remodeling, there are two processes that involve in bone homeostasis, osteoblastogenesis and osteoclastogenesis. The result from osteoblastogenesis is bone formation and the result from osteoclastogenesis is bone resorption. There are evidences showing that PGE_2 can influence both processes of bone remodeling. However, the detail mechanism of how PGE_2 can induce both processes is still unclear.

During bone formation, it has been shown that PGE_2 could increase the number of osteogenic bone marrow stem cells (BMSCs) via EP4 receptor (Weinreb et al., 2006). PGE_2 could also induce osteogenic differentiation while reduced proliferation of human tendon stem cells (Zhang and Wang, 2012). In addition, PGE_2 increased cancellous bone formation in rat and stimulated osteogenic capacity of marrow cells both in vivo and in vitro (Keila et al., 2001).

In bone resorption, PGE_2 had been shown to reduce osteogenic differentiation by inhibiting ALP activity and mineralization of MC3T3-E1 osteoblast-like cells (Coetzee et al., 2009). PGE_2 also found increase the expression of sFRP-1, the Wnt antagonist that inhibit bone formation, suppressed ALP activity and decreased Beta-Catenin level in human osteoblast cell line (HOB) (Bodine et al., 2005). Moreover, PGE_2 increased the expression of human osteoclast inhibitory lectin (hOCIL) mRNA, an osteoclast formation inhibitor (Hu et al., 2004) resulting in the decrease bone resorption.

In peridontium, it has been demonstrated that mechanical stress could induce production of PGE_2 in periodontal ligament cells (PDL) (Kanzaki et al., 2002; Mayahara et al., 2012). Increase PGE_2 in HPDL cells treated with mechanical stress resulted in the increase of osteoclast formation in vitro. Moreover, normal human osteoblast when receiving stress showed a better support for osteoclastogenesis when co-cultured with RAW264.7 cells (the osteoclast precursor cell line) (Mayahara et al., 2012). These results indicate the correlation between mechanical stress, PGE_2 and osteoclastogenesis.

Factors involved in regulation of osteogenic differentiation

1. Runt-related transcription factor 2 (RUNX2) or Core-binding factor alpha1 (CBFA1)

RUNX2 or CBFA1 is a member of the RUNX family of transcription factors that possess a Runt DNA-binding domain. The RUNX family consists of RUNX1, RUNX2, RUNX3, which differ in their functions (Komori, 2003).

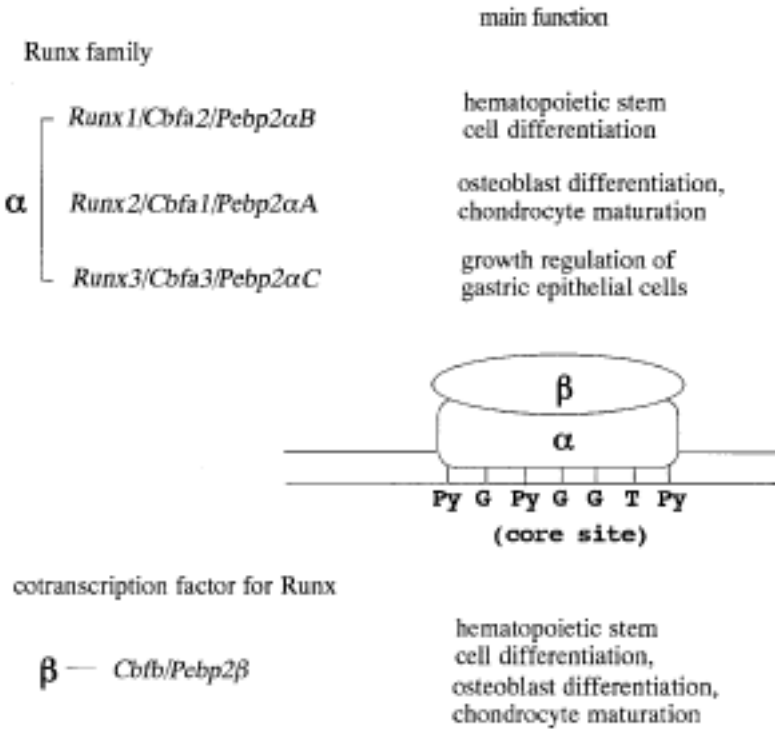

Figure 2.3 The RUNX family proteins

The figure 2.3 (Komori, 2003) shows the main functions of the RUNX family proteins. The function of RUNX protein requires the presence of core binding factor B (CBFB), a cotranscription factor that enhances the in vitro DNA binding of the runt protein (Ogawa et al., 1993).

RUNX2 is a protein encoded by the *RUNX2* gene in human. RUNX2 is a key transcription factor associated with osteoblast differentiation. It is essential for osteoblastic differentiation and skeletal morphogenesis and regulatory factors involved in skeletal gene expression (Komori et al., 1997; Otto et al., 1997). In RUNX2 null mice, no bone had been found indicated the important of this protein in bone formation (Komori et al., 1997; Otto et al., 1997). Moreover mesenchymal cells from RUNX2 null mice completely lack the ability to differentiate into osteoblasts, but retain the capacity to differentiate into adipocytes and chondrocytes (Kobayashi et al., 2000). These findings indicate that RUNX2 plays an essential role in the commitment of multipotent mesenchymal cells to the osteoblastic lineage (Fig. 2.4).

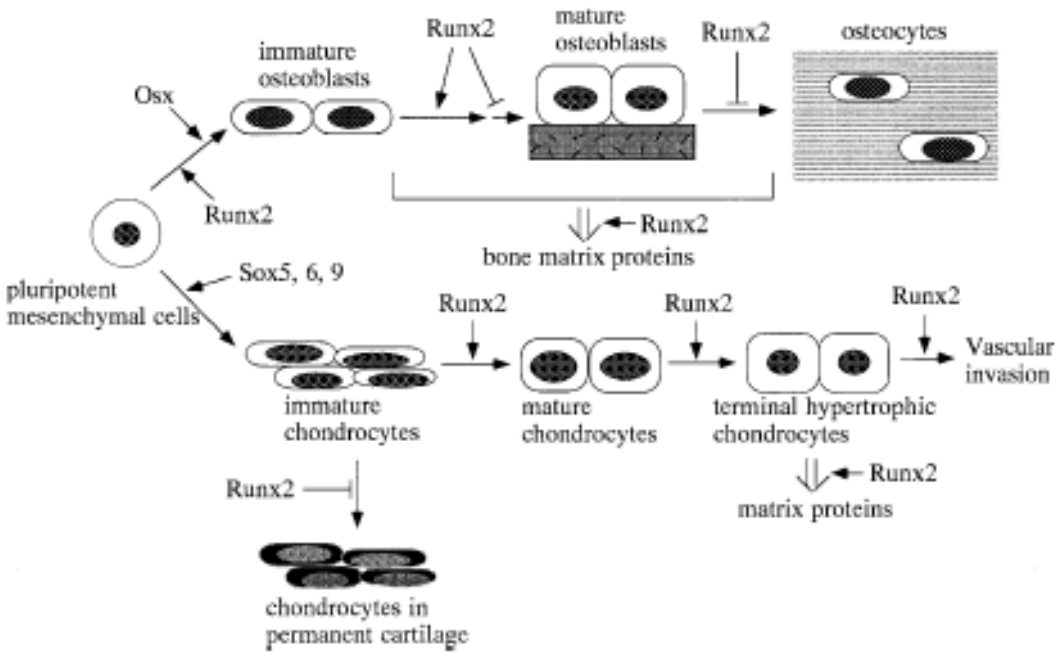

Figure 2.4 Functions of RUNX2 in osteogenic differentiation

The figure 2.4 (Komori, 2003) shows the functions of RUNX2 in osteogenic differentiation. RUNX2 drives pluripotent mesenchymal cells into osteoblast lineage, enhances osteoblast differentiation at an early stage, and inhibits osteoblast differentiation at a late stage. Moreover RUNX2 is also involved in bone matrix proteins synthesis.

