國立臺灣大學醫學院生物化學暨分子生物學研究所

碩士論文

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探討希樂葆在攝護腺癌細胞移動、侵襲以及間質蛋白酶活化的影響

Suppression of prostate cancer cell migration and invasion by celebrex through down-regulating matriptase activity

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口試委員會審定書

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本論文係盧盈潔君（學號 R98442014）在國立臺灣大學醫學院生物化學暨分子生物學研究所完成之碩士學位論文，於民國一百年七月十九日承下列考試委員審查通過及口試及格，特此證明

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盈潔 民國一百年七月於台大生化所

中文摘要

攝護腺癌的致死率在西方男性世界中排名第二位；在台灣，攝護腺癌的發生率以及死亡率亦有逐年增加的趨勢。有些研究指出發炎與攝護腺癌的產生以及惡化有關。希樂葆 (Celebrex) 是一種亞型環氧化酶 (Cyclooxygenase-2, COX-2) 抑制物，近年研究指出希樂葆具有抗發炎、抗癌以及預防癌症產生 (chemoprevention) 的功能。然而，希樂葆是如何抑制攝護腺癌細胞侵襲的分子機制尚未清楚了解。在本篇研究裡，我們建立了攝護腺癌細胞侵襲能力進化的細胞模式 (PC-3 以及 M2I2 PC-3 細胞)，並發現幾個發炎相關蛋白，亞型環氧化酶、磷酸化 JUN 激酶以及第一型介白素在 M2I2 PC-3 細胞的表現量增加。進而檢測了希樂葆對於攝護腺癌 PC-3 細胞的生長、移動與侵襲的影響。實驗結果指出希樂葆可有效地抑制攝護腺癌細胞的生長、移動與侵襲能力。為了更進一步研究希樂葆抑制攝護腺癌細胞移動與侵襲的分子機制，我探討希樂葆對於間質蛋白酶 (Matriptase) 可能的影響。間質蛋白酶是第二型嵌膜絲胺酸蛋白酶，近年來研究指出異常活化的間質蛋白酶與許多癌症的演進有相關聯，包括攝護腺癌。本篇實驗結果指出，希樂葆可以降低間質蛋白酶的表現以及釋出到細胞外的量；此量的降低，主要藉由抑制間質蛋白酶基因的表達以降低其表現量。更進一步地，在兩株不具亞型環氧化酶的攝護腺癌 DU-145 和 LNCaP 細胞中，希樂葆一樣可以藉由降低間質蛋白酶活化及表現量來抑制這兩種癌細胞的移動與侵襲能力。同時發現，在所有市面上可購得的非類固醇抗炎藥物中，希樂葆是最具潛力的藥物可抑制攝護腺癌細胞的間質蛋白酶活化。同時亞型環氧化酶主要產物 PGE_2 可刺激間質蛋白酶的活化。為了更進一步了解 PGE_2 如何促進間質蛋白酶的活化，我使用不同的 EP 接受器抑制物並發現 EP1 接受器可能參與間質蛋白酶的活化。整體來說，本論文實驗結果指出希樂葆具有抑制間質蛋白酶功能以及壓抑攝護腺癌細胞侵襲的能力。因此希樂葆是一個未來具有潛力的藥物，可用以抗攝護腺癌或預防此癌症的產生。

Abstract

Prostate cancer is the second leading cause of cancer-related death in men of the western world. In Taiwan, the incidence and mortality of prostate cancer (PCa) have been rising progressively in recent years. Several studies have showed that inflammation is involved in the development and progression of PCa. Celebrex, a COX-2 specific inhibitor, has been shown with anti-inflammatory, anti-carcinogenic, and chemopreventive effects. However, the molecular mechanism how celebrex suppresses PCa cell invasion is not well understood. In this study, we established a PC-3 cell invasion progression model (parental and M2I2 PC-3 cells) and found that several inflammation-associated proteins, COX-2, p-JNK and IL-1β were up-regulated in M2I2 PC-3 cells. The results showed that celebrex could significantly suppress PCa cell migration and invasion, at least in part, due to down regulation of a tumor-promoting serine protease matriptase at the gene expression and activation levels. Similarly, celebrex also can execute its anti-cancer properties in two COX-2-null PCa DU-145 and LNCaP cells, *via* a similar mechanism as shown in PC3 cells. Furthermore, PGE2, a main product of COX-2, could induce matriptase activation and its EP1 receptor was identified to be involved in matriptase activation in PCa cells. Taken together, the data indicated that celebrex exhibits a suppressive effect on PCa cell migration and invasion, at least in part, by down-regulating matriptase function.

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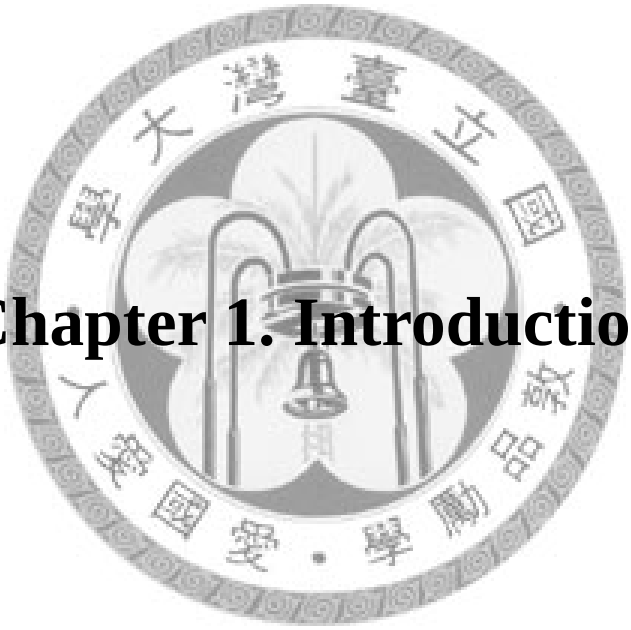


Chapter 1. Introduction

1.1 Prostate cancer

Prostate cancer is one of the most prevalent malignancies affecting men worldwide (1). In Taiwan, the incidence of prostate cancer has been rising in recent years with seventh leading cause of cancer deaths in 2010 (2). When cancer cells are confined within the capsule, patients are often treated with prostatectomy or radiation to remove or destroy the cancer lesions (3). Alternatively, some prostate cancer patients adopt androgen ablation therapy since most of early-staged prostate cancers are hormone-dependent. However, hormone-refractory prostate cancer frequently emerges during this therapy (4, 5). These recurrent prostate cancers become aggressive with high metastatic potentials and poor prognosis. At present, there is no efficacious therapy for advanced prostate cancer with hormone-refractory or metastatic phenotype (6). Thus, new molecular targets or drugs are needed to develop useful therapeutic approaches for treatment prostate cancer.

1.2 Inflammation in prostate carcinogenesis

Inflammation is a homeostatic response to tissue injury or infection, by recruiting inflammatory cells to clean up damaged tissue or to sequester pathogens (7). It has been reported that approximately 20% of all human cancers are caused by chronic inflammation, including prostate cancer (8). Exposure to environmental factors such as

infectious agents and dietary carcinogens, and hormonal imbalances lead to injury of the prostate and the development of proliferative inflammatory atrophy (PIA). PIA is a focal atrophic lesion and associated with chronic inflammation. In tissues, PIA is often directly adjacent to the lesions of prostatic intraepithelial neoplasia (PIN) and prostate cancers. Epithelial cells in PIA lesions usually highly express many molecular signs of stress, such as glutathione S-transferase P1 (GSTP1), glutathione S-transferase A1 (GSTA1), and cyclooxygenase-2 (COX-2) (9). Up-regulation of these molecules can increase genetic instability that might then induce high-grade PIN and early prostate cancer development, even for the cancer progression.

1.3 Cyclooxygenase-2 and cancer progression

Cyclooxygenase (COX) is a rate-limiting enzyme to catalyze the conversion of arachidonic acid to prostanoids, including prostaglandins and thromboxane A_2 (TXA_2) (10). Two isoforms of COX enzyme, COX-1 and COX-2, have been identified with a similar enzymatic activity (11). COX-1 is expressed commonly in most tissues to generate prostaglandins that modulate normal physiological functions, such as maintenance of the gastric mucosa and regulation of renal blood flow. COX-2, on the other hand, is not routinely expressed in most tissues, but is induced by a wide spectrum of growth factors and pro-inflammatory cytokines (12). Many studies suggest an

important role of COX-2 in pathophysiology of inflammation and carcinogenesis (12-14). Increased amounts of COX-2 are frequently found in both premalignant tissues and malignant tumors, including colon, breast, prostate and lung tumor (14). Prostaglandin E_2 (PGE_2) is the most abundant product of COX-2 that is found in various human malignancies. PGE_2 exerts its cellular effects *via* binding to its cognate E-prostanoid (EP) receptors (EP1-4). EP receptors belong to a family of transmembrane G-protein coupled receptors (15). EP1 is a $G\alpha_q$-coupled receptor that promotes calcium mobilization and PKC activation. EP2 and EP4 are coupled to $G\alpha_s$ and can activate adenylate cyclase, whereas EP3 is a $G\alpha_i$-coupled receptor that inhibits adenylate cyclase (16). Several lines of evidence reported that PGE_2 signaling can promote tumor growth and angiogenesis (17), and COX-2 affects many carcinogenic processes including angiogenesis (13), apoptosis (18), immunosuppression (19), and invasiveness (20). Thus, COX-2 may represent a potential therapeutic target for prostate cancer.

1.4 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used as effective anti-inflammatory, antipyretic and analgesic drugs. All traditional NSAIDs (e.g., aspirin) are able to inhibit both COX-1 and COX-2. However, long-term use of traditional NSAIDs, which inhibit both COX-1 and COX-2, is associated with serious

gastrointestinal side effects, such as ulceration and perforation of the gastric mucosa. These side effects have been attributed to the inhibition of COX-1, which mediates gastroprotective prostaglandin production. These observations led to the design and synthesis of a new class of NSAIDs that specifically inhibit COX-2 for suppressing inflammation (21). Despite the canonical anti-inflammatory activity of NSAIDs, several recent studies indicated that NSAIDs might be beneficial in the treatment of cancers (12, 15, 22). In fact, it has been reported that a reduction of 15~20% in the risk of prostate cancer in regular users of NSAIDs compared with non-users (22). Moreover, using COX-2 specific inhibitor in the human prostate tumor cell line DU-145 resulted in a decreased secretion of MMP-2 and MMP-9 (20). NSAIDs can inhibit angiogenesis through increased endothelial cell apoptosis, inhibition of endothelial cell migration and recruitment of inflammatory cells, all of which have been associated with growth inhibition and attenuation of the metastatic potential of cancer cells (23).

1.5. Celebrex (Celecoxib)

Celebrex is a COX-2 specific inhibitor that is developed as an alternative to aspirin. It is currently available for clinical use in the United States for the treatment of chronic arthritic conditions such as rheumatoid arthritis and osteoarthritis. On the other hand, they show promising not just for pain but also for cancer prevention. The US Food and

Drug Administration (FDA) approved daily doses of celebrex for reducing colon cancer risk in people with a rare genetic disease called familial adenomatous polyposis (FAP) (12). Pharmacological and genetic reports have indicated that a significant component of the anti-cancer properties of NSAIDs is due to their ability to inhibit the COX-2 enzyme. In addition, some mechanisms independent of COX-2 have been recently proposed to be involved in the anti-neoplastic effects of celebrex (23).

1.5.1 COX-2-independent effects of celebrex

Many studies have reported that celebrex reduces cell proliferation, migration and induces apoptosis in prostate cancer cells (24, 25). Molecular mechanisms involved in celebrex-induced cell cycle arrest are at least partly due to inhibition of protein kinase B (PKB/Akt) or its upstream kinase phosphoinositide-dependent kinase 1 (PDK-1) (26). This is because a celebrex analogue that lacks COX-2 inhibitory activity also can inhibit PDK-1 and PKB activity. Although these inhibitory effects may be independent of COX-2, the molecular mechanisms for celebrex to inhibit PKB or PDK-1 is still unclear. It has been shown that PKB can induce anti-apoptotic effects by phosphorylating procaspase 9 to prevent caspase 9 activation and then keep the pro-apoptotic protein BAD stay inactive (27). Thus, inhibition of PKB by celebrex promotes the activation of caspase 9 and BAD, leading to apoptosis. Moreover, celebrex also alters the expression

and activity of matrix metalloproteases (MMPs). Treatment with celebrex inhibited the activity and secretion of MMP-2 and MMP-9 in lung adenocarcinoma cells, and concurrent with inhibition of cell migration and invasion (28). The expression of MMP-2 and MMP-9 depends on activated PKB in human glioblastoma cells, and inhibition of PKB phosphorylation by celebrex leads to reducing these cell invasion (29). It indicated that inhibition of PDK-1 and PKB/AKT appears to play a role in celebrex-induced apoptosis, cell cycle arrest and down-regulation of angiogenesis and metastasis.

1.6 Matriptase

Matriptase was first isolated in the conditioned media from human breast cancer cells due to its gelatinolytic activity and was therefore thought to be involved in the degradation of the extracellular matrix (ECM) including fibronectin and laminin (30, 31). Matriptase, also named as membrane-type serine protease 1 (MT-SP1) (32), tumor-associated differentially expressed gene-15 (TADG-15) (33), suppression of tumorigenecity 14 (ST-14) (34), is a membrane-anchored serine protease. The orthologe of matriptase in mice is also known as epithin. Matriptase has been detected in various epithelial tissues and epithelial-derived cell lines.

1.6.1 Domain structure of matriptase

Matriptase is a 855 amino acid protein with a molecular mass of 94.7 kDa (35). It is consisted of an intracellular N-terminus (residues 1-54) followed by a transmembrane domain, a SEA domain (residues 86-201), two tandem CUB domains (residues 214-334 and 340-447), four LDL receptor (LDLR) domains (residues 452-486, 487-523, 524-561, and 566-604) and a C-terminal catalytic domain (residues 614-855) (36). The protein structure of matriptase is shown in Figure I. The N-terminal signal anchor of matriptase, which is not removed during the protein synthesis, and guides the protease to have a type II integral membrane domain as a linker between its cytoplasmic N-terminus and extracellular C-terminus (37).

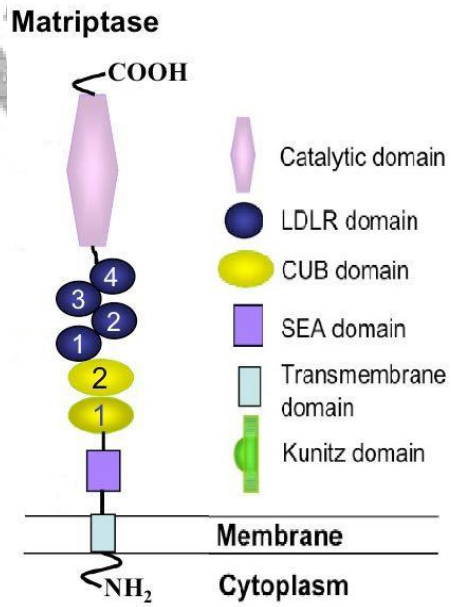


Figure I. The structure of matriptase [modified from (37)].

The stem regions of matriptase including SEA domains, CUB domains and LDLR domains, are non-catalytic and may function in protein-protein interactions or play a role in matriptase's subcellular localization, activation, inhibition, and substrate recognition (38, 39). Four potential N-linked glycosylation sites are also found in matriptase (Asn109, 302, 485, and 772). Glycosylation on Asn^{302} and Asn^{772} have been shown to be important for the protease activation (38). The C-terminus of matriptase consists of a serine protease catalytic triad (His^{656}, Asp^{711} and Ser^{805}) which are essential for its proteolytic activity and autoactivation (40, 41).

1.6.2 Proteolytic processing and activation of matriptase

Matriptase is synthesized as an inactive, single-chain polypeptide. The activation of matriptase requires two sequential endoproteolytic cleavages following a simultaneous interaction with its cognate inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1). The first cleave of matriptase zymogen occurs at G149 at SEA domain within endoplasmic reticulum or Golgi apparatus (42) by unknown proteolytic activity or possibly by nonenzymatic hydrolysis of the peptide bond to produce a 70-kDa single-chain, latent form of matriptase (43). After G149 processing, matriptase requires HAI-1 for its intracellular trafficking *via* the interaction of LDLR domains on each protein (44). Upon activation, matriptase undergoes an autocatalytic cleavage at

Arg614 within C-terminal catalytic domain to convert a single-chain latent form to a two-chain form of active matriptase with 26 kDa serine protease domain linked to the fourth LDLR domain through a disulfide bond (45). After activation, the active matriptase is quickly inhibited by HAI-1 with a formation of 120 kDa complexes. Then, the matriptase-HAI-1 complex is shed with a molecular mass of 95 or 110 kDa to conditioned media or extracellular environments, *via* an unknown mechanism. The processes of matriptase activation, inhibition and shedding are shown in Figure II.

