ผลของทรอมบินและพาร์วันต่อการแสดงออกของออสติโอโพรติจิลินและแรงค์ไลแกน
ต่อเซลล์เพาะเลี้ยงจากเอ็นยึดปริทันต์ของมนุษย์

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THE EFFECT OF THROMBIN AND PAR-1 ON OPG AND RANKL

EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS

Miss Uthaiwan Arayatrakoollikit

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Oral Biology

Faculty of Dentistry

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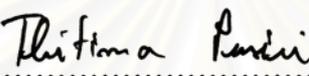
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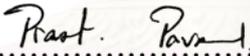
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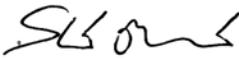
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อุทัยวรรณ อารยะตระกูลลิขิต : ผลของทรอมบินและพาร์วันต่อการแสดงออกของออสติโอโพรติจิลินและแรงค์ไลแกนต่อเซลล์เพาะเลี้ยงจากเอ็นยึดปริทันต์ของมนุษย์. (THE EFFECT OF THROMBIN AND PAR-1 ON OPG AND RANKL EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS) อ. ที่ปรึกษา : รศ.ทพ.ดร.ประสิทธิ์ ภวสันต์, อ.ที่ปรึกษาร่วม : รศ.ทพญ.ทัศนีย์ ยงชัยตระกูล 104 หน้า.

ทรอมบินมีอิทธิพลต่อพฤติกรรมทางชีวภาพของเซลล์เพาะเลี้ยงจากเอ็นยึดปริทันต์ของมนุษย์และมีบทบาทสำคัญในการเกิดการซ่อมแซมของกระดูกในระยะแรก ออสติโอโพรติจิลิน (โอพีจี) และแรงค์ไลแกนเป็นสารสองตัวที่มีบทบาทสำคัญในการควบคุมการเกิดสมดุลของกระดูก สมดุลของโอพีจีและแรงค์ไลแกนในเนื้อเยื่อเป็นปัจจัยหนึ่งที่มีบทบาทสำคัญในกลไกการเกิดการทำลายและการสร้างใหม่ของเนื้อเยื่อแข็ง อย่างไรก็ตามยังไม่เคยมีรายงานการศึกษาถึงบทบาทของทรอมบินที่มีต่อสมดุลของโอพีจีและแรงค์ไลแกนในเซลล์เพาะเลี้ยงจากเอ็นยึดปริทันต์ของมนุษย์ ดังนั้นในการศึกษาครั้งนี้ ได้ทำการศึกษาผลของทรอมบินที่มีต่อการแสดงออกและการสังเคราะห์โปรตีนโอพีจีและแรงค์ไลแกนในเซลล์เพาะเลี้ยงจากเอ็นยึดปริทันต์ของมนุษย์ รวมทั้งศึกษาถึงการส่งสัญญาณผ่านทรอมบินรีเซพเตอร์หรือพาร์ และกลไกการส่งสัญญาณต่าง ๆ ภายในเซลล์ ผลการศึกษาพบว่าทรอมบินที่ความเข้มข้น 0.1 ยูนิตต่อมิลลิลิตร สามารถกระตุ้นให้เกิดการสังเคราะห์โปรตีนโอพีจีเพิ่มขึ้นอย่างชัดเจน ซึ่งผลดังกล่าวสามารถถูกยับยั้งได้ด้วยสารไซโคลเฮกซิไมด์ แต่ไม่สามารถยับยั้งได้ด้วยสารแอคติโนมัยซินดี ผลจากการใช้สารยับยั้งต่อฟอสฟาติดิลอิโนสิทอลทรีไคเนส (พีไอทรีเค, แอลวาย294002) และแมมมาเลียนทาร์เก็ทอ็อฟราปาไมซิน (เอ็มทอร์) พบว่าสามารถยับยั้งการเพิ่มขึ้นของโปรตีนโอพีจีได้ และยังพบว่าแอลวาย 294002 สามารถยับยั้งการกระตุ้นการทำงานของเอเคที (ระดับฟอสโฟเอเคที)ได้เช่นเดียวกัน นอกจากนี้ผลการทดลองยังพบว่าพาร์วันแอนตาโกนิสท์สามารถยับยั้งการเพิ่มโอพีจีจากการเหนี่ยวนำของทรอมบิน โดยสรุป งานวิจัยในครั้งนี้แสดงให้เห็นว่าทรอมบินกระตุ้นการเพิ่มขึ้นของโปรตีนโอพีจีในเซลล์เพาะเลี้ยงจากเอ็นยึดปริทันต์ของมนุษย์เป็นไปได้ที่จะเกิดผ่านทางพาร์วัน และเป็นกลไกที่เกิดภายหลังกระบวนการทรานสคริปชันถ่ายทอดสัญญาณภายในเซลล์ผ่านทางพีไอทรีเค เอเคที และเอ็มทอร์ ตามลำดับ การศึกษาครั้งนี้แสดงให้เห็นว่าทรอมบินน่าจะมีบทบาทสำคัญในการควบคุมสมดุลของกระดูกและการซ่อมแซมของกระดูกผ่านทางระบบแรงค์ไลแกนและโอพีจี

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UTHAIWAN ARAYATRAKOOLLIKIT : THE EFFECT OF THROMBIN AND PAR-1 ON OPG AND RANKL EXPRESSION IN HUMAN PERIODONTAL LIGAMENT CELLS.   THESIS ADVISOR : ASSOC. PROF. PRASIT PAVASANT, D.D.S., Ph.D., THESIS COADVISOR : ASSOC. PROF. TUSSANEE YONGCHAITRAKUL, D.D.S., M.Sc., 104 pp.

Thrombin influences the biological behavior of human periodontal ligament cells (HPDL) and plays multiple roles in the early stage of bone healing. Osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) are two key molecules that regulate bone homeostasis. The balance of RANKL and OPG in the tissue is one of the factors significant in the mechanism of hard tissue destruction and remodeling. However, the specific role of thrombin on balance of RANKL and OPG has not been studied. In the present study, we investigated the effect of thrombin on RANKL and OPG synthesis. The participation of PAR-1 and the signaling pathways mediate by thrombin are focused. Our result showed that thrombin profoundly induced protein synthesis of OPG at 0.1 U/ml. The inductive effect was inhibited by cycloheximide, but not by indomethacin. Phosphatidylinositol 3'kinase (PI3K) inhibitor, LY294002 and mammalian target of rapamycin (mTOR) inhibitor, rapamycin, exerted the inhibitory effect on the thrombin induced OPG synthesis.   In addition, the thrombin-induced OPG synthesis was inhibited by protease-activated receptor (PAR) -1 antagonist.   Activation of phospho-Akt (p-Akt) was observed and the effect was abolished by LY294002. In conclusion, this study was the first to demonstrate that thrombin induce OPG synthesis in HPDL cells post-transcriptionally, possibly through PAR-1.   The regulation was through PI3K/Akt and mTOR pathway.   This finding suggests that thrombin may play a significant role in alveolar bone repair and homeostasis of periodontal tissue, partly through RANKL/OPG system.

Field of study Oral Biology   Student's signature...Uthaiwan Arayatrakoollikit

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CHAPTER I

INTRODUCTION

Periodontal disease is a chronic infection of periodontium resulting in the destruction of soft and hard connective tissue. It has been shown that gram negative anaerobic bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*) and *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) are major pathogens causing the disease. However, recent evidences indicate that the interaction between host cells and bacterial products also involves in the process of tissue destruction. Human periodontal ligament (HPDL) cells are the host cells that play roles in this process. The cells increase secretion as well as activation of matrix metalloproteinases (MMPs) in response to products from *P. gingivalis* and *A. actinomycetemcomitans* indicating the role of soft tissue destruction.

In addition, HPDL cells also involve bone resorption as they express receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG), two major molecules that function in osteoclastogenesis. RANKL, upon binding to RANK on the cell surface of osteoclasts, functions to induce differentiation, activation and survival of osteoclasts, whereas OPG, a decoy receptor for RANKL, functions to inhibit osteoclastogenesis (Tsuda et al., 1997). Thus, balance of RANKL and OPG in the tissue is one of the factors significant in the mechanism of hard tissue destruction and remodeling (Simonet et al., 1997; Lerner, 2004). The cells upregulate RANKL expression in response to lipopolysaccharides (LPS) which may lead to osteoclastogenesis. Increased ratio of the mRNA expression of RANKL/OPG has been found in patients with periodontitis (Lu et al., 2006; Bostanci et al., 2007a,

2007b).   In addition, application of OPG has been demonstrated to reduce bone destruction, suggesting the important role of RANKL/OPG in homeostasis of alveolar bone (Jin et al., 2007).

Major goals of periodontal therapy are to control periodontal inflammation and also to induce repair and regeneration of periodontal tissue (Takata, 1994). Basic cellular and molecular mechanisms of periodontal wound healing, however, are not yet fully understood. Wound healing presents as a complex, continuous process of inflammation, granulation tissue formation, matrix formation and final remodeling, in which fibroblasts, epithelial cells, endothelial cells, osteoblasts and inflammatory cells are involved (Wikesjo et al., 1992). Blood clotting systems have been demonstrated to be involved in certain pathobiological processes, including inflammation and wound healing following tissue injury (Colvin, 1986). Interestingly, the gingival and periodontal bleeding and coagulation characteristics of periodontal disease indicate that thrombin is likely to be generated via the coagulation cascade on the surface of locally damaged vessels of periodontal tissue. The thrombin-induced fibrin network can, therefore, provide a good substrate for the migration, attachment and growth of adjacent periodontal ligament cells (Polson and Proye, 1983). Clinically, if a blood clot in wound defect is stable and is later replaced by cells derived from PDL, repair and regeneration of cementum, PDL and fibrous attachment will be attainable (Wikesjo et al., 1992; Tatakis, 1992).

In addition to the anti-coagulant effects, thrombin can promote numerous cellular effects including chemotaxis, proliferation, extracellular matrix turnover and release of cytokines.   Thrombin also stimulates the production and secretion of

extracellular matrix proteins and influences connective tissue remodeling processes (Duhamel-Clerin et al., 1997; Chambers et al., 1998).

For mineral tissue, thrombin is able to influence the biological behavior of osteoblasts and plays multiple roles in the early stages of bone healing (Pagel et al., 2006). It has been shown to stimulate proliferation, migration, prostaglandin releasing and calcium mobilization in osteoblast-like cell lines or primary culture of osteoblasts (Lerner et al., 1989; Tatakis et al., 1989; Abraham et al., 1999; Karp et al., 2005). These results suggest a role of thrombin in bone repair.

Recently, thrombin-rich plasma and platelet-rich plasma, which contain thrombin have been used successfully for periodontal regeneration (Kassolis et al., 2000). However, the action of thrombin in periodontal tissue is still uncertain. Thrombin has been shown to stimulate proliferation and protein synthesis in HPDL cells (Chan et al., 1998). In addition, it could promote collagen gel contraction in gingival fibroblasts (Jeng et al., 2006). Although there are few reports regarding to the role of thrombin in periodontal tissue, the data suggested the possibility that thrombin may play a role in repair and healing of periodontal tissue.

The cellular actions of thrombin could be attributed to the activation of its receptor, protease-activated receptor (PAR), which is widely distributed in various kinds of tissues including gingival and periodontal ligaments. The family of PARs currently includes four members: PAR-1, PAR-2, PAR-3, and PAR-4. Thrombin is the physiological activator of PAR-1, PAR-3, and PAR-4 (Coughlin, 2000; O'Brien, Williams and Marshall, 2001); however, PAR-2 is activated by multiple trypsin-like serine proteases but not by thrombin (Coughlin and Camerer, 2003). PARs play important roles in normal physiology and disease; however, in many cases,

the particular protease and PAR that function in specific cellular process especially in periodontal tissue regeneration remain to be defined.

It has been demonstrated that PAR-1 is expressed by osteoblasts, and mediates thrombin-induced proliferation of these cells (Abraham et al., 1998; Abraham and MacKie, 1999; Song et al., 2005). Thrombin also stimulated osteoclastic bone resorption *in vitro* (Gustafson and Lerner, 1983; Hoffmann et al., 1986). Thrombin may participate in the pathological processes of pulpal and periodontal diseases since it can induce prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) production both of which species may mediate bone resorption (Chan et al., 1998; Chang et al., 1998). Interestingly, some evidences have been provided that HPDL cells express thrombin receptor (Chan et al., 1998), suggesting that these cells are potential targets to become activated by thrombin. However, the precise distribution of different PAR in HPDL cells and their specific roles following their activation by thrombin in periodontal tissue regeneration are still not clear.

Since both thrombin and RANKL/OPG ratio are important components of the process of tissue repair, we hypothesized that thrombin could affect the balance of RANKL/OPG in HPDL cells. The current study was undertaken to investigate the effect of thrombin on OPG synthesis. The participation of PAR-1 and the signaling pathways mediated by thrombin are focused.

RESEARCH OBJECTIVES

1. To investigate the effect of thrombin on the balance of OPG/RANKL expression in HPDL cells.

2. To determine the signaling pathway of RANKL and OPG expression in HPDL cells mediated by thrombin.

3. To elucidate whether changes of RANKL and OPG expression in HPDL cells induced by thrombin occur through PAR-1

RESEARCH HYPOTHESIS

1. Thrombin has effect on RANKL and OPG expression in HPDL cells.

2. Changes of OPG expression in HPDL cells induced by thrombin occur through PI3K signaling pathway.

3. Changes of OPG expression in HPDL cells induced by thrombin occur through PAR-1

EXPECTED BENEFIT

We anticipate that the results from this line of investigation would provide a better understanding of the mechanism controlling the periodontal tissue regeneration process. Our goal is to gain sufficiency knowledge basis in pathobiology of periodontal disease and the repair potential of tissue to develop new and specific therapeutic methods that will reverse the destructive consequence of the periodontal disease in the future.

CHAPTER II

REVIEW OF RELATED LITERATURE

Thrombin is a pluripotent serine protease that plays a central role in hemostasis following tissue injury by converting soluble plasma fibrinogen into an insoluble fibrin clot and by promoting platelet aggregation (Fenton, 1988; Tatakis, 1992; Altieri, 1993). The thrombin-induced fibrin network provides a good substrate for the migration, attachment and growth of adjacent cells (Polson and Proye, 1983). However, recent evidence revealed that thrombin is not only a clotting protease serving as both pro- and anti-coagulant molecule but also appears to play multifunctional roles related to inflammation, allergy, tumor growth, metastasis, tissue remodeling and probably wound healing (Cirino et al., 2000; Coughlin, 2000, 2001; Macfarlane, 2001). Thrombin exerts cellular effects via G-protein coupled proteased-activated receptors (PARs). Among the four known PAR receptors, thrombin activates PAR-1, PAR-3 and PAR-4, but not PAR-2 which is activated by other proteases (Vu et al., 1991; Couglin, 2000; O'brien, Williams and Marshall, 2000).

In addition to the anti-coagulant effects, thrombin can promote numerous cellular effects including chemotaxis, proliferation, extracellular matrix turnover and release of cytokines. Thrombin also stimulates the production and secretion of extracellular matrix proteins and influences connective tissue remodeling processes (Duhamel-Clerin et al., 1997; Chambers et al., 1998). Thrombin has been shown to stimulate the proliferation of many cells including endothelial cells, smooth muscle cells, and fibroblasts (Berk et al., 1991; Dawes et al., 1993; Herbert et al., 1994;

Chang et al., 1998, 1999). However, few studies have directly investigated the effects of thrombin on the behavior of dental cells such as HPDL cells. The study of Chan and his colleagues (1998) demonstrated that thrombin promoted growth of HPDL cells in a dose dependent manner, suggesting that this molecule may play a role in the homeostasis of periodontal tissue. Homeostasis of periodontium also requires balance of RANKL and OPG, two major molecules that play the important roles in osteoclastogenesis. It was shown that HPDL cells express RANKL and OPG. Changes in the level of RANKL and OPG expression in HPDL cells have been reported in periodontal disease and orthodontic tooth movement indicating the significant of HPDL cells in these phenomenons.

The background and interaction of thrombin, thrombin receptor (PAR), OPG-RANKL, and related article are presented in this review.

Wound Healing

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers involving the interactions of different cell types that vary depending on the type of tissue involved. It employs an intercalating array signaling peptides such as growth factors and cytokines that control and regulate a co-ordinated cellular and extracellular matrix remodeling to repair the lesion. Wound healing is characterized by three interrelated and overlapping phases: inflammation, tissue formation/stabilization and tissue remodeling (Cohen et al., 1992; Verderio et al., 2004) (Figure 2.1).

1. Inflammation :

Following tissue injury, which is probably the most well characterized process, the wound is normally filled by a blood clot which re-establishes hemostasis and serves as a temporary matrix for the migration of cells from adjacent tissue. Thrombin is generated from prothrombin via the extrinsic or the intrinsic coagulation pathway to induce blood clot formation and arrest bleeding (Altieri et al., 1993). Thrombin is important in these processes, not only because of its hemostatic effect after tissue injury, but also because of its effective release by fibrin matrix, leukocytes, and endothelium during inflammatory conditions. Fibrinogen is cleaved into fibrin and the framework for completion of coagulation process is formed. Fibrin provides the structural support for cellular constituents of inflammation.

Cells within the thrombus, predominantly activate platelets, release many wound healing mediators (such as platelet-derived growth factor (PDGF), platelet factor IV, transforming growth factor-beta (TGF-β and vasoactive amines) leading to the inflammatory response by the recruitment and activation of macrophages and fibroblasts (Verderio et al., 2004). PDGF is chemotactic for fibroblasts and along with TGF-β is a potent modulator of fibroblastic mitosis leading to collagen fibril construction in later phases. TGF-β also facilitates polymorphonuclear leukocytes (PMN) migration from surrounding blood vessels. These cells cleanse the wound, clearing it of debris (Verderio et al., 2004).

As the process continues, monocytes also extrude from the blood vessels. There are termed macrophages that continue the cleansing process and manufacture various growth factors. The macrophages orchestrate the multiplication of endothelial cells with the sprouting of new blood vessels, the duplication of smooth muscle cells,

and the creation of the milieu created by the fibroblast. Macrophages have a key role in phagocytosis of microorganism and remnants of the extracellular matrix (ECM) and in the secretion of wound healing cytokines such as PDGF, vascular endothelial growth factor (VEGF), TGF-β, tumor necrotsis factor (TNF) and also basic and acidic fibroblast growth factor (FGF), which mediate the transition between inflammation and tissue repair (McCawley and Matrisian, 2001).

2. Tissue formation

Fibroblasts are key cells in the formation of new tissue in the wound space. After migrating into the wound area, they synthesize new ECM with gradual replacement of the provisional matrix with a collagen matrix. Several matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) are expressed during wound healing and are responsible for matrix remodeling by degrading existing matrix in/around the wound edge and by creating a path for cell migration while new matrix is deposited (McCawley and Matrisian, 2001).

Endothelial cells, stimulated by the local secretion of angiogenic factors such as those belonging to the epidermal growth factor family (EGF) and the FGF family, are responsible for the neovascularization in the wounded area in the granulation tissue, thus supporting the nutritional requirement of proliferating fibroblast cells (Singer et al., 1999).

3. Tissue remodeling

The final phase of wound healing is to replace the injured tissue by parenchymal cells of the same type. Fibroblasts stop producing collagen and the

remodeling process occurs, for example, type III collagens are replaced with type I collagens and cartilages are replaced with bone.

Therefore, the first important phase in the wound healing is hemostasis which induces the completion of healing process.
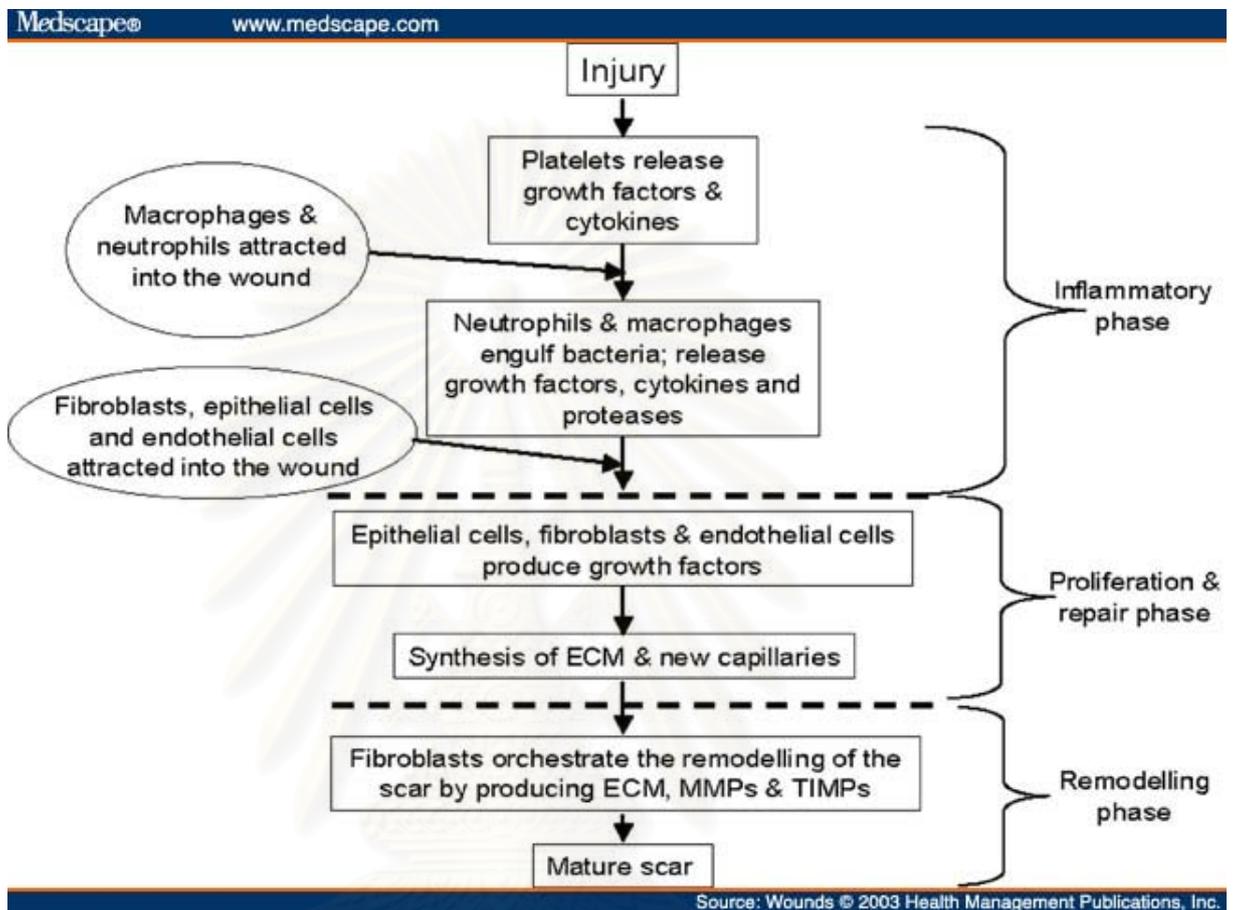
Figure 2.1 Scheme of the wound healing processes. Wound healing is characterized by three interrelated and overlapping phases: inflammatory phase, proliferation and repair phase and remodeling phase.