2. Osteocalcin (OCN)

OCN, also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP), is a non-collagenous protein found in bone and dentin. In human, this gene is encoded by the *BGLAP* gene (Cancela et al., 1990; Puchacz et al., 1989). OCN is secreted by osteoblasts and has been proposed to play a role in metabolic regulation (Lee et al., 2007). It is also implicated in bone mineralization and calcium ion homeostasis. As OCN is produced mainly by osteoblasts, it is often used as a

marker for the bone formation process. It has been observed that higher serum-osteocalcin levels are relatively well correlated with increases in bone mineral density (BMD) during treatment with anabolic bone formation drugs for osteoporosis, such as Teriparatide. In many studies, OCN is used as a preliminary biomarker on the effectiveness of a given drug on bone formation.

3. Alkaline phosphatase (ALP)

ALP is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called dephosphorylation. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphatase (Tamás et al., 2002). In humans, alkaline phosphatase is present in all tissues throughout the entire body, but is particularly concentrated in liver, bile duct, kidney, bone, and the placenta. Elevated ALP indicates that there could be the active bone formation occurring as ALP is a by product of osteoblast activity (such as the case in Paget's disease of bone). Levels are also elevated in people with untreated Celiac Disease (Pruessner, 1998).

4. Osterix (OSX)

OSX or Transcription factor SP7 is a protein that in human is encoded by the *SP7* gene (Nakashima et al., 2002). SP7 is a C2H2-type zinc finger transcription factor of the SP gene family and a putative master regulator of bone cell differentiation (Gao et al., 2004). The evidence showed that OSX null mice

completely lack bone but cartilage was normal (Zhang, 2012), indicating the importance of OSX in bone formation. Evidences suggested that OSX may function down stream of RUNX2 in the regulation of osteogenic differentiation (Celil et al., 2005).

5. Dentin matrix acidic phosphoprotein 1 (DMP1)

DMP1 is a protein encoded by the *DMP1* gene (Aplin et al., 1995; Hirst et al., 1997). Dentin matrix acidic phosphoprotein is an extracellular matrix protein that belong to the small integrin binding ligand N-linked glycoprotein (SIBLINGs) family. This protein is present in several cell types of bone and tooth tissues and its function in the process of mineralization. Mice lacking DMP1 showed the defect in mineralization indicating the critical role of this protein for proper mineralization of bone and dentin (Ling et al., 2005). The protein contains a large number of acidic domains, multiple phosphorylation sites, a functional arg-gly-asp cell attachment sequence, and a DNA binding domain. During osteoblast maturation, DMP1 was phosphorylated, secreted into the extracellular matrix and regulate mineralized matrix formation. Mutations in the gene are known to cause autosomal recessive hypophosphatemia, a disease that manifests as rickets and osteomalacia. The gene structure is conserved in mammals. Two transcript variants encoding different isoforms have been described for this gene.

However, since DMP1 contain the DNA binding domain, function of DMP1 within the cells was speculated. Recently, DMP1 has been shown to function as a nuclear protein. It has been shown that, in undifferentiated osteoblasts, nuclear

localization of DMP1 could regulates the expression of osteoblast-specific genes (Siyam et al., 2012).

6. Sclerostin

Sclerostin is a protein that in human is encoded by the *SOST* gene (Balemans et al., 2001; Brunkow et al., 2001). Sclerostin is a secreted glycoprotein containing a C-terminal cysteine knot-like (CTCK) domain. Cystein-knot domain has been found in a superfamily of Cys-knot growth factor related proteins such as TGF-beta, PDGF, BMP, glygoprotein hormone (FSH, LH, TSH) that can function as growth factor, morphogen and hormone (Isaacs, 1995; Vitt et al., 2001). The structure and sequence of sclerostin show a similarity to the DAN (differential screening-selected gene aberrative in neuroblastoma), a group of Cys-knot proteins that belong to the family of bone morphogenetic protein (BMP) antagonists. Sclerostin is produced by the osteocyte and has anti-anabolic effects on bone formation (Callewaert et al., 2010; Keller and Kneissel, 2005). Sclerostin was originally believed to be a non-classical Bone morphogenetic protein (BMP) antagonist. Recent evidence suggested that sclerostin could interact with LRP5/6 receptors, therefore inhibiting the Wnt signaling pathway (Choi et al., 2009) as well as the bone formation. The results indicated the function of Sclerostin as an antagonist of bone formation (Li et al., 2005). Although the underlying mechanisms are still unclear, it is believed that the function of sclerostin is mediated by Wnt signaling, not BMP signaling pathways (Krause et al., 2010; van Bezooijen et al., 2007).

7. TWIST-related protein (TWIST)

TWIST is a basic helix-loop-helix transcription factor that comprises of TWIST-related protein1 (TWIST1) and TWIST-related protein2 (TWIST2) or Dermo1. TWIST1 is a protein that in human is encoded by the *TWIST1* gene (Bourgeois et al., 1996; Dollfus et al., 2001). TWIST2 is a protein that is encoded by the *TWIST2* gene (Li et al., 1995; Perrin-Schmitt et al., 1997). It is thought that during osteoblast development this protein may inhibit osteoblast maturation and maintain cells in a preosteoblast phenotype. TWIST has been proposed to function as an antagonist of RUNX2 that inhibit function of RUNX2 in osteogenic differentiation induction (Bialek et al., 2004; Yousfi et al., 2002). Report by Bialek demonstrated that TWIST can directly interact with RUNX2 protein and inhibit function of RUNX2 (Bialek et al., 2004), therefore, TWIST has been considered as inhibitor of osteogenic differentiation. Genetic mutations in human *TWIST* gene have been identified in Saethre-Chotzen syndrome that cause TWIST haploinsufficiency and premature cranial suture fusion (el Ghouzzi et al., 1997; Howard et al., 1997) as a result of increased bone formation (Yousfi et al., 2001). *In vitro* molecular silencing of *TWIST1* in murine mesenchymal stem cells promoted osteoblast gene expression and matrix mineralization (Miraoui et al., 2010). Consistent with these findings, reduction of TWIST1 by antisense oligonucleotide increased bone cell maturation (Lee et al., 1999) while over-expression of TWIST1 in osteoblasts attenuated the osteogenic differentiation (Danciu et al., 2012).

CHAPTER III
RESEARCH METHODOLOGY

Cell culture

Human periodontal ligament (HPDL) cells were obtained from healthy periodontal ligament tissue of non-carious, freshly extracted third molars removed for orthodontic reasons. The protocol has been approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Informed consent was obtained from each patient. Briefly, teeth were rinsed with sterile phosphate-buffered saline. The periodontal ligament tissue was removed from the middle third of the root, harvested on 60-mm culture dishes and cultured with growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL of penicillin, 100 µg/mL of streptomycin and 5 µg/mL of amphotericin B) in a humidified atmosphere of 95% air, 5% CO2 at 37°C. Each preparation was prepared from one molar. Cells from passage 3 of three different donors were used in the experiments, which were run in triplicate. Media and supplements were from Gibco BRL (Carlsbad, CA, USA).

