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จุฬาลงกรณ์มหาวิทยาลัย

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IRON REGULATES MMP-9 EXPRESSION IN ORAL SQUAMOUS CARCINOMA CELL LINES

Mrs. Ruchadaporn Kaomongkolgit

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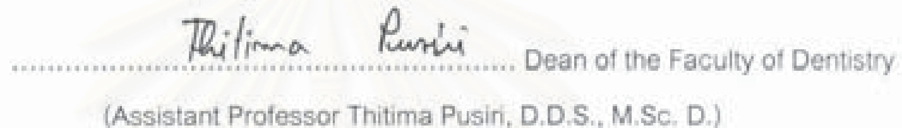
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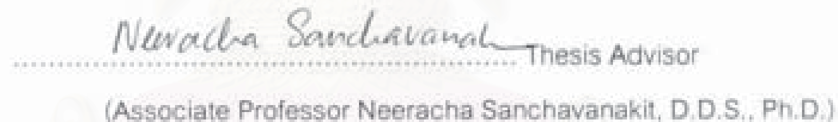
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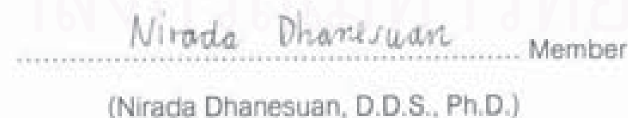
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รัชดาภรณ์ เค้ามงคลกิจ : ผลของเหล็กต่อการควบคุมการแสดงออกของเอ็มเอ็มพี-9 ในเซลล์มะเร็งช่องปากชนิดสแควมัสเซลล์คาร์ซิโนมา (IRON REGULATES MMP-9 EXPRESSION IN ORAL SQUAMOUS CARCINOMA CELL LINES) อ. ที่ปรึกษา: รศ.ทญ.ดร. นีรชา สารชวนะกิจ, อ. ที่ปรึกษาร่วม: ผศ.ดร. พูลลาภ ชีพสุนทร, 135 หน้า.

มะเร็งชนิดสแควมัสเซลล์คาร์ซิโนมาที่พบบริเวณศีรษะและลำคอเป็นมะเร็งที่พบว่ามีอัตราการลุกลามและการแพร่กระจายออกไปยังอวัยวะอื่นๆสูง เป็นสาเหตุของการเกิดความพิการและการตายที่พบได้ทั่วโลก ระดับการแสดงออกที่เพิ่มขึ้นของเอมไซม์เมทริกซ์เมทอลโลโปรทิเนส (เอ็มเอ็มพี)-9 พบว่าเกี่ยวข้องกับการลุก-ลามและการแพร่กระจายของมะเร็งชนิดสแควมัสเซลล์คาร์ซิโนมาที่พบบริเวณศีรษะและลำคอ นอกจากนี้มีรายงานการวิจัยเพิ่มมากขึ้นที่แสดงถึงความสัมพันธ์ระหว่างสภาวะที่มีเหล็กสะสมมากเกินกับการดำเนินไปของโรคมะเร็ง แต่อย่างไรก็ตามยังไม่มีการศึกษาถึงบทบาทของเหล็กต่อการควบคุมการแสดงออกของเอ็ม-เอ็มพี-9 ในมะเร็งชนิดสแควมัสเซลล์คาร์ซิโนมาที่พบบริเวณศีรษะและลำคอ ดังนั้นการวิจัยนี้จึงได้ศึกษาผลของเหล็กต่อการแสดงออกของเอ็มเอ็มพี-9 ในเซลล์มะเร็งชนิดสแควมัสเซลล์คาร์ซิโนมาที่พบบริเวณศีรษะและลำคอได้แก่ โอเอ็ม-2 และเอชเอ็น-22 โดยใช้เหล็กในรูปของเฟอริคแอมโมเนียมซิเตรต ผลการวิเคราะห์โดยใช้เทคนิครีเวิสทรานคริปชั่นโพลีเมอร์เรสเชนรีเอชั่นและเจลาตินไซม์โมกราฟีพบว่า เฟอริคแอมโมเนียมซิเตรตที่ความเข้มข้น 15 ไมโครกรัมต่อมิลลิลิตร เพิ่มการแสดงออกของเอ็มเอ็มพี-9 ในเซลล์ไลน์ทั้งสองชนิดในลักษณะที่สัมพันธ์กับความเข้มข้นที่มากขึ้น ผลการศึกษาจากการใช้สารยับยั้งที่มีความจำเพาะต่อโปรทีนเอิร์ค1/2 และเอเคที แสดงให้เห็นว่าเหล็กควบคุมการแสดงออกของเอ็มเอ็มพี-9 โดยผ่านทางการกระตุ้นเอิร์ค1/2 และเอเคที โดยเอิร์ค1/2 เป็นโปรทีนที่ถูกกระตุ้นก่อนเอเคที การวิเคราะห์โดยใช้เทคนิคอิเล็คโตรโฟเรติกมอบิลิตีชิฟพบว่าเหล็กเหนี่ยวนำการแสดงออกของเอ็มเอ็มพี-9 โดยการกระตุ้นแอคทิเวเทคโปรทีน-1 เนื่องจากการศึกษาโดยใช้แอนติบอดี้ที่มีความจำเพาะต่อทรานเฟอรินรีเซพเตอร์-1 พบว่าไม่สามารถยับยั้งผลของเฟอริคแอมโมเนียมซิ-เตรตในการกระตุ้นเอ็มเอ็มพี-9 แสดงว่าเซลล์รับรู้เหล็กโดยผ่านทางตัวรับสัญญาณที่ไม่ใช้ทรานเฟอรินรีเซพ-เตอร์ โดยสรุป งานวิจัยนี้เป็นการศึกษาแรกที่แสดงให้เห็นว่าเหล็กเพิ่มการแสดงออกของเอ็มเอ็มพี-9 ในเซลล์มะเร็งชนิดสแควมัสเซลล์คาร์ซิโนมาที่พบบริเวณศีรษะและลำคอ โดยเหล็กอาจจะเป็นอีกหนึ่งในหลายปัจจัยที่มีบทบาทในการเพิ่มการแสดงออกของเอ็มเอ็มพี-9 ซึ่งพบว่ามีความสำคัญในการพัฒนาและดำเนินไปของโรคมะเร็งชนิดสแควมัสเซลล์คาร์ซิโนมาที่พบบริเวณศีรษะและลำคอ

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RUCHADAPORN KAOMONGKOLGIT: IRON REGULATES MMP-9 EXPRESSION IN ORAL SQUAMOUS CARCINOMA CELL LINES. THESIS ADVISOR: ASSOC. PROF. NEERACHA SANCHAVANAKIT, D.D.S., Ph.D., THESIS CO-ADVISOR: ASST. PROF. POONLARP CHEEPSUNTHORN, B.Sc., M.S., Ph.D., 135 pp.

Head and neck squamous cell carcinoma (HNSCC) is a highly invasive cancer with distant metastasis and a cause of great morbidity and mortality worldwide. Over-expression of matrix metalloproteinase (MMP)-9 is implicated in the invasion and metastasis of HNSCC. There are increasing evidence of an association between iron overload and cancer progression. However, the effect of iron on MMP-9 expression in HNSCC has not been studied. In the present study, we examined the effect of iron on MMP-9 expression in HNSCC cell lines, OM-2 and HN-22. Ferric ammonium citrate (FAC), a source of iron, at 15 μg/ml increased MMP-9 expression in both cell lines in a dose dependent manner as shown by reverse transcription polymerase chain reaction and gelatin zymography analyses. Studies using specific inhibitors of extracellular signal-regulated kinase (ERK) 1/2 and Akt demonstrated that iron regulated MMP-9 through ERK1/2 and Akt, and ERK1/2 was an upstream activator of Akt. Analysis of electrophoretic mobility shift assay revealed that iron induced MMP-9 expression by activation of activated protein-1 (AP-1). Since, application of neutralizing antibody against transferrin receptor-1 could not abolish the stimulated MMP-9 expression, this result suggested that iron uptake was non-transferrin dependent. In conclusion, this study was the first to demonstrate that MMP-9 was up-regulated by iron in HNSCC cell lines. We suggest that iron may be one of several factors that cause an increase of MMP-9 which is necessary for the development and progression of HNSCC.

Field of study Oral Biology

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TABLE OF CONTENTS

Title Page (Thai).. i

Title Page (English).. ii

Abstract (Thai).. iv

Abstract (English).. v

Acknowledgements.. vi

Table of Contents... vii

List of Tables x

List of Figures.. xi

List of Abbreviations.. xiii

CHAPTER I: INTRODUCTION.. 1

CHAPTER II: REVIEW OF THE LITERATURE.. 5

TUMOR PROGRESSION.. 5

MATRIX METALLOPROTEINASES.. 7

MMP classification... 8

Structure of MMPs.. 9

Regulation of MMPs... 13

1. Transcriptional regulation of MMPs.. 13

2. Activation of latent MMPs... 13

3. Inhibition of active MMPs.. 17

MATRIX METALLOPROTEINASE-9... 19

Structure of MMP-9... 20

Regulation of MMP-9... 23

1. Transcriptional regulation of MMP-9... 23

2. Activation of latent MMP-9... 31

3. Inhibition of active MMP-9.. 35

MMP-9 and head and neck squamous cell carcinoma........................ 37

GENERAL OVERVIEW OF IRON UPTAKE AND METABOLISM…………………. 40

Iron distribution and homeostasis in the body………………………….... 41

Iron absorption and transport…………………………………………….... 43

Cellular uptake of iron……………………………………………………….47

Cellular iron storage and export……………………………………………52

Regulation of cellular iron metabolism……………………………………..53

IRON AND MMPs……………………………………………………………….. 57

IRON AND CANCER…………………………………………………………… 58

CHAPTER III: MATERIALS AND METHODS……………………………………………62

Cell culture and cell treatment……………………………………………….. 62

Cytotoxicity test (MTT assay)…………………………………………………. 63

Gelatin zymography…………………………………………………………... 64

Enzyme-Linked Immunosorbent Assay (ELISA)……………………………… 66

RNA extraction………………………………………………………………… 66

Reverse transcription polymerase chain reaction (RT-PCR)……………….. 67

Western blot analysis…………………………………………………………. 68

Nuclear protein extraction…………………………………………………….. 69

Electrophoretic mobility shift assay (EMSA)………………………………….. 70

Statistic analysis……………………………………………………………….. 71

CHAPTER IV: RESULTS……………………………………………………………………72

FAC increased MMP-9 expression in OM-2 and HN-22 cells…………………72

The effect of FAC on the expression levels of Tf, TfR1, and DMT-1…..………77

HN-22 cells responded to FAC using a non-transferrin dependent pathway…. 78

FAC regulated MMP-9 expression through ERK1/2 and PI3K/Akt signaling pathways………………………………………………………………………… 84

ERK1/2 activation is up-stream regulation of Akt in FAC increased MMP-9 expression……………………………………………………………………….. 89

FAC regulated AP-1 DNA binding activity………………………………………… 92

CHAPTER V: DISCUSSION AND CONCLUSION……………………………………..... 96

REFERENCES………………………………………………………………………………106

VITA………………………………………………………………………………………..135

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

Table 1 Classes of MMPs and MMP nomenclature.. 9

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figure 2.1 Matrix metalloproteinases (MMPs) in tumor progression....... 7

Figure 2.2 Domain structure of MMPs... 12

Figure 2.3 Stepwise activation of MMPs.. 16

Figure 2.4 The schematic structure of MMP-9...................................22

Figure 2.5 Comparison of the 3D structures of MMP-2 and MMP-9.....................22

Figure 2.6 Potential regulatory elements in the promoter region of the human MMP-9 gene..23

Figure 2.7 General schematic representation of signaling pathways regulating MMP-9 expression.. 29

Figure 2.8 Positive and negative regulators of MMP-9 expression.................. 31

Figure 2.9 The activation network of MMP-9 by MMPs................ 34

Figure 2.10 MMP cascade of zymogen activation in pro-MMP-9 activation......... 35

Figure 2.11 Iron distribution in the adult human body................................ 43

Figure 2.12 The cellular mechanisms of intestinal iron absorption.......................44

Figure 2.13 Regulation of cellular iron uptake...49

Figure 2.14 Consensus sequence and representation of the stem-loop structure of a human iron responsive element (IRE).. 55

Figure 2.15 Molecular regulations of iron-responsive mRNAs............................ 56

Figure 4.1 The cytotoxicity of FAC on HN-22 and OM-2 cells.............................73

Figure 4.2 FAC increased MMP-9 expression in OM-2 and HN-22 cells...................74

Figure 4.3 FAC increased MMP-9 expression in OM-2 and HN-22 cells analyzed by gelatin zymography and RT-PCR 75

Figure 4.4 Effect of FAC on TIMP-1 expression in HN-22 cells analyzed by ELISA.... 76

Figure 4.5 Effect of FAC on Tf, TfR1 and DMT1 expressions in HN-22 cells………... 77

Figure 4.6 The effect of holo-transferrin and apo-transferrin on viability of HN-22 cells…………………………………………………………………... 80

Figure 4.7 The effect of neutralizing antibody against human transferrin receptor on viability of HN-22 cells………………………………………………... 81

Figure 4.8 The effect of holo-transferrin and apo-transferrin on MMP-9 expression in HN-22 cells……………………………………………………………..... 82

Figure 4.9 HN-22 cells responded to FAC by using non-transferrin dependent pathway…………………………………………………………………... 83

Figure 4.10 FAC increased MMP-9 expression via ERK1/2 signaling pathway…......... 85

Figure 4.11 FAC increased MMP-9 expression via PI3K/Akt signaling pathway……... 87

Figure 4.12 Activation of ERK1/2 and Akt on MMP-9 expression by FAC………….... 90

Figure 4.13 FAC was not mediated MMP-9 expression by enhancing NF-κB binding in HN-22 cells……………………………………………………....93

Figure 4.14 Enhanced AP-1-binding-mediated MMP-9 expression by FAC in HN-22 cells……………………………………………………………….94

Figure 5.1 Schematic representation of signal pathway in HNSCC in response to FAC………………………………………………………………………104

LIST OF ABBREVIATIONS

ABCG2	ATP-binding cassette protein, subfamily G, member 2
AP-1	activator protein-1
AP-2	activator protein-2
α2M	alpha-2-macroglobulin
APMA	4-aminophenylmercuric acetate
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
Ca^{2+}	calcium ion
cDNA	complementary DNA
Cp	ceruloplasmin
CTAP-III	connective tissue activating peptide-III
DCT-1	divalent cation transporter-1
Dcytb	duodenal cytochrome B
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxane
DMT-1	divalent metal transporter-1
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
ETS	erythroblastosis twenty six
FAC	ferric ammonium citrate
FBS	fetal bovine serum
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
Fe-NTA	ferric nitrilotriacetate
FLVCR	feline leukaemia virus C receptor protein
FPN-1	ferropontin-1

สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย

GAPDH	glyceraldehyde 3 phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GRO-α	growth-related oncogene-alpha
HCP-1	heme carrier protein-1
Hp	ferroxidase hephaestin
$HgCl_2$	mercury chloride
HGF	hepatocyte growth factor
HH	hereditary hemochromatosis
HIF-1	hypoxia-inducible factor-1
HNSCC	head and neck squamous cell carcinoma
HO-1	heme oxygenase-1
ICAM-1	intercellular adhesion molecule-1
IFN-α	interferon-alpha
Ig	immunoglobulin
IMP	integrin mobiferrin pathway
IL-1	interleukin-1
IL-2Rα	interleukin-2 receptor alpha
IRE	iron responsive element
IRF-1	interferon regulatory factor-1
IREG-1	iron regulated gene-1
IRP	iron regulatory protein
ISRE	interferon-stimulated regulatory element
JNK	c-Jun N-terminal kinase
KiSS-1	metastin or human malignant melanoma metastasis suppressor
KRE-M9	keratinocyte differentiation factor-1 regulatory element
LIP	labile iron pool
LPS	lipopolysaccharide
LRP	low-density lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
MEF	myocyte enhancer factor
MMPs	matrix metalloproteinases

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

MT-MMPs — membrane-type- matrix metalloproteinases

MTP-1 — metal transporter protein-1

NGAL — neutral gelatinase-associated lipocalin

Nramp2 — natural resistance associated macrophage protein-2

NF-κB — nuclear factor-kappa B

NIP — nuclear inhibition protein

NTBI — non transferrin–bound iron

$OH^{\cdot}$ — hydroxyl radical

PAF — platelet-activating factor

PAGE — polyacrylamide gel electrophoresis

PBS — phosphate buffered saline

PDGF — platelet-derived growth factor

PEA-3 — polyoma enhancer activator -3

PF-4 — platelet factor-4

PI3K — phosphatidyl inositol 3-kinase

PKC — protein kinase C

PMA — phorbol 12-myristate 13-acetate

PVDF — polyvinylidene fluoride

Ref-1 — redox factor-1

RECK — reversion-inducing-cysteine-rich protein with Kazal motifs

RGD — Arg-Gly-Asp

RME — receptor-mediated endocytosis

ROS — reactive oxygen species

RT-PCR — reverse transcription polymerase chain reaction

SCC — squamous cell carcinoma

SDS — sodium dodecylsulphate

SFM — serum free medium

SP-1 — stimulating protein-1

STAT-1 — signal transducer and activator of transcription-1

TBE — tris borate-EDTA

TGF-β — transforming growth factor-beta

สถาบันวิทยบริการ
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Tf	transferrin
TfR	transferrin receptor
TIE	transforming growth factor-β inhibitory element
TIMPs	tissue inhibitor of matrix metalloproteinases
TFPI-2	tissue factor protease inhibitor-2
TPA	12-*O*-tetradecanoylphorbol-13-acetate
tPA	tissue plasminogen activator
TNF-α	tumor necrosis factor-alpha
uPA	urokinase plasminogen activator
UTR	untranslated region
VEGF	vascular endothelial growth factor
Zn^{2+}	zinc ion

CHAPTER I
INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy and causes morbidity and mortality worldwide. In the western world, HNSCC represents 5% of newly diagnosed cancers, whereas it accounts for about 40% of all malignancies in Southeast Asia (Parkin et al., 2005). HNSCC is characterized by local invasion, lymphatic dissemination, and distant metastasis in 30-40% of cases (Vokes et al., 1993). Despite improvements in diagnosis and treatments of cancer, the biological factors and mechanisms underlying dissemination of HNSCC have not yet been fully elucidated. Therefore, overall prognosis of patients remains poor (Massano et al., 2006).