(Available from: http://www.medscape.com)

Thrombin

Thrombin is an important effector protease of the coagulation cascade that leads to formation of a hemostatic plug. Thrombin is thought to act near the site at which it is generated and it is activated when circulating coagulation factors in the blood plasma make contact with tissue factor. Tissue factor is a membrane protein that is usually produced by cells that are separated from blood (i.e., epithelial cells). However, it is also expressed at low levels on circulating monocytes and microparticles from leukocytes. Tissue factor is associated with the activation of zymogen factorX by factor VIIa. Factor Xa together with its cofactor Va subsequently converts prothrombin to the active enzyme. Thus, plasma coagulation can only take place when the vascular integrity is damaged (Coughlin, 2000). Thrombin causes shape change of endothelial cells and increases permeability of endothelial cell layers. However, recent evidences reveal that thrombin is not only a clotting proteinase serving as both a procoagulant and anticoagulant molecule but also appears to play multifunctional roles related to inflammation, allergy, tumor growth, metastasis, tissue remodeling, thrombosis, and probably wound healing (Cirino et al., 2000; Coughlin, 2000,2001; Macfarlane, 2001) (Figure 2.2).
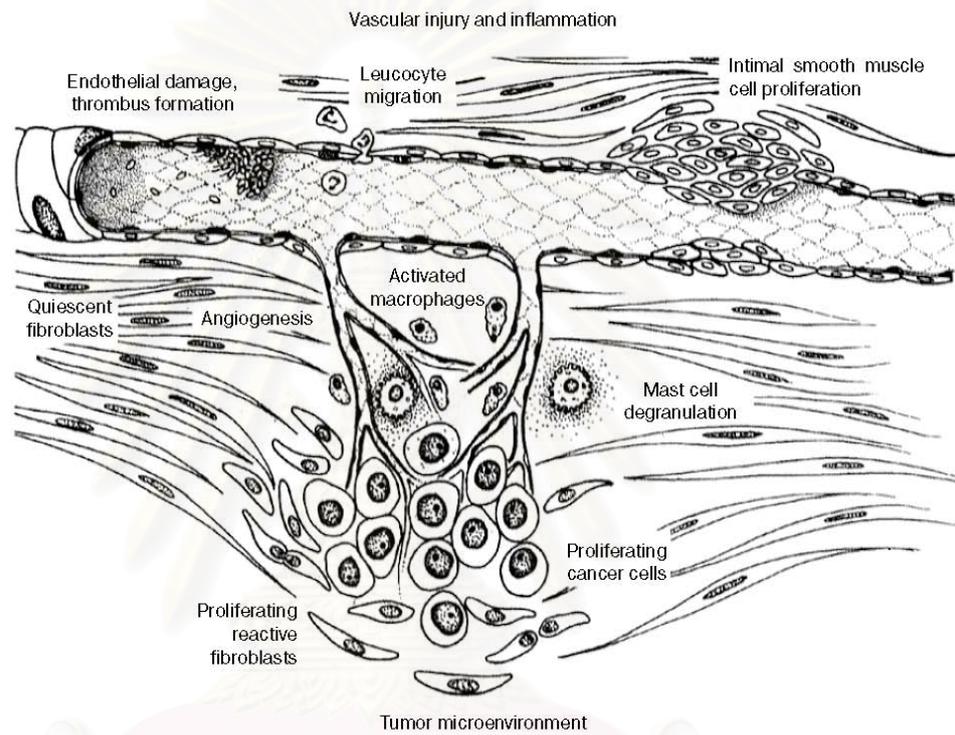
An illustrative representation of PAR-1 mediated cellular effects during vascular injury, inflammation, and within the tumor microenvironment

Figure 2.2 An illustrative representation of cellular effects of thrombin during vascular injury, inflammation, and within tumor microenvironment (Derian et al., 2002)

Thrombin structure

The 26,929 bp cDNA and gene structure for human prothrombin were determined in 1983 (Degen et al., 1983). The prothrombin gene is located on chromosome 11 and is organized into 14 exons with 13 intervening sequences. Prothrombin is synthesised in the liver as a pre-propeptide and undergoes a number of post-translational processes prior to secretion. It is converted to thrombin following tissue injury by cleavage at two sites by factor Xa. The resultant 39 kDa thrombin comprises two chains crosslinked by four disulphide bonds that house a narrow groove containing the catalytic triad consisting of His-57, Asp-102 and Ser-205 (Figure 2.3). This groove is hydrophobic and exhibits a preference for apolar amino acids preceding arginine at a thrombin susceptible bond such as Leu Asp Pro Arg/Ser (where/represents the cleavage site). The high specificity of thrombin towards its substrates and receptors is conferred by its unique anion binding exosite. In addition, thrombin possesses three specificity pockets which interact with inhibitors (Goldsack et al., 1998).
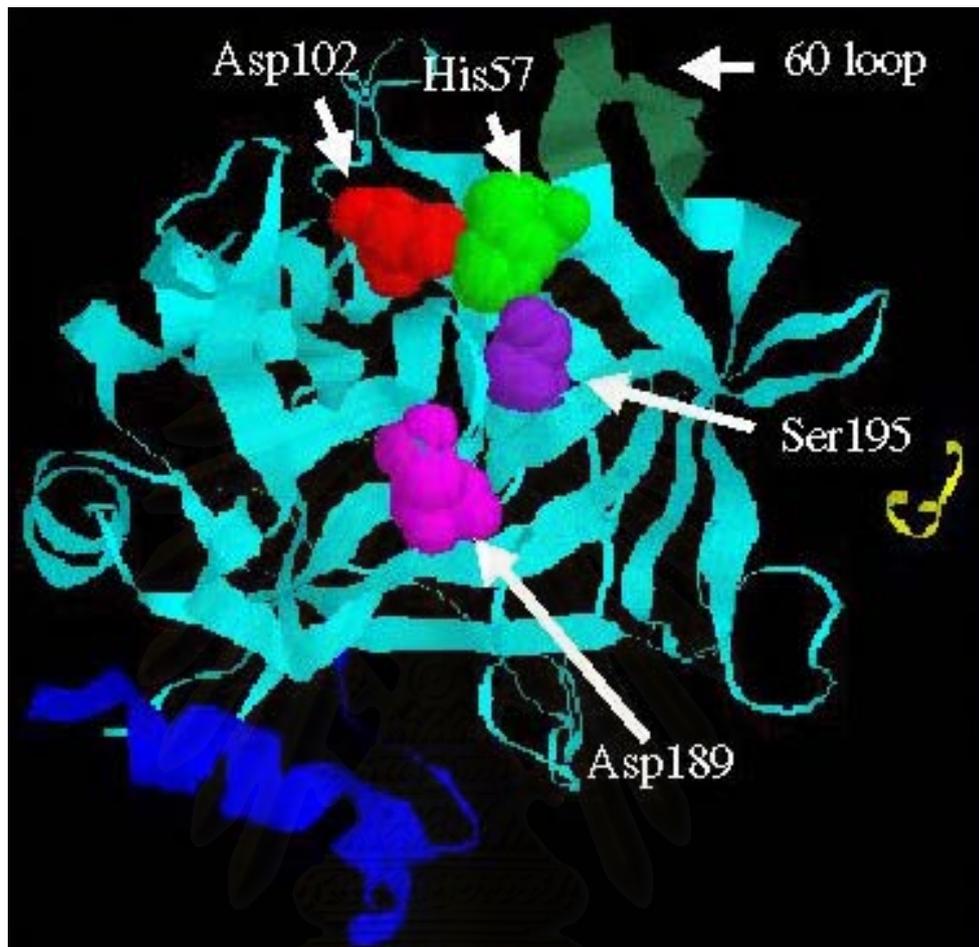
Figure 2.3 The Crystal structure of thrombin. Thrombin is a serine protease with an overall ellipsoid-like structure and is constituted of a small A-chain (blue) and a catalytic B-chain (green). In B chain, there is a catalytic triad, composed of His-57, Asp-102 and Ser-195, which is a cleavage site of thrombin. (Available from: http://opbs.okstate.edu/)

Biological function of thrombin

    1.   Role in blood coagulation cascade

Thrombin generation from prothrombin occurs in the region of vessel and tissue damage at the initial stage of repair, when a fibrin clot is forming and inflammation is developing. Inflammation leads to the activation of blood cells, endothelium and subendothelium of vessels. Under these conditions, thrombin is involved in the adhesion and recruitment of leokocytes, platelet activation and cell proliferation. The proinflammatory effect of thrombin plays an important role in tissue repair, and also in pathologies that are related to chronic activation of coagulation cascade.

Thrombin plays a key role in blood coagulation system. Together with conversion of fibrinogen to fibrin, activation of blood coagulation factors V, VIII, XI and XIII, and platelet aggregation, thrombin regulates hemostasis by activation of the anticoagulant system of protein C and inhibition of fibrinolysis (through activation of TAFI, thrombin –activated fibrinolysis inhibitor) (Figure 2.4). Thrombin activates various cell types in and outside blood participating in the processes of development, regulation of vessel tone, inflammation, tissue repair, atherosclerosis, carcinogenesis and many others (Dugina et al., 2002).

    2.   Role of thrombin in inflammation and tissue repair

In addition to promoting platelet aggregation, thrombin also stimulates platelets to release mediators including thromboxane A2, platelet factor 4, PDGF and TGF-β (Goldsack et al., 1998). Thrombin also plays a major role in trafficking of

inflammatory cells into sites of injury and is chemotactic for a number of different cell types including, monocytes, macrophages and neutrophils. It also influences several key processes of tissue repair. For example, thrombin stimulates expression of endothelial cell surface adhesion molecules and enhances vascular permeability and leucocyte extravasation. These activated endothelial cells release vasoactive mediators including prostacyclin, endothelin-1 and nitric oxide (Magazine and Srivastava, 1996). Furthermore, thrombin stimulates proliferation in a number of cells such as fibroblasts (Figure 2.5).

Thrombin has been shown to stimulate the proliferation of many cells including endothelial cells, vascular smooth muscle cells, epithelial cells, neuronal cells, immune cells, fibroblasts and osteoblasts. (Berk et al., 1991; Dawes et al., 1993; Herbert et al., 1994; Chang et al., 1998; Lafay, 1998; Abraham and Mackie, 1999; Madamanchi et al., 2001). It has been demonstrated that PAR-1 was expressed by osteoblasts, and mediates thrombin-induced proliferation of these cells (Abraham et al., 1998; Abraham and Mackie, 1999; Song et al., 2005). Thrombin also stimulated osteoclastic bone resorption *in vitro* (Gustafson and Lerner, 1983; Hoffmann et al., 1986).

However, few studies have directly investigated the effects of thrombin on the behavior of dental cells. It has been demonstrated that thrombin can promote cell proliferation in dental pulp cells (Chang et al., 1998, 1999; Gruber et al., 2004), gingival fibroblasts (Chang et al., 2001), HPDL cells (Chan et al., 1998) and osteoblast cells (Song et al., 2005). Thrombin may participate in the pathological processes of pulpal and periodontal diseases since it can induce PGE_2 and PGI_2 production both of which species may mediate bone resorption (Chan et al., 1998;

Chang et al., 1998, 1999). In summary, thrombin exerts a number of receptor-mediated cellular effects which may play vital roles in coagulation, inflammation and wound healing (Figure 2.6).
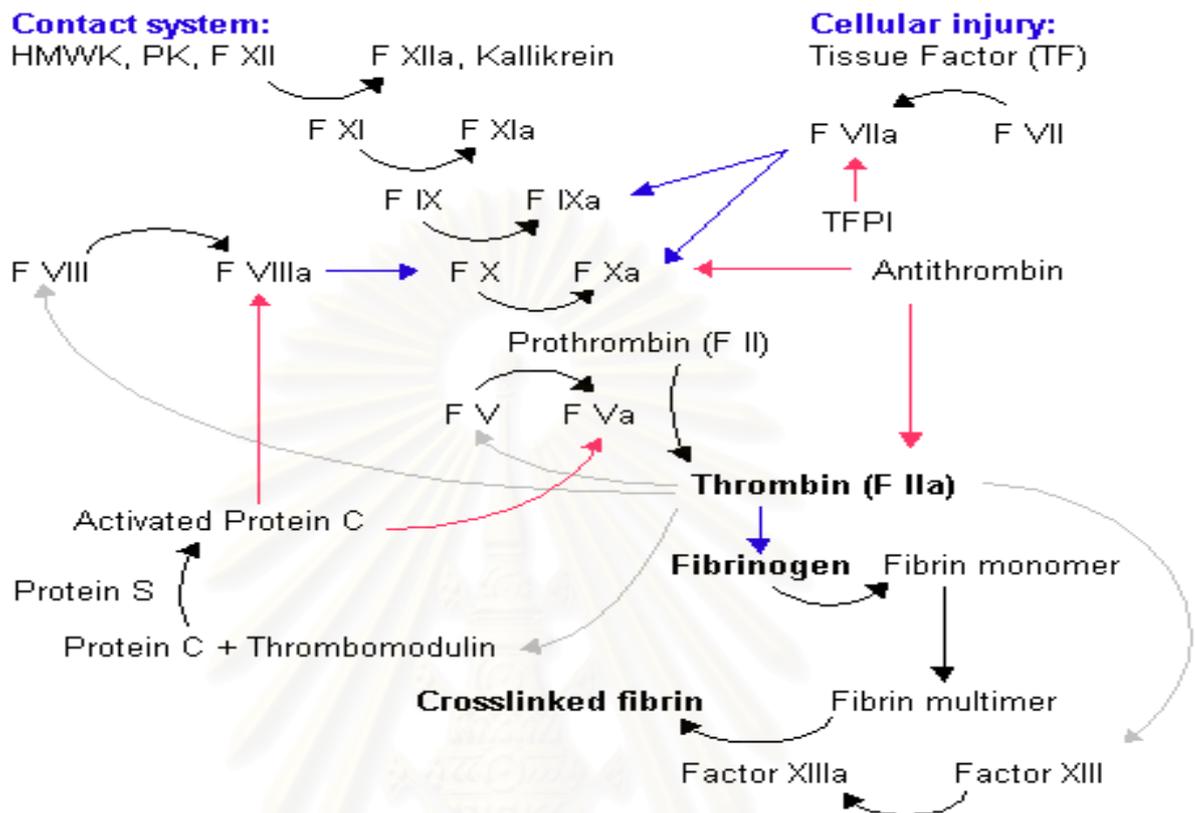
Figure 2.4 The coagulation cascade. Following damage to the blood vessel endothelium Tissue Factor (TF) is released, this then forms a complex with FVIIa (TF-FVIIa) this activates FIX and FX. FVII itself is activated by thrombin, FXIa, plasmin, FXII and FXa. The activation of FXa by TF-FVIIa is almost immediately inhibited by tissue factor pathway inhibitor (TFPI). FXa and its cofactor FVa form the prothrombinase complex which activates prothrombin to thrombin. Thrombin has a large array of functions. Its primary role is the conversion of fibrinogen to fibrin, the building block of the hemostatic plug. In addition, it activates other components of coagulation cascade including FV and FVII and their inhibitor protein C (in the presence of thrombomodulin), and it activates FXIII, which form the covalent bonds that crosslink the fibrin polymers from the activated monomers. (Legend; HWMK =

High molecular weight kininogen. PK = prekallikrien. TFPI = Tissue factor pathway inhibitor. Black arrow = conversion/activation of factor. Red arrows = action of inhibitors. Blue arrow – reactions catalysed by activated factor. Grey arrow = various function of thrombin.) (Available from: http://www.biocrawler.com/encyclopedia/)
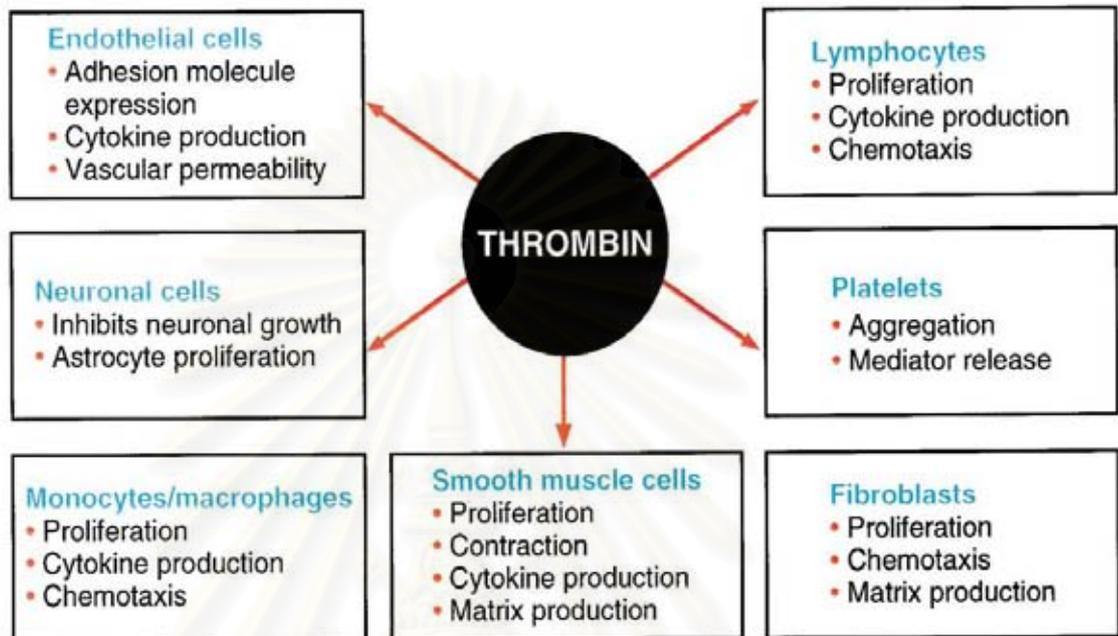
Figure 2.5 Cellular effects of thrombin. Thrombin stimulates a variety of cellular effects, most of which are critical to tissue repair. (Goldsack et al., 1998)

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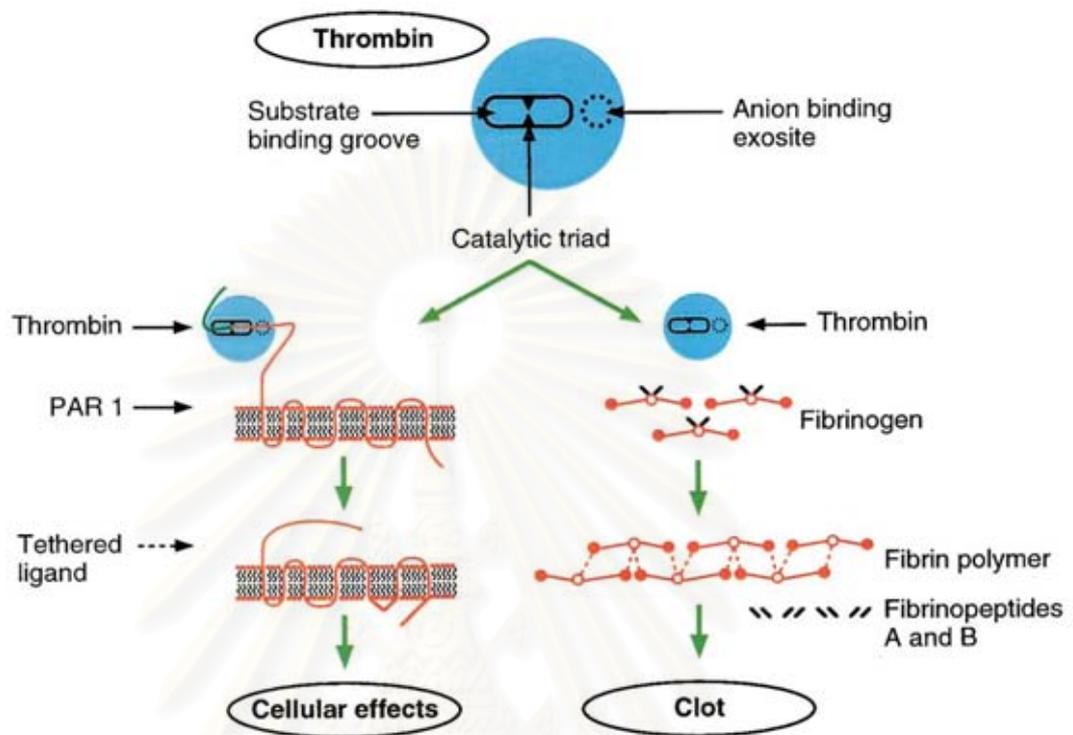
Figure 2.6 Structure and function of thrombin. This diagram shows a schematic representation of thrombin and its interaction with the cellular PAR-1 receptor and fibrinogen. The interaction between thrombin and PAR-1 initiates a variety of cellular effects. The interaction between thrombin and fibrinogen is the final step of the coagulation cascade leading to the fibrin clot. Thrombin has several discrete sites. The anion binding exosite of thrombin lies within the deep groove and binds its substrates. The substrate binding sites within this deep groove orientates thrombin's substrates (PAR-1 or fibrinogen) enabling cleavage at the catalytic site at an Arg/Ser susceptible bond. (Goldsack et al., 1998)

Thrombin receptor or protease-activated receptor (PAR)

Thrombin exerts its cellular effects via G-protein coupled receptors (GPCR). The unique mechanism of receptor activation was discovered to involve the proteolytic unmasking of an N-terminal receptor sequence that becomes a tethered ligand, binding to and activating receptor signaling (Vu et al., 1991). Based on this mechanism of activation, the receptor for thrombin has been referred to as a 'protease-activated receptor', assigned the acronym PAR by the International Union of Pharmacology (Hollenberg and Compton, 2002).

Four PAR family members have been identified to date (Figure 2.7). In 1991, the first thrombin receptor present in human platelets was cloned and its unique activation mechanisms were described. This receptor belongs to a large superfamily of G-protein-couple seven trans-membrane domain receptors and is now known as PAR-1. Another member of the PAR family, PAR-2 was found in 1994. PAR-3 and PAR-4 were cloned as the second and third thrombin receptors in 1997 and 1998, respectively.

Thrombin receptors or PARs belong to a new subfamily of GPCRs with seven trans-membrane domains activated via proteolytic cleavage by serine proteases (Dery et al., 1998; Coughlin, 2000; Macfarlane et al., 2001; Hollenberg and Compton, 2002) (Figure 2.8). PAR-1, PAR-3, and PAR-4 are targets for thrombin, trypsin, or cathepsin G (Vu et al., 1991; Ishihara et al., 1998; Kahn et al., 1998; Xu et al., 1998). In contrast, PAR-2 is resistant to thrombin, but can be activated by trypsin, mast cell tryptase, factor Xa, acrosin, gingipain, and neuronal serine proteinases (Dery et al., 1998; Coughlin, 2000, Macfarlane et al., 2001) (Table 1).

The well-known thrombin receptor PAR-1 has been identified as deriving from human platelets. PAR-1 is widely distributed among cells and tissues, consistent with thrombin's widespread effects described in *in vitro* studies. Thus, regulation of PAR-1 expression and/or activity presents an important approach to modulate thrombin's action. PAR-2 is the only PAR described to date that is not activated by thrombin. The most widely studies of proteolytic activators of PAR-2 are trypsin and tryptase (Corvera et al., 1997; Mirza et al., 1997; Molino et al., 1997). The cell and tissue distribution of PAR-2, like PAR-1, is widespread with significant expression in pancreas, colon, kidney, small intestine, and liver. PAR-2 is notably absent from platelets. Furthermore, PAR-3 has recently been cloned and its expression occurs mainly in the human bone marrow, adrenal gland, and lymph node. It is absent from human platelets. An additional protease activator receptor, PAR-4, has also been cloned and its expression noted and confirmed in human platelets, lung, pancreas, and small intestine.
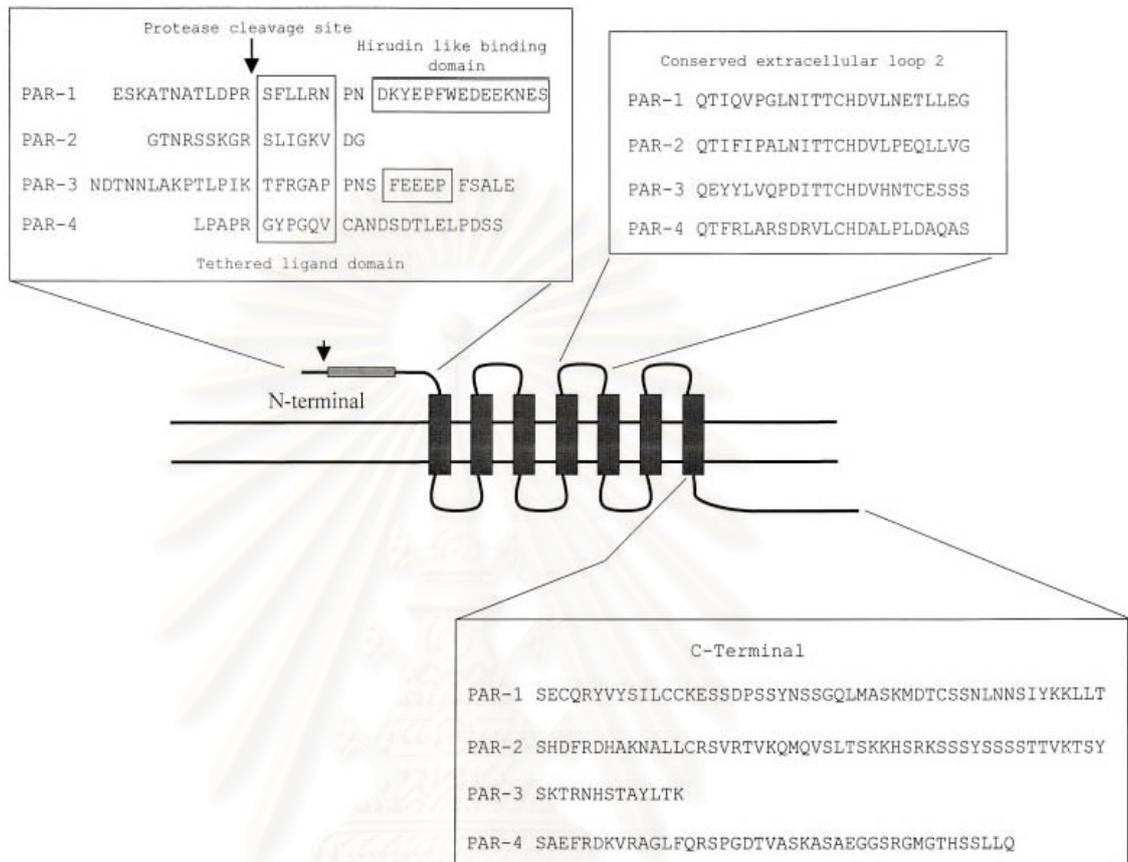
Figure 2.7 Structure and functional domain of PAR-1, PAR-2, PAR-3 and PAR-4. The key areas of PAR receptor activation are highlighted. The N terminus cleavage domain, the extracellullar loop 2 (ECL-2), and the C terminal tail. (Macfarlane et al., 2001)
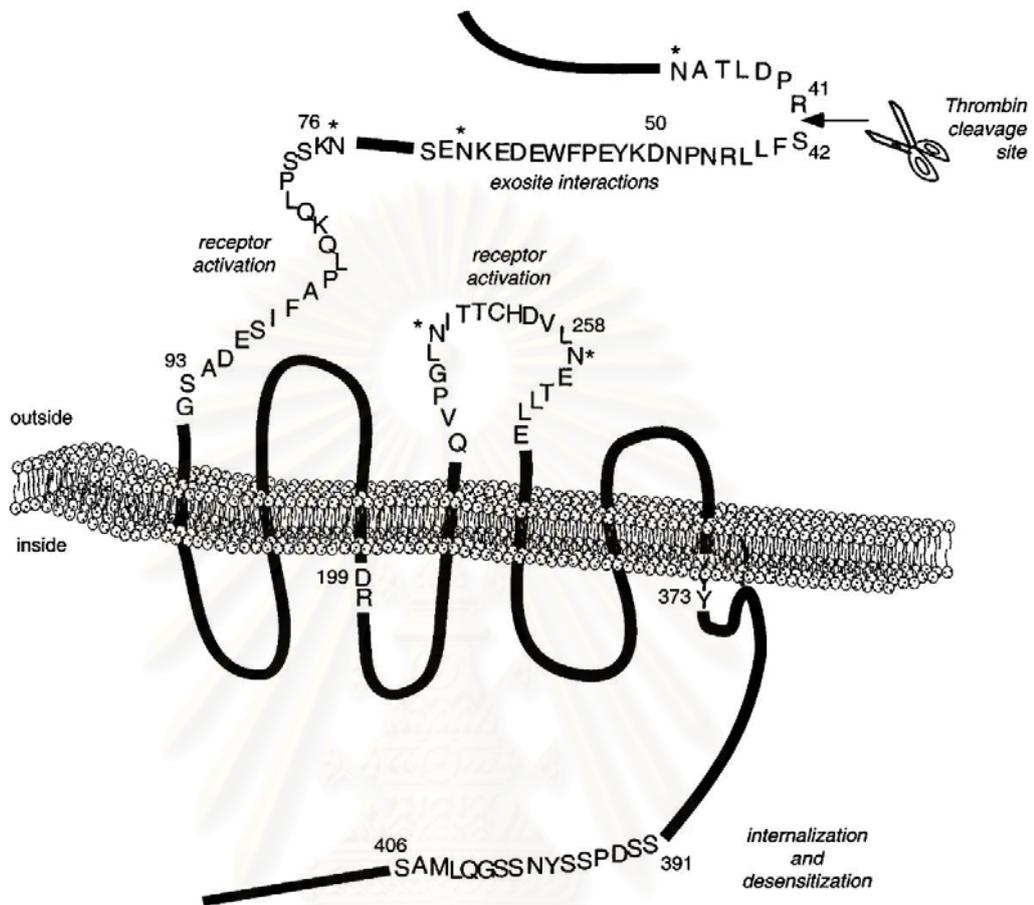
Figure 2.8 Structure and features of seven transmembrane GPCR. Cartoon of human PAR-1 highlighting domains thought to be involved in receptor activation and interactions with thrombin. The N terminus cleavage domain and the hirudin-like binding domain, the extracellullar loop 2 (ECL-2) where tethered ligand/receptors interaction occurs, and the C terminal tail that is involved in desensitization and some aspects of intracellular signal (O'Brien et al., 2001)

	PAR₁	PAR₂	PAR₃	PAR₄
Amino acid composition	425 aa (h)	397 aa (h)	374 aa (h)	385 aa (h)
Tethered ligand sequence	SFLLR (h) SFFLR (m, r)	SLIGKV (h) SLIGRL (m, r)	TFRGAP (h) SFNGGP (m)	GYPGQV (h) GYPGKV (m)
Selective agonist peptide	TFLLR-NH₂ TFRIFD[a]	SLIGKV-NH₂ (h) SLIGRL-NH₂ (m, r)	None known	GYPGKV-NH₂ GYPGQV-NH₂ AYPGKF-NH₂
High-affinity ligands	Thrombin	Trypsin, tryptase, trypsin-2	Thrombin[b]	Thrombin, trypsin
Low-affinity ligands	Trypsin; FVIIa/TF/FXa; granzyme A, plasmin	Matriptase/MT-serine protease 1; Der p3; Der p9; FVIIa/TF/FXa; cockroach proteases	Trypsin > factor Xa	Cathepsin G, factors VIIa/X
Inhibitory proteases	Cathepsin G, proteinase-3; elastase; chymase	Elastase, chymase	Cathepsin G	Unknown
Selective antagonists	Trans-cinnamoyl-parafluoro-Phe-Paraguanidino-Phe-Leu-Arg-Arg-NH₂ Mercaptopropionyl-Phe-Cha-Arg-Lys-Pro-Lys-Pro-Asn-Asp-Lys-NH₂ Non-peptide antagonists: RWJ56110 and RWJ58259	None known	None known	Trans-cinnamoyl-YPGKF-NH₂

The letters denote the amino acid sequences of peptides in one letter code: aa, amino acids; h, human; m, mouse; r, rat; NH₂, respective amides.
[a] TFRIFD is the *Xenopus* thrombin receptor tethered ligand domain (the human PAR₁ tethered ligand domain sequence, SFLLRN, activates both PAR₁ and PAR₂).
[b] Proteinase cleavage but PAR₃ does not generate a calcium signal.

