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MOLECULAR MECHANISMS OF THROMBOXANE A_2 RECEPTOR-MEDIATED INVASION IN LUNG CANCER CELLS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By

Xiuling Li

Lexington, Kentucky

Director: Dr. Hsin-Hsiung Tai, Professor of Pharmaceutical Sciences

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

MOLECULAR MECHANISMS OF
THROMBOXANE A_2 RECEPTOR-MEDIATED INVASION
IN LUNG CANCER CELLS

Thromboxane A_2 receptor (TP) has been shown to play important roles in multiple aspects of cancer development including regulation of tumor growth, survival and metastasis. Molecular mechanisms of TP mediated cancer cell invasion remain to be identified. TP agonist, I-BOP, significantly elevated several matrix metalloproteinases (MMPs) including MMP-1, MMP-3, MMP-9 and MMP-10 in A549 human lung adenocarcinoma cells overexpressing TPα (A549-TPα) or TPβ (A549-TPβ). Signaling pathways of I-BOP-induced MMP-1 expression were examined in further detail as a model system for MMPs induction. Signaling molecules involved in I-BOP-induced MMP-1 expression were identified by using specific inhibitors including small interfering (si)-RNAs of signaling molecules and promoter reporter assay. The results indicate that I-BOP-induced MMP-1 expression is mediated by protein kinase C (PKC), extracellular signal-regulated kinase (ERK)-activator protein-1(AP-1) and ERK-CCAAT/enhancer-binding protein β (C/EBPβ) pathways. I-BOP-induced cellular invasiveness of A549-TPα cells was blocked by, GM6001, a general inhibitor of MMPs. Knockdown of MMP-1 and MMP-9 by their respective siRNA partially reduced I-BOP-stimulated A549-TPα cells invasion suggesting that other MMPs induced by I-BOP were also involved.

Furthermore, secreted MMP-1 in conditioned media from I-BOP-treated A549-TPα cells (CM-I-BOP) autocrinely induced monocyte chemoattractant protein-1 (MCP-1) expression. The induction of MCP-1 by MMP-1 in A549 cells was via activation of protease-activated receptor 2 (PAR2) instead of commonly assumed PAR1. This conclusion was reached from the following findings: (1) expression of MCP-1 induced by trypsin, a PAR2 agonist, was inhibited by a PAR2 antagonist. (2) expression of MCP-1 induced by MMP-1 and by CM-I-BOP was blocked by a PAR2 antagonist but not by other PAR antagonists; (3) expression of MCP-1 induced by MMP-1 and by CM-I-BOP was attenuated significantly by pretreatment of cells with PAR2-siRNA.

Finally, MCP-1 also can be induced by direct activation of TP in a SP1 involved mechanism. CM-I-BOP enhanced MCP-1-dependent migration of RAW 264.7

macrophages. Co-culture of A549 cells with RAW 264.7 macrophages induced expression of MMPs, VEGF and MCP-1 genes, and increased the invasive potential in A549 cells.

My studies provide molecular mechanisms by which TP-mediated cancer cell invasion and suggest that TP is a potential anti-cancer drug target.

Key words: Thromboxane A_2 Receptor, Invasion, Matrix Metalloproteinases, Monocyte Chemoattractant Protein-1, Protease-Activated Receptor

Xiuling Li

May 14, 2012

MOLECULAR MECHANISMS OF
THROMBOXANE A_2 RECEPTOR-MEDIATED INVASION
IN LUNG CANCER CELLS

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF ABBREVIATIONS xiii

1. Introduction 1

1.1 Thromboxane A_2 (TXA_2) and its biological function 1

1.1.1 Discovery of TXA_2 and its biosynthesis 1

1.1.2 Physiology and pathophysiology of TXA_2 3

1.2 Thromboxane A_2 receptor (TP) and its signal transduction 3

1.3 TXA_2 signaling in cancer progression 8

1.3.1 Thromboxane A_2 synthase (TXAS), TP and cancer prognosis 8

1.3.2 Mechanisms of TXA_2 signaling in carcinogenesis 9

1.3.2.1 Cell survive, proliferation and apoptosis 9

1.3.2.2 Cell migration, invasion and metastasis 10

1.3.2.3 Angiogenesis 12

1.4 Cancer invasion and metastasis 13

1.5 Matrix metalloproteinases (MMPs) in cancer invasion and metastasis 17

1.5.1 Association of COX -2 and MMPs in cancer invasion and metastasis 18

1.5.2 MMPs regulation 19

1.6 Protease-activated receptors (PARs) and cancer progression and invasion 20

1.6.1 PARs and their activation 20

1.6.2 PAR1 and cancer progression and invasion 22

1.6.3 PAR2 and cancer progression and invasion 22
1.7 Monocyte chemoattractant protein 1(MCP-1) and cancer progression and metastasis 24
1.7.1 MCP-1 discovery 24
1.7.2 MCP-1 regulation 24
1.7.3 MCP-1/CCR2 expression and activity 25
1.7.4 MCP-1 and cancer progression and metastasis 26
1.7.4.1 Direct effects 26
1.7.4.2 Indirect effects 27
1.8 Tumor associated macrophages (TAMs) and cancer invasion 29
1.8.1 TAMs as prognostic marker for human cancer 29
1.8.2 TAMs enhance invasiveness of cancer cells 30
2. Purpose of this dissertation 32
3. Materials and methods 33
3.1 Reagents and antibodies 33
3.2 Cell lines and culture 34
3.2.1 Wild-type cell lines 34
3.2.2 Establishment of A549-TPα and A549-TPβ cell lines 35
3.3 Cell treatment and Western blot analysis 35
3.4 Protein precipitation 36
3.5 Reverse transcription-polymerase chain reaction (RT-PCR) 36
3.6 Zymographic assay 38
3.7 Conditioned media and activation of MMPs 38

3.8 Transient transfection 38

3.9 Small interfering (si)-RNA treatment 39

3.10 Real-time PCR analysis of MCP-1 mRNA 39

3.11 Promoter constructs and site-directed mutagenesis 40

3.12 Luciferase assay 41

3.13 Preparation of nuclear extract 42

3.14 Macrophage migration assay 42

3.15 Co-culture experiments 43

3.16 Invasion assay 43

3.17 Statistical analysis 44

4. Results 45

4.1 Overexpression of TP increases invasion of human lung cancer cell A549 45

4.1.1 Ectopic expression of TPα and TPβ in A549 cells by lentiviral approach 45

4.1.2 TP agonist induces decreases in E-cadherin levels in A549-TPα and TPβ cells 47

4.1.3 TP agonist induces morphological changes of A549-TPα and TPβ cells 48

4.1.4 TP agonist induces invasion of A549-TPα and TPβ cells 49

4.2 Increased expression of MMPs mediates TP agonist-induced invasion in A549-TPα cells 50

4.2.1 Induction of MMPs expression by TP agonist in both A549-TPα and TPβ cells 50

4.2.2 Upregulation of MMP-1 is dependent on PKC and MAPK/ERK signaling 56

4.2.3 AP-1 as a downstream signal of PKC and MAPK/ERK pathways 58

4.2.4 C/EBPβ contributes to I-BOP-induced MMP-1 expression 65
4.2.5 Elevated MMPs promote TP-mediated cell invasion 70
4.3 MMP-1 released by activation of TP induces expression of MCP-1 from lung cancer cells by activation of PAR2 73
4.3.1 Conditioned media from TP agonist-treated A549-TPα cells induces the release of MCP-1 from A549 cells 73
4.3.2 MMP-1 mediates the release of MCP-1 by conditioned media from TP agonist-treated A549-TPα cells 75
4.3.3 MMP-1-induced MCP-1 release is PAR2 dependent 78
4.4 Activation of TP increases MCP-1 expression and recruits macrophages to promote invasion of lung cancer cells 86
4.4.1 Induction of MCP-1 expression by activation of TP 86
4.4.2 SP1 is important for TPα-mediated MCP-1 expression 91
4.4.3 TP agonist-induced MCP-1 exhibits chemotactic effects on macrophages ... 97
4.4.4 Co-culture of RAW 264.7 macrophages induces expression of metastatic genes by A549 cells 99
4.4.5 Co-culture of RAW 264.7 macrophages induces morphological changes and invasion of A549 cells 104
5. Discussion 107
5.1 Increased expression of MMPs mediates TP agonist-induced invasion in A549-TPα cells 107
5.2 MMP-1-mediated release of MCP-1 by conditioned media from TP agonist-treated A549-TPα cells is through activation of PAR2 112

5.3 Activation of TP increases MCP-1 expression and recruits macrophages to promote invasion of lung cancer cells 114

6. Summary and conclusions 120

7. References 122

VITA 154

LIST OF TABLES

Table 1. Primers used for RT-PCR 37

Table 2. Primers used for real-time PCR 40

Table 3. Primers used for generation of site-directed mutagenesis 41

LIST OF FIGURES

Figure 1. Schematic presentation of thromboxane A_2 synthesis..... 2

Figure 2. Structures of thromboxane A_2 receptor α and β proteins 4

Figure 3. Structures of thromboxane A_2 receptor agonists and antagonist..... 5

Figure 4. G protein coupling of thromboxane A_2 receptor and signal transduction..... 7

Figure 5. The metastatic cascade 14

Figure 6. Cancer cell invasion process 16

Figure 7. Mechanism of activation of proteinase-activated receptors by proteinases 21

Figure 8. Transcription factors in MCP-1 promoter 25

Figure 9. Mechanisms of MCP-1 on cancer progression..... 28

Figure 10. Characterization of A549-TPα and A549-TPβ cells 46

Figure 11. Effects of TP activation on E-cadherin expression 47

Figure 12. Effects of TP activation on cell morphological changes 48

Figure 13. Effects of TP activation on cell invasion..... 49

Figure 14. Effects of I-BOP on MMPs expression and activity 51

Figure 15. Effects of I-BOP on MMP-1 expression 53

Figure 16. Effects of different inhibitors on I-BOP-induced MMP-1 expression 57

Figure 17. AP-1 is a downstream signal of PKC and MAPK/ERK pathways 60

Figure 18. C/EBPβ contributes to I-BOP-induced MMP-1 expression..... 66

Figure 19. Elevated MMPs promote I-BOP-induced cell invasion 71

Figure 20. CM-I-BOP induces release of MCP-1 from A549 cells..... 74

Figure 21. MMP-1 mediates CM-I-BOP-induced MCP-1 from A549 cells 75

Figure 22. PAR2 antagonist blocks MMP-1-induced MCP-1 release from A549 cells... 80

Figure 23. PAR-APs induce MCP-1 release from A549 cells 81

Figure 24. PARs antagonists block PARs agonists-induced MCP-1 release from A549 cells 82

Figure 25. MMP-1 and PAR2-AP induce the transcription of MCP-1 83

Figure 26. PAR2 antagonist blocks CM-I-BOP-induced MCP-1 release from A549 cells 84

Figure 27. PAR2 siRNA suppresses MMP-1 and CM-I-BOP-induced MCP-1 release from A549 cells 85

Figure 28. Effects of I-BOP on MCP-1 expression in A549-TPα cells 87

Figure 29. I-BOP-induced MCP-1 protein expression in H157 and H460 cells 89

Figure 30. Comparison of I-BOP with other prostanoids on the induction of MCP-1 in A549-TPα and H460 cells 90

Figure 31. Effects of TP antagonist and several protein kinases inhibitors on I-BOP-induced MCP-1 expression 92

Figure 32. Effects of SP1 inhibition on I-BOP-induced MCP-1 expression at protein levels 93

Figure 33. Effects of SP1 inhibition on I-BOP-induced MCP-1 expression at mRNA levels 94

Figure 34. SP1 protein levels are increased in the nuclei of A549-TPα cells following I-BOP stimulation 95

Figure 35. Regulation of MCP-1 promoter activity by SP1 96

Figure 36. Effects of A549-TPα conditioned medium on the migration of RAW 264.7 macrophages 98

Figure 37. Diagram of a transwell co-culture system 100

Figure 38. Transwell co-culture of RAW 264.7 macrophages induces morphological changes of A549 cells 101

Figure 39. Co-culture of macrophages induces metastatic gene expression by A549 cells 102

Figure 40. Co-culture of macrophages decreases E-cadherin expression by A549 cells 103

Figure 41. Diagram of a direct co-culture system..... 105

Figure 42. Direct co-culture of macrophages induces morphological changes of A549 cells 105

Figure 43. Co-culture of macrophages induces invasion of A549 cells 106

Figure 44. Proposed pathways of TP-mediated MMP-1 expression 111

Figure 45. Illustration of a tentative mechanism for TP-mediated invasion of cancer cells 119

LIST OF ABBREVIATIONS

A549-TPα = A549 cells stably overexpressing TPα receptor

A549-TPβ = A549 cells stably overexpressing TPβ receptor

AA = arachidonic acid

AC = adenylyl cyclase

AP-1 = activator protein-1

APRIL = a proliferation-inducing ligand

bFGF = basic fibroblast growth factor

C/EBPβ = CCAAT/enhancer-binding protein β

CM-I-BOP = conditioned media from I-BOP-treated A549-TPα cells

COX1/2= cyclooxygenase 1or 2

CSF1= colony-stimulating factor 1

CSF2= colony-stimulating factor 2

CTGF= connective tissue growth factor

CXCL1= Chemokine (C-X-C motif) ligand 1

Cyr61= Cysteine-rich, angiogenic inducer, 61

DAG = diacylglycerol

DMEM = Dulbecco modified Eagle's medium

ECM = extracellular matrix

EGFR= epidermal growth factor receptor

EMT = epithelial-mesenchymal transition

ERK = extracellular signal-regulated kinase

GAPDH = glyceraldehydes-3-phosphate dehydrogenase

GPCR = G-protein coupled receptor

HB-EGF = heparin-binding epidermal growth factor

IFN-γ = interferon gamma

IGF-1 = insulin-like growth factor1

IL-6 = interleukin-6

IL-8= Interleukin 8

MAPK = mitogen-activated protein kinase

MCP-1= monocyte chemoattractant protein-1

MMP = matrix metalloproteinase

NF-κB = Nuclear factor-kappaB

NK = nature killer

NSCLC = non-small cell lung cancer

PDGF = platelet-derived growth factor

PKA = protein kinase A, cAMP dependent protein kinase

PKC = protein kinase C, calcium dependent protein kinase

PLC = phospholipase C

PVDF = polyvinylidene fluoride

PGDS = prostaglandin D_2 synthase

PGES= prostaglandin E_2 synthase

$PGF_{2\alpha}$ S= prostaglandin $F_{2\alpha}$ synthase

PGIS= prostaglandin I_2 synthase

PI-3K = phosphoinositide-3-kinase

RT-PCR = reverse transcription polymerase chain reaction

SAPK/JNK = stress-activated protein kinase/c-Jun N-terminal kinase

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

siRNA = small interfering RNA

TCA = trichloroacetic acid

TAM = tumor associated macrophage

TNFα = tumor necrosis factor alpha

TP = thromboxane A_2 receptor

TXA_2 = thromboxane A_2

TXAS = thromboxane synthase

PAR= protease-activated receptor

VEGF= vascular endothelial growth factor

1. Introduction

1.1 Thromboxane A_2 (TXA_2) and its biological function

1.1.1 Discovery of TXA_2 and its biosynthesis

Thromboxane A_2 (TXA_2) was originally discovered by Piper and Vane as a rabbit aorta contracting substance (RCS) in 1969 [1]. The release of RCS was reduced by steroidal and non-steroidal anti-inflammatory drugs suggesting the association of RCS with prostaglandins [2]. In 1975, Hamberg *et al.* reported that RCS actually was TXA_2 derived from arachidonic acid (AA) and prostaglandin G_2 (PGG_2) [3]. Because TXA_2 has a very short half-life of only 34 seconds in aqueous solution, they proposed a chemical structure of TXA_2 from indirect experiments by trapping it with nucleophiles such as CH_3O-. This structure was finally confirmed by Bhagwat *et al.* in 1985 [4].

TXA_2 is one of the five primary prostanoids generated from AA through cyclooxygenase (COX) pathway. The biosynthesis of TXA_2 and other prostaglandins involves three sequential steps (Figure 1):

(1) Release of AA from cell membrane phospholipids by phospholipase A_2 (PLA_2).

(2) Conversion of AA to prostaglandin H_2 (PGH_2) by COX-1 and COX-2.

(3) Isomerization of PGH_2 to TXA_2 and other PGs by their specific synthases or isomerases.

The second step is believed to be the rate-limiting step for generation of TXA_2 and other PGs. Two isoforms of COX have been described. COX-1 is constitutively expressed and is involved in normal physiological functions, such as platelet aggregation, gastric mucosa protection and renal functions, whereas, COX-2 is an inducible isoform

associated with pathological functions such as inflammation, cardiovascular disease and cancer.

The enzyme converted PGH_2 to TXA_2 is thromboxane A_2 synthase (TXAS), which was first found in platelet microsomes [5], then was reported to express in many other tissues such as lung, platelets, kidney, stomach, duodenum, colon and spleen [6, 7].

As mentioned above, TXA_2 is very unstable and rapidly, non-enzymatically degraded into a stable but inactive thromboxane B_2 (TXB_2) form.

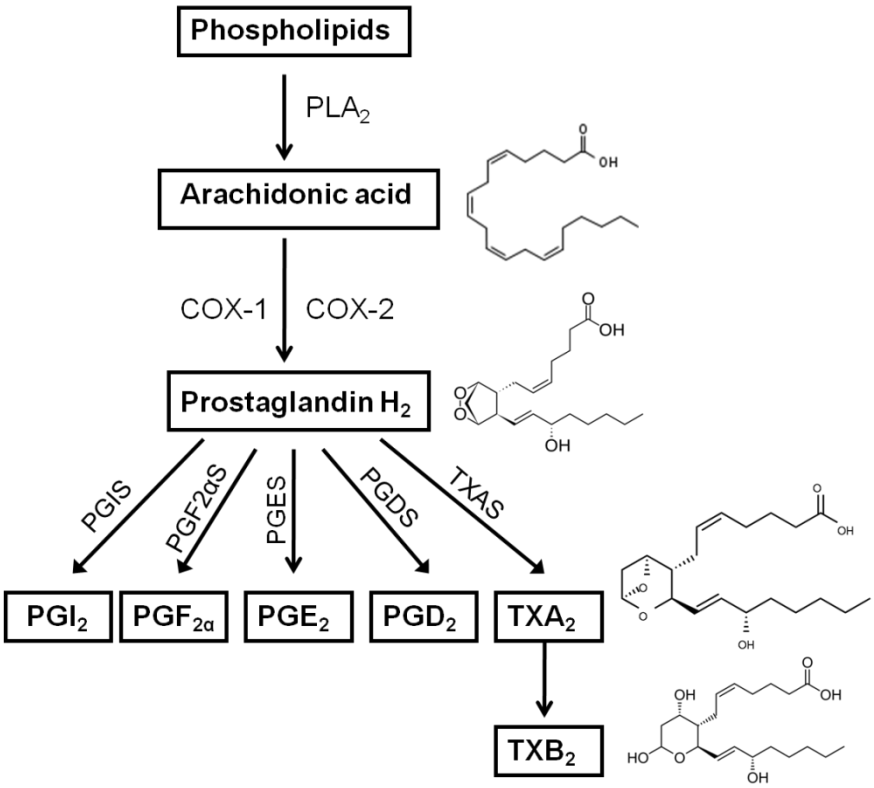

Figure 1. Schematic presentation of thromboxane A_2 synthesis

PLA_2 catalyzes phospholipids to form arachidonic acid (AA). Then AA is metabolized by COX-1 and COX-2 to form prostaglandin H_2 (PGH_2), which is converted into PGs by specific synthases.

1.1.2 Physiology and pathophysiology of TXA_2

TXA_2 has a broad range of bioactivities. It induces vascular smooth muscles contraction, revealing its contribution to hypertension. It also causes contraction of bronchial smooth muscles, which may result in asthma. TXA_2 is a potent platelet activator, leading to platelet shape change, secretion and aggregation, thus promoting thrombus formation and thrombosis. In addition, it has been shown that TXA_2 stimulates the proliferation of several types of cells including oligodendrocytes [8], smooth muscle cells [9] and lung cancer cells [10]. Moreover, TXA_2 is implicated to be involved in allergies, inflammation, acquired immunity modulation, atherogenesis, angiogenesis, and metastasis of cancer [11].

1.2 Thromboxane A_2 receptor (TP) and its signal transduction

TXA_2 exerts its biological activities through its cell surface receptor, the T-prostanoid (TP) receptor. TP is widely distributed in many tissues and organs, including brain, eye, thymus, heart, lung, aorta, liver, uterus, intestine, kidney, placenta, and platelets [11]. TP belongs to G-protein-coupled-receptor (GPCR) superfamily and is expressed as two isoforms in human, named TPα and TPβ. These TP isoforms arise from a single gene via alternative splicing of mRNA and share the first 328 amino acids, but differ in their C-terminal tails with TPβ longer than TPα (Figure 2) [12]. The ligand binding domain of TP resides mainly in the extracellular region of the protein, therefore no differences were observed in the ligand binding between TPα and TPβ, whereas there are some variances in the G-protein coupling of these two isoforms. TPα is coupled to Gs and TPβ is coupled to Gi.

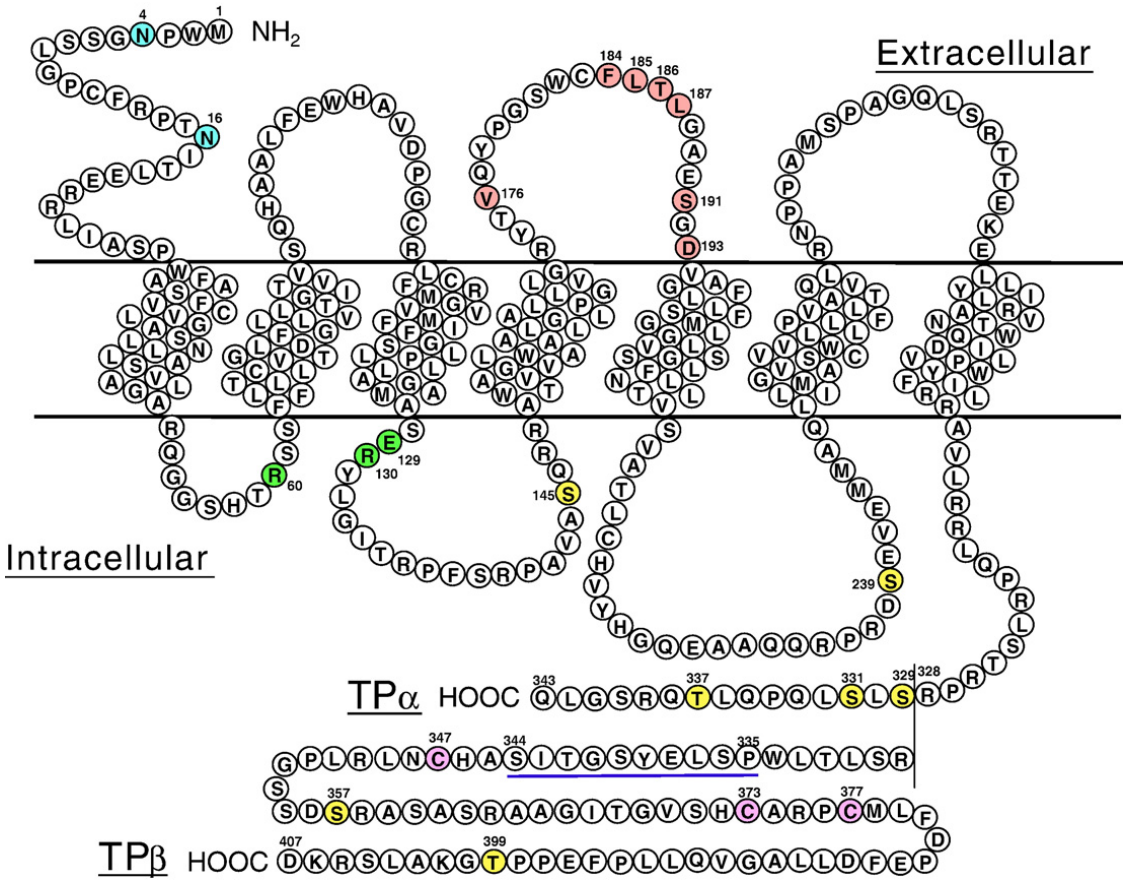

Figure 2. Structures of thromboxane A_2 receptor α and β proteins

Due to the very unstable property of TXA_2, its mimetics instead of TXA_2 itself are used by researchers to study TP-mediated events. U46619 and I-BOP are two synthetic TP specific agonists and SQ29584 is a specific TP antagonist.

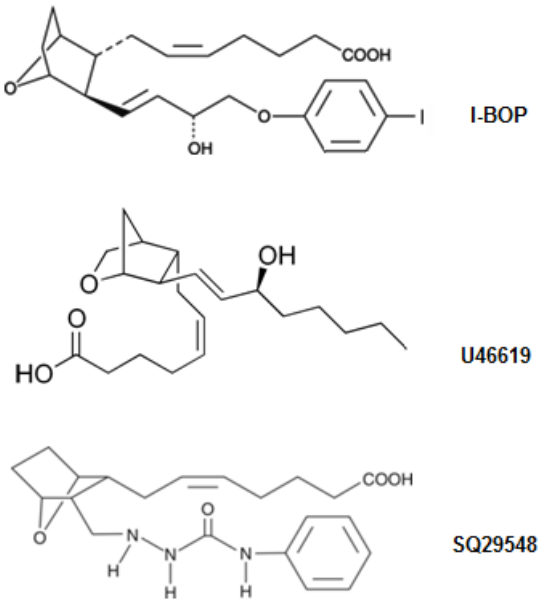

Figure 3. Structures of thromboxane A_2 receptor agonists and antagonist

TP-mediated signal transduction is mostly achieved through activation of its coupled G proteins. Gq was the first found trimeric G protein coupled to TP to activate phospholiase C-β (PLC-β) [13], resulting in the accumulation of inositol triphosphate (IP3)/diacylglycerol (DAG), subsequent mobilization of intracellular calcium and activation of PKC. In addition to Gq, TP has been shown to be coupled with other G proteins, including G12 family G proteins (G12 and G13) [14], Gi [15], Gs [16] and Gh [17], thus activating multiple signaling pathways. G12 and G13 have been shown to

activate RhoGEF, following the activation of Rho-mediated signaling [18]. TP-mediated contraction of rat caudal arterial smooth muscle and migration of prostate cancer cells were reported to depend on RhoA/ROCK pathway [19, 20]. Both Gi and Gs regulate cyclic AMP (cAMP) levels but in opposite directions. Gi inhibits adenylyl cyclase (AC), thus decreasing cAMP levels, and Gs stimulates AC, thus increasing cAMP levels. The activity of protein kinase A (PKA) is regulated by the levels of cAMP. It has been shown that TPα couples to Gs, TPβ couples to Gi, and elevation of cAMP were observed in HEK or CHO cells expressing TPα but not TPβ [21, 22]. Therefore, TPα and TPβ may have distinct signal pathways to regulate cellular events. Using CHO cells stably expressing TPα or TPβ, Miyosawa *et al.* found that TPα not TPβ-mediated activation of the extracellular signal regulated kinase1/2 (ERK1/2) was dependent on PKA activation [23]. This contradicted with an earlier report that PKA was involved in ERK activation in both HEK cells stably expressing TPα or TPβ [24]. This might be explained by the assumption that the levels of signaling molecules downstream of TP vary in different cell types [23]. Moreover, many studies indicated that TP-mediated signaling evens differ in different cell types and tissues [25]. Gh, which is a dimeric G protein, activates phospholipase C-δ (PLC-δ), subsequent production of IP3 and DAG, a similar response to that mediated by Gq. Although Gh couples to both TPα and TPβ, only TPα-Gh association results in a PLC-dependent IP3 signaling [17]. Dissociated from Gα subunit, the activated Gβγ can also mediate signaling transduction through activation of class I_B phosphatidylinositol 3-kinase (PI3K) and ERK1/2. Although there is evidence that PI3K inhibitor blocked TP-mediated activation of ERK, Gβγ subunits did not seem to be involved in this regulation, suggesting the existence of other mechanisms than activating

G proteins for TP-mediated signal transduction. Further studies showed that TPα and TPβ directly interact with PI3K class 1_A p85 adaptor subunit to regulate ERK1/2 activation [24]. Furthermore, activation of TP may transactivate epidermal growth factor receptor (EGFR) through a src-dependent mechanism, leading to the activation of ERK1/2 [24, 26]. Moreover, it was reported that 14-3-3ζ as a scaffold protein to associate with TP and raf-1, therefore, mediates TP signal-regulated ERK1/2 activation [27]. G protein coupling of TP and signal transduction is summarized in Figure 4 [11].