Many proteases are involved in the progression of cancer. Matrix metalloproteinases (MMPs) are the principal group of extracellular matrix (ECM)-degrading enzymes, responsible for the degradation of almost all components of the ECM (Visse and Nagase, 2003). In particular, over-expression and activation of MMP-9 (Gelatinases B, 92-kDa type IV collagenase) are strongly associated with tumor aggressiveness and decrease survival of patients with HNSCC (Riedal et al., 2000; O-Charoenrat et al., 2001). Additional studies have shown predominant up-regulation of MMP-9 at the invasive front (Ondruschka et al., 2002). Initially, MMP-9 was believed to facilitate the breakdown of type IV collagen, the major component of basement membrane, thus aiding tumor invasion. However, recent evidence suggested that MMP-9 regulates bioavailability and activities of various growth factors and cytokines which affect the immune response and angiogenesis, resulting in

growth initiation and maintenance of primary and metastatic tumors (Chambers and Matrisian, 1997). The regulation of MMP-9 has been shown to depend upon a large number of cytokines, growth factors, and other stimuli including iron (Mook el al., 2004). Iron has been reported to induce MMP-9 secretion in human skin fibroblasts (Campo et al, 2006), mouse macrophages in developing atherosclerosis (Lee et al., 2003), and serum of patients with severe chronic venous disease (Zamboni et al., 2005), resulting in matrix degradation. However, no evidence exists regarding regulation of MMP-9 expression in cancer.

Iron is an essential element for fundamental biological processes, since iron-containing enzymes catalyze a number of reactions such as energy metabolism, cell respiration, DNA synthesis, and regulation of cell growth and differentiation. On the other hand, iron exerts its toxicity through a series of reactions with reactive oxygen species (ROS) by generating the highly toxic hydroxyl radicals ($OH^{\cdot}$), which cause DNA damage and various pathological conditions including cancer (Toyokuni, 1996). Iron contributes to cancer development, not only as a cancer initiator but also as a cancer promoter, by facilitating cancer cell growth and suppressing the host defense activities. So living organisms develop strict regulatory processes for iron metabolism to prevent iron overload condition (Van et al., 1984; Le and Richardson, 2002). Various types of tumors have been reported to readily take up iron as seen by the higher iron content in pre-malignant and malignant tissues of colorectal, liver and laryngeal cancers than that in normal tissues (Bonkovsky, 1991; Durak et al., 1994; Labropoulou et al., 2004). Interestingly, there was a report of a positive correlation of serum ferritin, which represented the total body iron, with

advanced stage of head and neck cancer (Maxim and Veltri, 1986). In addition, patients with iron overload condition from genetic diseases such as hemochromatosis and neonatal β-thalassemia, were reported to contain higher iron accumulation in the tissues of head and neck region including oral mucosa, salivary glands, and oropharyngeal tissues (Vaiopoulos et al., 1995; Smith et al., 2004). Since excess iron could support cancer progression, high level of iron, either from genetic or acquired conditions such as blood transfusion, anemia and chronic liver disease, may increase the rate of cancer progression. Therefore, the present study aims to investigate the modulation and the molecular mechanism of MMP-9 regulation by iron in head and neck squamous carcinoma cells.

Hypothesis

Iron up-regulates MMP-9 expression in HNSCC cell lines.

Specific Aims

1. To investigate the effect of iron on MMP-9 expression in HNSCC cell lines.

2. To elucidate the molecular mechanism that iron modulates MMP-9 expression in HNSCC cell lines.

CHAPTER II
REVIEW OF THE LITERATURE

TUMOR PROGRESSION

Tumor progression is a complex, multistage process by which normal cells undergo genetic changes that result in phenotypic alterations and the acquisition of the ability to metastasis by which a subset or individual cancer cell disseminates from a primary tumor to distant secondary organs or tissues. Tumor cells fulfill their metastatic potential, which allow them to escape from the primary tumor, migrate and invade surrounding tissues, enter the vasculature, circulate and reach the secondary sites, extravasate, and establish metastatic foci (Hanahan and Weinberg, 2000; Chambers et al., 2002; Pantel and Brakenhoff, 2004). During metastasis, tumor cells are involved in numerous interactions with the ECM, growth factors and cytokines associated with the ECM, basement membranes, and the microenvironment of the secondary site. Although many factors regulate malignant tumor growth and spread, interactions between a tumor and its surrounding microenvironment result in the production of important protein products that are crucial to each step of tumor progression (Pantel and Brakenhoff, 2004). Several regulatory pathways are either altered or aberrantly expressed to render tumor cells the ability to successfully accomplish each and all steps of the tumor progression (Deryugina and Quigley, 2006).

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Various types of proteases have been found to participate directly or indirectly in the ECM turnover during the metastatic process such as serine, cysteine, MMPs and aspartic proteases (Zucker et al., 2000). However, MMPs are the principal ECM-degrading enzymes, MMPs are able to degrade almost all of the component of ECM, including the basement membrane. Basement membrane composes of type IV collagen, laminin, entactin, and proteoglycans, and serves as a barrier between epithelium and connective tissue compartments (Yurchenco and Schittny, 1990). Disruption of basement membrane integrity, a feature of invasive tumors, allows tumors to spread locally and distantly. It was initially believed that MMPs, via breakdown of the physical barrier, were primarily involved in tumor invasion, entry and exit of tumor cells from the circulation, and local migration at metastatic sites. There are growing evidence showing that MMPs have expanded roles. They are important for the creation and maintenance of a microenvironment that facilitates growth and angiogenesis of tumors at primary and metastatic sites by regulating access to growth factors and cytokines from ECM surrounding the growing tumor, either directly or via proteolytic cascade (Figure 2.1) (Chambers and Matrisian, 1997; Nelson et al., 2000).

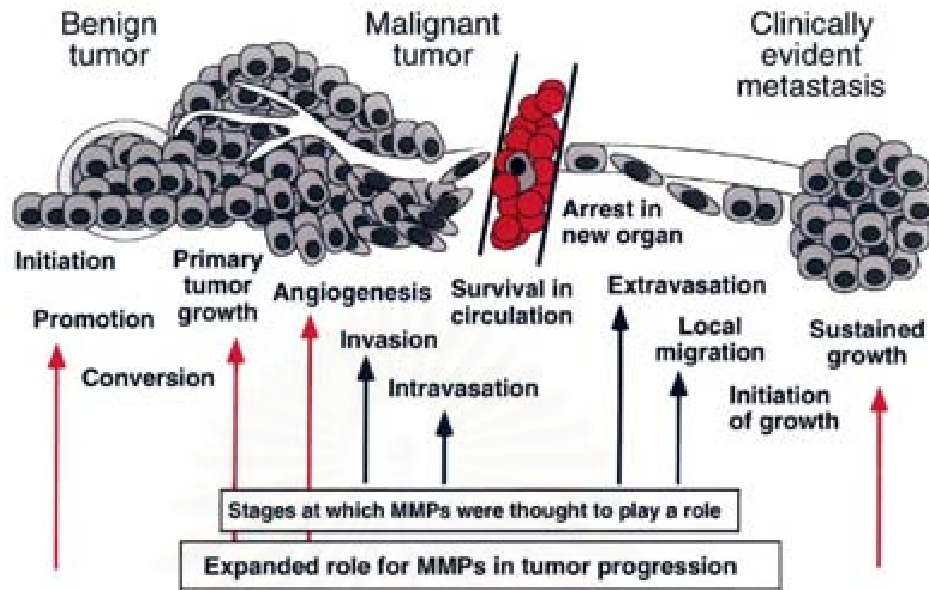

Figure 2.1 Matrix metalloproteinases (MMPs) in tumor progression. The cellular changes that occur as normal cells become benign, malignant, and metastatic tumor are depicted (Nelson et al., 2000).

MATRIX METALLOPROTEINASES (MMPS)

MMPs are a family of enzymes with 28 members identified to date. All are extracellular (predominantly secreted pericellularly, and some membrane-bound) endopeptidases requiring zinc ion (Zn^{2+}) for their activities. The first discovered MMP was a collagenase-1 (MMP-1) in the tadpole undergoing metamorphosis in 1962 (Gross and Lapiere, 1962). Major functions of MMPs are degradation and remodeling of all components of ECM (Chambers and Matrisian, 1997). Moreover, MMPs play a vital role in the control of signals by matrix molecules, which regulate cell proliferation, differentiation,

and cell death. Physiologically, these enzymes play a role in normal tissue remodeling events such as embryonic development, organ morphogenesis, angiogenesis, ovulation, hair follicle growth, mammary gland involution, bone remodeling, infection, inflammation and wound healing (Chamber and Matrisian, 1997). On the other hand, their expressions appear to contribute to various pathological processes including rheumatoid arthritis, osteoarthritis, pulmonary emphysema, atherosclerosis, neurological diseases, and tumor progression and metastasis (Chambers and Matrisian, 1997; Nagase and Woessner, 1999; Parks and Shapiro, 2001). MMPs are produced by a variety of cells, including neutrophils, macrophages, chondrocytes, endothelial cells, epithelial cells, fibroblasts, and various types of cancer cells (Parks and Shapiro, 2001).

1. MMP classification

MMPs have been categorized in four subgroups on the basis of their substrate specificity: collagenases, gelatinases, stromelysins, and matrilysins. As the list of MMPs is growing, a numbering system is adapted and MMPs are classified now according to their structure and substrate specificity. Eight structural classes are recognized, five classes of MMPs being secreted and three being membrane-type MMPs (MT-MMPs) (Tabel1 and Figure 2.2) (Mook et al., 2004; Visse and Nagase, 2003).

Tabel 1 Classes of MMPs and MMP nomenclature

Structural class	Names and synonyms	MMP nomenclature
Secreted MMPs		
Minimal domain	**Matrilysin**, matrin, PUMPI, small uterine metalloproteinase	MMP-7
	Matrilysin 2, endometase	MMP-26
Simple hemopexin domain	**Collagenase-1**, interstitial collagenase, fibroblast collagenase, tissue collagenase	MMP-1
	Collagenase-2, neutrophil collagenase, PMN collagenase, granulocyte collagenase	MMP-8
	Collagenase-3	MMP-13
	Stromelysin 1, transin-1, proteoglycanase, pro-collagenase activating protein	MMP-3
	Stromelysin 2, transin-2	MMP-10
	Metalloelastase, macrophage elastase, macrophage metalloelastase	MMP-12
	Collagenase-4 (no human homologue)	MMP-18
	RASI-1, MMP-18[a]	MMP-19
	Enamelysin	MMP-20
	None	MMP-27
Furin activated and secreted	**Stromelysin 3**	MMP-11
	Epilysin	MMP-28
Gelatin-binding	**Gelatinase A**, 72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase	MMP-2
	Gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase	MMP-9
Vitronectin-like insert	Homologue of *Xenopus* XMMP	MMP-21
MT-MMPs		
Transmembrace	MT1-MMP	MMP-14
	MT2-MMP	MMP-15
	MT3-MMP	MMP-16
	MT5-MMP	MMP-24
GPI-linked	MT4-MMP	MMP-17
	MT6-MMP	MMP-25
Type II Transmembrance	Cysteine arry MMP (CA-MMP), femalysin, MIFR, MMP-21/MMP-22	MMP-23

[a] When MMP-19 was cloned it was called MMP-18. However, an MMP from Xenopus had already received that name and therefore this MMP is now calling MMP-19 (Mook et al., 2004).

2. Structure of MMPs

The primary structure of MMPs contains three common structurally well-preserved domain motifs: signal peptide domain, propeptide domain, and catalytic domain (Figure 2.2) (Visse and Nagase, 2003; Das et al., 2003; Klein et al., 2004). All MMPs contain signal peptide domain or secreting leader sequence that is necessary to signal secretion (John and Tuszynski, 2001). The propeptide domain (amino-terminal) is about 80 amino acids length and responsible for the latency of the enzyme. The propeptide domain contains a

unique highly conserved PRCGVPDV sequence whose proteolytic cleavage is required for enzyme activation (Woessner, 1994; Nagase and Woessner, 1999). The cysteine residue in this sequence interacts with the catalytic zinc atom in the active site, prohibiting activity of the MMPs. To activate the MMPs, the cysteine–zinc interaction has to be disrupted that called the cysteine switch (Van Wart and Birkedal-Hansen, 1990; Springman et al., 1990). Finally, the catalytic domain of MMPs is responsible for substrate hydrolysis. The structure of the catalytic domains of MMPs is quite similar with subtle structural differences among the five substrate groups. Together with the differences in domain structure, these subtle structural differences in the catalytic domain are thought to control the characteristic specificity for substrates of MMPs (Nagase and Woessner, 1999). This domain contains two zinc ions and at least one calcium ion (Ca^{2+}) coordinated to various residues (Massova, et al., 1998). One of the two zinc ions is present in the active site and involved in the catalytic processes of MMPs. The second zinc ion and the calcium ions are necessary for maintaining of the dimensional structure of MMPs (Chakraborti et al., 2003). Differences from other MMPs, MMP-2 and MMP-9 have three repeats of fibronectin type II-like domain inserted in the catalytic domain responsible for substrate binding (Visse and Nagase, 2003). The majority of MMPs have additional domains including a hemopexin-like domain. The hemopexin-like domain (carboxy-terminal) has a four-bladed propeller structure, with a disc-like structure in the middle containing a calcium ion and this domain linked to the catalytic domain by a hinge region. The function of the hemopexin-like domain is not fully understood, but it is generally contributed to substrate specificity and inhibitor binding.

However, in membrane-type MMPs, the hemopexin-like domain contains a transmembrane domain for anchoring the protein in the membrane and it is required for the dimerization of MT1-MMP and MMP-9 (Toth et al., 2003). Whereas the hemopexin-like domain in MMP-2 binds to TIMP (tissue inhibitor of matrix metalloproteinase)-2 resulting in the activation process (Morgunova, 1999). All MMPs, except MMP-7, MMP-26, and MMP-23, contain the hemopexin-like domain. Where as MMP-23, also called cysteine array MMP, this enzyme lacks the hemopexin-like domain, instead, it has a cysteine-rich in the cysteine array region follow by immunoglobulin (Ig) G-like domain. It is proposed to be a type II membrane protein harboring the transmembrane domain in the N-terminal part of the propeptide domain (Pei et al., 2000). The furin cleavage site, a recognition motif for intracellular furin-like serine proteinases which between propeptide and catalytic domains that allows intracellular activation by these proteinases, is found in MMP-11, six of MT-MMPs, MMP-21, MMP-23 and MMP-28. There are six MT-MMPs: four are type I transmembrane proteins and contain a short cytoplasmic domain (MMP-14, MMP-15, MMP-16, and MMP-24), and two are glycosylphosphatidylinositol (GPI) anchored proteins (MMP-17 and MMP-25) (Itoh et al., 1999; Kojima et al., 2000). In case of MMP-21 contains vitronectin insert that has not been found in other MMPs (Visse and Nagase, 2003).

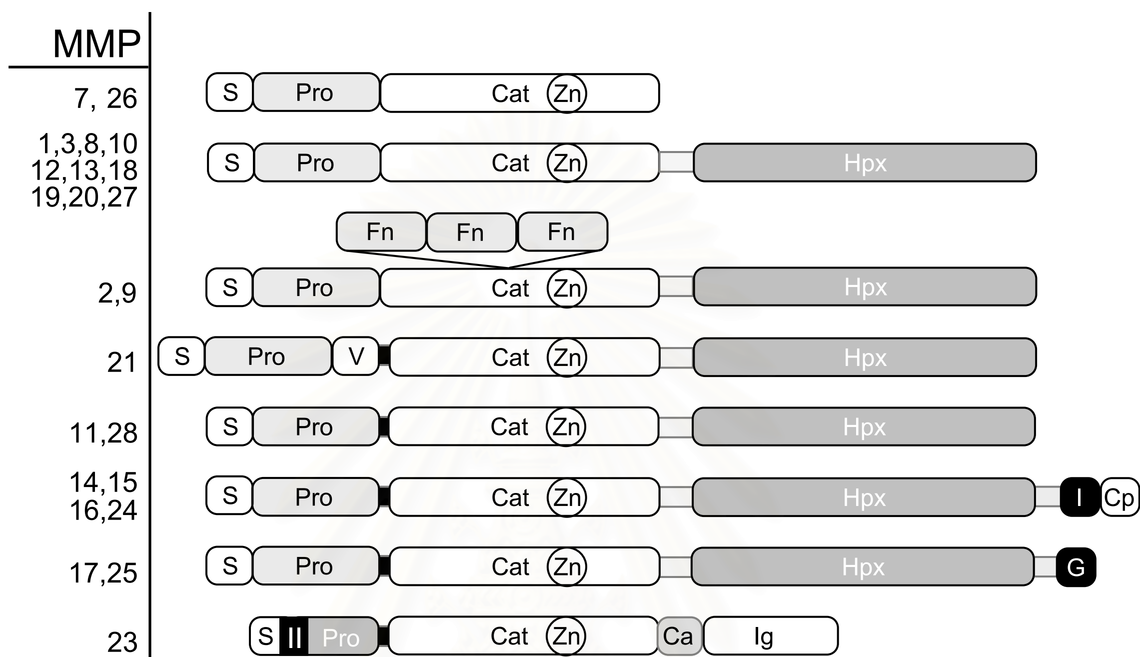

Figure 2.2 Domain structure of MMPs : S, signal peptide; Pro, propeptide; Cat, catalytic domain; Zn, active-site zinc; Hpx, hemopexin like domain; Fn, fibronectin type II like repeated domain; V, vitronectin insert; I, type I transmembrane domain; II, type II transmembrane domain; G, GPI anchor; Cp, cytoplasmic domain; Ca, cysteine array region; and Ig, IgG-like domain. A furin cleavage site is depicted as a black band between propeptide and catalytic domain (Visse and Nagase, 2003).

3. Regulation of MMPs

MMPs are highly regulated enzymes and this regulation includes at least three different levels: transcriptional regulation, activation of latent MMPs and inhibition of active MMPs (Curran and Murray, 2000; Sternlicht and Werb, 2001).