Table 1 Pharmacology of protease-activated receptors and their agonists.

(O'Brien et al., 2001)

Mechanism of PAR activation

Interestingly, PARs are activated by a unique mechanism: proteases activate PARs by proteolytic cleavage within the extracellular N terminus of their receptors, thereby exposing a novel "cryptic" receptor-activating N-terminal sequence that remaining tethered, binds to and activates the receptor (Figure 2.9) within the same receptor (Vu et al., 1991). Specific residues (about six amino acids) within this tethered ligand domain are believed to interact with extracellular loop 2 and other domains of the cleaved receptor, resulting in activation. This intramolecular activation process is followed by coupling to G proteins and the triggering of a variety of downstream signal transduction pathways. Several studies during the past few years have also demonstrated that several mechanisms exist to regulate stimulation and termination of PAR-initiated signaling (Dery et al., 1998; Coughlin, 2000; Macfarlane et al., 2001; Hollenberg and Compton, 2002). Importantly, the availability of PARs at the cell surface is governed by trafficking of the receptor from intracellular stores, and the signaling properties depend on the presence of G proteins and G protein-coupled receptor kinases (GRKs) that modify activity.

For PAR-1, PAR-2, and PAR-4, it is well established that short synthetic peptides or PAR-activating peptides (PAR-APs) designed on their proteolytically revealed tethered ligand sequences can serve as selective receptor agonists (Table 1). PAR-3 is unique among the PARs in that it is not activated by synthetic pectides representing its tethered peptide ligand. Thus, the functional role of PAR-3 remains unclear. Some PAR-APs activate more than one PAR, and they activate receptors at concentrations in the micromolar range as compared with nanomolar potencies of the

proteinases themselves (Scarborough et al., 1992; Blackhart et al., 2000). Although the PAR1-AP, SFLLRN-NH2, also activates PAR-2, PAR2-APs, like SLIGRL-NH2, are not capable of activating other PARs. Unfortunately, the relatively low potency (10 to 100 µm EC50) and susceptibility to aminopeptidases limit the utility of the PAR-APs in some bioassay systems (Coller et al., 1993). Recently, modified synthetic agonist peptides with higher potency, resistance to aminopeptidases, and greater receptor selectivity have been developed and characterized. These receptor-selective agonists are of use to study the consequences of activating PARs in *vivo* (Table 1) (Dery et al., 1998; Macfarlane et al., 2001; Hollenberg and Compton, 2002). So far, antagonists for PAR-1, PAR-2 and PAR-4 have been synthesized, but are not yet available for PAR-3.
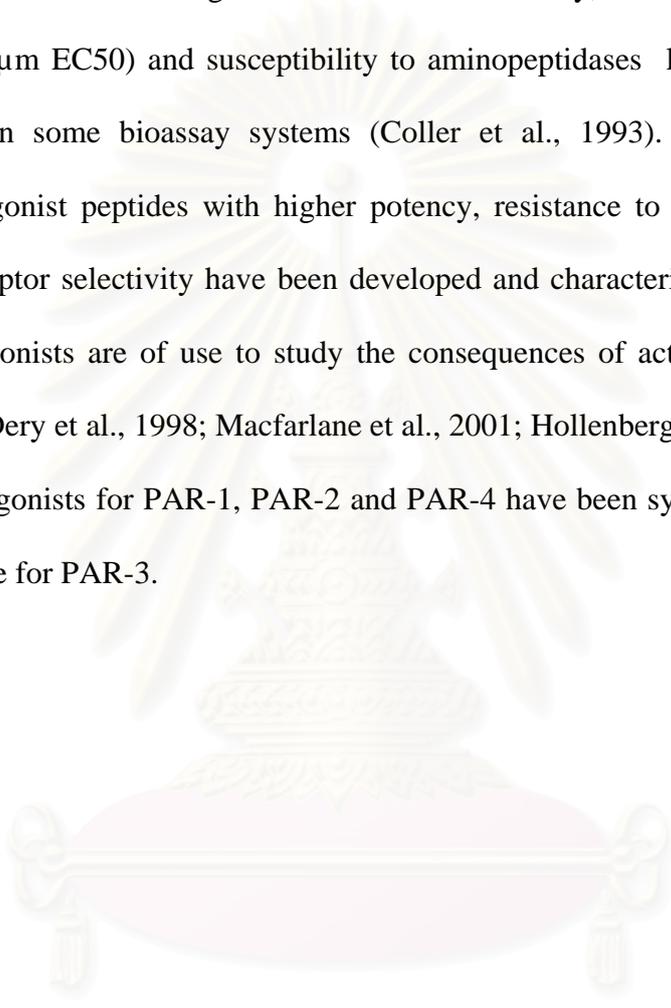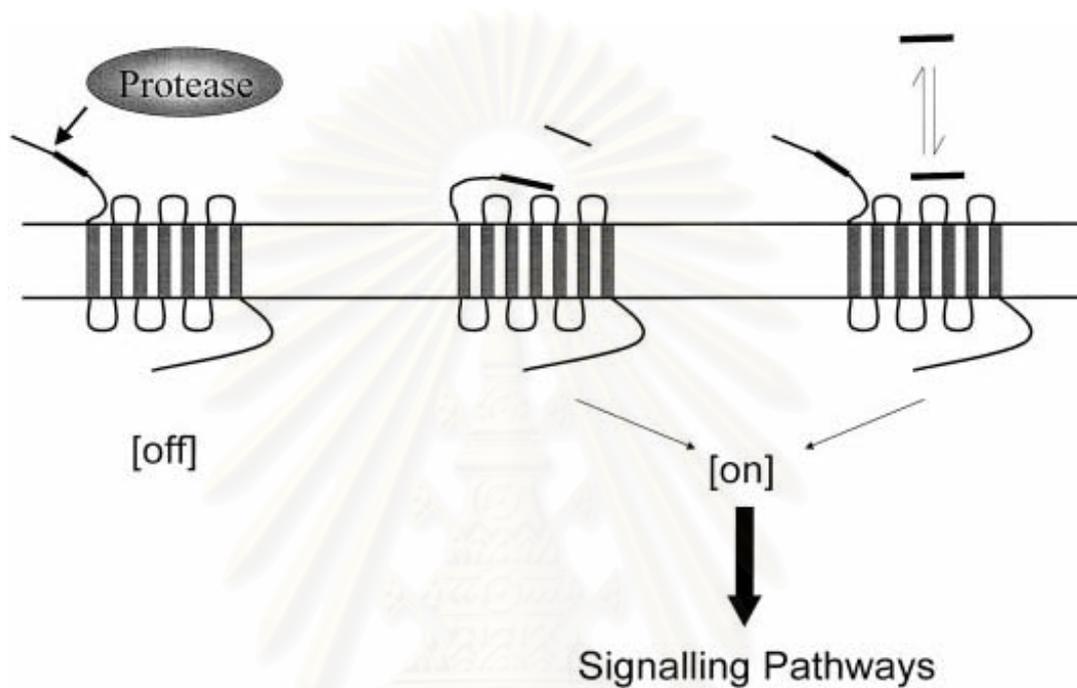
Figure 2.9 Protease activation of PARs. Proteolytic cleavage the N-terminus generates a new tethered ligand designated by the filled section that interacts with extracellular loop 2 of the receptor. A peptide sequence derived from the N-terminus is able to activate the receptor in the absence of protease-mediated cleavage of the N terminus. (Macfarlane et al., 2001)

PAR-1 mediating cellular signaling

The mechanism of PAR activation was initially established for PAR-1 (Vu et al., 1991) and appears to be a general paradigm for other PARs. PARs most likely elicit signaling responses according to the classic paradigm established for other GPCRs. That is, upon ligand activation of PARs, conformational changes in the receptor promote interaction with heterotrimeric G proteins at the plasma membrane. Considerable progress has been made in identifying the subtypes of G proteins that interact with PAR-1, and the role of these subunits in signaling and physiological regulation has been studied in knockout mice (Offermans, 2001). In common with several other GPCRs, PAR-1 interacts with multiple G protein subtypes (MacFarlane et al., 2001). PAR-1 interacts with several α-subunits, in particular Gq11α, G12/13α and Giα, which accounts for the pleotropic action of its ligands (Figure 2.10).

A major pathway of coupling is through Gqα (Hung et al., 1992). Several early studies indicated that PAR-1 couples to inhibition of cAMP accumulation through Giα and stimulates phospholipase C (PLC), catalyzed hydrolysis of phosphoinositides to stimulate production of inositol trisphosphate and diacylglycerol through Gq11α. Together, Ca^{2+} and PKC activate numerous pathways, including Ca^{2+}-regulated protein kinases and mitogen-activated protein (MAP) kinases. More recent studies have illustrated coupling of PAR-1 to G12/13 (Offermanns et al., 1994); however, whether activation of PAR-1 modulates G12/13 effectors such as Rho GEFs and PLC remains to be determined. The extent to which PAR-1 couples to each of these pathways in a particular cell type presumably depends upon the G protein and effecter repertoire expressed in the cell.

In contrast to PAR-1, studies that directly assess the coupling of other PARs to distinct G protein subtypes have not been reported. However, activation of PAR-2, PAR-3, and PAR-4 cause increases in inositol phosphates and mobilization of Ca^{2+} in a variety of cell types (Bohm et al., 1996; Ishihara et al., 1998; Shapiro et al., 2000), suggesting that these receptors are capable of activating Gq and/or Gi signaling responses.

Gβγ subunits of heterotrimeric G proteins couple PAR-1 to may other pathways, notably activation of phosphatidylinositol 3-kinase (PI3K). PI3K thus links PAR-1 to changes in cytoskeletal structure, cell motility, survival, and mitogenesis. For instance, in astrocytes, the effects of PAR-1 agonists on activation of extracellular signal response kinases (ERKs) 1/2 and proliferation are strongly inhibited by wortmannin, which blocks PI3K (Wang et al., 2002).

There has been considerable interest in understanding the mechanisms by which PAR-1 couples to the MAP kinase cascades, given the important mitogenic role of thrombin. Several MAP kinase "signaling modules" have been characterized in mammalian cells (Widman et al., 1999) with a common organization: a MAP kinase kinase kinase (a serine-threonine kinase) phosphorylates and activates MAP kinase kinase (threonine-tyrosine kinase), which in turn phosphorylates MAP kinase (serine-threonine kinase). MAP kinases in turn regulate multiple substrates in the cytoplasm and nucleus. The MAPK ERK1/2 module plays a critical role in cell proliferation and differentiation.
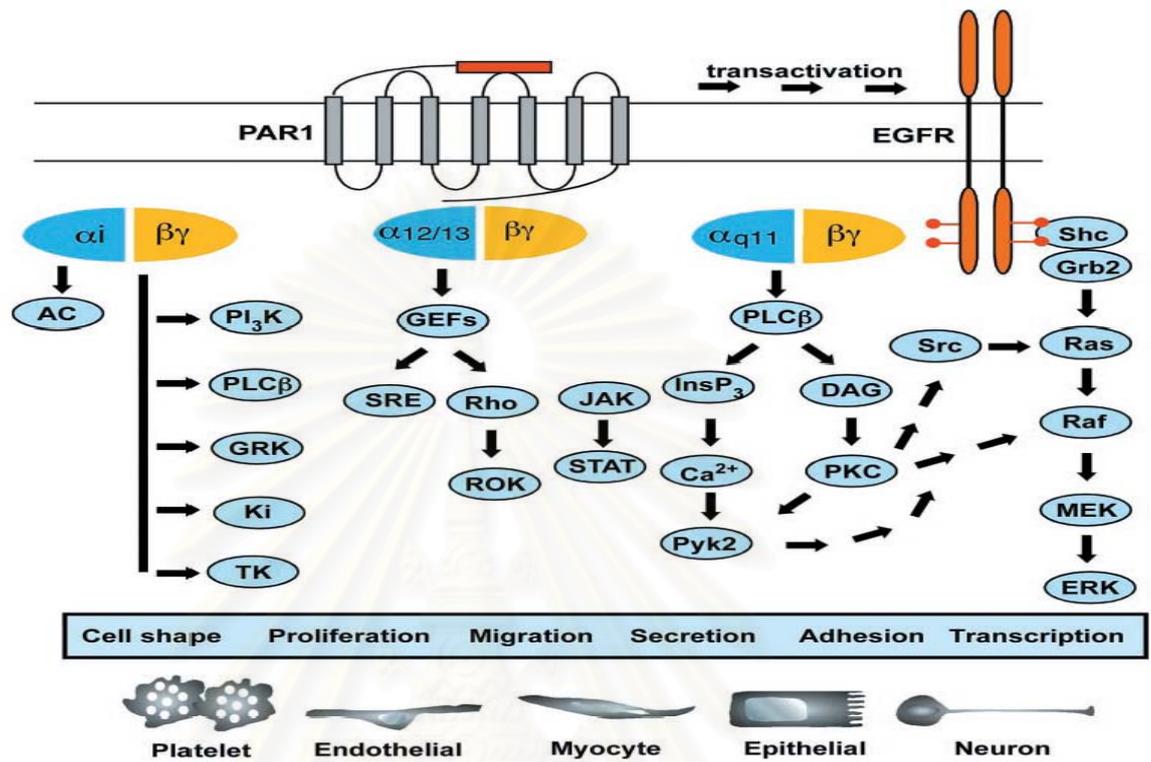
Figure 2.10 Summary of PAR-1 signal transduction. PAR-1 couples to Giα, G12/13α, and Gq11α. Giα inhibits adenylyl cyclase (AC) to reduce cAMP. G12/13α couples to guanine nucleotide exchange factors (GEF), resulting in activation of Rho, Rho-kinase (ROK), and serum response elements (SRE). Gq11α activates phospholipase Cβ (PLCβ) to generate inositol trisphosphate, which mobilizes Ca^{2+}, and diacylglycerol (DAG), which activates protein kinase C (PKC). PAR-1 can activate the mitogen-activated protein kinase cascade by transactivation of the EGF receptor, through activation of PKC, PI3K, Pyk2, and other mechanisms. Gβγ subunits couple PAR-1 to other pathways, such as activation of GRKs, potassium channels (Ki), and nonreceptor tyrosine kinases (RTK) (Coughlin, 2000).

Role of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) in osteoclastogenesis

Bone is a dynamic tissue that is permanently remodeled as an intrinsic mechanism to regenerate itself and as a means to integrate the various chemical, hormonal, and biomechanical external stimuli. At the cellular level, bone remodeling follows a sequence of repetitive cycles of bone resorption by osteoclasts and bone formation by osteoblasts. (Manalagas and Jilka, 1995; Roodman, 1996; Suda et al., 1997). Osteoblast-osteoclast contact, intercellular communication, and co-ordination are crucial for differentiation, activation, and function of both cell lineages in order to accomplish longitudinal growth, generate new bone during fracture repair, achieve peak bone mass, and preserve bone during life.

While a large number of hormones, growth and inflammatory factors, and cytokines are known to regulate osteoclast formation and function, significant new understanding of osteoclastogenesis has recently been made with the discovery that new members of the TNF family of ligands and receptors play a crucial role in osteoclast formation and activity and may be the molecular mediators of many of these other regulators.

The first component identified for a novel pathway regulating bone resorption and remodeling was OPG, which was discovered in mice via a genomics-based approach. The protein named "osteoprotegerin" based on its ability to protect bone. Independent of this effort, scientists in Japan isolated a protein secreted by cultured human fibroblasts that proved to be identical to OPG (Tsuda et al., 1997; Yasuda, Shima, Nakagawa, Mochizuki et al., 1998). This molecule was referred to as

osteoclastogenesis inhibitory factor (OCIF) based on its ability to suppress osteoclastogenesis *in vitro*. OPG/OCIF was shown soon thereafter to also suppress osteoclast activity (Hakeda et al., 1998; Yasuda, Shima, Nakagawa, Yamaguchi et al., 1998; Burgess et al., 1999), survival (Akatsu et al., 1998; Lacey et al., 2000), and adhesion to bone surfaces (O;Brien et al., 2000, 2001). The name OPG was officially adopted by the nomenclature committee of the American society of Bone and Mineral Research (ASBMR). The preferred nomenclature and synonyms for the OPG/RANK/RANKL pathway are described in Table 2. Structural domains of native OPG are described in Figure 2.11

OPG is an atypical member of the TNF receptor family in that it is a secreted protein with no transmembrane domain and no direct signaling properties (Simonet et al., 1997; Tsuda et al., 1997; Yasuda, Shima, Nakagawa, Mochizuki et al., 1998). Secretion of OPG is mediated by a 21-residue signal peptide. OPG has three major structural motifs including four cysteine-rich TNF-R domains, a heparin binding domain, and two death domain homologous (DDH) regions. The DDH regions within native OPG have yet to be associated with any functional role (Yamaguchi et al., 1998), although recent evidence suggests this domain contributes to the formation of homodimers. Unlike the intracellular DDH regions found in apoptosis inducing proteins such as Fas, the DDH regions in OPG reside in an extracellular environment because OPG is secreted. OPG contains four cysteine-rich TNF-R homologous domains that were necessary and sufficient for binding to its target RANKL and for osteoclast inhibition in cell culture studies. The heparin binding domain is also involved in homodimer formation, but deletion of this region did not reduce the ability of OPG to inhibit osteoclastogenesis *in vitro*

(Schneeweis et al., 2005). OPG exists as monomeric and homodimeric forms, and in one study these two forms were shown to have similar potency for inhibiting osteoclastogenesis *in vitro* (Tomoyasu et al., 1998). However, more recent analyses using analytical ultracentrifugation to more accurately identify monomer versus homodimer forms revealed that the OPG homodimer had 1000-fold greater affinity for RANKL than the monomeric form (Schneeweis et al., 2005). This result suggests that the dimeric form of OPG is probably a more potent RANKL inhibitor than the monomeric form. To enhance the pharmacologic activity of native OPG, numerous constructs have been created wherein the signal peptide, heparin binding domain and DDH domains were removed and the remaining peptide was fused to the Fc domain of human immunoglobulin IgG1 (eg. Fc-OPG (Morony et al., 1999; Bekker et al., 2001; Smith et al., 2003) (Figure 2.11). The Fc fusion partner maintains the potent dimeric nature of OPG while significantly increasing its circulating half-life. The great majority of published studies describing the use of OPG in animals have relied on these types of recombinant OPG fusion proteins, while a few early studies demonstrated that native OPG was also capable of suppressing bone resorption in mice. The major biological sources of OPG that modulate bone resorption are not well established. Osteoblasts or cells of that lineage have been shown to produce OPG (Hofbauner et al., 1998; Gori et al., 2000; Udawaga et al., 2000). The physical proximity of osteoblasts and osteoclasts during bone remodeling creates opportunities for osteoblast-derived regulation of bone resorption. However, the production of OPG by cultured osteoblasts was shown to increase with cell differentiation, suggesting that mature osteoblasts are not well suited to support osteoclastogenesis (Gori et al., 2000). OPG is also produced by endothelial cells (Collin-Osdoby et al.,

2001), vascular smooth muscle cells (Oleson et al., 2005) and other cells (Haynes et al., 2003). The high concentrations of OPG extracted from healthy and diseased human blood vessels suggest that vascular cells might be major contributors to the circulating pool of OPG. Circulating OPG would still have significant potential to inhibit bone resorption by suppressing the differentiation of circulating osteoclast precursors (Shalhoub et al., 1999). The ability of OPG to suppress bone resorption and increase bone mass is related to its ability to bind via its TNF-R domains to TNF domains within its natural ligand, now known as RANKL.