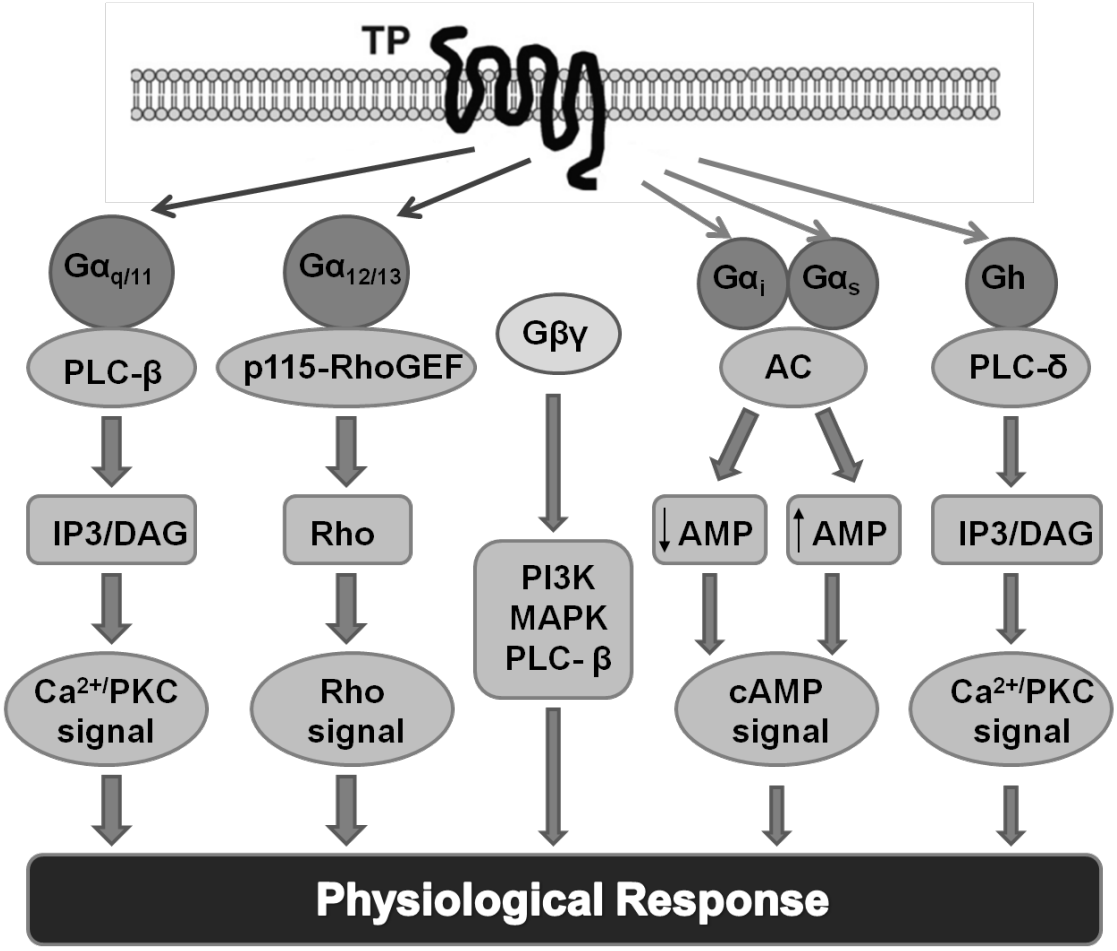

Figure 4. G protein coupling of thromboxane A_2 receptor and signal transduction

1.3 TXA_2 signaling in cancer progression

Early in 1981, TXA_2 has already been reported to stimulate melanoma tumor cells proliferation, suggesting its potential role in tumor progression [28]. Then, over the past three decades, more and more evidence indicated the important role of TXA_2 signaling in malignancy.

1.3.1 Thromboxane A_2 synthase (TXAS), TP and cancer prognosis

TXAS and TP are two main components of TXA_2 pathway, and both were observed to increase in tumor tissues from 46 patients with prostate cancer [29]. The levels of TXAS and TP were significantly associated with higher Gleason scores and pathologic stages of the tumors [29]. A large study employing 120 human breast tumor tissues found that TP was highly expressed in aggressive tumors (grade 3 and above) and linked with higher mortality and worse prognosis [30]. In addition, TXAS and TP were expressed at significantly higher levels in human colorectal carcinoma compared with normal tissues [31, 32]. Furthermore, Watson and his colleagues reported that the expression of TXAS was significantly elevated in invasive bladder cancer, and negatively correlated with patient survival. TP protein not mRNA expression was also increased in invasive bladder cancer tissues compared to paired normal tissues [33]. One following study from the same group further identified that it was the TPβ isoform highly expressed in bladder cancer tissues and correlated well with poor prognosis for patients [34]. Moreover, high levels of TXB_2, the stable nonenzymatic hydrolysis product of TXA_2, were detected in lung cancer tissues compared to normal lung tissues, suggesting the increased TXAS activity in lung cancer [35]. More recently, Cathart *et al*. examined the expression profile of TXAS in non-small cell lung cancer (NSCLC) in 204 patients, and

confirmed that TXAS and TXB_2 were significantly higher in tumor tissues than the matched normal tissues [36]. Although TXAS was not a prognostic factor, this study indicated that TXAS could promote tumor progression and serve as a potential therapeutic target for treatment of NSCLC [36].

1.3.2 Mechanisms of TXA_2 signaling in carcinogenesis

To understand the mechanisms underlying the effects of TXA_2 signaling on cancer progression, both *in vitro* and *in vivo* studies were conducted by many researchers to investigate the role of TXA_2 pathway in cancer biology. The results showed that TXA_2 signaling can modulate cancer progression in many aspects including tumor cells proliferation, apoptosis, migration, invasion, angiogenesis and the metastatic process.

1.3.2.1 Cell survive, proliferation and apoptosis

TXA_2 has been found to contribute to survival, proliferation and growth of several types of cancer cells including lung cancer, colorectal cancer, brain cancer and bladder cancer cells. It was reported that in nude mice tumor xenograft model, tumors from A549 cells with ectopic TPα expression exhibited faster growth rate than those tumors from the control A549 cells [37]. Using another lung cancer cell line H157 which expressed endogenous TP, we identified that Nurr1 is a target gene of TXA_2 signaling. The expression of Nurr1 is critical for TP-mediated H157 cell proliferation [10]. Huang *et al.* recently reported that carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) increased the production of TXAS and TXA_2 *in vivo* and in lung cancer cells *in vitro*. The increased TXA_2 may subsequently activate CREB via PI3K/Akt and ERK pathways, thereby contributing to the NNK-induced survival and growth of lung cancer cells [38]. TXAS was found highly expressed in human colonic cancer cell lines.

Proliferation of these cells was inhibited by antisense oligonucleotide for TXAS as well as TP antagonists, indicating a critic role of TXA_2 in colon cancer growth [31]. Furegrelate, a specific TXAS inhibitor, significantly inhibited glioblastoma growth through inducing proapoptotic, antiproliferative, and anti-angiogenic effects. Further, the inhibition of TXAS also increased the sensitivity to conventional alkylation chemotherapy *in vivo* [39]. Moreover, TXAS inhibitors and TP antagonists-induced apoptosis and increased sensitivity to chemotherapeutic agents were also found in bladder cancers [34, 40].

1.3.2.2 Cell migration, invasion and metastasis

The most important difference between benign and malignant tumors is that the malignant tumors spread to other parts of the body through the lymphatic system or bloodstream (a process called metastasis), while benign tumors do not. Tumor cells migration and invasion are initial steps in metastasis. Within prostate cancer tissues, TXAS and TP were found highly expressed in the areas of perineural invasion which is a major mechanism by which tumor spread outside the prostate, indicating the involvement of TXA_2 signaling in metastasis of prostate cancer [29, 41]. Further studies showed that TXAS and TXA_2 signaling mediated migration and invasion in prostate cancer cells [20, 41]. The mechanism by which TXA_2 drives these processes is related to Rho signaling. TP is known to couple to G_{12} family of heterotrimeric G proteins ($G\alpha_{12}$ and $G\alpha_{13}$). Kelly *et al.* reported that expression of activated G_{12} and G_{13} promoted prostate cancer cells invasion through activation of the RhoA family of G proteins [42]. They further observed that inhibition of G_{12} signaling blocked TXA_2-induced invasion of prostate cancer cells suggesting the G_{12}-RhoA signaling is involved in TP-mediated cell invasion

[42]. Later, Nie and colleagues demonstrated that activation of TP regulated prostate cancer cell motility and cytoskeleton reorganization through inducing cell contraction via activation of RhoA [20]. A recent study revealed that in prostate cancer cells PC3 and LNCaP, both TPα and TPβ constitutively interact with protein kinase C-related kinases (PRK1), which is a RhoA effector. Activation of TPs enhanced PRK1 activation and disruption of PRK1 impaired TP-mediated cell migration [43]. In addition to prostate cancer, expression levels of TXAS were highly elevated in selected migration-advantaged glioma cells. TXAS inhibitors, dazmegrel and furegrelate, reduced the migration rate of these cells to the rate of the parental cells, suggesting TXAS may influence the motility of glioma cells [44]. Further, TXB_2 was detected in a wide range of glioma cell lines and the relative expression of which correlated with migration of these cell lines. Treatment with TXAS specific inhibitors resulted in decreased migration rate and intercellular adhesion, indicating TXA_2 signaling may represent a novel strategy for anti-invasive therapies for glial tumors [45]. As mentioned above, TXAS and TP especially TPβ were highly expressed in invasive bladder cancer tissues compared with their normal paired tissues and correlated with poor prognosis [33, 34]. Ectopic expression of TPβ in normal bladder epithelial cell line significantly increased migration and invasion [34]. The mechanistic study revealed that TP receptor agonist stimulated bladder cancer cell migration through both β-arrestin 2 and $G\alpha_{12}$ signaling [34]. Moreover, TXAS was also implied to be involved in lung cancer metastasis since TXAS inhibitor significantly inhibited pulmonary metastasis in mice intravenously injected Lewis lung carcinoma [46].

1.3.2.3 Angiogenesis

In order to grow beyond a certain size and to metastasize, tumors need to recruit blood vessels to supply oxygen and nutrients. Angiogenesis is the growth of new blood vessels from the existing vasculature and is critical for tumor growth and progression. TXA_2 modulates angiogenesis at different levels.

The expression of TXAS was reported to positively correlate to the production of angiogenic factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in NSCLC [47], indicating TXA_2 may enhance angiogenesis, thereby accelerating cancer progression. It was demonstrated that TXA_2 induced expression of VEGF at both mRNA and protein levels in human lung cancer cells. Xenografts of A549-TPα cells increased tumor growth rate and vascularization in nude mice [37]. Similar results were also obtained from mice inoculated with C26-TXAS cells, which are colon adenocarcinoma cells overexpressing exogenous TXAS [48].

TXA_2 not only has an autocrine effect on tumor cells to release pro-angiogenic factors but also directly act on endothelial cells to promote angiogenesis. The pro-angiogenic factor bFGF and VEGF stimulated the release of TXA_2 from endothelial cells up to five-fold [46]. TXA_2 mimetic U46619 increased endothelial cells migration and the inhibitors of TXAS or antagonist of TP blocked bFGF-induced endothelial cells migration *in vitro* and angiogenesis *in vivo* [46, 49]. Furthermore, treatment of rat aortic explants with U46619 significantly enhanced vessel sprouting whereas treatment with TXA_2 inhibitors showed a significant decrease in vessel sprouting [50], which was not rescued by adding VEGF indicating the essential contribution of TXA_2 to angiogenesis.

There are numerous studies indicating the role of TXAS, TXA_2 and TP in cancer progression, and some mechanisms were also revealed in these studies. However, regarding the relationship between TXA_2 and cancer invasion and metastasis, most of these reports are focused on TXAS. As shown in Figure 1, prostaglandins and thromboxane synthetic pathways are complex and influenced by each other. Inhibition of TXAS will increase the production of other PGs which either as tumor suppressor (PGI_2) or promoter (PGE_2). Therefore the approach of inhibiting TXAS will result in other effects related with the generation of other PGs. In this dissertation, I used approaches that only interfere with TXA_2/TP and no other PGs pathways to identify the key components of TXA_2/TP signaling-promoted cancer invasion, and thus providing additional mechanisms for validating TP as a target of intervention to block cancer invasion and metastasis.

1.4 Cancer invasion and metastasis

Prevention of cancer metastasis is the major goal of cancer therapy as metastasis is account for 90% of death in patients with malignancy. The process that cancer cells break off from a primary tumor and spread to other parts of the body is metastasis. Metastasis is a very complicated process still not well understood. The classic view of cancer metastasis consists a complex succession of cell-biological events starting from epithelial cells in primary tumors: (1) invade locally into surrounding extracellular matrix (ECM) and the host stroma, (2) intravasate into blood and lymph vessels, (3) survive in the circulation and transport through the vasculature, (4) arrest in the capillary beds of distant organs, (5) extravasate from vessels into the parenchyma of distant tissues, (6) survive in the new microenvironment and establish micrometastases, and (7) proliferate within the

organ parenchyma, therefore generating the metastatic colonization and clinically detectable macroscopic metastases (Figure 5) [51]. To complete all the metastatic processes, cancer cells have to overcome the physiological barriers in each step. This is regulated by molecular pathways operating within cancer cells and also the cooperation between stroma cells and cancer cells. Each step in the metastatic cascade can be rate limiting. Here, we only focus on the initial step of metastasis, local invasion.

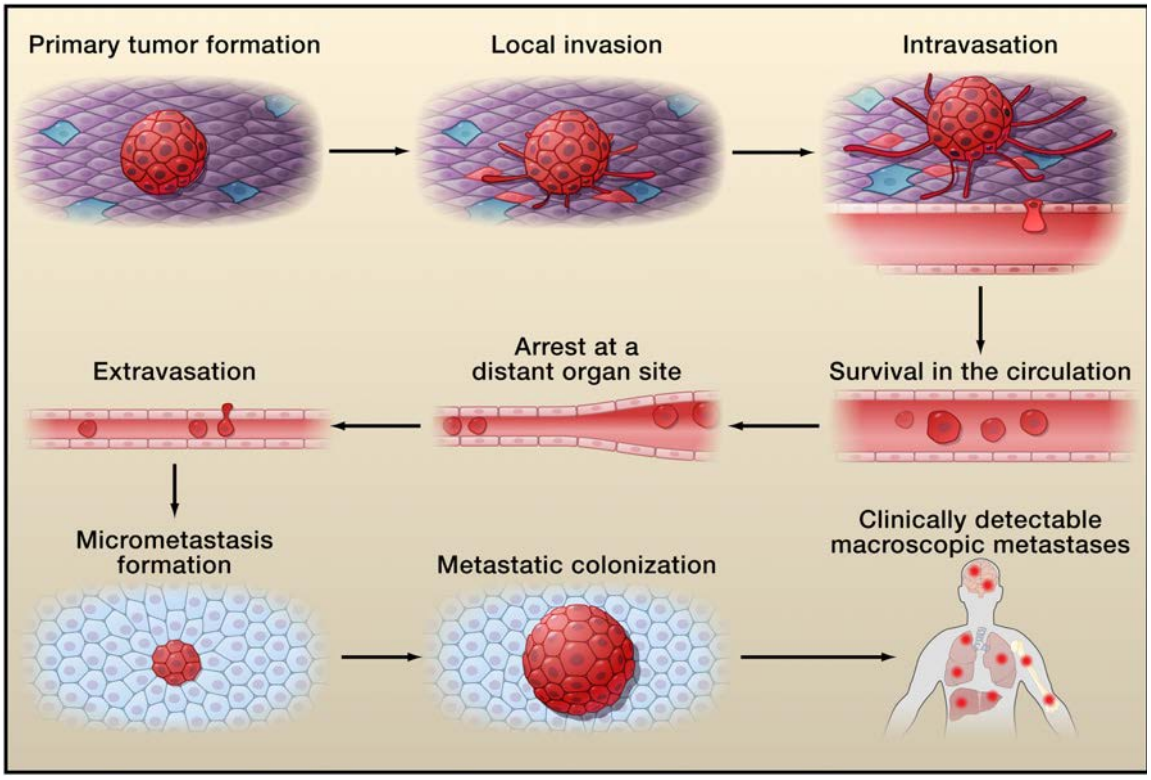

Figure 5. The metastatic cascade

Invasion is a process that cancer cells break away from a primary tumor and enter the surrounding tissues. During this process, cancer cells lose cell-cell adhesion, acquire a migratory phenotype, penetrate the basement membrane, and invade the interstitial matrix (Figure 6) [52].

Most solid tumors are derived from epithelial cells which are closely attached to each other to form a rigid cell sheets. Cadherins, a family of intercellular adhesion molecules, play important roles in keeping cells together. E-cadherin, one subtype in this family, has been demonstrated to play a crucial role in epithelial cell-cell adhesion. It has been implicated in carcinogenesis due to its partially or entirely missing in human epithelial cancers [53]. Loss of E-cadherin resulting in tumor cells invasion has been shown in E-cadherin knockout mouse models [54]. Indeed, repression of E-cadherin is a critical molecular feature of epithelial-mesenchymal transition (EMT), which is a biological program defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. Cells that have undergone EMT often disassemble their adherens junctions, lose polarity, acquire spindle-shaped morphology, enhance migration, induce mesenchymal proteins, as well as various proteinases. Therefore, EMT is a critical early event in cancer invasion and metastasis [55].

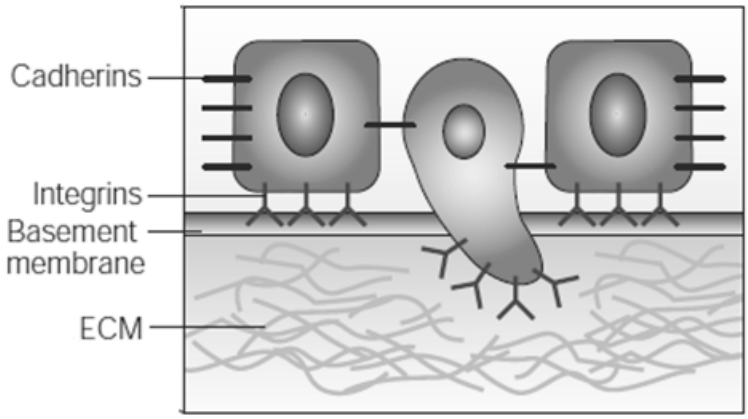

Figure 6. Cancer cell invasion process

At the onset of invasion process, cancer cells lose E-cadherin-dependent cell-cell adhesion, acquire a migratory phenotype, penetrate the basement membrane, and invade the interstitial matrix.

Loss of adhesion enables cells to move, whereas loss of the basement membrane ultimately allows the invasion of cancer cells into the stromal compartment. Basement membrane is a thin ECM layer which separates the epithelium from the interstitial stroma. In addition to the structural function, basement membrane also plays an important role in signal transduction through integrin-mediated cell-marix interaction, leading to the alterations in cell polarity, proliferation, survival, invasion and metastasis[52]. To breach the basement membrane and invade the interstitial matrix cancer cells release a variety of proteolytic enzymes such as collagenases, cathepsins, and plasminogen activators. Among these enzymes, matrix metalloproteinases (MMPs) play major roles in proteolytic degradation of basement membrane and ECM remodeling due to their ability to degrade a

multitude of substrates. MMPs also release growth factors sequestered in the ECM to stimulate cancer cell proliferation and angiogenesis [56, 57].

Once cancer cells have dissolved basement membrane, they invade the stroma where they encounter a variety of stromal cells including fibroblasts, endothelial cells, and various bone marrow-derived cells (BMDCs) such as myeloid cell-derived suppressor cells, mesenchymal stem cells, macrophages, as well as other immune cells [58]. These stromal cells in tumor microenvironment secrete growth factors or proteases that further enhance tumor cells proliferation, invasion and metastasis. For example, tumor associated macrophages (TAMs) produced EGF to activate EGFR signaling in the malignant epithelial cells, thereby enhancing their invasive potential [59]. Similarly, the expression of cathepsin and MMP-9 proteases by TAMs further augments carcinoma cells invasion [60, 61]. More detailed background about TAMs and the roles they played in cancer invasion will be introduced later.

1.5 Matrix metalloproteinases (MMPs) in cancer invasion and metastasis

As mentioned above, MMPs are the major players in degrading basement membrane and ECM, the physiological barriers that must be overcome by cancer cells for successful invasion and metastasis. MMPs are a family of zinc-dependent proteases which contain 24 members in human, and collectively, they are capable of degrading all known ECM components. Elevation of MMPs has been found in various cancer types and considered as biomarkers for poor prognosis [62]. Therefore, understanding molecular pathways leading to upregulation of MMPs in cancer is important for the treatment of cancer invasion and metastasis.

1.5.1 Association of COX -2 and MMPs in cancer invasion and metastasis

COX-2, the key enzyme in TXA_2 synthetic pathway, was found to be associated with MMPs expression to increase metastasis of several types of cancer. Byun *et al*. reported that there was a positive correlation between COX-2 and MMP-2 expression, and the latter might contribute to cancer progression and reduced survival in NSCLC patients [63]. COX-2 inhibitor blocked liver metastasis of colorectal cancer cells due to suppression of MMP-9 activity [64]. Moreover, inhibition of COX-2 decreased invasion and metastasis in breast cancer via decrease in multiple MMPs [65]. In light of these findings, no reports were found to describe the relationship between TP and MMPs in cancer progression. Since TXA_2 is one of the major prostanoids downstream of COX-2 pathway, it is proposed that TXA_2 may also mediate cancer cell invasion through upregulation of MMPs.

MMP-1, the first member of MMPs family to be identified, was found to be associated with poor prognosis in a wide variety of advanced cancers including lung cancer [66, 67]. Low levels of both MMP-1 and COX-2 were positively correlated with survival in NSCLC [68]. Most recently, protease-activated receptor 1(PAR1) was identified as a novel substrate for MMP-1[69]. MMP-1/PAR1 axis has been shown to mediate invasion of breast cancer and melanoma cells [69, 70] providing further evidence for the role of MMP-1 in cancer invasion and metastasis.

In this dissertation, I mainly focused on the role of MMP-1 in TP-mediated cancer cell invasion, and further to identify the signaling pathways involved in TP-mediated MMP-1 expression which serve as a model for TP-mediated regulation of other MMPs.

1.5.2 MMPs regulation

MMPs are synthesized as pro-zymogens which are either secreted into extracellular space or in membrane-bound form. MMPs activity can be regulated at multiple levels such as activation by cell surface proteins [71] and inhibition by tissue inhibitors of metalloproteinases (TIMPs) [72]. However, aberrantly high expression of MMPs has major impact on ECM turnover, thereby contributing to the pathology of diverse diseases including cancer, and this is predominantly controlled at the levels of MMPs gene expression.

Based on the compositions of cis-acting elements in their promoters, MMPs have been roughly grouped into three categories [73]. Group 1 consists of the majority of the MMPs: MMP-1, 3, 7, 9, 10, 12, 13 and 19 which contain a TATA box and an AP-1 site in the proximal region of their promoters. Group 2 consists of MMP-8 and MMP-11, which contain a TATA box but lack the AP-1 site. Group 3 consists of MMP-2 and MMP-14 which lack both the TATA box and the AP-1 site. This classification indicates the types of extracellular signaling that activate them.

Indeed, a variety of signal transduction pathways are involved in MMPs gene regulation [74]. The mitogen activated protein kinase (MAPK) pathways including ERK pathway as well as the p38 MAPK and c-Jun N-terminal kinase (JNK) pathways regulate expression of several MMPs genes such as MMP-1[75-77], MMP-3[78, 79], MMP-9 [80, 81] and MMP-13[82]. Nuclear factor-kappaB (NF-κB) pathway can impact MMPs gene expression through direct transactivation or induction of intermediary genes. For example, RelA, A subfamily of NF-κB proteins, may activate NF-κB co-factor Bcl-3 expression [83], and that Bcl-3 in turn activates MMP-1 gene expression [84]. MMP-9

promoter contains a canonical NF-κB binding site that could directly bind RelA to activate gene transcription [85]. Other signaling pathways such as Smad and STAT regulate MMPs gene expression via interaction with other transcription factors. Smad3 interacted with JunB and Runx-2 for TGF-β-induced MMP-13 expression is an example of this mechanism [86]. STAT3 does not directly bind the MMP-1 promoter, but it can augment MMP-1 transcription through induction of AP-1 proteins such as c-fos [87]. In contrast to STAT3, STAT1 mediates interferon (INF)-γ -repressed IL-1β induction of MMP-13 via sequestration of the transcriptional co-activator CBP/p300 [88]. In summary, MMPs gene regulation is a complex process which involves the integration of multiple signaling pathways and transcriptional factors.

1.6 Protease-activated receptors (PARs) and cancer progression and invasion

1.6.1 PARs and their activation

Protease-activated receptor (PAR) is a unique family of GPCR, which consists of four members: PAR1, PAR2, PAR3 and PAR4. Each PAR carries its own ligand, which is in a masked state until exposed by receptor cleavage. The exposed ligand binds intramolecularly to the receptor to trigger multiple signaling cascades. A certain proteases can activate PARs. Thrombin can cleave and activate PAR1, PAR3 and PAR4. PAR2 is cleaved by trypsin, mast cell tryptase, tissue factor/factor VIIa/factor Xa but not by thrombin. Small synthetic peptides corresponding to the tethered ligand domain are developed as PAR agonist peptides (PAR-APs), which activate PARs without receptor cleavage [89, 90] (Figure 7).

PARs mediate a wide variety of physiological and pathophysiological responses including coagulation, inflammatory responses, repair, mucosal protection, neurogenic inflammation and pain transmission, etc. The role of PARs in cancer progression has also been revealed [91].

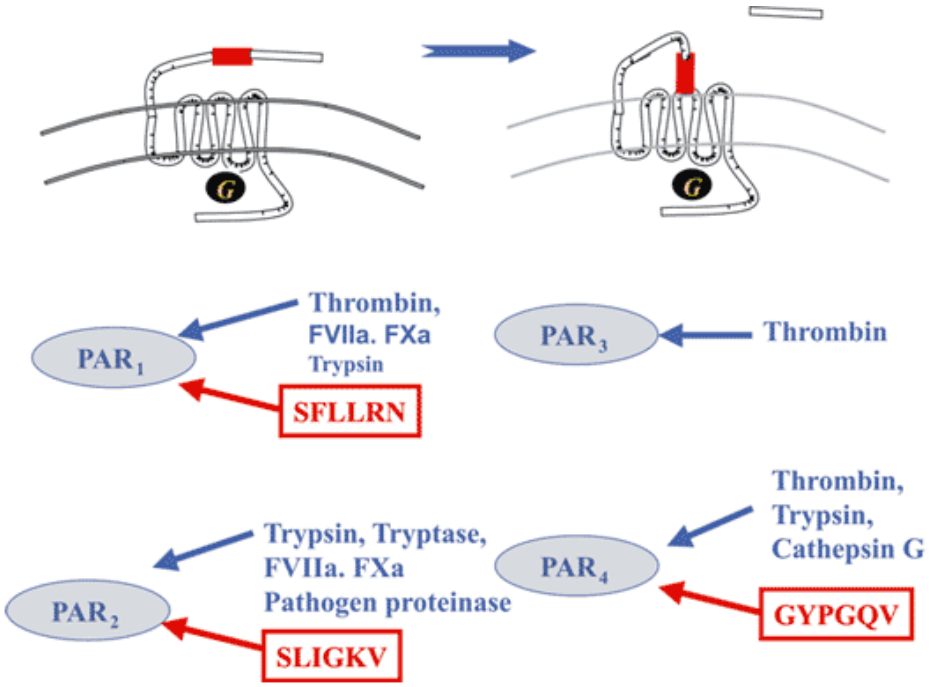

Figure 7. Mechanism of activation of proteinase-activated receptors by proteinases

Four members of the family: PAR1, PAR2, PAR3 and PAR4, proteinases responsible for PARs activation (in blue) and tethered ligand peptidic sequences (red boxes) that can be used to activate each receptor.