3.1 Transcriptional regulation of MMPs

MMPs can be regulated at the level of gene expression by growth factors, cytokines, hormones, full length and fragmented ECM, trace elements, and drugs via signal transduction pathways (Chamber and Matrisian, 1997; Nagase and Woessner, 1999; Thomas et al., 1999; Nelson et al., 2000). These factors cause variable patterns of expression in different tissues and have variable effects on the different MMP family members, complicating the understanding of gene regulation of MMPs in both physiological and pathological states. Despite a vast array of research in MMP regulation, transcriptional activation is not fully understood (John and Tuszynski, 2001).

3.2 Activation of latent MMPs

All MMPs are produced as inactive enzymes and are activated by disruption of cysteine-zinc interaction as called the cysteine switch. This interaction must be broken by proteolytic cleavage or conformational modification that leads to exposure of the active site (Van Wart and Birkedal-Hansen, 1990). MMPs can be activated *in vitro* by chemical agents, such as thiol-modifying agents (4-aminophenylmercuric acetate; APMA), mercury chloride

($HgCl_2$), and N-ethylmaleimide), oxidized glutathione, sodium dodesylsulphate (SDS), chaotropic agents, and Reactive Oxygen Species (ROS) (Figure 2.3) (Visse and Nagase, 2003). Low pH and heat treatment can also lead to activation (Nagase, 1997). Proteolytic activation of MMPs is an *in vivo* mechanism and stepwise in many cases (Figure 2.3) (Visse and Nagase, 2003). The initial proteolytic attack occurs inside the propeptide region. Once a part of the propeptide is removed, this probably destabilizes the cysteine–zinc interaction, which allows the intermolecular processing by partially activated MMP intermediates or other active MMPs. Thus, the final step in the activation is conducted by an MMP (Visse and Nagase, 2003).

Activation of MMPs by plasmin is a relevant pathway *in vivo* (Chakraborti et al., 2003). Plasmin is generated from plasminogen by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) bound to a specific cell surface receptor. Plasmin has been reported to activate MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, and MMP-13 (Lijnen, 2001) and these activated MMPs can participate in processing other MMPs (Chakraborti et al., 2003).

Most MMPs are secreted from cells and activated extracellularly. However, in MMPs those have a furin cleavage site as MMP-11, six of MT-MMPs (MMP-14, MMP-15, MMP-16, MMP-24, MMP-17, and MMP-25), MMP-23 and MMP-28, are activated intracellularly by furin proteinase. Because these proteins are secreted as active enzymes, their gene expression and inhibition by endogenous inhibitors would be critical for the regulation of activity (Pei and Weiss, 1995; Visse and Nagase, 2003).

Moreover, MMPs can also be activated by fragments or components of ECM. For example, MMP-2 bound to elastin, underwent a fast autoactivation (Emonard and Hornebeck, 1997). Recently, binding of MMP-9 to gelatin was shown to induce MMP-9 activation via a non-proteolytic mechanism, resulting in the acquisition of catalytic activity without removal of the propeptide domain (Bannikov et al., 2002).

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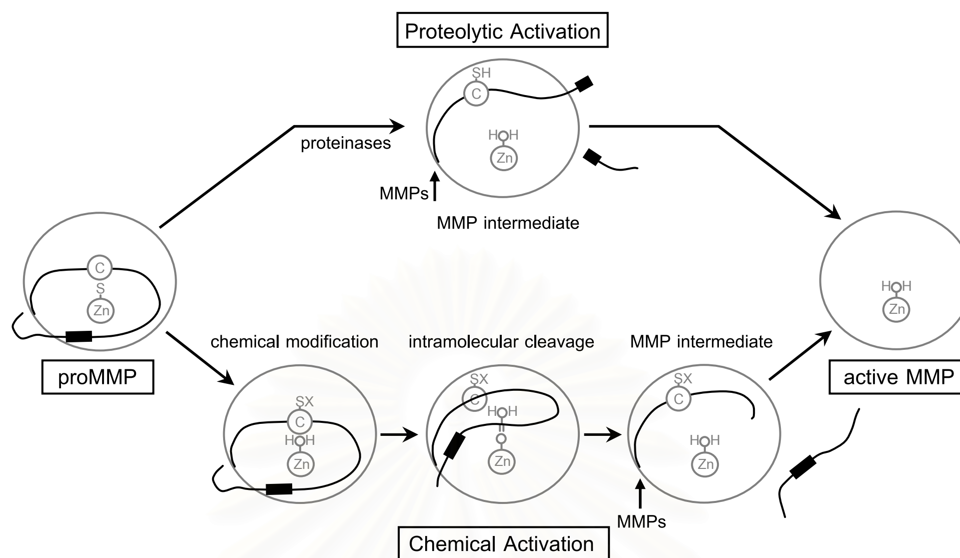

Figure 2.3 Stepwise activation of MMPs. MMPs produced as inactive enzymes can be activated by proteinases (upper pathway) or by chemical modification (lower pathway). The catalytic domain is represented as a gray circle, with the active-site containing the catalytic-site (Zn). The propeptide domain is schematically shown as a black line containing the bait region (black rectangle) and the cysteine switch (C). SH indicates the sulfhydryl of the cysteine. Activation by proteinases is mediated by cleavage of the bait region; this partly activates the MMPs. Full activation is achieved by completed removal of the propeptide domain by intermolecular processing. Chemical activation relies on modification of the cysteine switch sulfhydryl (SX), resulting in partial activation of the MMP by intramolecular cleavage in the propeptide domain. Full activity results from the removal of the remainder of the propeptide domain by intermolecular processing (Visse and Nagase, 2003).

3.3 Inhibition of active MMPs

Various physiological agents can have an inhibitory effect on MMPs. Several natural inhibitors of MMPs exist, but the most common inhibitors are α2-macroglobulin (α2M) and TIMPs (John and Tuszynski, 2001). The α2M is a broad-spectrum protease inhibitor that is commonly expressed in the plasma and produced by the liver (Stetler-Stevenson, 1999). α2M is a homotetramer, built up of two pairs of non-covalently associated dimers. Each subunit contains a bait domain with cleavage sites for almost all known endopeptidases and a receptor-binding domain. When endopeptidases cleave the four bait domains α2M undergoes conformational change resulting in the irreversible entrapment of one or two molecules of the endopeptidases. At the same time, the receptor-binding domains are exposed on the surface and the total complex undergoes rapid endocytosis by binding to the low-density lipoprotein receptor-related protein (LRP) (Kolodziej et al, 2002). α2M has a large molecular weight, 780 kDa preventing the molecule from entering into tissue spaces (Birkedal-Hansen et al., 1993; John and Tuszynski, 2001). TIMPs are much smaller molecules (size~ 21-29 KDa) and expressed in various tissues and fluids. There are four members of the mammalian TIMP family; TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (Gomez et al., 1997). The amino-terminal domain presence in all TIMP molecules is responsible for the MMP inhibitory activity. TIMPs form high affinity, non-covalent complexes with catalytic domain of all active MMPs in a 1:1 stoichiometric ratio. TIMP-1 and TIMP-2 are endogenous inhibitors of all types of active MMPs whereas TIMP-3 and TIMP-4 are inhibitors of some MMPs (Klein et al., 2004). A third known natural inhibitor of MMPs is endostatin. Endostatin

is a cleavage product of type XVIII collagen. Under physiological conditions, level in the circulation is low, whereas level of endostain is increased during cancer progression (Digtyar et al., 2007). Endostatin is able to inhibit MMP-2, MMP-9, and MMP-13 activation including their activities *in vitro*, but the precise mechanism of inhibition is not known (Lee et al., 2002; Nyberg et al., 2003). Several other proteins have been reported to inhibit MMPs. Tissue factor pathway inhibitor-2 (TFPI-2) is a serine protease inhibitor that inhibits MMP-1 and MMP-13 to degrade triple-helical collagen within human atheroma (Herman et al., 2001). In addition, TFPI-2 also reduced the activity of MMP-2 and MMP-9 (Herman et al., 2001). Membrane-bound β-amyloid precursor protein has also been shown to inhibit MMP-2 (Miyazaki et al., 1993). However, the mechanisms of MMP inhibition by these proteins are not known.

MATRIX METALLOPROTEINASE-9 (MMP-9)

The MMP-9 gene locates on human chromosome 20q11.1-13.1 (Daniels et al., 1996). MMP-9 cDNA was first cloned in 1989 from transformed human fibroblasts (Wilhelm et al., 1989). Subsequently it was discovered that the so-called type IV collagenase of neutrophils is the product of the same gene (Devarajan et al., 1992). MMP-9 has been cloned in many mammalian species, and homology exists between the cow, rabbit, rat, mouse, and human (Atkinson and Senior, 2003). MMP-9 is produced by keratinocytes, monocytes, macrophages, polymorphonuclear leukocytes, and a large variety of malignant cells (Westermarck and Kahari, 1999). The role of MMP-9 in physiological conditions is mainly achieved by a remodeling of ECM components in many processes such as in reproduction, growth and development, angiogenesis, inflammation and wound healing (Van den Steen et al., 2002). MMP-9 knock-out mice exhibit no obvious phenotypic defects. However, these mice exhibited a delayed long bone growth associated with an abnormally thickened growth plate, which was accompanied by delayed vascularization and ossification. This abnormality was detected only during development, and normal appearance of bone was seen by 8 weeks of age, probably the deficiency was compensated by others MMPs (Vu et al., 1998). Additionally, MMP-9 knock-out mice are resistant to subepidermal blistering in experimental bullous pemphigoid, an autoimmune blistering disease, because of the lack of MMP-9 in neutrophils (Liu et al., 1998).

Most of MMPs have been implicated in cancer either in early, progressive or metastatic stages. MMP-9, (Gelatinases B, 92-kDa type IV collagenase) is of particular interest with respect to the development and progression of cancer. Historically, interest in this enzyme was based on the capacity of this enzyme to degrade type IV collagen, a major component of basement membrane, which considered being essential in tumor invasion and metastasis (Bergers et al., 2000; Huang et al., 2002). Furthermore, this enzyme has been shown to play a key role in tumor cell growth, angiogenesis and tumor progression, by promoting ECM degradation and the processing of cytokines, growth factors, hormones, and cell receptors (Nguyen et al., 2001; Egeblad and Werb, 2002). MMP-9 expression increases in metastatic cancers when compared to benign tumors and non-invasive cancers, and there is compelling *in vitro* and *in vivo* evidence for the role of MMP-9 in tumor progression (Lakka et al., 2005; Katori et al., 2006).

1. Structure of MMP-9

Human MMP-9 is synthesized as a proenzyme with a molecular mass of 92 kDa. In addition to having the prototypic structure of MMPs, it contains fibronectin type II-like repeats within its catalytic domain (Figure 2.4 and Figure 2.5) (Ram et al., 2006; Opdenakker et al., 2001). The structure of the fibronectin type II-like repeats consist of two short double-strand anti-parallel β-sheets, approximately perpendicular to each other and to three large irregular loops (Elkins et al., 2002). This repeats are responsible for substrate binding, which bind to gelatin, type I and IV collagen, laminin, and elastin (Allan et al., 1995; Shipley

et al., 1996). The activation status of MMP-9 is important, because pro-MMP-9 binds with higher affinity to type I collagen and gelatin with lower affinity to type IV collagen compared with active MMP-9 (Allan et al., 1995). MMP-9 also contains a type V collagen-like domain that is highly glycosylated, which may affect substrate specificity and resistance to degradation (Atkinson and Senior, 2003). The sugars extend this region of the protein to act as a spacer between the zinc binding domain of MMP-9 and the carboxy terminal hemopexin-like domain. The biological significance of the spacer region remains elusive so far. It might, for instance, function in the colateral association of multiple MMP-9 molecules, favoring or preventing specific clipping of hemopexin-like domain and its ligands or binding of the enzyme to specific surfaces (Shipley et al., 1996). The hemoplexin-like domain of MMP-9 consists of a four-bladed β-propeller structure with a pseudo-four-fold symmetry. The blades are composed of antiparallel four β-stranded β-sheet structures. This domain contains a single cysteine-cysteine bond and plays a role in substrate recognition, interaction with TIMPs in inhibition of MMP-9 activity, and binding of the enzyme to ECM or cell surface (Nagase and Woessner, 1999). Pro-MMP-9 can form a stable complex with either TIMP-1 or TIMP-3 via the carboxy terminal hemoplexin-like domain, with the higher-affinity binding to TIMP-1 (Olson et al., 1997). Indeed, pro-MMP-9 is the only MMP that forms a high-affinity complex with TIMP-1, however, the role of the complex has not yet been elucidated (Fridman et al., 2003).

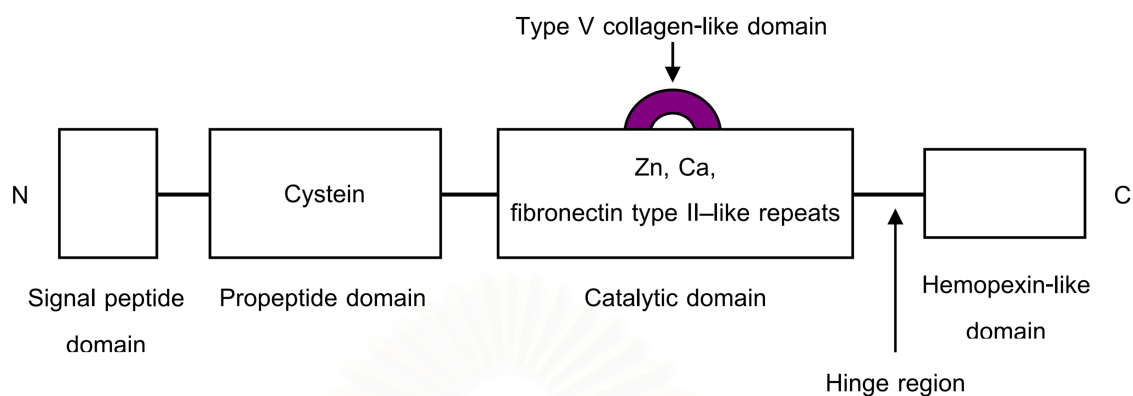

Figure 2.4 The schematic structure of MMP-9 (Ram et al., 2006).

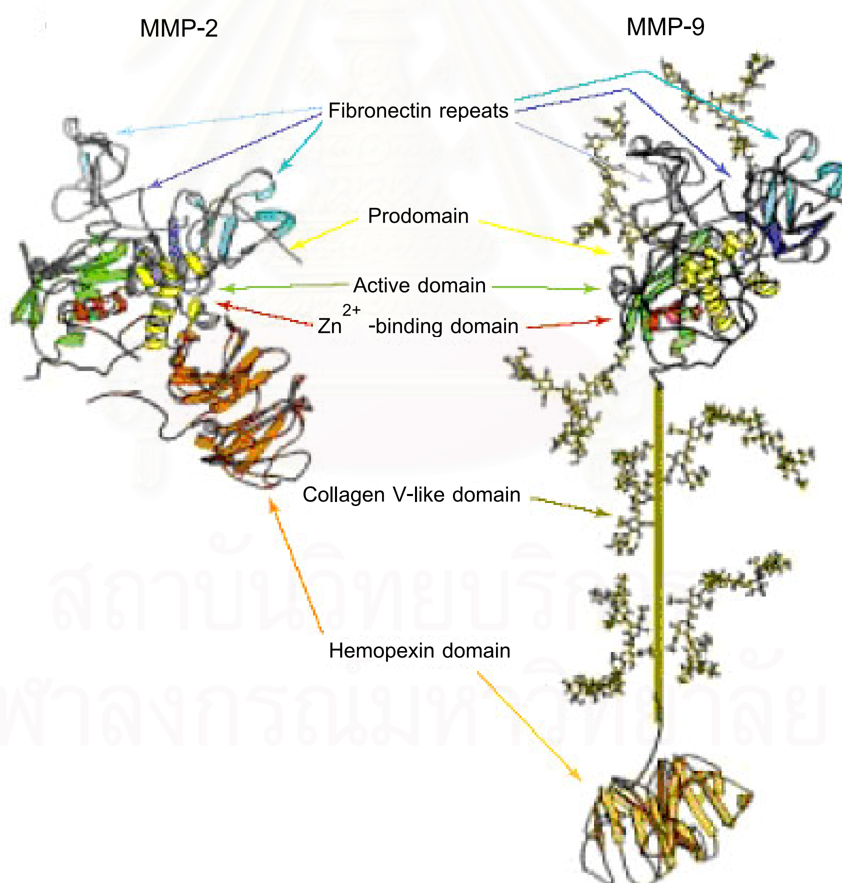

Figure 2.5 Comparison of the 3D structures of MMP-2 and MMP-9 (Opdenakker et al., 2001).

2. Regulation of MMP-9

2.1 Transcriptional regulation of MMP-9

The MMP-9 promoter is in a 2-kb 5' flanking region that contains several transcription factor binding motifs including activator protein-1 (AP-1) and activator protein-2 (AP-2), nuclear factor kappa B (NF-κB) binding sites, stimulating protein-1 (SP-1) and polyoma enhancer activator-3 (PEA-3) sites, GT-boxes, interferon-stimulated regulatory element (ISRE), transforming growth factor-β inhibitory element (TIE), keratinocyte differentiation factor-1 regulatory element (KRE-M9), and nuclear inhibition protein (NIP) binding sites (Huhtala et al., 1991; Sato and Seiki, 1993). However, some of them; AP-1, NF-κB, PEA-3, and SP-1 has been reported having the responsibility on the major physiological responses (Figure 2.6) (Mook et al., 2004).

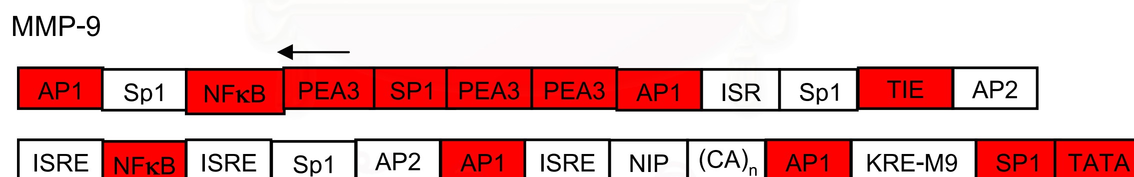

Figure 2.6 Potential regulatory elements in the promoter regions of the human MMP-9 gene. The relative positions of the regulatory elements, indicated as boxes, are not drawn to scale. Regulatory elements with established physiological roles are depicted in dark grey (Mook et al., 2004).