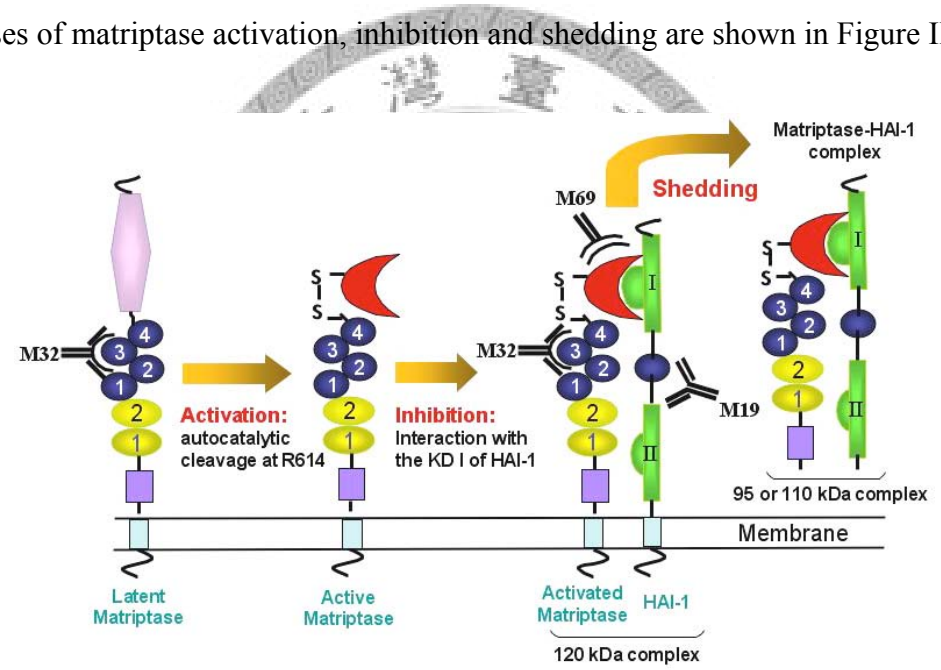


Figure II. The process of matriptase activation, inhibition and shedding [modified from (37)].

To analyze the protein levels of matriptase, HAI-1 and the complexes they form, three monoclonal antibodies (M32, M69 and M19) are generated by Dr. Chen-Yong Lin's group (45). M32 is an anti-total matriptase monoclonal antibody that recognizes the third LDLR domain and can detect a 120 kDa matriptase-HAI-1 complex, 95 kDa

full-length matriptase, 95 kDa of the catalytic domain of matiptase-HAI-1 complex and 70 kDa latent form of matriptase. M69 is an anti-activated matriptase monoclonal antibody that is able to distinguish the activated matriptase from its latent part, and mainly detect 120 kDa matriptase-HAI-1 complexes. M19 is an anti-HAI-1 monoclonal antibody which can recognize a 55 kDa free (uncomplexed) HAI-1 and the HAI-1 in the complex with matriptase. In addition, 95 kDa and 110 kDa of matriptase-HAI-1 complexes also can be detected in conditioned media by M32, M69 and M19 antibodies (46). The patterns of matriptase and HAI-1 detected by these three monoclonal antibody are showed as Figure III.

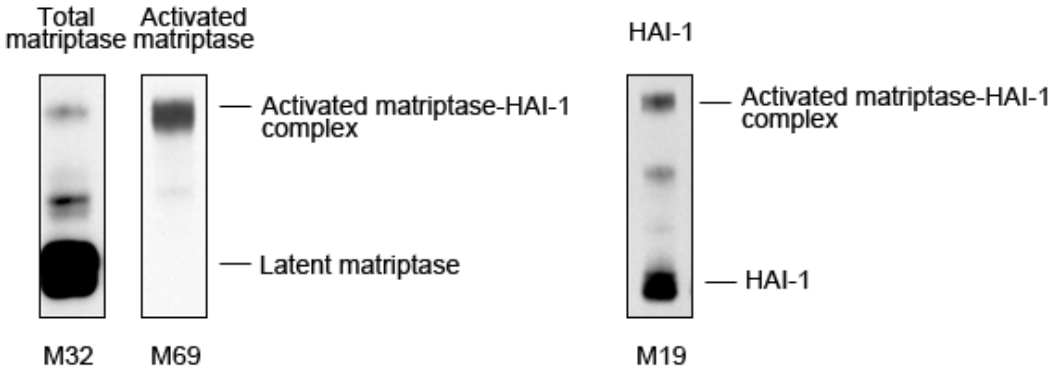


Figure III. Monoclonal antibodies for the detection of matriptase, HAI-1 and their complex in PC-3 cells. [modified from Ya-Yun Lin's thesis (2010)]

1.6.3 Relationship between HAI-1 and matriptase

Hepatocyte growth factor activator inhibitor-1 (HAI-1) is encoded by the s*erine protease inhibitor Kunitz type 1 (SPINT1)* gene (47). HAI-1 has 478 amino acids with a

calculated molecular mass of 55 kDa and serves as a protease inhibitor for HGFA or matriptase (48). HAI-1 has not an only inhibitory function but also a chaperon activity for matriptase's biosynthesis, intracellular trafficking and activation (44). The existence of HAI-1 is thought to ensure that matriptase can be quickly inactivated after activation to prevent cells from uncontrolled matriptase activity and incorrect signaling (38). The ratio of matriptase to HAI-1 has been shown to be shifted towards matriptase in late cancer stage and the imbalance has been proposed to promote the proteolytic activity of matriptase, which has been implicated in cancer malignancy (49).

1.6.4 Physiological functions of matriptase

Matriptase is strongly expressed in epithelial tissues, such as stomach, pancreas, gallbladder, colon, and prostate (50, 51). Physiologically, matriptase has been proposed to play an important role in the maintenance of epithelial structures (52, 53) and in tissue remodeling (30, 31). Moreover, matriptase activity is required for the activation of a membrane-bound serine protease, prostasin (54). The study about matriptase-deficient mice suggests that matriptase is essential in the formation of epidermal barrier and lack of matriptase may cause fatal dehydration within 48 hrs after birth (55, 56). Embryonic or postnatal ablation of matriptase in epithelial tissue caused severe organ dysfunction, which was associated with increased permeability, loss of tight junction-

associated proteins, and generalized epithelial demise (55). Taken together, the function of matriptase is proposed to be implicated in epithelial cell homeostasis including proliferation, differentiation, and migration.

1.6.5 Role of matriptase in human cancer and tumor progression

Matriptase has been shown to be over-expressed in variety of epithelia-derived human tumors, including prostate (57, 58), breast (59), colon (74), stomach (60), ovarian (61) and renal cancer (62). In human cancer, matriptase has been proposed as an oncogenic protein with function to promote tumor cell invasion and metastasis (35, 48). In prostate cancer, matriptase expression is significantly increased in malignant tumors compared to adjacent normal parts, and the expression levels of matriptase are correlated with tumor grades (63). In colorectal cancer cells, matriptase overexpression is shown to significantly enhance their invasiveness (64). There is also extensive evidence that the matriptase level is augmented by either deregulation, stabilization or overexpression in a variety of tumor tissues (65). These studies show that elevated levels of matriptase can promote cancer cell invasion and suggest a direct involvement of the protease in the development and progression of cancer. Matriptase exhibits a trypsin-like proteolytic activity which can activate a number of proteins, including protease-activated receptor-2 (PAR-2), MMP3, prostasin, single-chain urokinase-type

plasminogen activator (uPA) and the proform of hepatocyte growth factor (proHGF) (53, 66). In the matriptase transgenic mice, matriptase overexpression promoted the tumorigenecity and carcinogen-induced tumor formation (67). Taken together, it indicates that dysregulation of matriptase promotes the development and progression of human cancer and it may serve as a new potential target for cancer therapies.

1.7 Research motivation

Prostate cancer is rising progressively in recent years in Taiwan and one of the major causes of cancer-related death in the western world. Metastatic progression of prostate cancer is one of the main causes for low survival rate. Since inflammation has been proposed to promote in the prostate carcinogenesis and disease progression, in this study, I was interested in examining the effect of the anti-inflammation by using a NSAID, celebrex, on prostate cancer cell proliferation, migration and invasion. Several study aims were addressed as follows. (1) To analyze the effect of celebrex on prostate cancer cell proliferation, migration and invasion. (2) To isolate a serine protease affected by celebrex in prostate cancer cells. (3) To delineate whether the reduction of activated matriptase by celebrex leads to decreasing prostate cancer cell migration and invasion. (4) To investigate whether celebrex was through a COX-2-independent mechanism to inhibit prostate cancer cell migration and invasion. From these studies,

the information will provide some insights for the potential of a current clinically used drug, celebrex, in prostate cancer chemoprevetion and chemotherapy.

Chapter 2. Materials and methods

2.1 Materials

2.1.1 Cell lines

PC-3, DU-145 and LNCaP cells were originally obtained from ATCC. Human prostate cancer PC-3 cells were isolated from a bone metastasis (68). Human prostate cancer DU-145 cells were isolated from a brain metastasis (69). Androgen-responsive human prostate carcinoma LNCaP cells were isolated from lymph node metastasis (70).

2.1.2 Antibodies

(1) Matriptase and HAI-1: monoclonal M32 (total matriptase), M69 (activated matriptase), and M19 (HAI-1) mAbs were a gift from Dr. Chen-Yong Lin, Greenebaum Comprehensive Cancer Center, Department of Biochemistry and Molecular Biology, University of Maryland, Baltimore, MD 21201

(2) Anti-β-actin Ab: Sigma, MO, USA

(3) Anti-COX-2 Ab: Cayman Chemical, USA

(4) Anti-iNOS Ab: GeneTex, Taiwan

(5) Anti-IL-1β Ab: Abcam, USA

(6) Anti-phospho-SAPK/JNK Ab: Cell Signaling, USA

(7) Anti-V5 Ab: Invitrogen, CA, USA

(8) HRP-conjugated Goat anti-mouse IgG: Jackson Immuno Research, USA

(9) HRP-conjugated Goat anti-rabbit IgG: Jackson Immuno Research, USA

2.1.3 Enzymes

(1) SuperScriptTM Ⅲ Reverse Transcriptase: Invitrogen, USA

(2) Taq polymerase: Bioman, Taiwan

2.1.4 Reagents

(1) 2-Mercaptoethanol: Sigma-Aldrich, USA

(2) 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB): Sigma-Aldrich, USA

(3) 100 bp DNA marker: Bioman, Taiwan

(4) Agarose: Uni-Region, USA

(5) Ampicillin: Sigma-Aldrich, USA

(6) Bovine serum albumin: Sigma-Aldrich, USA

(7) Celebrex: Sigma-Aldrich, USA

(8) Chloroform: Sigma-Aldrich, USA

(9) Crystal violet: Sigma-Aldrich, USA

(10) Diethyl pyrocarbonate (DEPC)-H_2O: Invitrogen, USA

(11) Dulbecco's Modified Eagle Medium (DMEM): Gibco, USA

(12) Dimethyl sulfoxide (DMSO): Sigma-Aldrich, USA

(13) ECL (enhanced chemiluminescence): Thermo, IL, USA

(14) Fetal bovin serum (FBS): Gibco, USA

(15) Gelatin: Sigma-Aldrich, USA

(16) Geneticin (G418): Gibco, USA

(17) Glycine: J.T. Baker, USA

(18) Glutamine: Sigma-Aldrich, USA

(19) Isopropanol: Sigma-Aldrich, USA

(20) L-161982: Cayman Chemical, USA

(21) L798106: Cayman Chemical, USA

(22) Lipofectamine 2000™: Invitrogen, USA

(23) Matrigel: BD biosciences, USA

(24) Methanol: Sigma-Aldrich, USA

(25) OPTI-MEM: Gibco, USA

(26) Penicillin/streptomycin: Gibco, USA

(27) Phosphate buffered saline (PBS): Gibco, USA

(28) Plasmid Midi Kit: Geneaid, Taiwan

(29) Prestained protein ladder: Fermentas, USA

(30) Prostaglandin E_2 (PGE_2): Cayman Chemical, USA

(31) RPMI-1640 medium: Gibco, CA, USA

(32) SC51322: Cayman Chemical, USA

(33) Sodium pyruvate: Sigma-Aldrich, USA

(34) TEMED: J.T. Baker, USA

(35) Triton X-100: J.T. Baker, USA

(36) Trizol: Invitrogen, USA

(37) Tris-Base: J.T. Baker, USA

(38) Tris-HCl: J.T. Baker, USA

(39) Trypsin-EDTA: Invitrogen, USA

(40) Tween-20: Fluka, USA

2.2 Methods

2.2.1 Cell culture

PC-3 cells were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin in a humidified, 5% CO_2, 37 °C incubator. DU-145 and LNCaP cells were maintained in RPMI-1640 medium supplemented with 5% FBS, 2 mM glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin in a humidified, 5% CO_2, 37 °C incubator.

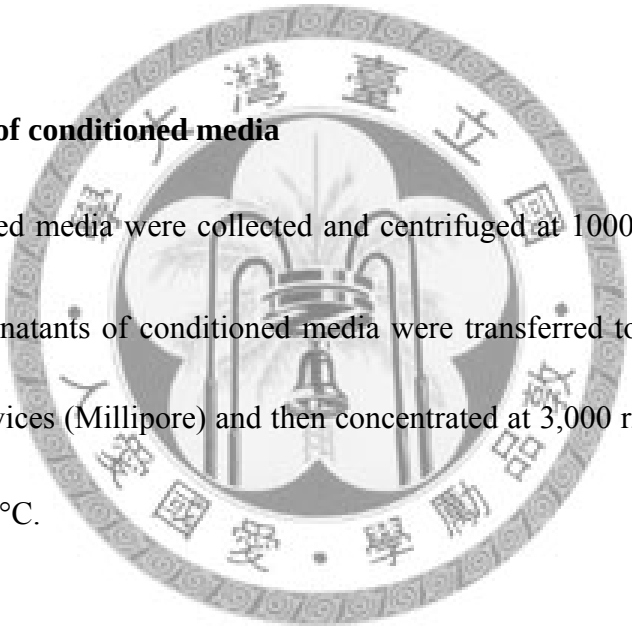
2.2.2 Celebrex and PGE_2 treatment

Celebrex (Sigma-Aldrich, USA) and PGE_2 (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and stored in a dark colored eppendorf at -20 °C. The stock solution was further diluted to the indicated concentration at each experiment. Before the treatments, cells were grown up to 80% confluence and then exposed to celebrex or PGE_2 at different concentrations and for various time periods. DMSO was used for adjustment to make each set of treatment with an equal amount of solvent.

2.2.3 Protein extraction

To prepare cell lysates, cells were washed twice with ice-cold PBS and lysed in a

lysis buffer (As shown in 2.3 Buffer). Lysed cells on petri dishes were scratched by a scraper, then collected to an eppendorf and kept on ice for 10 min. Cell lysates were centrifuged at 13,000 r.p.m., 4 °C, for 20 minutes. After centrifugation, the supernatant were collected. Protein concentrations of the supernatants were determined by spectrophotometry with Protein Assay reagent (Bio-Rad) and calculated by a BSA standard curve using the Microsoft Excel program.



2.2.4 Preparation of conditioned media

The conditioned media were collected and centrifuged at 1000 r.p.m., 4 °C, for 5 minutes. The supernatants of conditioned media were transferred to Amicon® Ultra-4 centrifuge filter devices (Millipore) and then concentrated at 3,000 r.p.m. centrifugation for 30 minutes at 4 °C.

2.2.5 Western blot analysis

For matriptase western blotting by M32 and M69 mAbs, and HAI-1 detection by M19 mAb. Equal amounts of cell lysates were taken and mixed with 5X sample buffer without any reducing reagent for SDS-PAGE. For other samples, equal amounts of cell lysates were mixed with 6X protein loading dye with 5% 2-Mercaptoethanol and boiled for 10 min. The samples were separated by 10% SDS-PAGE and transferred to

nitrocellular membranes (Whatman, USA) within Towbin transfer buffer at 300 mA for 3 hrs in a 4 °C refrigerator. The protein-transferring efficiency can be checked by staining nitrocellular membranes with Ponceau S dye. After staining, Ponceau S dyes were cleaned out using TBST buffer. The membranes were blocked with 5% skim milk in TBST at room temperature for 1 hr and then incubated with primary antibodies in blocking buffer at 4°C, shaking overnight. Primary antibodies were then discarded and the membranes were washed with TBST four times, 10 min per wash. The membranes were then incubated with secondary antibodies conjugated with Horseradish peroxidase (Jackson, USA) in blocking buffer at room temperature for 1 hr. Secondary antibody solutions were removed and the membranes were washed by TBST four times, 10 min per wash. The target proteins on membranes were visualized using an Enhanced Luminol Reagent *Plus* (Perkin Elmer, U.S.A.) and detected by a luminescent image analyzer with a CCD camera (LAS-4000; Fujifilm, Japan).

2.2.6 Cell viability assay and IC_{50} determination

PC-3 or DU-145 cells were seeded at densities of $3x10^5$ and $5x10^4$ cells/cm^2 in 24-well plates in 10% FBS DMEM or 5% FBS RPMI-1640 medium and maintained in a 37 °C, 5% CO_2 incubator. LNCaP cells were seeded at densities of $1.5x10^6$ and $1x10^5$ cells/cm^2 within 5% FBS RPMI-1640 medium at 37 °C in a 5% CO_2 incubator. Next

day, celebrex was added at indicated concentrations. After 16-hour treatment, 0.5 mg/ml MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were added into each well and incubated with cells for 2 hr at a 37 °C incubator. The oxidization form of MTT, formazan with a purple color, was then dissolved in DMSO and measured at 540 nm using a spectrophotometer. Each set of experiments was done in triplicate. The results were statistically calculated and represented as mean ± S.D.

2.2.7 Cell growth assay

$1x10^4$ of PC-3 or DU-145 cells were seeded in each well of 24-well plates in 10% FBS DMEM or 5% FBS RPMI-1640 medium and cultured at a 37 °C, 5% CO_2 humidified incubator. Twenty-four hours after seeding, cells were treated with indicated concentrations of celebrex. Control cells were treated with DMSO (solvent control). The media were refreshed every day. Cell viability was measured using MTT methods at each time point. Each set of experiment was performed in triplicate. The data were statistically calculated with normalization to day 0 and shown as mean ± S.D.