1.6.2 PAR1 and cancer progression and invasion

PAR1 is overexpressed in a wide range of cancers, such as melanoma, high-grade gliomas, colon cancer, prostate cancer and invasive breast cancer. The contributions of PAR1 to cancer invasion and metastasis have been demonstrated in many studies. Even-Ram *et al.* showed a direct correlation between the levels of PAR1 expression and the degree of invasiveness in established breast cancer cell lines as well as in human breast tissue specimens [92]. Transfection of functional PAR1 is sufficient to promote the invasion of MCF-7 cells, which are normally noninvasive breast carcinoma cells without PAR1 expression, both in vitro and in a xenograft nude mouse model [69]. In addition, antisense of PAR1 reduced the invasiveness of aggressively metastatic breast cancer cells [69, 92].

Recently, the identification of novel PAR1 activating protease, MMP-1, connects the extracellular proteolytic activity with membrane receptor-mediated signal in cancer progression. MMP-1/PAR1 axis has been identified to mediate invasion and metastasis of breast cancer [69, 93], melanoma [70] and ovarian cancer [94, 95]. Whether MMP1 targets other PARs or other MMPs cleave PARs remains open for further investigation.

1.6.3 PAR2 and cancer progression and invasion

PAR2 is another dominant PAR mediating protease signaling in tumor cells. Factor VIIa and Xa-induced migration and invasion of invasive breast cancer cells is mediated by PAR2 [96, 97]. Using mouse mammary tumor virus-polyoma middle T (PyMT) mouse model which mimics important aspects of human breast cancer pathology, Versteeg *et al.* demonstrated that PAR2, not PAR1, plays a critical role for spontaneous breast cancer development [98].

PAR2 promotes cancer progression through various mechanisms. Activation of PAR2 induces proliferation of gastric and colon cancer cells through trans-activation of EGFR and ERK1/2 activation mechanisms [99, 100]. PAR2 mediates migration of breast cancer cells through β-arrestin-dependent ERK1/2 activation [101]. TF-VIIa-PAR2 signaling in breast cancer cells induced a broad array of pro-angiogenic factors and immune regulators such as VEGF [102], Cyr61, VEGF-C, CTGF, CXCL1, IL-8, CSF1 and CSF2 [103]. Upregulation of IL-8 by TF-VIIa-PAR2 signaling resulted in breast cancer cell migration and invasion [97]. PAR2 agonist-triggered IL-8 release may also promote pancreatic cancer progression [104].

Since PARs participate in regulation of inflammatory cytokines and related genes, which are important for cancer progression. The question that whether PARs signaling could recruit inflammatory cells, such as monocyte/macrophages, neutrophils, and T cells to modulate cancer growth and metastasis needs to be addressed. PAR1-mediated production of chemokine monocyte chemoattractant protein 1(MCP-1) is critical for natural killer cells and macrophages recruitments in a mouse heart-to-rat transplant model [105]. Activation of PAR1or PAR2 induces MCP-1 expression was also reported in human lung carcinoma cell line A549 [106]. These studies suggest that MCP-1 may link PARs and inflammatory cells in caner progression.

1.7 Monocyte chemoattractant protein 1(MCP-1) and cancer progression and metastasis

1.7.1 MCP-1 discovery

MCP-1(nomenclature name is chemokine (C-C motif) ligand 2, CCL2; alternative name are MCAF, JE, SMC-CF, HC-11) is a small cytokine belonging to CC chemokine family. MCP-1 was initially discovered as the product of the JE gene from murine fibroblasts treated with PDGF [107, 108]. Human MCP-1 protein was isolated and purified by two groups in 1989 [109-111], they identified this novel cytokine as the product of human JE gene [112, 113]. Human MCP-1 is produced as a protein precursor which contains a 23 amino acid of signal peptide. The mature MCP-1 comprises 76 amino acids corresponding for a predicted molecular weight of 8.7 kDa. However, SDS-PAGE analysis showed that MCP-1 secreted by human cells migrated as 13 and 15 kDa proteins [110]. This may be due to the post-translational modification of MCP-1with O-linked carbohydrate processing [114]. Nearly half of murine MCP-1 protein displays a molecular weight of 30-35 kDa due to high glycosylation.

1.7.2 MCP-1 regulation

MCP-1 is a small inducible chemokine encoded by CCL2 gene, which located on human chromosome 17. A variety of cytokines, such as platelet-derived growth factor (PDGF) [115], tumor necrosis factor alpha (TNFα) [116], interferon gamma (IFN-γ) [117], and IL-1β [118, 119] can induce MCP-1 expression at transcriptional level.

Several cis-acting transcription regulatory elements binding sites on MCP-1 promoter have been mapped (Figure 8). The distal part of MCP-1 promoter contains two NF-κB binding sites, which are required for TNFα-induced MCP-1 expression, but not

essential for PDGF-stimulated expression [115]. The proximal part of MCP-1 promoter is essential for all aspects of MCP-1 gene regulation and is sufficient for PDGF- and INF-γ-induced expression [115, 117]. The proximal region contains a GC box, which binds transcription factor SP1 to regulate both constitutive and stimulus-induced transcriptional activity. Two AP-1 and one NF-κB sites also sit in the proximal region. They are responsible for endothelin-1-mediated MCP-1 regulation in human airway smooth muscle cells [120].

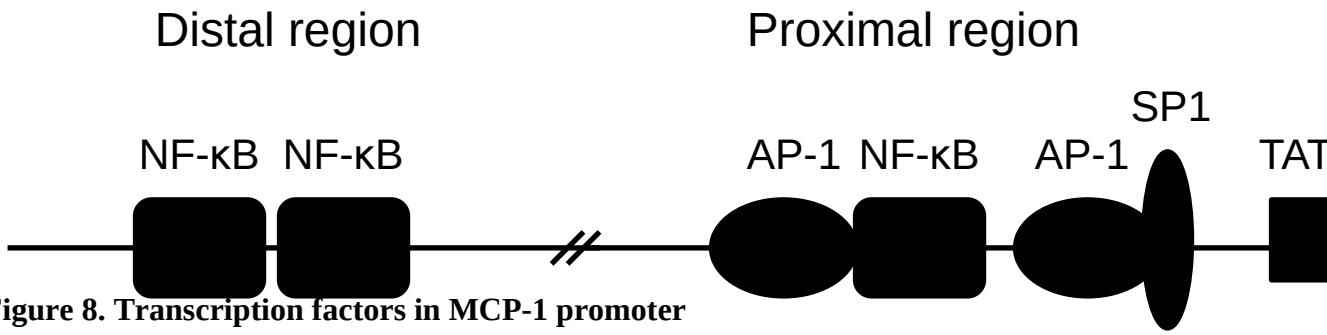

Figure 8. Transcription factors in MCP-1 promoter

1.7.3 MCP-1/CCR2 expression and activity

MCP-1 is expressed by many cell types and organs either constitutively or after stimulation by cytokines or growth factors. Both *in vitro* and *in vivo* studies demonstrated that MCP-1 is a potent chemoattractant for monocytes, T lymphocytes, nature killer (NK) cells, basophils, and dendritic cells [121]. MCP-1 exerts its effects through its receptor CCR2. There are two isoforms of CCR2, named CCR2A and CCR2B, which differ only in their C-terminal tails. CCR2 expression is not as broad as MCP-1. CCR2A mainly expresses by mononuclear cells and vascular smooth muscle cells. CCR2B is the predominant isoform expressed by monocytes and activated NK

cells. MCP-1 is very specific and binds only to CCR2 with a high binding affinity (Kd < 1nM). The other MCPs (MCP-2, MCP-3, and MCP-4 in human, MCP-2, MCP-3, and MCP-5 in mouse) also bind CCR2 with high affinity leading to the uncertainty of whether MCP-1 is essential in recruitment of monocytes *in vivo.* A study using MCP-1-deficient mice showed that loss of MCP-1 alone is sufficient for impairment of monocyte trafficking *in vivo* and other MCPs do not increase to compensate for MCP-1's absence [122] suggesting the critical role of MCP-1 in monocytes recruitment.

1.7.4 MCP-1 and cancer progression and metastasis

MCP-1 is expressed by a variety of cancer types suggesting its role in cancer progression. MCP-1 promotes tumorigenesis and metastasis through two mechanisms: (1) directly acts on tumor cells to influence cancer behavior, and (2) indirectly acts on tumor microenvironment by recruiting macrophage to tumor cells, therefore promoting tumor growth, angiogenesis and metastasis.

1.7.4.1 Direct effects

Direct effects of MCP-1on tumor cell physiology have been demonstrated in prostate cancer cells. Both MCP-1 and CCR2 were found to be expressed by prostate cancer cell lines, including LNCaP, C4-2B and PC3 cells. MCP-1 induces proliferation and invasion of PC3 and LNCaP cells in both autocrine and paracrine manners [123, 124]. Suppression of tumor- and host-derived MCP-1 by its neutralizing antibody attenuates prostate tumor growth and metastasis *in vivo* [124]. Moreover, CCR2 expression is correlated with the malignant state of prostate cancer. The more aggressive the cancer cells, the higher levels the CCR2 expression [125]. In addition to prostate cancer, MCP-1 also induces migration of breast cancer cell MCF-7 and malignant glioma

cells *in vitro* [126, 127]. Multiple myeloma (MM) cells, malignant plasma cells in bone marrow, express a high level of CCR2 compared with normal plasma cells [128]. CCR2 mediates migration of MM cells to osteoclasts which generate high levels of MCP-1 and several other MCPs. Osteoclasts in turn produce various growth factors, such as insulin-like growth factor1 (IGF-1), interleukin-6 (IL-6) and a proliferation-inducing ligand (APRIL) to support MM cells survival, growth and drug resistance [129].

1.7.4.2 Indirect effects

A large body of research has indicated the indirect effects of MCP-1 on cancer progression. Actually, MCP-1 is originally isolated from tumor-derived chemoattractants [111] which mediate the infiltration of macrophage into tumors to either suppress or enhance tumor growth. MCP-1 associated with mononuclear cell infiltrate inhibiting tumor growth has been reported [130, 131]. However, the anti-tumor effect of MCP-1 is unlikely to be its major influence on tumor regulation based on the biological and epidemiological considerations [132].

There is growing evidence supported the notion that MCP-1-mediated macrophage infiltration promotes tumor progression. In a clinical study, the levels of MCP-1 in primary breast cancers were found to be correlated significantly with the accumulation of tumor associated macrophages (TAMs) [133], which are capable of affecting tumor progression in a variety of aspects. After 6 years of follow up, those breast cancer patients with high levels of MCP-1 had a significantly shorter relapse free survival. This clinical association might be explained by mechanistic studies using mouse model that MCP-1 secreted by breast tumor cells recruits inflammatory monocytes which produce VEGF to promote tumor cells extravasation and lung metastasis [134, 135]. MCP-1 was

reported to directly influence prostate cancer growth and metastasis [123]. It also can enhance prostate tumor growth and angiogenesis indirectly by recruitment of monocytes and TAMs into the tumor microenvironment [136]. Moreover, tumor cells derived from renal cancer recruit tumor-infiltrating lymphocytes (TIL) by secreting MCP-1 and IL-8, which suggests MCP-1's indirect effect on renal cancer progression [137]. CC chemokines including MCP-1 and MIP-1b were found to be associated with macrophage infiltration in human NSCLC tumors. Those patients with recurrence of disease had higher macrophage infiltration in their initial tumors [138]. In addition to tumor cells themselves, tumor associated stromal cells such as endothelial cells, fibroblasts and macrophages also produced a significant amount of MCP-1 to increase TAM infiltration and maintain inflammation, therefore promoting tumor progression [139, 140]. The two mechanisms of MCP-1 on caner progression is summarized in Figure 9 [132].

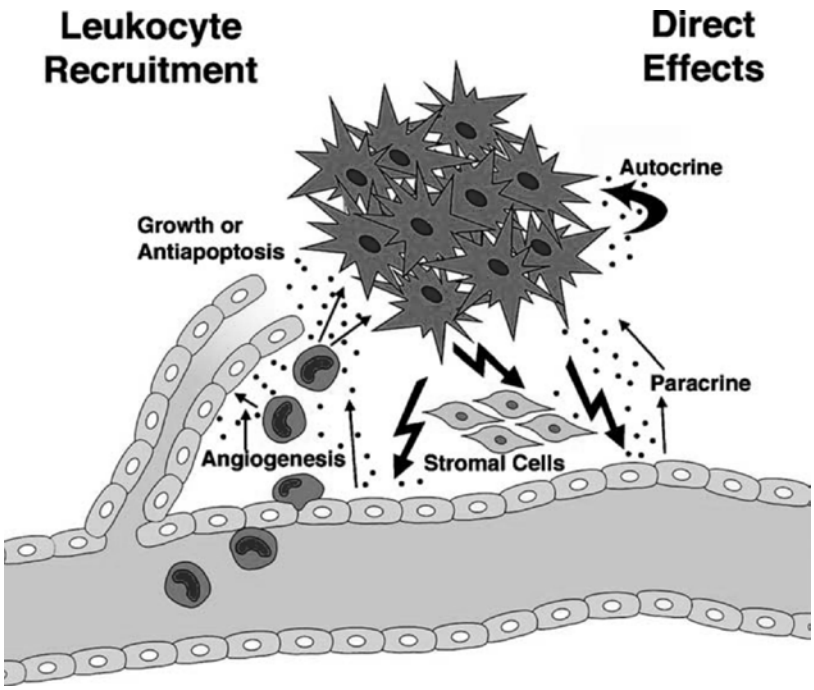

Figure 9. Mechanisms of MCP-1 on cancer progression

1.8 Tumor associated macrophages (TAMs) and cancer invasion

Solid tumors are comprised of not only malignant cells but also many other non-malignant cell types including fibroblasts, endothelial cells, and immune cells such as neutrophils, dendritic cells, macrophages, eosinophils and mast cells [141, 142]. Macrophages are the most abundant immune infiltrates in tumors and these macrophages are termed as tumor associated macrophages (TAMs). TAMs are mostly derived from the peripheral blood monocytes and recruited by tumor cells secreted chemokines and growth factors such as CCL2 (MCP-1), CCL3, CCL4, CCL5, CSF-1 and VEGF [143, 144].

1.8.1 TAMs as prognostic marker for human cancer

A large body of clinical evidence showed that increased accumulation of TAMs are associated with poor prognosis for a variety of cancer types including prostate, breast, ovarian, cervical, bladder, endometrial, and esophageal cancers [133, 145-149]. For lung cancer, the data obtained from clinical studies seem controversial. Two groups reported that higher levels of macrophages were significantly correlated with lower survival rates for patients with NSCLC [138, 150]. However, the results reported by Toomey *et al.* showed that there was no association between macrophages infiltration and prognosis of NSCLC [151]. These conflicting observations may derive from the different methods used to assess macrophage infiltration, or the different tumor grades and stages in these studies. In addition, macrophages within different locations of tumor may have different effects on the outcome of patients. Welsh *et al.* found that the numbers of stroma macrophages were negatively associated with NSCLC patient's survival time, whereas the numbers of tumor islet macrophages were positively associated with patient's survival time [152]. Indeed, the impact of macrophages on tumor is more complicated.

Macrophages have been classified into two major groups: M1 and M2 macrophages. M1 macrophages possess pro-inflammatory properties, whereas M2 macrophages have anti-inflammatory phenotype and promote tumor progression [153]. TAMs have been identified as M2 macrophages [154]. Within the tumor islet, both M1 and M2 macrophages could be present. Those macrophages associated with favorable outcome in NSCLC patients were identified as M1 macrophages [155] . M2 macrophages may function the opposite way. Zeni *et al*. [156] reported that the percentage of IL-10-positive macrophages within tumor islet was higher in patients with late stage of NSCLC and lymph node metastasis compared with those patients with lower stage of NSCLC and without metastasis, and these macrophages also predicted the worse overall survival rate. High expression of IL-10 is one of the characteristic of M2 phenotype [157], thus this study suggested that these macrophages behaved as M2-polarized TAMs, and thereafter promoted progression of NSCLC.

1.8.2 TAMs enhance invasiveness of cancer cells

TAMs promote cancer progression in many aspects, including stimulation of tumor growth, induction of angiogenesis, and promotion of tumor cells migration and invasion. TAMs enhancement of cancer cells invasion through several mechanisms. First, macrophages-derived proteases could disrupt the extracellular matrix, and therefore allow cancer cells to invade the surrounding tissues. For example, TAMs secreted cathepsin B, one of cysteine-type lysosomal proteases, to promote lung metastasis of breast cancer as well as the invasion of pancreatic tumor *in vivo* [60, 158]. Other proteases such as MMP-9 [61, 159] and urokinase-type plasminogen activator [160], a serine protease, are also secreted by TAMs, and both of them are involved in TAM-enhanced cancer cells

invasion. In addition to proteases, TAMs also produce growth factors such as EGF and TGFβ which directly act on carcinoma cells to promote their invasion [59, 161]. Furthermore, TAMs could increase invasion of cancer cells by inducing the epithelial-mesenchymal transition (EMT). Lin *et al.* reported that activated macrophages may stimulate the migration and invasion of HepG2 human hepatocellular carcinoma cells via c-Src and EGFR-dependent downregulation of E-cadherin and β-catenin at the adherens junctions [162]. Moreover, TAMs interact with cancer cells to upregulate a wide array of genes in cancer cells, such as IL-6, NF-κB, ICAM-1, MMP-1, MMP-9, VEGF-A, VEGF-C, etc. and these genes are involved in angiogenesis, growth, adhesion, invasion, and metastasis [163]. In return, macrophages could also be activated by cancer cells to secrete growth factors, cytokines and proteases, subsequently enhanced cancer invasion and progression [164, 165].

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2. Purpose of this dissertation

TXA_2-TP signaling axis is involved in multiple steps of cancer progression including cancer invasion and metastasis. Therefore, the purpose of this dissertation is to identify novel cellular and molecular mechanisms by which TP mediates cancer cell invasion. I am particularly interested in identifying key molecular mediators associated with TP-mediated invasion of lung cancer cells and with specific focus on the investigation of TP-mediated signal transduction pathways leading to the expression of some of these molecules. Another purpose is to explore the interactions and relationship among these mediators and how they exert their functions in facilitating TP-mediated cancer cell invasion.

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3. Materials and methods

3.1 Reagents and antibodies

I-BOP, SQ29548, PGD_2, PGE_2 and $PGF_{2\alpha}$ were from Cayman Chemical (Ann Arbor, MI). LY294002, GF109203X and SP600125 were from Calbiochem (San Diego, CA). Phorbol-12-Myristate-13-Acetate (PMA), U0126, SB203580 and MG132 were from Alexis Biochemicals (San Diego, CA). SCH79797 was from Tocris Bioscience (Ellisville, MO). ENMD-1068 was from Enzo Life Sciences (Farmingdale, NY). Agonist peptides of PARs: PAR1, TFLLR-NH_2; PAR2, SLIGRL-NH_2; PAR3, TFRGAP-NH_2; PAR4, GYPGQV-NH_2, as well as antagonist peptide of PAR4, trans-cinnamoyl-YPGKF (tc-Y-NH_2) were supplied by Peptides International (Louisville, KY). Recombinant human MMP-1 and MMP-3 were purchased from PeproTech (Rocky Hill, NJ). MMP-1-siRNA, MMP-9-siRNA, C/EBPβ-siRNA, PAR2-siRNA and control-siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture medium, heat-inactivated fetal bovine serum (FBS), restriction enzymes, SuperScript II reverse transcriptase, penicillin G and Lipofectamine 2000 were supplied by Invitrogen or Gibco (Carlsbad, CA). TransIT-2020 Transfection Reagent was from Mirus Bio LLC (Madison, WI). Site-directed mutagenesis kit was from Stragene (La Jolla, CA). Centrifugal filter devices were purchased from Millipore (Billerica, MA). CytoSelect 96-well cell migration and invasion assay kits were supplied by Cell Biolabs (San Diego, CA). TRI Reagent, trypsin, soybean trypsin inhibitor, RS-102895, mithramycin A, geldanamycin and other biochemicals and chemicals were from Sigma-Aldrich (St. Louis, MO). pcDNA3-MMP-9 expression plasmid was a kind gift of Dr. R. Fridman of the Wayne State University (Detroit, MI). All lentivirus related plasmids were kind gifts of Dr.

Louis Hersh of the University of Kentucky (Lexington, KY). Human MMP-1 promoter /luciferase reporter plasmids pGL3-2942 and pGL3-2942ΔCEBP were kindly supplied by Dr. Matthew P. Vincent of Dartmouth Medical School (Hanover, NH). Human MCP-1-CAT reporter plasmid was kindly provided by Dr. Bassel E. Sawaya of Temple University (Philadelphia, PA).

The primary antibodies used in this study were MMP-1, MMP-3, MMP-9 (NeoMarkers, Fremont, CA), PAR-2, SP1, E-cadherin, Phospho-ERK1/2, C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA), Phospho-c-Jun (Ser73), Phospho-C/EBPβ (Thr235), ERK1/2 (Cell Signaling Technology, Danvers, MA), MCP-1 (PeproTech, Rocky Hill, NJ), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (generated in house). Horseradish peroxidase (HRP)-linked goat anti-mouse and rabbit secondary antibodies were supplied by Invitrogen (Carlsbad, CA).

3.2 Cell lines and culture

3.2.1 Wild-type cell lines

A549, H460 and H157 non-small cell lung cancer cells were maintained in RPMI 1640 medium. Mouse RAW 264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM). 293 FT cells were used for lentivirus production and maintained in DMEM supplemented with 0.1mM non-essential amino acids. All these cells lines were from American Type Culture Collection (Manassas, VA) and grown in their corresponding cell culture media with supplemented 10% FBS, 100 U/ml penicillin G and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2.

3.2.2 Establishment of A549-TPα and A549-TPβ cell lines

TPα and TPβ were cloned into pCSC-SP-PW lentiviral vector, transfected into 293 FT cells with packaging plasmids, pMDL-gp.RRE, pRSV.Rev and pVSVG using Lipofectamine 2000 following the manufacture's protocol. Similarly, GFP was cloned into pCSC-SP-PW vector and used as a control plasmid. The medium was replaced with fresh DMEM medium containing 10% FBS after 12 h transfection. Supernatants containing virus particles were collected 36 h after the start of transfection, filtered through 0.45 µm filter, and then were used to infect A549 cell line. A549 cells infected with virus particles encoding GFP, TPα or TPβ were harvested in three separate pools and designated as control A549 cells, A549-TPα and A549-TPβ cells accordingly. These infected cells were cultured until stably expressing the encoding genes. All these cells were maintained in RPMI1640 medium supplement with 10% FBS, 0.1 mg/mL streptomycin and 100U/mL of penicillin G at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

3.3 Cell treatment and Western blot analysis

Cells were plated in 12-well plates to achieve ~ 80% confluence, and then were starved in RPMI 1640 medium without FBS for 24 h before stimulation. For inhibitor study, cells were pretreated with the respective inhibitors at working concentrations or control (0.1% DMSO) for 30 min in serum-free medium prior to stimulation. After a certain time of treatment, cells were collected and lysed with lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM NaF, 5 mM pyrophosphate, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). Conditioned media were

collected and concentrated via trichloroacetic acid (TCA)-acetone method. Western blot analysis was carried out as described previously [166]. Briefly, proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat milk in Tris-buffered saline at room temperature for 1 h and incubated with primary antibodies overnight at 4°C. The membranes were then incubated with horseradish peroxidase-linked goat anti-mouse or rabbit secondary antibodies for 1 h at room temperature and developed using enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Cardiff, UK). All membranes were stripped using stripping buffer before reprobing with anti-glyceraldehyde-3-phosphate dehydrogenase or anti-ERK1/2 antibodies to ensure equal protein loading. Band intensities were quantified using NIH Image J software and normalized to those of GAPDH.

3.4 Protein precipitation

Proteins in media samples were precipitated by using trichloroacetic acid (TCA)-acetone method. Briefly, TCA was added to media at a final concentration of 10%. Proteins were precipitated for 4 h on ice. Precipitated protein was centrifuged at 5,000 x *g* for 30 min, washed three times in cold acetone, and air dried. Pellets were frozen at -80°C until analyzed.

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

Total RNA was isolated from cells using the TRI Reagent and reverse-transcribed using the SuperScript II reverse transcriptase following the manufacturer's instructions. PCR conditions were 2 min at 95°C followed by 35 cycles (23 cycles for MCP-1 and β-

actin) of 95°C for 1 min, 50-55°C for 30s, 72°C for 30s. The PCR product of each sample was analyzed by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. The primers used in the PCR reactions are summarized in Table1.

Table 1. Primers used for RT-PCR

NAME	SEQUENCE
TPα	5'-GAGATGATGGCTCAGCTCCT-3' (forward) 5'-CTACTGCAGCCCGGAGCG-3' (reverse)
TPβ	5'-CTCAGCTCCTGGGGATCAT-3'(forward) 5'-GTCAAATTCAGGGTCAAAGAGCA-3'(reverse)
MMP-1	5'-CATTGATGGCATCCAAGC-3'(forward) 5'-CCGGACTTCATCTCTGT-3'(reverse)
MMP-3	5'-GTTAGGAGAAAGGACAGTGGTCCTG-3'(forward) 5'-GGCATAGGCATGGGCCAAAACATT-3'(reverse)
MMP-9	5'-GGCGCTCATGTACCCTATGT-3'(forward) 5'-TCAAAGACCGAGTCCAGCTT-3'(reverse)
MMP-10	5'-GTCACTTCAGCTCCTTTCCT-3'(forward) 5'-ATCTTGCGAAAGGCGGAACT-3'(reverse)
MMP-13	5'-AGTGGTAAGAATAGTAGATGTG-3' (forward) 5'-GGCCGATCATATATTCAATAAGT-3' (reverse)
MCP-1	5'-ATAGCAGCCACCTTCATTCC-3' (forward) 5'-TTCCCCAAGTCTCTGTATCT-3' (reverse)
VEGF	5'-GGATGTCTATCAGCGCAGCTAC-3' (forward) 5'-TCACCGCCTCGGCTTGTCACATC-3' (reverse)
β-actin	5'-GGCATGGGTCAGAAGGATTCC-3'(forward) 5'-AGCACAGCCTGGATAGCAACG-3' (reverse)

3.6 Zymographic assay

Media samples were concentrated 20-fold using the centrifugal filter devices, then applied to 10% polyacrylamide gel containing 0.1% gelatin (detecting MMP-2, 9) or 12% polyacrylamide gel containing 0.1% casein (detecting MMP-1, 3). Samples were electrophoresed at 4°C under a non-reducing condition. The gel was washed with 2.5% Triton X-100 for 30 min twice at room temperature to remove the SDS and incubated in the developing buffer [100 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, 0.05% 23 lauryl ether (Brij-35)] at 37°C for 36 h. Gelatinolytic and caseinolytic proteinase activities were revealed by coomassie blue staining.