Treatment

HPDL cells were seeded in twelve-well plates at a density of 37,500 cells/cm^2 for 24 hours. Cells were starved in serum-free medium for 8 hours and then the medium was changed into either growth or osteogenic medium (growth medium supplemented with 50 µg/mL ascorbic acid, 250 nM Dexamethasone, and 5 mM

beta-glycerophosphate (Sigma-Aldrich Chemical, St Louis, MO, USA). Cells were treated with 0-1 μM of prostaglandin E2 (PGE_2) (Cayman Chemical, Ann Arbor, MI, USA), 0-1 μM of Butaprost, an EP2 agonist (Cayman Chemical, Ann Arbor, MI, USA), 0.6-42 nM of Sulprostone, an EP1, 3 agonist (Tocris Bioscience, Ellisville, MO, USA) and 1.25-2.5 nM of TCS2510, an EP4 agonist (Tocris Bioscience, Ellisville, MO, USA).

For inhibitory experiments, cells were cultured with 20 μM of a PKA inhibitor (Adenosine 3′, 5′-cyclic Monophosphorothioate, Rp-Isomer, Triethylammonium Salt; Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA) for 30 minutes prior to stimulation with PGE_2.

After 24 hours treatment, total RNA was extracted and subjected to quantitative real-time polymerase chain reaction analysis (qPCR). Protein secreted from the cells was determined after 2 or 4 days by Western blot analysis. The effective dose was selected and used for the rest of the experiments.

Transfection of small inhibitory RNA

HPDL cells were grown in six-well plates and cultured in medium without antibiotics, to 70–80% confluence. Cells were then treated, according to the manufacturer's instructions, with the mixed solution of small interfering RNA oligonucleotides specific to E prostanoid receptor type 2 (Santa Cruz Biotechnology, Dallas, Texas, USA). Control small interfering RNA (Santa Cruz Biotechnology, Dallas, Texas, USA) was used as a control treatment. The transfection with the small inhibitory RNA was done 24 h prior to PGE_2 treatment.

Osteogenic differentiation

Cells were seeded at the same density as described above in twenty-four-well plates and maintained in an osteogenic medium. The medium was changed every 48 h. Osteoblast marker gene expressions and mineral deposition was investigated using the methods described below.

MTT assay

MTT assay was used to determine cytotoxicity and cell proliferation. Cells were seeded at the same density as described above in twenty-four-well plates. After overnight incubation, the cells were exposed to 0-1 μM of PGE_2 for 24 or 72 hours. Then the medium was removed, and 1 mL MTT solution was added and incubated for 30 minutes at 37°C. The purple formazan precipitate was extracted by using 1 mL DMSO and glycine buffer on a shaker at room temperature. The solution was measured by using an absorbance microplate reader (BioTek ELx800, BioTek Instruments Inc., Winooski, Vermont, USA) at absorbance 570 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using Isol-RNA Lysis Reagent (5Prime, Gaitherburg, MD, USA) according to the manufacturer's instructions. One microgram of each RNA sample was converted to cDNA by avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) for 1.5 h at 42°C. Subsequently, polymerase chain reaction was performed. The primers were designed following the reported sequences from GenBank. The oligonucleotide sequences of the primers used were shown in table 3.1. The mRNA level of GAPDH was served as internal control.

Polymerase chain reaction amplification was performed using Taq polymerase (Invitrogen, Vila Guarani, SP, Brazil) in a reaction volume of 25 µL containing 25 pmol of primers and 1 µL of reverse transcription product. The amplification profile is 94°C for 1 min, hybridization at 60°C for 1 min and extension at 72°C for 2 min. The polymerase chain reaction was performed in a DNA thermal cycler (Biometra, Göttingen, Germany). The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining. The signals were quantified using an image analyzer (Vilber Lourmat, Marne-la-Vallee, France).

Table 3.1 The primers used for RT-PCR

Gene	Forward sequence (5'-3')	Reverse sequence(5'-3')	Accession No
EP1	CAT CCT ACT GCG CCA GGC CG	CCA GGC GCT CGG TGT TAG GC	NM_000955.2
EP2	CTT ACC TGC AGC TGT ACG	GAT GGC AAA GAC CCA AGG	NM_000956.3
EP3	GAG CAC TGC AAG ACA CAC AC	GAT CTC CAT GGG TAT TAC TGA CAA	NM_198719.1
EP4	CCT GCA GCA CGT CGG ATG CT	GGG CCT CTG CTG TGT GCC AA	NM_000958.2
RANKL	CCA GCA TCA AAA TCC CAA GT	CCC CTT CAG ATG ATC CTT C	NM_033012.2
OPG	TCA AGC AGG AGT GCA ATC G	AGA ATG CCT CCT CAC ACA GG	NM_002546.3
RUNX2	CCC CAC GAC AAC CGC ACC AT	CAC TCC GGC CCA CAA ATC	NM_057179.2
OCN	CTT TGT GTC CAA GCA GGA GG	CTG AAA GCC GAT GTG GTC AG	NM_199173.4
ALP	CGA GAT ACA AGC ACT CCC ACT TC	CTG TTC AGC TCG TAC TGC ATG TC	NM_000478.3
OSX	GCC AGA AGC TGT GAA ACC TC	GAC AGC AGG GGA CAG AAA AG	NM_152860.1
DMP1	CAG GAG CAC AGG AAA AGG AG	CTG GTG GTA TCT TGG GCA CT	NM_004407.3
SOST	ACT TCA GAG GAG GCA GAA ATG G	CAA GGG GGA ATC TTA TCC AAC TTT C	NM_025237.2
TWIST1	TCT TAC GAG GAG CTG CAG ACG CA	ATC TTG GAG TCC AGC TCG TCG CT	NM_000474.3
TWIST2	GCT GCG CAA GAT CAT CCC	GTA GCT GCA GCT GGT CAT C	NM_033012.2
GAPDH	TGA AGG TCG GAG TCA ACG GAT	TCA CAC CCA TGA CGA ACA TGG	NM_002046.3

Quantitative realtime polymerase chain reaction (qPCR)

QPCR was used to quantitate the mRNA expression levels. QPCR was performed in a Lightcycler Nano realtime PCR system (Roche Applied Science, Indianapolis, IN, USA) using sybergreen system (Roche Applied Science, Indianapolis,

IN, USA). Amplification was performed as follows: 40 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. Reaction product was quantified with GAPDH as the reference gene. The primer sequences were shown in table 3.2.

Table 3.2 The primers used for qPCR

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Accession no.
RANKL	TCA GCC TTT TGC TCA TCT CAC TAT	CCA ACC CCG ATC ATG GT	NM_033012.2
OPG	AGC TGC AGT ACG TCA AGC AGG A	TTT GCA AAC TGT ATT TCG CTC TGG	NM_002546.3
RUNX2	ATG ATG ACA CTG CCA CCT CTG A	GGC TGG ATA GTG CAT TCG TG	NM_057179.2
OCN	CTT TGT GTC CAA GCA GGA GG	CTG AAA GCC GAT GTG GTC AG	NM_199173.4
TWIST1	TCT TAC GAG GAG CTG CAG ACG CA	ATC TTG GAG TCC AGC TCG TCG CT	NM_000474.3
TWIST2	GCT GCG CAA GAT CAT CCC	GTA GCT GCA GCT GGT CAT C	NM_033012.2
GAPDH	TCA TGG GTG TGA ACC ATG AGA A	GGC ATG GAC TGT GGT CAT GAG	NM_002046.3

Mineralization assay

The cells were fixed with cold methanol for 10 min, washed with deionized water and stained with 1% Alizarin Red S solution for 3 min at room temperature. To quantify the amount of calcium deposition, the staining was eluted with 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate at room temperature for 15 min and the absorbance was read at 570 nm.