The promoter of MMP-9 is under the control of a variety of inducers, such as tumor promoters, growth factors, cytokines, oncogene products, and physiological substances, including metal ions, ROS, and hormones (Mook et al., 2004; Burrage et al., 2006). Neutrophils, in contrast to other cell types, have shown a rather exceptional MMP-9 expression pattern, which is synthesized and expressed during maturation in the bone marrow and the MMP-9 is stored within specific granules from which it is readily released (Cowland and Borregaard, 1999; Atkinson and Senior, 2003). Therefore, stimulation of mature neutrophils does not result in up-regulation of MMP-9 synthesis, but may induce release of the enzyme by degranulation. Basal expression of MMP-9 is low in most cell types, but the expression is highly responsive to the stimuli. The induced level can be more than a 100-fold of the basal expression level. The transcription is also regulated in a tissue-specific manner (Frisch and Morisaki, 1990; Harendza et al., 1995). An inducer of MMP-9 in one cell type may be down-regulating this enzyme in another cell type or in the same cell type when co-induced with other factors (Van den Steen et al., 2002).

Tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β have been found to up-regulate MMP-9 in cultured human fibroblasts (Wong et al., 2001). Furthermore, MMP-9 in fibroblasts in up-regulated by IL-1α alone or synergistically with either phorbol 12-myristate 13-acetate (PMA), platelet- derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) (Van den Steen et al., 2002). NF-κB and/or AP-1 have been shown to regulate MMP-9 gene expression following TNF-α treatment of osteosarcoma cells, fibrosarcoma cells, SCC cells, vascular smooth muscle cells, salivary gland cells, and bronchial epithelial

cells (Sato and Seiki, 1993; Beppu et al., 2002; Bond et al., 2001; Azuma et al., 2000; Hozumi et al., 2001). Besides IL-1 and TNF-α, other growth factors and cytokines have also been shown to exert their up-regulatory effect on MMP-9 expression such as IL-17, hepatocyte growth factor (HGF), interferon-alpha (IFN-α), Transforming growth factor-beta (TGF-β), whereas IL-4, IL-10, IFN-γ, and IFN-β have been reported to exert inhibitory effect on basal or stimulated MMP-9 expression in various cell types (Van den Steen et al., 2002).

Stimulation of MMP-9 expression by phorbol ester was reported to be both p38-kinase and AP-1 (-79 bp)-dependent (Simon et al., 1998). MMK6 was shown to be involved in AP-1-dependent MMP-9 promoter activity (Simon et al., 2001). Mitogen-activated protein kinase (MAPK) activity was essential for MMP-9 expression in oncogenic transformed rat embryo cells and in tumorigenic SCC cells, which display constitutive activation of both extracellular signal-regulated kinase (ERK) and/ or c-Jun N-terminal kinase (JNK) (Gum et al., 1997; Himelstein et al., 1997; Simon et al., 1999). In the SCC cells, phorbol ester induced MMP-9 secretion was required stimulation of the p38 MAPK pathway (Simon et al., 1998; Simon et al., 2001).

Moreover the modulation of MMP-9 expression by various cell-cell contact settings *in vitro* has been reported in human, murine, bovine, and rat cells (Van den Steen et al., 2002). Endothelial cells directly up-regulate the expression of MMP-9 in monocytes and in T cells via intercellular adhesion molecule-1 (ICAM-1) (Amorino and Hoover, 1998; Mostafa et al., 2001; Aoudjit et al., 1998; Lou et al., 1999). Activated T cells were capable of inducing MMP-9 expression in monocytes and mast cells through direct intercellular contact (Malik et

al., 1996; Baram et al., 2001). In addition, the interaction of CD40 on monocytes or smooth muscle cells with CD40 ligand (gp39) on T cells was shown to stimulate monocytic and smooth muscle cell MMP-9 production (Malik et al., 1996; Schonbeck et al., 1997). E-cadherin-mediated cell-cell contacts were involved in the down-regulation of MMP-9 mRNA and protein levels in mouse squamous carcinoma cells (Llorens et al., 1998).

Because the gelatinases play a major role in ECM turnover by degrading gelatin, collagen types IV, V, VII, X, and XI, elastin, laminin, fibronectin, and proteoglycan core protein, the main adhesive event that regulates their expressions is the contact of cells with ECM components. These interactions are mediated by αβ integrin receptors on the cell surface that recognize specific sequences in the matrix proteins, such as the Arg-Gly-Asp (RGD) motif in fibronectin (Werb et al., 1989). For example, expression of MMP-9 was induced by intact fibronectin in lymphoid tumor cells and in human SCC cells through mediation by the αv integrin subunit (Esparza et al., 1999; Vacca et al., 2001; Thomas et al., 2001). Various integrin-mediated pathways for the production of MMP-9 are activated during tumor development to facilitate cell invasion. For instance, a role for α3β1 integrin in maintaining MMP-9 production by transformed epithelial cells and mammary carcinoma cells was found in normal primary keratinocytes (Larjava et al., 1993; DiPersio et al., 2000; Morini et al., 2000). Fibronectin, by binding to the α3β1 integrin, stimulates the expression of MMP-9 through increasing AP-1/DNA binding activity and c-Fos protein expression via the activation of phosphatidyl inositol 3-kinase (PI3K) and ERK signaling pathways in human non-small cell lung carcinoma (Han et al., 2006).

Many hormones or hormone-mimicking compounds have been shown to modulate expression of MMP-9 in different mammalian cell types *in vitro*. Up-regulation of MMP-9 was induced by estradiol in mesangial cells (Potier et al., 2001). Similarly, MMP-9 appears to respond positively to parathyroid hormone, and 1, 25-dihydroxyvitamin D3 (Meikle et al., 1992). Dexamethasone, a synthetic glucocorticoid that potently down-regulates the immune response, was found to inhibit PMA-induced MMP-9 expression in fibrosarcoma cells by promoting translocation of the glucocorticoid receptor from the cytosol to the nucleus, which downmodulates AP-1 activity (Cowland and Borregaard, 1999). Additionally, dexamethasone blocked TNF-α-induced MMP-9 expression in SCC cells by targeting NF-κB (Beppu et al., 2002). Moreover, it was found that trace elements including iron all influenced the regulation of MMP-9 (Lee et al., 2003; Campo et al., 2006).

Most of the regulatory mechanisms mediated by those stimuli occur primarily at the transcriptional level and are initiated by the binding of the stimulating factor to cell surface receptor. Signals exerted by extracellular stimuli are transmitted to the nucleus. A major mechanism for this signal transduction involves activation of serine-threonine kinases related to the MAPK superfamily, p38 kinase, JNK and ERK (Robinson and Cobb, 1997; Chang and Karin, 2001). The p38 kinases and JNKs are generally activated in response to inflammatory cytokines, osmotic stress and apoptotic signals. ERKs generally respond to cytokines, growth factors and phorbol esters. As a consequence, a group of protein kinases [MAPK kinase kinases (MAPKKKs)] phosphorylates MAPK kinases (MAPKK) which phosphorylates and activates MAPK then active MAPKs translocate to the nucleus and

activate a series of transcription factors that interact with transcription factor binding sites in MMP-9 promoters (Figure 2.7) (Van den Steen et al., 2002; Mook et al., 2004). Moreover, MMP-9 expression can be mediated via protein kinase C (PKC), PI3K (Thant et al., 2000; Kermorgant et al., 2001; Bischof et al., 2002), and NF-κB signaling pathways (Westermarck and Kahari, 1999; Sanceau et al., 2002).

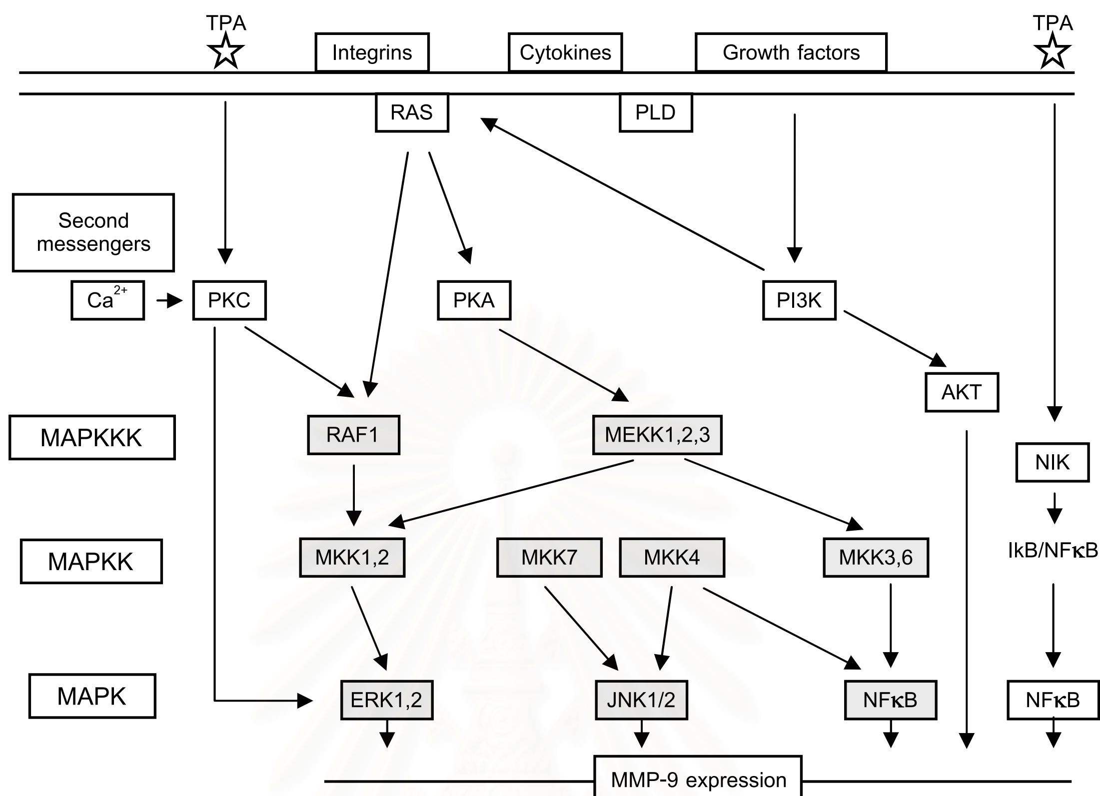

Figure 2.7 General schematic representation of signaling pathways regulating MMP-9 expression (Mook et al., 2004).

Activation of transcription factors can either up-regulate or down-regulate MMP-9 expression. This is dependent on which transcription factor is activated but can also be dependent on the cell type. MMP-9 expression is regulated by binding of multiple factors to their responsive elements (Figure 2.8) (Mook et al., 2004). NF-κB up-regulates MMP-9 transcription via TNF-α signaling pathway due to a recognition sequence at -600 bp (Crowe et al., 2001). Interferons down-regulate MMP-9 expression by activation of interferon regulatory factor-1 (IRF-1) and signal transducer and activator of transcription-1 (STAT-1),

which compete with binding of NF-κB (Sanceau et al., 2002). KiSS-1, metastin or human malignant melanoma metastasis suppressor, diminishes MMP-9 expression by reducing NF-κB binding to the promoter (Yan et al., 2001). Active c-fos inhibits MMP-9 expression via the AP-1 site at -79 bp (Crowe and Brown, 1999), whereas signals which activate JNK1 or active c-jun stimulate MMP-9 expression via AP-1 at -79 bp (Crowe et al., 2001). Thus, opposite effects on MMP-9 expression can be mediated via the same recognition site, depending on which binding proteins are activated. This was also found for ETS (erythroblastosis twenty six) transcription factors. ETS-2 induces MMP-9 promoter activity whereas myocyte enhancer factor (MEF), another ETS transcription factor, reduces MMP-9 promoter activity probably due to competition for the same ETS site (Seki et al., 2002).

In addition, modulation of mRNA stability has been observed to be involved in the regulation of MMP-9 gene expression in response to inducers. For example, treatment of monocytic precursor U937 cells with PMA stimulated MMP-9 expression at the transcriptional level, and subsequent exposure to bacterial lipopolysaccharide (LPS) increased the half-life of MMP-9 mRNA (Saarialho-Kere et al., 1993). TGF-β1 up-regulated MMP-9 in human prostate cancer cell lines through increased mRNA stability by de novo synthesis of mRNA-stabilizing proteins (Sehgal and Thompson, 1999).

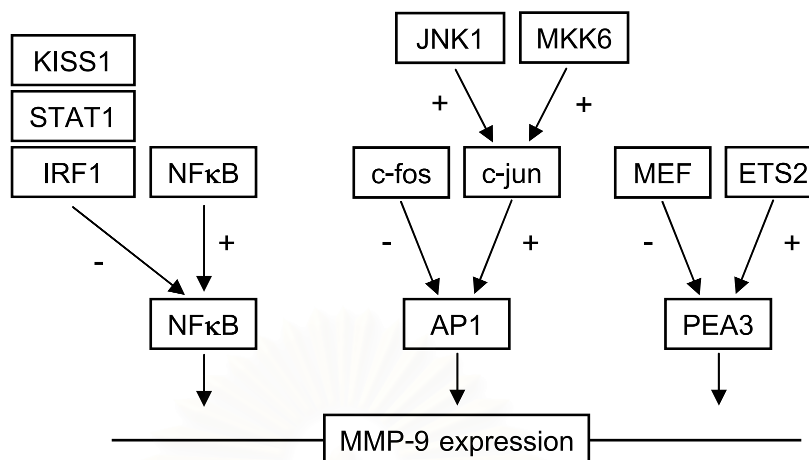

Figure 2.8 Positive and negative regulators of MMP-9 expression.

(+) Up-regulation; (-) down-regulation of MMP-9 expression

(Mook et al., 2004)

2.2. Activation of MMP-9

Like all MMPs, MMP-9 is produced in a latent form that requires activation for enzymatic activity. The activation of MMP-9 has been studied in details and both proteolytic and non-proteolytic activation mechanisms have been described. A variety of proteases, those belong to the families of MMPs and serine proteinases, can cleave the propeptide domain of MMP-9 at specific sites resulting in active MMP-9 (Fridman et al., 2003). MMP-1, MMP-2, MMP-3, MMP-7, MMP-10, and MMP-13 have been reported to activate MMP-9 (Figure 2.9) (Fridman et al., 2003; von Bredow et al., 1998; Knauper et al., 1997; Van den Steen et al., 2002). MMP-3 is one of the most effective MMP-9 activator (Goldberg, 1992). It generates the fully active 82-kDa species via an inactive intermediate form of 85-kDa and

the active 82-kDa species is further processed to a 64-kDa active species by being removal of the C-terminal end (Ogata et al., 1992). Nevertheless, removal of the carboxy terminal region of MMP-9 may alter sensitivity to TIMP-1 inhibition, as this region is the high-affinity binding site for TIMP-1 (Okada et al., 1992). Activation of MMP-9 by other MMPs involves cell surface, because the activated MMPs are known to associate directly or indirectly with the cell surface. For example, MMP-3 is activated by plasmin, which in turn is generated from plasminogen by uPA bound to the uPA receptor on the plasma membrane. This process can lead to MMP-9 activation via plasmin activated MMP-3 (Ramos-De Simone et al, 1999). The activation of MMP-9 by MMP-2 and MMP-13 are also the result of a cascade of zymogen activation initiated on the cell surface by MT1-MMP as depicted in Figure 2.10 (Fridman et al., 2003). Indeed, MMP-2 is activated by MT1-MMP and both MT1-MMP and MMP-2 are able to activate MMP-13 (Knauper et al., 1996). It is thought that the overall activity of these proteases at the cell surface can accomplish the efficient degradation of interstitial collagen, which is initiated by the collagen-degrading enzymes (MT1-MMP and MMP-13) and is followed by the gelatinase activities of MMP-2 and MMP-9.

Several serine proteinases can also activate MMP-9 including cathepsin G, trypsin, and tissue kallikrein (Desrivires et al., 1993; Duncan et al., 1998; Lijnen, 2001). Recent studies demonstrated that a tissue-associated chymotrypsin-like proteinase is responsible for activation of MMP-9 in skin tissues of patients with chronic unhealed wounds (Han et al., 2002). Pancreatic trypsin-2 isolated from a human carcinoma cell line was shown to be an

effective activator of MMP-9 (Sorsa et al., 1997). Both α- and β-chymases derived from mast cells are also MMP-9 activators (Cousens et al., 1999).

Recent studies have suggested the existence of an alternative mechanism for pro-MMP-9 activation by disengagement of the propeptide domain from the active site without the participation of proteolytic enzymes in tissue sections of human placenta. This process may occur via oxidative modification of the cysteine side-chain thiol, which would diminish its ability to serve as an effective ligand to the catalytic zinc ion (Okamoto et al., 2001), or via conformational changes induced by binding to substrate (Bannikov et al., 2002). Either process may result in activation without removal of the inhibitory propeptide domain and consequently generate enzymatic activity in the absence of a noticeable change in molecular mass. Recently, binding of MMP-9 to gelatin or type IV collagen was shown to induce MMP-9 activation via a non-proteolytic mechanism, resulting in the acquisition of catalytic activity without removal of propeptide domain, this process has been proposed to be a novel activation mechanism for MMP-9 *in vivo* (Bannikov et al., 2002). The activation of MMP-9 can also be performed by chemical such as organomercurials, urea and detergents. Interestingly, it was also found that MMP-9 can be activated by ROS, such as hypochlorous acid (Peppin and Weiss, 1986).