3.7 Conditioned media and activation of MMPs

A549-TPα cells were plated into 10 cm dishes to achieve ~ 80% confluence next day, then media were switched to 10 ml serum-free RPMI1640. After 24 h starving, cells were treated with vehicle (0.1% ethanol) or 50 nM I-BOP for another 24 h. Then, conditioned media were collected and passed through 30 kDa and 10 kDa centrifugal filters sequentially to separate MMPs and MCP-1(MCP-1 molecular weight is 13-15 kDa). The remains on each filter were resolved in 1.5 mL serum-free medium and stored at -80°C until used. MMPs in conditioned media were activated using 10 µg/mL trypsin for 1 h at 37°C, followed by adding 40 µg/mL soybean trypsin inhibitor to neutralize the trypsin.

3.8 Transient transfection

A549-TPα cells were transfected with pcDNA3 or pcDNA3-MMP-9 expression plasmid using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. Media were collected for further use after transfection for 48 h.

3.9 Small interfering (si)-RNA treatment

A549-TPα cells were transiently transfected with control siRNA or C/EBPβ, MMP-1, MMP-9 siRNA at concentration of 50 nM using Lipofectamine 2000. Cells were treated with siRNA for 24 h prior to stimulation by 50 nM I-BOP for another 24 h. For PAR-2 knockdown experiment, control A549 cells were transiently transfected with control siRNA or PAR2-siRNA at concentration of 100 nM using Lipofectamine 2000. Cells were treated with the siRNA for 72 h prior to stimulation by MMP-1 or conditioned media for another 16 h.

3.10 Real-time PCR analysis of MCP-1 mRNA

The real-time PCR was performed with human MCP-1 and β-actin (as an internal standard)-specific primers (Table 2). All real-time PCR reactions were carried out in a final volume of 50 μL and were performed in duplicate for each cDNA sample in the ABI PRISM 7700 Sequence Detection System according to the manufacturer's protocol. The optimized reaction consisted of 25 μL of iTaq SYBRGreen Supermix with ROX, 0.02 U/μL of Uracil-N-glycosylase, 3 μL of diluted cDNA templates, and 200 nM of each specific forward and reverse primer. The PCR protocol was 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Specificity of the amplification was checked by melt-curve analysis. Relative levels of mRNA expression were calculated according to Pfaffl method [167]. Individual expression values were normalized by comparison with β-actin mRNA expression.

Table 2. Primers used for real-time PCR

NAME	SEQUENCE
MCP-1	5’- ATAGCAGCCACCTTCATTCC-3’(forward) 5’- ATCCTGAACCCACTTCTGCT-3’ (reverse)
β-actin	5’- AGAAAATCTGGCACCACACC-3’ (forward) 5’- AGAGGCGTACAGGGATAGCA-3’ (reverse)

3.11 Promoter constructs and site-directed mutagenesis

The MMP-1 promoter construct pGL3-512 was generated from pGL3-2942 by KpnI digestion. A site-directed mutagenesis kit was used to mutate the core sequences of three AP-1 sites in the pGL3-512 construct: -429m: 5’-TCAGTCA-3’ to 5’-ggAGTCA-3’; -181m: 5’-TTAATCA-3’ to 5’-ggAATCA-3’; -73m: 5’-TGAGTCA-3’ to 5’-ggAGTCA-3’. Human MCP-1-CAT reporter plasmid was reconstructed into pGL3 luciferase reporter vector. A site-directed mutagenesis kit was used to mutate the core sequence of SP1 binding site in the reconstructed luciferase reporter plasmid: SP1 wild-type (SP1wt), 5'-CCGCCC-3' to SP1 mutant (SP1m), 5'-CCGggg-3'. Primers used for generation of these mutants were list in Table 3.

Table 3. Primers used for generation of site-directed mutagenesis

NAME	SEQUENCE
-429m forward	GCTGGGGGAGCTGAACTGGAGTCAGTACAGGAGCCGAACAGCC
-429m reverse	GGCTGTTCGGCTCCTGTACTGACTCCAGTTCAGCTCCCCCAGC
-181m forward	CGCACACATCTTGTTTGAAGGGAATCATGACATTGCAACACC
-181m reverse	GGTGTTGCAATGTCATGATTCCCTTCAAACAAGATGTGTGCG
-73 forward	GGATGTTATAAAGCAGGAGTCAGACACCTCTGGCTTTCTGG
-73 reverse	CCAGAAAGCCAGAGGTGTCTGACTCCTGCTTTATAACATCC
SP1m forward	CCCTCCTCCTGCTTGACTCCGGGGTCTCTCCCTCTGCCCGC
SP1m reverse	GCGGGCAGAGGGAGAGACCCCGGAGTCAAGCAGGAGGAGGG

3.12 Luciferase assay

Cells were seeded in 12-well plate and transfected with pGL3-MMP-1 or pGL3-MCP-1 reporter plasmids using TransIT-2020 Transfection Reagent. After 24 h of transfection, cells were incubated with I-BOP or vehicle control (0.1% ethanol) for additional 18 h. Thereafter, cells were collected and further detected by using a microplate luminometer (MTX lab systems, Vienna, VA). The luciferase activity in cell lysate was determined as described previously [166].

3.13 Preparation of nuclear extract

Nuclear proteins were prepared as described previously [168]. In brief, A549-TPα cells were scraped into cold phosphate buffered saline (PBS) and centrifuged at 1000 rpm for 2 min. Pellets were resuspended in buffer A [10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 15 min, then centrifuged at full speed in a tabletop centrifuge. The nuclear pellets were lysed for 20 min on ice in buffer B (20 mM HEPES-KOH, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF), and centrifuged for 2 min as above. The supernatant containing nuclear proteins was frozen at -80°C until analyzed.

3.14 Macrophage migration assay

RAW 264.7 macrophages migration was assessed by using CytoSelect 96-well cell migration assay kit (Cell Biolabs, San Diego, CA) following the manufacturer's instruction. In brief, ~ 1.0×10^5 cells in serum free medium were seeded onto the top well of a Trans-well insert (8 µm polycarbonate nucleopore filters), and the bottom well was supplemented with 150 µL serum free medium or A549-TPα conditioned medium through a10 kDa cut-off membrane prepared in **2.7**. After 12 h of stimulation, cells that had migrated through the filter were detached and dyed with CyQuant GR dye solution. The fluorescence was measured at 480/520 nm. For antibody neutralization study, 150 µL serum free medium or conditioned medium was incubated with MCP-1 neutralizing antibody (5 µg) or isotype control antibody (5 µg) for 2 h at 37°C before added to the lower chamber. For inhibition study, RAW 264.7 cells were incubated with MCP-1

receptor (CCR2) antagonist RS-102895 (10 μM) for 30 min before stimulation with the conditioned medium.

3.15 Co-culture experiments

For transwell co-culture system, control A549 or A549-TPα cells were plated into a six-well culture plate, and RAW 264.7 cells were seeded onto transwell inserts (with a 0.4 μm pore size) top on another six-well plate. After 24 h, replace the medium of A549 and RAW cells with fresh serum free medium and move the RAW 264.7 transwell inserts onto the six-well plate where A549 cells were seeded. After 12 h of co-culture, A549 cells were pictured and collected for RT-PCR analysis. For direct co-culture, control A549 cells which express GFP were plated with or without RAW 264.7 cells into a 12-well plate and cells were pictured after a certain periods. Diagrams of two co-culture systems were given in Figure 38 and Figure 42. The images of cells were captured using a Kodak digital camera under Olympus Tokyo CK inverted microscope.

3.16 Invasion assay

For I-BOP-induced invasion of A549-TPα and TPβ cells, the invasion assay was assessed by using CytoSelect 96-well cell invasion assay kit following the manufacturer's instruction. In brief, ~ 1.0×10^5 cells in serum free medium were seeded onto the ECM protein coated filters, and the lower wells were supplement with medium containing 0.1% FBS. I-BOP or GM6001 were directly added to the cell suspension. After incubation for 24 h, cells that had penetrated through the filter were detached and dyed with CyQuant GR dye solution. The fluorescence was measured at 480/520 nM.

For macrophages-promoted invasion of control A549 cells, the invasion assay was carried out following the instructions of Cultrex 24-well Transwell BME cell invasion assay (Trevigen). Briefly, a 24-well unit with 8 µm polycarbonate nucleopore filters (Corning) evenly coated with 100 µL basement membrane extract coating solution (Trevigen) at 37 °C for 4 h. Control A549 cells (2×10^5) expressing GFP and RAW 264.7 macrophages (1×10^5) in serum free medium were placed in the upper compartment, and 50% RPMI 1640 plus 50% DMEM medium supplied with 0.1-0.5% FBS was added to the lower compartment. After 24 h incubation, cells that had not invaded were removed with a cotton swab. Cells that had invaded the lower surface of the membrane were observed under a fluorescence microscope. Invaded cells in three randomly selected fields were counted.

3.17 Statistical analysis

The differences between each group were expressed as mean ± SD. Statistical significance was assessed by Student's *t* test. Differences were considered statistically significant when P values were ≤ 0.05.

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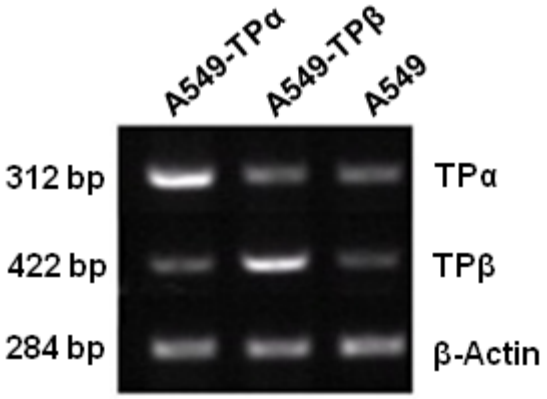
4. Results

4.1 Overexpression of TP increases invasion of human lung cancer cell A549

4.1.1 Ectopic expression of TPα and TPβ in A549 cells by lentiviral approach

It has been reported that TP agonists can initiate signaling transductions by activating endogenously expressed TPα/β in some lung and prostate cancer cells [10, 20]. In order to explore whether TPα and TPβ function differently we chose to overexpress each receptor in human lung A549 cells which express low levels of endogenous TP and were shown no response to TP agonist [166]. The approach of using liposome-mediated DNA transfection method to increase expression of TP in A549 cells encountered limited success. The approach of using lentiviral vectors to deliver TPα and TPβ cDNAs into A549 cells was therefore attempted. A lentiviral vector harboring GFP was used as a control. Stably transduced clones and populations (cell pools) were collected and designated as control A549, A549-TPα, and A549-TPβ cells. The cell pools were used in this study to avoid deriving from the specificity of a single clone. The expression of TPα and TPβ in these cells were determined by RT-PCR. As shown in Figure 10a, A549-TPα cells expressed a significantly higher level of TPα than A549-TPβ and control A549 cells. Similarly, A549-TPβ cells expressed highest level of TPβ. Although there are low levels of endogenous expression of TPα and TPβ in A549 cells, their levels are not adequate to mediate ERK responses to TP agonist, I-BOP, stimulation. As shown in Figure 10b, I-BOP induced significant phosphorylation of ERK in both A549-TPα and A549-TPβ cells but not in control A549 cells.

(a)



(b)

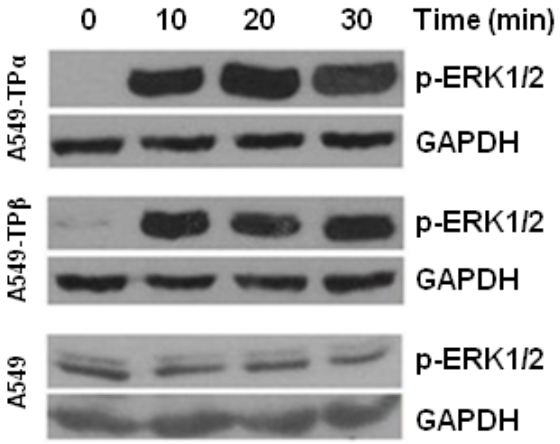


Figure 10. Characterization of A549-TPα and A549-TPβ cells

(a) RT-PCR identification of over-expressed TPα and TPβ mRNA in A549-TPα and A549-TPβ cells. Cells total RNA was isolated. RT-PCR was carried out as described in Materials and methods. (b) I-BOP-induced phosphorylation of ERK1/2 in A549-TPα and A549-TPβ cells. Cells were starved for 24 h before treatment with 50 nM I-BOP at indicated time. Western blot was performed as described in Materials and methods. GAPDH was used as the protein loading control. Data are representative of three independent experiments.

4.1.2 TP agonist induces decreases in E-cadherin levels in A549-TPα and TPβ cells

Tumor cell invasion is associated with the disruption of cell-cell adhesion and the movement of cells across the ECM. E-cadherin-catenin complex is critical for cell adhesiveness and maintenance of normal tissue architecture. Reduction of E-cadherin is tightly linked with cell invasion [169]. We examined the effects of TP agonists, I-BOP and U46619, on the expression of E-cadherin in our established A549-TPα and TPβ cells. Results showed that activation of TPα and TPβ significantly reduced E-cadherin expression (Figure11).

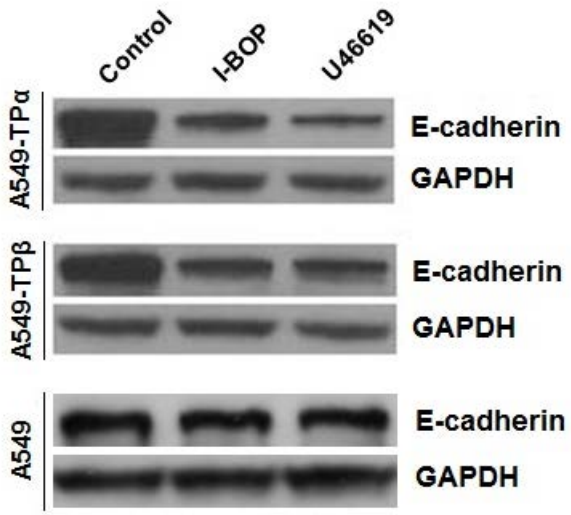


Figure 11. Effects of TP activation on E-cadherin expression

A549-TPα and TPβ cells were starved for 24 h before treated with 50 nM I-BOP or 1μM U46619 for another 48 h. The expression of E-cadherin was detected by Western blot analysis. Data are representative of three independent experiments.

4.1.3 TP agonist induces morphological changes of A549-TPα and TPβ cells

To acquire the invasive properties, tumor cells change their epithelial morphology to scattered spindle-shaped morphology which is usually associated with the loss of E-cadherin. In this study, when stimulated with I-BOP, A549-TPα and TPβ cells became more scattered and exhibited elongated protrusions, indicating their invasive potential (Figure 12).

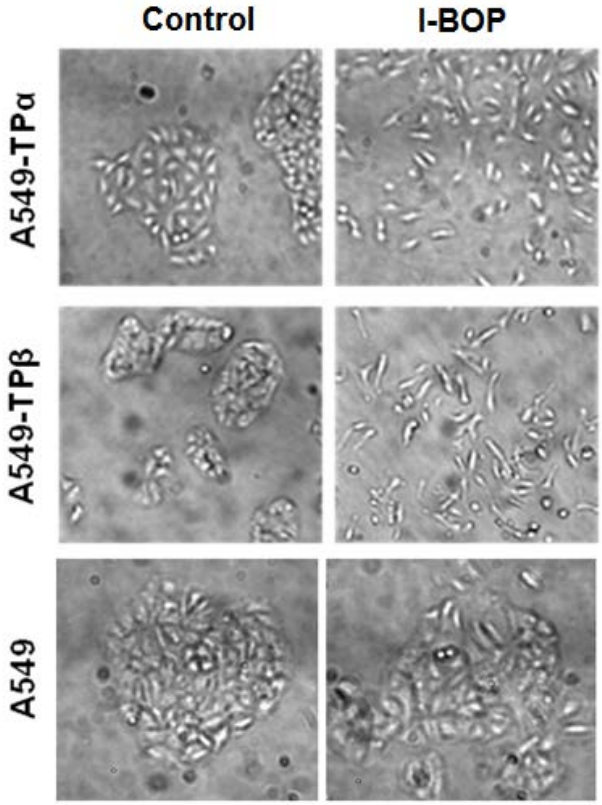


Figure 12. Effects of TP activation on cell morphological changes

A549 control, A549-TPα and A549-TPβ cells were treated with 10 nM I-BOP or vehicle control (0.1% ethanol) for 48h. Cells were visualized with Zeiss Axiovert S100 inverted microscope. The images were captured using a Kodak digital camera.

4.1.4 TP agonist induces invasion of A549-TPα and TPβ cells

Next, we sought to determine whether I-BOP affects A549-TPα and A549-TPβ cells invasion by using transwell invasion assay. As shown in Figure 13, I-BOP at 10 nM induced significant increase in both A549-TPα and A549-TPβ cellular invasiveness. Nonetheless, it has no effect on control A549 cells.

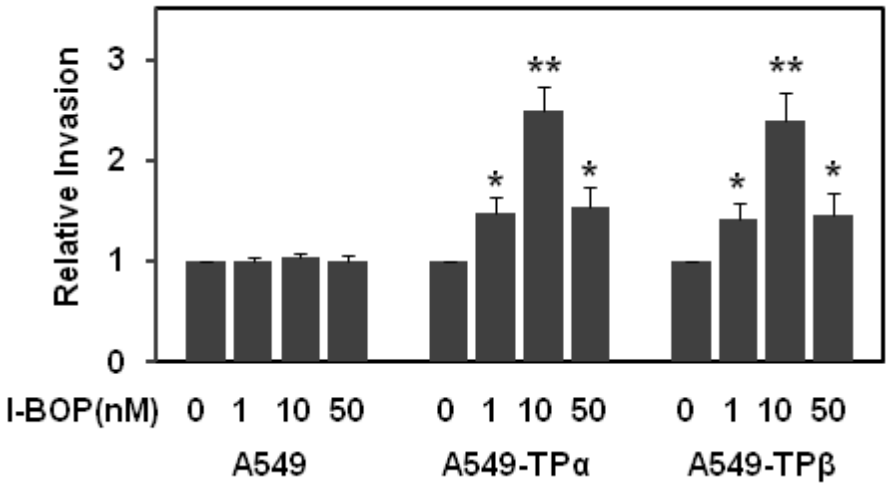


Figure 13. Effects of TP activation on cell invasion

The invasiveness of A549 control, A549-TPα and A549-TPβ cells under indicated concentrations of I-BOP was assayed as described in Materials and methods. Three separate experiments and triplicate wells each were performed. Data are presented as invasion relative to the vehicle control-treated cells. * $P < 0.05$; ** $P < 0.01$.

4.2 Increased expression of MMPs mediates TP agonist-induced invasion in A549-TPα cells

4.2.1 Induction of MMPs expression by TP agonist in both A549-TPα and TPβ cells

Induction of MMPs expression by I-BOP in A549-TPα, A549-TPβ and the control A549 cells was examined. Using semi-quantitative RT-PCR, we found that transcription of MMP-1, MMP-3, MMP-9 and MMP-10 genes was significantly induced by I-BOP in A549-TPα and A549-TPβ cells but not in the control A549 cells (Figure 14a). I-BOP-induced increase in secretion of MMP-1 and MMP-9 in the media of A549-TPα and A549-TPβ cell cultures was determined by Western blot analysis and zymographic assay (Figure 14b. MMP-3 can also be detected by casein zymography and it has a similar molecular weight as MMP-1). The expression patterns were comparable for A549 cells expressing either TPα or TPβ. Therefore, A549 cells expressing TPα were primarily used for in depth study.

MMP-1 is the first member of the MMP family being identified and studied in greater detail. Many signaling pathways targeted to MMP-1 gene are conserved in other MMP genes. Therefore, subsequent studies were focused on TP-mediated MMP-1 expression and regulation in A549-TPα cells. I-BOP induced strong and sustained expression of MMP-1 mRNA as shown in Figure 15a. MMP-1 mRNA level increased rapidly at 1 h and reached maximum by 8 h following I-BOP stimulation and remained high over a 24 h period. The levels of protein expression in conditioned media determined by Western blot analysis correlated well with the levels of mRNA analyzed by RT-PCR. Dose-dependent study indicated that I-BOP at 1 nM was sufficient to induce MMP-1 expression in A549-TPα cells. I-BOP at 50 nM induced the maximal expression

(Figure 15b). The induction of MMP-1, MMP-3 and MMP-10 by I-BOP was also found in another lung cancer cell line H460 (Figure 15c) which expresses endogenous TP receptors, indicating the induction of MMPs was not simply due to the artificial expression of exogenous TP receptors.

(a)

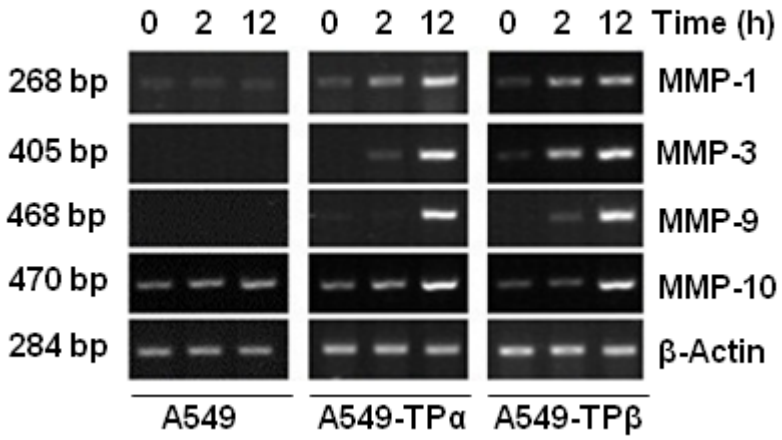


Figure 14. Effects of I-BOP on MMPs expression and activity

(a) I-BOP-induced transcription of several MMPs in A549-TPα and A549-TPβ cells. Cells were starved for 24 h before treatment with 50 nM I-BOP for 2 h or 12 h and then total RNA was isolated. RT-PCR was carried out as described in Materials and methods. Data are representative of three independent experiments.

(b)

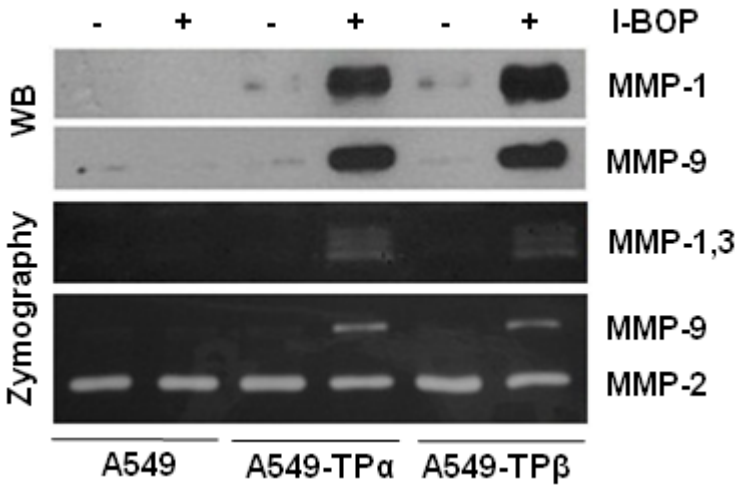


Figure14. Effects of I-BOP on MMPs expression and activity

(b) I-BOP-induced protein secretions of MMP-1and MMP-9 in A549-TPα and A549-TPβ cells. Cells were treated with 50 nM I-BOP for 24 h. Protein expression of MMP-1 and MMP-9 in the conditioned media were detected by Western blot (upper panel) and zymography (lower panel) as described in Materials and methods. MMP-3 can also be detected by casein zymography and it has a similar molecular weight as MMP-1. Data are representative of three independent experiments.

(a)

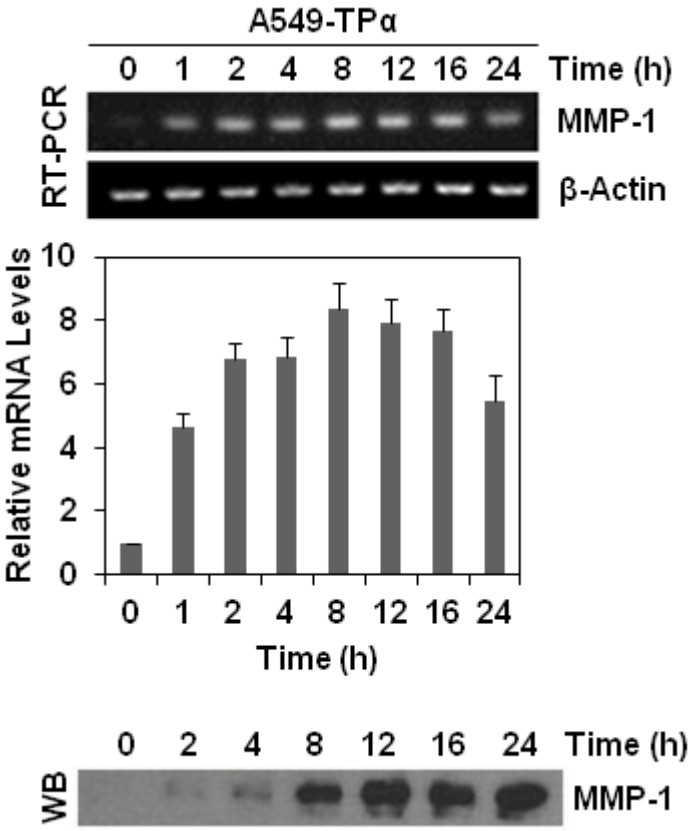


Figure 15. Effects of I-BOP on MMP-1 expression

(a) Time-dependent effects of I-BOP on MMP-1 expression. A549-TPα cells were treated with 50 nM I-BOP at indicated time. RT-PCR was carried out to identify mRNA levels of MMP-1. Densitometric analysis of each band was made. The ratio of MMP-1 to β-actin densities at 0 h was normalized to 1.0. Protein expression of MMP-1in the conditioned medium was detected by Western blot analysis. Data are representative of three independent experiments.

(b)

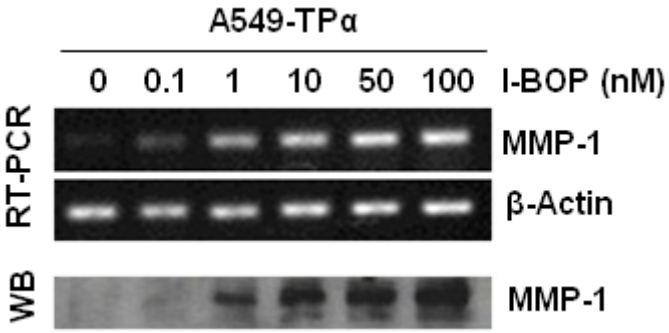


Figure 15. Effects of I-BOP on MMP-1 expression

(b) Dose-dependent effects of I-BOP on MMP-1 expression. A549-TPα cells were treated with indicated concentrations of I-BOP for 12 h. MMP-1 mRNA level was determined by RT-PCR. MMP-1 protein expression in the conditioned medium was detected by Western blot analysis. Data are representative of three independent experiments.

(c)

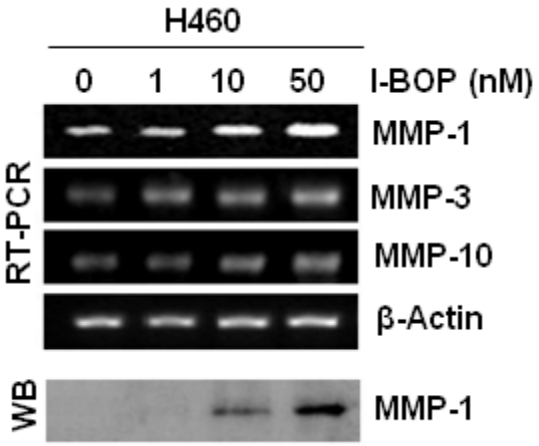


Figure 15. Effects of I-BOP on MMP-1 expression

(c) I-BOP-induced MMPs expression in H460 cells. H460 cells were treated with indicated concentrations of I-BOP for 8 h. MMP-1, MMP-3 and MMP-10 mRNA levels and MMP-1 protein expression were determined as described above. Data are representative of three independent experiments.