Protein extraction and Western blot analysis

Protein was extracted with radioimmuno-precipitation (RIPA) buffer (50 mM Tris/HCL, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate) containing cocktail protease inhibitors or phosphatase inhibitors (1 mM sodium vanadate, 50 mM NaF) (Sigma-Aldrich Chemical, St Louis, MO, USA). Protein concentrations were measured using BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and equal amounts of protein samples were subjected to electrophoresis on a 12% sodium dodecyl sulfate–polyacrylamide gel and subsequently transferred onto nitrocellulose membrane. The membrane was incubated with primary antibody against RANKL (dilution 1:300, R & D Systems, Minneapolis, MN, USA) or Actin (dilution 1:2000, Chemicon International, Temecula, CA, USA) or TWIST1 (dilution 1:250, Abcam, Cambridge, United Kingdom) or RUNX2 (dilution 1:1000, R & D Systems, Minneapolis, MN, USA) or GAPDH (dilution 1:2000, Chemicon International, Temecula, CA, USA) at 4°C. The membranes were then incubated with biotinylated secondary antibody, followed by peroxidase-labeled streptavidin. The signal was captured by chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) and quantified using an image analyzer.

Measurement of osteoprotegerin (OPG) by enzyme-linked immunosorbent assay (ELISA)

The amount of OPG secreted in culture medium was measured by using OPG ELISA kit (Human Osteoprotegerin/TNFRSF11B DuoSet; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. The optical density was

determined at 450 nm, using an absorbance microplate reader. Data were expressed as fold increase of OPG over control.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using statistical software (SPSS, Chicago, IL, USA). Scheffe's test was used for post hoc analysis ($p < 0.05$).

CHAPTER IV
RESULTS

PGE_2 increased RANKL/OPG expression ratio in HPDL cells

We first investigated cytotoxicity of PGE_2 in HPDL cells. HPDL cells were cultured in osteogenic medium with or without various concentrations of PGE_2 varies from 0.001 to 1 μM. All doses of PGE_2 showed no toxicity to the cells as indicated by MTT assay after 3 days culture as in Fig.4.1 A.

Next we examined the effect of exogenous PGE_2 on both RANKL and OPG expressions. HPDL cells were treated with various concentrations of PGE_2 as described above in growth medium. After 1 and 2 days determined the mRNA and protein expressions of RANKL and OPG. The resulted indicated that PGE_2 up-regulated RANKL/OPG ratio at both mRNA (Fig.4.1 B) and protein (Fig.4.1 C-D) levels.

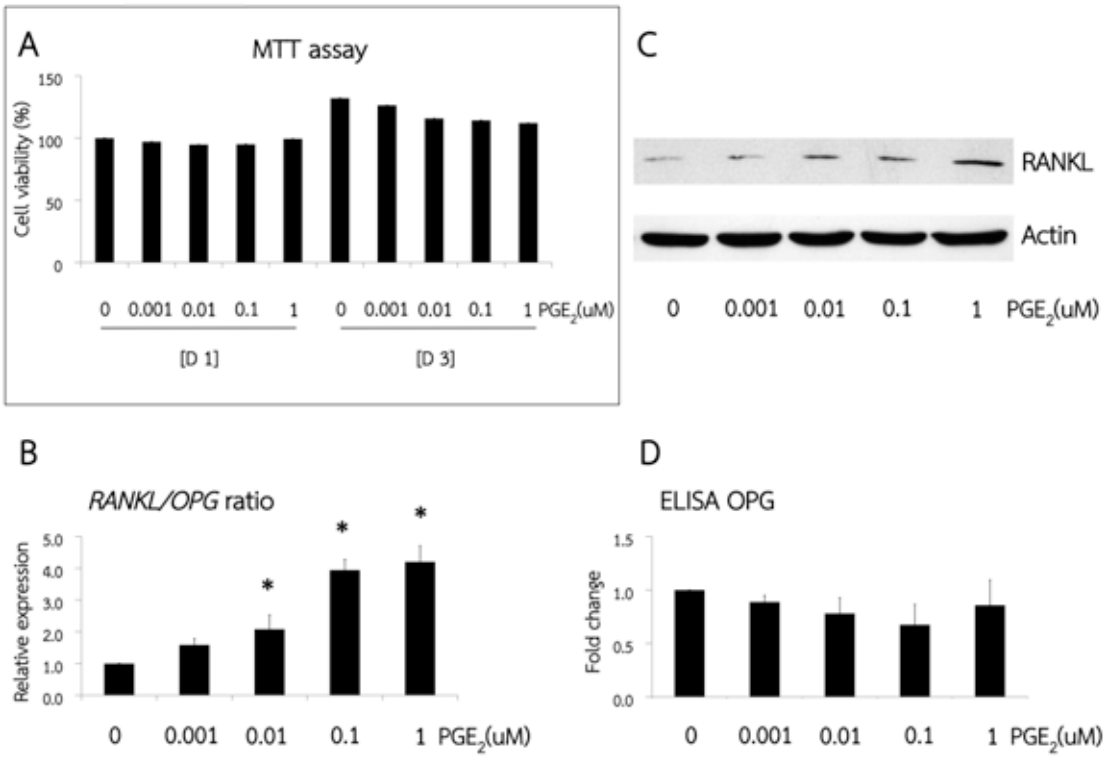

Figure 4.1 PGE_2 had no cytotoxicity and increased RANKL/OPG ratio in HPDL cells.

HPDL cells were cultured in osteogenic medium in the presence of 0.001-1 μM of PGE_2 for 1 and 3 days. Cytotoxicity was determined by MTT assay (A). Cells were treated with 0.001-1 μM of PGE_2 in growth medium for 1-2 days. Expressions of RANKL and OPG were measured using RT-PCR (B) after 1 day and Western blot analysis (C) or ELISA (D) after 2 days. The results were shown as average + S.D. from three experiments. Asterisks indicated the statistical significance with $p<0.05$. The experiments were done in triplicate using 3 HPDL lines from three donors.

PGE2 inhibited in vitro calcification of HPDL cells

HPDL cells were cultured in osteogenic medium with or without PGE_2 as described above for 18 days. In vitro calcification was determined using Alizarin Red S staining as shown in Fig.4.2. The results indicated that exogenous PGE_2 could suppress the in vitro calcification of HPDL cells in a dose dependent manner. The amount of calcium deposition has been quantitated by eluing the staining with 10% cetylpyridinium chloride monohydrate and read the absorbance at 570 nm (Fig.4.2). The quantitative results supported the decrease of calcium staining and the significant inhibitory effect could be found starting from 0.01 μM of PGE_2.

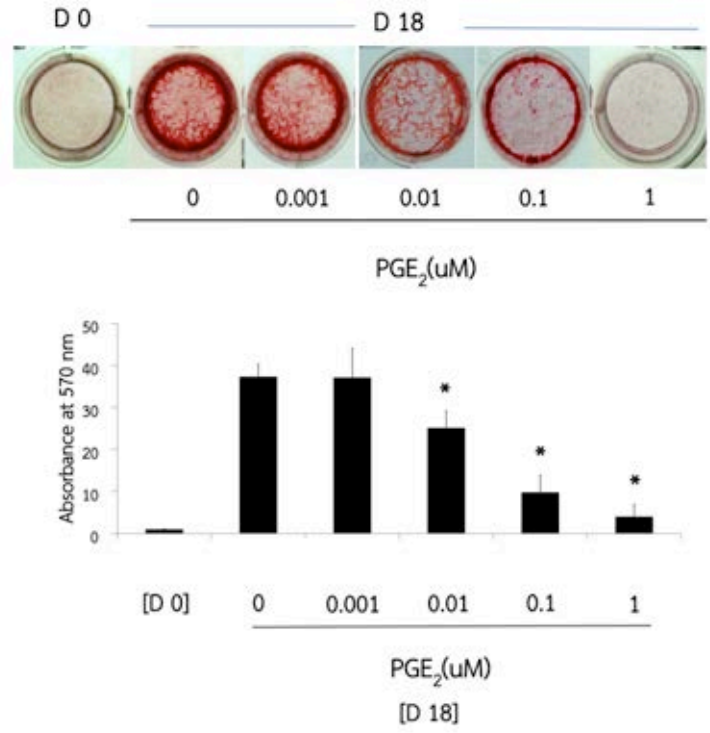

Figure 4.2 PGE_2 inhibited osteogenic differentiation of HPDL cells in vitro.