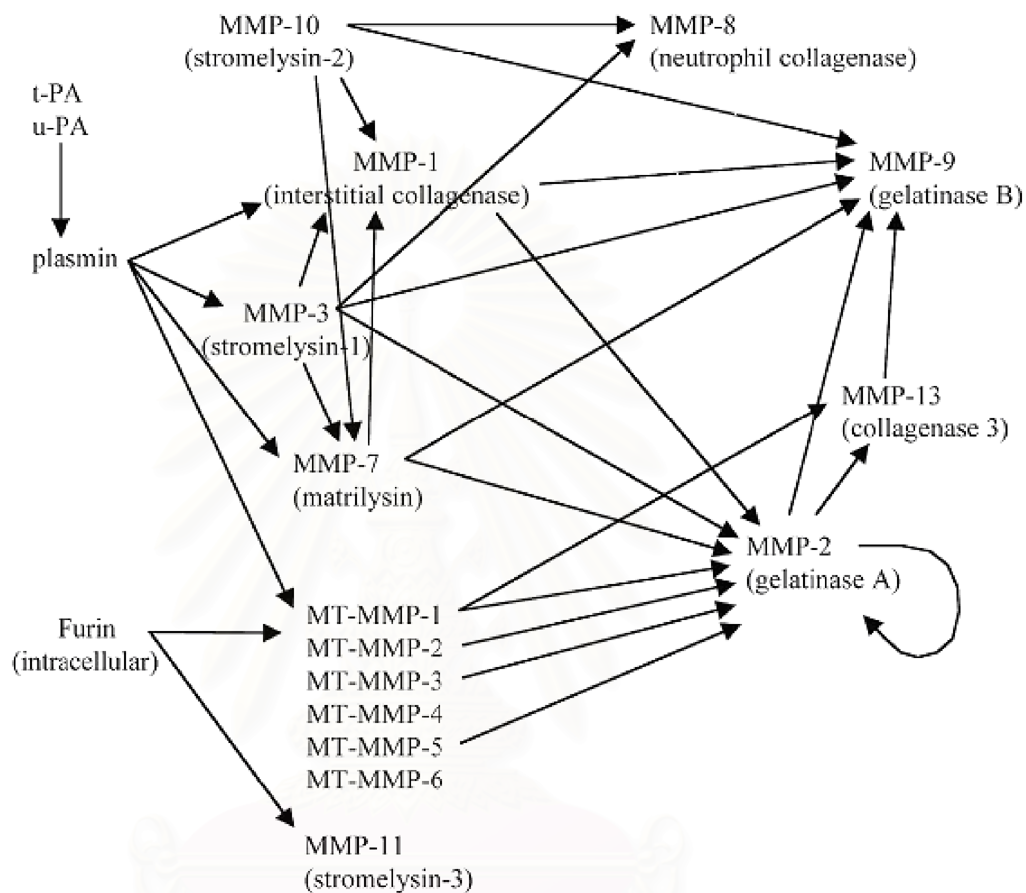

FIGURE 2.9 The activation network of MMP-9 by MMPs. Possible pathways for the activation of MMP-9 by other MMPs are shown. Arrows from one enzyme to another indicate that the active form of the first enzyme converts the proform of the second enzyme to its active form, which is indicated (Van den Steen et al., 2002).

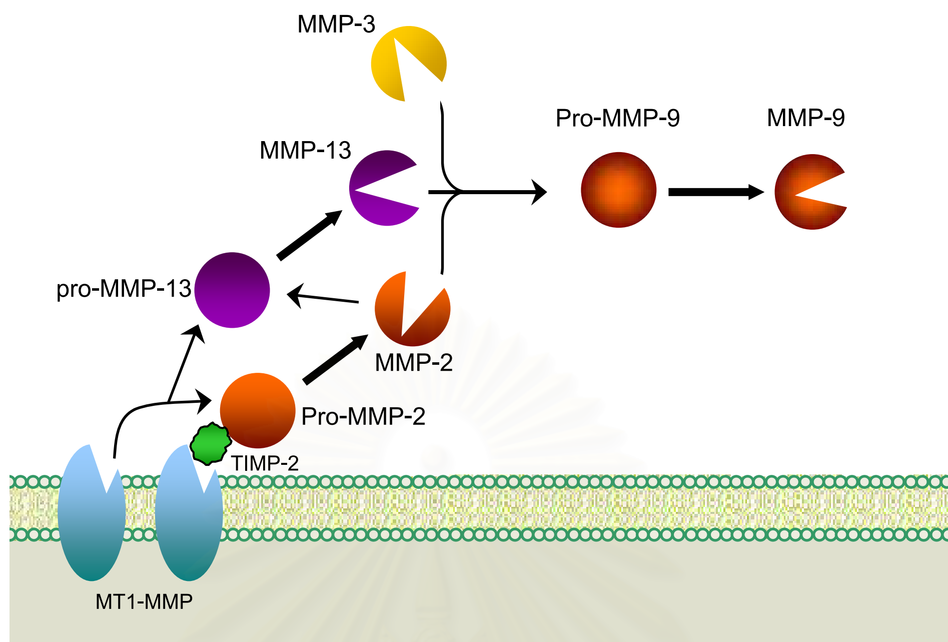

Figure 2.10 MMP cascade of zymogen activation involved in pro-MMP-9 activation. Pro-MMP-9 can be activated by several MMPs including MMP-2, MMP-3 and MMP-13 (Fridman, et al., 2003).

2.3. Inhibition of MMP-9 activity

The predominant circulating inhibitor of MMP-9 is α2-macroglobulin. Active forms of MMP-9 are trapped by α2-macroglobulin and removed from the circulation via scavenger receptors. TIMPs all have affinity for MMP-9, but typically TIMP-1. Generally, TIMP-1 forms non-covalent complex with active MMP-9 and inhibit MMP-9 activity. Moreover, TIMP-1 can form complex with the carboxy terminal of the proenzyme as well as the catalytic domain of the active form (Goldberg et al., 1992). The reason for this dual association is unknown (Atkinson and Senior, 2003). Thrombospondins and tissue factor protease inhibitor-2 (TFPI-

2) also can bind and inactivate MMP-9 (Egeblad and Werb, 2002; Herman et al., 2001). Recent evidence indicates that binding of MMP-9 to the cell surface protein may also negatively regulate enzyme function. These membrane proteins are reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) and low-density lipoprotein receptor-related protein (LRP) (Herz and Strickland, 2001). RECK, a GPI-anchored glycoprotein, was shown to inhibit the enzymatic activity of MMP-9, but the mechanism by which RECK inhibit MMP-9 activity is unclear (Takahashi et al., 1998). LRP is responsible for the internalization of a variety of ligands including MMP-9, which was shown to bind with high affinity to purified LRP either as a free enzyme or in complex with TIMP-1. The MMP-9 or MMP-9/TIMP-1 complex is internalized and followed by degradation of complex in a chloroquine-dependent mechanism (Hahn-Dantona et al., 2001). Moreover, MMP-9 is inhibited by pharmaceutical inhibitors, these inhibitors can be divided into four basic categories: peptidomimetics, non-peptidomimetics, tetracycline derivatives, and bisphosphonates (Klein et al., 2004). Peptidomimetics, such as Batimastat and Marmistat, contain a sequence that resembles MMP substrates, but are relatively nonspecific (Bramhall et al., 2002). The non-peptidomimetics, such as Prinomastat, are synthesized to resemble the catalytic pocket of MMPs (Shalinsky et al., 1999). The tetracycline derivatives can decrease MMP production and activity (Bonomi, 2002). Finally, bisphosphonates, such as Clodronate, the mechanism of action of the bisphosphonates is not known yet, but they have various inhibitory effects on MMPs, including inhibition of MMP activity (Hidalgo and Eckhardt, 2001).

3. MMP-9 in head and neck squamous cell carcinoma (HNSCC)

The ability of MMP-9 to degrade type IV collagen, major component of basement membrane, which enables tumor cells to migrate and colonize host tissues, appears to be particularly important in cancer progression. Although the main function of MMP-9 is involved in ECM degradation, it is notable that MMP-9 also alters and modulates biological functions of cytokines and growth factors by its degradative capacity, resulting in promotion of tumor growth, angiogenesis, metastasis, and suppression immune responses (Duffy et al., 2000; Nguyen et al., 2001; Deryugina and Quigley, 2006). For example, MMP-9 plays a role in platelet-activating factor (PAF)-induced angiogenesis (Ko et al., 2005). MMP-9 can proteolytically activate TGF-β which promotes tumor invasion and angiogenesis (Yu and Stamenkovic, 2000). The activation of MMP-9 by α-chymase was associated with the angiogenesis switch in a mouse model of squamous epithelial carcinogenesis (Cousens et al., 1999). Additionally, MMP-9 also appears to activate releasing of tissue-bound fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) that facilitates tumor cell growth and angiogenesis (Bergers et al., 2000; Fingleton, 2003). Cyclic peptides containing the sequence, which are potent and selective inhibitor of MMP-9, have been shown to inhibit tumor and endothelial cell migration *in vitro* and tumor growth and invasion *in vivo* (Koivunen et al., 1999). Moreover, MMP-9 is involved in various processes that decrease immune responses. The immunosuppressive role of MMP-9 in cancer was demonstrated by suppression of T-cell proliferation through disruption of signaling mediated by IL-2 receptor alpha (IL-2Rα) (Sheu et al., 2001). Since active TGF-β is an important

inhibitor of T-cell responses to tumors, MMP-9, an activator of TGF-β, can contribute to inhibition of T-cell responses (Gorelik and Flavell, 2001). Growth-related oncogene alpha (GRO-α), connective tissue activating peptide-III (CTAP-III), and platelet factor-4 (PF-4), cytokines that attract neutrophils and activate leukocytes, are degraded into multiple fragments by MMP-9, thereby also decreasing the immune response (Van den Steen et al, 2000). Furthermore, ICAM-1, CD44 and type IV collagen are cell surface docking molecules for MMP-9 (Fiore et al., 2002). Therefore, localization of MMP-9 at the cell surface due to ICAM-1 and subsequent activation of MMP-9 can result in ICAM-1 shedding, thus increasing resistance of cancer cells to be killed by immune cells in HL-60 cancer cells (Kridel et al., 2001). MMP-9 through all of these data is certainly one of the key enzymes involved in tumor invasion, metastasis, and progression.

HNSCC is the sixth most common cancer in the world and accounts for 90% of malignant neoplasia of the upper respiratory system (Parkin et al., 2005). The biological aggressiveness of HNSCC reflected in its ability to metastasize to regional lymph node. Metastasis of HNSCC to the cervical lymph node is considered the most unfavorable prognostic factor in terms of survival (Mamelle et al., 1994). Despite recent advances in the management of local advanced HNSCC, the overall survival of patient has improved only marginally over the past three decades and overall prognosis of patients remains poor (Hunter et al., 2005; Massano et al., 2006). A better understanding of the molecular mechanisms underlying the development of HNSCC may help to identify targets for pharmacological intervention in this disease (Nagpal and Das, 2003).

Over-expression of MMP-9 has been identified in a large proportion of SCC arising in the head and neck (Nyberg et al., 2002; Franchi et al., 2002; Tsai et al., 2003; Katayama et al., 2004; Kato et al., 2005). Activation of MMP-9 was significantly higher in malignant tissues as compared to adjacent normal tissues of this cancer (Patel et al., 2005). In addition, the correlative studies of MMP/TIMP expression suggested the potential role of MMP-9 in progression and metastasis of human HNSCC (O-charoenrat et al., 1999). Elevated levels of MMP-9 correlate with the invasiveness of HNSCC (Kawamata et al., 1998; Thomas et al., 2001; Impola et al., 2004) and over-expression of MMP-9 mRNA is associated with progression of oral dysplasia to cancer (Jordan et al., 2004). Furthermore, high level of MMP-9 has been associated with poor prognosis of oral cancer patients with lymph node metastases (Katayama et al., 2004). Additional studies in human tumors have shown that MMP-9 is up-regulated in diffuse invasive HNSCC specimens, with predominant localization to the invasive front (Juarez et al., 1998). In culture, HNSCC cell lines with high levels of MMP-9 show greater invasion capacities compared to those with low levels of expression (Robinson et al., 2003). Modulation of MMP-9 expression via phobol ester treatment leads to enhanced invasive behavior in an *in vitro* study (Hong et al., 2000), whereas blocking MMP-9 expression *in vivo* reduces invasion in an orthotopic murine model of HNSCC (Simon et al., 1998). These data support that MMP-9 is one of important determinants of invasive phenotype in HNSCC.

GENERAL OVERVIEW OF IRON UPTAKE AND METABOLISM

Iron is an essential nutritional element playing a vital role in many cellular processes such as oxygen transport, cellular respiration, electron transport, DNA synthesis, ATP generation, cell proliferation and differentiation (Crichton and Ward, 1992; Richardson and Ponka, 1997). In fact, without iron, cells are unable to proceed from the G1 to the S phase of the cell cycle, since iron appears to play a critical role in the expression regulation of a number of molecules that control cell cycle progression (Siah et al., 2005). Furthermore, in many cell lines iron is involved in the regulation of PKC gene expression, which plays a vital role in signal transduction pathways (Gao et al, 1999). Iron also plays a role in the immune system, neurotransmitter system, muscle function, energy metabolism (Beard, 2001) and gene regulations including in MMP expression and activation (Gardi et al., 2002; Lee et al., 2003; Zamboni et al., 2005).

Maintaining iron availability for cellular metabolism and growth is critical to the survival of both prokaryotes and eukaryotes (Jurado, 1997). Iron exists in two redox states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), and it can readily convert from one to the other by either losing or gaining an electron. This property of iron makes it useful in all of metabolic reactions that require a loss or gain of electrons (Andrews, 2000; Srai et al., 2002). However, with this property, it makes toxicity by generating free radicals when iron is present in excess (McCord, 1998). As a consequence, living organisms develop strictly regulation processes for iron transport, uptake and storage. The balance among these

mechanisms is essential for live (Richardson and Ponka, 1997).

There are two types of iron in the diet. Firstly, heme iron derived from hemoglobin and myoglobin which served as oxygen carriers in the erythroid tissue and muscle, respectively. Heme iron represents a small fraction of total iron in the diet which very well absorbed. Secondly, non-heme iron or inorganic iron is found abundantly in vegetables. The most prevalent forms of non-heme iron in metalloproteins are iron-sulfur clusters, such as 2Fe-sS, 3Fe-4S, and 4Fe-4S (Carpenter and Mahoney, 1992; Sharp and Srai, 2007).

1. Iron distribution and homeostasis in the body

The human body contains approximately 3–5 g of iron (45–55 mg/kg of body weight in adult women and men, respectively), distributed as illustrated in Figure 2.11. The majority of body iron (~60–70%) is utilized within hemoglobin in the circulating red blood cells. Other iron-rich organs are the liver and muscles. Approximately 20–30% of body iron is stored in hepatocytes and in reticuloendothelial macrophages, to a large extent within ferritin and its degradation product, hemosiderin. The remaining body iron is primarily localized in cytochromes, and iron containing enzymes. Since human does not possess any physiological pathway for iron excretion. Thus, body iron homeostasis is regulated at the level of iron absorption. Disregulated iron absorption leads to iron deficiency or overload. It is believed that three regulatory cues contribute to the maintenance of iron homeostasis (Andrews, 1999; Finch, 1994). The first is called “dietary regulator”. A healthy individual absorbs daily 1–2 mg of iron from the diet, which compensates nonspecific iron

losses by cell desquamation in the skin and the intestine. In addition, menstruating women physiologically lose iron from the blood (Siah et al., 2005). It has been known that after the ingestion of a dietary iron bolus, absorptive enterocytes are resistant in acquiring additional iron for several days. This phenomenon, also described as “mucosal block”, probably results from the accumulation of intracellular iron. A second signal, called “stores regulator”, controls iron uptake in response to body iron stores. It is well established that in iron-deficient conditions, iron absorption is significantly stimulated by two to three folds. When iron stores are replenished, iron absorption returns to basal levels. It has been hypothesized that this type of regulation requires the programming of precursor cells in the duodenal epithelium after sensing plasma Tf saturation. A third signal, called” erythropoietic regulator”, modulates iron absorption in response to erythropoiesis. Because most of the body’s iron is utilized by the bone marrow for hemoglobinization of red blood cells, therefore this signal has a dominant function in the control of iron homeostasis in the body (Ganz, 2003). Erythropoiesis requires approximately 30 mg iron/day, which is mainly provided by the recycling of iron via reticuloendothelial macrophages. These macrophages ingest senescent red blood cells and release iron to circulating Tf. The pool of Tf-bound iron (~3 mg) is very dynamic and undergoes more than 10 times daily recycling (Papanikolaou and Pantopoulos, 2005).

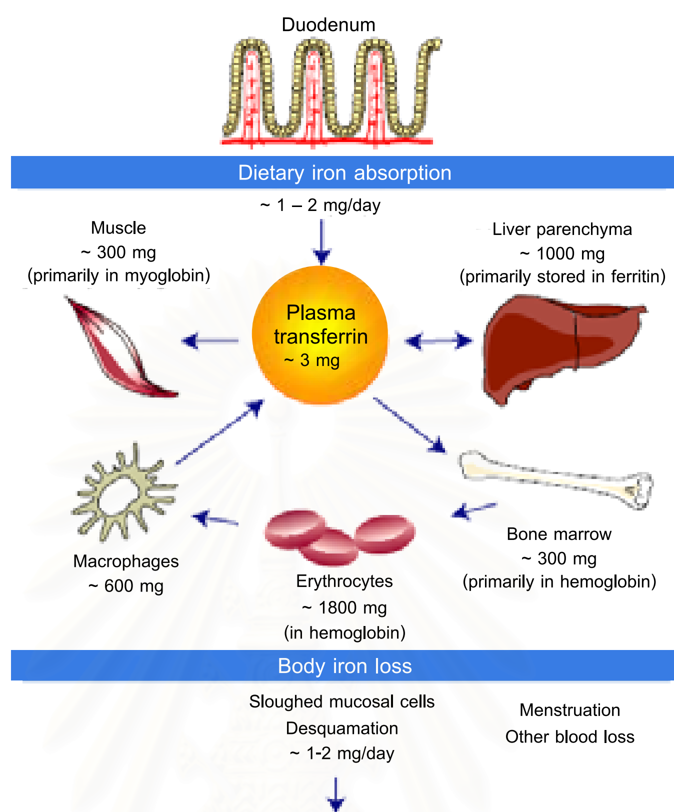

Figure 2.11 Iron distribution in the adult human body

(Papanikolaou and Pantopoulos, 2005)

2. Iron absorption and transport

An average daily diet contains approximately 15 mg of iron, from which only 1–2 mg is absorbed. Both heme and non-heme iron are absorbed in the duodenum, though their transports across the apical membrane of the enterocytes occur through totally independent pathways (Figure 2.12) (Conrad and Umbreit, 2000; Sharp and Srai, 2007).