4.2.2 Upregulation of MMP-1 is dependent on PKC and MAPK/ERK signaling

Signal transduction pathways leading to the expression of MMP-1 were investigated using specific inhibitors of signaling molecules. As shown in Figure 16, SQ29548, a TP antagonist, blocked totally I-BOP-induced MMP-1 expression at both mRNA and protein levels as expected. GF109203X, a PKC inhibitor, attenuated almost completely MMP-1 expression at both mRNA and protein levels. LY294002, a phosphoinositide-3-kinase (PI-3K) inhibitor, had no inhibitory effect on MMP-1 induction.

Downstream signaling pathway, mitogen-activated protein kinase (MAPK) pathway was also examined. A549-TPα cells were treated with MAPK/ERK inhibitor (U0126), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) inhibitor (SP600125) and p38 MAPK inhibitor (SB203580) at previously established inhibitory concentrations[166]. Only U0126 completely blocked I-BOP-induced MMP-1 expression. Other inhibitors exhibited no effects as shown in Figure 16. These data indicate that upregulation of MMP-1by I-BOP is PKC involved, MAPK/ERK dependent, SAPK/JNK and p38 MAPK independent process.

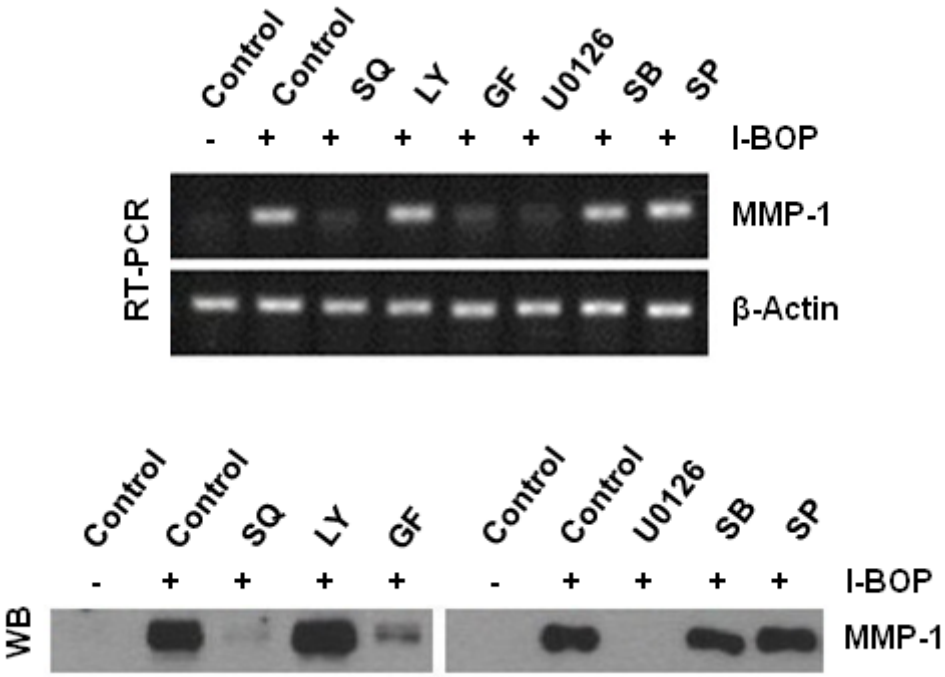


Figure 16. Effects of different inhibitors on I-BOP-induced MMP-1 expression

A549-TPα cells were treated with various inhibitors for 30 min before incubation with 50 nM I-BOP for 12 h. RT-PCR (upper panel) and Western blot (lower panel) were performed to determine MMP-1 mRNA level in cells and protein secretion in the conditioned medium, respectively. Data are representative of three independent experiments. Inhibitor concentration used: 10 μM SQ29548 (SQ), 10 μM LY294002 (LY), 0.5 μM GF109203X (GF), 10 μM U0126, 10 μM SB203580 (SB), or 10 μM SP600125 (SP).

4.2.3 AP-1 as a downstream signal of PKC and MAPK/ERK pathways

AP-1, composed of c-Jun and c-Fos families, is a known transcriptional regulator of MMP-1 expression[73]. We sought to determine whether PKC and MAPK/ERK signaling pathways mediate its activation and therefore regulate TP-induced MMP-1 expression.

Time course studies indicated that c-Jun was strongly activated by I-BOP within 10 min. The phosphorylation of c-Jun maintained at a maximal level from 30 min to 1 h, and remained high even at 2 h (Figure 17a upper panel). The protein expression of c-Fos was significantly induced by I-BOP at 1 h and reached a peak level at 2 h (Figure 17a lower panel). The rapid activation of c-Jun and c-Fos is consistent with the rapid induction of MMP-1 mRNA as shown in Figure 15a. These data suggest that there is a positive correlation between I-BOP-induced AP-1 activation and MMP-1 expression.

Cells were further treated with PKC and ERK inhibitors to test if they inhibit I-BOP-induced c-Jun/c-Fos activation. The results showed that PKC inhibitor, GF109203X, attenuated significantly c-Jun/c-Fos activation. ERK inhibitor, U0126, also significantly repressed c-Jun activation and totally blocked c-Fos expression (Figure 17b). GF109203X had no effect on I-BOP-induced ERK activation suggesting that PKC-induced activation of c-Jun/c-Fos was not likely to be mediated by MAPK/ERK (Figure 17b). PMA, a PKC activator, stimulated the activation of c-Jun/c-Fos providing further evidence that PKC was involved in I-BOP-induced AP-1 activation (Figure 17c). Although JNK inhibitor partially blocked I-BOP-induced phosphorylation of c-Jun, it has no inhibitory effect on I-BOP-induced c-Fos expression (Figure 17d).

To further confirm the involvement of PKC-AP-1 and ERK-AP-1 signal cascades in MMP-1 induction, luciferase reporter bearing a fragment of MMP-1 gene promoter which contains putative AP-1 sites was employed to examine their roles. In addition to the common ~-70 bp AP-1 site in all Group 1 MMP promoters, the 512 bp length of MMP-1 promoter construct contains two other putative AP-1 sites at -181 bp and -429 bp [170]. I-BOP induced a significant increase in luciferase activity which was presumably due to the activation of AP-1 proteins. Site-directed mutagenesis of AP-1 sites at -73 or -181 bp of MMP-1 promoter significantly decreased I-BOP-induced luciferase activity. However, mutation of AP-1 site at -429 bp has no effect on I-BOP induction of MMP-1 promoter activity indicating that the -73 and -181 AP-1 sites in the proximal region of MMP-1 promoter are more important for its transcription (Figure 17e). Inhibitors of PKC and ERK significantly attenuated the increase in luciferase activity induced by I-BOP stimulation (Figure 17f) supporting the notion that PKC and MAPK/ERK signaling pathways may converge at the AP-1 elements to regulate I-BOP-induced MMP-1 gene expression.

(a)

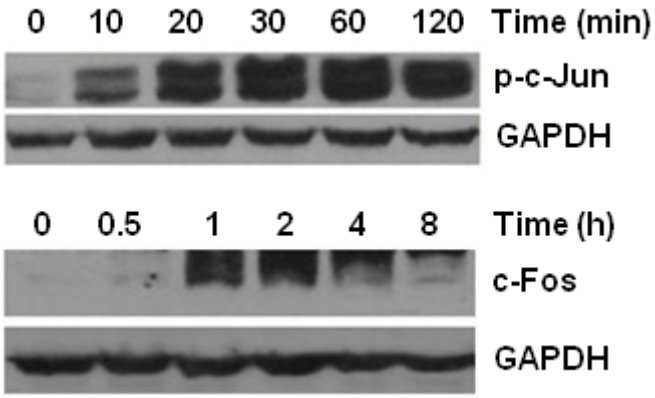


Figure 17. AP-1 is a downstream signal of PKC and MAPK/ERK pathways

(a) Time-dependent effect of I-BOP on phosphorylation of c-Jun and induction of c-Fos. A549-TPα cells were treated with 50 nM I-BOP at indicated time. Western blot analysis was performed to detect p-c-Jun and c-Fos. Data are representative of three independent experiments.

(b)

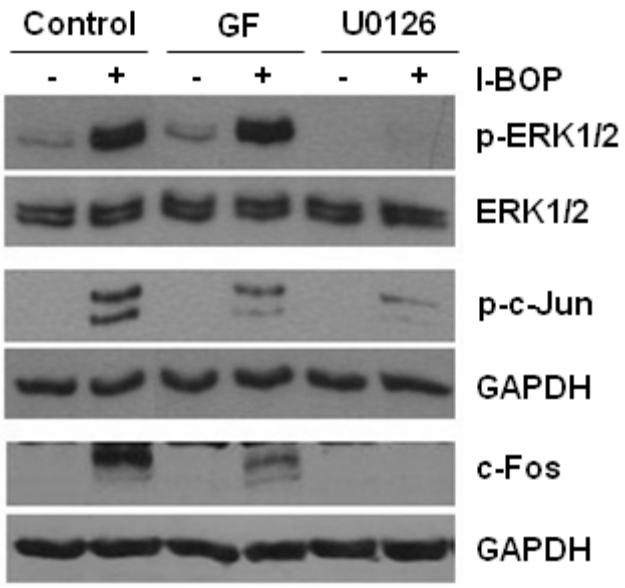


Figure 17. AP-1 is a downstream signal of PKC and MAPK/ERK pathways

(b) Effect of PKC and ERK inhibitors on I-BOP-induced phosphorylation of c-Jun and the expression of c-Fos. A549-TPα cells were treated with 0.5 μM GF109203X (GF) or 10 μM U0126 for 30 min before incubation with 50 nM I-BOP for 30 min (detecting p-c-Jun and p-ERK1/2) or 2 h (detecting c-Fos), followed by Western blot analysis with corresponding primary antibodies to detect p-ERK1/2, p-c-Jun and c-Fos. Data are representative of three independent experiments.

(c) (d)

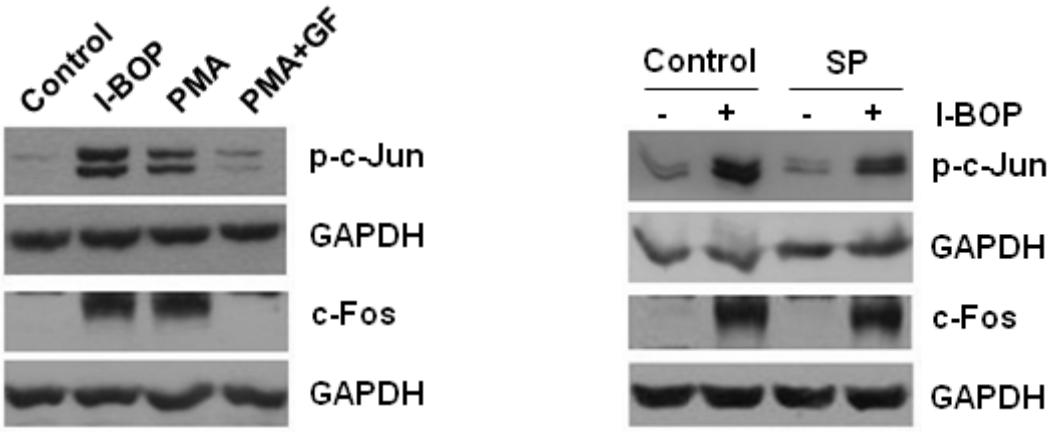


Figure 17. AP-1 is a downstream signal of PKC and MAPK/ERK pathways

(c) Effect of PMA on phosphorylation of c-Jun and expression of c-Fos. Cells were treated with 0.5 µM GF109203X (GF) for 30 min before incubation with 100 nM PMA for 30 min (detecting p-c-Jun) or 2 h (detecting c-Fos), followed by Western blot analysis to detect p-c-Jun and c-Fos. Data are representative of three independent experiments.

(d) Effect of JNK inhibitor on I-BOP-induced phosphorylation of c-Jun and expression of c-Fos. Cells were treated with 10 µM SP600125 (SP) for 30 min before incubation with 50 nM I-BOP for 30 min (detecting p-c-Jun) or 2 h (detecting c-Fos), followed by Western blot analysis to detect p-c-Jun and c-Fos. Data are representative of three independent experiments.

(e)

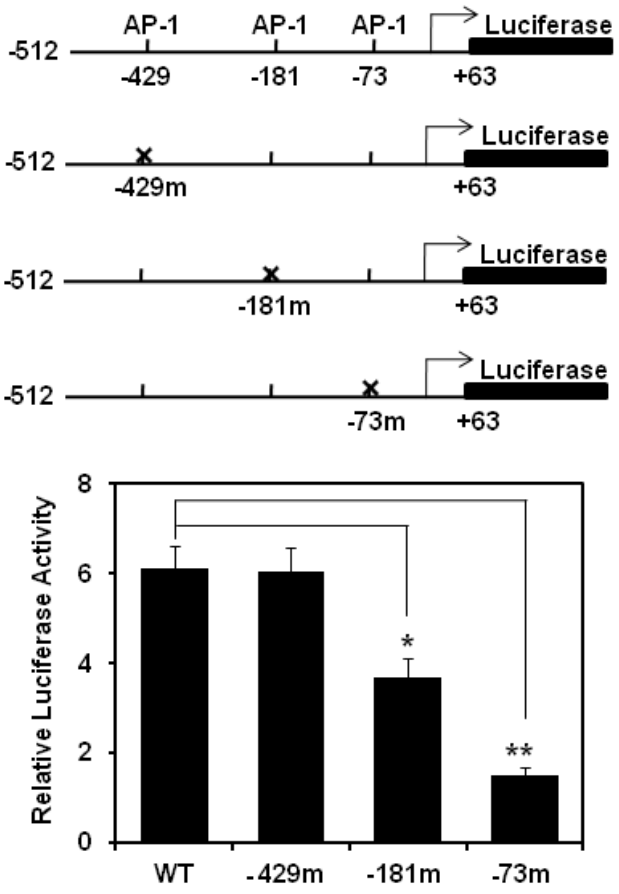


Figure 17. AP-1 is a downstream signal of PKC and MAPK/ERK pathways

(e) AP-1 sites are important for MMP-1 gene activation. Luciferase reporter plasmids bearing 512 bp fragment of MMP-1 promoter with wild type or mutated AP-1 sites were transfected into A549-TPα cells. After 24 h of transfection, cells were treated with 50 nM I-BOP or vehicle control (0.1% ethanol) for additional 18 h. Cell lysates were then prepared. Luciferase activity was assayed as described in Materials and methods. Data were shown as fold change relative to the vehicle control. Values were means ± SD of three independent experiments. Statistical analysis was performed by Student's t-test. * $P < 0.05$; ** $P < 0.01$.

(f)

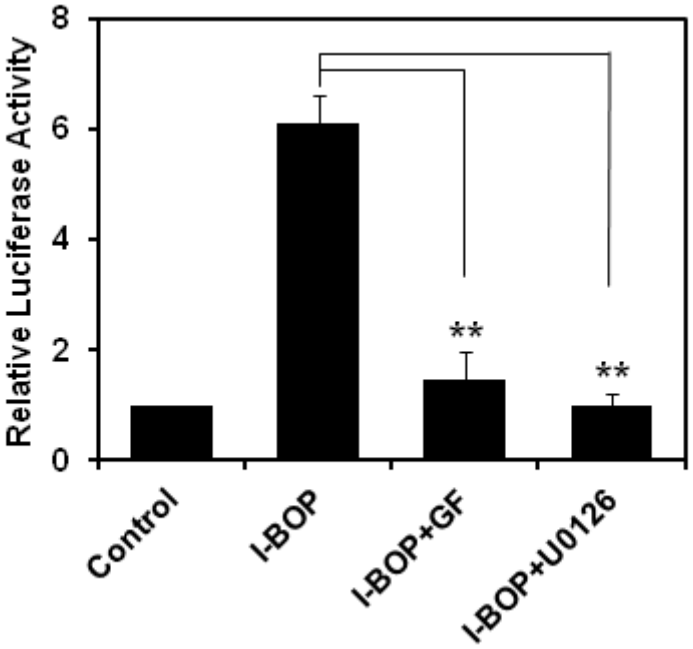


Figure 17. AP-1 is a downstream signal of PKC and MAPK/ERK pathways

(f) Effect of PKC and ERK inhibitors on I-BOP-induced MMP-1 transcription. Luciferase reporter construct containing 512 bp of MMP-1 promoter was transfected into A549-TPα cells. After transfection for 24 h, cells were treated with 0.5 μM GF109203X (GF) or 10 μM U0126, followed by stimulation with 50 nM I-BOP or vehicle control (0.1% ethanol) for 18 h. Luciferase activity was assayed and data were presented as fold change relative to the vehicle control. Values were means ± SD of three independent experiments. Statistical analysis was performed by Student's t-test. ** $P < 0.01$.

4.2.4 C/EBPβ contributes to I-BOP-induced MMP-1 expression

I-BOP-induced MMP-1 mRNA did not reach the maximal level until 8 h suggesting the possibility that other transcription factors activated later by I-BOP may participate in the regulation of MMP-1 transcription. CCAAT/enhancer-binding protein β (C/EBPβ) was identified to contribute to the increase in MMP-1 transcription by IL-1β in chondrocytes and A549 cells [171, 172]. To establish whether C/EBPβ play a role in I-BOP-mediated MMP-1 expression, we first set out to determine the activation of C/EBPβ by I-BOP using Western blot analysis. Both the expression and phosphorylation of C/EBPβ increased in response to I-BOP stimulation (Figure 18a). The maximal increase occurred at 8 h concurrent with the maximal level of MMP-1 mRNA in I-BOP treated cells (Figure 15a and Figure 18a), indicating the possibility that C/EBPβ may modulate MMP-1 gene expression. The effect of C/EBPβ inhibition on I-BOP-mediated MMP-1 expression was then examined. C/EBPβ siRNA at 50 nM significantly knocked down C/EBPβ protein expression and phosphorylation in I-BOP treated cells as compared with the control siRNA (Figure 18b). The knockdown of C/EBPβ partially blocked I-BOP-induced MMP-1 mRNA and protein expression, indicating the involvement of C/EBPβ in MMP-1 regulation.

Furthermore, luciferase reporter assay was used to demonstrate the contribution of C/EBPβ to I-BOP-induced MMP-1 transcription. C/EBPβ-binding element is located at -2,921 bp of human MMP-1 promoter. Deletion mutation of this binding site resulted in ~70% reduction of basal promoter activity and ~40% decrease in relative fold of I-BOP-induced promoter activity compared with the wild type (Figure 18c) indicating that C/EBPβ is critical for constitutive and I-BOP-induced MMP-1 expression in A549-TPα

cells. The upstream signaling pathways leading to the activation of C/EBPβ in I-BOP treated cells were further examined. ERK has been reported to phosphorylate C/EBPβ at Thr-235 to increase its transcriptional activity [173]. Our Western blot results showed that ERK inhibitor, U0126, abrogated both I-BOP-induced expression and phosphorylation of C/EBPβ (Figure 18d) suggesting that C/EBPβ-mediated MMP-1 regulation is ERK-dependent.

(a)

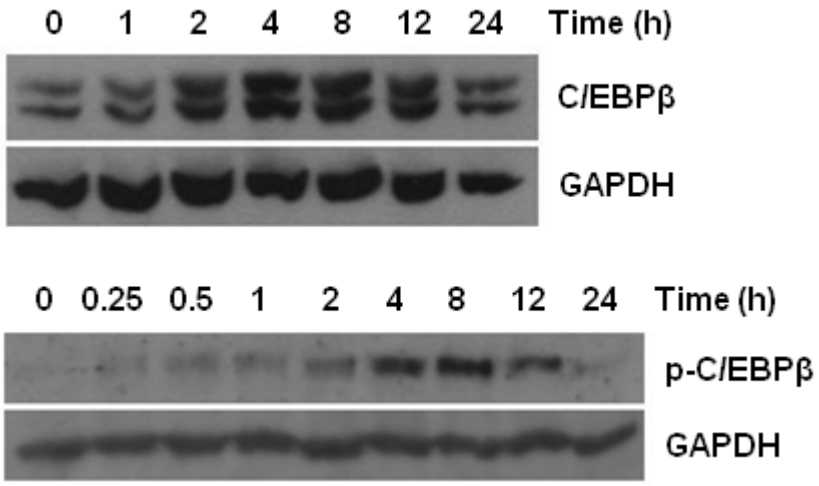


Figure 18. C/EBPβ contributes to I-BOP-induced MMP-1 expression

(a) Time-dependent effect of I-BOP on expression and phosphorylation of C/EBPβ. A549-TPα cells were treated with 50 nM I-BOP at indicated time. Western blot analysis was performed to detect both the expression and phosphorylation of C/EBPβ. Data are representative of three separate experiments.

(b)

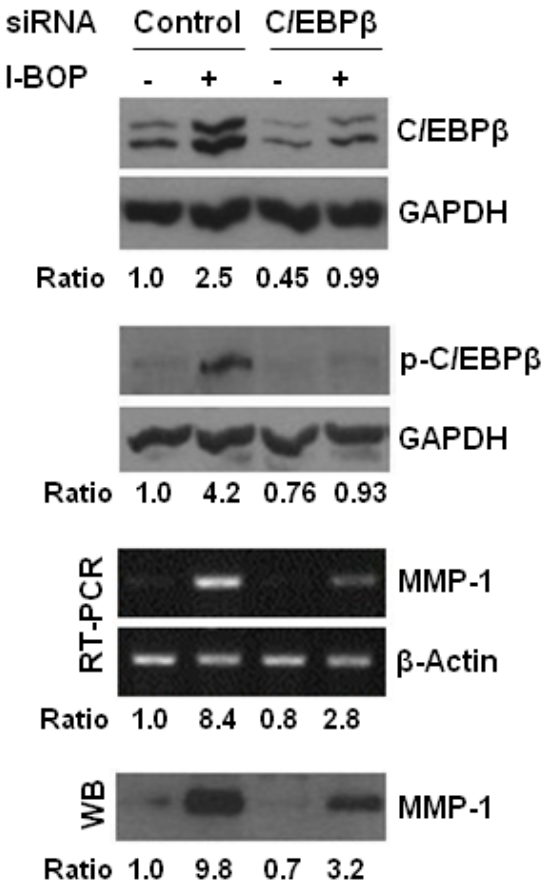


Figure 18. C/EBPβ contributes to I-BOP-induced MMP-1 expression

(b) Effect of C/EBPβ knockdown on I-BOP-induced MMP-1 expression. A549-TPα cells were transfected with 50 nM control siRNA or C/EBPβ siRNA. After transfection for 24 h, cells were treated with vehicle or 50 nM I-BOP for 8 h (detecting C/EBPβ and p-CEBP/β) or 12 h (detecting MMP-1). Western blot analysis was carried out to detect the expression of C/EBPβ, p-C/EBPβ in cell lysates and the secretion of MMP-1 in the conditioned medium. RT-PCR was performed to identify MMP-1 mRNA level as described in Materials and methods. Densitometric analysis of each band was made. The value of vehicle-treated control was normalized as 1.0.

(c)

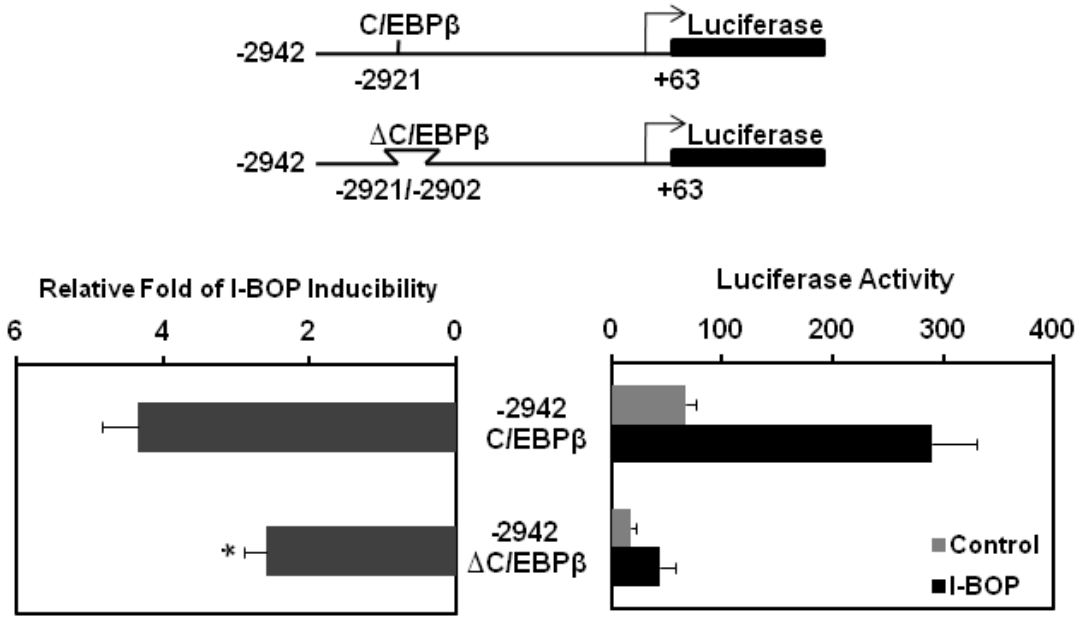


Figure 18. C/EBPβ contributes to I-BOP-induced MMP-1 expression

(c) C/EBPβ-mediated regulation of MMP-1 promoter activity. A549-TPα cells were transfected with luciferase reporter plasmids containing 2,942 bp of MMP-1 promoter sequence or same length promoter with an internal deletion of the CEBPβ site located at -2,921bp. After transfection for 24 h, cells were treated with vehicle control or 50 nM I-BOP for additional 18 h. Luciferase activity was assayed and data are presented as relative light unit (right panel). The relative fold of I-BOP inducibility of wild and mutant promoters was compared (left panel). * $P < 0.05$.

(d)

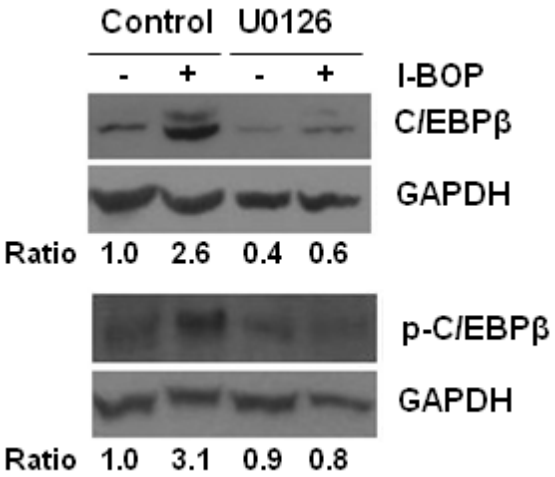


Figure 18. C/EBPβ contributes to I-BOP-induced MMP-1 expression

(d) I-BOP-induced C/EBPβ activation is ERK-dependent. A549-TPα cells were treated with 10 μM U0126 for 30 min before stimulation with vehicle control or 50 nM I-BOP for 8 h. CEBPβ and p-CEBPβ were assayed by Western blot analysis. Densitometric analysis of each band was made. The value of vehicle-treated control was normalized as 1.0. Data are representative of three separate experiments.

4.2.5 Elevated MMPs promote TP-mediated cell invasion

It is believed that MMP family proteins are associated with cancer invasive potential [174]. To investigate whether I-BOP could increase cell invasion through upregulation of MMPs, *in vitro* invasion assay was performed. As shown in Figure 14, I-BOP at 10 nM induced significant increase in both A549-TPα and A549-TPβ cellular invasiveness. Nonetheless, it has no effect on A549 control cells. GM6001, a general MMPs inhibitor, blocked both basal and I-BOP-induced invasiveness of A549-TPα cells (Figure 19a). To further validate the role of I-BOP-induced MMPs in cell invasion, A549-TPα cells were treated with siRNA of MMP-1 and MMP-9 and then analyzed for cellular invasiveness. Total inhibition of MMP-1 and MMP-9 expression resulted in ~40% and ~60% decrease in invasion, respectively (Figure 19b). These findings suggest that TP-mediated A549 cell invasion is at least partially dependent on MMP-1 and MMP-9 induction.