HPDL cells were cultured in osteogenic medium in the presence of 0.001-1 μM of PGE_2 for 18 days [D18]. In vitro calcification was determined by Alizarin Red S staining. The staining of D 0 culture was used as background. Graph below showed the staining quantitation by elute the staining with 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate at room temperature for 15 min and the absorbance was read at 570 nm. The results were shown as average + S.D. from three experiments. Asterisks indicated the statistical significance with $p<0.05$. The experiments were done in triplicate using 3 HPDL lines from three donors.

PGE2 upregulated osteoblastogenesis related gene markers

HPDL cells were treated with PGE_2 as described in Fig.4.2 for 10 days. The mRNA expressions were analyzed by RT-PCR analysis. The genes that were investigated included: osteoblast markers; *RUNX2*, *OCN*, *OSX*, *ALP*, *DMP1* and inhibitor of osteogenic differentiation markers; *TWIST1*, *TWIST2*, *SOST*. Interestingly, PGE_2 upregulated almost all the genes in osteoblast markers group despite the inhibitory effect of PGE_2 on in vitro calcification. Corresponded to the inhibitory effect on in vitro calcification, PGE_2 upregulated the expression of *TWIST1,* an inhibitior of RUNX2, but not *TWIST2* and *SOST*, in a dose dependent manner as shown in Fig.4.3 A.

To quantitate the level of gene expressions, realtime PCR was performed. The results showed the upregulation of both *RUNX2* and *TWIST1* but not *OCN* and *TWIST2* in dose dependent manner. The results were in agreement with those from conventional PCR. The 4-fold and 10-fold increase of *RUNX2* and *TWIST1* were detected, repectively in cell cultured with 1 μM of PGE_2 (Fig.4.3 B).

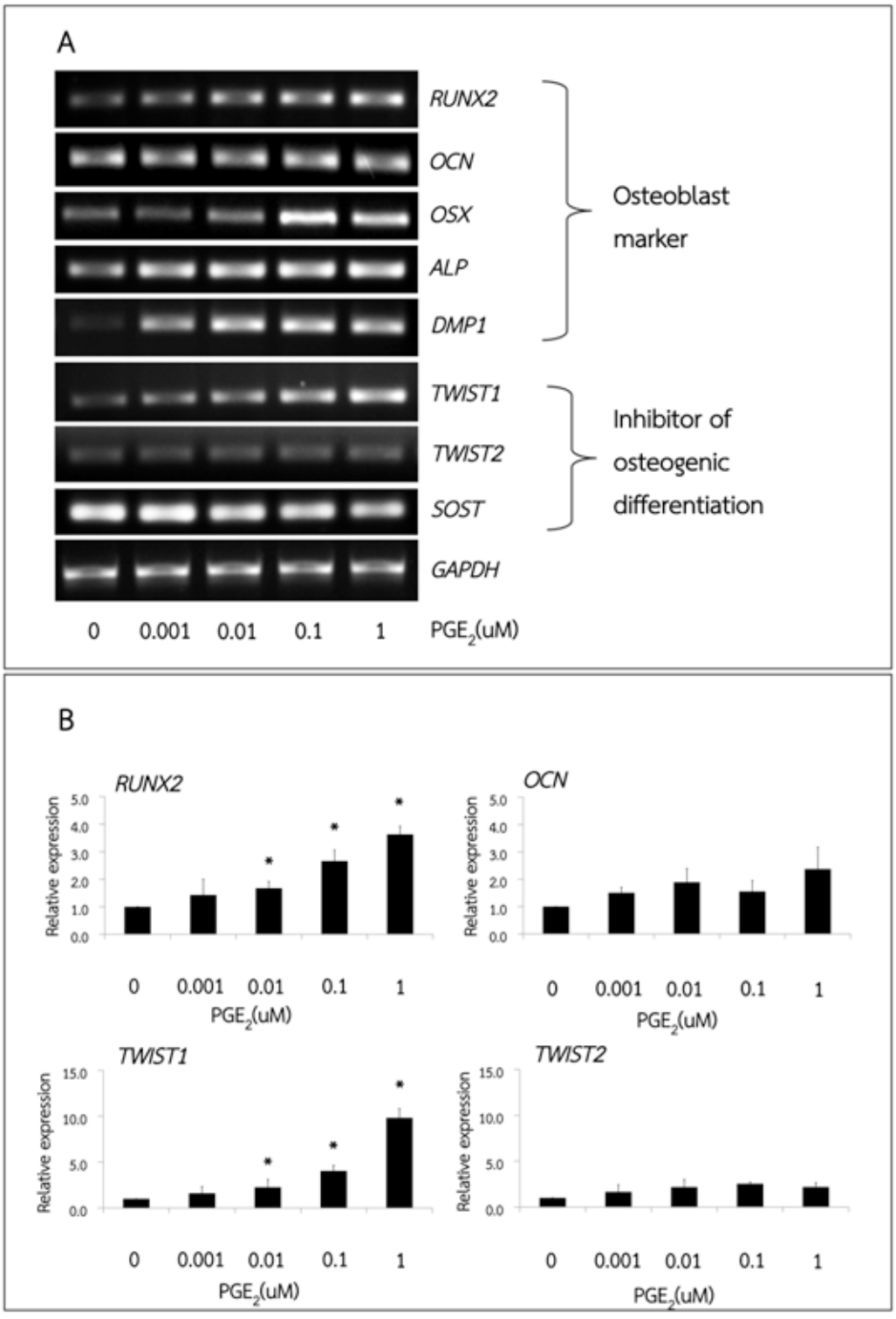
A
RUNX2
OCN
OSX
ALP
DMP1
TWIST1
TWIST2
SOST
GAPDH
Osteoblast marker
Inhibitor of osteogenic differentiation
0 0.001 0.01 0.1 1 PGE2(uM)
B
RUNX2
OCN
TWIST1
TWIST2
Relative expression
0 0.001 0.01 0.1 1
PGE2(uM)

Figure 4.3 PGE_2 induced both osteoblast marker and inhibitor of osteogenic differentiation mRNA expressions.

HPDL cells were treated with 0.001-1 μM of PGE_2 in osteogenic medium for 10 days. Then collected the mRNA and analyzed by both conventional (A) and realtime (B) PCR. The results were shown as average + S.D. from three experiments. Asterisks indicated the statistical significance with $p<0.05$. The experiments were done in triplicate using 3 HPDL lines from three donors.

PGE_2 increased both protein and mRNA expressions of RUNX2 and TWIST1

To investigate the short term effect of PGE_2, HPDL cells were cultured for 1 day with 0.001-1 μM of PGE_2 and the expressions of RUNX2 and TWIST1 were examined by qPCR and Western blot analysis. Results from Fig.4.4 A-B indicated that PGE_2 could increase both *RUNX2* and *TWIST1*, but not *TWIST2*, in HPDL cells. The increased expression of both RUNX2 and TWIST1 proteins was confirmed by Western blot analysis as shown in Fig.4.4 C. However, the detectable level of both proteins could be seen after 4 day in culture.