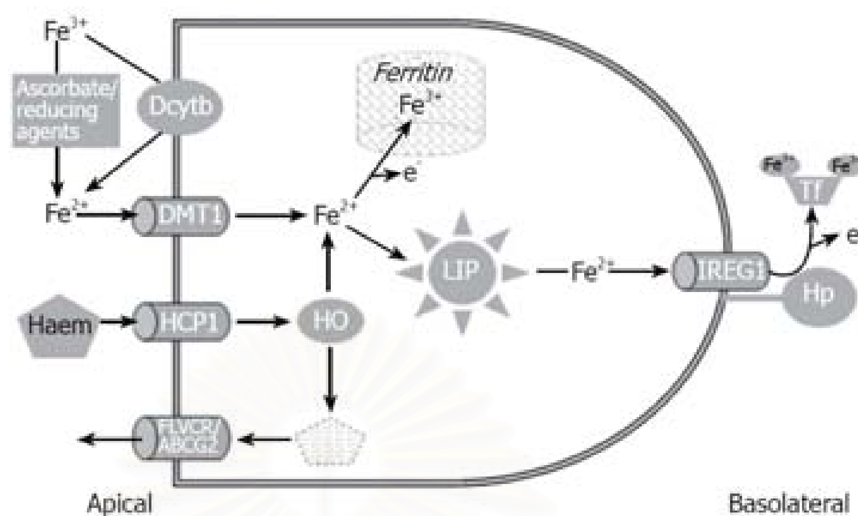

Figure 2.12 The cellular mechanisms of intestinal iron absorption (Sharp and Srai, 2007).

The majority of dietary inorganic iron enters the gastrointestinal tract in the ferric form. However, Fe^{3+} is thought to be essentially non-bioavailability and, therefore, it must first be converted to ferrous iron prior to absorption. There are numerous dietary components capable of reducing Fe^{3+} to Fe^{2+}, including ascorbic acid, and amino acids such as cysteine and histidine (Han et al., 1995). It is believed that the action of these dietary reducing agents takes place in the acidic environment of the gastric lumen (Srai et al., 2002; Sharp and Srai, 2007). However, ferric iron reaching the duodenal enterocytes may still be reduced by the cells endogenous reducing activity. Dietary ferric iron is reduced to ferrous iron by iron-regulated ferric reductase, duodenal cytochrome B (Dcytb), which is highly expressed on the brush border membrane of enterocytes (Mckie et al., 2001). Following reduction either by Dcytb or dietary reducing agents, the resulting Fe^{2+} becomes a substrate for the divalent metal transporter-1 (DMT-1) also known as the divalent

cation transporter-1 (DCT-1), and natural resistance associated macrophage protein-2 (Nramp-2) (Gunshin et al., 1997; Fleming et al., 1997; Dunn et al., 2006). DMT-1 also transports other divalent metal cations, including zinc, manganese, nickel, copper, cadmium, and cobalt, but the intracellular pathway is not known (Wang et al., 2002). The relatively low pH of the proximal duodenum together with the acidic microenvironment at the brush border membrane stabilize iron in the ferrous form and provide a rich source of protons that are essential for driving iron uptake across the apical membrane of the intestinal epithelium (Andrews et al., 1999; Canonne-Hergaux et al., 1999).

Some investigators have proposed that Fe^{3+} might be absorbed by intestinal enterocytes via a mechanism that is distinct from DMT-1. In this model, ferric iron, which is insoluble at physiological pH, is released from the food matrix in the acidic environment of the stomach, and is chelated by mucins on the duodenal brush border surface, which maintain the iron in the ferric state. Ferric enters the enterocyte across the apical membrane via interaction with β3-integrin and mobilferrin (a calreticulin homologue), also call the integrin mobiferrin pathway (IMP). This pathway appears to be unique for ferric iron and is not shared with other metals (Conrad et al., 1993). In the cytosol, this complex combines with flavin monooxygenase and β2-microglobulin to form a larger complex (approximately 520 kDa) known as paraferritin, which has ferric reductase activity resulting in the conversion of the absorbed Fe^{3+} to Fe^{2+} (Umbreit et al., 1996; Umbreit et al., 1998). Recent evidence suggests that the paraferritin complex may also contain DMT-1, which may permit the delivery of ferrous iron to intracellular organelles (Umbreit et al., 2002).

Another source of dietary iron is heme, a molecule that consists of a protoporphyrin ring that binds iron. Heme results from the breakdown of hemoglobin and myoglobin found in meat products, and it is thought to be internalized through the recently identified receptor heme carrier protein-1 (HCP-1) (Shayeghi et al., 2005). HCP-1 is expressed at high levels in the duodenum and, upon binding of heme to HCP-1 on the cell surface, the complex is internalized by receptor-mediated endocytosis. It is suggested that the resultant endosomal vesicles migrate to the endoplasmic reticulum, where iron is liberated from heme by the heme oxygenase-1 (HO-1) that is found on the reticulum surface (Shayeghi et al., 2005). Whereas, the ATP-binding cassette protein (ABCG2), and the feline leukemia virus C receptor protein (FLVCR) mediate heme efflux and may act to remove bilirubin formed as a by-product of heme degradation from the enterocytes (Quigley et al., 2004).

Once in the enterocyte, iron has one of three fates. First, it can be immediately utilized in the many cellular processes for which it is essential. Second, if body iron stores are high, iron may be diverted into the storage protein ferritin, and lost when the cell is shed at the villus tip. Third, iron passes into the labile iron pool (LIP) and is subsequently exported out of the enterocyte via a pathway which requires the ferroxidase hephaestin (Hp), and the basolateral iron transporter, ferropontin-1/metal transporter protein-1 (FPN-1/MTP-1) (Donovan et al., 2000; Abbond and Haile, 2000), also found in literature as iron regulated gene-1 (IREG-1) (Mckie et al., 2001). The Fe^{2+} is then exported through the basolateral membrane of the enterocyte to the interstitial space by the exporter IREG-1 and oxidized by the Hp to Fe^{3+}. Once extracellular, iron is then bound with high affinity by the

serum iron-transport protein transferrin (Tf) and taken into the circulation to all the cells and tissues (Qian et al., 1997; Gomme and McCann, 2005). Transferrin is a glycoprotein with homologous N-terminal and C-terminal iron binding domains, which comprise of two forms, iron-loaded Tf (holo-Tf) and iron-depleted Tf (apo-Tf). Apo-Tf binds transferrin receptor (TfR) with 500-fold lower affinity than holo-Tf (Conrad and Umbreit, 2000).

3. Cellular uptake of iron

The uptake of iron by individual cells in the body is regulated by total body iron level and intracellular iron level. Iron is largely transported into non-intestinal nucleated cells by the classical pathway utilizing the Tf-dependent pathway (Inman and Wessling-Resnick, 1993; Conrad et al., 1994).

It has become clear that both normal and neoplastic cells can obtain iron from Tf by a variety of different mechanisms. After iron is converted to the ferric form, two atoms of ferric (di-ferric) bind to apo-Tf and then transported through the serum to target tissues and binding to TfR-1. Two isoforms of TfR have been identified, TfR-1 and TfR-2. Human TfR-2 is 45% identical with TfR-1 in the extracellular domain, but TfR-2 mRNA dose not contain iron responsive element sequences and is apparently not regulated by intracellular iron levels (Kawabata et al., 1999). Transferrin receptor 2, like TfR-1, binds Tf in a pH-dependent manner, but it has a 25-30 fold lower affinity for Tf than TfR-1. However, TfR-2 distribution differs from TfR-1, it is restricted to hepatocytes, duodenal crypt cells, and erythroid cells, suggesting a more specialized role in iron metabolism (Trinder and Baker,

2003).

Transferrin/TfR-1 assembly is internalized into a clathrin-coated pit that assisted by receptor-mediated endocytosis (RME) with a proton-pumping for lowering the pH. At the low pH, near 5, ferric ion is dissociated from Tf and converted to ferrous ion by a putative oxidoreductase in the endosome. The ferrous is then transported out of the endosome by DMT-1 (Gunshin et al., 1997) into the cytosol where it is either utilized for heme synthesis or stored in the iron storage protein ferritin. The TfR-1 and apo-Tf are then recycled by returning to plasma membrane. In the extracellular space, where the pH is 7.4, the affinity of TfR for apo-Tf is immeasurably low (Richardson and Ponka, 1997). Thus, apo-Tf is released and both apo-Tf and TfR can be recycled for another iron transport (Figure 2.13). The entire cycle is complete within a few minutes and 100-200 such cycles are experienced by Tf during its lifetime in the circulation (Gomme and McCann, 2005). Additionally, in several cell types, such as hepatocytes, melanoma cells and some other neoplastic cells, additional non-receptor-mediated mechanisms exist that may also play an important role in the uptake of iron from Tf, possibly by adsorptive pinocytosis (Richardson and Baker, 1992; Trinder et al., 1996). It has been postulated that a surface mechanism of iron uptake from Tf was mediated by a membrane-bound oxidoreductase (a di-ferric Tf reductase). However, the role of this process in iron uptake from Tf remains controversial and requires further investigation (Richardson and Ponka, 1997).

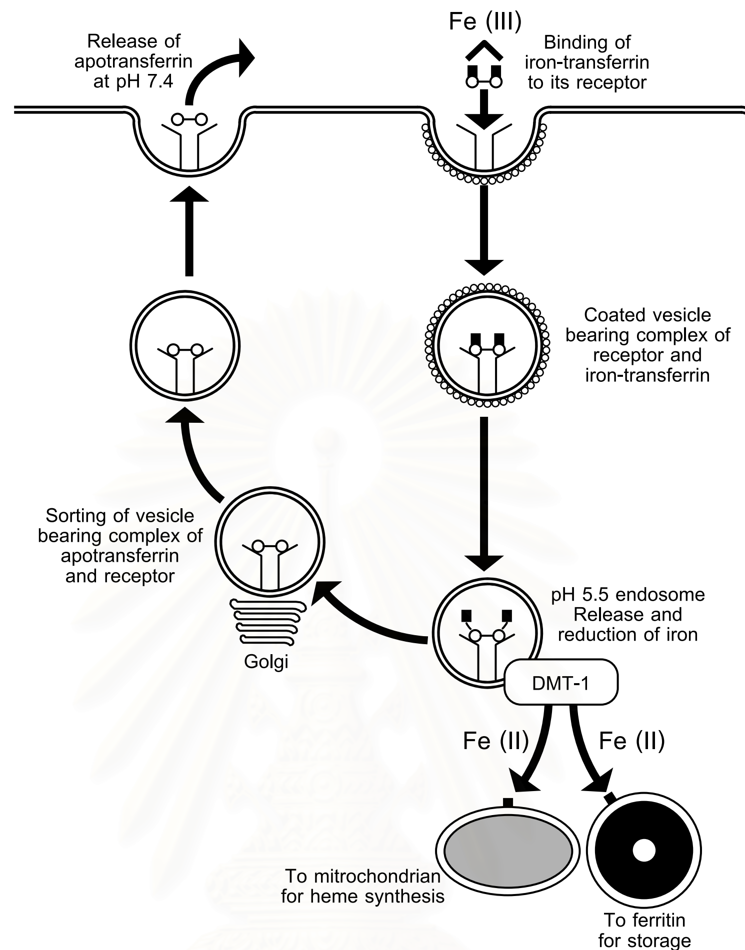

Figure 2.13 Regulation of cellular iron uptake. Two atoms of ferric iron (Fe^{3+}) bind to transferrin, and then this complex binds to the transferrin receptor-1 on the cell membrane. They are internalized into a clathrin-coated pit and formed pH-lowering endosome by a proton-pumping. At the low pH, iron is free from transferrin and then crosses the endosomal membrane as Fe^{2+} via the divalent metal transporter-1 (DMT-1). Iron-depleted transferrin is then returned to the cell surface where, again, encounterring a pH of 7.4, releasing transferrin for another cycle of iron transport (Aisen, 2004).

Although Tf-dependent iron uptake probably predominates under normal circumstances, pathological iron overload leads to supersaturation of Tf iron binding sites and circulating free iron. As Tf becomes saturated in overload states, excess iron is also found as non-transferrin-bound iron (NTBI) and is likely to play an important role in iron overload conditions. Several lines of evidence indicate that NTBI is reduced and transported across cell membrane via DMT-1 (Randell et al., 1994; Hentze et al., 2004).

There is strong evidence that cells must also express a Tf-independent iron transport system. For example, anemic mice and humans that lack of Tf showed iron overload in parenchymal tissues such as liver and spleen (Trenor et al., 2000). Similarly, in hereditary hemochromatosis (HH) even though has the saturation of Tf binding capacity, iron is cleared from plasma by uptake into parenchymal tissues. Furthermore, TfR knockout mice die in an early embryonic period, but there are some developing tissues, suggesting an alternative iron uptake mechanism (Levy et al., 1999). The recent study suggested that the neutrophil gelatinase-associated lipocalin (NGAL/24p3)–mediated iron delivery might be most important for tissue and organ development, at a point before circulation of Tf and expression of TfR is established (Levy et al., 1999). Neutrophil gelatinase-associated lipocalin is created by neutrophils and epithelial cells. It has been implicated in the transport of small organic molecules such as retinol, prostaglandins, fatty acids including iron (Yang et al., 2002). Iron can be complexes by NGAL in a form that is internalized and trafficked to endosomes with acidic pH (Goetz et al., 2002; Kaplan, 2002).

Tissue culture cells also possess a similar capability to uptake inorganic iron free of Tf (Hodgson et al., 1995; Graham et al., 1998). Several reports have characterized the import of iron independent of the Tf-mediated pathway as shown in HeLa cells, fibroblasts, lymphocytes, and bronchial epithelial cells (Kaplan et al., 1991; Inman and Wessling-Resnick, 1993; Conrad et al., 1994; Wang et al., 2002). In one of the best studied cell lines, separated pathways for transports of non Tf ferric and ferrous into tissue cultured cells were demonstrated; erythroleukemia-like K562 cells transport ferric iron by the β3-integrin-mobilferrin pathway or IMP (Conrad et al, 1994). Ferric iron is bound to a cell surface β3-integrin and transferred to a calreticulin-like chaperone protein called mobilferrin (Conrad et al., 1990; Conrad et al., 1993).

Two forms of iron, ferric and ferrous compound, have been used in cell culture studies (Huang et al., 2002; She et al., 2002; Underwood et al., 2003; Juang, 2004; Ohgami et al., 2005; Baldys et al., 2007). Ferric iron is not soluble at physiological pH and must be chelated to remain soluble above pH 3. In the intestine, the chelator mucin is available (Conrad et al., 1991), but in tissue culture systems a chelator must be added, usually citrate or nitrilotriacetic acid, to solubilize the ferric iron. The concentrations of chelators must be controlled or else the chelators both compete with cells for iron and can cause cell damage. Tissue culture cells also directly transport ferrous iron, which is soluble at physiological pH but rapidly oxidized to ferric iron in an aerobic environment. A reducing agent, often ascorbic acid, must be added to maintain transiently the ferrous valence. It is believed that the ferrous pathway in non-intestinal cells is the same as in the intestine and that the iron is

transported via DMT-1 (Gunshin et al., 1997; Conrad et al., 2000).

4. Cellular iron storage and export

Once iron enters the cell, the portion that is not needed for immediate use is stored by ferritin, a spherical protein cage which can accommodate up to 4500 iron atoms (Theil, 2003). In vertebrates the ferritin molecule is assembled from 24 polypeptide subunits of two ferritin types, heavy- (H) and light- (L) ferritin. The two subunits appear to have different functions in iron metabolism. H-ferritin has been linked to iron utilization, while L-ferritin is more closely associated with cellular iron storage (Levenson and Tassabehji, 2004). The ratio of H to L subunits varies depending on tissue type, physiologic status and changes in response to inflammation and infection. Ferritin has enzymatic properties, converting Fe^{2+} to Fe^{3+} as iron is internalized and packed into the mineral core. Degradation of ferritin and concomitant iron release helps to mobilize iron for cellular utilization. Little is known about how ferritin is degraded, but both lysosomal and proteosome pathways have been implicated (Hentze et al., 2004). The storage of iron in this molecule protects the cells from the damaging effects of free iron and also keeps it sequestered in a bioavailability form.

There is evidence to indicate that iron is also released from non-intestinal cells, and this process is accelerated by the serum ferroxidase ceruloplasmin (Cp). Ceruloplasmin converts Fe^{2+} to Fe^{3+} that is loaded onto Tf for transport in the plasma (Levenson and Tassa-behji, 2004).

5. Regulation of cellular iron metabolism

Proteins involved in iron uptake, storage, utilization, and export must be regulated in a coordinated fashion. The signals and regulatory mechanisms that orchestrate their expression involve modulation of transcription, mRNA stability, translation, and posttranslational modifications (Hentze et al., 2004). Iron responsive elements (IREs) regulate the stability and translation of a number of mRNAs that code for proteins involved in iron metabolism such as both the heavy (H) and light (L) subunits of the iron storage protein ferritin, the TfR, DMT-1, and FPN-1. Iron responsive elements are found in the 3' or 5'-untranslated region (UTR) of these iron-regulated mRNAs (Gunshin et al, 1997; Canonne-Hergaux et al, 1999). There is a IRE sequence producing a stem-loop or hairpin structure (Figure 2.14), there appears to be heterogeneity in these sequences, permitting functional diversity of IRE function (Gdaniec et al., 1998; Ke et al., 1998). The location of the IRE, in either the 3' or 5' sequence of the mRNA determines the effect of iron on protein translation. IRE function is conferred by the presence of iron regulatory proteins (IRP) that bind to the hairpin structures in iron-responsive mRNAs. Under conditions of low iron availability, IRPs bind to IREs. When an IRP is bound to IRE in the 5'-UTR of an mRNA, translation is blocked. Conversely, if the IRE is localized to the 3'-UTR, binding enhances mRNA stability and results in increased translation. Two examples of this differential regulation by IRE–IRP is the synthesis of the iron storage protein ferritin and the iron uptake receptor, TfR (Figure 2.15). Low iron availability (-Fe) results in binding of IRPs to the five IREs in the 3'-UTR of TfR mRNA. This increases TfR mRNA stability increases translation. Thus, cellular iron

uptake is increased. At the same time, IRPs bind to the single 5'-UTR on ferritin mRNA, blocking its translation and reducing the capacity for iron storage. Conversely, increases in iron (+Fe) result in the formation of an iron–sulfur cluster that binds the IRPs and prevents IRP binding to IREs (Figure 2.15). The end result would be to decrease iron uptake and increase iron storage under conditions of high iron availability.