2.2.8 Migration and invasion assays

The effect of celebrex on the invasion and migration of cancer cells was evaluated by matrigel-coated or uncoated transwell assays. For invasion assay, 3 μg matrigels (BD

biosciences, USA) were diluted in 100 μl serum-free medium, added on the upper chamber of each transwell and air-dried overnight. Transwell chambers without any coating were used for migration assay. Before seeding, PC-3, DU-145 or LNCaP cells were starved for 24 hrs. 1 x 10^5 cells ($3x10^5$ cells/cm^2) of serum-starved PC-3 and DU-145 cells in 200 μl serum-free media were seeded onto the upper chamber of a transwell with different concentrations of celebrex and PGE_2. DMSO was used as control. 5 x 10^5 cells ($1.5x10^6$ cells/cm^2) of serum-starved LNCaP cells in 200 μl serum-free medium were seeded onto the upper chamber of a transwell with different concentrations of celebrex. Control cells were treated with DMSO. Lower chambers of transwells were added with 750ul DMEM or RPMI-1640 medium with 10% FBS as chemoattractants containing the same concentrations of celebrex and PGE_2 as that in the upper chamber. The durations for PC-3, DU-145 and LNCaP cell migration and invasion were 16, 16 and 24 hours. Cells were then fixed with methanol for 15 minutes and stained with crystal violet for 1 hour. The cells on the upper surface of transwells were wiped off with a cotton swab. Invaded or migrated cells on the lower surface of transwells were photographed (100x) under a light microscope (Nikon TS-100) and analyzed with a Nikon digital sight (DS-U2) software system. Each set of experiments was performed in triplicate. The results were statistically calculated and presented as mean ± S.D.

2.2.9 Real-time PCR

PC-3 cells were seeded at a density of $5x10^4$ cells per 60-mm petri dish. Twenty-four hours after seeding, cells were treated with different concentrations of celebrex. Control cells were treated with DMSO. After 16-hour treatment, cells were lysed by 500 μl Trizol (Invitrogen, U.S.A.) reagent on ice for 5 minutes, and then 200 μl chloroform (Sigma, MO, USA) were added to extract RNA into an aqueous phase. For phase separation, the mixture was centrifuged at 13,000 r.p.m., 4 °C for 15 minutes. After centrifugation, the upper-phase solution was transferred to a new eppendorf. RNA was precipitated by mixing the RNA extract with 500 μl of isopropanol (Sigma, USA). After centrifugation at 13,000 r.p.m at 4°C for 15 minutes, RNA pellets were washed with 500 μl 75% of ethanol, centrifuged again at 13,000 r.p.m. for 5 minutes and air-dried in a hood until the pellet was invisible. Twenty μl of DEPC-H_2O (Invitrogen, U.S.A.) were added to dissolve the RNA pellet and the RNA concentrations were detected by spectrophotometer with O.D. 260. To generate the cDNA from mRNA, 5 μg of total RNA were mixed with 50 μM oligo-dT, 10 mM dNTP mixture, 0.1 M dithiothreitol (DTT) at 65 °C for 10 minutes, and then incubated on 4 °C for primer annealing. The mixture was then incubated with 4 μl First Strand buffer (5X), 1 μl DTT (0.1 μM) and 1 μl SuperScript^{TM}Ⅲ Reverse Transcriptase (Invitrogen, CA, USA) at 50 °C for 1 hour elongation. Then, the mixtures were heated at 70 °C for 15 minutes to

inactivate the reaction. Real-time PCR was performed using Power SYBR Green real-time PCR system and ABI StepOne^{TM} detection system (Applied Biosystems).

The primers for real-time PCR are as follows:

Matriptase	Forward	5'-ATCGCCTACTACTGGTCTGAG-3'
(161 bp)	Reverse	5'- GTTTTGGAGTCCGTGGGGAAA-3'
HAI-1	Forward	5'- CCACGCTGGTACTATGAC-3'
(107 bp)	Reverse	5'- GCTAGAATGCACTCTTCT-3'
GAPDH	Forward	5'-TCAACGACCACTTTGTCAAGCT-3'
(156 bp)	Reverse	5'-GTGAGGGTCTCTCTCTTCCTCTTG-3'

2.2.10 Zymography

The activities of secreted matrix metalloproteinases in conditioned media were assayed by gelatin zymography. PC-3 cells were seeded at a density of $5x10^4$ cells per 60-mm dish in 10% FBS DMEM medium. After 24 hours, PC-3 cells were treated with celebrex or DMSO (solvent control) in serum-free DMEM for 16 hours. The conditioned media were collected and centrifuged at 1000 r.p.m., 4 °C, for 5 minutes. After centrifugation, the supernatants of the conditioned media were collected and concentrated using Amicon® Ultra-4 centrifuge filter devices (Millipore) at 3,000 r.p.m. at 4 °C for 30 minutes. Without boiling and reducing, the samples underwent

electrophoresis in 8% polyacrylamide gels [polyacrylamide gel electrophoresis (PAGE)] containing 0.1% w/v gelatin and 0.1% sodium dodecyl sulfate (SDS) in 125 V for 3 hours in a 4 °C refrigerator. After electrophoresis, the gel was incubated with a renaturation buffer (50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2.5% v/v Triton X-100) at 37 °C for 15 min three times (50 r.p.m). After protein renaturation, the buffer was decanted and the gel was incubated with a developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$) at 37 °C for 16 hrs with gentle agitation (30 r.p.m). 2 mM EDTA was added to inhibit matrix metalloproteinases activity (control). Gels were then stained with Coomassie brilliant blue R (0.1% w/v) and destained in 30% methanol and 10% acetic acid. Gelatinolytic activity appeared as a clear band on a blue background. The bands were imaged by a luminescent image analyzer with a CCD camera (LAS-4000; Fujifilm, Japan).

2.2.11 Transfection

One day before transfection, cells were seeded up with 70% confluence per 6-well dish and incubated overnight. Next day, cells were rinsed with PBS twice and then transfected by the transfection solution, a mixture of plasmids with Lipofectamine 2000 (Invitrogen, USA). The transfection solutions were prepared by mixing 4 μg of plasmid DNA with 6 μl Lipofectamine 2000 in 500 μl OPTI-MEM (Gibco, USA) at room

temperature for 30 min before transfection. 6 hours after transfection, OPTI-MEM medium were refreshed with culture medium. The stable pools were selected by 400 μg/ml geneticin for two weeks and maintained in regular media supplemented with 400 μg/ml geneticin and cells with less than 10 passages were used for experiments.

2.3 Buffer

Cell lysis buffer

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3V0_4
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% Glycerol
1 mM PMSF
Protease inhibitor cocktail

5X protein loading dye

1 M Tris-base	31.25 mL
10% SDS	62.5 mL
Glycerol	100 mL
Bromophenol blue	250 mg

Adjustment with ddH_2O to 250 mL (pH=6.8)

6X protein loading dye

Tris-base	1.36 g
SDS	3.6 g
Glycerol	l 9 mL
Bromophenol blue	0.018 %
2-Mercaptoethanol	1.62 mL

Adjustment with ddH_2O to 30 ml (pH=6.8)

10X Western blotting running buffer

Tris-base	30.2 g
Glycine	187.6 g
SDS	10 g
Adjustment with d.d.H_2O to	1 L (pH=8.3)

1X Towbin transfer buffer

Tris-base	3.63 g
Glycine	17.3 g
Methanol	240 mL
Adjustment with d.d.H_2O to	1.2 L

Blocking buffer

Skim milk powder	2.5 g
1X TBST	50 mL

10X TBS

NaCl	87.67 g
Tris-base	24.22 g
Adjustment with d.d.H_2O to	1 L (pH=7.4)

1xTBST

10X TBS	100 mL
Tween-20	1 mL
Adjustment with d.d.H_2O to	1 L

Stripping buffer

0.5M Tris-HCl	6.25 mL
2-Mercaptoethanol	500 l
10% SDS	10 mL
Adjustment with ddH_2O to	50 mL

Chapter 3. Results

3.1 Correlation of inflammation-associated protein expression and matriptase activation in a PC-3 cell invasion progression model

We established a PC-3 cell invasion progression model (Figure 1A) using transwell selection approaches with migration twice and invasion twice from the parental PC-3 cells. As shown in Figure 1B, the migration and invasion abilities of M2I2 cells were increased by approximately 50%, compared to the parental PC-3 cells. Since several lines of evidence showed that inflammation is involved in the development and progression of prostate cancer (8), I examined several inflammation-associated protein expression, including COX-2, IL-1β and p-JNK, in PC-3 and M2I2 cells by western blotting. The data (Figure 1C) show that the levels of COX-2, IL-1β and p-JNK protein expression were increased in M2I2 cells, compared to the parental PC-3 cells. Since matriptase has been identified as an oncogenic membrane-anchored serine protease for cancer malignancy (44) and matriptase expression is correlated with the progression of prostate cancer (64), I further investigated the total protein and activated levels of matriptase in PC-3 and M2I2 cells by western blot analysis using anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. As shown in Figure 1D, the levels of activated matriptase (a complex of active matriptase and its cognate inhibitor HAI-1) were increased in M2I2 cells, compared to PC-3 cells. Taken together, these results showed that inflammation-associated protein expression and matriptase activation are

up-regulated during the progression of PC-3 cells with highly migratory and invasive potentials.

3.2 Effect of celebrex on the cell viability of PC-3 cells

It has been shown that the high level of COX-2 expression is associated with advanced prostate cancer (71). To examine the role of COX-2 signaling in the progression of prostate cancer and the activation of matriptase, I first tested the effect of celebrex, a COX-2 specific inhibitor, on the cell viability of prostate cancer PC-3 cells. PC-3 cells were treated with different concentration of celebrex (0.01-1000 μM) for 16 hrs and the cell viability was assessed by MTT assays. As shown in Figure 2, celebrex reduced the viabilities of PC-3 cells in a dose-dependent manner. Moreover, celebrex exhibited various half-maximal inhibitory concentrations (IC_{50}) according to the cell densities. The IC_{50} values of celebrex for PC-3 cells with the cell densities of $5x10^4$ and $3x10^5$ cells/cm^2 were 107.9 μM and 688.7 μM, respectively. These results indicated that a high IC_{50} of celebrex to PC-3 cells was associated with a high cell density. Thus, celebrex had an inhibitory effect on PC-3 cell viability. The IC_{50} of celebrex for the cell viability was dependent on cell density.

3.3 Inhibitory effect of celebrex on PC-3 cell growth.

To further examine the effect of celebrex on the cell growth of PC-3 cells, PC-3 cells were treated with the different concentrations of celebrex (5 and 25 μM) for 0, 2, 4 and 6 days. Control cells were treated with equal amount of DMSO. At each time point, the amounts of PC-3 cells were assessed by MTT assays. As shown in Figure 3A, 5 μM celebrex had a marginal effect to decrease the growth of PC-3 cells while 25 μM celebrex significantly reduced the cell growth. Taken together, the results indicated that celebrex had a growth-inhibitory effect on PC-3 cells.

3.4 Celebrex inhibited the motility and invasion of PC-3 cells.

To further investigate whether celebrex can decrease prostate cancer cell migration and invasion, PC-3 cells were treated with different concentrations of celebrex and their cell migration and invasion were examined by transwell assays. The results (Figure 3B and 3C) showed that celebrex marginally reduced approximately 30% cell migration after 5 μM celebrex treatment and suppressed 70% cell migration upon 25 μM celebrex treatment. However, the invasion ability of PC-3 cells were decreased approximately by 60% after 5 μM celebrex treatment and significantly reduced up to 80% upon 25 μM celebrex treatment. To determine that the effect of celebrex on PC-3 cell migration and invasion was not due to cytotoxic effect, I examined the cytotoxic effect of celebrex on

PC-3 cells. PC-3 cells with a density of $3x10^5$ cells/cm^2 were treated with 5 and 25 μM celebrex for 16-hour and counted by trypan blue exclusion methods. The results (Figure 3D) showed that there was no significant cytotoxicity on PC-3 cells after 5 and 25 μM celebrex treatment. Taken together, the data indicated that celebrex ably suppressed PC-3 cell migration and invasion in a dose-dependent manner.

3.5 Celebrex reduced matriptase and HAI-1 shedding and protein expression in PC-3 cells.

To investigate whether celebrex suppressed PC-3 cell migration and invasion was *via* inhibiting matriptase activation, I examined the effect of celebrex on the total protein and activation levels of matriptase as well as HAI-1, matriptase's aognate inhibitor, using western blotting with anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. As shown in Figure 4A, celebrex ably decreased the whole levels of matriptase including latent matriptase and activated matriptase in a dose-dependent manner. The decrease of activated matriptase by celebrex was further validated by the western blotting with an anti-activated matriptase Ab (M69). Interestingly, the total protein levels of HAI-1 were also reduced by celebrex. It has been proposed that matriptase activation is followed by the shedding of the matriptase-HAI-1 complex with a molecular mass of 95 or 110 kDa to extracellular

environments (72). The shedding of matriptase-HAI-1 complex has been thought as a mechanism to remove active matriptase from cells (46). To identify whether the reduced levels of matriptase and HAI-1 by celebrex were through celebrex's effect on promoting the shedding of matriptase and HAI-1, the conditioned media after celebrex treatment were collected and examined by western blotting with anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. The data (Figure 4B) showed that celebrex reduced the levels of matriptase-HAI-1 complexes and HAI-1 in the conditioned media in a dose-dependent manner. Taken together, celebrex can inhibit the activation and shedding of matriptase and HAI-1 in prostate cancer cell, suggesting that the decrease of cellular activated matriptase upon celebrex treatment may not be due to the protein shedding.

3.6 Celebrex down-regulated gene expression of matriptase and HAI-1 in PC-3 cells.

Since the data (Figure 4A and 4B) showed that celebrex can reduce the total protein levels of matriptase and HAI-1 in PC-3 cells, I further examined whether celebrex was through altering the gene expression of matriptase and HAI-1, leading to decrease their protein levels. With real-time RT-PCR, the data (Figure 4C) showed that celebrex significantly reduced the gene expression of matriptase and HAI-1 in the cells.

Thus, the data taken together from Figure 4A and 4C indicated that the decreased levels of matriptase and HAI-1 proteins by celebrex were, at least in part, *via* transcriptional regulation.

3.7 Celebrex decreased MMPs activity in PC-3 cells.

Since it has been shown that matrix metalloproteinases (MMPs) and serine proteases play important roles in cancer cell invasion in many cancers (73, 74), I also analyzed the effect of celebrex on MMPs activity by gelatin zymography. As shown in Figure 5, the activity of secreted MMP-9 but not MMP-2 was significantly suppressed by celebrex in a dose-dependent manner.

3.8 Prostaglandin E_2 (PGE_2) increased the motility and invasion of PC-3 cells.

Since celebrex is a COX-2 specific inhibitor, I further examined the effect of PGE_2, the most abundant product of COX-2 in human malignancies, on the PC-3 cell migration and invasion by transwell assays. The data (Figure 6A and 6B) showed that the migration and invasion abilities of PC-3 cells were significantly induced approximately by 50% after 50 nM PGE_2 treatment. Upon 100 nM PGE_2 treatment, the invasion ability of PC-3 cells was increased approximately by one fold; while the migration ability of PC-3 cells was enhanced up to two folds. Taken together, these

results indicated that PGE_2 can increase the migration and invasion of PC-3 cells.

3.9 PGE_2 induced the levels of matriptase activation and HAI-1 but reduced the shedding of matriptase and HAI-1 in PC-3 cells.

I then further investigated the effect of PGE_2 on the protein and activation levels of matriptase by treating PC-3 cells with the 100 nM PGE_2 for 0, 0.5, 1, 2, 4, 8 and 16 hrs. Control cells were treated with DMSO. The protein and activation levels of matriptase were assessed using western blotting with an anti-total matriptase (M32) mAb. As shown in Figure 7A, PGE_2 was able to induce matriptase activation. Then, I examined the effect of 50 and 100 nM PGE_2 on the protein and activation levels of matriptase by western blotting with anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. The data (Figure 7B) showed that PGE_2 ably increased the levels of activated matriptase and HAI-1 in a dose-dependent manner. I further analyzed if PGE_2 increased the levels of matriptase-HAI-1 complex and HAI-1 through suppressing the shedding of both proteins. The conditioned media after PGE_2 treatment were collected and examined by western blotting with anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. The data (Figure 7C) showed that the shedding of matriptase-HAI-1 complex and HAI-1 was reduced by PGE_2 in a dose-dependent manner. Taken together, the increased level of cellular

activated matriptase upon PGE_2 treatment may be at least partly due to PGE_2-suppressing the shedding of matriptase-HAI-1 complexes into the conditioned media.

3. 10 Effect of EP antagonists on matriptase shedding and protein expression.

It has been reported that PGE_2 exerts its cellular effects *via* binding to its cognate receptors (EP1-4) (10). To further explore the mechanism of celebrex-suppressed matriptase activation, I used an EP1 receptor antagonist SC51322, an EP3 receptor antagonist L798106 and an EP4 receptor antagonist L161982 to treat PC-3 cells and analyzed their effects on the protein and activation levels of matriptase using western blotting with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. The data (Figure 8) showed that EP1 receptor antagonist was able to suppress the activation and shedding of matriptase in PC-3 cells, while EP3 and EP4 antagonists could enhance the shedding of matriptase.

3.11 Celebrex down-regulated matriptase expression and activation partly *via* COX-2-independent pathway in PC-3 cells.

It has been reported that celebrex has some inhibitory function *via* a COX-2 independent fashion in spite of its effect on inhibiting COX-2 (23). Since the data

(Figure 4A and 7B) showed that celebrex was able to reduce matriptase expression, but PGE_2 has no significant effect on matriptase protein levels, I further investigated whether celebrex reduced matriptase expression and activation may be partly through a COX-2 independent pathway. PC-3 cells were treated with 25 μM celebrex, 100 nM PGE_2, or both. The total protein and activation levels of matriptase were analyzed by western blotting with anti-total matriptase (M32) and anti-activated matriptase (M69). As shown in Figure 9, PGE_2-induced matriptase was also ably inhibited by celebrex. In other words, PGE_2 could not rescue celebrex-inhibited matriptase activation. These results suggested that the inhibition of matriptase expression and activation by celebrex may be partly *via* a COX-2-independent pathway.