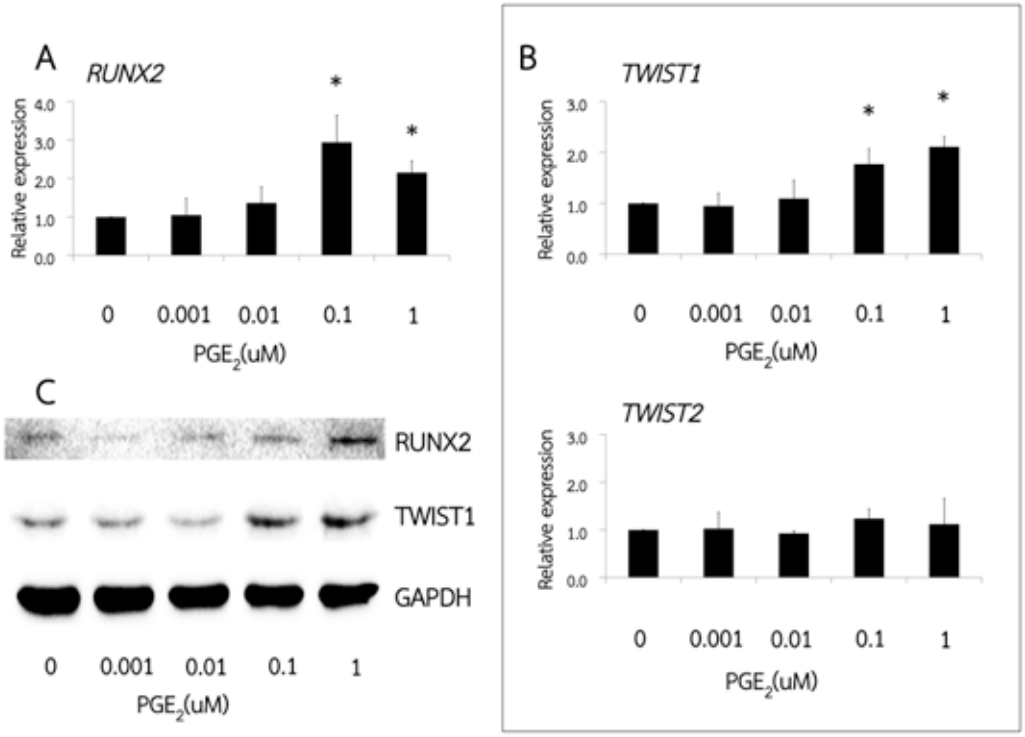

Figure 4.4 PGE_2 increased both protein and mRNA expression of RUNX2 and TWIST1.

HPDL cells were treated with 0.001-1 μM of PGE_2 in osteogenic medium. After 1 day the mRNA expressions were analyzed by qPCR (A-B) and the protein expressions were analyzed by Western blot analysis (C) after 4 days. The results were shown as average + S.D. from three experiments. Asterisks indicated the statistical significance with $p<0.05$. The experiments were done in triplicate using 3 HPDL lines from three donors.

EP2 was involved in PGE_2-induced *RUNX2* and *TWIST1* expression

HPDL cells expressed all EP receptors as shown in Fig.4.5 E. To further investigate the role of EP receptor, cells were cultured with EP agonists for 1 day and the expressions of *RUNX2* and *TWIST1* were examined by qPCR (Fig.4.5 A). Among EP agonists used, only EP2 agonist could significantly increase the expression of both *RUNX2* and *TWIST1*. Furthermore, increase expression of both *RUNX2* and *TWIST1* by EP2 agonist was found in a dose dependent manner but not EP4 agonist (Fig.4.5 B). The gene silencing approach using siRNA against EP2 was performed to further support the role of EP2 receptor. Transfection with siEP2 could inhibit the expression of *EP2* more than 70%, while control-siRNA had no effect (Fig.4.5 D). The results from Fig.4.5 C revealed that the inductive effect of PGE_2 on both *RUNX2* and *TWIST1* expressions was attenuated in HPDL cells transfected with siEP2.

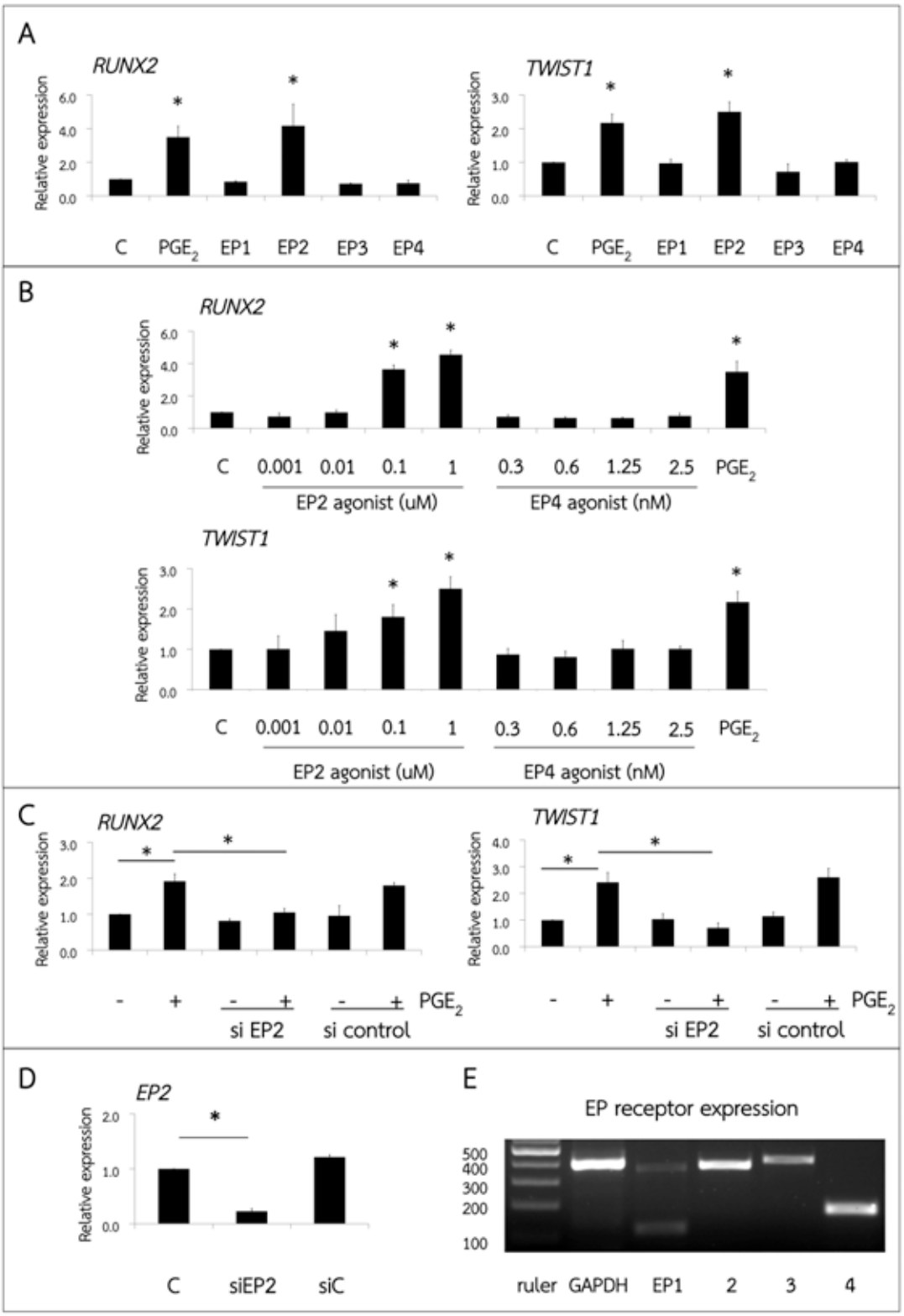
A
RUNX2
Relative expression
6.0
4.0
2.0
0.0
C
PGE2
EP1
EP2
EP3
EP4
TWIST1
Relative expression
3.0
2.0
1.0
0.0
C
PGE2
EP1
EP2
EP3
EP4
B
RUNX2
Relative expression
6.0
4.0
2.0
0.0
C
0.001
0.01
0.1
1
0.3
0.6
1.25
2.5
PGE2
EP2 agonist (uM)
EP4 agonist (nM)
TWIST1
Relative expression
3.0
2.0
1.0
0.0
C
0.001
0.01
0.1
1
0.3
0.6
1.25
2.5
PGE2
EP2 agonist (uM)
EP4 agonist (nM)
C
RUNX2
Relative expression
3.0
2.0
1.0
0.0
-
+
-
+
-
+
PGE2
si EP2
si control
TWIST1
Relative expression
4.0
3.0
2.0
1.0
0.0
-
+
-
+
-
+
PGE2
si EP2
si control
D
EP2
Relative expression
2.0
1.0
0.0
C
siEP2
siC
E
EP receptor expression
500
400
300
200
100
ruler
GAPDH
EP1
2
3
4