(a)

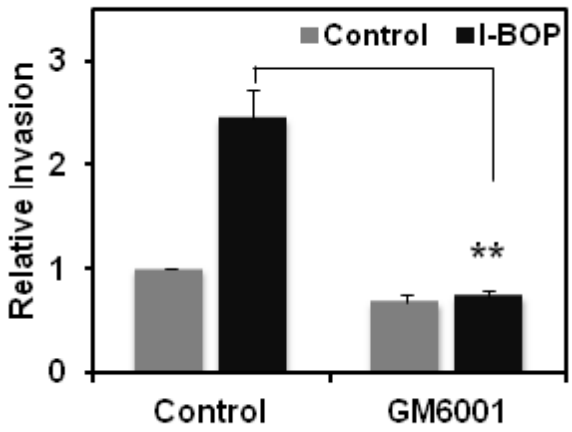


Figure 19. Elevated MMPs promote I-BOP-induced cell invasion

(a) Effect of MMPs inhibitor on I-BOP-induced invasion of A549-TPα cells. GM6001 at 10 μM was added in cell suspension before treatment with vehicle control or 10 nM I-BOP. Invasion assay was performed as described in Materials and methods. ** $P < 0.01$.

(b)

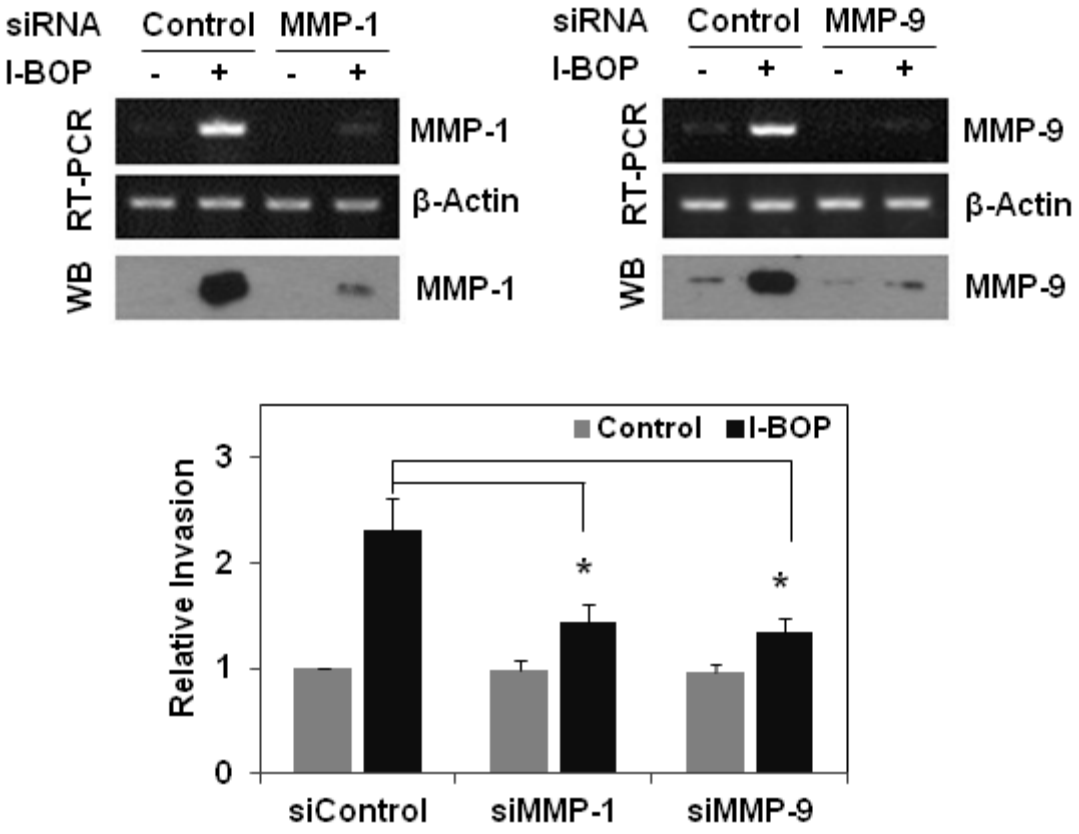


Figure 19. Elevated MMPs promote I-BOP-induced cell invasion

(b) Effect of MMP-1 and MMP-9 knockdown on I-BOP-induced invasion. A549-TPα cells were transfected with 50 nM control, MMP-1 or MMP-9 siRNA. After transfection for 24 h, cells were resuspended in serum-free media with vehicle control or 10 nM I-BOP. The mRNA and protein levels of MMP-1 and MMP-9 were measured by RT-PCR and Western blot analysis. Invasion assay was performed as described in Material and methods. Three separate experiments and triplicate wells each were performed. Data are presented as invasion relative to the vehicle control-treated cells. * $P < 0.05$.

4.3 MMP-1 released by activation of TP induces expression of MCP-1 from lung cancer cells by activation of PAR2

In addition to structural components of the ECM, MMPs can cleave non-ECM molecules. Protease-activated receptor 1(PAR1) was identified as a novel substrate for MMP-1[69]. MMP-1/PAR1 axis has been identified to mediate invasion and metastasis of various cancers including breast cancer [69, 93], melanoma [70] and ovarian cancer [94, 95]. MCP-1-mediated macrophage infiltration has been demonstrated to contribute to cancer metastasis too [132]. Increased release of MCP-1 from A549 cells by activation of PAR1 and PAR2 has been reported before [106]. Here we investigated whether MMPs released by TP activation may cleave and activate PARs to initiate other cascades that involved in cancer invasion and metastasis.

4.3.1 Conditioned media from TP agonist-treated A549-TPα cells induces the release of MCP-1 from A549 cells

To determine if MMPs induced by TP agonist can exert autocrine influences on cancer cell behavior, we first measured MCP-1 levels in A549 cells treated with conditioned media (CM) from A549-TPα cells. As shown in Figure 20, the CM from I-BOP-treated A549-TPα cells (CM-I-BOP) stimulated significantly the release of MCP-1 from control A549 cells compared with the CM from vehicle-treated A549-TPα cells (CM-Control). We found that activation of TP induced the expression and release of MCP-1 directly from A549-TPα cells (results are presented in **4.4**). To avoid the impact of TP on the induction of MCP-1, we used control A549 cells in the following studies.

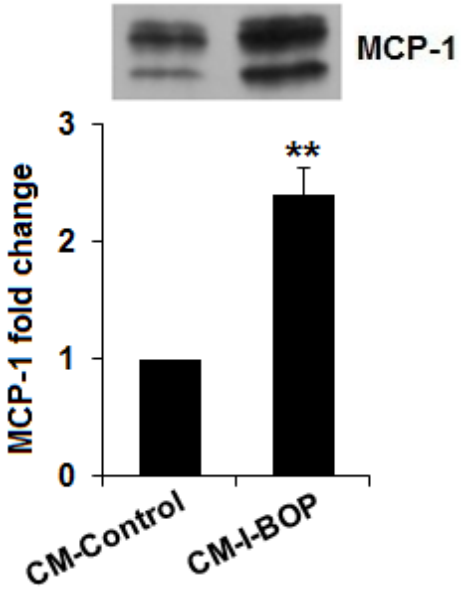


Figure 20. CM-I-BOP induces release of MCP-1 from A549 cells

A549 cells were incubated with 100 µL of concentrated conditioned media (CM) as described in Materials and methods for 16 h. CM-Control: CM from vehicle (0.1% ethanol)-treated A549-TPα cells; and CM-I-BOP: CM from I-BOP-treated A549-TPα cells. Western blot was conducted to determine the release of MCP-1 from A549-GFP cells as described in Materials and methods. Densitometric analysis of each band was made and results shown as fold change, ** $P < 0.01$ compared with CM-Control.

4.3.2 MMP-1 mediates the release of MCP-1 by conditioned media from TP agonist-treated A549-TPα cells

I-BOP induced an increase in protein levels of MMP-1, MMP-3 and MMP-9 in the media of A549-TPα cells (Figure 21a). Different approaches were used to examine which of these MMPs can induce MCP-1 expression. As shown in Figure 21b, the CM from A549-TPα cells over-expressing MMP-9 did not induce the release of MCP-1 from these cells. Recombinant human MMP-3 did not stimulate the expression of MCP-1 either. However, MMP-1 significantly increased the expression of MCP-1 (Figure 21c). Further, the CM from MMP-1-siRNA-treated A549-TPα cells, in which the induction of MMP-1 by I-BOP was significantly decreased, had less effect on MCP-1 release than those media from control siRNA-treated cells (Figure 21d). These data indicate that MMP-1 in the CM-I-BOP may be responsible for inducing the release of MCP-1.

(a)

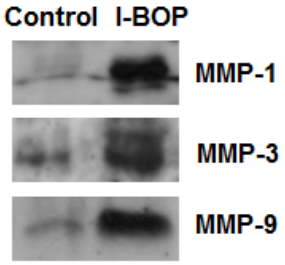


Figure 21. MMP-1 mediates CM-I-BOP-induced MCP-1 from A549 cells

(a) I-BOP induces MMP-1, MMP-3 and MMP-9 expression. The protein levels of MMP-1, MMP-3 and MMP-9 in CM from A549-TPα cells treated with 0.1% ethanol (control) or I-BOP were determined by Western blot analysis. Data are representative of three independent experiments.

(b) (c)

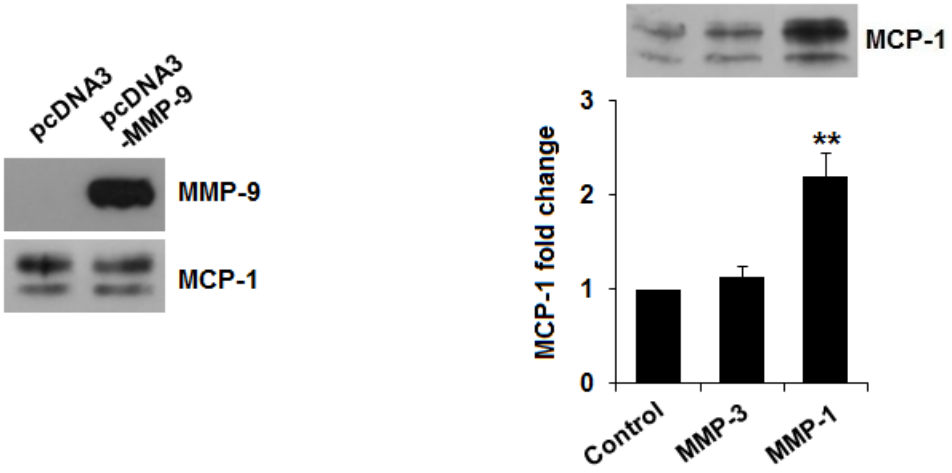


Figure 21. MMP-1 mediates CM-I-BOP-induced MCP-1 from A549 cells

(b) The effects of MMP-9 on MCP-1 release. A549-TPα cells were transfected with pcDNA3 and pcDNA3-MMP-9 plasmids. After 48 h transfection, conditioned media were collected and MMP-9 protein levels were determined by Western blot (upper panel). A549 cells were treated for 16 h with above CM. MCP-1 protein levels were determined by Western blot (lower panel). Results are representative of three independent experiments. (c) The effects of MMP-3 and MMP-1 on MCP-1 release. Control A549 cells were treated with PBS (control), 5 nM MMP-1 or 5 nM MMP-3 for 16 h. The release of MCP-1 was determined by Western blot. Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with control.

(d)

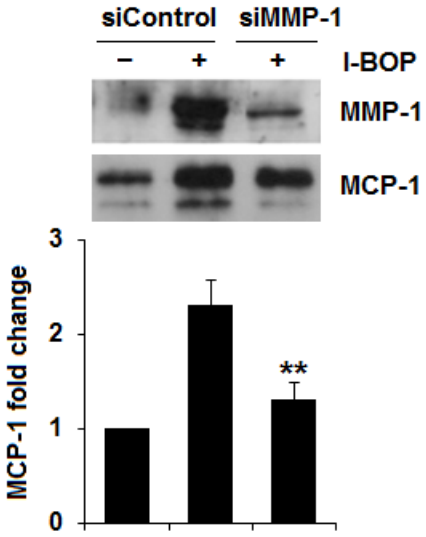


Figure 21. MMP-1 mediates CM-I-BOP-induced MCP-1 from A549 cells

(d) The effects of MMP-1 knockdown in CM on MCP-1 release. A549-TPα cells were treated with either control or MMP-1 siRNA as indicated in Materials and methods. Conditioned media were collected and MMP-1 protein levels were determined by Western blot (upper panel). A549 cells were treated for 16 h with the above CM. MCP-1 protein levels were determined by Western blot (lower panel). Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with siControl plus I-BOP. Results are representative of three individual experiments.

4.3.3 MMP-1-induced MCP-1 release is PAR2 dependent

To determine if MMP-1-induced MCP-1 expression in A549 cells was achieved by cleaving and activating PARs, PARs specific antagonists were employed in this study. Unexpectedly, PAR1 antagonist SCH79797 at 50 nM did not block MMP-1-induced MCP-1 expression. However, SCH79797 at the same concentration effectively inhibited TFLLR-NH_2, a PAR1-AP, -induced MCP-1 expression (Figure 22 and 24b). Similar results were obtained from the treatment with tc-Y-NH_2, a PAR4 antagonist peptide (Figure 22 and 24b). Interestingly, PAR2 antagonist ENMD-1068 completely inhibited MMP-1-induced MCP-1 expression (Figure 22). Comparing all PAR-APs, we found SLIGKV-NH_2, a PAR2-AP, induced the most effective release of MCP-1 and PAR3-AP showed no induction of MCP-1 from A549 cells (Figure 23). The concentration of each PAR-AP used here is all optimal for the maximum induction. Trypsin, a known PAR2 activating protease, also induced the expression of MCP-1 at 0.1μg/mL. Both inductions can be blocked totally by PAR2 antagonist (Figure 24a).

We also compared the increase of MCP-1 mRNA level by MMP-1 and PAR2-AP. Our RT-PCR results showed that a PAR2-AP induced more rapid expression of MCP-1 than did MMP-1. PAR2-AP already stimulated the expression of MCP-1 mRNA at 1 h, and the expression reached the peak at 2 h. Nonetheless, MMP-1-treated cells displayed a delayed increase in the expression of MCP-1 mRNA (Figure 25). In addition, CM-I-BOP-induced MCP-1 release was also blocked by antagonist of PAR2 but not those of other PARs, which is in consistent with the results obtained from MMP-1 treatment (Figure 26).

To further demonstrate that MMP-1-induced MCP-1 expression is mediated by PAR2, the siRNA of PAR2 was used to knock down its expression in A549 cells. As shown in Figure 27a, PAR2 siRNA at 100 nM successfully knocked down greater than 80% of PAR2 protein expression in A549 cells. The inductions of MCP-1 by MMP-1 and by CM-I-BOP in PAR2 knockdown cells were decreased by ~45% and ~40%, respectively (Figure 27b and 27c). These data indicate that MMP-1-induced release of MCP-1 from A549 cells is significantly mediated by PAR2.

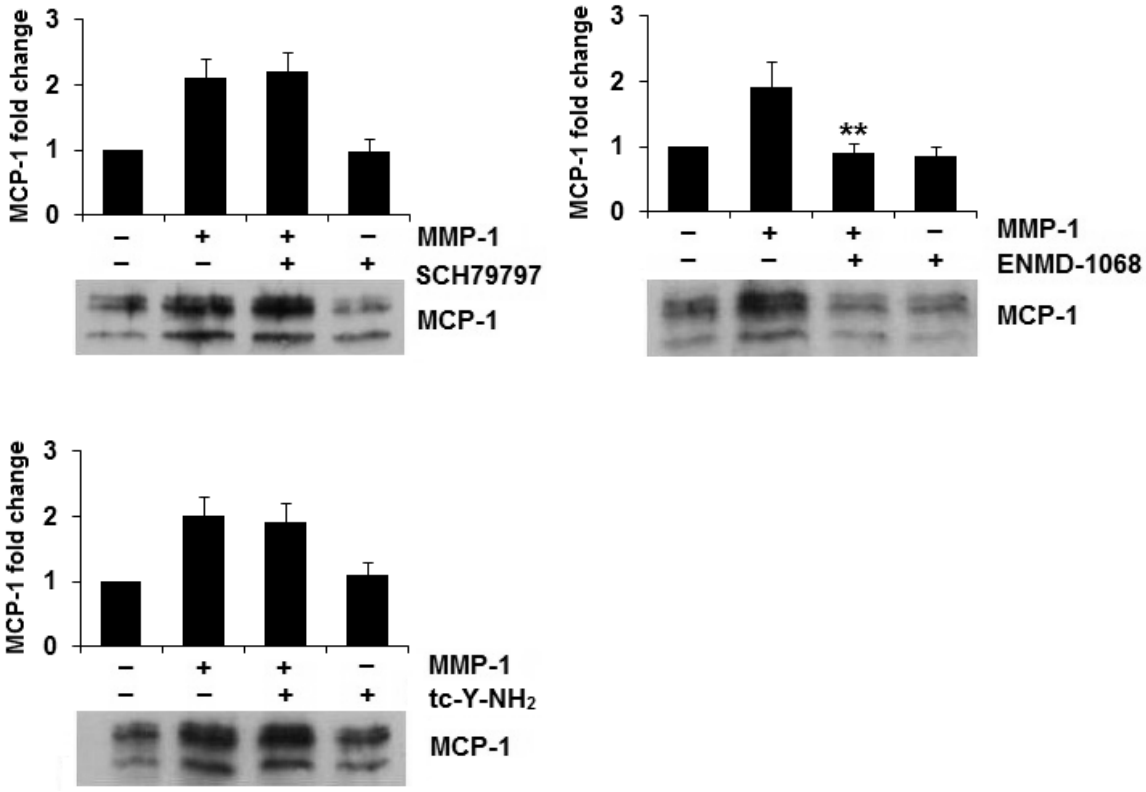


Figure 22. PAR2 antagonist blocks MMP-1-induced MCP-1 release from A549 cells

Cells were treated with different PARs antagonists at optimized inhibitory concentrations for 30 min before stimulation with 5 nM MMP-1 for 16 h. PARs antagonists: PAR1, 50 nM SCH79797; PAR2, 150 µM ENMD-1068; PAR4, 300 µM tc-Y-NH_2. MCP-1 protein levels were determined by Western blot. Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with MMP-1 alone.

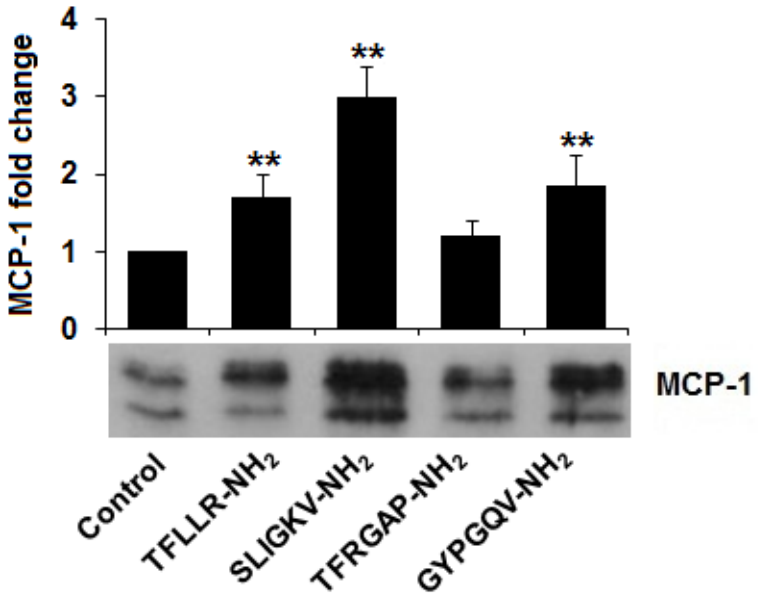


Figure 23. PAR-APs induce MCP-1 release from A549 cells

Cells were treated with different PAR-APs: PAR1, 300 µM TFLLR-NH_2; PAR2, 300 µM SLIGRL-NH_2; PAR3, 300 µM TFRGAP-NH_2; PAR4, 300 µM GYPGQV-NH_2, for 16 h. MCP-1 protein levels were determined by Western blot. Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with control.

(a) (b)

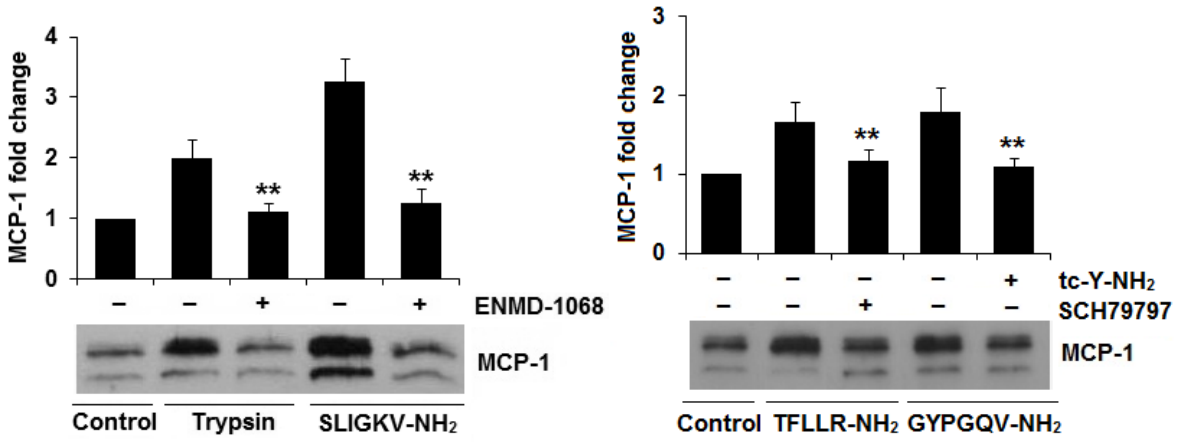


Figure 24. PARs antagonists block PARs agonists-induced MCP-1 release from A549 cells

(a) Cells were treated with or without 150 µM PAR2 antagonist, ENMD-1068, for 30 min before incubation with PAR2 agonist, 0.1µg/ml trypsin (optimized concentration for PAR2 activation) and 300 µM SLIGRL-NH_2, for 16 h. MCP-1 protein levels were measured by Western blot. Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with trypsin or SLIGRL-NH_2 alone.

(b) Cells were treated with or without PAR1 or PAR4 antagonist (50 nM SCH79797 for PAR1, 300 µM tc-Y-NH_2 for PAR4) for 30 min before stimulation with PAR1-AP, 300 µM TFLLR-NH_2 or PAR4-AP, 300 µM GYPGQV-NH_2, for 16 h. MCP-1 protein levels were determined by Western blot. Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with TFLLR-NH_2 or GYPGQV-NH_2 alone.

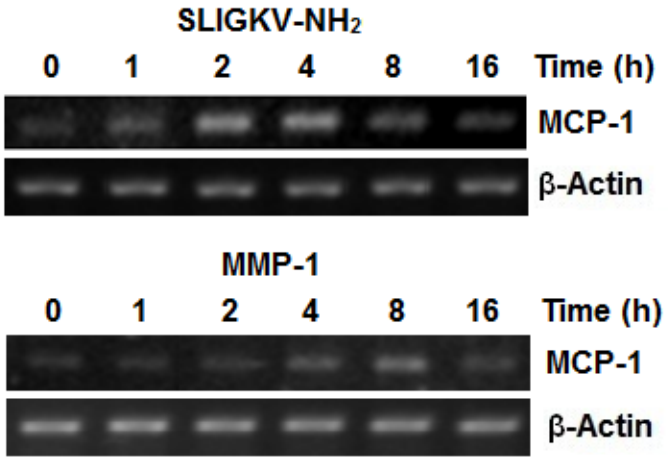


Figure 25. MMP-1 and PAR2-AP induce the transcription of MCP-1

Cells were treated with 300 μM PAR2-AP, SLIGRL-NH_2 or 5 nM MMP-1 at indicated time. RT-PCR was carried out to identify mRNA levels of MCP-1 in cells. Data are representative of at least three individual experiments.

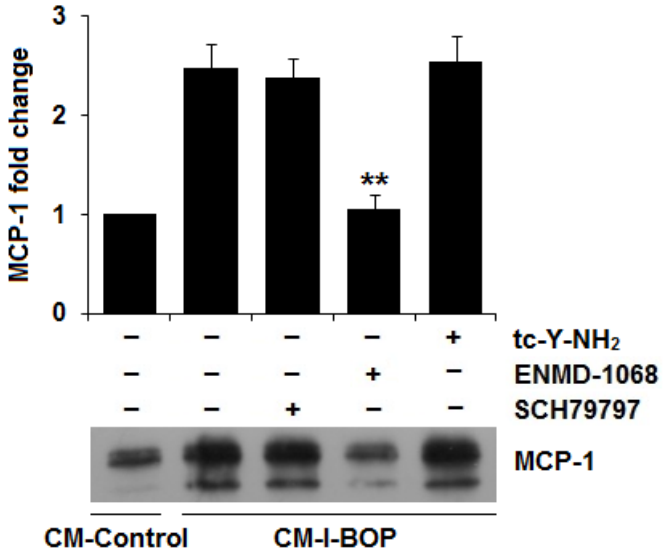
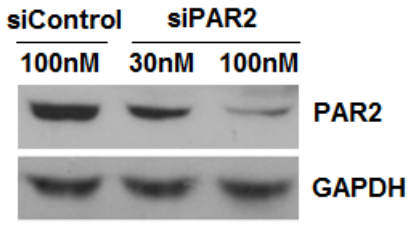


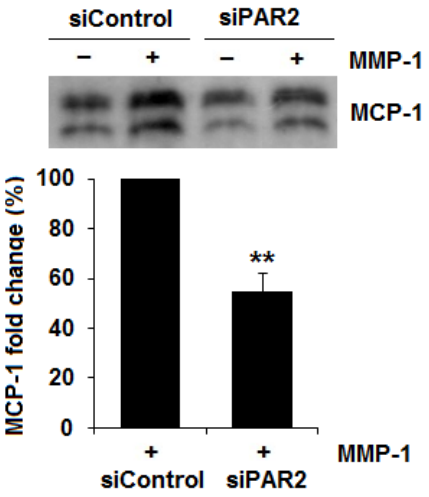
Figure 26. PAR2 antagonist blocks CM-I-BOP-induced MCP-1 release from A549 cells

Cells were treated with different PARs antagonists as described in Figure 23 for 30 min before stimulation with CM-I-BOP for 16 h. MCP-1 protein levels were determined by Western blot. Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with CM-I-BOP alone.

(a)



(b)



(c)

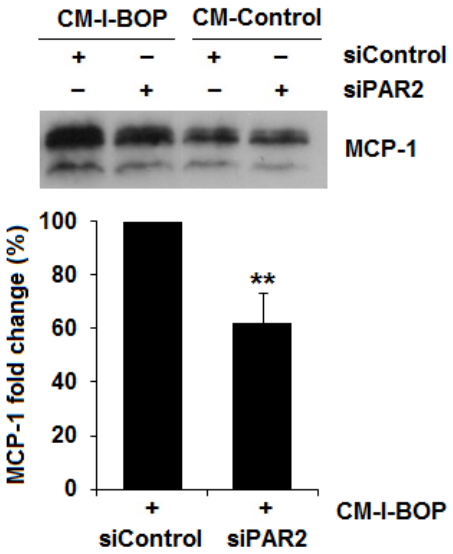


Figure 27. PAR2 siRNA suppresses MMP-1 and CM-I-BOP-induced MCP-1 release from A549 cells

(a) Cells were treated with control or PAR2-siRNA as described in Materials and methods. After treatment for 72 h, PAR2 protein levels were determined by Western blot analysis. (b) and (c) Control or PAR2-siRNA-treated cells were incubated with 5 nM MMP-1 or CM for 16 h. MCP-1 release were determined by Western blot. Densitometric analysis of each band was made and results were shown as fold change, ** $P < 0.01$ compared with siControl.