3.12 Effect of celebrex on the cytotoxicity of non-COX-2 expressed prostate cancer cells, DU-145 and LNCaP cells.

To further address that celebrex reduced matriptase expression and activation was through a COX-2-independent pathway, I examined the effect of celebrex on two non-COX-2 expressed prostate cancer cells, DU-145 and LNCaP cells. As shown in Figure 10A, COX-2 expression was hardly detectable in DU-145 and LNCaP cells by western blotting using an anti-COX-2 Ab, compared to PC-3 cells. Then, the effect of celebrex on th cell viability of DU-145 and LNCaP was assessed by MTT assays. The

data (Figure 10B and 10C) shown that celebrex was able to reduce the both viabilities of DU-145 and LNCaP cells in a dose-dependent manner. The IC_{50}values of celebrex for DU-145 cells were 96.3 μM for the cell density of $5x10^4$ cells/cm^2 and 639.6 μM for cell density of $3x10^5$ cells/cm^2. The IC_{50} values of celebrex for LNCaP cells were 117.6 μM for the cell density of $1x10^5$ cells/cm^2 and 527.8 μM for the cell density of $1.5x10^6$ cells/cm^2. Similar to PC-3 cells (Figure 2), the IC_{50} of celebrex for the viability of DU-145 and LNCaP cells was also dependent on cell density.

3.13 Inhibitory effects of celebrex on cell motility and invasion in DU-145 cells *via* down-regulating matriptase.

I further examined the effect of celebrex on the DU-145 cell migration and invasion by transwell assays. The results (Figure 11A and 11B) showed that the migration and invasion abilities of DU-145 cells were decreased approximately by 20% after 5 μM celebrex treatment and suppressed up to 50% upon 25 μM celebrex treatment. To investigate whether celebrex-suppressed DU-145 cell migration and invasion were *via* down-regulating matriptase, I examined the effect of celebrex on the protein levels of matriptase and HAI-1 by western blotting using anti-total matriptase (M32) and anti-HAI-1 (M19) mAbs. As shown in Figure 11C, celebrex decreased the levels of matriptase and HAI-1 in a dose-dependent manner. Taken together, celebrex

was able to suppress DU-145 cell migration and invasion partly due to reducing the protein level of matriptase.

3.14 Inhibitory effects of celebrex on LNCaP cell migration and invasion *via* suppressing the total and activated levels of matriptase.

I also examined the effect of celebrex on LNCaP cell migration and invasion using transwell assay. The results (Figure 12A and 12B) showed that the migratory and invasive cells of LNCaP cells were decreased approximately by 20% after 5 μM celebrex treatment and suppressed up to 40% with 25 μM celebrex treatment. Then, the effect of celebrex on the total and activated levels of matriptase was analyzed by western blotting using anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. As shown in Figure 12C, celebrex was able to reduce the activated level of matriptase in a dose-dependent manner. I also analyzed the effect of celebrex on the shedding of both matriptase and HAI-1 in LNCaP cells, by examining the shed proteins using western blotting with anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. As shown in Figure 12D, celebrex suppressed the shedding of matriptase-HAI-1 complex and HAI-1 into the conditioned media in a dose-dependent manner. Taken together, celebrex ably suppressed LNCaP cell migration and invasion, at least part due to down-regulation of matriptase.

3.15 Effect of celebrex and other NSAIDs on matriptase expression and activation in LNCaP cells.

To further investigate whether commercially available NSAIDs exhibited a similar effect on down-regulating matriptase, as celebrex, I then examined the effect of other NSAIDs including aspirin, Etoricoxib, Ibuprofen and Indomethacin on matriptase expression and activation by western blotting using anti-total matriptase (M32) and anti-activated matriptase (M69). The data (Figure 13) showed that the total and activated levels of matriptase were dramatically reduced by celebrex but not other NSAIDs in LNCaP cells. Taken together, the data indicated that celebrex was a potent inhibitor to down-regulate matriptase.

3.16 Role of matriptase on PC-3 cell migration and invasion.

Since matriptase has been shown to be overexpressed in variety of epithelia-derived human tumors, including prostate cancer (57, 58), we established the matriptase-overexpressing PC-3 cells by transfection and selection. The exogenous matriptase proteins produced by plasmids of matriptase-V5 tag were detected by western blotting analysis with anti-matriptase and anti-V5 Abs. As shown in Figure 14A, the protein levels of matriptase were increased in matriptase-overexpressing PC-3 cells. Then, I investigated the role of matriptase in the PC-3 cell migration and invasion by

transwell assays. Matriptase-overexpressing PC-3 cells exhibited strong migration and invasion capabilities, compared to the control PC-3 cells (Figure 14B). Taken together, the data indicated that matriptase was able to promote PC-3 cell migration and invasion.

3.17 Inhibitory effect of celebrex on matriptase-overexpressing PC-3 cell migration, invasion and matriptase activation.

I further examined the effect of celebrex on matriptase-overexpressing PC-3 cell migration and invasion. As shown in Figure 14C, celebrex ably reduced approximately 80% cell migration and suppressed 95% cell invasion. To identify whether the decreased matriptase-overexpressing PC-3 cell migration and invasion by celebrex were through celebrex's inhibitory effect on matriptase activation, I analyzed the effect of celebrex on matriptase activation in matriptase-overexpressing PC-3 cells using western blotting with anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs. As shown in Figure 14D, celebrex was able to suppress matriptase expression and activation. Taken together, these results indicated that celebrex exhibited an inhibitory potential to the prostate cancer cell PC-3 migration and invasion induced by matriptase overexpression.

Chapter 4. Discussion

Chronic inflammation has been proposed to be associated with tumorigenesis by promoting cellular transformation, survival, proliferation, invasion, angiogenesis, and metastasis (75, 76). Since several lines of evidence showed that inflammation is involved in the development and progression of prostate cancer (8, 77), in this study, I also observed that several inflammation-associated proteins, COX-2, p-JNK and IL-1β were up-regulated following a prostate cancer PC-3 invasion progression, which was established by serial transwell selection. Interestingly, an anti-inflammation NSAID Celebrex (a COX-2-specific inhibitor) was able to reduce the cancer cell proliferation, migration and invasion, suggesting that a reduction of inflammation signaling can inhibit the progression of human prostate cancer.

Expression of COX-2 is induced by many growth factors and pro-inflammatory cytokines in specific pathophysiological conditions (12). It has been reported that COX-2 is involved in the invasiveness (78, 79), anti-apoptosis (18), and angiogenesis (13) of colon and breast cancer. Recently, increased COX-2 expression has been associated with high prostate tumour grade (80). Consistent with this phenomenon, our data showed that the expression of COX-2 was enhanced in highly invasive PC-3 cells of the prostate cancer progression model, and correlated with the cell migration and invasion (Figure 1). Thus, this cancer cell model recapitulates the progression of human prostate cancer, and COX-2 may be a therapeutic target for prostate cancer.

To specifically inhibit COX-2 activity, several NSAIDs (e.g., aspirin, celebrex) have been developed for clinical uses as effective anti-inflammatory, antipyretic and analgesic drugs. Recent studies have provided several evidences that celebrex was recommended as chemotherapeutic and chemopreventive drugs for colon and prostate cancer (24, 81, 82). Celebrex has shown the inhibitory effects on the growth and invasiveness of colon cancer cells (83). In this study, I also found that celebrex could inhibit the cell proliferation, migration and invasion of prostate cancer cells and the IC_{50} of celebrex for the cell viability was dependent on cell density. Moreover, my data showed that celebrex inhibited the migration and invasion of PC-3 cells was not due to its effect on cell cytotoxicity. Thus, celebrex may be a potentially useful compound for prostate cancer therapy.

In addition to the involvement of matrix metalloproteinases (MMPs) in cancer metastasis (84-86), several recent studies revealed that pericellular serine proteases also play an important role in cancer metastasis (48, 64). In this study, I identified a membrane-anchored serine protease matriptase as a new target protein inhibited by celebrex. It has been shown that matriptase can promote the tumorigenesis, and correlated with prostate cancer progression (63) and involved in ErbB-2-induced prostate cancer cell invasion (87). I further found that celebrex could reduce PC-3 cell migration and invasion that was partly *via* decreasing the total and activated levels of

matriptase. The decreased protein level of matriptase by celebrex treatment was not due to its effect on promoting the matriptase shedding, but through the down-regulation of the gene expression. Moreover, my data also showed that the activity of secreted MMP-9 was also suppressed by Celebrex in prostate cancer cells. Thus, celebrex-reduced PC-3 cell migration and invasion was partly attributed to decrease of the total and activated matriptase as well as the activity of secreted MMP-9.

Prostaglandin E_2 (PGE_2) is the most abundant product of COX-2 and can induce several signal pathways to promote cell proliferation (88, 89), invasion (90), and angiogenesis (91) in cancer cells. In this study, I showed that PGE_2 could promote prostate cancer PC-3 cell migration and invasion. This increased PC-3 cell migration and invasion may be partly due to PGE_2-inducing matriptase activation. However, PGE_2 was unable to rescue celebrex-reduced matriptase expression and activation, suggesting that Celebrex-inhibited matriptase activation may be *via* a COX-2 independent pathway. This is further supported by the facts that celebrex exerts its anti-neoplastic effects *via* a COX-2-independent pathway (23, 26). Moreover, celebrex and its derivatives without any COX-2-inhibiting activity still had strong anti-carcinogenic activity (23, 92). In this study, my data further supported the importance of the COX-2-independent pathway affected by celebrex in suppressing matriptase and the invasion of prostate cancer cells, because celebrex also can execute its anti-cancer properties in two COX-2-null prostate

cancer cells, DU-145 and LNCaP cells. Similar to PC-3 cells, the suppression of these cell migration and invasion by Celebrex was partly *via* decreasing the total and activated matriptase. I further explored whether other commercially available NSAIDs had similar inhibitory effects as celebrex on matriptase and observed that the other NSAIDs were not as effective as celebrex to reduce the total and activated levels of matriptase, suggesting that celebrex is a potent inhibitor to down-regulate matriptase through inhibiting a COX-2-independent pathway.

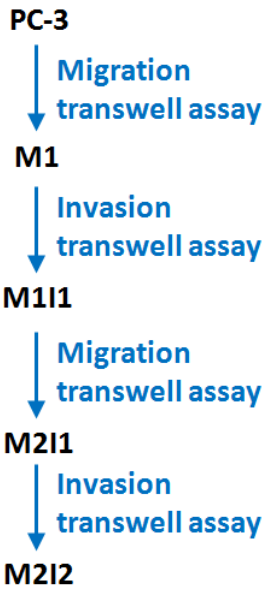
Numerous studies have demonstrated that PGE_2 and its EP receptors are implicated in promoting carcinogenesis in different types of cancer (93, 94). PGE_2 binds to the four EP receptors that are coupled to different G proteins and induce a variety of intracellular signaling cascades (95). It has been reported that PGE_2-induced VEGF secretion in prostate cancer cells is mediated through EP2-dependent cAMP signaling pathways (96). My result further identified EP1 receptor as a factor involved in matriptase activation because EP1 receptor antagonist was able to suppress the activation and shedding of matriptase in PC-3 cells. Since PKC has been shown as one of the important downstream molecules of EP1 receptor and a previous study has indicated that PKC is involved in matriptase activation (95, 97), these results taken together suggest that the reduction of PGE_2-matriptase activation by celebrex may be *via* inhibiting a EP1-mediated PKC signal pathway. The detailed molecular mechanisms in which

celebrex suppresses PGE_2-induced matriptase activation need more investigations.

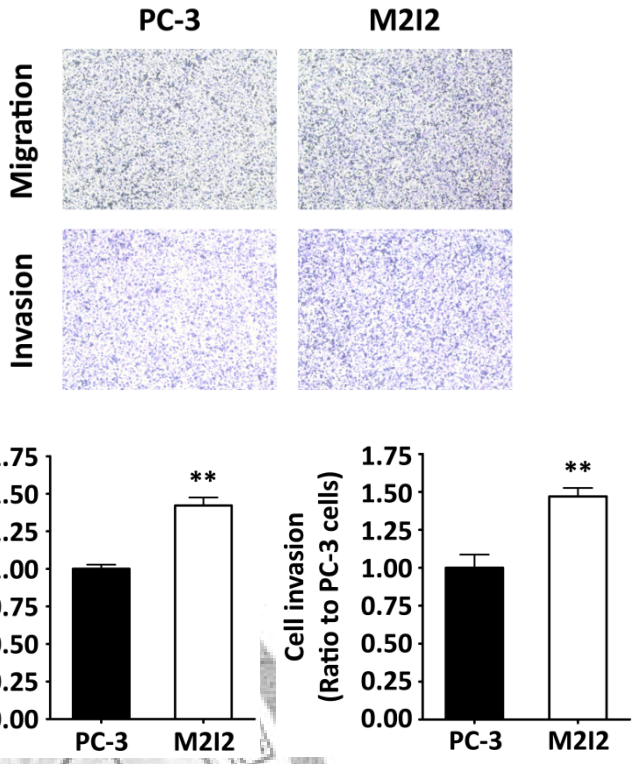
In conclusion, our data revealed that the effect of celebrex against the growth, migration and invasion of prostate cancer cells, was partly attributed to decrease the activated levels of matriptase and the gene expression. Therefore, this study provides some information that celebrex may be a potential option for prostate cancer treatment or chemoprevention in the future.

Chapter 5. Figures

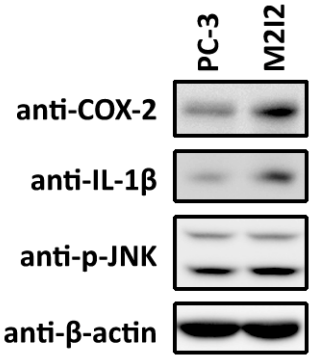
(A)


PC-3
Migration transwell assay
M1
Invasion transwell assay
M1I1
Migration transwell assay
M2I1
Invasion transwell assay
M2I2

(B)


PC-3
M2I2
Migration
Invasion
Cell migration (Ratio to PC-3 cells)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
PC-3
M2I2
Cell invasion (Ratio to PC-3 cells)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
PC-3
M2I2

(C)


PC-3
M2I2
anti-COX-2
anti-IL-1β
anti-p-JNK
anti-β-actin

(D)

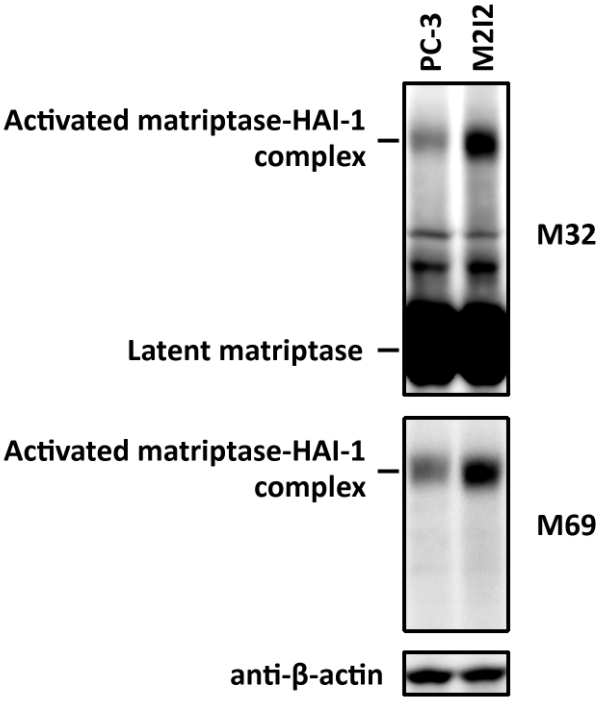
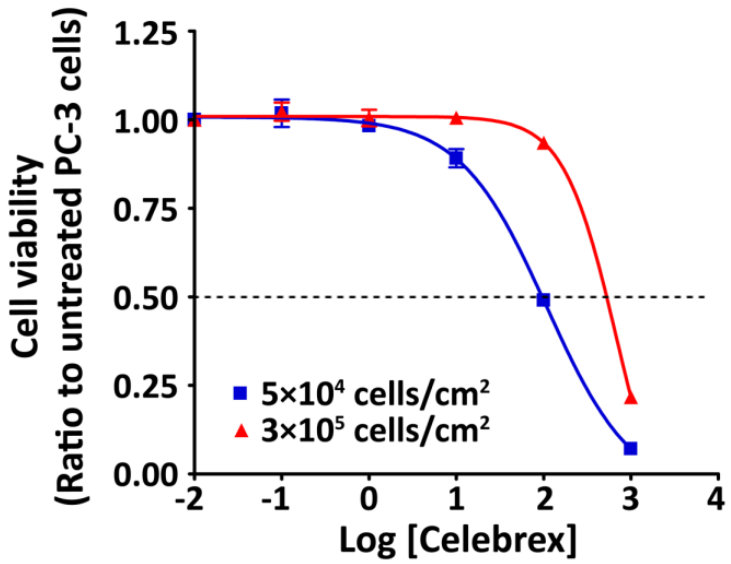

PC-3
M2I2
Activated matriptase-HAI-1 complex
M32
Latent matriptase
Activated matriptase-HAI-1 complex
M69
anti-β-actin

Figure 1. Establishment of prostate cancer PC-3 cell progression model and analysis of inflammation biomarkers and matriptase expression in these cells.