Figure 4.5 EP2 was involved in PGE_2-induced *RUNX2* and *TWIST1* expressions.

Cells were treated with EP1-4 agonists and the expression of *RUNX2* and *TWIST1* were determined by realtime PCR (A). Various doses of EP2 and 4 agonists were used to further confirm the effect on *RUNX2* and *TWIST1* mRNA expressions (B). In addition, EP2 silencing by using siRNA could abolish the inductive effect of PGE_2 on *RUNX2* and *TWIST1* expressions (C). The results were shown as relative expression from three experiments using three HPDL cells from three donors.

The inductive effect of PGE_2 on both *RUNX2* and *TWIST1* occurred via PKA pathway

Since EP receptor signaling is associated with cAMP-PKA signaling pathway (Breyer et al., 1998), the involvement of PKA in the inductive effect of PGE_2 was investigated. Cells were cutured with PGE_2 in the presence or absence of PKA inhibitor (Fig.4.6 A). The results indicated that PKA inhibitor could attenuate the inductive effect of PGE_2 on both *RUNX2* and *TWIST1* expressions. In addition, application of forskolin, the PKA activator, could upregulate *RUNX2* and *TWIST1* in a dose dependent manner (Fig.4.6 B), corresponded with the effect of PKA inhibitor.

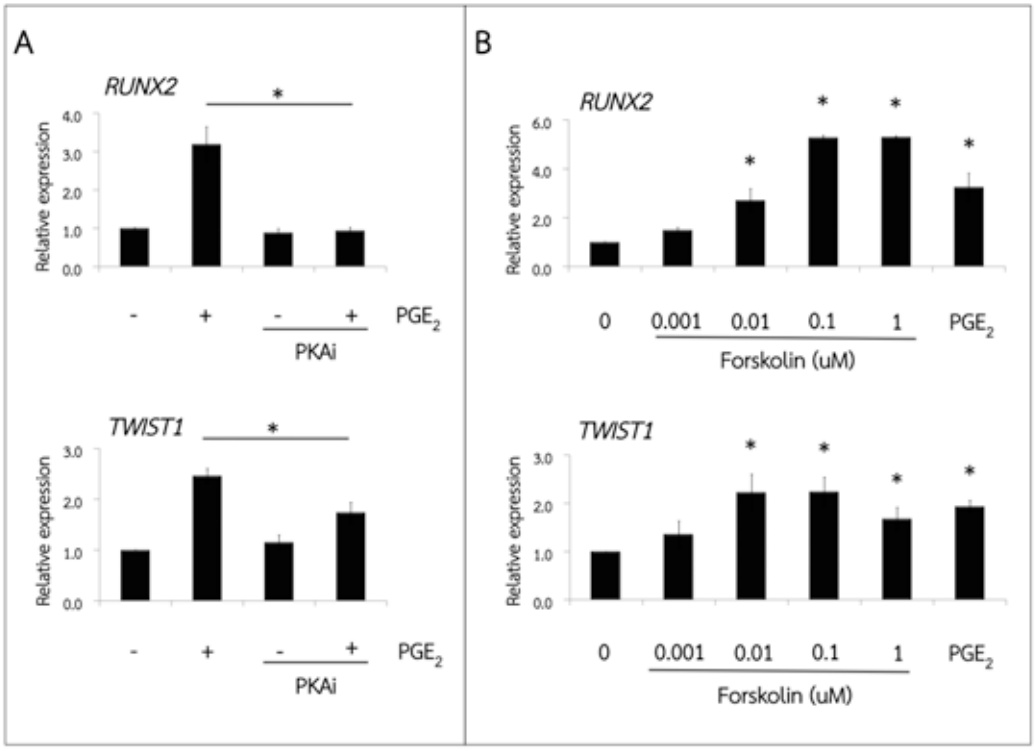

Figure 4.6 The inductive effect of PGE_2 occurred via PKA pathway.

Cells were treated with 1 μM of PGE_2 and the expressions of *RUNX2* and *TWIST1* was determined by realtime PCR. Application of PKA inhibitor (PKAi) could inhibit the inductive effect of PGE_2. Moreover, when cells were treated with PKA activator, forskolin, the upregulation of *RUNX2* and *TWIST1* was observed. The results were shown as relative expression from three experiments using three HPDL cells from three donors.

CHAPTER V
DISCUSSION AND CONCLUSION

The periodontal tissue, which functions as a cushion to absorb masticatory forces exerted upon the tooth, is populated with PDL cells. These cells possess several osteoblastic characteristics, such as the expression of alkaline phosphatase and the ability to generate in vitro mineral deposition. We previously reported that PDL cells responded to an applied pressure by releasing PGE_2 into the extracellular space (Luckprom et al., 2010; Wongkhantee et al., 2007). The results of the present study suggest a role of PGE_2 in the homeostasis of the periodontal ligament. PGE_2 might help to maintain the fibroblastic phase and inhibit the osteogenic differentiation of HPDL cells.

PGE_2 has been shown to exert both anabolic and catabolic function on bone. The inductive effect of PGE_2 on osteoclast formation has been reported (Kanzaki et al., 2002). PGE_2 is one of the potent inducers of RANKL, which plays an essential role in osteoclastogenesis via its interaction with RANK on osteoclast precursor surface. In the present study, we demonstrated that exogenous PGE_2 increased RANKL/OPG ratio (Fig.4.1) suggesting the role of PGE_2 on osteoclastogenesis in HPDL cells. Moreover, a role of PGE_2 on bone formation was also reported (Keila et al., 2001). PGE_2 has been shown to promote proliferation and differentiation of marrow stromal cells (Weinreb et al., 2006) in vitro, induced cancellous bone formation in a rat model (Keila et al., 2001) and induced both *RUNX2* expression and in vitro mineral deposition by rat marrow stromal cells (Yoshida et al., 2002). Interestingly, the results from the present study showed that PGE_2 inhibited osteogenic differentiation of HPDL cells in vitro

(Fig.4.2) while upregulated almost all osteoblastogenesis related gene marker expressions especially *RUNX2*, a key regulator of osteogenic differentiation (Komori et al., 1997; Otto et al., 1997) (Fig.4.3-4). The difference is likely due to the different cell types and species. Our previous results also indicate different responses between HPDL cell and mouse marrow stromal cells (Sooampon et al., 2013). In the latter study, capsaicin induced OPG expression by HPDL cells, whereas RANKL expression was induced by mouse marrow stromal cells. The exact mechanisms underlying these differences are still unclear. On the other hand, the results in this study showed that PGE_2 also upregulated *TWIST1,* an inhibitior of RUNX2 (Fig.4.3-4). This finding suggested that PGE_2 inhibited osteogenic differentiation of HPDL cells in vitro could occur via the upregulation of TWIST1.