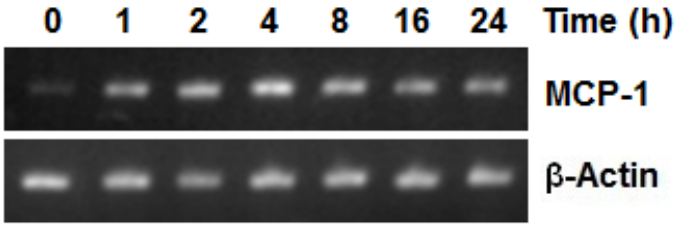
4.4 Activation of TP increases MCP-1 expression and recruits macrophages to promote invasion of lung cancer cells

In addition to stimulating MCP-1 expression through released MMP-1, activation of TP can directly induce a rapid and significant expression of MCP-1 in A549-TPα cells.

4.4.1 Induction of MCP-1 expression by activation of TP

To examine the effects of activation of TPα on the expression of MCP-1, A549-TPα cells were treated with TP agonist I-BOP at different time points. RT-PCR results showed that I-BOP induced a rapid and sustained expression of MCP-1 mRNA (Figure 28a). Quantitative real-time PCR assay was utilized to further determine the kinetics of MCP-1 induction. As shown in Figure 28b, the MCP-1 mRNA levels peaked at 4 h and still maintained a significant increase at 16 h after I-BOP treatment. The protein levels of I-BOP-induced MCP-1 expression were analyzed by Western blot assay. The accumulation of MCP-1 in conditioned medium reached the maximum level at 24 h following stimulation (Figure 28c). Further, the induction of MCP-1 by I-BOP was dose-dependent and the effect was significant even at 1 nM of I-BOP (Figure 28d). In addition, the induction of MCP-1 by I-BOP was also observed in H157 and H460 human lung cancer cells that express high endogenous levels of TP [10] (Figure 29). When compared with other prostanoids including PGD_2, PGE_2, and $PGF_{2\alpha}$, I-BOP induced the most significant expression of MCP-1 in A549-TPα and H460 cells (Figure 30). Moreover, other prostanoids induced the peak expression of MCP-1 at a higher concentration (1μM). These data show that TP plays an important role in the production of MCP-1 in lung cancer cells.

(a)



(b)

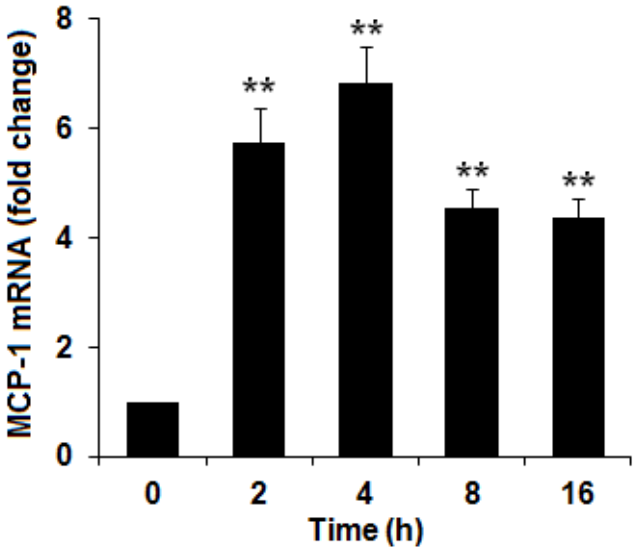
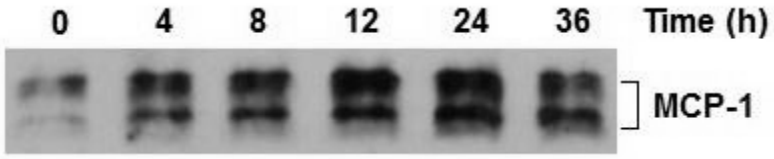


Figure 28. Effects of I-BOP on MCP-1 expression in A549-TPα cells

(a) I-BOP induced transcription of MCP-1. Cells were serum-starved for 24 h before treated with 50 nM I-BOP for the indicated time periods. RNAs were isolated and RT-PCR was carried out as described in Materials and methods. (b) MCP-1 mRNA induction in (a) was confirmed by quantitative real-time PCR. ** $P < 0.01$ compared with untreated cells.

(c)



(d)

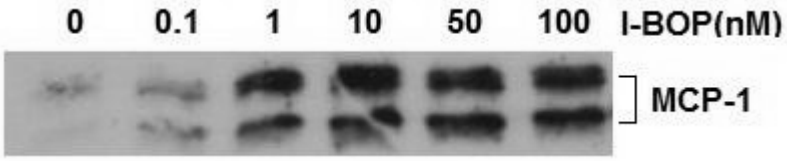


Figure 28. Effects of I-BOP on MCP-1 expression in A549-TPα cells

(c) Time-dependent effects of I-BOP on MCP-1 protein expression. After 24 h serum-starvation, cells were treated with 50 nM I-BOP for the indicated time periods. Media were collected and proteins in each medium were concentrated by trichloroacetic acid (TCA) precipitation as described in Materials and methods. Concentrated samples were dissolved in SDS-PAGE sample loading buffer and analyzed by Western blot. (d) Dose-dependent effects of I-BOP on MCP-1 protein expression. After 24 h serum-starvation, cells were treated with indicated dose of I-BOP for further 24 h. Media were collected and assayed as described in (c).

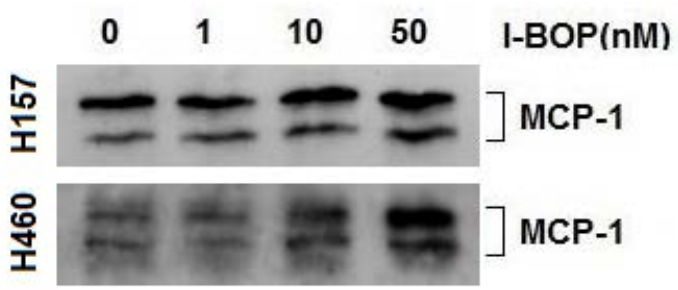


Figure 29. I-BOP-induced MCP-1 protein expression in H157 and H460 cells

After 24 h serum-starvation, cells were treated with indicated dose of I-BOP for further 24 h. Media were collected and assayed as described in Figure 28c. Data are representative of three independent experiments.

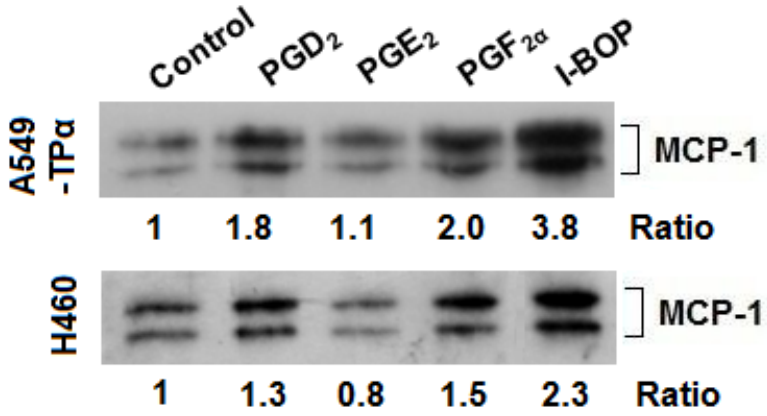


Figure 30. Comparison of I-BOP with other prostanoids on the induction of MCP-1 in A549-TPα and H460 cells

After 24 h serum-starvation, cells were treated with 50 nM I-BOP, 1μM PGD_2, 1μM PGE_2 or 1μM $PGF_{2\alpha}$ for 24 h. Media were collected and assayed as described in Figure 28c. Densitometric analysis of each band was made, and control untreated time point is normalized to 1. Data are representative of three independent experiments.

4.4.2 SP1 is important for TPα-mediated MCP-1 expression

Upon ligand binding TP activates several downstream signal transduction cascades including PKC, ERK and NF-κB pathways that are involved in MCP-1 induction by several cytokines [118, 119, 175]. To investigate whether these pathways are responsible for I-BOP-induced MCP-1 expression, several specific inhibitors were used. As shown in Figure 31, except TP antagonist, SQ29548, none of other inhibitors (GF109203X for PKC, U0126 for MEK/ERK, and MG132 for NF-κB pathways) has an effect on I-BOP-induced MCP-1 expression indicating these pathways are not involved in the induction. In the proximal region of MCP-1 promoter, there is a GC rich element, which can bind nuclear factor SP1 to activate transcription (Figure 8) [115]. To elucidate whether SP1 is critical for MCP-1 induction by I-BOP, we employed mithramycin A (MTM), a specific SP1inhibitor preventing SP1 from binding to its consensus GC rich sites [176], and geldanamycin (GA), an inhibitor of Hsp90 which was also reported to affect binding of SP1 to gene promoter [177]. Both inhibitors significantly suppressed the induction of MCP-1 by I-BOP at protein and mRNA levels (Figure 32 and 33). We further investigated the effects of I-BOP on SP1 translocation and expression. As shown in Figure 34, SP1 protein started to accumulate in nucleus after incubation with I-BOP for 30 min, and remained elevated at 4 h. I-BOP also induced the expression of SP1 at 2 h. Moreover, promoter-luciferase reporter bearing a fragment of 500 bp of human MCP-1 gene promoter was used to evaluate the contribution of SP1 to I-BOP-induced MCP-1 transcription. SP1-binding element is located at -115 bp of MCP-1 promoter. Site-directed mutagenesis of SP-1 site resulted in a significant decrease of I-BOP-induced

promoter activity compared with the wild type (Figure 35). These data reveal that TP-mediated MCP-1 expression is SP1 dependent.

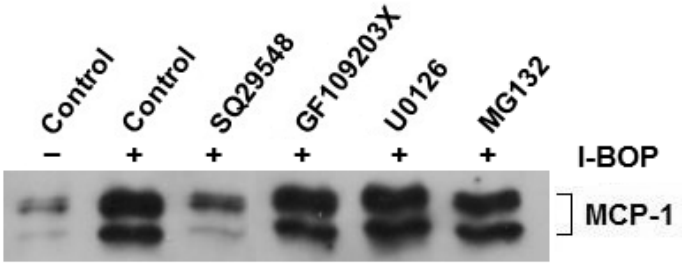


Figure 31. Effects of TP antagonist and several protein kinases inhibitors on I-BOP-induced MCP-1 expression

Cells were treated with TP antagonist SQ29548 (10 μM), PKC inhibitor GF109203X (0.5 μM), MEK inhibitor U0126 (10 μM), and NF-κB activation inhibitor MG132 (10 μM) for 30 min before incubation with 50 nM I-BOP for 24 h. Media were collected and assayed as described in Figure 28c.

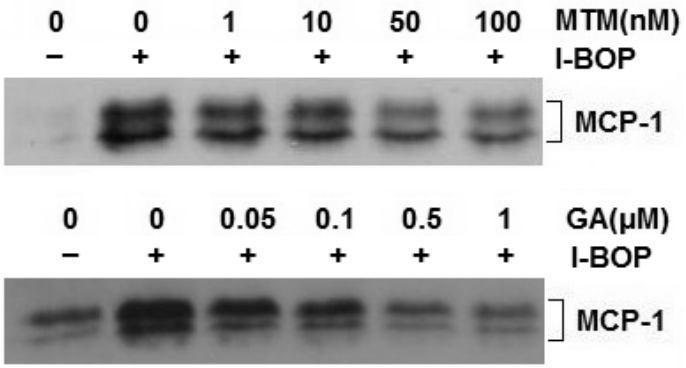


Figure 32. Effects of SP1 inhibition on I-BOP-induced MCP-1 expression at protein levels

Cells were treated with indicated concentrations of SP1 inhibitor mithramycin A (MTM) and Hsp90 inhibitor geldanamycin (GA) for 30 min before incubation with 50 nM I-BOP for 24 h. Media were collected and assayed as described in Figure 28c.

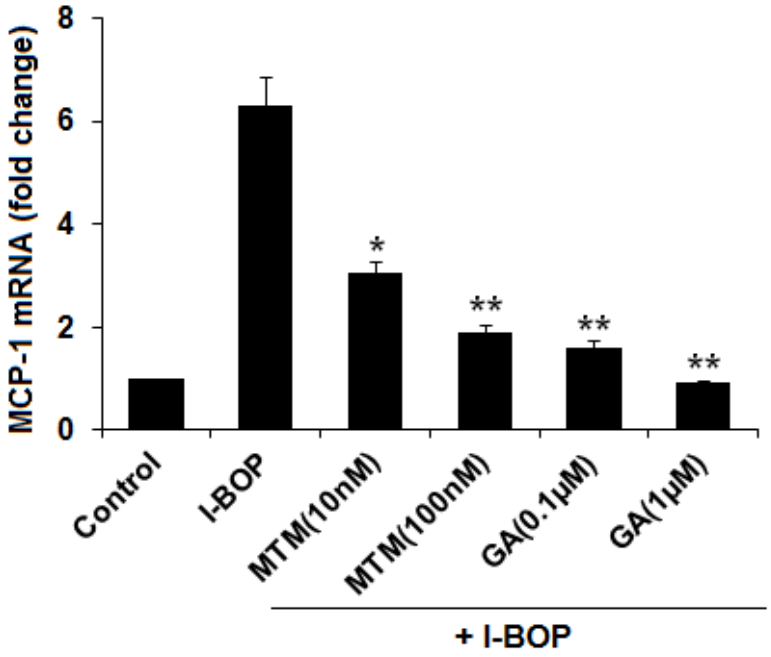


Figure 33. Effects of SP1 inhibition on I-BOP-induced MCP-1 expression at mRNA levels

Cells were treated with above inhibitors for 30 min before incubation with 50 nM I-BOP for 4 h. RNAs were isolated and real-time PCR was performed as described in Materials and methods. * $P < 0.05$ and ** $P < 0.01$ compared with I-BOP treated alone.

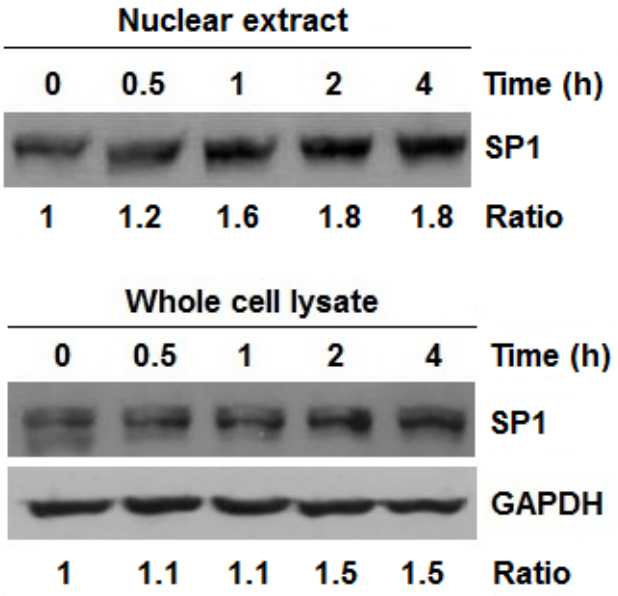


Figure 34. SP1 protein levels are increased in the nuclei of A549-TPα cells following I-BOP stimulation

Cells were treated with 50 nM I-BOP for the indicated time periods. Nuclear extract and whole cell lysate were prepared as described in Materials and methods. Protein level of SP1 was determined by Western blot. Densitometric analysis of each band was made and control untreated time point was normalized to 1.

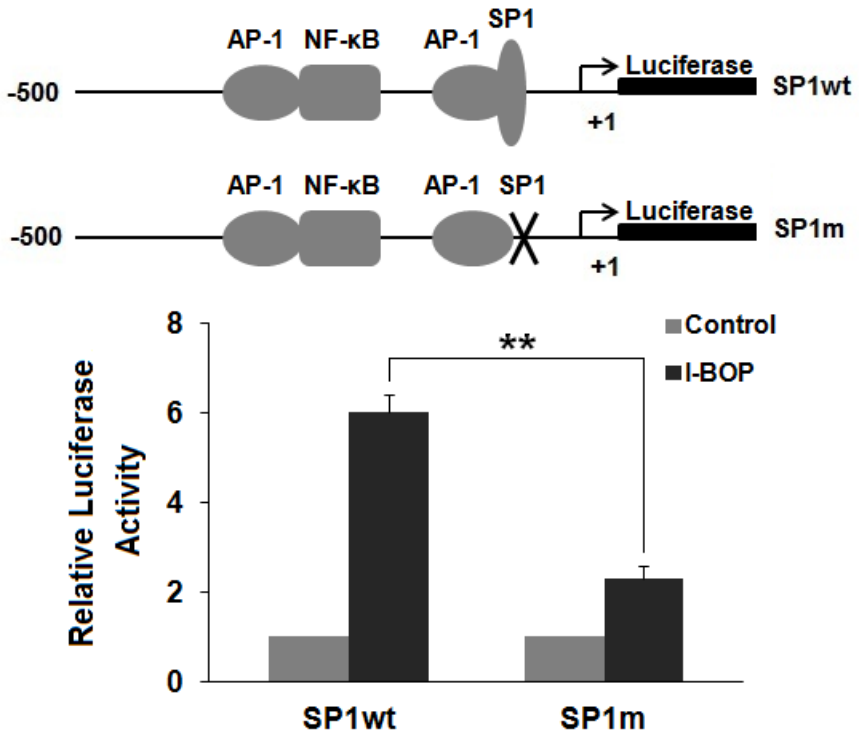


Figure 35. Regulation of MCP-1 promoter activity by SP1

A549-TPα cells were transfected with luciferase reporter plasmids containing 500 bp of MCP-1 promoter sequence with either wild type or mutated SP1 binding sites. After 24 h transfection, cells were treated with vehicle control (0.1% ethanol) or 50 nM I-BOP for additional 18 h in serum free medium**.** Luciferase assay was carried out as described in Materials and methods. ** $P < 0.01$.

4.4.3 TP agonist-induced MCP-1 exhibits chemotactic effects on macrophages

To determine the chemotactic property of I-BOP-induced MCP-1 on macrophages, we examined the effect of conditioned medium from A549-TPα cells treated with or without I-BOP on the migration of murine macrophage RAW 264.7 cells. As shown in Figure 36, conditioned media from I-BOP-treated cells significantly induced RAW 264.7 cells migration compared to the media from vehicle (0.1% ethanol)-treated cells. MCP-1 neutralizing antibody and RS-102895, antagonist of CCR2, significantly decreased the chemotactic potency of medium from I-BOP-treated A549-TPα cells. These data indicate that MCP-1 is a key chemotactic factor in A549-TPα cell culture which caused migration of macrophages.

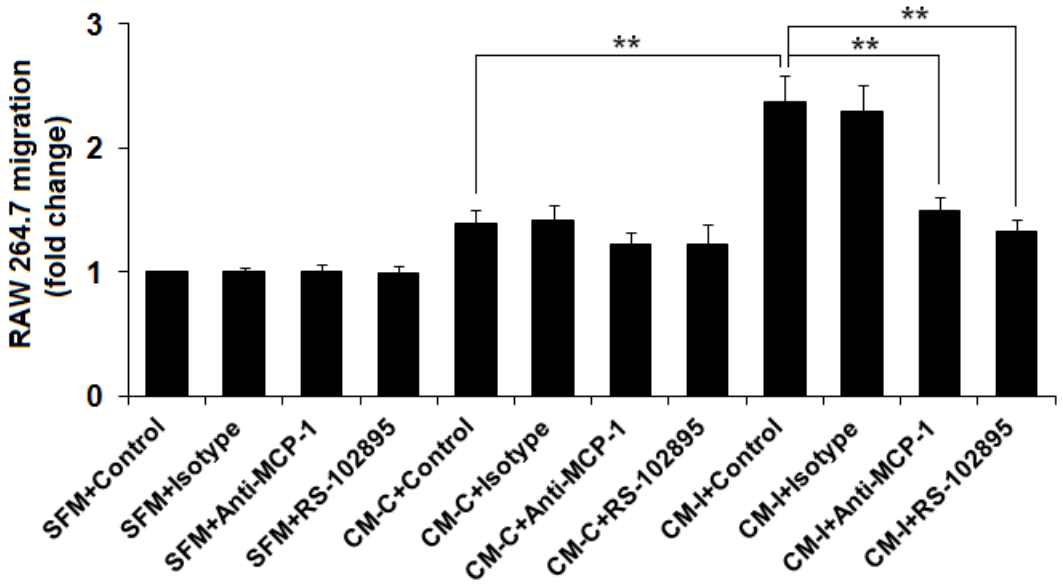


Figure 36. Effects of A549-TPα conditioned medium on the migration of RAW 264.7 macrophages

Chemotaxis assay of RAW 264.7 cells was carried out as described in Materials and methods. Preparation of conditioned medium (CM) from A549-TPα cells was also carried out as described in Material and methods section. CM-C is derived from vehicle-treated cells, and CM-I is derived from I-BOP-treated cells. SFM represents serum free medium. Rat IgG antibody which has no known specificity served as isotype control. The concentrations of both antibodies were 5μg/mL original CM. The concentration of RS-102895 was 10 μM. ** $P < 0.01$.

4.4.4 Co-culture of RAW 264.7 macrophages induces expression of metastatic genes by A549 cells

To elucidate the effects of macrophages on the invasiveness of A549 cells, we first used a trans-well co-culture system as shown in Figure 37. In this co-culture system, there is no cell-cell direct contact and cells communicate with each other through soluble proteins. Both control A549 and A549-TPα cells became more elongated and scattered after 12 h of co-culture with RAW264.7 cells than the cells grown alone (Figure 38). Furthermore, results from RT-PCR analysis showed that expressions of metastatic genes including several MMPs and VEGF were significantly elevated in either control A549 or A549-TPα cells co-cultured with macrophages (Figure 39). Macrophages also induced MCP-1 expression by control A549 or A549-TPα cells (Figure 39). As mentioned in **3.1.2**, Reduction of E-cadherin is tightly linked with cell migration and invasion [169]. Therefore, we examined whether macrophage-lung cancer cell interactions regulate E-cadherin expression in control A549 or A549-TPα cells. Indeed, co-culture with RAW 264.7 macrophages significantly reduced the levels of E-cadherin in either type of A549 cells (Figure 40). Collectively, these data indicate that co-culture with macrophages may increase migration and invasion potential of A549 cells.

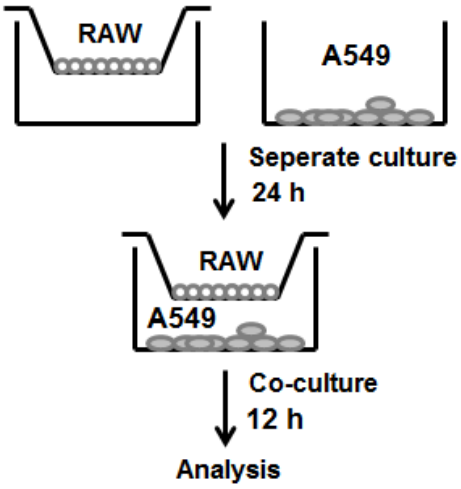


Figure 37. Diagram of a transwell co-culture system

RAW 264.7 cells and A549 cells were separately cultured for 24 h and then co-cultured in serum free medium for further 12 h or 24 h as described in Materials and methods.

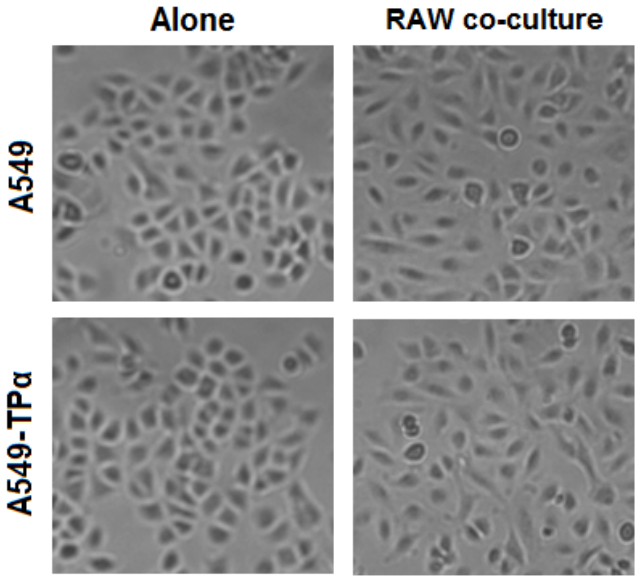


Figure 38. Transwell co-culture of RAW 264.7 macrophages induces morphological changes of A549 cells

Either control A549 or A549-TPα cells became more scattered and spindle shaped after culture with RAW 264.7 cells. A549 cells were pictured after 12 h of culture with or without RAW 264.7 cells as described in Figure 37.

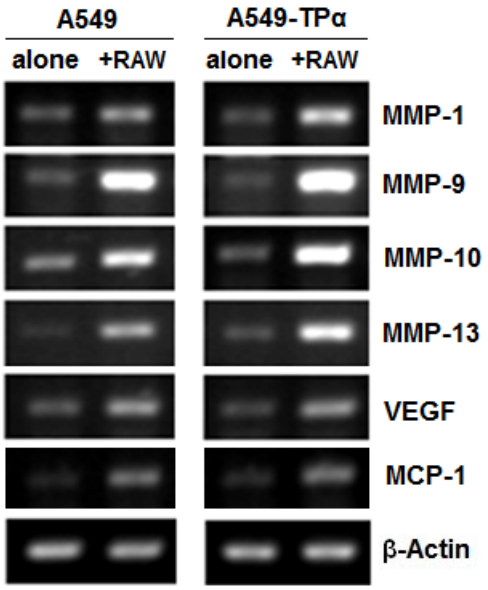


Figure 39. Co-culture of macrophages induces metastatic gene expression by A549 cells

The expressions of several metastatic genes in A549 and A549-TPα cells including MMPs, VEGF and MCP-1 mRNA after co-culture with macrophages were examined by RT-PCR as described in Materials and methods. Data are representative of three independent experiments.

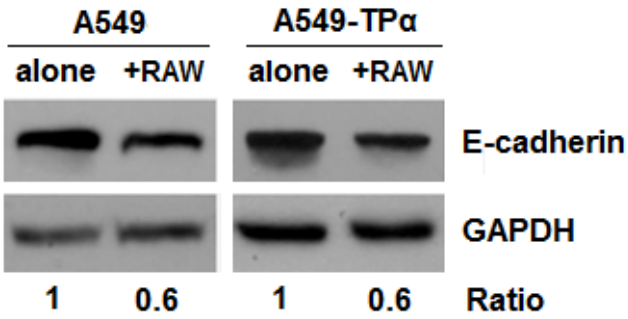


Figure 40. Co-culture of macrophages decreases E-cadherin expression by A549 cells

A549 cells were collected after 24 h of culture with or without RAW 264.7 cells as described in Figure 37. Protein level of E-cadherin was determined by Western blot as described in Material and methods. Results are a representative of three independent experiments.

4.4.5 Co-culture of RAW 264.7 macrophages induces morphological changes and invasion of A549 cells

To further examine if macrophages promote invasion of A549 cells, we next used another co-culture system as shown in Figure 41. In this system, cells communicate with each other via direct contact, which is closer to the physiological situation. As shown in Figure 42, after 12 h of co-culture with macrophages, control A549 cells exhibit elongated protrusions indicating the invasive potential. This morphology still sustained after 36 h of co-culture. In addition, in matrigel invasion assay, co-culture with macrophages induced a 2.5-fold increase in cellular invasiveness of control A549 cells through matrigel relative to those cells grown alone (Figure 43). These results indicate that macrophages attracted by MCP-1 released from A549 cells may stimulate cancer cells invasion.

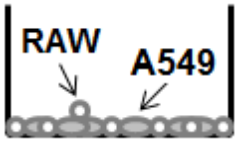


Figure 41. Diagram of a direct co-culture system

RAW 264.7 cells and A549 cells were plated into the same well of a culture plate.