(A) PC-3 cell progression model was established by serial transwell selection. (B) For invasion assay, 3 μg of matrigel were diluted in 100 μl serum-free medium, then added on the upper chamber of each transwell and air-dried overnight. No matrigel was coated on transwell for migration assay. 1×10^5 of serum-starved PC-3 cells or M2I2 cells in serum-free medium were seeded into the upper chamber of each transwell. After 16-hour incubation, the cells were fixed with methanol and stained with crystal violet. The lower surfaces of transwells were photographed under a light microscope (magnification, 100X). Migratory and invasive cell numbers were measured by ImageJ software and statistically calculated. Results were represented as mean ± SD (n=3). **: $P<0.01$. (C) Western blotting analysis of several inflammation biomarkers in PC-3 cells and M2I2 cells. PC-3 or M2I2 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 10% FBS DMEM at a 37℃, 5% CO_2 incubator. Twenty-four hrs after seeding, cell lysates were collected and analyzed by western blotting using anti-COX-2, anti-IL-1β and anti-p-JNK Abs. (D) Assessment of matriptase expression and activation in the PC-3 cell progression model by using anti-total matriptase (M32) and anti-activacted matriptase (M69) mAbs under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.

(A)



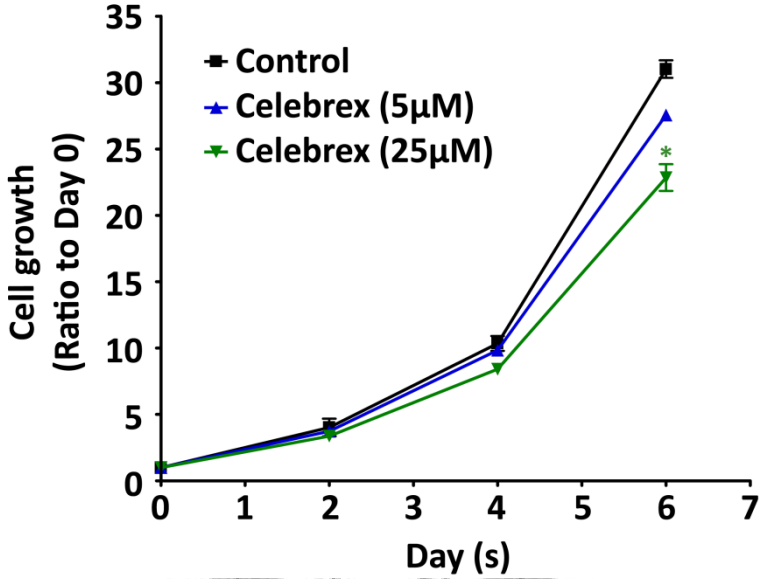
(B)

Cell density (cells/cm²)	IC_{50} (μM)
$5x10^4$	107.9
$3x10^5$	688.7

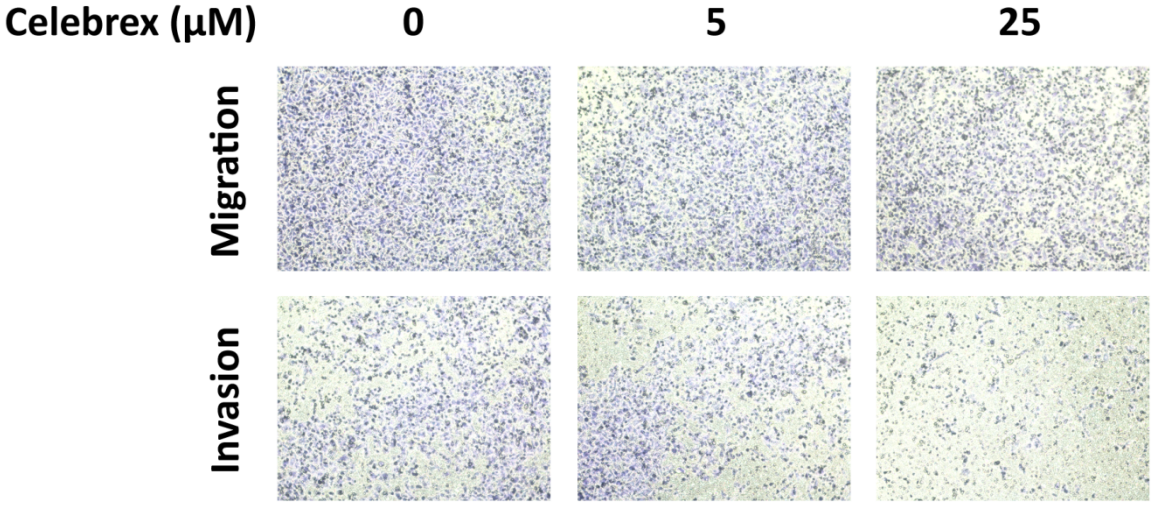
Figure 2. Effect of celebrex on the cell viability of PC-3 cells.

(A) PC-3 cells were seeded at the densities of 3×10^5 and 5×10^4 cells/cm^2 in 24-well plates. Next day, cells were treated with different concentrations (0.01-1000 μM) of celebrex for 16 hours at 37 °C in a 5% CO_2 incubator. The effect of celebrex on cell viability was determined by MTT assays. Each experiment was performed in triplicate. Values were represented as mean ± SD (n=3). (B) The IC_{50} values of celebrex for different cell densities of PC-3 cells were calculated and shown in the table.

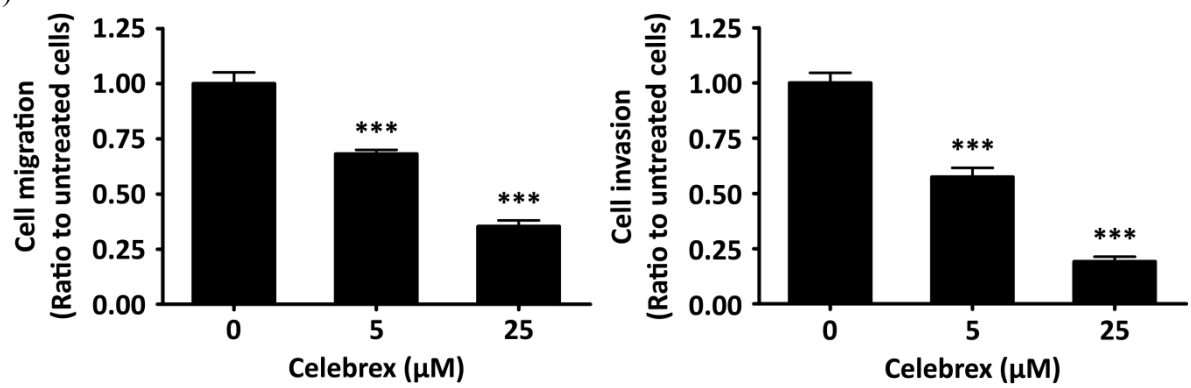
(A)


Control
Celebrex (5μM)
Celebrex (25μM)
Cell growth
(Ratio to Day 0)
Day (s)
*

(C)


Celebrex (μM)
0
5
25
Migration
Invasion

(D)


Cell migration
(Ratio to untreated cells)
1.25
1.00
0.75
0.50
0.25
0.00
***
***
0
5
25
Celebrex (μM)
Cell invasion
(Ratio to untreated cells)
1.25
1.00
0.75
0.50
0.25
0.00
***
***
0
5
25
Celebrex (μM)

(D)

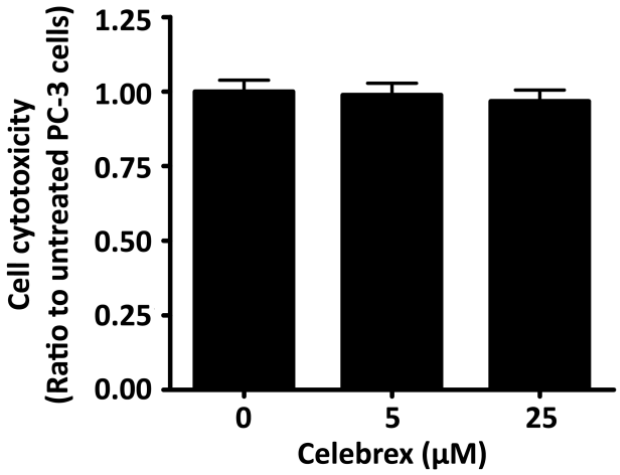
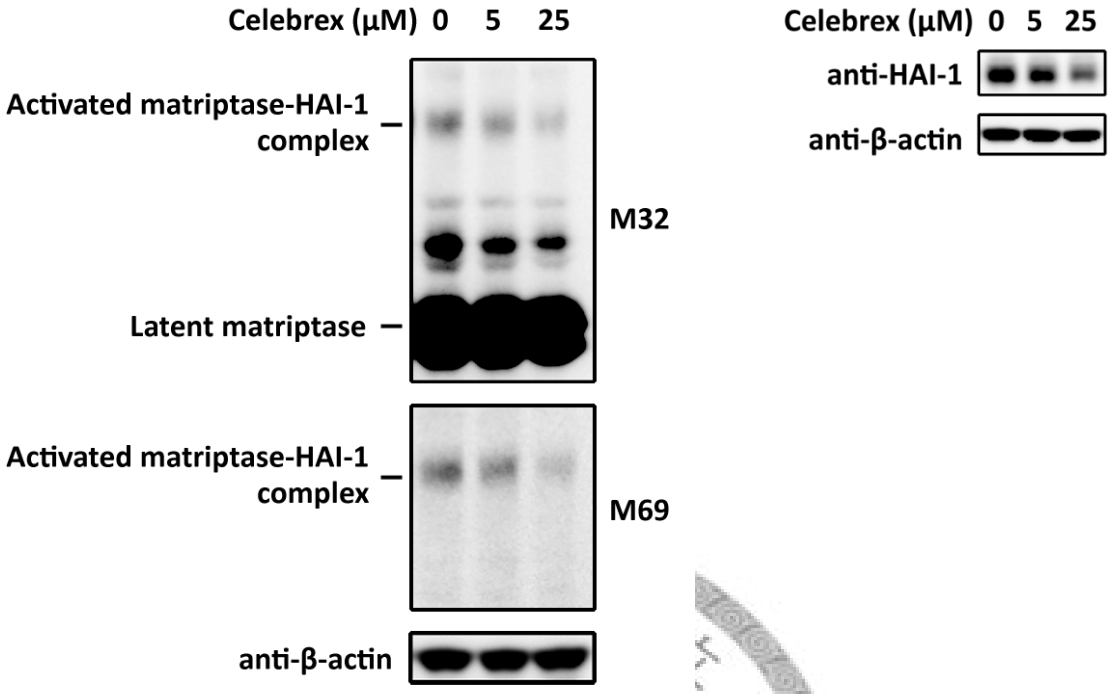


Figure 3. Effect of celebrex on PC-3 cell growth, migration and invasion.

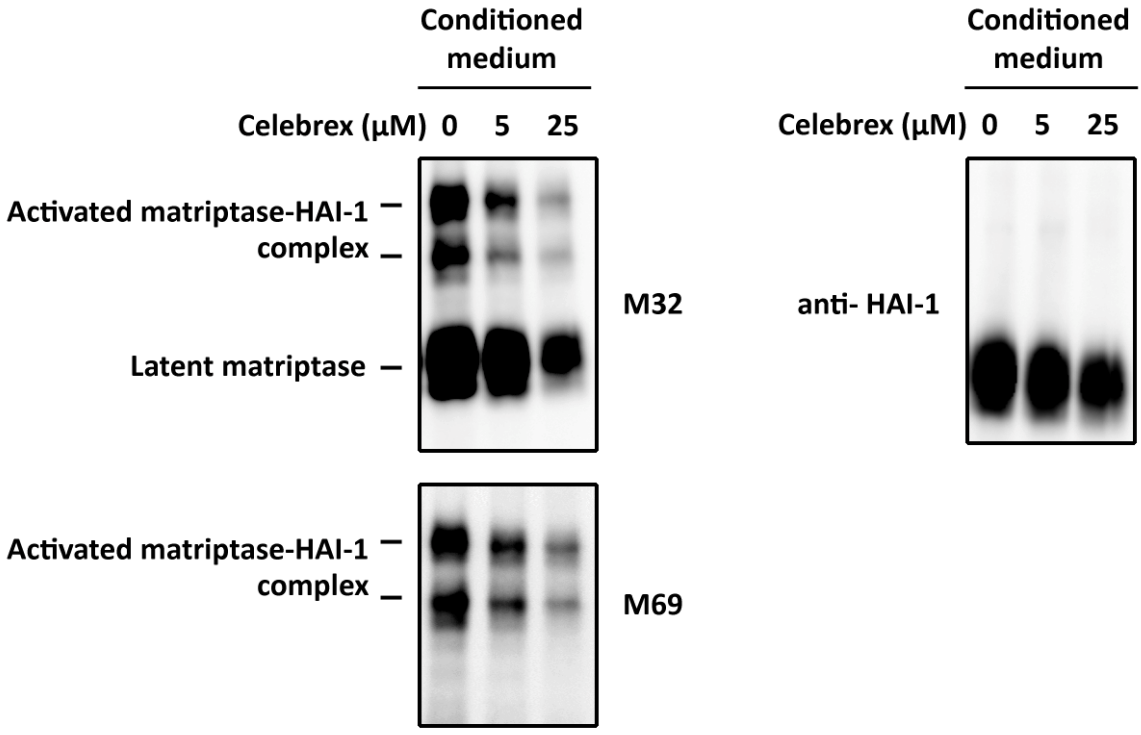
(A) Analysis of the effect of celebrex on PC-3 cell growth. 1×10^4 of PC-3 cells were seeded in each well of 24-well plates and cultured at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cell amount was measured using MTT assay as Day 0 and other sets of cells were treated with indicated concentrations of celebrex (0, 5 and 25 μM) for 2, 4 or 6 days and the media were refreshed every day. Cell amount was measured using MTT methods at each time point and normalized to Day 0. Values were represented as mean ± SD (n=3). *: $P<0.05$. (B) Analysis of the cytotoxic effect of celebrex on PC-3 cells. PC-3 cells were seeded at a density of 3×10^5 cells/cm^2 in 6-well plates. Next day, cells were treated with indicated concentrations of celebrex (0, 5 and 25 μM) for 16 hours at 37 °C in a 5% CO_2 incubator. After treatment, cells were trypsinized and the viable cell numbers were counted by a hemacytometer with a trypan blue exclusion method and normalized to untreated cells. (C) Examination of the celebrex effect on PC-3 cell migration and invasion. 1×10^5 (3×10^5

cells/cm^2) of serum-starved PC-3 cells in serum-free medium were seeded into the upper chamber of each transwell with indicated concentrations of celebrex (0, 5 and 25 μM). After 16-hour incubation, invaded cells were stained and photographed as described in Figure 1B. Each assay was performed in triplicate. (D) Invaded PC-3 cells were measured by ImageJ software and statistically calculated. Results were presented as mean ± SD (n=3). ***: $P<0.001$.

(A)


Celebrex (μM) 0 5 25
Activated matriptase-HAI-1 complex
Latent matriptase
M32
Activated matriptase-HAI-1 complex
M69
anti-β-actin
Celebrex (μM) 0 5 25
anti-HAI-1
anti-β-actin

(B)


Conditioned medium
Celebrex (μM) 0 5 25
Activated matriptase-HAI-1 complex
Latent matriptase
M32
Activated matriptase-HAI-1 complex
M69
Conditioned medium
Celebrex (μM) 0 5 25
anti- HAI-1

(C)

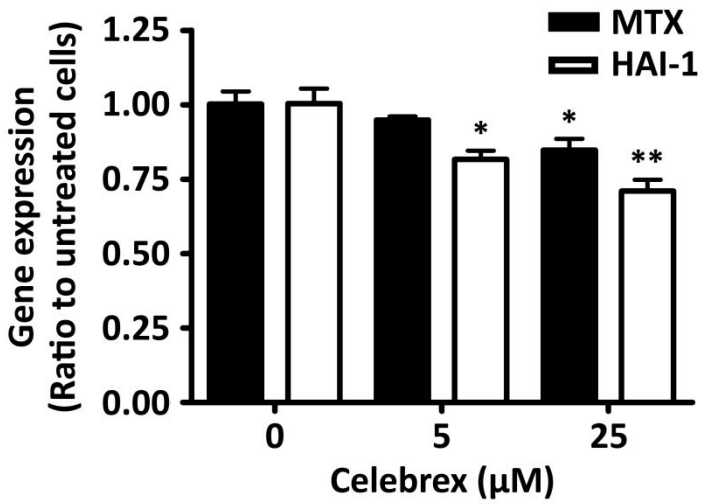
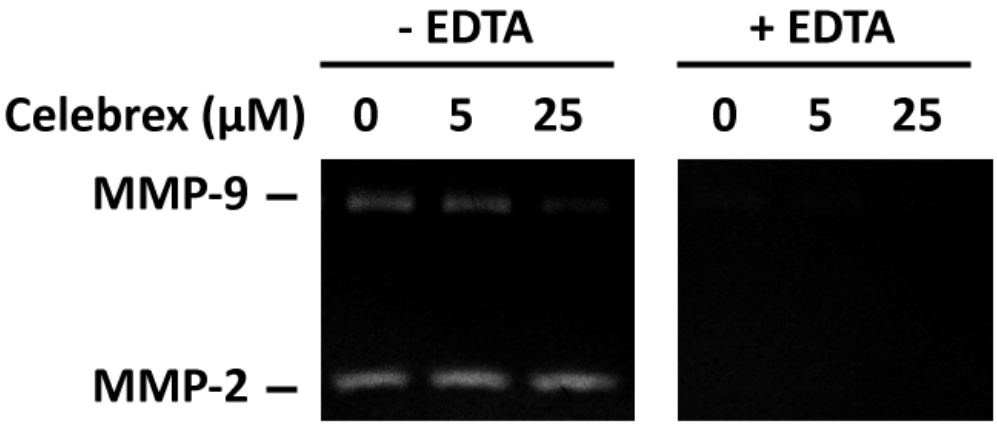


Figure 4. Effect of celebrex on matriptase and HAI-1 expression and shedding in PC-3 cells.