This study shows for the first time that PGE_2 can modulate *RUNX2* and *TWIST1* expressions, two key functional genes that regulate osteogenic differentiation, in HPDL cells. Despite the increased RUNX2 mRNA and protein expressions, PGE_2 inhibited in vitro mineral deposition by HPDL cells cultured in osteogenic medium. This inhibitory effect might due to the upregulation of *TWIST1*. In addition, the inductive function of PGE_2 on both genes proved to depend on the EP2-PKA signaling pathway.

PGE_2 generally exerts its effect via EP receptors. Among the 4 subtypes of EP receptor, EP2 and EP4 are the main receptors that participate in the hard tissue homeostasis. Application of EP2 and EP4 agonists had been demonstrated to either induce or inhibit bone formation (Kambe et al., 2012; Sakuma et al., 2000; Suzawa et al., 2000; Weinreb et al., 1999; Yoshida et al., 2002). Therefore, the role of each of these two receptors is still unclear. It has been reported that PGE_2 induced *RUNX2*

expression and mineralization via EP2 and EP4 in rat and mouse bone marrow cultures (Weinreb et al., 1999; Yoshida et al., 2002). In addition, EP2 and EP4 agonists could increase bone formation in animal model (Kambe et al., 2012; Yoshida et al., 2002). Moreover, EP2 and EP4 have been shown to participate in PGE_2-induced bone resorption (Li et al., 2000; Sakuma et al., 2000; Suzawa et al., 2000). Although an explanation for these contrasting findings is still lacking, it is possible that EP receptors have functional redundancy and the chemical agonist used in those experiments might activate more than one EP receptor.

In the present study, the EP4 agonist did not have any effect on the expressions of both *RUNX2* and *TWIST1*. Application of EP2 agonist, however, showed an effect similar to that seen with PGE_2 (Fig.4.5 A-B). The role of EP2 was confirmed by the use of silencing approach (Fig.4.5 C), indicating the importance of EP2 in HPDL cells in their response to PGE_2.

It has been demonstrated that PGE_2 activated EP2 receptor resulted in an increased level of cytosolic cAMP (Serra-Pages et al., 2012; Srivastava et al., 2012). Subsequently, cAMP is either activate adenylate kinase or act as transcription factor by translocating into the nucleus. In the present study, inhibition of PKA reduced the expressions of both *RUNX2* and *TWIST1*, and activation of PKA induced the expression of both genes (Fig.4.5), suggesting an EP2-PKA pathway. The role of EP2-PKA was reported in many biological phenomena, such as mast cell degranulation or IL-8 expression by colonic epithelial cells (Serra-Pages et al., 2012; Srivastava et al., 2012). Whether this pathway is involved in *RUNX2* and *TWIST1* expressions in other cells types need further investigation.

RUNX2 (or CBFA1) is a key regulator of osteogenic differentiation (Komori et al., 1997; Otto et al., 1997). In RUNX2 silencing mice, skeleton formation is almost absent due to a defective osteoblast differentiation. The finding that PGE_2 upregulates *RUNX2* suggests that the anabolic effect of PGE_2 might occur via this induction. Interestingly, despite an increased expression of RUNX2, in vitro mineral deposition was inhibited. It is possible that PGE_2 exerted this inhibitory effect via an upregulation of TWIST1, since TWIST1 has been demonstrated to be able to bind and inhibit RUNX2 function. Although TWIST could inhibit function of RUNX2, it had no effect on *RUNX2* expression (Bialek et al., 2004). Moreover, in *TWIST1*+/- mice, premature osteoblast differentiation as well as premature trabecular bone formation in E16 embryo was detected (Bialek et al., 2004). The inhibitory role of TWIST1 coincided with the observation that the expression of osteocalcin (OCN) was not increased. Since OCN is one of the target molecules of RUNX2, the explanation could be that TWIST1 binds to RUNX2 and thereby prevents the function of this transcription factor.

In conclusion, PGE_2 proved to regulate the mRNA and protein expressions of both RUNX2 and TWIST1. Our data strongly suggest the involvement of the EP2-PKA signaling pathway in this process. The balance between RUNX2 and TWIST1 may regulate the differentiation state of PDL cells. The level and duration of PGE_2 exposure may help regulate the behavior of PDL cells and help modulate the homeostasis of periodontal tissue.

Future studies

1. To further investigate the role of TWIST1 on PGE_2 inhibited osteogenic differentiation using gene silencing approach with shRNA.
2. To determine the correlation between ATP and TWIST1. Since our previous study found that ATP upregulated PGE_2 production and secretion (Luckprom et al., 2010).
3. To determine the correlation between PGE_2 and Notch Signaling in osteogenic differentiation of HPDL cells. Since our preliminary data showed that Notch suppressed *TWIST* mRNA expression (Osathanon et al., 2013).

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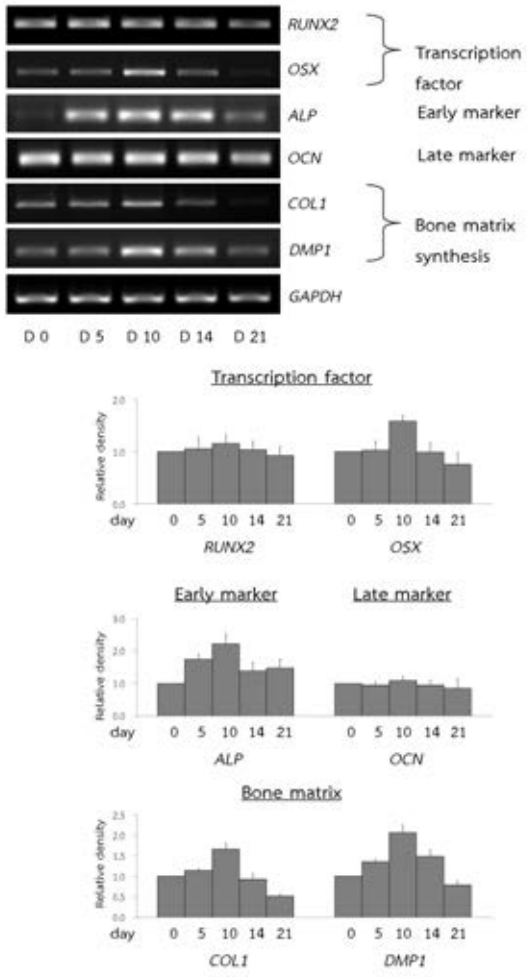
APPENDIX

APPENDIX



HPDL cells were cultured in osteogenic medium for 21 days. The expression of osteogenic differentiation markers was detected by RT-PCR at day 0, 5, 10, 14 and 21 cultures. The detected markers included *RUNX2* and osterix (*OSX*), two transcription factors that regulate osteogenic differentiation; *ALP* and *OCN*, early and late marker of osteogenic differentiation; *COL1* and *DMP1*, two major proteins found in bone matrix. The result showed the significant upregulation of *OSX* but not *RUNX2* at day-10. Moreover, increased of *ALP*, *COL1* and *DMP1* was also detected, but not the *OCN*.

VITA

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