RANKL has a storied history in bone biology that precedes its positive identification by more than a decade. As early as 1980 it was appreciated by Chambers et al that cells of the osteoblastic lineage might be involved in promoting osteoclast recruitment and activity (Chambers, 1980). A more formal hypothesis, and experimental confirmation in various model systems further supported this notion, but the identity of this "osteoclast activating factor" remained elusive. The eventual identification of this protein involved the use of OPG as a probe to recover an OPG binding protein that was initially referred to as osteoprotegerin ligand (OPGL) (Lacey et al., 1998). An identical protein (osteoclast differentiation factor, ODF) was independently identified from a cDNA library prepared from ST-2 stromal 6 cells (Yasuda, Shima, Nakagawa, Mochizuki et al., 1998). This cell line had been previously shown to support osteoclast formation in a manner that could be completely inhibited by OPG (Tsuda et al., 1997). While the essential role of OPGL/ODF in osteoclast biology was an important finding, this protein proved to be identical to one previously identified and referred to as receptor activator of nuclear factor κB ligand (RANKL) (Anderson et al., 1997).
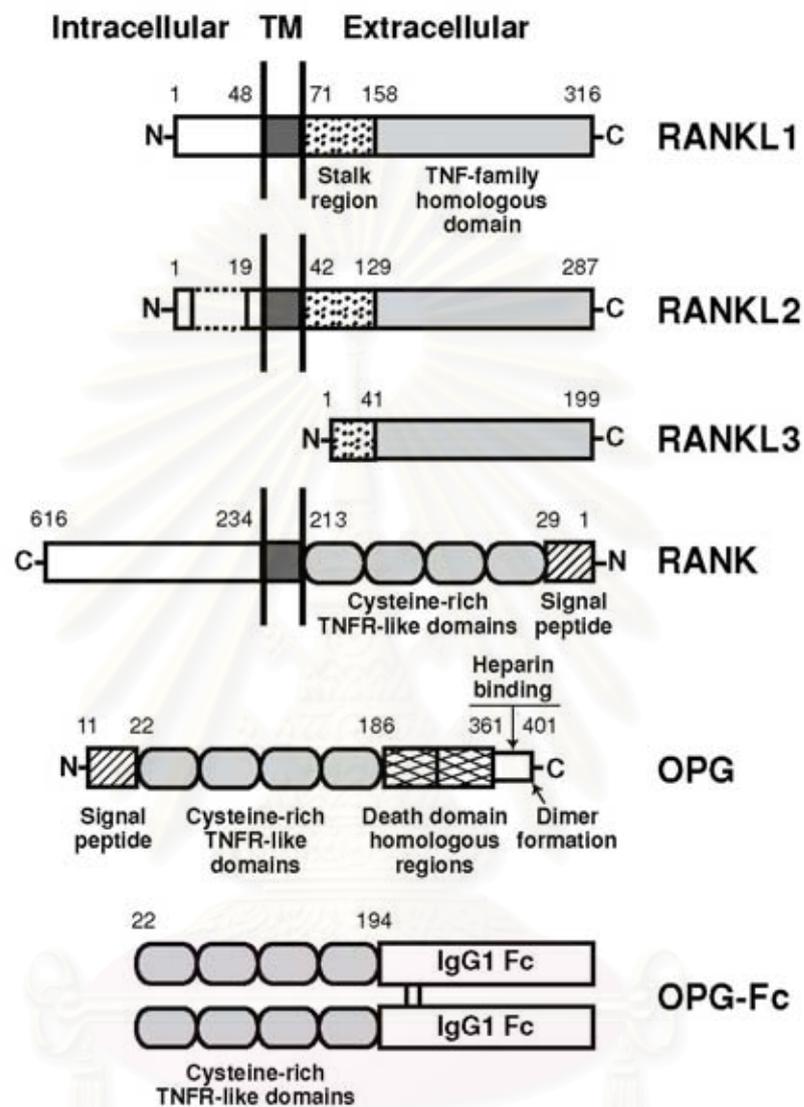
Figure 2.11 Protein structures of RANKL, RANK, OPG and OPG-Fc. **A-C:**
Three isoforms of RANKL are described, each of which possesses similar C-terminal
TNF-homology domains that are required for RANK activation. RANKL1 and
RANKL2 isoforms possess a transmembrane (TM) domain and a stalk region that
contains a proteolytic site (*arrow)* that allows for cleavage of RANKL from cell
surfaces. RANKL3 contains a truncated stalk region that possesses a proteolytic
cleavage site, the function of which is unclear because this isoform lacks a

transmembrane domain and is therefore secreted in the absence of proteolysis. **D-E:** RANK is a transmembrane protein with a large C-terminal cyotoplasmic domain and an amino-terminal extracellular domain. Both RANK and OPG contain aminoterminal signal peptides as well as cysteine-rich TNFR-like domains that bind to RANKL. OPG lacks a transmembrane domain, consistent with its secretion as a soluble protein. OPG also includes 2 death domain homologous (DDH) regions, the roles of which are unknown. A heparin binding domain at the carboxy-terminus of OPG limits the half-life and distribution of the molecule, and this domain is also involved in dimer formation. **F:** Human OPG-Fc is a recombinant fusion protein that has been used in human clinical trials and in the majority of preclinical animal studies. This construct includes amino acids 22-194 of native OPG, comprising the minimal TNFR-like domains that mediate RANKL inhibition. This fragment lacks the signal peptide, DDH regions and heparin binding domain of native OPG. The Fc fragment of human IgG1 was fused to the carboxy-terminus of this 22-194 fragment to maintain a dimeric molecule with a sustained circulating half-life. Numbers in figure represent amino acids. (Kearns et al., 2007)

Acronym	Synonym
Ligand	
RANKL	**RANK ligand**
OPGL	Osteoprotegerin ligand
ODF	Osteoclast differentiation factor
TRANCE	TNF-related activation-induced cytokine
SOFA	Stromal osteoclast–forming activity
TNFSF-11	TNF superfamily 11
Receptor	
RANK	**Receptor activator of NF-kB**
ODAR	Osteoclast differentiation and activation receptor
TNFRSF-11A	TNF superfamily receptor 11A
Decoy receptor	
OPG	**Osteoprotegerin**
OCIF	Osteoclastogenesis inhibitory factor
TR-1	TNF receptor-like molecule 1
FDCR-1	Follicular dendritic receptor 1
TNFRSF-11B	TNF superfamily receptor 11B

Table 2 Acronyms and synonyms for proteins from the OPG/RANK/RANKL pathway. Acronyms and synonyms in **bold** are ASBMR-preferred terms for bone biology applications. References can be found within original JBMR Manuscript. (Kearns et al, 2007)

RANKL, also referred to as tumor necrosis factor–related activation-induced cytokine (TRANCE) (Wong et al., 1997), was originally identified as a product of T cells. This TNF family member was able to promote dendritic cell survival and co-stimulation. The essential role of RANKL/TRANCE in bone resorption was confirmed soon thereafter. RANKL is the generally accepted acronym for this protein (Table 2). RANKL is a novel member of the TNF family of ligands. There are at least 3 forms of RANKL, two of which possess a transmembrane domain that positions the biologically active carboxy-terminus to the extracellular domain (ie. a type II transmembrane protein) (Figure 2.11). One of these forms, RANKL2, is a shorter alternative splicing variant of RANKL1. Both of these variants can remain on cell surfaces or can be proteolytically cleaved into soluble forms that possess osteoclast-stimulating activities within their TNF-homology domains (Ikeda et al., 2001). RANKL is produced by numerous cell types including cells of the osteoblast lineage (Bord et al., 2003; Nakamichi et al., 2007) and activated T-cells (Kong et al., 1999). T-cells express both soluble and membrane-bound forms of RANKL (Nakamichi et al., 2007), and both forms are implicated in focal bone erosions associated with inflammatory arthritis (Kong et al., 1999). Cells of the osteoblast lineage can express RANKL on their surface in a manner that facilitates osteoclastogenesis *in vitro* via cell-to-cell contact with osteoclast precursors. Differentiation of cultured osteoblasts was associated with reduced RANKL expression and decreased ability to support osteoclastogenesis (Deyama et al., 2000), suggesting that the mature bone forming osteoblast might not be capable of directing osteoclast activity via RANKL. Membrane RANKL has been suggested to be somewhat more potent than soluble RANKL in stimulating osteoclastogenesis *in vitro*

(Nakashima et al., 2000). However, soluble RANKL is measurable in the circulation and serum RANKL has been shown in some studies to increase with stimulated bone resorption (Ziolkowska et al., 2002; Nakamichi et al., 2007). Soluble recombinant RANKL is also capable of causing severe skeletal catabolism in mice and in rats (Yuan et al., 2007). These results indicate that soluble RANKL has the potential to be an important physiological and pathological mediator of bone resorption.

RANKL is involved in numerous aspects of osteoclast differentiation and function. RANKL was implicated in the fusion of osteoclast precursors into multinucleated cells, their differentiation into mature osteoclasts, their attachment to bone surfaces, their activation to resorb bone, and their continued survival by avoiding apoptosis. In most situations, RANKL probably relies on macrophage colony-stimulated factor (M-CSF, also known as CSF-1) as a co-factor for osteoclast differentiation (Itoh et al., 2000). However, it is interesting to note that unlike RANKL knockout mice, the osteoclast population recovers over time in mice lacking functional M-CSF (Begg et al., 1993). Recent preliminary evidence also suggests that RANKL can stimulate osteoclastogenesis and bone resorption in mice that lack functional M-CSF. These results suggest that RANKL plays a dominant role in the regulation of bone resorption, and no factor or combination of factors have been shown to be capable of restoring bone resorption when RANKL is absent.

The receptor that mediates all known activities for RANKL is called RANK (Figures 2.11). RANK is a homotrimeric TNF receptor family member that was initially discovered from a bone marrow-derived dendritic cell cDNA library. RANK was identified as a receptor that mediated the ability of RANKL to promote the survival of cultured dendritic cells (Anderson et al., 1997). An important role for

RANK in osteoclastogenesis was reported by Nakagawa and colleagues, who referred to this TNF receptor family member as osteoclast differentiation factor receptor (ODFR) (Nakagawa et al., 1998). The essential role for RANK in bone resorption was demonstrated soon thereafter by the high bone mass phenotype of RANK knockout mice, which were virtually devoid of osteoclasts (Dougall et al., 1998). RANK and RANKL knockout mice were virtual phenocopies of each other (Li et al., 2000), which indicated that RANK and RANKL had few if any roles beyond their mutual interactions.

The binding and activation of RANK involves direct interactions between the extracellular receptor binding domain of trimeric RANKL, and the extracellular cysteine-rich domains of trimeric RANK (Lam et al., 2001). This interaction is thought to cause oligomerization of RANK and the subsequent activation of several signal transduction pathways. A simplified overview of major signaling pathways is described in Figure 2.12. RANKL binding to RANK results in the recruitment of an adapter protein known as TNF ReceptorAssociated Factor-6 (TRAF-6) to specific sites within the intracellular domain of RANK. TRAF-6 acts a second messenger to activate various protein kinase pathways as well as transcription factors including NF-κB. Activated NF-κB translocates to the nucleus and upregulates the expression of c-fos, which then interacts with nuclear factor of activated T cells (NFAT)-c1 to induce the transcription of osteoclastogenic genes. This brief historical summary recaps a remarkable 3 year period in bone biology research (1997-1999), during which time the pieces to a long-sought picture of osteoclast biology were rapidly described in the scientific literature. The picture emerged from numerous independent laboratories around the world, using novel and distinct discovery

platforms and model systems. Most satisfying scientifically was the consistency of observations and conclusions between research groups regarding the functions and essential roles of OPG, RANK and RANKL. The consensus regarding this molecular triad is that OPG functions as a soluble decoy receptor by binding to RANKL, thereby preventing RANKL from binding and activating RANK (Figure 2.13). OPG inhibition of RANKL leads to the rapid arrest of osteoclast formation, attachment to bone, activation, and survival.
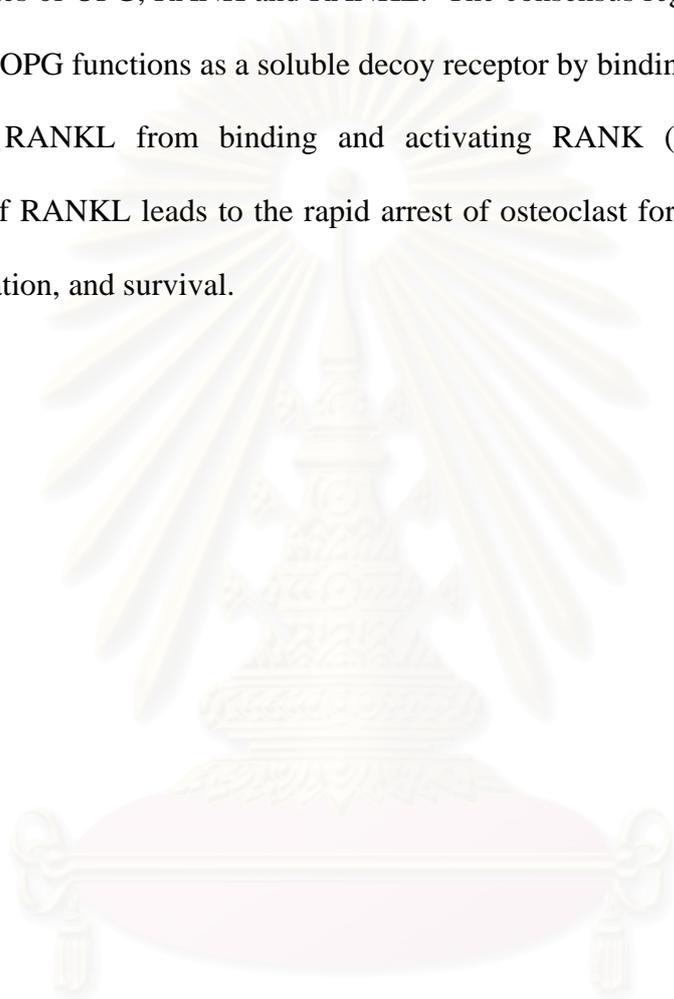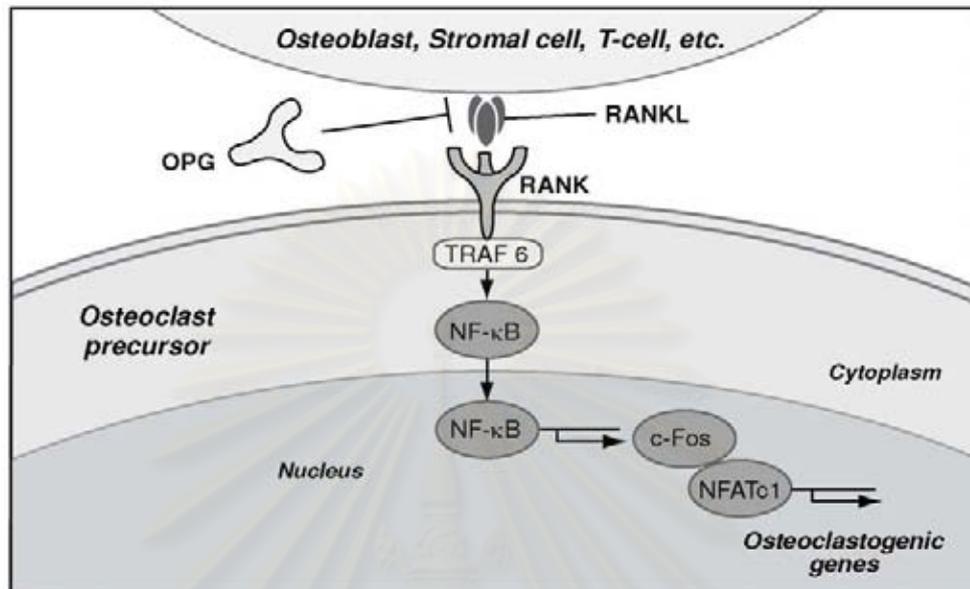
Figure 2.12 Essential signaling pathways activated by RANKL interactions with RANK. RANKL, which is produced by osteoblasts, stromal cells, T cells and other sources, activates RANK on the surface of osteoclasts and osteoclast precursors. RANK activation leads to the recruitment of the adaptor protein TRAF-6, leading to NF-κB activation and translocation of NF-κB to the nucleus. NF-κB increases c-Fos expression and c-Fos interacts with NFAT-c1 to trigger the transcription of osteoclastogenic genes. Activation of these pathways is prevented naturally by OPG, which prevents RANKL from activating RANK in the extracellular environment. RANKL-RANK interactions are also prevented by RANK-Fc (a truncated form of RANK that acts as a non-signaling decoy receptor) and by denosumab, a fully human monoclonal antibody that binds and inhibits RANKL. NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TRAF, tumor necrosis factor receptor associated factor. (Kearns et al., 2007)
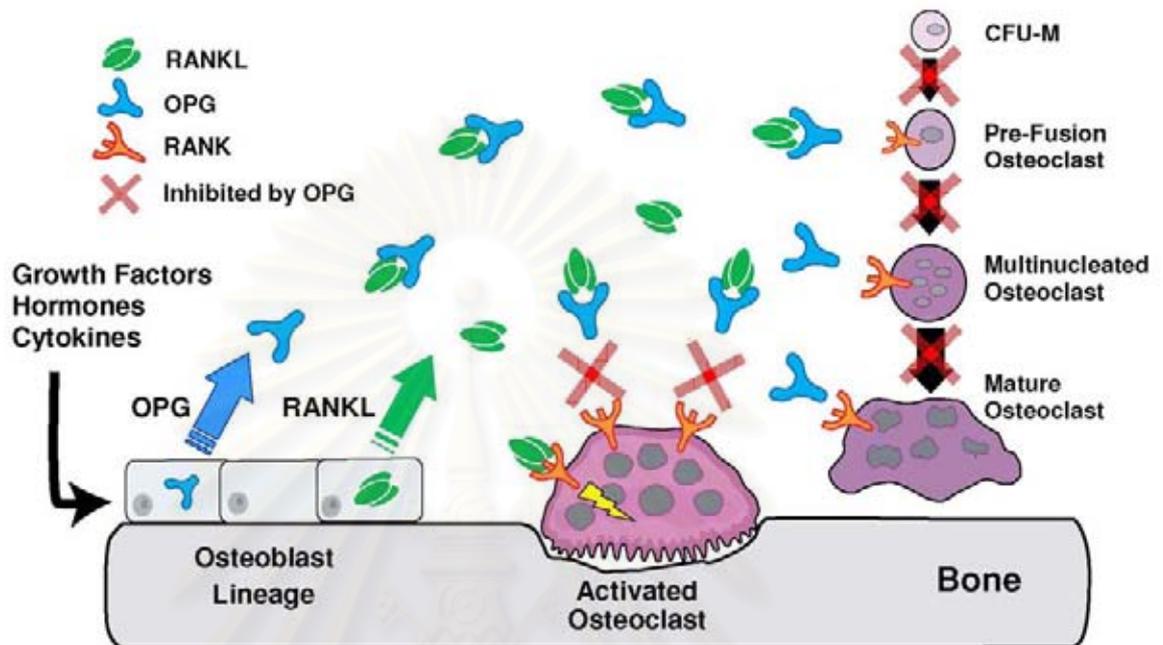
Figure 2.13 Mechanisms of action for OPG, RANKL and RANK. RANKL is produced by osteoblasts, bone marrow stromal cells, and other cells under the control of various proresorptive growth factors, hormones and cytokines. Osteoblasts and stromal cells produce OPG, which binds to and thereby inactivates RANKL. The major binding complex is likely to be a single OPG homodimer interacting with high affinity with a single RANKL homotrimer. In the absence of OPG, RANKL activates its receptor RANK, found on osteoclasts and pre-osteoclast precursors. RANK/RANKL interactions lead to pre-osteoclast recruitment, fusion into multinucleated osteoclasts, osteoclast activation and osteoclast survival. Each of these RANK-mediated responses can be fully inhibited by OPG. (Kearns et al., 2007)

Expression patterns and hormone/cytokine regulation of expression

With some notable exceptions, the expression patterns of OPG, RANK and RANKL genes would not obviously predict their biological roles. Expression of each gene product has been shown in numerous cells and tissues in which no known role for that gene product has been ascribed. OPG mRNA, for example, is highly expressed in the lung, heart, kidney and placenta (Simonet et al., 1997) and OPG has yet to demonstrate any physiological or pathological role in those organs. OPG mRNA is also expressed in liver, stomach, intestine, skin, bone, and most other human tissues (Yasuda, Shima, Nakagawa, Yamaguchi et al., 1998) with the exceptions of brain, skeletal muscle, thymocytes or peripheral blood lymphocytes (Yun et al., 1998). At the cell culture level, OPG is expressed by bone marrow stromal cells (Gori et al., 2000), fibroblasts (Yasuda, Shima, Nakagawa, Mochizuki et al., 1998), endothelial cells (Hsu et al., 1999), lymphoid cells (Yun et al., 1998), smooth muscle cells (Yun et al., 1998), and other cell types. RANK mRNA is expressed in bone, bone marrow, spleen, skeletal muscle, brain, heart, liver, lung, mammary tissue, and skin (Hsu et al., 1999; Fata et al., 2000) and at the cellular level by osteoclasts, osteoclast precursors, dendritic cells, chondrocytes, endothelial cells, fibroblasts and macrophages, and other cells (Nakagawa et al., 1998; Hsu et al., 1999; Min et al., 2003). RANKL is highly expressed in peripheral lymph nodes and bone, and also in spleen, thymus, Peyer's patches, intestine, brain, heart, skin, skeletal muscle, kidney, liver, lung, and in mammary tissue of pregnant mice (Lacey et al., 1998; Yasuda, Shima, Nakagawa, Mochizuki et al., 1998; Kartsogiannis et al., 1999; Fata et al., 2000). Cells that express RANKL include osteoblasts, bone marrow

stromal cells, activated T cells, B cells, fibroblasts, endothelial cells, chondrocytes, mammary epithelial cells (Wong et al., 1997; Yasuda, Shima, Nakagawa, Mochizuki et al., 1998; Collin-Osdoby et al., 2001; Hofbauer et al., 2001; Eghbali-Fatourechi et al., 2003). Numerous growth factors, hormones, cytokines and drugs have been shown to influence the expression of OPG and RANKL, and an excellent summary of these regulatory responses was described recently (Table 3). RANK tends to be minimally regulated, which is not unexpected because the modulation of OPG and/or RANKL levels is probably sufficient to control RANK's signaling output. Numerous molecules that stimulate bone resorption have been shown to upregulate RANKL expression and/or to downregulate OPG expression. Reciprocal regulation of RANKL and OPG is often observed, a phenomenon that might serve to amplify pro-resorptive signals. Evidence in numerous disease models and settings suggests that the ratio of RANKL:OPG represents an important determinant of bone resorption (Lee et al., 1999; Fazzalari et al., 2001 Ma et al., 2001; Morabito et al., 2004).

	OPG	RANKL	RANK
1,25-dihydroxyvitamin D	↑↓	↑	↑
Hormones			
Estrogen	↑	↓/—	
Testosterone	↑↓	—	
Glucocorticoid	↓	↑	
Parathyroid hormone	↓	↑	
Parathyroid hormone-related peptide	↓	↑	
Cytokines			
Interleukin-1	↑↓	↑	↑
Interleukin -4			↓/—
Interleukin -7		↑	
Interleukin -13	↑	↓	—
Interleukin -17	↓	↑	—
Tumor necrosis factor α		↑	
Interferon γ	↑	↑	↑
Prostaglandin E2	↓	↑	
Growth Factors			
Transforming growth factor β	↑	↑↓	—
Bone morphogenetic protein 2	↑		—

↑, increased expression; ↓, decreased expression; —, no change observed

Table 3 Regulation of OPG, RANKL and RANK expression (Kearns et al., 2007)

The mammalian target of rapamycin (mTOR)

mTOR is a serine/threonine kinase that controls many aspects of cellular physiology, including transcription, translation, cell size, cytoskeletal organization and autophagy (Hay and Sorrenberg, 2004; Beevers et al., 2006). Recent advances in the mTOR signaling field have found that mTOR exists in two heteromeric complexes, mTORC1 and mTORC2 (Figure 2.15). mTORC1 is composed of mTOR, regulatory associated protein of mTOR (Raptor), and mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) (Kim et al, 2002, 2003). This complex possesses the classic features of mTOR by functioning as a nutrient/energy/redox sensor and controlling protein synthesis (Hay and Sorrenberg, 2004). The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine) and oxidative stress (Fang et al., 2001). mTORC1 is inhibited by low nutrient levels, growth factor deprivation, reductive stress, caffeine, rapamycin and curcumin (Kim et al., 2002; Beevers et al., 2006). The two best characterized targets of mTORC1 are p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) (Hay and Sorrenberg, 2004). mTORC2 is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) (Sarbassov et al., 2004; Frias et al., 2006). mTORC2 has been shown to function as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, Rho A, Rac1, Cdc42 and protein kinase C α (PKCα) (Sarbassov et al., 2004). mTORC2 appears to be regulated by insulin, growth factors, serum and nutrient levels. Originally, mTORC2 was identified as a

rapamycin-insensitive entity, as acute exposure to rapamycin did not affect mTORC2 activity or Akt phosphorylation (Sarbassov et al., 2005).

Undoubtedly the best described function of mammalian TOR is its regulation of translation, and one mechanism by which mTOR regulates translation is by directly phosphorylating the key translation regulators p70 S6K1 and 4E-BP1 (Figure 2.14). Under basal conditions, S6K1 and 4E-BP1 are bound to a scaffolding protein, the eukaryotic initiation factor3 (eIF3) translation initiation complex; but upon stimulation, mTOR phosphorylates and activates S6K1 and 4E-BP1, causing their dissociation from eIF3 (Holz et al., 2005). The phosphorylation of S6K1 on T389 and 4E-BP1on T37 and T41 are often used as functional readouts of mTOR activity, since these sites are specifically phosphorylated by mTOR both *in vitro* and *in vivo* and are inhibited by rapamycin treatment (Brown et al., 1994; Brunn et al., 1997). After activation by mTOR, S6K1 phosphorylates the ribosomal protein S6, leading to an increase in translation of a subset of mRNAs. Originally it was thought that S6K1 enhanced the translation of mRNAs that contained specific, repetitive 50-terminal oligopolypyrimidine (5'TOP) tracts; but more recently it has been suggested that mTOR regulates 5'TOP mRNAs independently of S6K1 (Stolovich et al., 2002). mTOR also phosphorylates 4E-BP1, and in doing so it inhibits 4E-BP1 function. The net result of this action is to increase the translation of mRNAs with 7-methyl guanine caps, as 4E-BP1 is an inhibitor of the cap dependent initiation factor eIF4E. It is especially significant that mTOR leads to enhancement of translation through S6K1, 4E-BP1 and other molecules, since it has been postulated that mTOR may serve as a nexus that controls up to 15% of the bulk translation of the cell (Jefferies et al., 1994). Moreover, translation regulation – especially through eIF4E –directly increases the

size (and hence the growth) of cells via regulation of key growth promoting proteins such as c-Myc and cyclin D1 (Mamane et al., 2004).