PC-3 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 10% FBS DMEM at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cells were washed twice with PBS. Indicated concentrations of celebrex (0, 5, and 25 μM) in the serum-free DMEM were added to the media and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 16 hours. (A) After treatments, cells were collected and lyzed by lysis buffer. (B) The conditioned media were collected and concentrated by Ultra-4 centrifuge filter devices (Millipore) under 3,000 r.p.m. centrifugation at 4 °C for 30 minutes. Cell lysates and concentrated conditioned media were analyzed using anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. M32 and M69 mAbs were used for western blotting under a non-boiled and non-reduced condition. The cell lysate with

boiling and reducing reagent was used for western blot analysis with M19. A loading control was analyzed with an anti-β-actin Ab. (C) Analysis of the effect of celebrex on the gene expression of matriptase (MTX) and HAI-1 in PC-3 cells. 1×10^6 (5×10^4 cells/cm^2) of PC-3 cells were seeded in each 60-mm dish and maintained in 10% FBS DMEM at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, indicated concentrations of celebrex (0, 5 and 25 μM) were added to the media and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 4 hours. After the treatments, total RNAs were extracted by Trizol reagent and analyzed by real-time RT-PCR described in method 2.2.9. Results were statistically calculated and shown as mean ± S.D. *: $P<0.05$, **: $P<0.01$.

(A)



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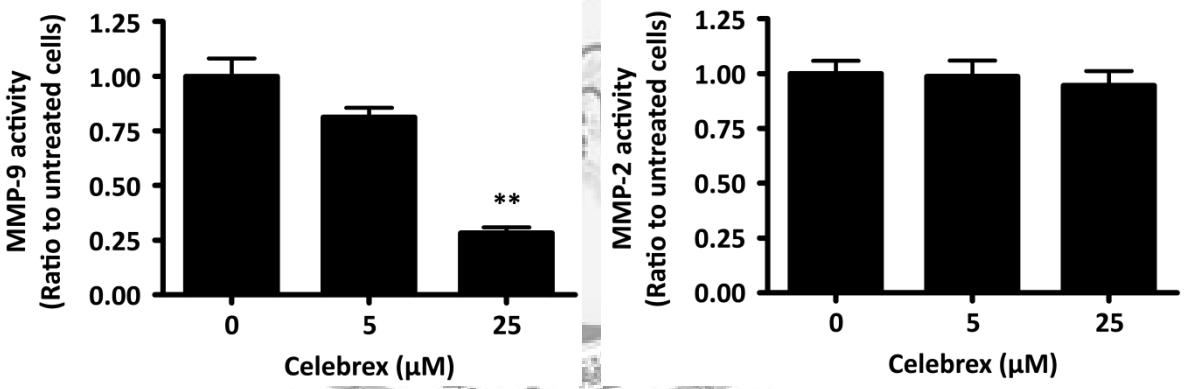
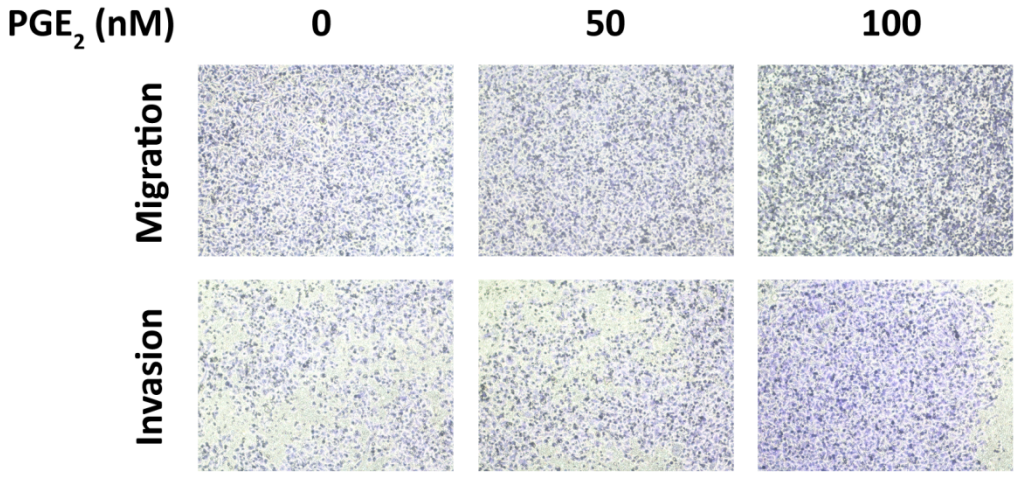


Figure 5. Analysis of the celebrex effect on MMPs activity by gelatin zymography.

PC-3 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 10% DMEM culture medium at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cells were washed twice with PBS. Indicated concentrations of celebrex (0, 5 and 25 μM) within serum-free DMEM media were added and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 16 hours. The conditioned media were collected and concentrated as described

in Figure 4B. Gelatinolytic activities of MMP-9 and MMP-2 in the conditioned media were determined by gelatin zymography described in method 2.2.10. Results were statistically calculated and presented as mean ± S.D. **: $P<0.01$.

(A)



(B)

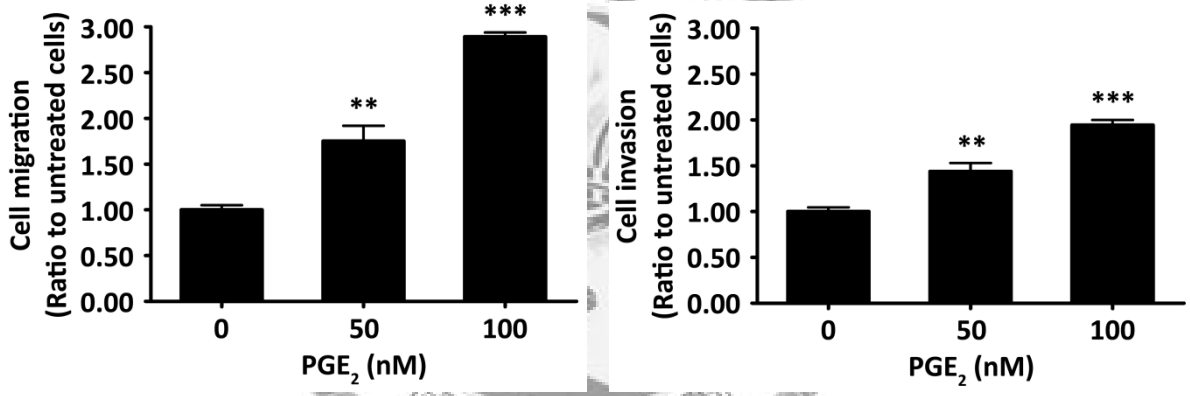
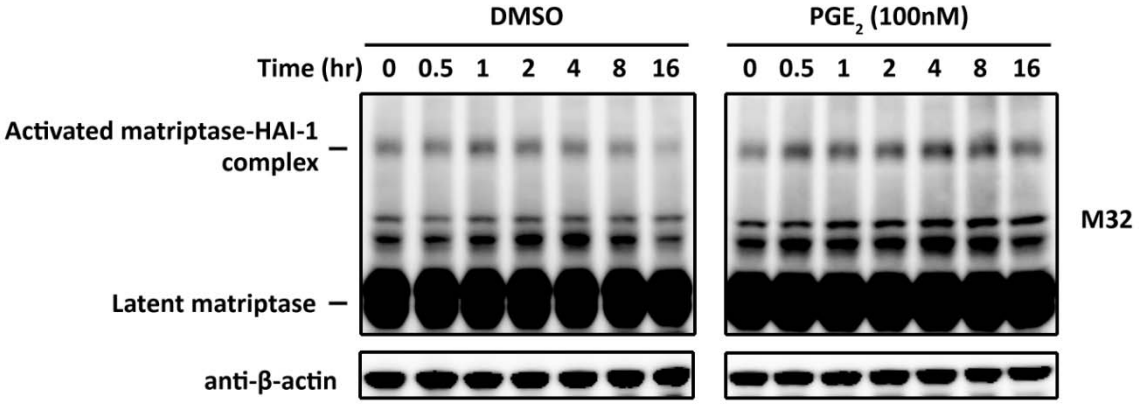


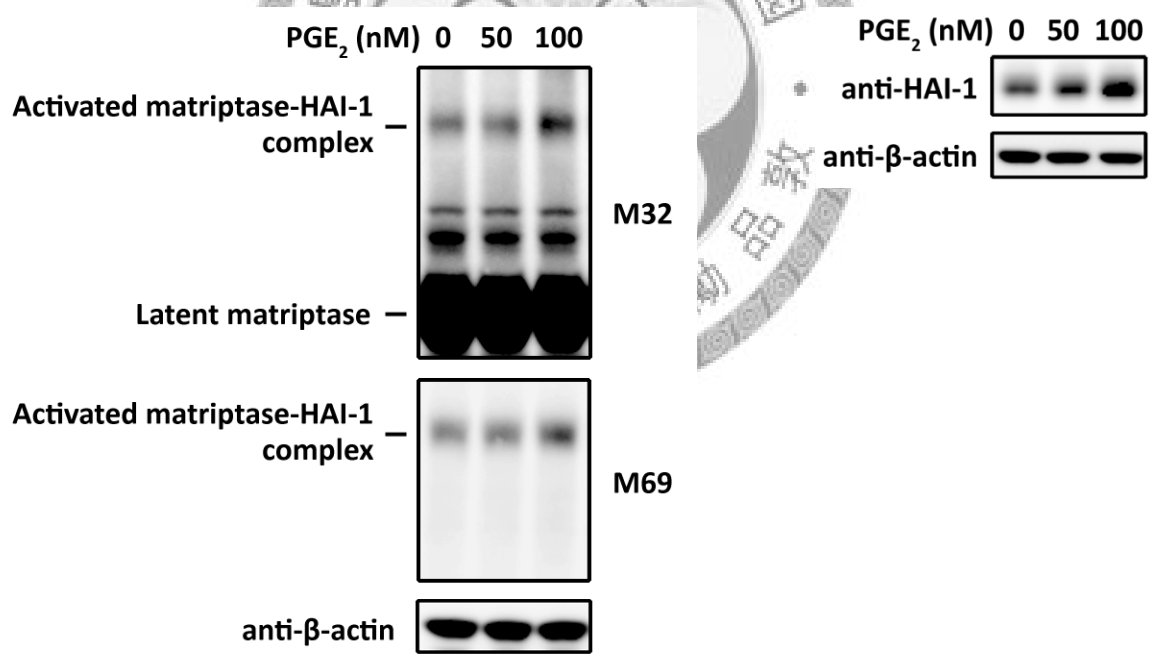
Figure 6. Effect of PGE_2 on PC-3 cell migration and invasion.

(A) 1×10^5 (3×10^5 cells/cm^2) of serum-starved PC-3 cells in serum-free medium were seeded into the upper chamber of each transwell with indicated concentrations of PGE_2 (0, 50 and 100 nM) for cell migration and invasion assays. After 16-hour incubation, invaded cells were stained by crystal violet and photographed as described in Figure 1B. (B) Invaded PC-3 cells were measured by ImageJ software and statistically calculated. Results were represented as mean ± SD (n=2). **: $P<0.01$, ***: $P<0.001$.

(A)


DMSO
PGE2 (100nM)
Time (hr) 0 0.5 1 2 4 8 16
0 0.5 1 2 4 8 16
Activated matriptase-HAI-1 complex
Latent matriptase
anti-β-actin
M32

(B)


PGE2 (nM) 0 50 100
Activated matriptase-HAI-1 complex
Latent matriptase
M32
Activated matriptase-HAI-1 complex
M69
anti-β-actin
PGE2 (nM) 0 50 100
anti-HAI-1
anti-β-actin

(C)

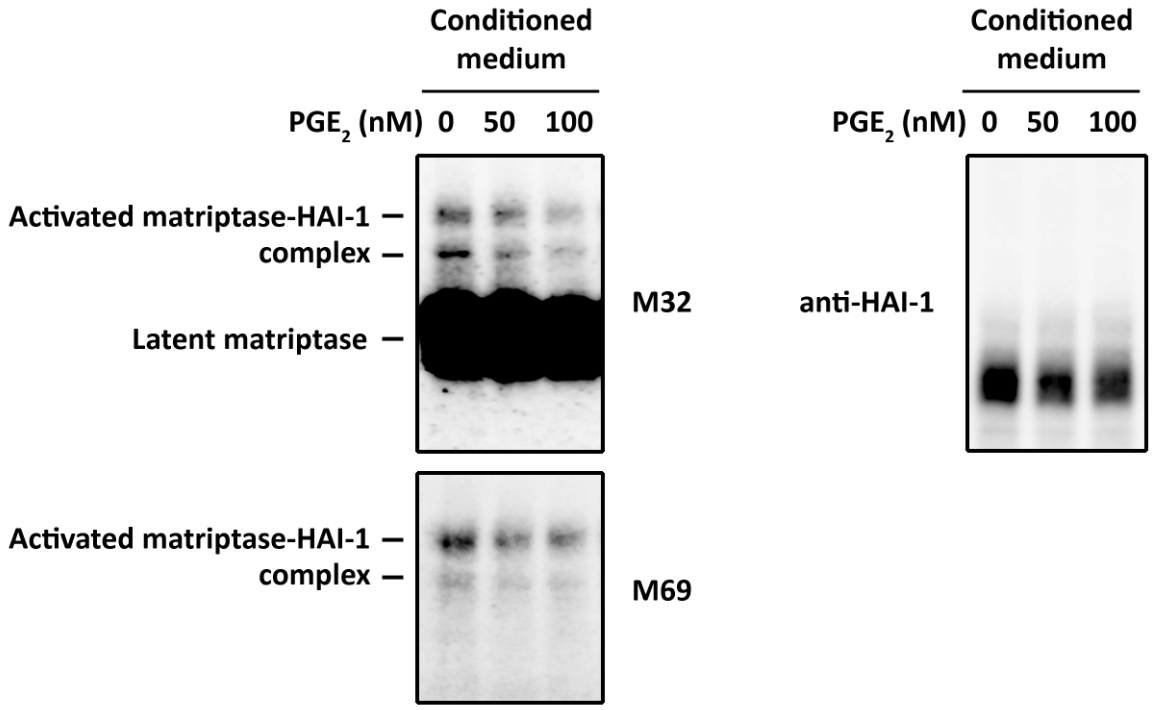
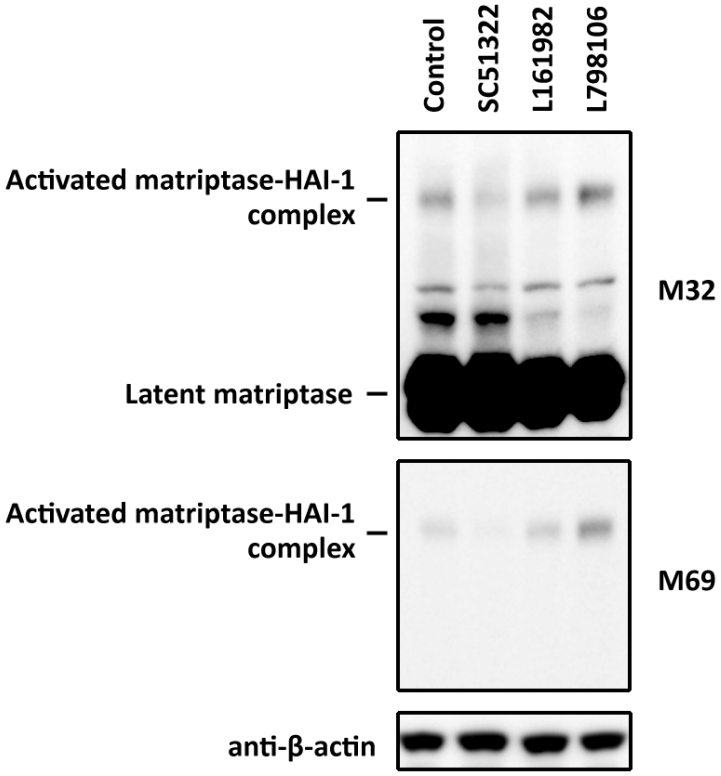


Figure 7. Effect of PGE_2 on matriptase and HAI-1 expression and shedding in PC-3 cells.

PC-3 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 10% FBS DMEM at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cells were washed twice with PBS. (A) PC-3 cells were treated with 100 nM PGE_2 in the serum-free DMEM and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 0, 0.5, 1, 2, 4, 8 or 16 hrs. At each time point, the levels of total matriptase and activated matriptase in the cells were assessed by western blotting using anti-total matriptase (M32). (B) Indicated concentrations of PGE_2 (0, 50, and 100 nM) in the serum-free DMEM were added to PC-3 cell culture media. After 16-hour treatment, cells were collected and lyzed by lysis buffer. (C) The conditioned

media were collected and concentrated as described in Figure 4B. Cell lysates and concentrated conditioned media were analyzed using anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. M32 and M69 mAbs were used for western blotting under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.