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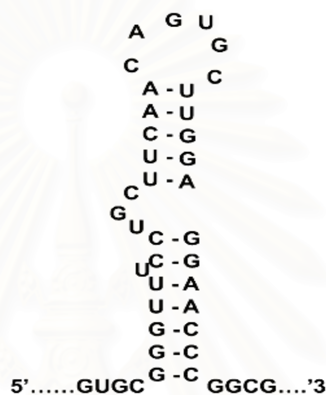

Figure 2.14 Consensus sequence and representation of the stem-loop structure of a human iron responsive element (IRE). Structures similar to this are found in the 3' and 5'-UTR of iron-responsive mRNAs and function to regulate mRNA stability and translation (Levenson and Tassabehji, 2004).

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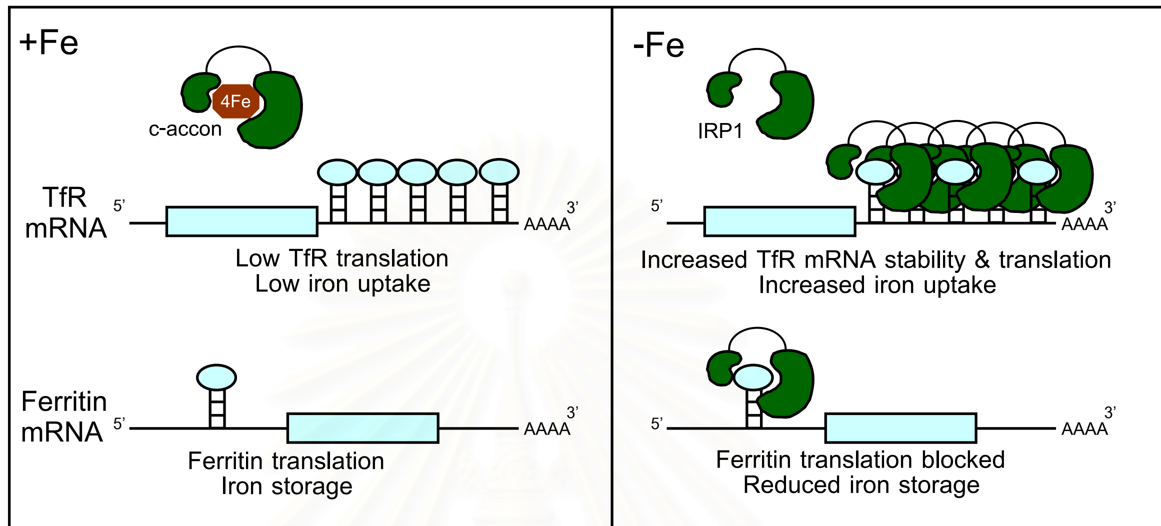

Figure 2.15 Molecular regulations of iron-responsive mRNAs. Regulation of the TfR and ferritin mRNAs by high (+Fe) and low (-Fe) iron availability. In +Fe conditions, iron associates with iron regulatory proteins (IRP; IRP-1 and IRP-2) in an iron–sulfur cluster that confers acconitase activity to IRP1. The resulting cytosolic acconitase (c-accon) is rendered unable to bind IREs permitting translation of the iron storage protein ferritin and inhibiting translation of TfR. Under conditions of low iron availability (-Fe), IRP conformation permits binding to IREs, enhancing the stability and translation of TfR mRNA and blocking the translation of ferritin mRNA (Levenson and Tassabehji, 2004).

IRON AND MMPs

Previous study has shown that non-cytotoxic doses (5-25 uM) of iron can stimulate collagen synthesis and higher doses of iron induced proliferation in hepatic stellate cells. Moreover, iron treatment increases MMP-2 activity, without affecting MMP-1 activity (Gardi et al, 2002). One study has shown significant changes in serum TIMP-1 and MMP concentrations in haemochromatosis and may contribute to hepatic fibrosis with progressive iron loading (George et al, 1998). The study focused on the involvement of ferrous/ferric iron on the role in mediating the UVB response and the iron–driven generations of $HO^{\cdot}$ and lipid peroxides were identified as early events in the downstream signaling pathway after UVB irradiation, and leading to the induction of MMP-1 and MMP-3 on RNA levels in cultured human dermal fibroblasts (Brenneisen et al, 1998). In 2001, Wenk reported that human dermal fibroblast were exposed to ferric citrate resulting in increased induction of MMP-9 as determined by enzyme-linked immunosorbent assay (ELISA) and this induction was completely suppressed by deferoxamine (Wenk et al, 2001). Recent study reported that the increased iron deposition in legs affected by chronic venous disease leading to iron release in the serum and the iron-driven pathway as a mechanism for MMP-9 hyper-expression leading to tissue lesion (Zamboni et al, 2005). Furthermore, treatment of murine macrophages with increasing concentrations of ferric ammonium citrate significantly enhanced the amount of MMP-9 secreted dose dependently, supporting the role of iron as a positive regulator of MMP-9 (Lee et al, 2003).

IRON AND CANCER

There is a growing body of evidence that suggests a role of iron in carcinogenesis and the malignant progression of cancers. Iron has been shown to favor neoplastic cell growth and display carcinogenic activity (Toyokuni, 1996; Weinberg, 1999; Huang, 2003). This is illustrated by the fact that a common complication of hereditary hemochromatosis is the development of hepatocellular carcinoma and other forms of cancer including esophageal, skin melanoma, and acute myeloid leukemia (Papanikolaou and Pantopoulos, 2005). Iron exerts its toxicity through a series of reactions with ROS called the modified Haber-Weiss or Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H_2O_2^{-} \rightarrow HO^{\cdot} + HO^{-}$), generating the highly toxic $HO^{\cdot}$, which represents one of the most important mechanisms in various pathological conditions such as cirrhosis, coronary heart disease, congestive heart failure. Hydroxyl radical can lead to DNA damage and impairment of normal DNA and protein synthesis and cell proliferation and thus has been thought to be causally involved in the multistep process of carcinogenesis (Toyokuni, 1996; Huang, 2003). However, the mechanisms for iron-induced neoplastic transformation are poorly characterized. It is clear that iron overload disrupts the redox balance of the cell and generates chronic oxidative stress, which modulates signaling networks related to malignant transformation (Benhar et al., 2002). Overload of iron leads to excess ROS causing oxidative stress and resulting in DNA damage and changes in gene expressions (Valko et al., 2006). Signal cascades stimulated by metal ions via ROS regulated by two distinct protein families, the MAPKs and

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the signaling factors that use cysteine motifs as a redox sensitive such as thioredoxin and redox factor1 (Ref-1) leading to activation of several redox-regulated transcription factors; AP-1, NF-κB, and hypoxia-inducible factor1 (HIF-1) (Valko et al., 2006).

Numerous laboratory experiments and epidemiological studies over the past few decades have observed that body iron stores and dietary iron intake have both been shown to be positively associated with subsequent risk of cancers such as colon cancer (Nelson, 1992; Wurzelmann et al, 1996;), colorectal cancer (Nelson et al., 1994; Labropoulou et al., 2004), lung cancer (Knekt, 1994), prostate cancer (Juang, 2004) and esophageal cancer (Chen et al., 1999). In addition, a significant association between high serum ferritin levels and neoplasia was reported in several studies (Marcus and Zinberg, 1975; Ola et al., 1995). In epidemiological studies, over-expression of TfR has been qualitatively described for various cancer (Hogemann-Savellano et al, 2003) and elevated TfR content in human prostate cancer cell lines (Keer et al., 1990). Furthermore, recent evidence has suggested that increased body iron stores as, indicated by high percentages of Tf saturation, and may be associated with an increased risk of cancer and cancer mortality (Glei et al., 2002; Mainous et al., 2005). Furthermore, increased body iron stores (as judged from serum ferritin level, Tf concentration, or Tf saturation) were associated with poor prognosis of several human malignant neoplasms (neuroblastoma, childhood Hodgkin's diseases, and acute lymphocytic leukemia). Besides, Toyokuni and Sagripanti demonstrated that ferric citrate efficiently induces oxidative single- and double-strand breaks in the plasmid DNA *in vitro* (Toyokuni and Sagripanti, 1993).

For animal studies, in 1959, Richmond reported for the first time that iron compound induce malignant tumors in animals. Repeated intramuscular injections of iron-dextran complex (widely used for the treatment of iron-deficiency anemia in adults and infants) caused spindle cell sarcoma or highly pleomorphic sarcoma in rats at the site of injections. He suggested that his observation was related to a high incidence of hepatoma following on the liver cirrhosis associated with hemochromatosis and that carcinogenic mechanism may be by blocking the antioxidant activity of vitamin E (Richmond, 1959). In 1982, Okada and Midorikawa first showed that an iron compound can induce malignant tumors at sites different from injections (Okada and Midorikawa, 1982). Yamada et al., reported that exposure of a rat liver epithelial cell line (RL34) to ferric nitrilotriacetate (Fe-NTA) induced extremely rapid neoplastic transformation (Yamada et al., 1990).

Moreover, tumor growth is enhanced by iron as observed in cell culture (Hann et al., 1990), animal (Siegers et al., 1992) and human studies (Edling, 1982; Mandishona et al., 1998). Many tumors readily take up iron as seen by the higher iron contents found in many premalignant and malignant tissues compared with that in normal tissue (Gorodetsky et al., 1986; Bonkovsky, 1991; Durak et al., 1994; Labropoulou et al., 2004). Base on the probable role of iron in tumor development, iron withdrawal strategies have been investigated in the management of human tumors (Donfrancesco et al, 1990; Weinberg, 1996). Iron chelators have been shown to inhibit growth of malignant cell lines from neuroblastoma (Becton and Bryles, 1988), hepatoma (Hann et al., 1990), Kaposi's sarcoma (Simonart et al., 2000), cervical carcinoma (Simonart et al., 2002), and oral squamous cell carcinoma (Lee et al.,

2006). Therefore overloaded of iron in the body involves in cancer in two major aspects. Firstly, iron can catalyze the production of ROS, and these species may be proximate carcinogens that involved in cancer initiation. Secondly, iron may be involved in cancer promotion by facilitating cancer growth and modulating local immune response by modifying immune equilibrium (Toyokuni, 1996; Weinberg, 1996; Huang, 2003).

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CHAPTER III
MATERIALS AND METHODS

1. Cell culture and cell treatment

OM-2, human gingival squamous carcinoma cell line, derived from metastatic site and HN-22, human epiglottic squamous carcinoma cell line were kindly provided by Professor Teruo Amagasa (Tokyo Medical and Dental University, Tokyo, Japan) and Professor Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institute of Health; NIDCR, NIH, USA), respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml amphotericin B at 37 °C in humidified atmosphere of 95% air, 5% CO_2. Medium and supplements were from GibcoBRL (Grand Island, NY, USA). Cells were seeded at $5x10^4$ cells per well of 24 well plate or $3x10^5$ cells per well of 6 well plate and used for various experimental purposes. Cells were treated with 0-50 μg/ml ferric ammonium citrate, FAC (Sigma, St. Louis, MO, USA), in serum free medium (SFM) for 24 hours to find the non-toxic dose for determining the effect of FAC on MMP-9 expression. A neutralizing antibody against human transferrin receptor (TfR) (Calbiochem, CA, USA), holo-transferin or apo-transferrin (Sigma, St. Louis, MO, USA) was added in the presence or absence of FAC for 24 hours to clarify the iron uptake pathway. To elucidate the iron regulatory pathway, cells were treated for 1 hour with either ERK1/2, p38 kinase, JNK, PI3K or Akt inhibitor (Calbiochem, CA, USA) and cultured in the presence

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or absence of FAC for 24 hours. Conditioned media were collected for analysis of MMP-9 by gelatin zymography and enzyme-linked immunosorbent assay (ELISA). Cells were collected for RT-PCR and Western blot analyses. Nuclear protein was extracted for electrophoretic mobility shift assay (EMSA).

2. Cytotoxicity test (MTT assay)

The cytotoxicity of FAC were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Denizot and Lang, 1986). This assay bases on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystal formation is proportional to the number of viable cells. Before terminating the experiment, the culture medium was aspirated, replaced with 0.5 µg/ml MTT solution and incubated for 10 minutes in the CO_2 incubator. The solution was then aspirated and 900 µl of dimethyl sulfoxane (DMSO) containing 100 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10) was added to dissolve the formazan crystals. Finally, after 10 minutes of rotary agitation, the absorbance of the solution at 540 nm was measured using Genesis10 UV-vis spectrophotometer (Thermo Spectronic, NY, USA). The viable cell number was calculated from the standard curve of cell number by plotting a scattergram of the absorbance value against the known number of cell.

3. Gelatin zymography

Secreted MMP-9 was analyzed by gelatin zymography (Heussen and Dowdle, 1980). Briefly, conditioned medium were collected and applied to 10% sodium dodesylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1% gelatin (Sigma, St. Louis, MO, USA). The loaded amount of conditioned medium was normalized according to the cell number analyzed by MTT assay. Electrophoresis was performed at a constant 125 volts for 90 minutes at room temperature. After electrophoresis, gels were incubated 10 minutes for three times with 2.5% Triton X-100 and then in buffer containing 50 mM Tris, 10 mM $CaCl_2$, 0.15 M NaCl, and 0.02% Briji-35 at 37 °C overnight. Finally, gels were stained with 0.1% Coomassie Brilliant Blue R250 in 7.5% acetic acid and 12.5% methanol for 30 minutes, and then destained in 7.5% acetic acid and 5% methanol until transparent bands were visible on dark blue background. The conditioned medium from U2OS, human osteosarcoma cell line, was used as known containing proMMP-9. Quantitative analysis of band density was performed using Scion Image analysis software (Scion, Frederick, MA, USA).

4. Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of MMP-9 in the conditioned medium was measured in duplicate using a commercialized ELISA kit, which recognized both human pro- and active- forms of MMP-9, according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN, USA). This

assay employed the quantitative sandwich enzyme immunoassay technique. The monoclonal antibody specific for MMP-9 has been pre-coated onto a microplate. Standards and samples were loaded into the wells, and MMP-9 was bound by immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for MMP-9 was added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells and the color developed in a proportion to the amount to total MMP-9 bound in the initial step. The color development was stopped and the intensity of the color was measured. Briefly, 100 µl of the diluent was added to each well and then 50 µl of standard or sample was added. After 2 hours of incubation at room temperature with constant shaking (500 ± 50 rpm), the reaction solution was discarded and the wells were washed 4 times with washing buffer. Polyclonal antibody against MMP-9 conjugated to horseradish peroxidase was then added to each well and incubated for another 1 hour on the shaker at room temperature. The solution was discarded and the wells were washed for 4 times. After that 200 µl of substrate solution was added to each well and the microplate was allowed to stand for 30 minutes at room temperature in the dark. After addition of 50 µl of stop solution, the optical density at 450 nm was measured by using a Genesis10 UV-vis spectrophotometer (Thermo Spectronic, NY, USA). The MMP-9 concentration was calculated from the standard curve of recombinant human MMP-9 provided by the manufacturer.

5. RNA extraction

Total RNA was extracted from cells using TRI REAGENT (MRC, Cincinnati, OH, USA) according to the manufacturer's instruction. Cells were lysed directly in a culture plate by adding 1 ml of TRI REAGENT in each well of 6-well plate and passing the cell lysate several times through a pipette tip. One milliliter of the homogenate was transferred to 1.5 ml microfuge tube and was kept for 5 minutes at room temperature allowing the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added into the homogenate. Microfuge tube was capped tightly and shaken vigorously for 15 seconds. The mixture was kept at room temperature for 2-15 minutes and then centrifuged at 12,000xg for 15 minutes at 4 °C. After centrifugation, the mixture was separated into the lower red of the phenol-chloroform phase, the white plaque of the interphase, and the upper colorless of the aqueous phase. RNA remains exclusively in the aqueous phase, whereas DNA and protein are in the interphase and phenol-chloroform phase. The aqueous phase was transferred to a fresh tube. RNA was precipitated by addition of an equal volume of isopropanol, incubation at room temperature for 5-10 minutes and centrifugation at 12,000xg for 8 minutes at 4 °C. Precipitated RNA formed a gel-like or white pellet on the bottom of the tube. The supernatant was removed and RNA pellet was washed with 1 ml of 75% ethanol. Ethanol was then removed by centrifugation at 7,500xg for 5 minutes at 4 °C. RNA pellet was briefly air-dried and dissolved in RNase-free water. RNA concentration was determined by measuring the optical density at 260 nm with a Genesis10 UV-vis

spectrophotometer (Thermo Spectronic, NY, USA). The quality of the RNA can be evaluated from the ratio of the absorbance at 260/ 280 being greater than 1.8.

6. **Reverse transcription polymerase chain reaction (RT-PCR)**

One microgram of total RNA was used to generate complementary DNA (cDNA) by reverse transcription reaction using AMV reverse transcriptase and oligo dT (Promega, Madison, WI, USA). The reaction was incubated at 42 °C for 90 minutes and then incubated at 99 °C for 2 minutes. Complementary DNA was amplified using Taq polymerase (Qiagen, Hilden, Germany). The thermal cycling condition comprised initial denaturation at 95 °C for 5 minutes, followed by denaturation at 94 °C for 30 seconds, annealing at 60 °C for 45 seconds, and extension at 72 °C for 90 seconds. Last cycle was followed by additional 10 minutes extension incubation at 72 °C. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a loading control. Complementary DNA was amplified 24 cycles for GAPDH, 26 cycles for Tf and TfR-1, 30 cycles for DMT-1 and TIMP-1, and 35 cycles for MMP-9. To amplify the cDNA fragments, the sequences of PCR primers were as follow: MMP-9 sense 5'-GACTCGGTCTTTGAGGAGCC-3', MMP-9 antisense 5'-GAACTCACGCGC CAGTAGAA-3', product size 263 bp; TIMP-1 sense 5'-AGTCAACCAGACCACCTTATACCA-3', TIMP-1 antisense 5'-TTTCAGAGCCTTGGAGGAGCTGGTC-3', product size 362 bp; Tf sense 5'-ATGTGGCCTTTGTCAAGCACT-3', Tf antisense 5'-GCTCATCATACTGGTCCCTGT CA-3', product size 450 bp; TfR-1 sense 5'-AAAATCCGGTGTAGGCACAG-3', TfR1

antisense 5'-CCTTAAATGCAGGGACGAA-3', product size 263 bp; DMT-1 sense 5'GGAGCAGTGGCTGGATTTAAGT-3', DMT-1 antisense 5'-CCACTCCCAGTCTAGCTGCA A-3', product size 391 bp ; GAPDH sense 5'-TGAAGGTC GGAGTCAACGGAT-3', and GAPDH antisense 5'-TCACACCCATGACGAACATGG-3', product size 376 bp. Analysis of amplicons was accomplished on 2% agarose containing ethidium bromide and visualized under UV transilluminator (UVtec, Cambridge, UK). The band intensity was determined by Scion Image analysis software (Scion, Frederick, MA, USA).