Although the regulation of transcription by major cellular signaling pathways has been extensively studied owing to the widespread use of microarray analysis, the study of the regulation of translation is far behind; and the relative contribution of translation versus transcription in modulating cellular phenotypesis only beginning to be studied. Since mTOR serves as an integration point that relays numerous upstream signals to the translation apparatus, it is essential to appreciate how it is regulated.
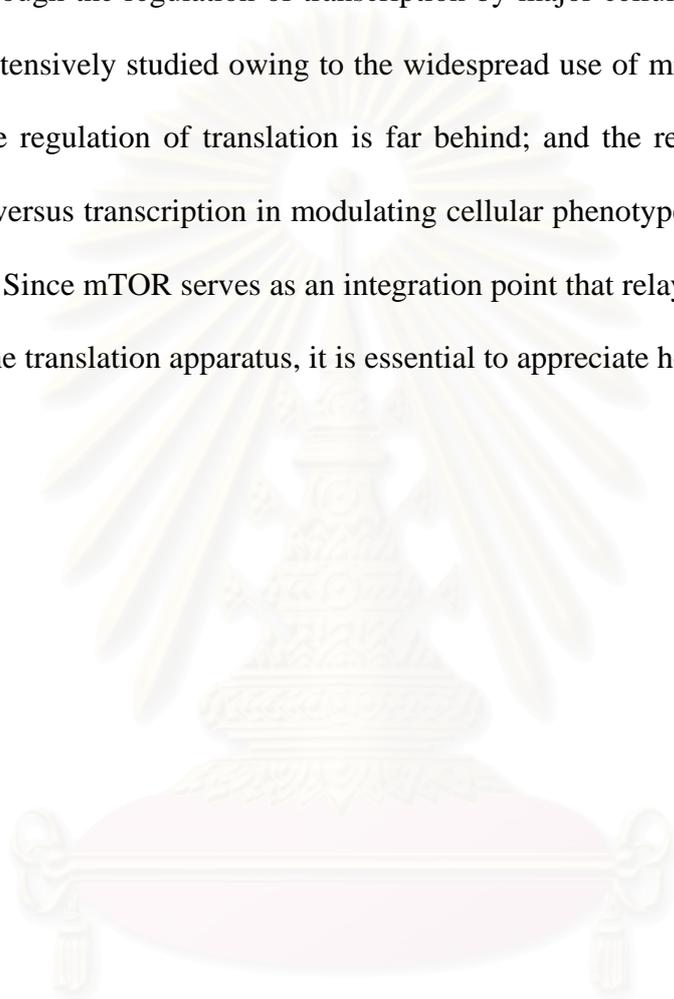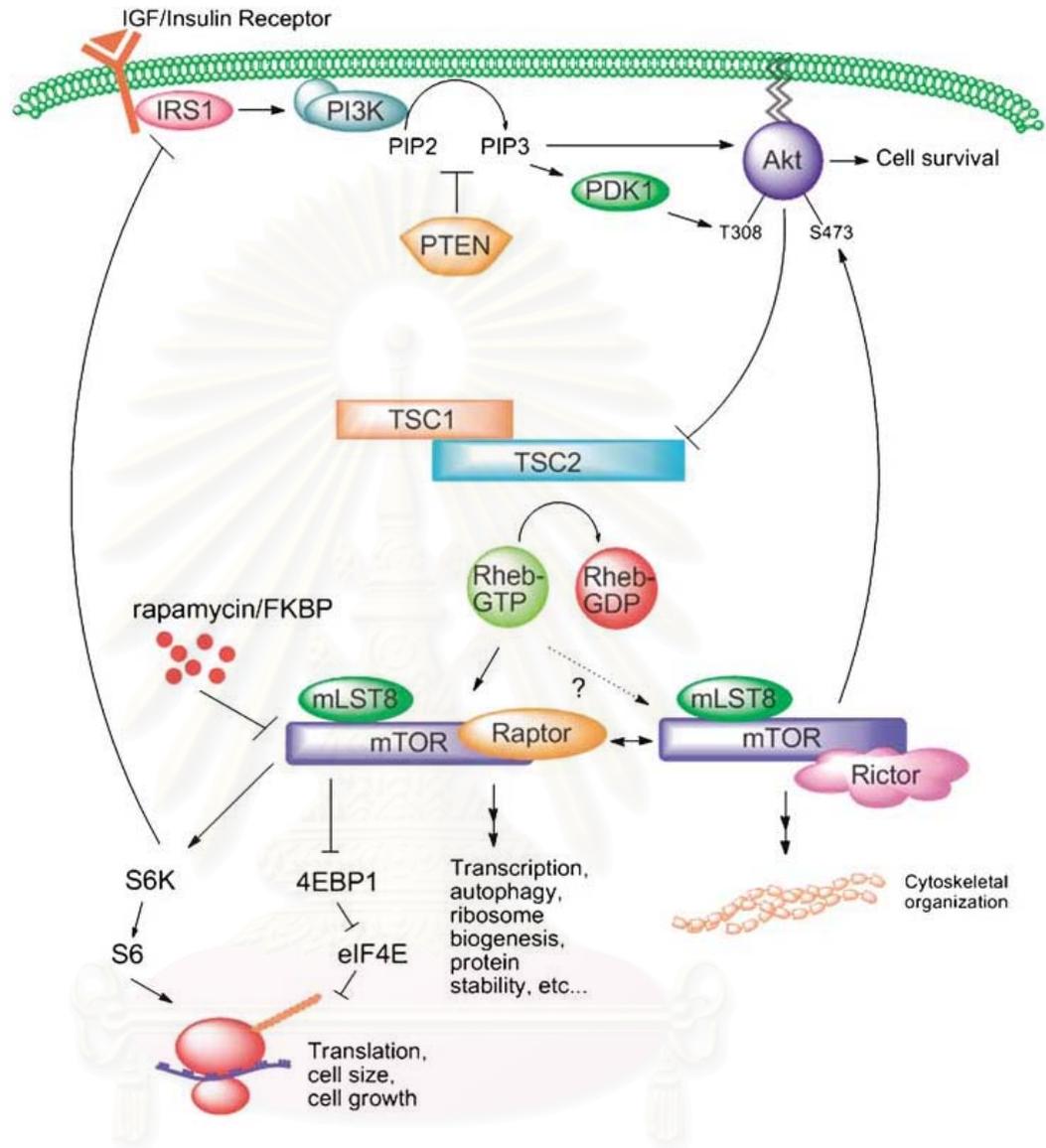
Figure 2.14 A model illustrating how mTOR fits within the insulin/Akt signaling network. Insulin or insulin-like growth factor initiates signaling at the insulin receptor, leading to activation of IRS1 and PI3K. The lipid products of PI3K, PIP3 (i.e. phosphatidylinositol 3,4,5 trisphosphate and phosphatidylinositol 3,4 bisphosphate), recruit both PDK1 and Akt to the plasma membrane. Akt is subsequently phosphorylated on T308 by PDK1 and by mTORC2 (consisting of mTOR, mLST8 and Rictor) on S473, leading to full activation. Akt then

phosphorylates numerous targets to promote growth and survival, including the TSC1/2 complex. By phosphorylating TSC2, Akt inactivates TSC2s GAP activity for the small G protein Rheb. GTP-bound Rheb is a potent activator of the rapamycin-sensitive complex mTORC1 (consisting of mTOR, mLST8 and Raptor), but it is unclear whether Rheb plays a role in regulating mTORC2. mTORC1 phosphorylates several targets, including the translation control proteins S6K1 and 4E-BP1. By phosphorylating and activating S6K1, mTORC1 initiates a negative feedback loop to modulate its own activity, via S6K-mediated reduction in the activation of PI3K (Corradetti and Guan, 2006).
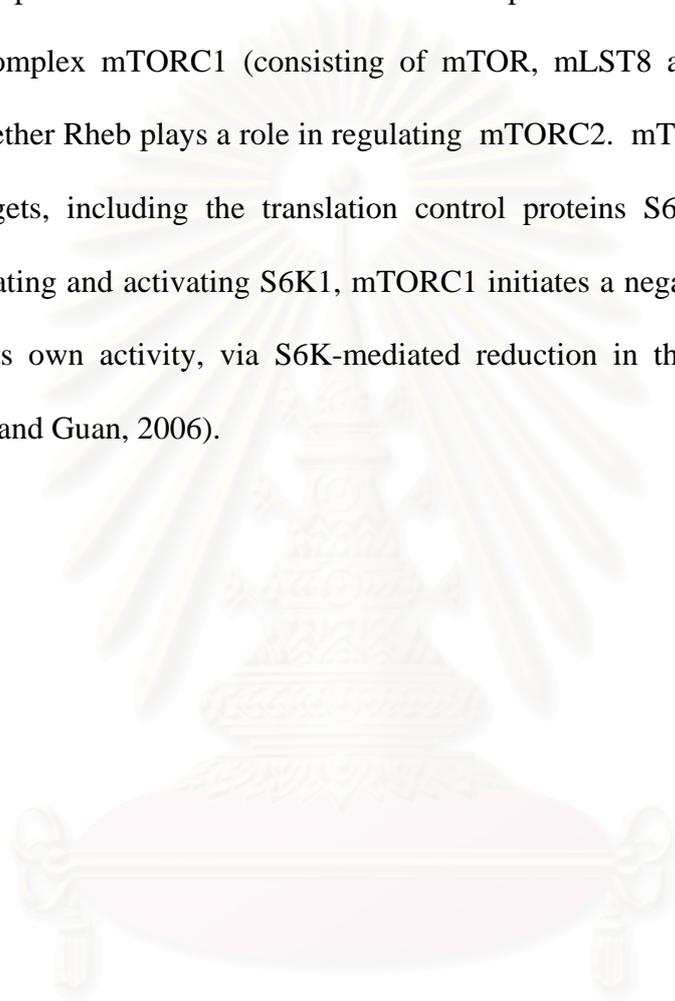
Thrombin, OPG and RANKL in HPDL cells

Thrombin has been shown to stimulate the proliferation of many cells including endothelial cells, smooth muscle cells, and fibroblasts (Berk et al., 1991; Dawes et al., 1993; Herbert et al., 1994; Chang et al., 1998). For mineral tissue, thrombin is able to influence the biological behavior of osteoblasts and plays multiple roles in the early stages of bone healing (Pagel et al., 2006). It has been shown to stimulate proliferation, migration, prostaglandin release and calcium mobilization in osteoblast-like cell lines or primary culture of osteoblasts (Lerner et al., 1989; Tatakis et al., 1989; Abraham and MacKie, 1999). These results suggest a role of thrombin in bone repair. However, few studies have directly investigated the effects of thrombin on the behavior of dental cells such as HPDL cells. The study of Chan et al. (1998) demonstrated that thrombin promotes growth of HPDL cells in a dose dependent manner, suggesting that this molecule may play a role in the homeostasis of periodontal tissue.

Homeostasis of periodontium also requires balance of RANKL and OPG, two major molecules that play the important roles in osteoclastogenesis. It was shown that HPDL cells express RANKL and OPG mRNA *in vitro* (Kanzaki et al., 2001). Changes in the level of RANKL and OPG expression in HPDL cells have been reported in periodontal disease and orthodontic tooth movement indicating the significant of PDL cells in these phenomenons.

Taken together, these data suggest that thrombin and PARs play an important role in tissue repair processes including in periodontal tissue repair. Although, several studies have also shown that HPDL cells express OPG and RANKL, two major

molecules that required for balance of periodontal tissue. The relation between thrombin and OPG-RANKL are not yet elucidated. This is an objective of my study to examine the effect of thrombin on HPDL cells. Since thrombin could affect the osteoclast activity, it is tempting to hypothesize that thrombin could affect the expression of RANKL and OPG expression. The study will also include the participation of PAR-1 in the thrombin-induced HPDL cells.
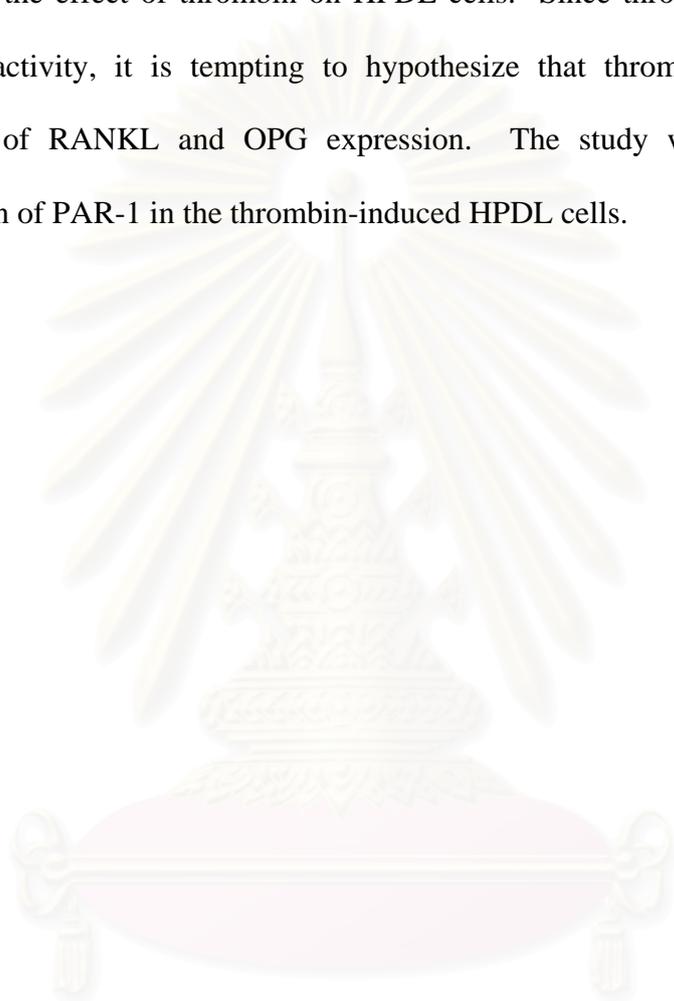
CHAPTER III

RESEARCH METHODOLOGY

1. Cell cultures

HPDL cells were obtained from healthy periodontal ligament tissue of non-carious, freshly extracted third molars, or tooth removed for orthodontic reasons at Faculty of Dentistry, Chulalongkorn University. All patients gave informed consent. Triplicate from 3 different people were done. All teeth were stored in Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, immediately after extraction. Medium and supplements were from Gibco BRL (Grand Island, NY, USA). The explant was from the middle third of the root and the HPDL tissue obtained. HPDL tissues were minces into 1 mm x 1 mm x 1 mm small pieces by surgical blade and placed on the 35 mm culture dish (Corning, New York, USA) and grown in DMEM supplemented with 10% fetal bovine serum. HPDL tissue cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. Tissue explants were maintained in DMEM supplemented with 10% fetal bovine serum. When HPDL cells migrated from the explants had become confluent, they were detached with 0.25% trypsin-EDTA and subcultured at a ratio of 1:3. The cell passages numbers from 3 to 6 were used in this study.

2.   Treatment of cells and inhibition

HPDL cells were seeded in six-well plates, at density of 200,000 cells per well. After deprivation of serum for 16 h, cells were stimulated with 0.001, 0.01 and 0.1 U/ml of thrombin (Sigma-Aldrich Chemical, St Louis, MO, USA). After 24 h, the RNA was extracted for reverse transcription polymerase chain reaction (RT-PCR) analysis. Cell protein extracts and the conditioned medium were collected from the parallel set of cultures for Western blot analysis after 48 h of treatment. The effective dose was selected and used for the rest of the experiments. To examine if the effect induced by thrombin was similar to that mediated through PAR-1, cultures were exposed to PAR-1 agonist peptide, SFLLRN (Peptides International Inc., KY, USA), which mimics the first six amino acids of the new amino terminus unmasked by receptor cleavage, functions as an agonist for PAR-1 and activates the receptor independently of thrombin and proteolysis. Media was collected for analysis.

The inhibitors used were indomethacin (10 µM, Sigma), actinomycin D (0.5 µg/ml, Calbiochem, EMD Biosciences, CA, USA), cycloheximide (1 µg/ml, Sigma), PI3K inhibitor, LY294002 (1.4 µM, Cayman Chemical, MI, USA), rapamycin (100 pM, Calbiochem) and PAR-1 antagonist, SCH 79797 dihydrochloride (100 nM, Tocris Bioscience, Bristol, UK). Cells were treated with each kind of inhibitors for 30 minutes prior to the addition of 0.1 U/ml of thrombin. Cell protein extraction and collection of medium were performed for further analyses. To examine the signaling molecules affected by thrombin, another set of cultures were treated with thrombin, with or without pre-incubated with the inhibitor, and the cell protein extracts were collected for Western blot analysis to detect active/phosphorylated Akt and total Akt.

3. Toxicity test (MTT assay)\

HPDL cells were plated in triplicate into 24 well plates, at initial density of 50,000 cell/well in DMEM with supplemented overnight. Culture medium were changed to serum free media containing various concentration of thrombin range from 0.001-1 U/ml and PAR-1-agonist peptide range from 10, 50 100 nM and culture for another 24 hours. Cell numbers under all conditions were measured by MTT assay method. Briefly, after remove cells from CO_2 incubator, HPDL cells were washed with PBS for remove all condition media. The medium was changed in DMEM without phenol red and 0.5 mg/ml of MTT solution was added into each well during the final 3 hour of incubation. The MTT solution can be reduced to insoluble formazan by mitochondrial dehydrogenase of variable cells. Finally the medium was removed. The insoluble formazan produced was lyzed with dimethyl sulfoxide (DMSO) and glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10), in 900 µl and 125 µl respectively, and measure the quantity of cells, using the Genesis 10 UV-vis spectrophotometer at wavelength 570 nm (Thermo Spectronic, Roche, NY, USA). The viable cell number was calculated from the standard curve of cell number by plotting a scattergram of the absorbance value against the known number of cells.

4. RNA isolation

After the treatment, total cellular RNA was extracted with TRI Reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to manufacturer's instructions. Briefly, 1 ml. of TRI reagent was added to each well, scraped the cell

layer and incubate for 5 minutes and then transfer TRI reagent into 1.5 ml. eppendorf, 200 µl of chloroform was added and shaken vigorously. The mixture was then centrifuged at 10,000 rpm for 15 minutes at 4 °C.

After centrifugation, the mixture was separated into the lower red of the phenol-chloroform phase, the white plaque of the interphase, and the upper colorless of the aqueous phase. RNA remained exclusively in the aqueous phase, whereas DNA and protein were in the interphase and phenol-chloroform phase. The aqueous phase was collected and added isopropanol in equal volume to precipitate RNA, incubation at room temperature for 10 minutes and centrifugation at 14,000 rpm for 15 minutes at 4°C. After centrifugation, precipitated RNA formed a gel-like or white pellet on the bottom of the tube. The supernatant was removed and RNA pellet was washed with 1 ml of 75% ethanol and then centrifugation at 7,500 rpm for 5 minutes at 4 °C.

RNA pellet was briefly air-dried and dissolved in nuclease free water and the amount of RNA was determined by measuring the optical density at 260 nm. using a Genesis10 UV-vis spectrophotometer (Thermo Spectronic). The quality of the RNA can be evaluated from the ratio of the absorbance at 260/280 nm being greater than 1.8.

5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

One µg of each RNA sample was converted to complementary DNA (cDNA) by a reverse transcription using an AMV (Avian myeloblastosis virus) reverse transcriptase (Promega, Madison, WI, USA) for 90 minutes at 42°C and then

incubated at 99 °C for 2 minutes. Subsequent to the reverse transcription, a polymerase-chain reaction was performed. The primers were prepared following the reported sequences from GenBank. Glyceraldehyde 3 phospate dehydrogenase (GADPH) was used as a loading control.

The oligonucleotide sequences of the primers were as follow:

RANKL sense 5'-GCCAGCTAGAAAACCACCAA-3' and antisense 5'-TGGATTTGCTTCCAGGCTCA-3' (PCR product, 603 bp); OPG sense 5'-TCAAGCAGGAGTGCAATCG-3' and antisense 5'-AGAATGCCTCCTCACACAGG-3' (PCR product, 342 bp) ; PAR-1 sense 5'-TCAACCGGAGTGTTTGTAGT-3' and antisense 5'-TTGAGGACGAGAGCAACTAC-3' (PCR product, 395 bp); PAR-2 sense 5'-GGTAAGGTTGATGGCACATC-3' and antisense 5'-TGGTCTGCTTCACGACATAC-3' (PCR product, 509 bp); PAR-3 sense 5'-GAAAGCCCTCATCTTTGCAG-3'and antisense 5'-AGGTGAAAGGATGGACGATG-3' (PCR product, 598 bp); PAR-4 sense 5'-TCTATGGTGCCTACGTGCCCA-3' and antisense 5'-TTATGAGGACACCACCCACACTG-3' (PCR product, 381 bp); GAPDH sense 5'-TGAAGGTCGGAGTCAACGGAT-3' and antisense 5'-TCACACCCATGACGAACATGG-3' (PCR product, 376 bp).

The PCR was performed using Tag polymerase (Qiagen, Hilden, Germany) with a PCR volume of 25 μl. The mixtures contained 25 pmol of primers and 1μl of RT product. The PCR working conditions were set initially denaturation at 95°C for 5 minutes, followed by denaturation at 94°C for 30 seconds, primer annealing at 60°C for 45 seconds, and chain elongation at 72°C for 90 seconds. Last cycle was followed by additional 10 minutes extension incubation at 72°C. The PCR was performed on a DNA thermal cycler (Tpersonal, Whatman Biometra, Goettingen, Germany). The

amplified DNA was then electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide fluorostaining. An intensity of each band was determined by Scion Image analysis software (Scion Corporation, Frederick, Maryland, USA).

6. Western blot analysis

The amount of OPG was determined from the culture medium. The medium was collected and lyophilized to concentrate. The lyophilized was dissolved in sample buffer, boiled and subject to SDS-PAGE. To detect RANKL, cell protein extracts were prepared on ice using radioimmunoprecipitation (RIPA) buffer (50 mM Tris/HCL, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate) containing cocktail protease inhibitors (Sigma); 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4. To detect phospho-Akt or total Akt, cells were lysed in PhosphoSafe extraction reagent (EMD Biosciences, Madison, WI, USA) according to the manufacturer's instruction.

The protein extraction was assayed from the cells by following procedures. The conditioned medium was aspirated from HPDL cells and cells were rinsed twice with phosphate buffered saline (PBS). Then, 80 µl of RIPA buffer or PhosphoSafe extraction reagent was added in each well of 6-well plate and incubated for 5 minutes at room temperature. Extract was transferred to 1.5 ml eppendorf and centrifuge at 14,000 rpm for 10 minutes at 4°C to pellet cellular debris. Supernatant was transferred to a fresh tube and stored at -80°C. The total amount of proteins was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein from each sample were boiled and subjected to sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition, along with prestained high molecular weight standards (Bio-Rad, Hercules, CA, USA). The proteins were then transferred onto the nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA). The membrane was incubated in blocking buffer (5% non-fat dry milk, 0.1% Tween 20 in deionized water) at room temperature for 1 hour. Then, the membrane was stained overnight with primary antibody for RANKL (R&D System, Inc, Minneapolis, MN , USA), OPG (R&D System), β-actin (Chemicon international, Temecula, CA), phospho-Akt and total Akt (Cell Signaling Technology, Inc., Denvers, MA, USA) at the dilution of 1:1000 in blocking buffer at 4°C. After extensively washed with PBS, the membrane was incubated with biotinylated-secondary antibody (Sigma) for 30 minutes at room temperature, and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 30 minutes, respectively. The protein bands were detected using a commercial chemiluminescence system (Pierce) and were exposed on CL-X Posture film (Pierce). The band intensity was determined by Scion Image analysis software (Scion). β-Actin served as internal control for normalization purpose.

7. Data analysis

All experiments were repeated at minimum of three times. All data are expressed as mean±SD. The data presented in some figures were from a representative experiment, which was qualitatively similar to the replicated experiments, statistical differences between groups were determined using one-way

analysis of variance (SPSS version 11), and Scheffe's test was used for post-hoc analysis. P value of less than 0.05 was considered statistically significance.

CHAPTER IV

RESULTS

Thrombin induces the expression of OPG and RANKL

HPDL cells were cultured for 24 hours with various concentration of thrombin ranging from 0.001-1 U/ml and the cytotoxicity of thrombin was determined by using MTT assay. As shown in Figure 4.1, thrombin ranging from 0.001-1 U/ml was not toxic to HPDL cells. However, we found that thrombin at 1 U/ml acted as mitogen on HPDL cells.

The effective dose of thrombin on OPG and RANKL expression was determined. HPDL cells were stimulated with 0.001-0.1 U/ml of thrombin. After the treatment, the RNA or protein extracts were collected along with the conditioned medium as described in research methodology. The results demonstrated that thrombin increased the mRNA expression of RANKL, but not that of OPG, dose dependently (Figure 4.2). The relative density compared with control was showed in Figure 4.2 B. We found that the protein expression of both RANKL and OPG increased (Figure 4.2 C). It is noticeable that the protein level of OPG was not concurred with that of the mRNA. The level of OPG increased dose dependently and was pronounced at the concentration of 0.1 U/ml. The ratio of OPG/RANKL intensity was calculated and the result demonstrated that OPG/RANKL ratio significantly increased over the control as shown in Figure 4.2 D. The concentration of 0.1 U/ml was then selected for the rest of the experiments.

To examine whether thrombin differently regulated RANKL and OPG expression, indomethacin, a non-specific cyclo-oxygenase (COX) inhibitor, was applied to the cultures 30 minutes before addition of thrombin. The result demonstrated that indomethacin was capable of blocking thrombin-induced expression of RANKL but not that of OPG (Figur 4.3). The result indicated that regulation of RANKL and OPG by thrombin was mediated through different pathways. Regulation of RANKL via COX and PGE_2 has been established, we thus focus only on the regulation of OPG synthesis.