(A)


Control
SC51322
L161982
L798106
Activated matriptase-HAI-1 complex
Latent matriptase
M32
Activated matriptase-HAI-1 complex
M69
anti-β-actin

(B)

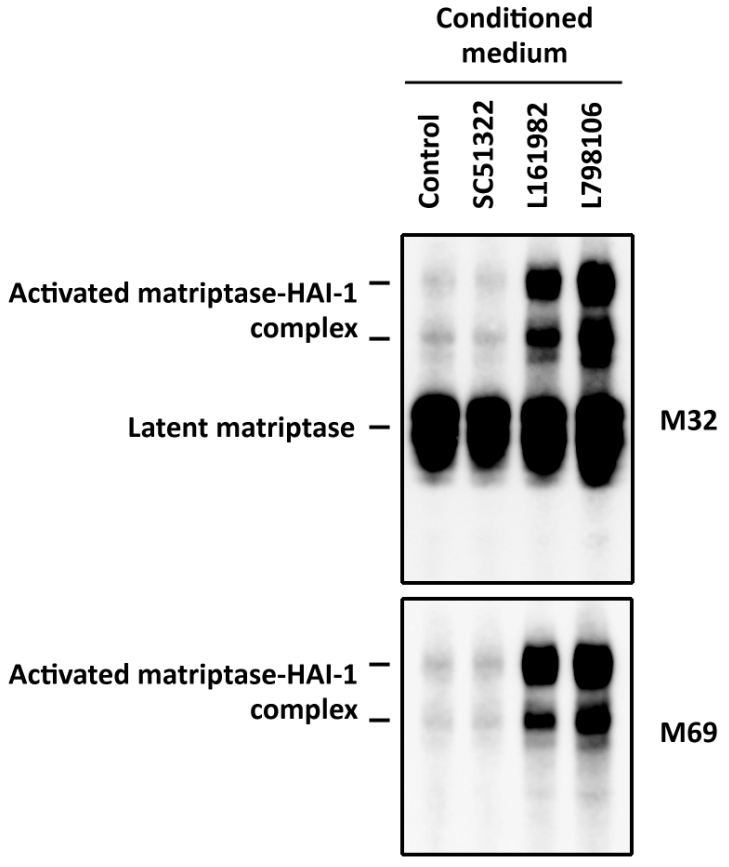

Conditioned medium
Control
SC51322
L161982
L798106
Activated matriptase-HAI-1 complex
Latent matriptase
M32
Activated matriptase-HAI-1 complex
M69

Figure 8. Effect of EP antagonists on matriptase expression and activation in PC-3 cells.

PC-3 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 10% FBS DMEM at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cells were washed twice with PBS. Indicated concentrations of an EP1 antagonist (SC51322, 10 μM), an EP4 antagonist (L161982, 10 μM) and an EP3 antagonist (L798106, 10 μM) in the serum-free DMEM were added to the media and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 16 hours. (A) After treatments, cell lysates were collected and analyzed by western blotting. (B) The conditioned media were collected and concentrated as described in Figure 4B. Cell lysates and concentrated conditioned media were analyzed using anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.

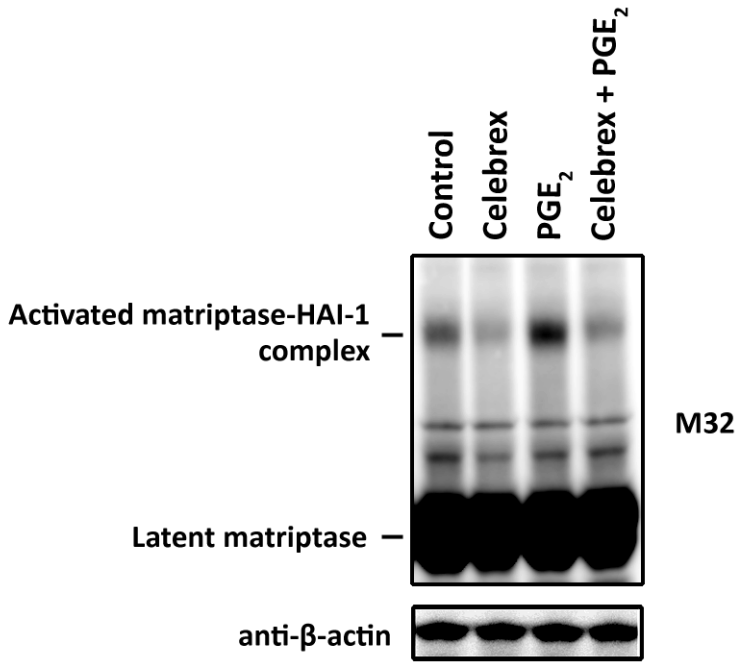
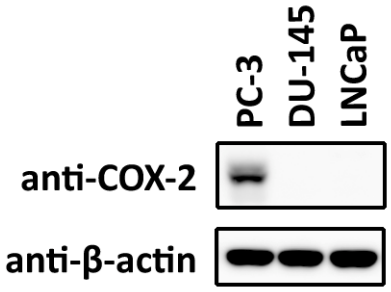


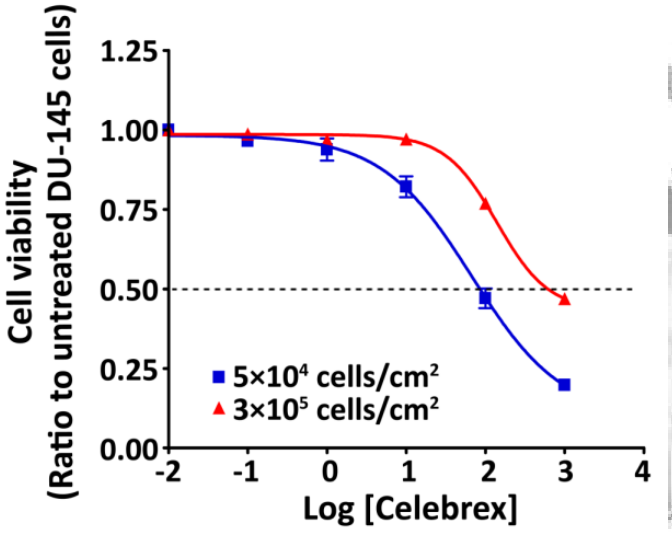
Figure 9. Analysis of the of PGE_2 and celebrex effect on matriptase in PC-3 cells.

PC-3 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 10% FBS DMEM at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cells were washed twice with PBS. Cells were treated with celebrex (25 μM), PGE_2 (100 nM) or both. After 16-hour treatments, cell lysates were collected and analyzed by western blotting using anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.

(A)

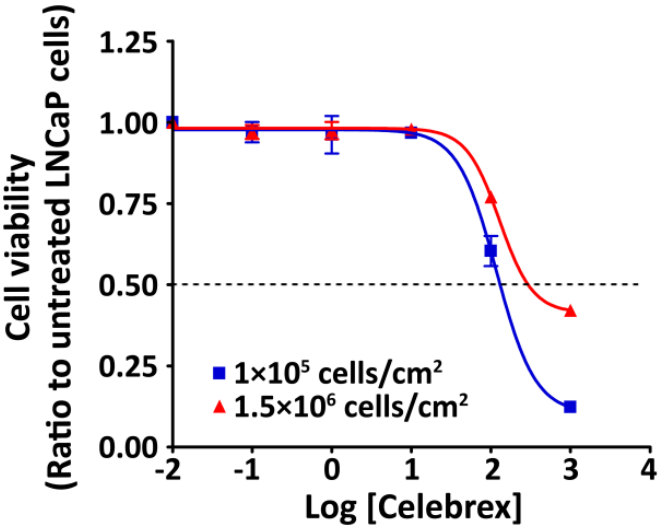


(B)



Cell density (cells/cm²)	IC_{50} (μM)
$5x10^4$	96.3
$3x10^5$	639.6

(C)

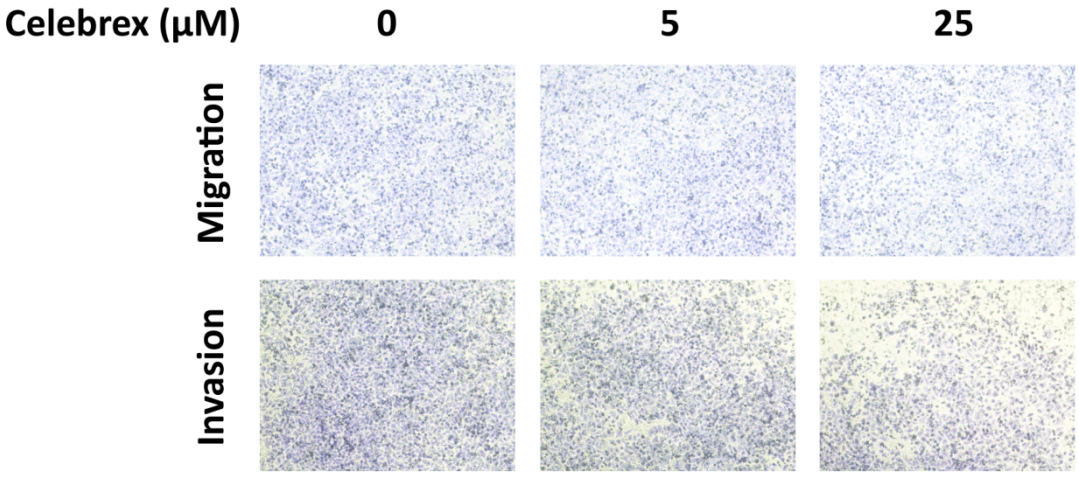


Cell density (cells/cm²)	IC_{50} (μM)
$1x10^5$	117.6
$1.5x10^6$	528.7

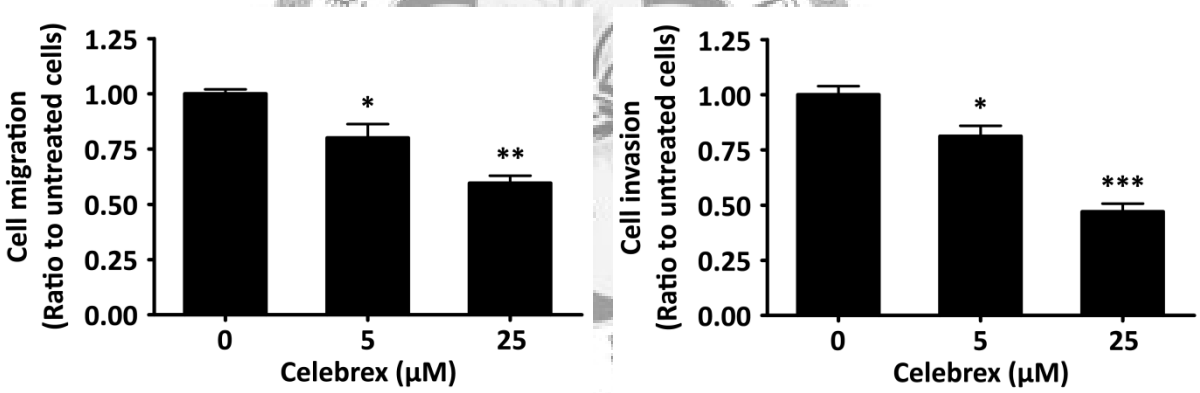
Figure 10. Examination of COX-2 expression in PC-3, DU-145 andLNCaP cells and the effect of celebrex on the cell viability of DU-145 and LNCaP cells.

(A) Western blotting analysis of COX-2 expression in PC-3, DU-145 and LNCaP cells. PC-3 cells or DU-145 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 10% FBS DMEM or 5% FBS RPMI-1640 medium at a 37℃, 5% CO_2 incubator. LNCaP cells were seeded at a density of 1×10^5 cells per 60-mm dish with 5% FBS RPMI-1640 medium at a 37℃, 5% CO_2 incubator. Twenty-four hrs after seeding, cell lysates were collected and analyzed by western blotting using anti-COX-2 Ab. β-actin was used as control with immunoblots with an anti-β-actin Ab. (B) Analysis of the effect of celebrex on the cell viability of DU-145 cells. DU-145 cells were seeded at the densities of 3×10^5 and 5×10^4 cells/cm^2 in 24-well plates. (C) Analysis of the effect of celebrex on the cell viability of LNCaP cells. LNCaP cells were seeded at the densities of 1.5×10^6 and 1×10^5 cells/cm^2 in 24-well plates. Next day, cells were treated with different concentrations of celebrex for 16 hours at 37 °C in a 5% CO_2 incubator. The effect of celebrex on cell viability was determined by MTT assays. Each assay for the effect of celebrex on cell viability was performed in triplicate. The IC_{50} values of celebrex for different cell densities of DU-145 or LNCaP cells were calculated and shown in right panel of each figure. Values were represented as mean ± SD (n=3).

(A)


Celebrex (μM)
0
5
25
Migration
Invasion

(B)


Cell migration
(Ratio to untreated cells)
1.25
1.00
0.75
0.50
0.25
0.00
*
**
0
5
25
Celebrex (μM)
Cell invasion
(Ratio to untreated cells)
1.25
1.00
0.75
0.50
0.25
0.00
*
***
0
5
25
Celebrex (μM)

(C)

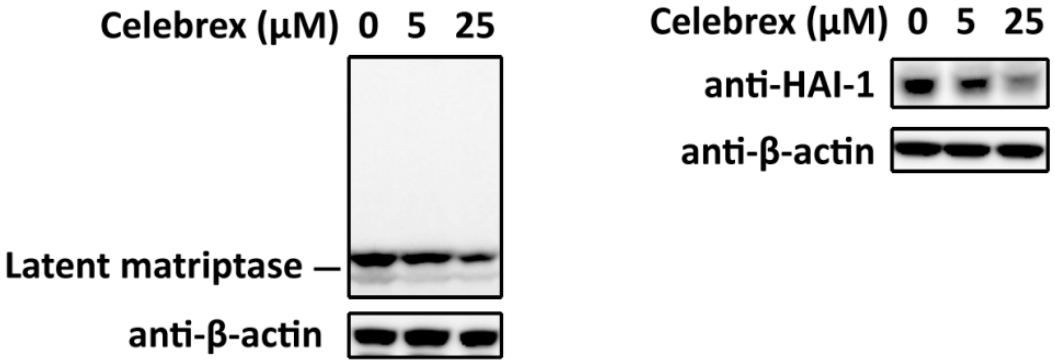
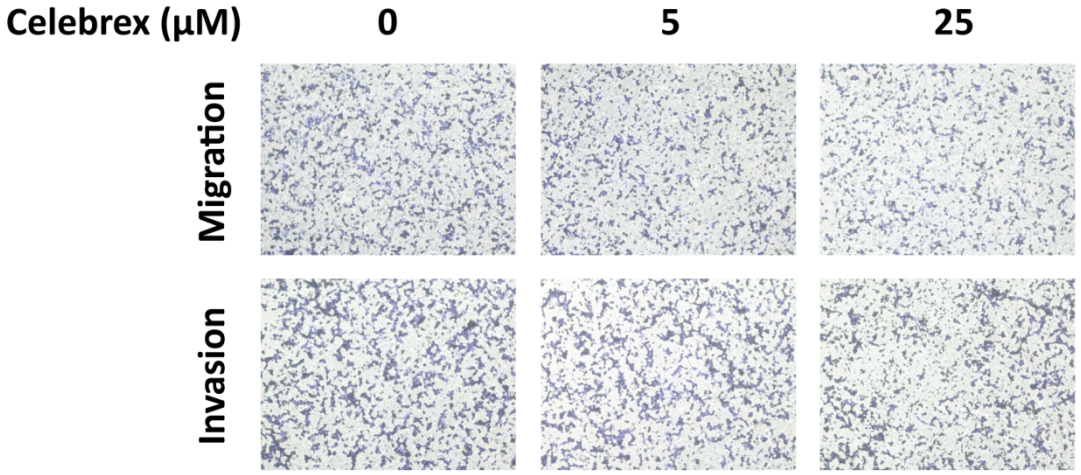

Celebrex (μM) 0 5 25
Latent matriptase
anti-β-actin
Celebrex (μM) 0 5 25
anti-HAI-1
anti-β-actin

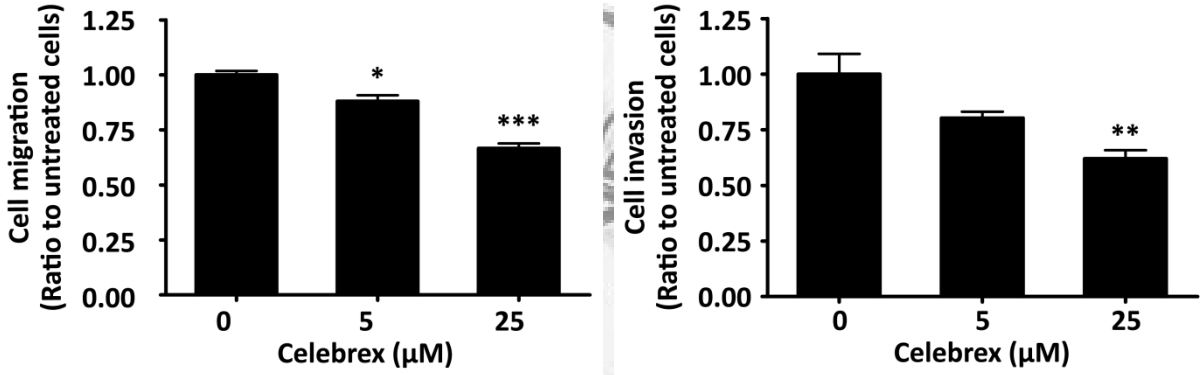
Figure 11. Effect of celebrex on DU-145 cell migration, invasion and on the expression of matriptase and HAI-1.

(A) Analysis of the effect of celebrex on DU-145 cell migration and invasion. 1×10^5 (3×10^5 cells/cm^2) of serum-starved DU-145 cells in serum-free medium were seeded into the upper chamber of each transwell with indicated concentrations of celebrex (0, 5 and 25 μM). After 16-hour incubation, migratory and invasive cells were stained and photographed as described in Figure 1B. (B) Invaded DU-145 cells were measured by ImageJ software and statistically calculated. Results were represented as mean ± SD (n=3). *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$. (C) DU-145 cells were seeded at a density of 5×10^4 cells per 60-mm dish and maintained in 5% FBS RPMI-1640 medium at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cells were washed twice with PBS. Indicated concentrations of celebrex (0, 5, and 25 μM) in the serum-free RPMI-1640 medium were added to the media and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 16 hours. After treatments, cell lysates were collected and analyzed by western blotting using anti-total matriptase (M32) and anti-HAI-1 (M19) mAbs. M32 mAb were used for western blotting under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.