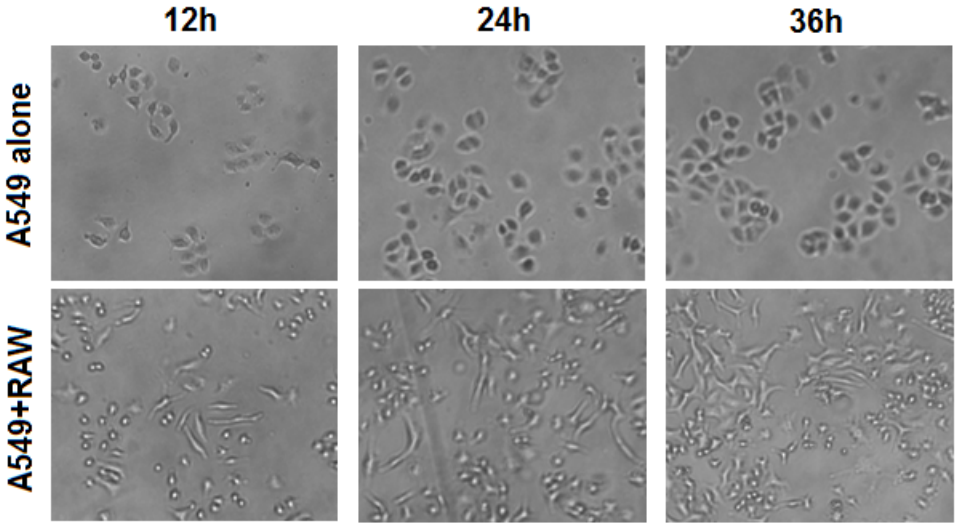


Figure 42. Direct co-culture of macrophages induces morphological changes of A549 cells

A549 cells exhibit elongated shape after culture with RAW 264.7 macrophages. A549 cells were plated into a 12-well plate with or without RAW 264.7 cells as described in Figure 41. Cells were pictured at the indicated time points. In the lower panel, those small cells exhibiting round shape were RAW 264.7 cells. Results are representative of three independent experiments.

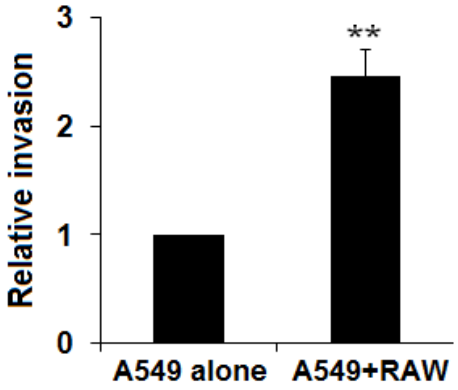


Figure 43. Co-culture of macrophages induces invasion of A549 cells

Invasion assay of A549 cells was carried out as described in Material and methods. ** $P < 0.01$.

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5. Discussion

5.1 Increased expression of MMPs mediates TP agonist-induced invasion in A549-TPα cells

There is much evidence that COX-2 and one of its derived products, prostaglandin E_2 (PGE_2) mediate invasion and metastasis of various cancers through upregulation of MMPs [63-65, 178]. TXA_2, another product downstream of COX-2, is also reported to be involved in cancer invasion and metastasis [46, 179]. However, there are few data on the connection between TXA_2-TP and MMPs in promotion of cancer invasion and metastasis. In this study, it was demonstrated that one TXA_2 mimetic, I-BOP, induced significantly the expression of several MMPs, such as MMP-1, MMP-3, MMP-9 and MMP-10 in both A549-TPα and A549-TPβ cells. This study is the first to establish the association between TP and MMPs in cancer; it also provides the evidence that TP mediates cellular invasive potential by inducing MMPs.

To study the mechanisms of I-BOP-induced MMPs expression, MMP-1 was chosen as a model system to explore the roles of nuclear factors and signal transduction pathways in TP-mediated MMPs gene expression. Using specific pharmacological inhibitors of various signaling molecules which were reported to be activated in TP-mediated signaling transductions [10, 26, 180], we identified PKC and MAPK/ERK but not SAPK/JNK, P38 MAPK or PI3K pathways are involved in I-BOP-stimulated MMP-1 expression. Recently, Uchiyama *et al*. reported that both MMPs and PKC inhibitors inhibited TP agonist U46619-induced shedding of heparin-binding epidermal growth factor (HB-EGF)[181], suggesting the involvement of PKC in TP-mediated MMPs expression or activation.

We further explored downstream targets of PKC and ERK pathways in the regulation of MMP-1 expression. As we mentioned in **1.5.1**, based on the compositions of cis-acting elements in their promoters, MMPs have been roughly grouped into three categories. Group 1 consists of the majority of the MMPs: MMP-1, 3, 7, 9, 10, 12, 13 and 19 which contain a TATA box and an AP-1 site in the proximal region of their promoters. In the present study, MMPs induced by I-BOP belong to Group 1, suggesting that transcription factor AP-1 can be activated and involved in TP-mediated signaling. It has been previously reported that ERK-AP1 pathway play an important role in cytokine and growth factor stimulation of expression of MMPs. Constitutive activation of ERK transactivates the MMP-1 promoter through phosphorylation and activation of AP-1 and Ets proteins [182]. EGF activation of the ERK pathway can activate multiple MMP genes simultaneously, including MMP-1[75], MMP-3[78], MMP-7, MMP-9[80] and MMP-14 [183]. PKC has also been shown to regulate MMPs expression through activation of AP-1[184]. Activation of AP-1 by TP agonist has been found in human vascular smooth muscle cells and endothelial cells [185, 186]. In this study, we found that I-BOP induced significantly the phosphorylaton of c-Jun and the expression of c-Fos, the major subunits of AP-1 transcription factors. Inhibition of PKC and ERK repressed the induction of p-c-Jun and c-Fos, suggesting that PKC and ERK pathways regulate MMP-1 expression through targeting at AP-1 factors. Both ERK and JNK can directly activate c-Jun according to others report [187]. We also detected the activation of JNK in I-BOP-treated cells and inhibition of JNK partially blocked I-BOP-induced p-c-Jun. However, JNK inhibitor neither decreased the induction of MMP-1 nor changed the expression of c-Fos, suggesting that activation of c-Jun alone is not sufficient to induce

TP-mediated MMP-1 expression. Activation of both c-Jun and c-Fos is required for TP-mediated MMP-1 regulation. This is consistent with the findings of Hu *et al*. [188]who demonstrated that EGF and platelet-derived growth factor (PDGF) could not induce the expression of MMP-1 and MMP-3 even though these growth factors induced significantly the expression of c-Jun, JunB and other AP-1 proteins in c-fos-deficient cell lines. To further define the role of AP-1 in I-BOP-induced MMP-1 transcription, we mutated three AP-1 sites separately in the region close to the transcription start site of MMP-1 promoter. The results showed that the proximal AP-1 site, -73 bp relative to the transcription start site, is critical to I-BOP-induced MMP-1 transcription.

In addition to AP-1, our results indicated that transcription factor C/EBPβ was also involved in I-BOP-induced MMP-1 expression. Previously, it was demonstrated that IL-1β and IL-17 induced MMP-1 expression through ERK-dependent induction and phosphorylation of C/EBPβ on Thr-235[171, 172, 189]. We showed that inhibition of ERK blocked I-BOP-induced expression and phosphorylation of C/EBPβ and knockdown of C/EBPβ by siRNA decreased I-BOP-induced MMP-1 expression. These results support the contention that C/EBPβ plays an important role in inducing MMP-1 expression. It has been shown that there are several silencer elements in the region between -1,653 and -2,672 bp of MMP-1 promoter which suppress the transcription of MMP-1[190]. The C/EBPβ site located at -2,921 bp is outside of the region of repression. Therefore, deletion mutation of this C/EBPβ site significantly decreased the basal and I-BOP-induced MMP-1transcription. In addition to MMP-1, MMP-3 and MMP-10 also contain multiple putative C/EBP sites in their promoters. Silencing C/EBPβ decreased IL-1β-induced MMP-3 and MMP-10 gene expression [172]. Our previous study

indicated that C/EBP plays an important role in I-BOP-stimulated COX-2 transcription [166]. Taken together, these data suggest that the role of C/EBP signaling pathways in TP-mediated transcriptional regulation of cancer related genes cannot be overlooked.

Our data show that I-BOP increased invasiveness of A549-TPα and A549-TPβ cells maximally at low concentration (10 nM) although a higher concentration was needed to induce maximal expression of MMPs (Figure 13). This could be due to the fact that a higher concentration of TP agonist reduced tumor cell migration [20]. It seems that TP-mediated cell migration is in a temporal and spatial manner [20]. Further studies are required to provide a plausible explanation for the poor correlation.

In our studies, MMP-1 and MMP-9 function as mediators of I-BOP-induced cell invasion which are supported by other reports that short hairpin RNA of MMP-1 reduced invasiveness of breast cancer cells *in vitro* and prevent lung metastasis of melanomas [191, 192], and inhibition of MMP-9 blocked chronic lymphocytic leukemia B-cell invasion through matrigel [193]. These results may be due to the degradation of ECM by MMPs. Nevertheless, MMP-1 was recently found to activate protease activated receptor-1(PAR-1) to promote invasion of breast cancer and melanoma cells [69, 70]. MMP-9 has also been found to display anti-apoptotic effects on tumor cells that are independent of its catalytic activity [194]. These newly discovered functions of MMP-1 and MMP-9 may also be involved in TP-mediated cell invasion.

Moreover, COX-2 and MMP-1 were found to be part of the clinically validated lung metastasis gene signature (LMS) genes. Knockdown of COX-2 and MMP-1/2 genes synergistically inhibited metastasis of breast cancer [195]. We previously demonstrated that I-BOP significantly increased COX-2 expression in A549-TPα cells. The induced

COX-2 produced more prostaglandins and TXA_2 as a result of positive feedback [166]. Taken together, these studies suggest that targeting TP may prevent metastasis of cancer effectively via suppression of both COX-2 and MMP-1 production.

GM6001, a general inhibitor of MMPs, inhibited both basal and I-BOP-induced invasion. These results suggest that MMPs, other than MMP-1 and MMP-9, such as MMP-3 and MMP-10 which are also induced by I-BOP are involved in TP-mediated cell invasion. Elevated levels of these MMPs were previously reported to be critical for cancer invasion and metastasis [196-198].

In summary, our findings indicate that TXA_2-TP mediates cancer cell invasion through upregulation of several MMPs. PKC-, ERK-AP-1 and ERK-C/EBPβ pathways are involved in TP-mediated MMP-1 expression. Figure 44 depicts signaling pathways for TP-mediated expression of MMPs.

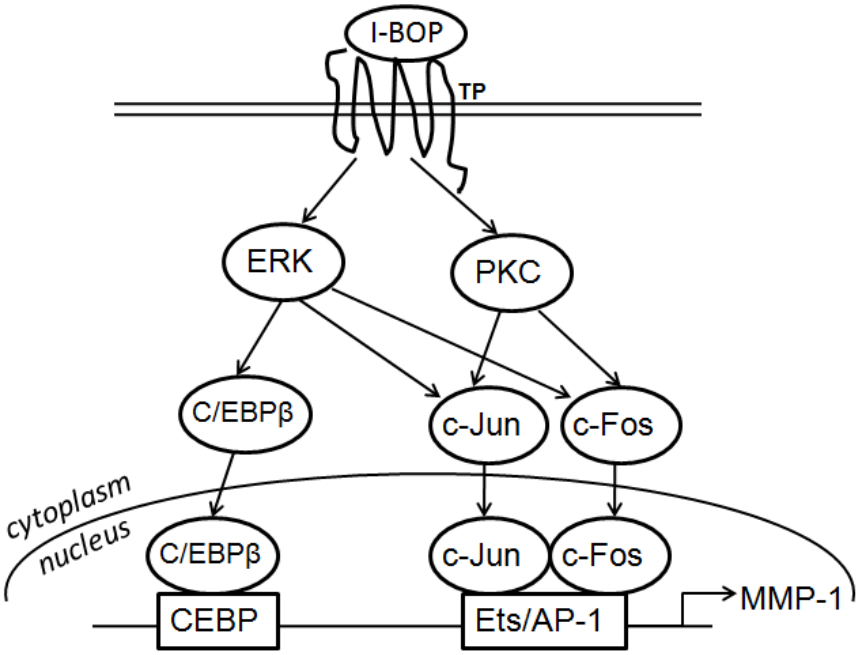

Figure 44. Proposed pathways of TP-mediated MMP-1 expression

5.2 MMP-1-mediated release of MCP-1 by conditioned media from TP agonist-treated A549-TPα cells is through activation of PAR2

MMP-1 was reported to promote melanoma progression through both its collagenase activity and its PAR1 activating function [70]. MMP-1/PAR1 signaling induces a variety of genes which can contribute to melanoma invasion and metastasis. Here, it was demonstrated that the increased MMP-1 could induce the expression of MCP-1, a potential biomarker for tumor invasion and metastasis, by autocrinely acting on cancer cells surface receptor PAR2 in A549 cells.

Except for MMP-1, activation of TPα also induces MMP-3 and MMP-9 expression. MMP-3 and MMP-1 have been reported to induce macrophage MMP-9 expression in TNF-α and COX-2 dependent manner [199]. However, in our system neither MMP-3 nor MMP-9 can stimulate the release of MCP-1 from A549 cells, indicating the specific function of MMP-1 in MCP-1 gene induction. MMP-1 also induces an array of genes expression via PAR1 in endothelial cells in addition to melanoma cells [200]. However, MMP-1 and thrombin, a known PAR1 ligand, induce expression of different subsets of pro-angiogenic genes. PAR1 antagonist, SCH79797 cannot inhibit MMP-1-induced VEGFC, NOTCH4, AKT1 and ANGPT2 genes expression in endothelial cells [200]. Moreover, MMP-1-induced FGFR2, IGF1, SERPINB5 and S100A4 genes expression have not been associated with thrombin/PAR1 signaling in melanoma cells [70]. These studies indicate that MMP-1 may target other receptors than PAR1 to induce these genes expression. Our data showed that antagonist of PAR2, but not that of PAR1 or PAR4, inhibits MMP-1-induced MCP-1 release from A549 cells indicating the involvement of PAR2 in this process. The concentration of SCH79797 we used was 50 nM, which was

sufficient to block PAR1-AP-induced MCP-1 release. The same concentration also effectively blocked MMP-1-induced gene expressions in melanoma cells [70]. This is also supported by studies on MCP-1 induction using different PAR-APs. Although PAR1-AP and PAR4-AP can increase the expression of MCP-1, PAR2-AP induced more significantly MCP-1 release compared with them ($p<0.001$). Furthermore, Asokananthan *et al.* [201] reported that agonist peptides of PAR1, PAR2 and PAR4 but not that of PAR3 stimulated the release of other cytokines IL-6 and IL-8 from A549 cells with a rank order of potency of PAR2>PAR4>PAR1, which is in the same order as that for MCP-1 induction in our studies.

PAR2 has been shown to play an important role in cancer progression. PAR2 mediates tumor or stromal cells-derived proteases to shape tumor microenvironment by inducing various growth factors, pro-angiogenic and immune modulating cytokines [202]. It has been found that PAR2 signaling in breast cancer cells induced an array of pro-angiogenic factors and immune regulators, such as VEGF [102], Cyr61, VEGFC, CTGF, CXCL1, IL8, CSF1 and CSF2 [103]. Although activation of PAR1 also up-regulated some of those factors in breast cancer cells, PAR2 was the major mediator for the induction of immune regulators CXCL1, IL8 and CSF2. MCP-1, another immune regulating cytokine, is often up-regulated together with IL-8 by extracellular stimuli [203, 204]. Thus, PARs-mediated MCP-1 expression may also mainly depend on PAR2 activation.

PAR-APs activate PARs by directly binding with their respective receptors without the cleavage process. Accordingly, the induction of MCP-1 mRNA by SLIGKV-NH_2, a PAR2-AP, was rapid. However, MMP-1 induced a delayed MCP-1 transcription

suggesting that MMP-1 may behave like other proteases by proteolytic unmasking of the tethered ligand first before the activation of PAR2. How MMP-1 cleaves and activates the N-terminus of PAR2 needs to be determined further.

Induction of MCP-1 by thrombin/PAR1 signaling has been observed in different cell types [106, 205, 206]. Similarly, induction of pro-angiogenic gene expressions by thrombin and MMP-1 was found also via PAR1 in human microvessel endothelial cells [200]. However, it appears that thrombin and MMP-1 cleave and activate PAR1 in a different manner. Thrombin cleaves PAR1 at R41-S42 and exposes SFLLRN sequence to activate PAR1, whereas MMP-1 cleaves PAR1 at two different locations (F87-I88 and L44-L45). Cleavage at L44-L45 results in decreasing or abolishing the functional activity of SFLLRN sequence [207]. Consequently, MMP-1 may also cleave and activate other PARs than PAR1. PAR2 appears to be an ideal target of MMP1 since antagonist of PAR2, ENMD-1068, but not that of PAR1 or PAR4 was able to block MMP-1 induced MCP-1 expression.

In conclusion, our studies, for the first time, provide the evidence that MMP-1-induced MCP-1 expression is dependent on PAR2 activation in A549 cells. Although the mechanism of how MMP-1 activates PAR2 remains unknown, we linked TXA_2/TP signaling with PARs signaling in lung cancer cells by TP-mediated MMP-1 release.

5.3 Activation of TP increases MCP-1 expression and recruits macrophages to promote invasion of lung cancer cells

In addition to MMP-1, released by TP activation, can induce MCP-1 expression in A549 cells, TP agonist can directly stimulate the production of MCP-1 in A549-TPα

cells. In this study, we also suggest that MCP-1 may attract macrophages to cancer cells, thereby promoting their invasion.

TXA_2 has been shown to induce MCP-1 expression in vascular endothelial cells and also other cytokines in WISH cells (an amnion epithelium-derived cell line) [208, 209]. In addition, a recent report showed that TXAS inhibitor ozagrel suppressed MCP-1 and IL-8 gene expression and inflammatory cell accumulation in a lung injury model indicating the role of TXA_2 in regulation of these processes [210]. Cancers are considered as chronic wounds never heal. Therefore, they share certain characteristics. Several CC chemokines including MCP-1 were found to be highly expressed in human NSCLC tumor tissues and the levels of MCP-1 also significantly correlated to macrophage infiltration [138]. These findings led to the hypothesis that TXA_2 might be involved in lung cancer development via MCP-1-mediated macrophage recruitment. Especially compared to other prostanoids, TXA_2 agonist induced the most expression of MCP-1 in both H460 and A549-TPα cells indicating a significant contribution of TXA2-TP axis to MCP-1 production.

Mechanisms of regulation of MCP-1 by I-BOP were further studied. There are several cis-acting transcriptional regulatory elements including NF-κB, AP1 and SP1 sites in the promoter region of human MCP-1 gene (Figure 8). Expression of MCP-1 induced by IL-1β was regulated by PKC-dependent NF-κB activation in smooth muscle cells [118]. U46619, another TP agonist, was also reported to induce MCP-1 expression via PKC-dependent induction of NF-κB and AP-1 binding activity in vascular endothelial cells [208]. Activation of PKC, NF-κB and AP-1 by TP-mediated G protein signaling has been found in several other cell types including A549 cells [166]. However, in this study,

neither PKC inhibitor GF109203X nor NF-κB activation inhibitor MG132 had any effect on I-BOP-induced expression of MCP-1. As presented in **4.2**, I-BOP-induced MMP-1 expression was mediated by PKC and ERK-dependent AP-1 activation in the same cell lines. Nevertheless, inhibition of ERK did not block I-BOP-induced MCP-1 expression either, suggesting AP-1 was not involved in MCP-1 regulation by I-BOP in A549-TPα cells. These data indicated that separate signaling pathways were involved in the expression of downstream targets induced by I-BOP activation of TP. It is also possible that MCP-1 regulation is tissue-specific, and NF-κB and AP-1 are not important for MCP-1 expression in lung epithelial cancer cells. A separate study in fibroblast 3T3 cells showing that the expression of MMP-1 induced by growth factors was c-Fos-dependent, whereas that of MCP-1 was not provides support to our proposition. Moreover, it has been reported that PDGF induction of MCP-1 transcription requires SP1 but not NF-κB and AP-1 response elements in its promoter region in fibroblast [115]. Therefore, we propose that in cancer cells I-BOP may regulate MCP-1 expression in a way similar to how PDGF does in fibroblast. We observed that SP1 was increased in nuclei after I-BOP stimulation and SP1 inhibitor blocked I-BOP-induced MCP-1 expression. Furthermore, using GC-rich promoter region of 12(S)-lipoxygenase as a probe, Chang and his colleagues identified that Hsp90 interacts with SP1 and modulates its promoter binding ability [177]. In the present study, indirect inhibition of SP1 by Hsp90 inhibitor geldanamycin blocked I-BOP-induced MCP-1 expression at protein as well as mRNA levels. Moreover, the results obtained from MCP-1 promoter-luciferase reporter assay further indicate the critical role of SP1 binding site in MCP-1 regulation by I-BOP. Although the upstream signals by which TP regulates SP1 expression and translocation

need further studies, the results presented here demonstrated that I-BOP induced MCP-1 expression in A549-TPα cells by SP1-dependent but NF-κB and AP-1-independent mechanism.

As mentioned in **1.8.1**, macrophages have been classified into M1 and M2 two major groups. M1 macrophages inhibit tumor progression, whereas M2 macrophages promote tumor progression. Both M1 and M2 macrophages could be present in tumor islet. Here, we employed RAW264.7 macrophages to study the interactions between lung cancer cells and macrophages. RAW 264.7, a murine macrophage cell line, can be polarized into either M1 or M2 macrophages [211]. Interactions of RAW 264.7 macrophages with human cancer cells such as lymphoma and breast cancer cells have been reported in previous studies [212, 213]. Furthermore, human MCP-1 and mouse MCP-1 which is truncated at the C terminus are highly homologous. Indeed, human MCP-1 even has a higher chemotactic potency to murine monocytes than full length of murine MCP-1 due to the less glycosylation of human MCP-1 C terminus [214]. Therefore, RAW 264.7 macrophages can serve as an appropriate cell line to study TP-mediated interactions between lung cancer cells and macrophages.

Macrophages contribute to various aspects of cancer progression such as cancer cell proliferation, survival, invasion, metastasis and angiogenesis. In this study, co-culture of macrophages with A549 cells stimulated gene expressions of several MMPs and VEGF by A549 cells. It was reported that A549-TPα cells induced tumor formation and angiogenesis in nude mice through induction of VEGF expression [37]. In **3.2** we also showed that I-BOP-induced invasion of A549-TPα cells was mediated by increased expression of several MMPs such as MMP-1 and MMP-9. Here, macrophages induced

these genes, and thus enhanced the effects of TP-mediated angiogenesis and invasion of cancer cells. MCP-1 was also induced in A549 cells by co-culture with macrophages forming a positive feedback loop that might reinforce the recruitment of more macrophages to promote cancer cell invasion.

Tumors are very heterogeneous and contain numerous subpopulations of cells [215] that may express different levels of TP. We show here that once TXA_2 initiates a signaling cascade leading to the release of MCP-1 from tumor cells expressing high levels of TP, the subsequent recruited macrophages could increase the invasion of tumor cells regardless of their TP levels. Therefore, TP expands its influence on tumor progression through MCP-1-mediated macrophage recruitment.

In summary, TP-mediated interactions and relationships among MMP-1, PAR2 and MCP-1, as well as macrophages leading to an increased invasive potential of carcinoma cells is illustrated in Figure 45.

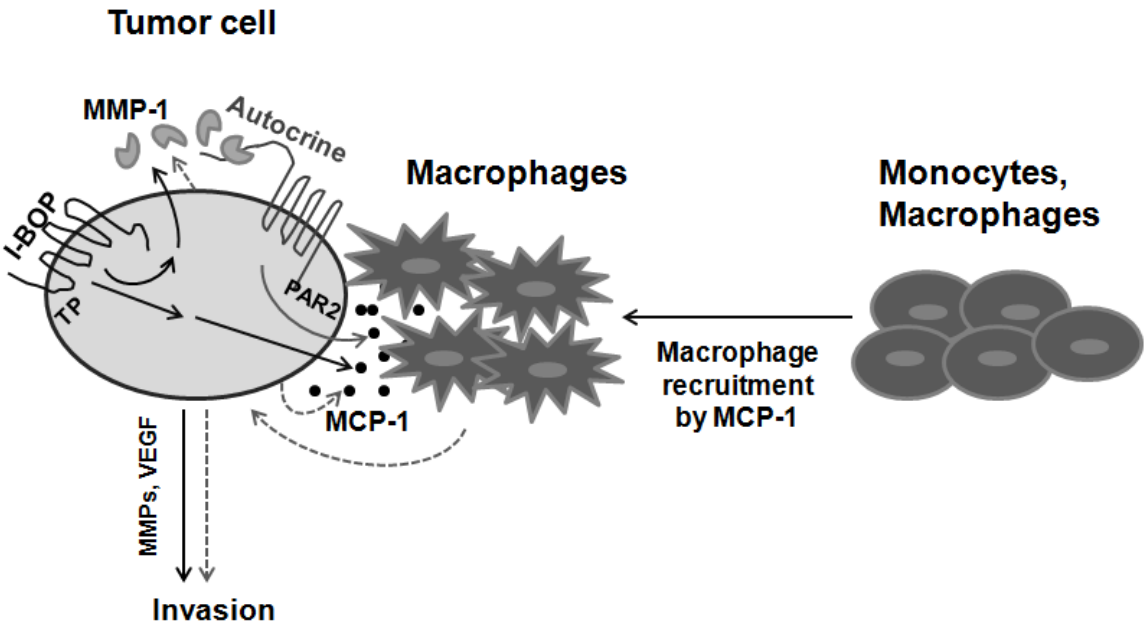

Figure 45. Illustration of a tentative mechanism for TP-mediated invasion of cancer cells

Black arrows (→) represent pathways mediated by direct activation of TP. Gray arrow (→) represents pathway mediated by PAR2 activation. Dashed arrows (⇢) represent pathways activated by macrophages.

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6. Summary and conclusions

In this dissertation, I investigated the cellular and molecular mechanisms by which thromboxane A2 receptor-mediated cancer cell invasion. The findings of this dissertation research are summarized below:

(1) Using human lung adenocarcinoma cells A549 overexpressing TPα or TPβ as a model system, we found that activation of either isoform of TP decreased the expression of cell-cell adhesion molecule E -cadherin, changed epithelial cell morphology to scattered spindle-shaped morphology, and induced invasion in these cells.

(2) Further studies revealed that activation of TPs induced expression of several MMPs including MMP-1, MMP-3, MMP-9, and MMP-10. MMPs general inhibitor and siRNA of MMP-1 and MMP-9 blocked TP agonist-induced invasion of A549-TPα cells indicating the positive correlation of MMPs to TP-mediated cancer cell invasion. We also identified several key signal transduction pathways involved in MMP-1 gene regulation including ERK-AP1, PKC-AP1, and ERK-C/EBPβ pathways.

(3) PAR2 was identified as a novel target of MMP-1 which induced MCP-1 expression in A549 cells. Conditioned media from I-BOP-treated A549-TPα cells (CM-I-BOP) also induced MCP-1 expression via MMP-1. PAR2 antagonist and siRNA blocked both MMP-1 and CM-I-BOP-induced MCP-1 expression.

(4) Activation of TP could directly induce the release of MCP-1via SP1 dependent pathway. MCP-1 recruited macrophages which in turn stimulated the expression of several metastatic genes including MMPs and VEGF, therefore, enhanced the

invasion of A549 cells. Macrophages also induced a further generation of MCP-1 by A549 providing a positive feedback loop to recruit more macrophages to enhance cancer cell invasion.

(5) Finally, the rapid expression of MCP-1 induced by activation of TPα is followed by delayed expression of MCP-1 either induced by MMP-1or macrophages to ensure continued supply of chemotactic MCP-1 for recruitment of macrophages and for promotion of invasion of cancer cells.

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7. References

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