To determine if the effect of thrombin on OPG synthesis was post-transcriptional, actinomycin D and cycloheximide, inhibitors of mRNA transcription and protein biosynthesis, were used, respectively. HPDL cells were exposed to thrombin in the absence or presence of either actinomycin D or cycloheximide for 24 hours. We found that the increased OPG induced by thrombin was abolished by cycloheximide (Figure 4.4), suggesting the post transcriptional regulation.
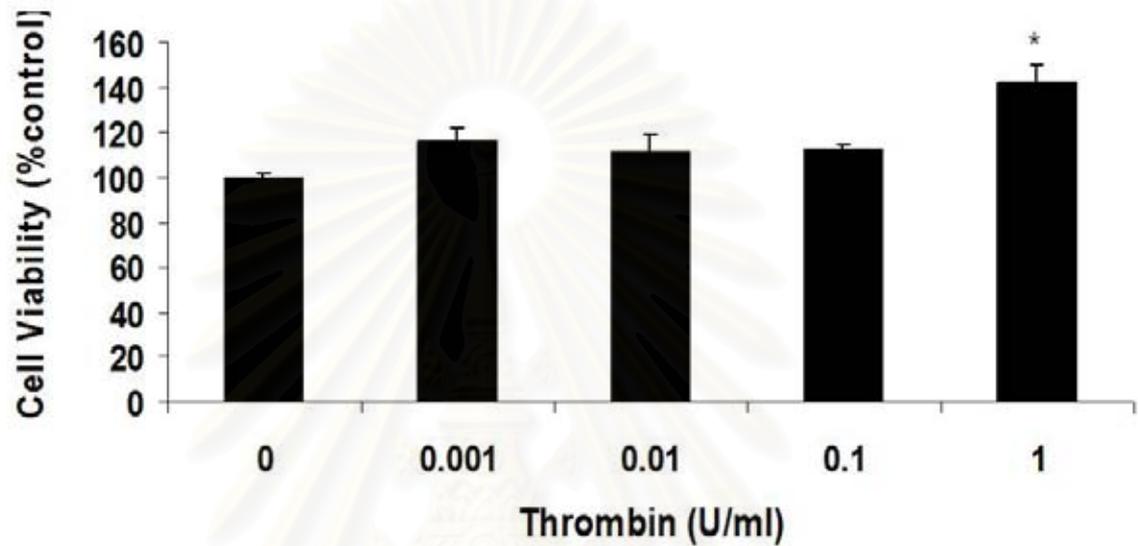
Figure 4.1 The cytotoxicity of thrombin on HPDL cells. HPDL cells were treated with various concentration of thrombin (0.001-1 U/ml) for 24 hours. Cell viability was determined by using MTT assay. No significant change of cell number was observed when cells were treated with 0.001, 0.01 and 0.1 U/ml of thrombin. Nevertheless, HPDL cells number were significantly increased when treated with 1 U/ml of thrombin. Results are expressed as percentage of the control and mean $\pm$ SD from three different experiments. * $p < 0.05$ compared with the non-treated condition
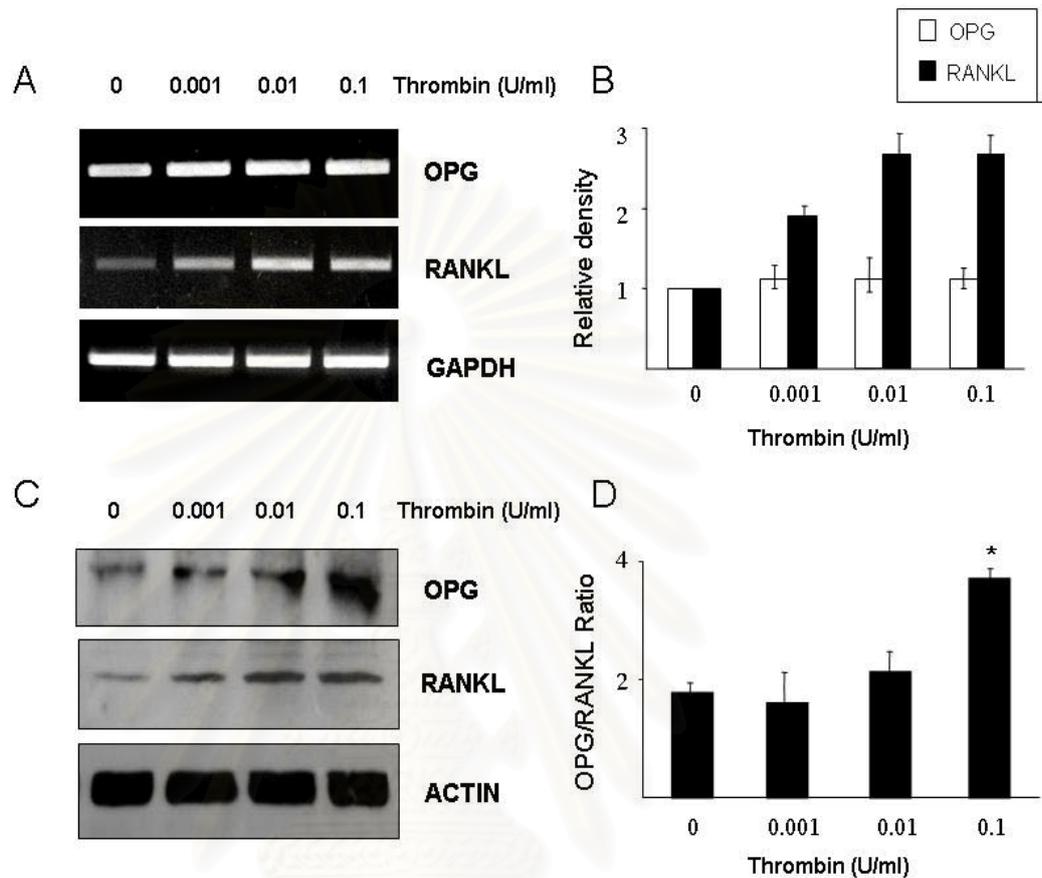
Figure 4.2 Effect of thrombin on mRNA expression of OPG and RANKL (A) and protein synthesis (C) in HPDL cells. Cells were treated with 0.001, 0.01, 0.1 U/ml of thrombin for 24 hours in serum-free condition. (A) The polymerase chain reaction (PCR) products revealed the up-regulation of RANKL, but not of OPG, in a dose dependent manner. The average density $\pm$ standard deviation (SD) of the PCR product from three gels is shown in the bar chart (B). (C) Western blot analysis revealed the dose dependently increase of RANKL and OPG after 48 hours of treatment. The relative density was presented as graphs in (D). The results are expressed as mean $\pm$

SD from three different experiments. * $p < 0.05$ compared with the non-treated condition. GAPDH, glyceraldehydes-3-phosphate dehydrogenase.
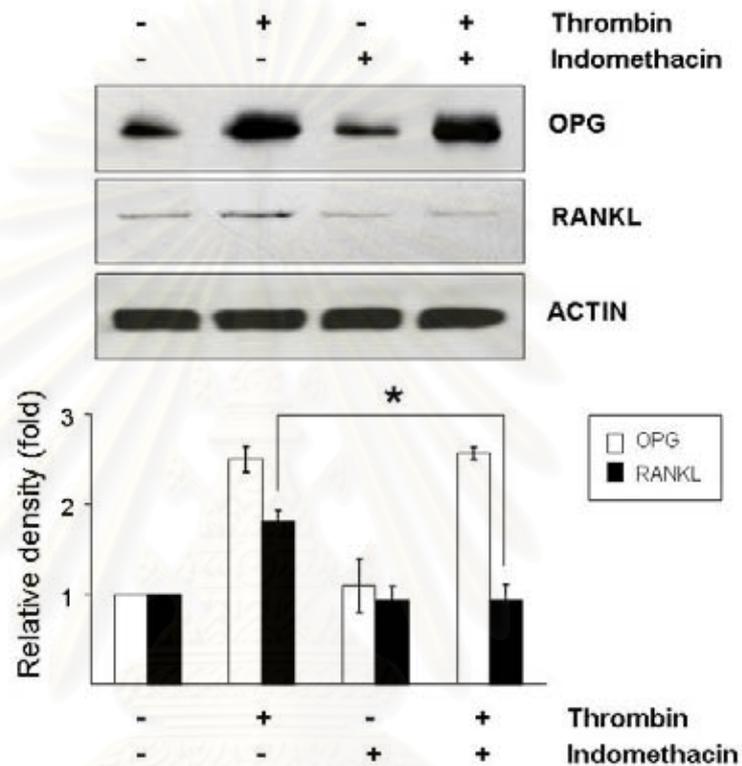
Figure 4.3 Effect of indomethacin on protein expression of OPG and RANKL. HPDL cells were exposed to 10 µM of indomethacin 30 min prior to addition 0.1 U/ml of thrombin for 24 hours. Western blot analysis showed that indomethacin exerted the inhibitory effect on thrombin-induced RANKL, but not on thrombin-induced OPG. The charts represent the average density $\pm$ SD of each band normalized to actin, from three independent experiment. * $p < 0.05$ compared with thrombin-treated control, -, without thrombin or indomethacin; +, with thrombin or indomethacin.
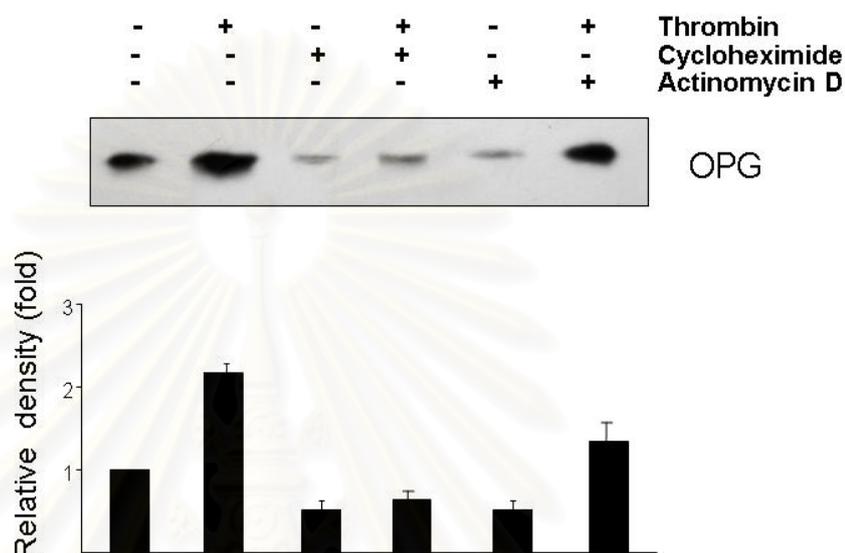
Figure 4.4 Effect of cycloheximide and actinomycin D on thrombin-induced protein expression of OPG in HPDL cells. Cells were pre-incubated with either cycloheximide (1 µg/ml) and actinomycin D (0.5 µg/ml) 30 min prior to treatment with 0.1 U/ml of thrombin for 48 hours. The thrombin-induced protein expression of OPG was attenuated by cycloheximide, but not by actinomycin D. The relative density was presented as graphs and the results are expressed as mean $\pm$ SD from three different experiments. * $p < 0.05$ compared with thrombin-treated control. -,without thrombin or inhibitor; +,with thrombin or inhibitor.

Thrombin induces OPG synthesis through PI3K/mTOR pathway

To investigate the kinase signaling pathways involved in thrombin-induced OPG synthesis, either LY294002, the specific PI3K inhibitor, or rapamycin, a potent inhibitor of mTOR, was applied to quiescent cells for 30 minutes before addition of thrombin. The results showed that both LY294002 and rapamycin exerted the inhibitory effect on the thrombin-induced OPG synthesis (Figure 4.5 and 4.6). Phosphorylation of Akt (p-Akt) was also examined and was found to be increased in response to thrombin. In addition, the increase of p-Akt could be inhibited by LY294002 (Figure 4.7), providing the evidence that thrombin mediated its effect through PI3K/Akt-mTOR signaling pathway.
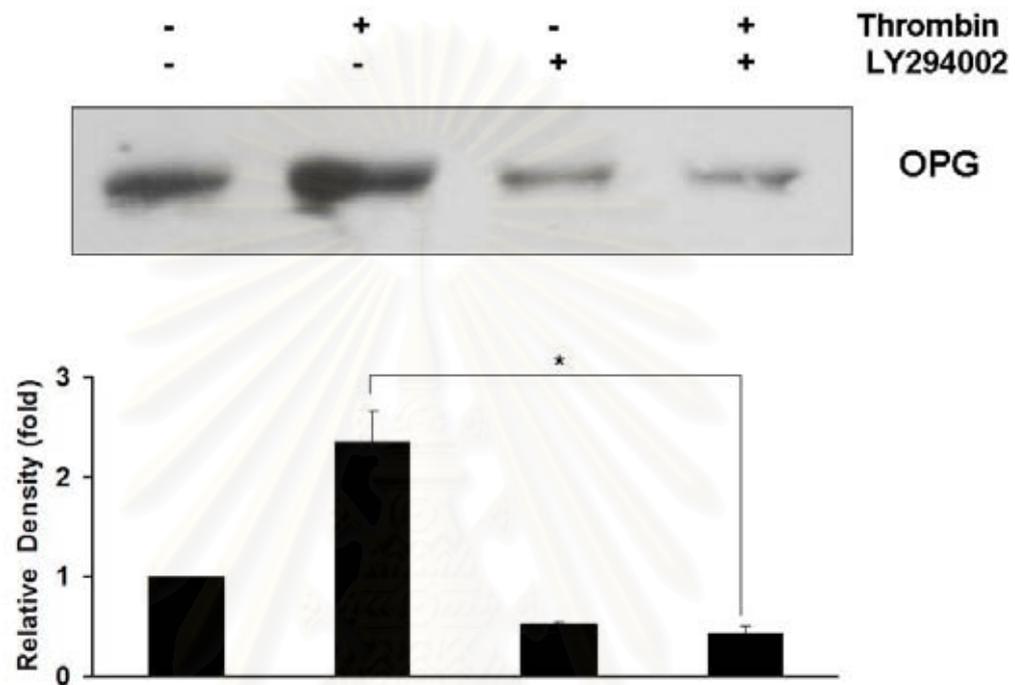
Figure 4.5 Inhibitory effect of LY294002 on thrombin-induced protein expression of OPG in HPDL cells. Cells were pre-incubated with LY294002 for 30 minutes before the addition of 0.1 U/ml of thrombin for 48 hours. After 48 hours, the media was collected, lyophilized and prepared for western blot analysis. LY294002 could inhibit the increasing of thrombin-induced OPG. The relative density was presented as graphs and the results are express as mean $\pm$ SD from three different experiments. * $p < 0.05$ compared with thrombin-treated control. -, without thrombin or LY294002; +,with thrombin or LY294002.
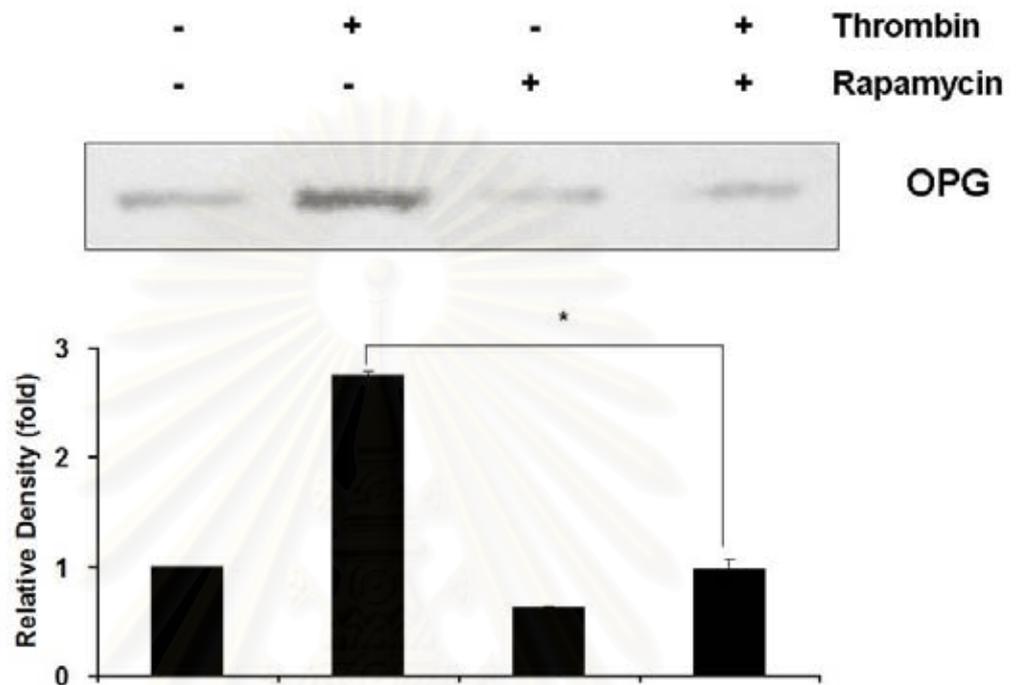
Figure 4.6 Inhibitory effect of rapamycin on thrombin-induced protein expression of OPG in HPDL cells. Cells were pre-incubated with rapamycin for 30 minutes before the addition of 0.1 U/ml of thrombin for 48 hours. After 48 hours, the media was collected, lyophilized and prepared for western blot analysis. rapamycin could inhibit the increasing of thrombin-induced OPG. The relative density was presented as graphs and the results are express as mean $\pm$ SD from three different experiments. * $p < 0.05$ compared with thrombin-treated control. -, without thrombin or rapamycin; +, with thrombin or rapamycin.
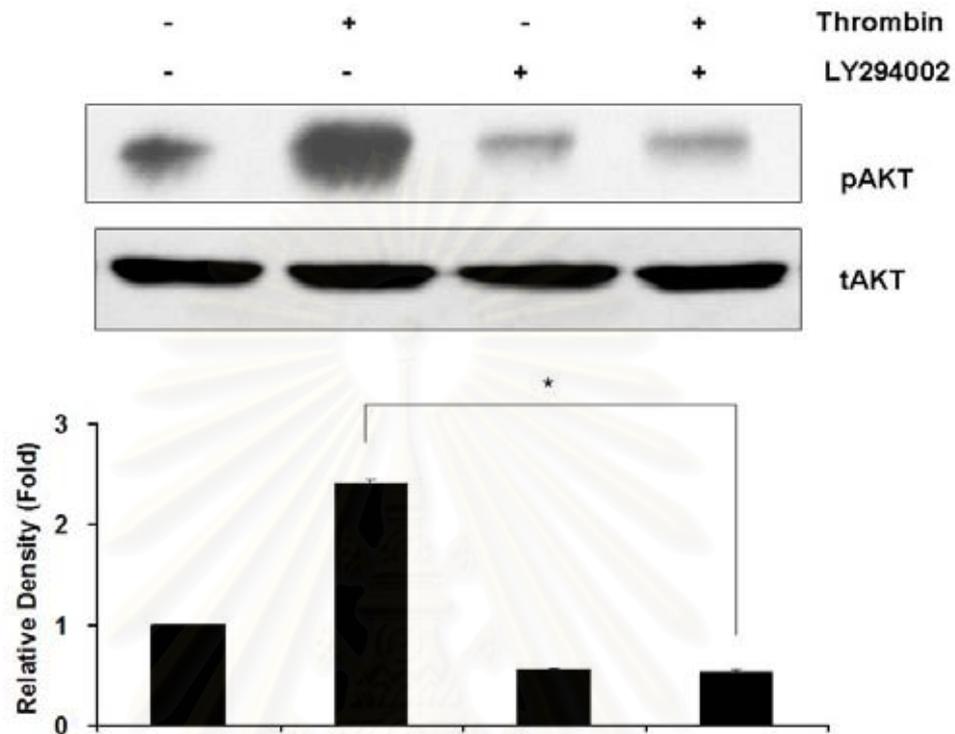
Figure 4.7 Inhibitory effect of LY294002 on thrombin-induced protein expression of Phospho-Akt (pAKT) in HPDL cells. Cells were pre-incubated with LY294002 for 30 minutes before the addition of 0.1 U/ml of thrombin. Cellular protein extracts were collected under ice-cold condition for western blot analysis after 5 minutes of treatment. The activation of p-Akt mediated by thrombin was observed and the activation was inhibited by LY294002. The bar charts represent the average band density of p-Akt normalized to its total protein (t-Akt). * $p < 0.05$ compared with thrombin-treated control. -, without thrombin or LY294002; +, with thrombin or LY294002.

HPDL cells express PAR-1, PAR-2 and PAR-3

To determine the existence of PARs on HPDL cells, the expression of 4 types of PARs were investigated. The result showed that HPDL cells expressed PAR-1, PAR-2 and PAR-3, but not PAR-4 (Figure 4.8).
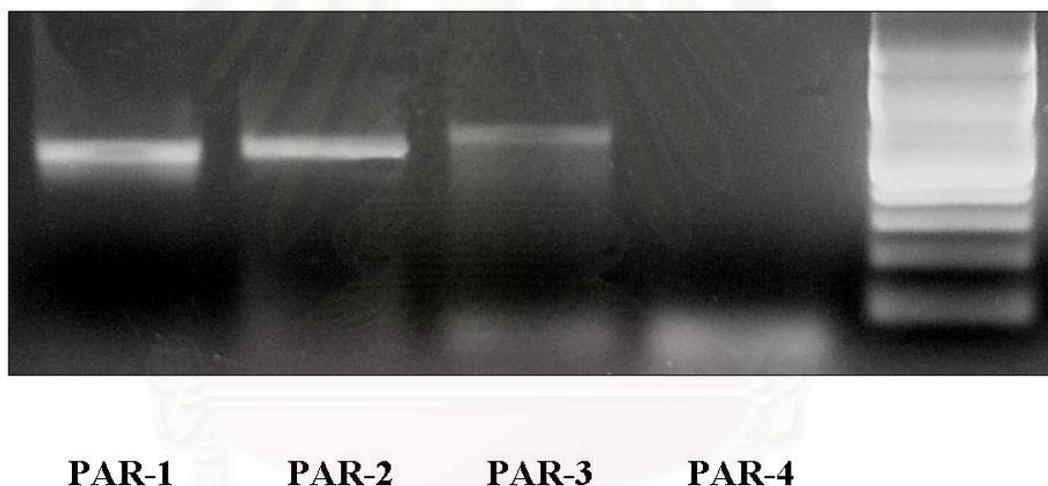
PAR-1   PAR-2   PAR-3   PAR-4

Figure 4.8 Expression profile of PAR-1, PAR-2, PAR-3 and PAR-4 on HPDL cells. RT-PCR analysis of PAR expression profile. RNA was extracted, reverse-transcribed and amplified with specific primers for each kind of PARs. Amplification products were separated on 1.5 % agarose gel, stained with ethidium bromide, and were photographed Lane 1, PAR-1; Lane2, PAR-2; Lane 3, PAR-3; Lane 4, PAR-4; 100 bp ladder, respectively

Thrombin stimulation involves PAR-1

Since proteolytic cleavage of four isoforms of PARs by thrombin has been proposed, the existence of PARs on HPDL cells was explored. PAR-1 antagonist was used to investigate the involvement of PAR-1. The result in Figure 4.10 showed that PAR-1 antagonist could diminish the increase of OPG. To confirm that PAR-1 may contribute to the regulation of OPG synthesis, PAR-1 agonist peptide was used to mimic the effect of thrombin. PAR-1 agonist appeared to enhance the OPG synthesis dose dependently (Figure 4.9). In addition, the effect could be blocked by LY294002 (Figure 4.11). These data suggested that PAR-1 was involved in the enhancement of OPG synthesis mediated by thrombin.
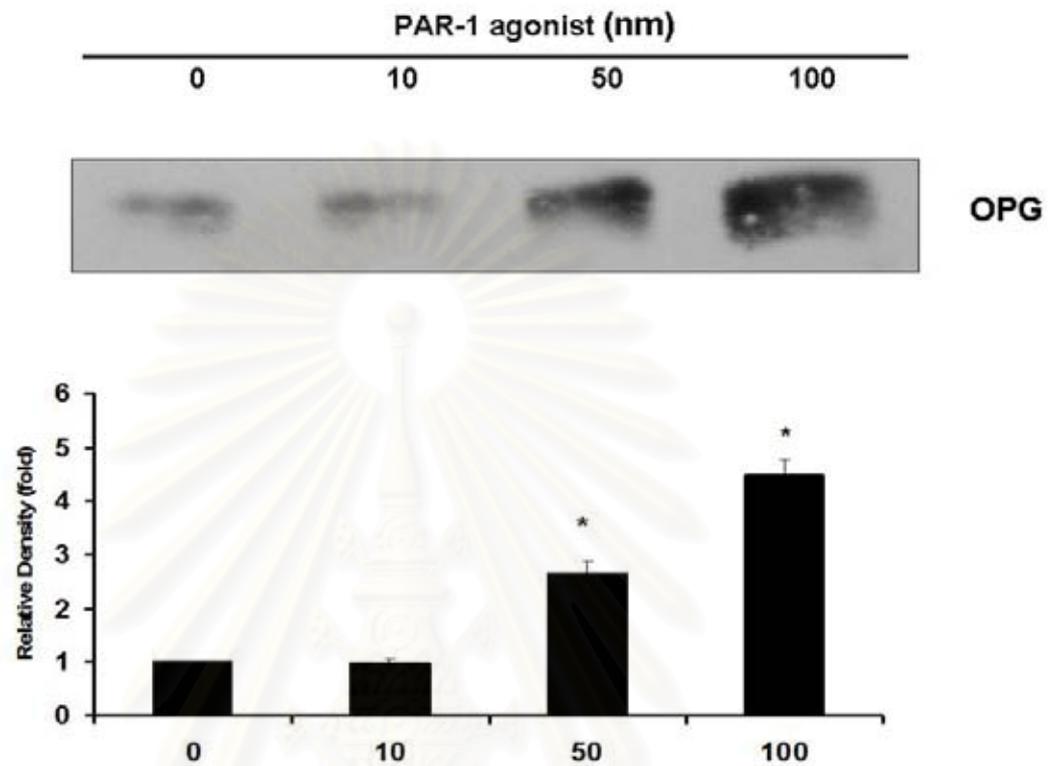
Figure 4.9 Effect of PAR-1 agonist on protein expression of OPG in HPDL cells. Cells were treated with 0, 10, 50 and 100 nM of PAR-1 agonist in serem-free condition. After 48 hours, the media was collected, lyophilized and prepared for western blot analysis. The result revealed the dose dependently increase of OPG after 48 hours of treatment. The relative density was presented as graphs and the results are express as mean $\pm$ SD from three different experiments. * $p < 0.05$ compared with non-treated condition.
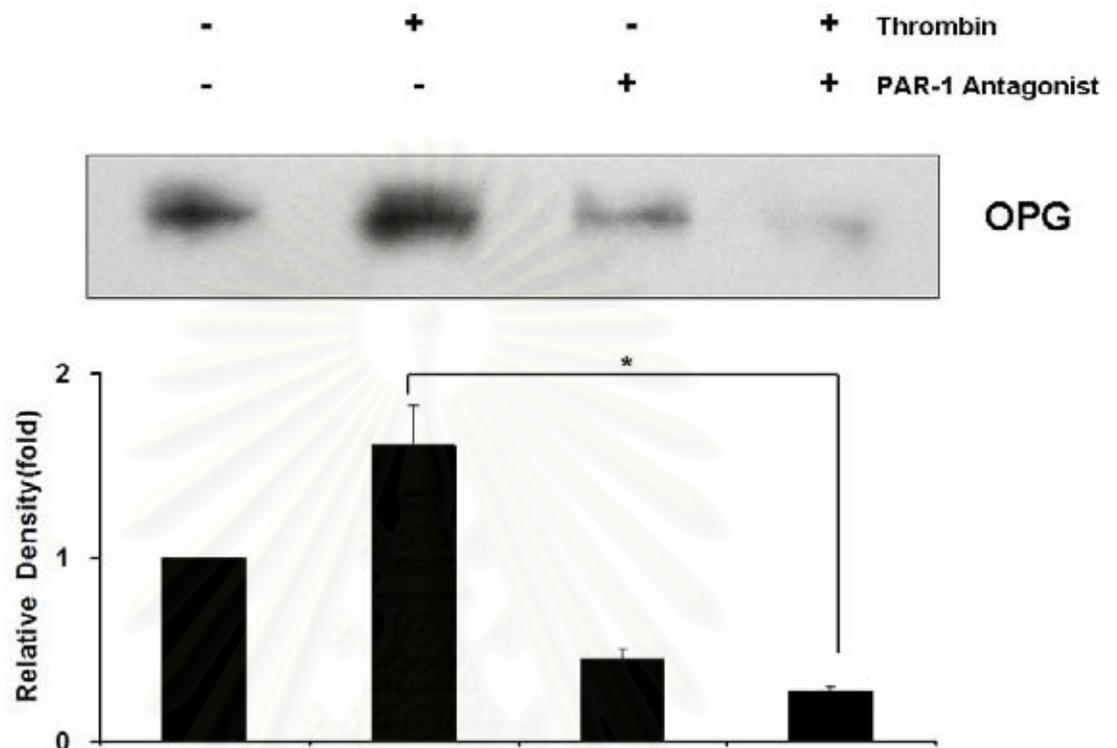
Figure 4.10 Inhibitory effect of PAR-1 antagonist on the thrombin-induced protein expression of OPG in HPDL cells. Cells were exposed to PAR-1 antagonist (100 nM) for 30 minutes before addition of 0.1 U/ml of thrombin. After 48 hours, the media was collected, lyophilized and prepared for western blot analysis. PAR-1 antagonist could inhibit the increased level of OPG mediated by thrombin. The relative density was presented as graphs and the results are express as mean $\pm$ SD from three different experiments. * $p < 0.05$ compared with thrombin-treated control. -, without thrombin or PAR-1 antagonist; +, with thrombin or PAR-1 antagonist.
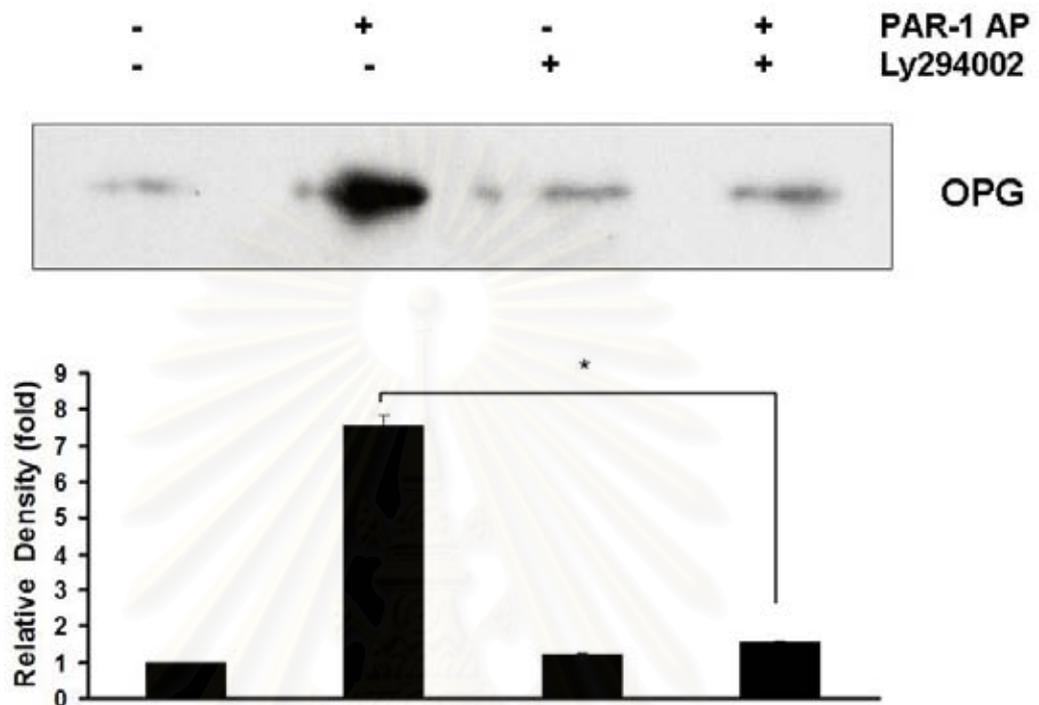
Figure 4.11 Inhibitory of LY294002 on PAR-1 induced protein expression of OPG. Cells were incubated with LY294002 30 minutes before addition of PAR-1 agonist (100nM) for 48 hours. After 48 hours, the media was collected, lyophilized and prepared for western blot analysis. LY294002 could inhibit the increased level of OPG mediated by PAR-1 agonist. The relative density was presented as graphs and the results are express as mean $\pm$ SD from three different experiments. * $p < 0.05$ compared with thrombin-treated control. -, without PAR-1 agonist or LY294002; +, with PAR-1 agonist or LY294002.