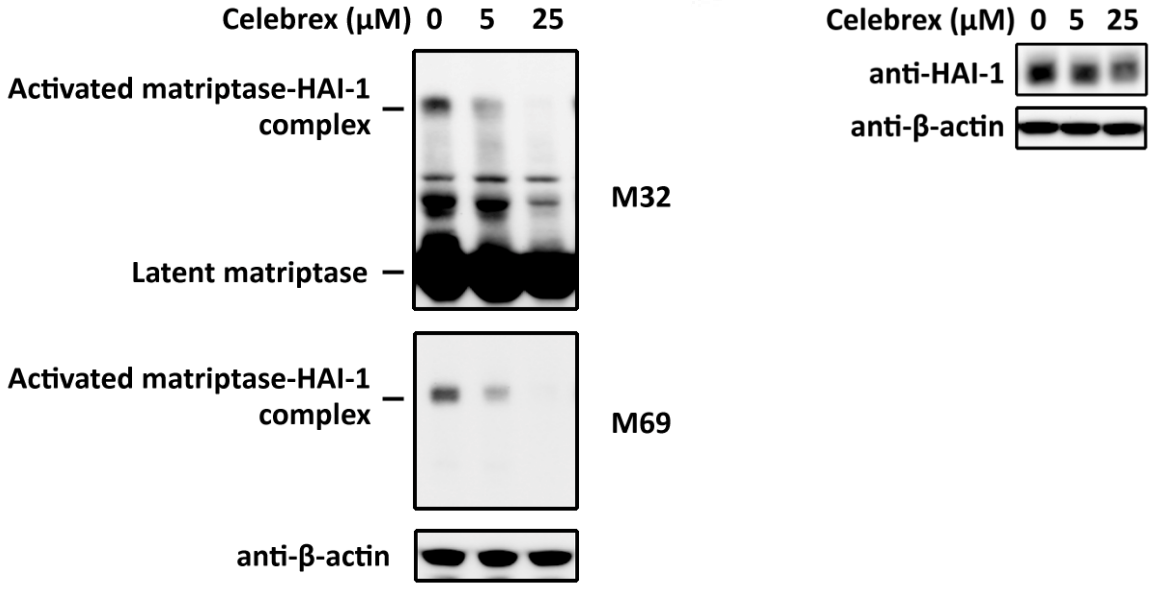
(A)


Celebrex (μM)
0
5
25
Migration
Invasion

(B)


Cell migration
(Ratio to untreated cells)
1.25
1.00
0.75
0.50
0.25
0.00
*
***
0
5
25
Celebrex (μM)
Cell invasion
(Ratio to untreated cells)
1.25
1.00
0.75
0.50
0.25
0.00
**
0
5
25
Celebrex (μM)

(C)


Celebrex (μM) 0 5 25
Activated matriptase-HAI-1 complex
Latent matriptase
M32
Activated matriptase-HAI-1 complex
M69
anti-β-actin
Celebrex (μM) 0 5 25
anti-HAI-1
anti-β-actin

(D)

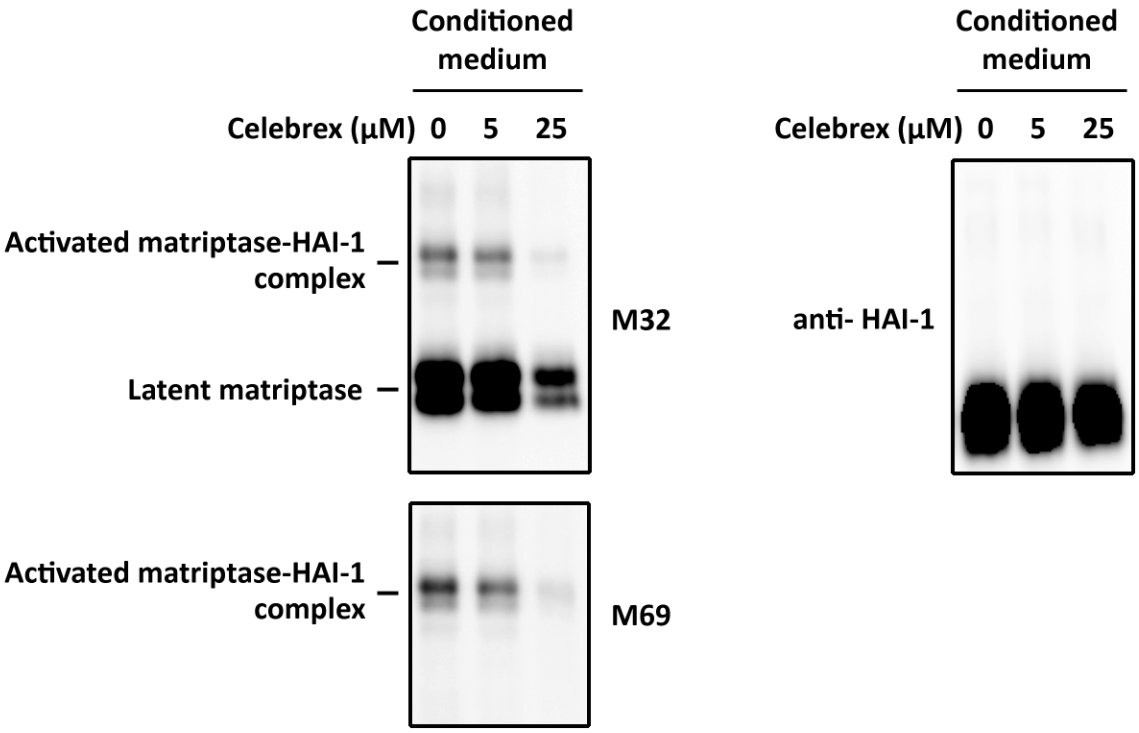


Figure 12. Effect of celebrex on LNCaP cell migration, invasion and on the expression and shedding of matriptase and HIA-1.

(A) Analysis of the effect of celebrex on LNCaP cell migration and invasion. 5×10^5 (1.5×10^6 cells/cm^2) of serum-starved LNCaP cells in serum-free medium were seeded into the upper chamber of each transwell with indicated concentrations of celebrex (0, 5 and 25 μM). After 16-hour incubation, invaded cells were stained and photographed as described in Figure 1B. (B) Statistically calculation of LNCaP cell migration and invasion. Invaded LNCaP cells were measured by ImageJ software. Results were statistically calculated and presented as mean ± SD (n=3). *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$. (C) Analysis of the celebrex effect on the expression of matriptase and HAI-1 in LNCaP cells. LNCaP cells were seeded at a density of

1×10^5 cells per 60-mm dish and maintained in 5% FBS RPMI-1640 medium at a 37 °C, 5% CO_2 incubator. Forty-eight hours after seeding, cells were washed twice with PBS. Indicated concentrations of celebrex (0, 5, and 25 μM) in the serum-free RPMI-1640 medium were added to the media and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 16 hours. After treatments, cell lysates were collected and analyzed by western blotting. (D) Effect of celebrex on the shedding of matriptase and HAI-1 in LNCaP cells. The conditioned media were collected and concentrated as described in Figure 4B. Cell lysates and concentrated conditioned media were analyzed using anti-total matriptase (M32), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. M32 and M69 mAbs were used for western blotting under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.

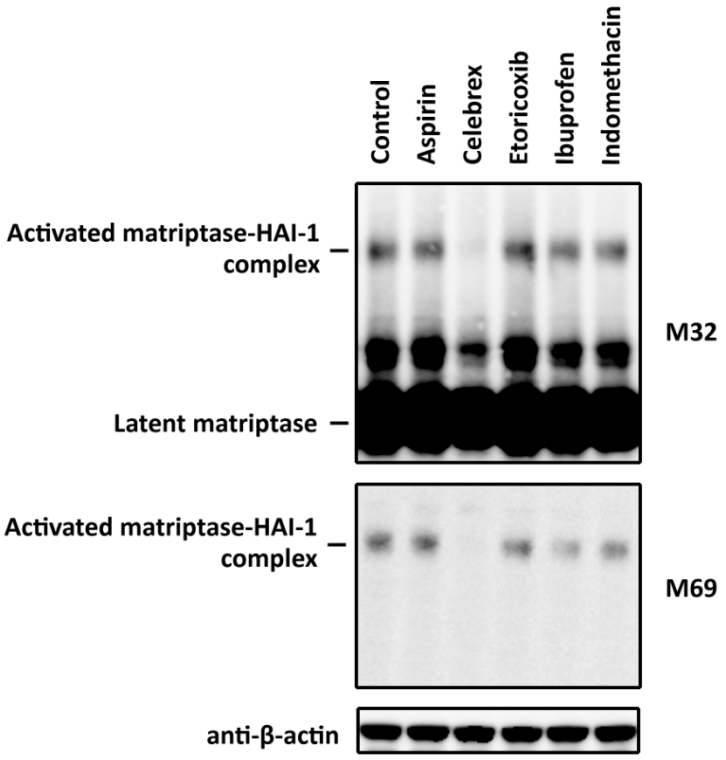
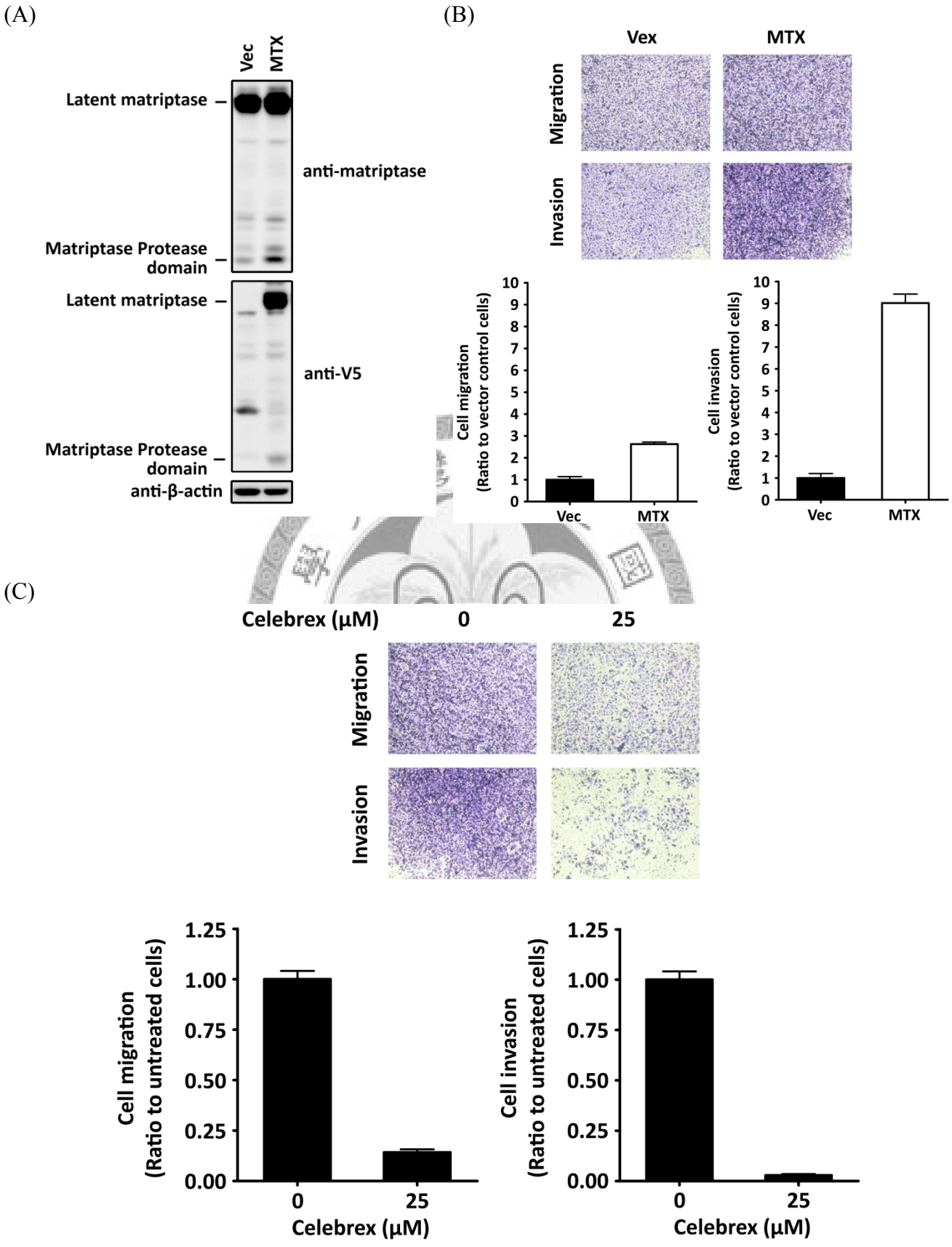


Figure 13. Effect of different NSAIDs on matriptase expression and activation in LNCaP cells.

LNCaP cells were seeded at a density of 1×10^5 cells per 60-mm dish and maintained in 5% FBS RPMI-1640 medium at a 37 °C, 5% CO_2 incubator. 48 hours after seeding, cells were washed twice with PBS. Indicated concentrations of aspirin (50 μM), celebrex (25 μM), Etoricoxib (25 μM), Ibuprofen (25 μM) and Indomethacin (25 μM) in the serum-free RPMI-1640 medium were added to the media and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 16 hours. After treatments, cell lysates were collected and analyzed by western blotting using anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.


(A)
Vec
MTX
Latent matriptase
anti-matriptase
Matriptase Protease domain
Latent matriptase
anti-V5
Matriptase Protease domain
anti-β-actin
(B)
Vex
MTX
Migration
Invasion
Cell migration (Ratio to vector control cells)
Cell invasion (Ratio to vector control cells)
Vec
MTX
(C)
Celebrex (μM)
0
25
Migration
Invasion
Cell migration (Ratio to untreated cells)
Cell invasion (Ratio to untreated cells)
Celebrex (μM)

(D)

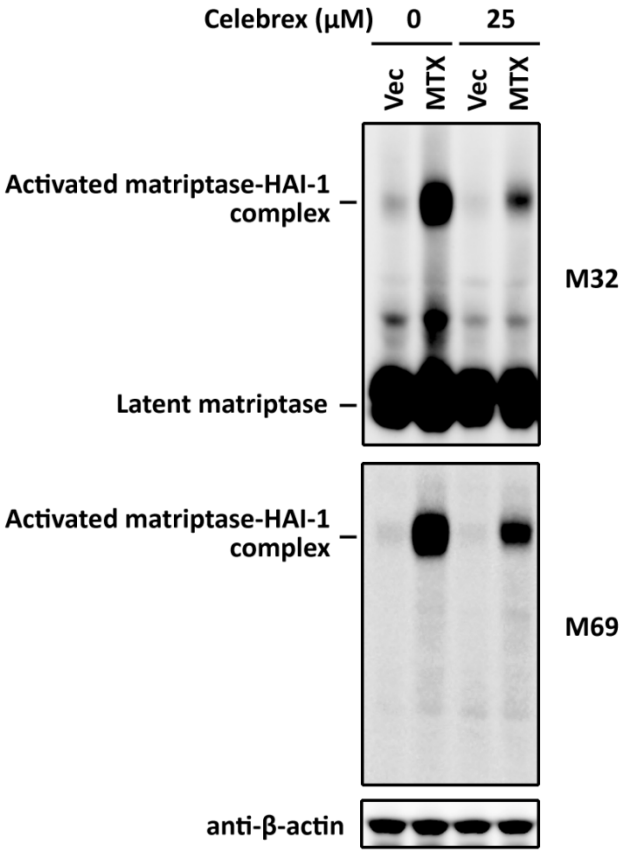


Figure 14. Establishment of MTX-overexpressing PC-3 cells and the effect of celebrex on MTX-overexpressing PC-3 cell migration, invasion and matriptase activation.

(A) Western blotting analysis of MTX-overexpressing PC-3 cells. PC-3 cells were seeding in 6-well plates and then were transfected with MTX-V5 plasmids. Control cells were transfected with vector (pcDNA 3.1) alone. After transfection, stable pools of transfectants were selected by 400 g/ml G418. Stable pools of transfectants were harvested for western blotting assays with anti-matriptase and an anti-V5 Ab. A loading control was analyzed with an anti-β-actin Ab. (B) Analysis of the role of matriptase on PC-3 cell migration and invasion. 1×10^5 (3×10^5 cells/cm^2) of serum-starved Vec cells or MTX cells in serum-free medium were

seeded into the upper chamber of each transwell. After 16-hour incubation, invaded cells were stained and photographed as described in Figure 1B. Invaded cells were measured by ImageJ software. Results were statistically calculated and presented as mean ± SD (n=2). (C) Analysis of the effect of celebrex on MTX-overexpressing PC-3 cell migration and invasion. 5×10^5 (1.5×10^6 cells/cm^2) of cells in serum-free medium were seeded into the upper chamber of each transwell with indicated concentrations of celebrex (0 and 25 μM). After 16-hour incubation, invaded cells were stained and photographed as described in Figure 1B. Invaded cells were measured by ImageJ software. Results were statistically calculated and presented as mean ± SD (n=2). (D) Analysis of the effect of celebrex on matriptase activation in MTX-overexpressing PC-3 cell. Cells were seeded at a density of 1×10^5 cells per 60-mm dish and maintained in 10% FBS DMEM medium at a 37 °C, 5% CO_2 incubator. Twenty-four hours after seeding, cells were washed twice with PBS. Indicated concentrations of celebrex (0 and 25 μM) in the serum-free DMEM medium were added to the media and the cells were cultivated at a 37 °C, 5% CO_2 incubator for 16 hours. After treatments, cell lysates were collected and analyzed by western blotting using anti-total matriptase (M32) and anti-activated matriptase (M69) mAbs under a non-boiled and non-reduced condition. A loading control was analyzed with an anti-β-actin Ab.

Chapter 6. References

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