CHAPTER V

DISCUSSION AND CONCLUSION

The balance of RANKL and OPG plays a significant role in bone homeostasis. They are two key molecules that regulate osteoclast formation, activation and survival. The present study provided new evidence that thrombin may involve in the homeostasis of alveolar bone and periodontium by regulating the balance of RANKL and OPG synthesized in HPDL cells.

Our results clearly demonstrated that thrombin differentially regulated RANKL and OPG expression. Upon activation, thrombin increased both RANKL mRNA and protein synthesis. No change in the level OPG mRNA was observed but the protein synthesis was significantly increased. Cycloheximide and actinomycin D were used in this experiment to clarify that the mechanism of thrombin-induced OPG synthesis was post-transcriptional mechanism. Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus* (Wool, 1966). Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis thus blocking translational elongation. Actinomycin D is an antineoplastic agent that inhibits RNA synthesis by binding to guanine residues and inhibiting DNA-dependent RNA polymerase. Our result from Western blot analysis showed that cycloheximide completely abolished the thrombin-induced OPG protein synthesis. In contrast, actinomycin D could not inhibit the effect of thrombin on OPG synthesis. These findings suggest that the effect of thrombin on OPG expression in HPDL cells is post transcriptional regulation.

Although both RANKL and OPG synthesis increased in response to thrombin, the ratio of OPG/RANKL was higher over control, suggesting the anabolic role of thrombin in alveolar bone homeostasis via HPDL cells. The increased ratio of OPG/RANKL supports the concept that thrombin may participate in bone tissue repair. High amount of OPG may be significant in the reduction of bone resorption as demonstrated in osteoporosis model (Hamdy, 2006). The increase ratio of OPG/RANKL by thrombin in HPDL cells seems to be contrast with previous reports on the catalytic effect of thrombin. It has been shown that thrombin increased release of ^{45}Ca from the rat/mouse calvarial bone explants (Lerner et al., 1988). The difference may depend on either different cell types or different models used in the experiments. Induction of bone resorption from thrombin was resulted from the release of prostaglandins and this effect could be inhibited by indomethacin, a cyclooxygenase inhibitor. Since PGE_2 has been reported to be a potent inducer of RANKL in osteoblasts (Kobayashi et al., 2005), the resorbing activity found after the thrombin application may be resulted from the increased level of RANKL.

In this study, indomethacin could not suppress OPG synthesis, indicates that regulation of OPG synthesis induced by thrombin in HPDL is a COX-independent mechanism. This result is different from the previous one reported by Marklund et al. (1994), whose report suggested that thrombin stimulated releasing of PGE_2 at 10 U/ml. However, our study used lower concentration (0.1 U/ml of thrombin). It is possible that the response of HPDL cells depend on doses of thrombin. Our results here suggested that low dose of thrombin (0.1 U/ml or less) increased OPG synthesis. The finding supports our hypothesis that thrombin may play a role in periodontal tissue repair, partly through the balance of OPG/RANKL. Nevertheless, the

significance of the thrombin-induced OPG synthesis *in vivo* requires further investigation.

Our result also shown that thrombin did not exhibit mitogenic effect on HPDL cells. However, Chan and his colleagues in 1998 found that thrombin can stimulate the growth of HPDL cells in a dose dependent manner (Chan et al., 1998). Thrombin has been shown to enhance the proliferation of smooth muscle cells (Dawes et al., 1993), endothelial cells and fibroblasts (Herbert et al., 1994), osteoblasts (Suzuki et al., 1996), and marrow stromal cells (Tarzami et al., 2006). The difference is possibly due to the different dose of thrombin and cell types used in this study. The dose of thrombin used in the present study (0.1 U/ml) is lower than that of previous studies, whilst, the mitogenic effect of thrombin was reported to be approximately 1-10 U/ml (Chan et al., 1998).

Although a number of candidate signaling pathways have been proposed, the signaling events that thrombin mediated OPG synthesis have never been fully elucidated. The data presented here define the important role of PI3K/Akt/mTOR in thrombin-induced OPG synthesis in HPDL cells. The ability of LY294002, a specific PI3K inhibitor, to completely abolish thrombin-induced OPG synthesis by HPDL cells suggested that PI3K is a key regulator of this response. This finding was supported by the demonstration of phosphorylation of Akt. In response to thrombin, pAkt was increased and could be inhibited by LY294002, providing the evidence that thrombin mediated its effect through PI3K/Akt signaling pathway. The involvement of PI3K is in consistent with previous results regarding to the effect of thrombin in several cell types, such as the induction of vascular endothelial growth factor (VEGF)

in retinal pigment epithelial cells (Bian et al., 2007) or the induction of IL-8 in prostate cancer cells (Liu, 2000).

In many cell types, the protein kinase Akt has been identified as a major downstream effector of PI3K (Burgering and Coffer, 1995; Franke et al., 1995; Kandel and Hay, 1999; Brazil and Hemmings, 2001; Lawlor and alessi, 2001). In this study, we showed that LY294002 completely abolished thrombin-induced phosphorylation of Akt. This finding suggests that thrombin stimulates the phosphorylation of Akt in a PI3K dependent manner. Several signaling molecules have been implicated as a downstream target of Akt including mammalian target of rapamycin (mTOR). mTOR is a multidomain protein that function as a regulator of the translational machinery and the signal from PI3K/mTOR has been shown to regulate cell growth, size and proliferation in many cell types (Martin and Blenis, 2002; Rajan et al., 2003; Martin et al., 2004; Takahashi, 2005; Parent et al., 2007). In this study, inhibitory effect of both LY294002 and rapamycin on OPG synthesis indicates that thrombin mediated its effect through PI3K/Akt/mTOR signaling pathway.

This study showed that HPDL cells express PAR-1, PAR-2 and PAR-3, but not PAR-4, which is consistent with the report from Chan and his colleagues (Chan et al., 1998) that HPDL cells express thrombin receptor. However, they did not mention that HPDL cells express which PARs member. In regard to receptors, thrombin has been shown to communicate with cells through its proteolytic action on PAR-1, which was considered to be thrombin receptor (Coughlin, 2000). PAR-3 and PAR-4, except PAR-2, also function as thrombin receptors. However, function of PAR-3 required the presence of PAR-4 (Nakanishi-Matsui et al., 2000). In addition, non-PAR

thrombin receptor was reported (Pagel et al., 2003). Taken all data together, these data suggest that the action of thrombin on OPG synthesis may occurs via PAR-1.

In conclusion, thrombin can stimulate OPG synthesis in HPDL cells post-transcriptionally, resulting in the increase of OPG/RANKL ratio. Thrombin is likely to exert its action through PAR-1 and PI3K/Akt/mTOR signaling pathways. These results suggested that thrombin may play a significant role in alveolar bone repair and keep homeostasis of periodontal tissue, partly through the balance of RANKL and OPG.
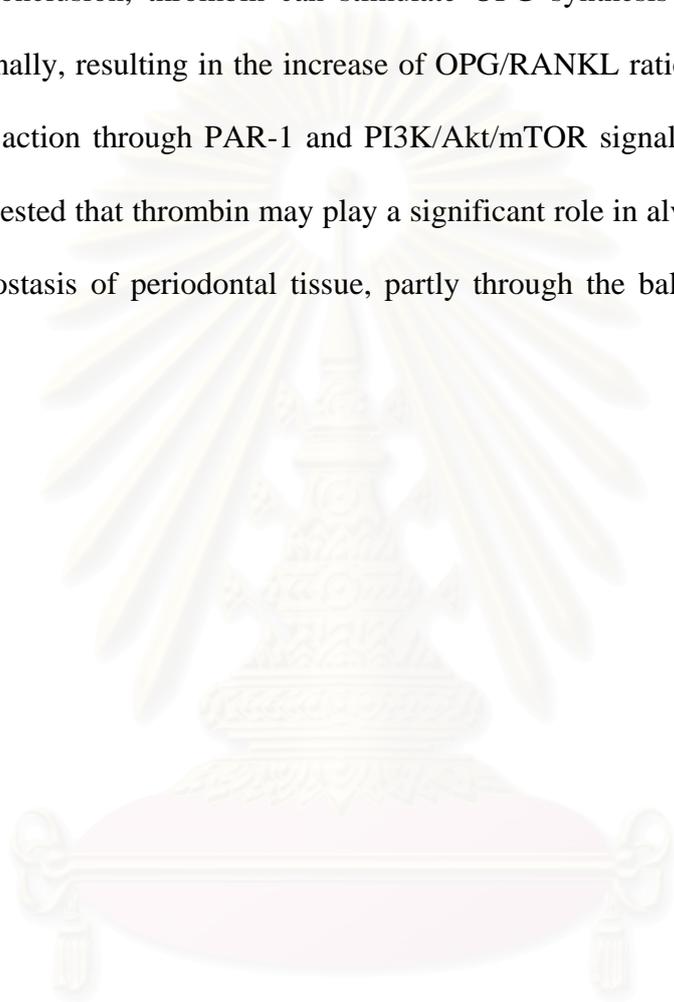
Future Studies

Although outside the scope of this thesis, a number of new arisen directions could be performed.

1. To determine the downstream target of PI3K/Akt/mTOR signaling pathway in thrombin-induced OPG synthesis.

2. To elucidate whether the effect of thrombin in HPDL cells occur via PAR-1 activation.

3. To determine the differentiation of osteoclast modulated by thrombin-treated HPDL cells.
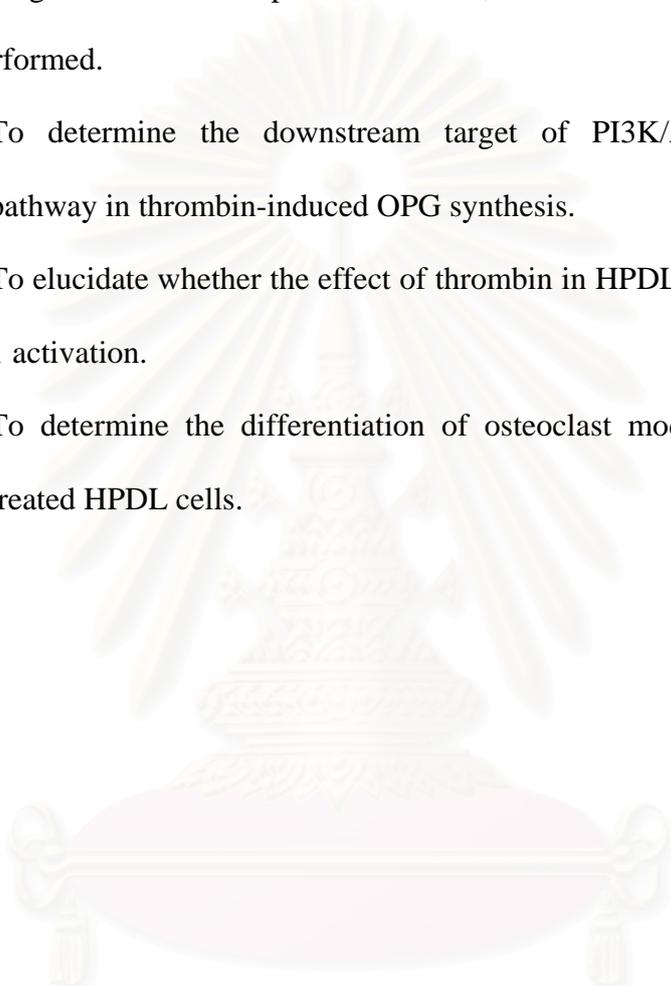
REFERENCES

Abraham, L. A., Jenkins, A. L., Stone, S. R., Mackie, E. J. (1998). Expression of the thrombin receptor in developing bone and associated tissues. **J Bone Miner Res**. 13(5) (May): 818-827.

Abraham, L. A., MacKie, E. J. (1999). Modulation of osteoblast-like cell behavior by activation of protease-activated receptor-1. **J Bone Miner Res**. 14(8) (Aug): 1320-1329.

Akatsu, T., Murakami, T., Nishikawa, M., Ono, K., Shinomiya, N., Tsuda, E., et al. (1998). Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclast. **Biochem Biophys Res Commun.** 250(2) (Sep 18): 229-234.

Altieri, D. C. (1993). Coagulation assembly on leukocytes in transmembrane signaling and cell adhesion. **Blood.** 81(3) (Feb 1): 569-579.

Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., et al. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. **Nature.** 390(6656) (Nov 13): 175-179.

Beevers, C. S., Li, F., Liu, L., Huang, S. (2006). Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. **Int J Cancer.** 119(4) (Aug 15): 757-764.

Begg, S. K., Radley, J. M., Pollard, J. W., Chisholm, O. T., Stanley, E. R., Bertoncello, I. (1993). Delayed hematopoietic development in osteopetrotic (op/op) mice. **J Exp Med.** 177(1) (Jan 1): 237-242.

Bekker, P. J., Holloway, D., Nakanishi, A., Arrighi, M., Leese, P. T., Dunstan, C. R. (2001). The effect of a single dose of osteoprotegerin in postmenopausal women. **J Bone Miner Res.** 16(2) (Feb): 348-360.

Berk, B. C., Taubman, M. B., Griendling, K. K., Cragoe, E. J., Jr., Fenton, J. W., Brock, T. A. (1991). Thrombin-stimulated events in cultured vascular smooth-muscle cells. **Biochem J.** 274 (Pt 3)(Mar 15): 799-805.

Bian, Z. M., Elner, S. G., Elner, V. M. (2007). Thrombin-induced VEGF expression in human retinal pigment epithelial cells. **Invest Ophthalmol Vis Sci.** 48(6) (Jun): 2738-2746.

Blackhart, B. D., Ruslim-Litrus, L., Lu, C. C., Alves, V. L., Teng, W., Scarborough, R. M., et al. (2000). Extracellular mutations of protease-activated receptor-1 result in differential activation by thrombin and thrombin receptor agonist peptide. **Mol Pharmacol.** 58(6) (Dec): 1178-1187.

Bohm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G., Bunnett, N. W. (1996). Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. **J Biol Chem.** 271(36) (Sep 6): 22003-22016.

Bord, S., Ireland, D. C., Beavan, S. R., Compston, J. E. (2003). The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. **Bone.** 32(2) (Feb): 136-141.

Bostanci, N., Ilgenli, T., Emingil, G., Afacan, B., Han, B., Toz, H., et al. (2007). Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. **J Clin Periodontol.** 34(5) (May): 370-376.

Bostanci, N., Ilgenli, T., Emingil, G., Afacan, B., Han, B., Toz, H., et al. (2007). Differential expression of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin mRNA in periodontal diseases. **J Periodontal Res.** 42(4) (Aug): 287-293.

Brazil, D. P., Hemmings, B. A. (2001). Ten years of protein kinase B signalling: a hard Akt to follow. **Trends Biochem Sci.** 26(11) (Nov): 657-664.

Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., et al. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. **Nature.** 369(6483) (Jun 30): 756-758.

Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Hosoi, H., Houghton, P. J., et al. (1997). Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. **Science.** 277(5322) (Jul 4): 99-101.

Burgering, B. M., Coffer, P. J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. **Nature.** 376(6541) (Aug 17): 599-602.

Burgess, T. L., Qian, Y., Kaufman, S., Ring, B. D., Van, G., Capparelli, C., et al. (1999). The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. **J Cell Biol.** 145(3) (May 3): 527-538.

Chambers, R. C., Dabbagh, K., McAnulty, R. J., Gray, A. J., Blanc-Brude, O. P., Laurent, G. J. (1998). Thrombin stimulates fibroblast procollagen production via proteolytic activation of protease-activated receptor 1. **Biochem J.** 333 (Pt 1)(Jul 1): 121-127.

Chambers, T. J. (1980). The cellular basis of bone resorption. **Clin Orthop Relat Res.** (151) (Sep): 283-293.

Chan, C. P., Lin, C. P., Chang, M. C., Hsieh, C. C., Hsu, C. C., Lin, C. L., et al. (1998). Effects of thrombin on the growth, protein synthesis, attachment, clustering and alkaline phosphatase activity of cultured human periodontal ligament fibroblasts. **Proc Natl Sci Counc Repub China B.** 22(4) (Oct): 137-143.

Chang, M. C., Chan, C. P., Wu, H. L., Chen, R. S., Lan, W. H., Chen, Y. J., et al. (2001). Thrombin-stimulated growth, clustering, and collagen lattice contraction of human gingival fibroblasts is associated with its protease activity. **J Periodontol.** 72(3) (Mar): 303-313.

Chang, M. C., Jeng, J. H., Lin, C. P., Lan, W. H., Tsai, W., Hsieh, C. C. (1999). Thrombin activates the growth, cell-cycle kinetics, and clustering of human dental pulp cells. **J Endod.** 25(2) (Feb): 118-122.

Chang, M. C., Lin, C. P., Huang, T. F., Lan, W. H., Lin, Y. L., Hsieh, C. C., et al. (1998). Thrombin-induced DNA synthesis of cultured human dental pulp cells is dependent on its proteolytic activity and modulated by prostaglandin E2. **J Endod.** 24(11) (Nov): 709-713.

Cirino, G., Napoli, C., Bucci, M., Cicala, C. (2000). Inflammation-coagulation network: are serine protease receptors the knot? **Trends Pharmacol Sci.** 21(5) (May): 170-172.

Cohen, I., Diegelmann, R., Lindblad, W. (1992). **Wound healing:Biochemical and Clinical Aspects.** Philadelphia: WB Saunders.

Coller, B. S., Springer, K. T., Scudder, L. E., Kutok, J. L., Ceruso, M., Prestwich, G. D. (1993). Substituting isoserine for serine in the thrombin receptor activation peptide SFLLRN confers resistance to aminopeptidase M-induced cleavage and inactivation. **J Biol Chem.** 268(28) (Oct 5): 20741-20743.

Collin-Osdoby, P., Rothe, L., Anderson, F., Nelson, M., Maloney, W., Osdoby, P. (2001). Receptor activator of NF-kappa B and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. **J Biol Chem.** 276(23) (Jun 8): 20659-20672.

Colvin, R. (1986). **Wound healing processes in hemostasis and thrombosis.** . Edinburge: Churchill publishingstore.

Corradetti, M. N., Guan, K. L. (2006). Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? **Oncogene.** 25(48) (Oct 16): 6347-6360.

Corvera, C. U., Dery, O., McConalogue, K., Bohm, S. K., Khitin, L. M., Caughey, G. H., et al. (1997). Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. **J Clin Invest.** 100(6) (Sep 15): 1383-1393.

Coughlin, S. R. (1998). Sol Sherry lecture in thrombosis: how thrombin 'talks' to cells: molecular mechanisms and roles in vivo. **Arterioscler Thromb Vasc Biol.** 18(4) (Apr): 514-518.

Coughlin, S. R. (2000). Thrombin signalling and protease-activated receptors. **Nature.** 407(6801) (Sep 14): 258-264.

Coughlin, S. R. (2001). Protease-activated receptors in vascular biology. **Thromb Haemost.** 86(1) (Jul): 298-307.

Coughlin, S. R., Camerer, E. (2003). PARticipation in inflammation. **J Clin Invest.** 111(1) (Jan): 25-27.

Dawes, K. E., Gray, A. J., Laurent, G. J. (1993). Thrombin stimulates fibroblast chemotaxis and replication. **Eur J Cell Biol.** 61(1) (Jun): 126-130.

Degen, S. J., MacGillivray, R. T., Davie, E. W. (1983). Characterization of the complementary deoxyribonucleic acid and gene coding for human prothrombin. **Biochemistry.** 22(9) (Apr 26): 2087-2097.

Derian, C. K., Damiano, B. P., D'Andrea, M. R., Andrade-Gordon, P. (2002). Thrombin regulation of cell function through protease-activated receptors: implications for therapeutic intervention. **Biochemistry (Mosc).** 67(1) (Jan): 56-64.

Dery, O., Corvera, C. U., Steinhoff, M., Bunnett, N. W. (1998). Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. **Am J Physiol.** 274(6 Pt 1) (Jun): C1429-1452.

Deyama, Y., Takeyama, S., Koshikawa, M., Shirai, Y., Yoshimura, Y., Nishikata, M., et al. (2000). Osteoblast maturation suppressed osteoclastogenesis in coculture with bone marrow cells. **Biochem Biophys Res Commun.** 274(1) (Jul 21): 249-254.

Dougall, W. C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., et al. (1999). RANK is essential for osteoclast and lymph node development. **Genes Dev.** 13(18) (Sep 15): 2412-2424.

Dugina, T. N., Kiseleva, E. V., Chistov, I. V., Umarova, B. A., Strukova, S. M. (2002). Receptors of the PAR family as a link between blood coagulation and inflammation. **Biochemistry (Mosc).** 67(1) (Jan): 65-74.

Duhamel-Clerin, E., Orvain, C., Lanza, F., Cazenave, J. P., Klein-Soyer, C. (1997). Thrombin receptor-mediated increase of two matrix metalloproteinases, MMP-1 and MMP-3, in human endothelial cells. **Arterioscler Thromb Vasc Biol.** 17(10) (Oct): 1931-1938.

Eghbali-Fatourechi, G., Khosla, S., Sanyal, A., Boyle, W. J., Lacey, D. L., Riggs, B. L. (2003). Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. **J Clin Invest.** 111(8) (Apr): 1221-1230.

Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., Chen, J. (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. **Science.** 294(5548) (Nov 30): 1942-1945.

Fata, J. E., Kong, Y. Y., Li, J., Sasaki, T., Irie-Sasaki, J., Moorehead, R. A., et al. (2000). The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. **Cell.** 103(1) (Sep 29): 41-50.

Fazzalari, N. L., Kuliwaba, J. S., Atkins, G. J., Forwood, M. R., Findlay, D. M. (2001). The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. **J Bone Miner Res.** 16(6) (Jun): 1015-1027.

Fenton, J. W., 2nd. (1988). Regulation of thrombin generation and functions. **Semin Thromb Hemost.** 14(3) (Jul): 234-240.

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., et al. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. **Cell.** 81(5) (Jun 2): 727-736.

Frias, M. A., Thoreen, C. C., Jaffe, J. D., Schroder, W., Sculley, T., Carr, S. A., et al. (2006). mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. **Curr Biol.** 16(18) (Sep 19): 1865-1870.

Giangrande, P. L. (2005). **Coagulation[Online].** Available from: http://www.biocrawler.com/enclycopedia/Coagulation[2005, Jan 19]

Goldsack, N. R., Chambers, R. C., Dabbagh, K., Laurent, G. J. (1998). Thrombin. **Int J Biochem Cell Biol.** 30(6) (Jun): 641-646.

Gori, F., Hofbauer, L. C., Dunstan, C. R., Spelsberg, T. C., Khosla, S., Riggs, B. L. (2000). The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. **Endocrinology.** 141(12) (Dec): 4768-4776.

Gruber, R., Jindra, C., Kandler, B., Watzak, G., Fischer, M. B., Watzek, G. (2004). Proliferation of dental pulp fibroblasts in response to thrombin involves mitogen-activated protein kinase signalling. **Int Endod J.** 37(2) (Feb): 145-150.

Gustafson, G. T., Lerner, U. (1983). Thrombin, a stimulator of bone resorption. **Biosci Rep.** 3(3) (Mar): 255-261.

Hakeda, Y., Kobayashi, Y., Yamaguchi, K., Yasuda, H., Tsuda, E., Higashio, K., et al. (1998). Osteoclastogenesis inhibitory factor (OCIF) directly inhibits bone-resorbing activity of isolated mature osteoclasts. **Biochem Biophys Res Commun.** 251(3) (Oct 29): 796-801.

Hamdy, N. A. (2006). Osteoprotegerin as a potential therapy for osteoporosis. **Curr Rheumatol Rep**. 8(1) (Feb): 50-54.

Hay, N., Sonenberg, N. (2004). Upstream and downstream of mTOR. **Genes Dev**. 18(16) (Aug 15): 1926-1945.

Haynes, D. R., Barg, E., Crotti, T. N., Holding, C., Weedon, H., Atkins, G. J., et al. (2003). Osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathies and osteoarthritis and normal controls. **Rheumatology (Oxford)**. 42(1) (Jan): 123-134.

Herbert, J. M., Dupuy, E., Laplace, M. C., Zini, J. M., Bar Shavit, R., Tobelem, G. (1994). Thrombin induces endothelial cell growth via both a proteolytic and a non-proteolytic pathway. **Biochem J**. 303 (Pt 1)(Oct 1): 227-231.

Hofbauer, L. C., Dunstan, C. R., Spelsberg, T. C., Riggs, B. L., Khosla, S. (1998). Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines. **Biochem Biophys Res Commun**. 250(3) (Sep 29): 776-781.

Hofbauer, L. C., Shui, C., Riggs, B. L., Dunstan, C. R., Spelsberg, T. C., O'Brien, T., et al. (2001). Effects of immunosuppressants on receptor activator of NF-kappaB ligand and osteoprotegerin production by human osteoblastic and coronary artery smooth muscle cells. **Biochem Biophys Res Commun**. 280(1) (Jan 12): 334-339.

Hoffmann, O., Klaushofer, K., Koller, K., Peterlik, M., Mavreas, T., Stern, P. (1986). Indomethacin inhibits thrombin-, but not thyroxin-stimulated resorption of fetal rat limb bones. **Prostaglandins**. 31(4) (Apr): 601-608.

Hollenberg, M. D. (2003). Proteinase-mediated signaling: proteinase-activated receptors (PARs) and much more. **Life Sci**. 74(2-3) (Dec 5): 237-246.

Hollenberg, M. D., Compton, S. J. (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. **Pharmacol Rev**. 54(2) (Jun): 203-217.

Holz, M. K., Blenis, J. (2005). Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. **J Biol Chem**. 280(28) (Jul 15): 26089-26093.

Hsu, H., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., et al. (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. **Proc Natl Acad Sci U S A**. 96(7) (Mar 30): 3540-3545.

Hung, D. T., Wong, Y. H., Vu, T. K., Coughlin, S. R. (1992). The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. **J Biol Chem**. 267(29) (Oct 15): 20831-20834.

Ikeda, T., Kasai, M., Utsuyama, M., Hirokawa, K. (2001). Determination of three isoforms of the receptor activator of nuclear factor-kappaB ligand and their differential expression in bone and thymus. **Endocrinology**. 142(4) (Apr): 1419-1426.

Ishida, A., Fujita, N., Kitazawa, R., Tsuruo, T. (2002). Transforming growth factor-beta induces expression of receptor activator of NF-kappa B ligand in vascular endothelial cells derived from bone. **J Biol Chem**. 277(29) (Jul 19): 26217-26224.

Ishihara, H., Zeng, D., Connolly, A. J., Tam, C., Coughlin, S. R. (1998). Antibodies to protease-activated receptor 3 inhibit activation of mouse platelets by thrombin. **Blood**. 91(11) (Jun 1): 4152-4157.

Itoh, K., Udagawa, N., Matsuzaki, K., Takami, M., Amano, H., Shinki, T., et al. (2000). Importance of membrane- or matrix-associated forms of M-CSF and RANKL/ODF in osteoclastogenesis supported by SaOS-4/3 cells expressing recombinant PTH/PTHrP receptors. **J Bone Miner Res**. 15(9) (Sep): 1766-1775.

Jefferies, H. B., Reinhard, C., Kozma, S. C., Thomas, G. (1994). Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. **Proc Natl Acad Sci U S A**. 91(10) (May 10): 4441-4445.

Jeng, J. H., Lan, W. H., Wang, J. S., Chan, C. P., Ho, Y. S., Lee, P. H., et al. (2006). Signaling mechanism of thrombin-induced gingival fibroblast-populated collagen gel contraction. **Br J Pharmacol**. 147(2) (Jan): 188-198.

Jin, Q., Cirelli, J. A., Park, C. H., Sugai, J. V., Taba, M., Jr., Kostenuik, P. J., et al. (2007). RANKL inhibition through osteoprotegerin blocks bone loss in experimental periodontitis. **J Periodontol**. 78(7) (Jul): 1300-1308.

Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., et al. (1998). A dual thrombin receptor system for platelet activation. **Nature**. 394(6694) (Aug 13): 690-694.

Kandel, E. S., Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. **Exp Cell Res**. 253(1) (Nov 25): 210-229.

Kanzaki, H., Chiba, M., Shimizu, Y., Mitani, H. (2001). Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. **J Dent Res**. 80(3) (Mar): 887-891.

Karp, J. M., Tanaka, T. S., Zohar, R., Sodek, J., Shoichet, M. S., Davies, J. E., et al. (2005). Thrombin mediated migration of osteogenic cells. **Bone**. 37(3) (Sep): 337-348.

Kartsogiannis, V., Zhou, H., Horwood, N. J., Thomas, R. J., Hards, D. K., Quinn, J. M., et al. (1999). Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. **Bone**. 25(5) (Nov): 525-534.

Kassolis, J. D., Rosen, P. S., Reynolds, M. A. (2000). Alveolar ridge and sinus augmentation utilizing platelet-rich plasma in combination with freeze-dried bone allograft: case series. **J Periodontol**. 71(10) (Oct): 1654-1661.

Kearns, A. E., Khosla, S., Kostenuik, P. (2007). RANKL and OPG Regulation of Bone Remodeling in Health and Disease. **Endocr Rev**. (Dec 5):

Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., et al. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. **Cell**. 110(2) (Jul 26): 163-175.

Kim, D. H., Sarbassov, D. D., Ali, S. M., Latek, R. R., Guntur, K. V., Erdjument-Bromage, H., et al. (2003). GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. **Mol Cell**. 11(4) (Apr): 895-904.

Kong, Y. Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., et al. (1999). Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. **Nature**. 402(6759) (Nov 18): 304-309.

Lacey, D. L., Tan, H. L., Lu, J., Kaufman, S., Van, G., Qiu, W., et al. (2000). Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. **Am J Pathol**. 157(2) (Aug): 435-448.

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. **Cell**. 93(2) (Apr 17): 165-176.

Lafay, M., Laguna, R., Le Bonniec, B. F., Lasne, D., Aiach, M., Rendu, F. (1998). Thrombomodulin modulates the mitogenic response to thrombin of human umbilical vein endothelial cells. **Thromb Haemost**. 79(4) (Apr): 848-852.

Lam, J., Nelson, C. A., Ross, F. P., Teitelbaum, S. L., Fremont, D. H. (2001). Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. **J Clin Invest**. 108(7) (Oct): 971-979.

Lawlor, M. A., Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? **J Cell Sci**. 114(Pt 16) (Aug): 2903-2910.

Lee, S. K., Lorenzo, J. A. (1999). Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. **Endocrinology**. 140(8) (Aug): 3552-3561.

Lerner, U. H. (2004). New Molecules in the Tumor Necrosis Factor Ligand and Receptor Superfamilies with Importance for Physiological and Pathological Bone Resorption. **Crit Rev Oral Biol Med**. 15(2) 64-81.

Lerner, U. H., Gustafson, G. T. (1988). Blood coagulation and bone metabolism: some characteristics of the bone resorptive effect of thrombin in mouse calvarial bones in vitro. **Biochim Biophys Acta**. 964(3) (Mar 17): 309-318.

Lerner, U. H., Sahlberg, K., Ljunggren, O. (1989). Thrombin and bradykinin enhance prostaglandin production in human peripheral blood monocytes. **J Oral Pathol Med**. 18(4) (Apr): 246-250.

Li, J., Sarosi, I., Yan, X. Q., Morony, S., Capparelli, C., Tan, H. L., et al. (2000). RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. **Proc Natl Acad Sci U S A**. 97(4) (Feb 15): 1566-1571.

Liu, J., Schuff-Werner, P., Steiner, M. (2006). Thrombin/thrombin receptor (PAR-1)-mediated induction of IL-8 and VEGF expression in prostate cancer cells. **Biochem Biophys Res Commun**. 343(1) (Apr 28): 183-189.

Lu, H. K., Chen, Y. L., Chang, H. C., Li, C. L., Kuo, M. Y. (2006). Identification of the osteoprotegerin/receptor activator of nuclear factor-kappa B ligand system in gingival crevicular fluid and tissue of patients with chronic periodontitis. **J Periodontal Res**. 41(4) (Aug): 354-360.

Ma, Y. L., Cain, R. L., Halladay, D. L., Yang, X., Zeng, Q., Miles, R. R., et al. (2001). Catabolic effects of continuous human PTH (1--38) in vivo is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. **Endocrinology**. 142(9) (Sep): 4047-4054.

Macfarlane, S. R., Seatter, M. J., Kanke, T., Hunter, G. D., Plevin, R. (2001). Proteinase-activated receptors. **Pharmacol Rev**. 53(2) (Jun): 245-282.

Madamanchi, N. R., Li, S., Patterson, C., Runge, M. S. (2001). Thrombin regulates vascular smooth muscle cell growth and heat shock proteins via the JAK-STAT pathway. **J Biol Chem**. 276(22) (Jun 1): 18915-18924.

Magazine, H. I., Srivastava, K. D. (1996). Thrombin-induced vascular reactivity is modulated by ETB receptor-coupled nitric oxide release in rat aorta. **Am J Physiol**. 271(3 Pt 1) (Sep): C923-928.

Mamane, Y., Petroulakis, E., Rong, L., Yoshida, K., Ler, L. W., Sonenberg, N. (2004). eIF4E--from translation to transformation. **Oncogene**. 23(18) (Apr 19): 3172-3179.

Mann, K. G., Williams, E. B., Krishnaswamy, S., Church, W., Giles, A., Tracy, R. P. (1990). Active site-specific immunoassays. **Blood**. 76(4) (Aug 15): 755-766.

Manolagas, S. C., Jilka, R. L. (1995). Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. **N Engl J Med**. 332(5) (Feb 2): 305-311.

Marklund, M., Lerner, U. H., Persson, M., Ransjo, M. (1994). Bradykinin and thrombin stimulate release of arachidonic acid and formation of prostanoids in human periodontal ligament cells. **Eur J Orthod**. 16(3) (Jun): 213-221.

Martin, D., Rojo, A. I., Salinas, M., Diaz, R., Gallardo, G., Alam, J., et al. (2004). Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. **J Biol Chem**. 279(10) (Mar 5): 8919-8929.

Martin, K. A., Blenis, J. (2002). Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. **Adv Cancer Res**. 861-39.

McCawley, L. J., Matrisian, L. M. (2001). Matrix metalloproteinases: they're not just for matrix anymore! **Curr Opin Cell Biol**. 13(5) (Oct): 534-540.

Min, J. K., Kim, Y. M., Kim, Y. M., Kim, E. C., Gho, Y. S., Kang, I. J., et al. (2003). Vascular endothelial growth factor up-regulates expression of receptor activator of NF-kappa B (RANK) in endothelial cells. Concomitant increase of angiogenic responses to RANK ligand. **J Biol Chem**. 278(41) (Oct 10): 39548-39557.

Mirza, H., Schmidt, V. A., Derian, C. K., Jesty, J., Bahou, W. F. (1997). Mitogenic responses mediated through the proteinase-activated receptor-2 are induced by expressed forms of mast cell alpha- or beta-tryptases. **Blood**. 90(10) (Nov 15): 3914-3922.

Miura, M., Tanaka, K., Komatsu, Y., Suda, M., Yasoda, A., Sakuma, Y., et al. (2002). A novel interaction between thyroid hormones and 1,25(OH)(2)D(3) in osteoclast formation. **Biochem Biophys Res Commun**. 291(4) (Mar 8): 987-994.

Molino, M., Barnathan, E. S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., et al. (1997). Interactions of mast cell tryptase with thrombin receptors and PAR-2. **J Biol Chem**. 272(7) (Feb 14): 4043-4049.

Morabito, N., Gaudio, A., Lasco, A., Atteritano, M., Pizzoleo, M. A., Cincotta, M., et al. (2004). Osteoprotegerin and RANKL in the pathogenesis of thalassemia-induced osteoporosis: new pieces of the puzzle. **J Bone Miner Res**. 19(5) (May): 722-727.

Morony, S., Capparelli, C., Lee, R., Shimamoto, G., Boone, T., Lacey, D. L., et al. (1999). A chimeric form of osteoprotegerin inhibits hypercalcemia and bone resorption induced by IL-1beta, TNF-alpha, PTH, PTHrP, and 1, 25(OH)2D3. **J Bone Miner Res**. 14(9) (Sep): 1478-1485.

Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima, N., Yasuda, H., Yano, K., et al. (1998). RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. **Biochem Biophys Res Commun**. 253(2) (Dec 18): 395-400.

Nakamichi, Y., Udagawa, N., Kobayashi, Y., Nakamura, M., Yamamoto, Y., Yamashita, T., et al. (2007). Osteoprotegerin reduces the serum level of receptor activator of NF-kappaB ligand derived from osteoblasts. **J Immunol**. 178(1) (Jan 1): 192-200.

Nakanishi-Matsui, M., Zheng, Y. W., Sulciner, D. J., Weiss, E. J., Ludeman, M. J., Coughlin, S. R. (2000). PAR3 is a cofactor for PAR4 activation by thrombin. **Nature**. 404(6778) (Apr 6): 609-613.

Nakashima, T., Kobayashi, Y., Yamasaki, S., Kawakami, A., Eguchi, K., Sasaki, H., et al. (2000). Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. **Biochem Biophys Res Commun**. 275(3) (Sep 7): 768-775.

O'Brien, E. A., Williams, J. H., Marshall, M. J. (2000). Osteoprotegerin ligand regulates osteoclast adherence to the bone surface in mouse calvaria. **Biochem Biophys Res Commun**. 274(2) (Aug 2): 281-290.

O'Brien, E. A., Williams, J. H., Marshall, M. J. (2001). Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of mouse parietal bone and attach to the endocranial membrane. **Bone**. 28(2) (Feb): 208-214.

O'Brien, P. J., Molino, M., Kahn, M., Brass, L. F. (2001). Protease activated receptors: theme and variations. **Oncogene**. 20(13) (Mar 26): 1570-1581.

Offermanns, S. (2001). In vivo functions of heterotrimeric G-proteins: studies in Galpha-deficient mice. **Oncogene**. 20(13) (Mar 26): 1635-1642.

Offermanns, S., Laugwitz, K. L., Spicher, K., Schultz, G. (1994). G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. **Proc Natl Acad Sci U S A**. 91(2) (Jan 18): 504-508.

Olesen, P., Ledet, T., Rasmussen, L. M. (2005). Arterial osteoprotegerin: increased amounts in diabetes and modifiable synthesis from vascular smooth muscle cells by insulin and TNF-alpha. **Diabetologia**. 48(3) (Mar): 561-568.

Pagel, C. N., Sivagurunathan, S., Loh, L. H., Tudor, E. M., Pike, R. N., Mackie, E. J. (2006). Functional responses of bone cells to thrombin. **Biol Chem**. 387(8) (Aug): 1037-1041.

Parent, R., Kolippakkam, D., Booth, G., Beretta, L. (2007). Mammalian target of rapamycin activation impairs hepatocytic differentiation and targets genes moderating lipid homeostasis and hepatocellular growth. **Cancer Res**. 67(9) (May 1): 4337-4345.

Polson, A. M., Proye, M. P. (1983). Fibrin linkage: a precursor for new attachment. **J Periodontol**. 54(3) (Mar): 141-147.

Rajan, P., Panchision, D. M., Newell, L. F., McKay, R. D. (2003). BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. **J Cell Biol**. 161(5) (Jun 9): 911-921.

Roodman, G. D. (1996). Advances in bone biology: the osteoclast. **Endocr Rev**. 17(4) (Aug): 308-332.

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., et al. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. **Curr Biol**. 14(14) (Jul 27): 1296-1302.

Sarbassov, D. D., Guertin, D. A., Ali, S. M., Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. **Science**. 307(5712) (Feb 18): 1098-1101.

Scarborough, R. M., Naughton, M. A., Teng, W., Hung, D. T., Rose, J., Vu, T. K., et al. (1992). Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. **J Biol Chem**. 267(19) (Jul 5): 13146-13149.

Schneeweis, L. A., Willard, D., Milla, M. E. (2005). Functional dissection of osteoprotegerin and its interaction with receptor activator of NF-kappaB ligand. **J Biol Chem**. 280(50) (Dec 16): 41155-41164.

Shalhoub, V., Faust, J., Boyle, W. J., Dunstan, C. R., Kelley, M., Kaufman, S., et al. (1999). Osteoprotegerin and osteoprotegerin ligand effects on osteoclast formation from human peripheral blood mononuclear cell precursors. **J Cell Biochem**. 72(2) (Feb 1): 251-261.

Shapiro, M. J., Weiss, E. J., Faruqi, T. R., Coughlin, S. R. (2000). Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. **J Biol Chem**. 275(33) (Aug 18): 25216-25221.

Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., et al. (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. **Cell**. 89(2) (Apr 18): 309-319.

Singer, A. J., Clark, R. A. (1999). Cutaneous wound healing. **N Engl J Med**. 341(10) (Sep 2): 738-746.

Smith, B. B., Cosenza, M. E., Mancini, A., Dunstan, C., Gregson, R., Martin, S. W., et al. (2003). A toxicity profile of osteoprotegerin in the cynomolgus monkey. **Int J Toxicol**. 22(5) (Sep-Oct): 403-412.

Song, S. J., Pagel, C. N., Pike, R. N., Mackie, E. J. (2005). Studies on the receptors mediating responses of osteoblasts to thrombin. **Int J Biochem Cell Biol**. 37(1) (Jan): 206-213.

Stolovich, M., Tang, H., Hornstein, E., Levy, G., Cohen, R., Bae, S. S., et al. (2002). Transduction of growth or mitogenic signals into translational activation of TOP mRNAs is fully reliant on the phosphatidylinositol 3-kinase-mediated pathway but requires neither S6K1 nor rpS6 phosphorylation. **Mol Cell Biol**. 22(23) (Dec): 8101-8113.

Stuart, E. (2003). **Wound[Online].** Available from: http://www.medscape.com[2003, September 08]

Suda, T., Nakamura, I., Jimi, E., Takahashi, N. (1997). Regulation of osteoclast function. **J Bone Miner Res**. 12(6) (Jun): 869-879.

Suzuki, A., Kozawa, O., Shinoda, J., Watanabe, Y., Saito, H., Oiso, Y. (1996). Thrombin induces proliferation of osteoblast-like cells through phosphatidylcholine hydrolysis. **J Cell Physiol**. 168(1) (Jul): 209-216.

Takahashi, K., Murakami, M., Yamanaka, S. (2005). Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. **Biochem Soc Trans**. 33(Pt 6) (Dec): 1522-1525.

Takata, T. (1994). Oral wound healing concepts in periodontology. **Curr Opin Periodontol**. 119-127.

Tarzami, S. T., Wang, G., Li, W., Green, L., Singh, J. P. (2006). Thrombin and PAR-1 stimulate differentiation of bone marrow-derived endothelial progenitor cells. **J Thromb Haemost**. 4(3) (Mar): 656-663.

Tatakis, D. N. (1992). Blood coagulation factors in periodontal pathophysiology: a review with emphasis on the role of thrombin. **Semin Thromb Hemost**. 18(1) (Jan): 28-33.

Tatakis, D. N., Dolce, C., Dziak, R. (1989). Thrombin's effects on osteoblastic cells. I. Cytosolic calcium and phosphoinositides. **Biochem Biophys Res Commun**. 164(1) (Oct 16): 119-127.

Tomoyasu, A., Goto, M., Fujise, N., Mochizuki, S., Yasuda, H., Morinaga, T., et al. (1998). Characterization of monomeric and homodimeric forms of osteoclastogenesis inhibitory factor. **Biochem Biophys Res Commun**. 245(2) (Apr 17): 382-387.

Tsuda, E., Goto, M., Mochizuki, S., Yano, K., Kobayashi, F., Morinaga, T., et al. (1997). Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. **Biochem Biophys Res Commun**. 234(1) (May 8): 137-142.

Udagawa, N., Takahashi, N., Yasuda, H., Mizuno, A., Itoh, K., Ueno, Y., et al. (2000). Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. **Endocrinology**. 141(9) (Sep): 3478-3484.

Verderio, E. A., Johnson, T., Griffin, M. (2004). Tissue transglutaminase in normal and abnormal wound healing: review article. **Amino Acids**. 26(4) (Jul): 387-404.

Vu, T. K., Hung, D. T., Wheaton, V. I., Coughlin, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. **Cell**. 64(6) (Mar 22): 1057-1068.

Wang, H., Ubl, J. J., Stricker, R., Reiser, G. (2002). Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. **Am J Physiol Cell Physiol**. 283(5) (Nov): C1351-1364.

Wang, L., Luo, J., He, S. (2007). Induction of MMP-9 release from human dermal fibroblasts by thrombin: involvement of JAK/STAT3 signaling pathway in MMP-9 release. **BMC Cell Biol**. 814.

Widmann, C., Gibson, S., Jarpe, M. B., Johnson, G. L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. **Physiol Rev**. 79(1) (Jan): 143-180.

Wikesjo, U. M., Nilveus, R. E., Selvig, K. A. (1992). Significance of early healing events on periodontal repair: a review. **J Periodontol**. 63(3) (Mar): 158-165.

Wong, B. R., Josien, R., Lee, S. Y., Sauter, B., Li, H. L., Steinman, R. M., et al. (1997). TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. **J Exp Med**. 186(12) (Dec 15): 2075-2080.

Wool, I. G., Cavicchi, P. (1966). Insulin regulation of protein synthesis by muscle ribosomes: effect of the hormone on translation of messenger RNA for a regulatory protein. **Proc Natl Acad Sci U S A**. 56(3) (Sep): 991-998.

Xu, W. F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., et al. (1998). Cloning and characterization of human protease-activated receptor 4. **Proc Natl Acad Sci U S A**. 95(12) (Jun 9): 6642-6646.

Yamaguchi, K., Kinosaki, M., Goto, M., Kobayashi, F., Tsuda, E., Morinaga, T., et al. (1998). Characterization of structural domains of human osteoclastogenesis inhibitory factor. **J Biol Chem**. 273(9) (Feb 27): 5117-5123.

Yasuda, H., Shima, N., Nakagawa, N., Mochizuki, S. I., Yano, K., Fujise, N., et al. (1998). Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. **Endocrinology**. 139(3) (Mar): 1329-1337.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., et al. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. **Proc Natl Acad Sci U S A**. 95(7) (Mar 31): 3597-3602.

Yuan, Y. Y., Kostenuik, P. J., Ominsky, M. S., Morony, S., Adamu, S., Simionescu, D. T., et al. (2007). Skeletal deterioration induced by RANKL infusion: a model for high-turnover bone disease. **Osteoporos Int**. (Nov 24):

Yun, T. J., Chaudhary, P. M., Shu, G. L., Frazer, J. K., Ewings, M. K., Schwartz, S. M., et al. (1998). OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. **J Immunol**. 161(11) (Dec 1): 6113-6121.

Ziolkowska, M., Kurowska, M., Radzikowska, A., Luszczykiewicz, G., Wiland, P., Dziewczopolski, W., et al. (2002). High levels of osteoprotegerin and soluble receptor activator of nuclear factor kappa B ligand in serum of rheumatoid arthritis patients and their normalization after anti-tumor necrosis factor alpha treatment. **Arthritis Rheum**. 46(7) (Jul): 1744-1753.

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