7. Western blot analysis

Cells were lysed in PhosphoSafe extraction reagent (EMD Biosciences, Madison, WI, USA) according to the manufacturer's instruction. The conditioned medium was aspirated from cells and cells were rinsed once with phosphate buffered saline (PBS). Then, 100 µl of PhosphoSafe extraction reagent was added in each well of 6-well plate and incubated for 5 minutes at room temperature. Extract was transferred to 1.5 ml microfuge tube and centrifuged at 16,000xg for 5 minutes at 4 °C to pellet cellular debris. Supernatant was transferred to a fresh tube and stored at -70 °C. Total protein was determined by using the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein extract from each sample was separated by 12% SDS-PAGE at 20 mA for 60 minutes at room temperature and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA) at 15 volts for 2 hours at room temperature. The membrane was blocked in 5% skim milk,

0.1% Tween-20 at room temperature for 1 hour. Then, the membrane was incubated overnight with rabbit anti- human phospho ERK1/2 polyclonal antibody or mouse anti-human ERK1/2 monoclonal antibody (R&D System, Minneapolis, MN, USA) and rabbit anti-human phospho Akt (ser473) polyclonal antibody or rabbit anti-human Akt polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) at the dilution of 1:1000 in the blocking buffer at 4 °C. After extensive washing with PBS, the membrane was incubated with biotinylated goat anti-rabbit or goat anti-mouse antibody (Sigma, St. Louis, MO, USA) for 30 min at room temperature and peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA, USA) for 30 min, respectively. The protein bands were detected using SuperSignal West Pico chemiluminescence (Pierce, Rockford, IL, USA) and were exposed on CL-X Posture film (Pierce, Rockford, IL, USA). The band intensity was determined by Scion Image analysis software (Scion, Frederick, MA, USA). Actin served as internal control for normalization purpose.

8. Nuclear protein extraction

Nuclear extract was prepared by using NU-PER nuclear extract kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. The conditioned medium was removed and cells were rinsed with cold PBS. Cells were harvested in 500 µl of PBS, transferred into 1.5 ml microfuge tube and centrifuged at 2,000xg for 5 min at 4 °C. The supernatant was discarded and the pellet was dried. Hundred microliter of ice-cold CER I was added to the

cell pellet and then vortexed vigorously for 15 seconds to fully resuspend the cell pellet. The resuspension was later incubated on ice for 10 minutes. Then, 5.5 µl of ice-cold CER II was added up with vigorous shaking for 5 seconds. The mixture was placed on ice for 1 minute. After that, the mixture was vortexed vigorously for 5 seconds and centrifuged at 16,000xg for 5 minutes. The supernatant was removed and the insoluble pellet, which contained nuclei, was dissolved in 50 µl of ice-cold NER by vortexing for 15 seconds. The sample was vertexed for 15 seconds and placed on ice for 10 minutes alternately, for a total of 40 minutes. The tube was then centrifuged at 16000xg for 10 minutes at 4 °C and the supernatant, which contained the nuclear extract, was transferred to a clean pre-chilled tube. Nuclear protein concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL, USA) and stored at -70 °C.

9. Electrophoretic mobility shift assay (EMSA)

AP-1 and NF-κB binding activities were examined by EMSA using LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). Briefly, the AP-1 binding site of MMP-9 promoter (5'CGCTTGATGACTCAGCCGGAA3') (Squarize et al., 2006) and the NF-κB binding site of MMP-9 promoter (5'AGTTGAGGGGACTTTCCCAGGC3') (Takahra et al., 2004) were labeled with biotin. Each the binding reaction was performed in the binding buffer containing 2.5% glycerol, 5mM $MgCl_2$, 50ng/µl Poly dI-dC, 0.05% NP-40, nuclear extract and 20 fmol of labeled probe, and incubated for 20 minutes at room temperature.

Specific binding was controlled by competition with 100-fold excess of non-labeled AP-1 or NF-κB oligonucleotide probe. DNA-protein complex was run on a non-denaturing 6% polyacrylamide gel in 0.5xTris borate-EDTA (ethylenediamine tetraacetic acid) (TBE) buffer at 100 volts for 1 hour at 4 °C, and transferred to a positive charged nylon membrane (Whatman, Schleicher & Schuell Bioscience, NH, USA) at 100 volts for 30 minutes at 4 °C. DNA-protein complex was crosslinked to the membrane with UV transilluminator (UVtec, Cambridge, UK) for 20 minutes. Membrane was blocked in blocking buffer for 15 minutes with gentle shaking and incubated with horseradish peroxidase conjugated streptavidin for 15 minutes at room temperature. After four-time washing with wash solution, biotin-labeled oligonucleotide was detected by chemiluminescence on CL-X Posture film (Pierce, Rockford, IL, USA).

10. Statistical analysis

All experiments were repeated at minimum of three times. All data collected from MTT assay, gelatin zymography, ELISA, RT-PCR and Western blot analyses were expressed as mean ± S.D.. The data presented in some figures were from a representative experiment, which was qualitatively similar to the replicated experiments. Statistical significance was determined with independent *t*-test of triplicate. SPSS 10.0 was used for all statistical analysis. Difference at $p < 0.05$ was considered statistical significance.

CHAPTER IV
RESULTS

FAC increased MMP-9 expression in OM-2 and HN-22 cells

OM-2 and HN-22 cells were cultured for 24 hours in various concentrations of FAC ranging from 0-50 µg/ml and the cytotoxicity of FAC were determined by MTT assay. As shown in Figure 4.1, FAC at 50 µg/ml was cytotoxic to both cell lines. Figure 4.2A, gelatin zymography demonstrated that OM-2 and HN-22 cells constitutively expressed MMP-9. Stimulation with non-toxic dose of FAC increased MMP-9 expression in a dose-dependent manner with the significant response at 15 and 25 µg/ml; however, the effect on the enzymatic activation was not detected. Increase of MMP-9 expression by FAC was regulated at the transcription level as demonstrated by RT-PCR (Figure 4.2B). Figure 4.3, ELISA demonstrated that FAC increased MMP-9 expression in a dose-dependent manner and consistent with the results from gelatin zymography and RT-PCR (Figure 4.2A and B). Since, the MMP-9 activity could be blocked by TIMP-1; therefore the expression level of TIMP-1 in HN-22 was determined by RT-PCR as shown in Figure 4.4. There was no change in TIMP-1 expression when treated with15 µg/ml of FAC.

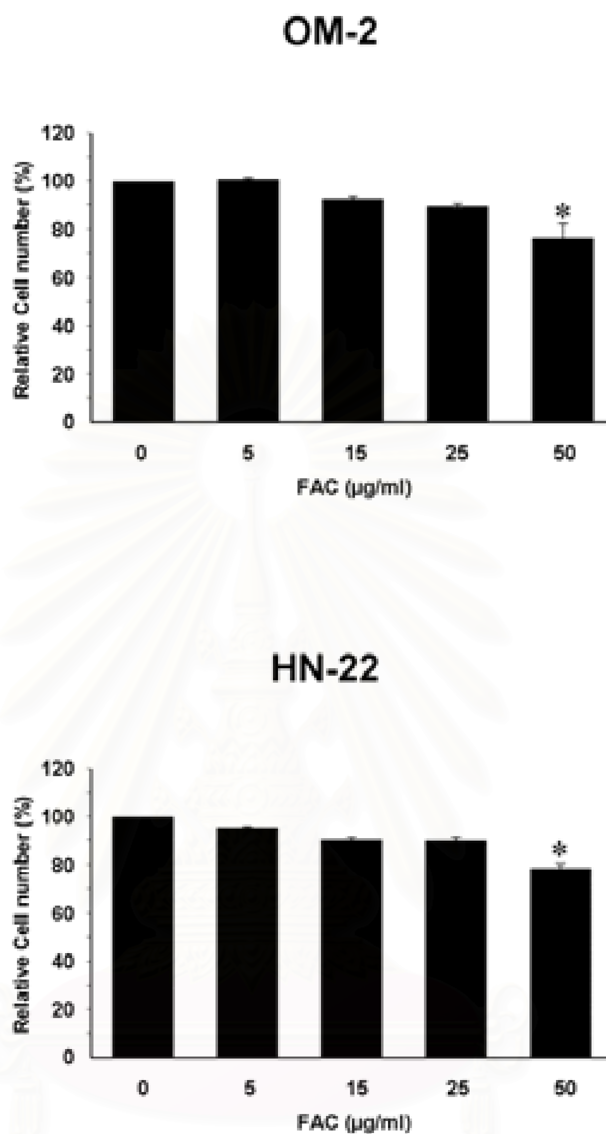

Figure 4.1 The cytotoxicity of FAC on HN-22 and OM-2 cells. Cells were treated with 0, 5, 15, 25, 50 μg/ml FAC for 24 hours and the viable cell number was determined by MTT assay. Histograms represent average percentage of cell number relative to that of the untreated (0 μg/ml) FAC from three independent experiments which performed in triplicate. * Indicate significant difference from untreated FAC (p<0.05).

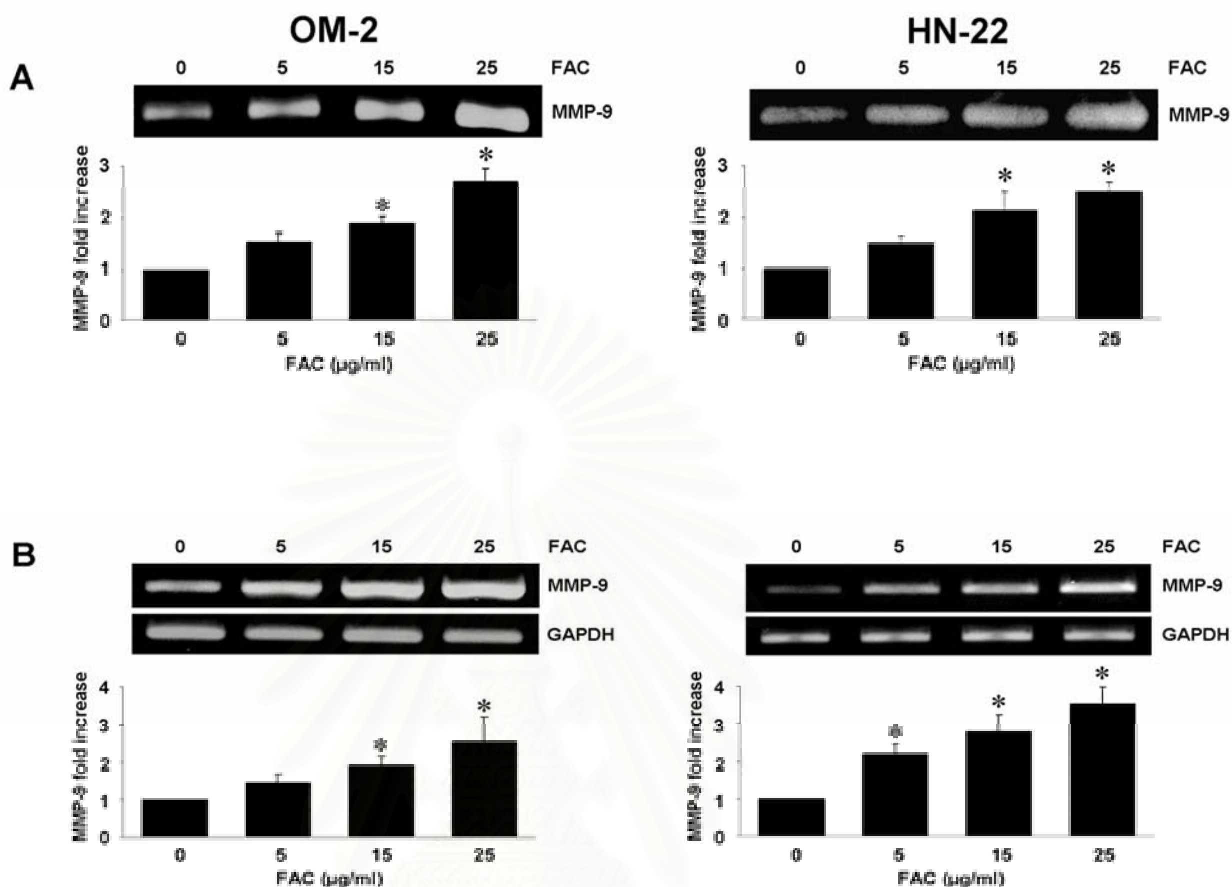

Figure 4.2 FAC increased MMP-9 expression in OM-2 and HN-22 cells analyzed by gelatin zymography and RT-PCR. Cells were treated with 5, 15, 25 µg/ml FAC for 24 hours. Conditioned media were subjected to gelatin zymography (A) and cells were extracted for RT-PCR (B). Amount of protein loading was normalized to the cell number which determined by MTT. Levels of MMP-9 mRNA were normalized to GAPDH. Histograms represent the average fold-increase of MMP-9 expression relative to that of the untreated (0 µg/ml) FAC from three independent experiments which performed in triplicate. * Indicate significant difference from untreated FAC (p<0.05).

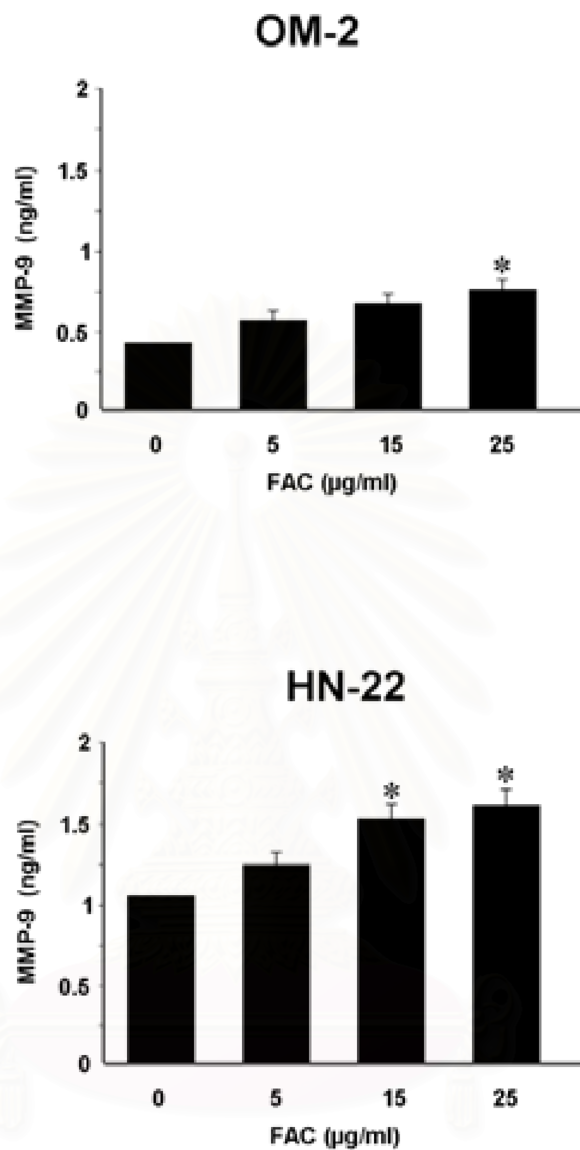

Figure 4.3 FAC increased MMP-9 expression in OM-2 and HN-22 cells analyzed by ELISA. Cells were treated with 5, 15, 25 µg/ml FAC for 24 hours. Conditioned media were subjected to ELISA. Histograms represent the average levels of total MMP-9 (ng/ml) from three independent experiments which performed in triplicate. * Indicate significant difference from untreated FAC (p<0.05).

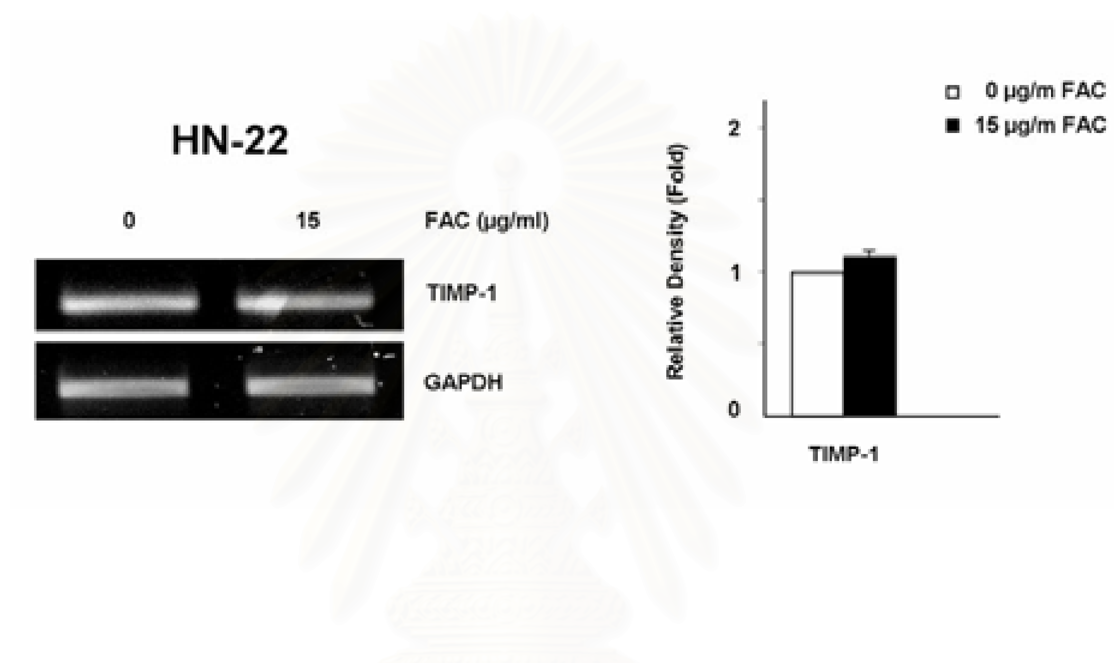

Figure 4.4 Effect of FAC on TIMP-1 expression in HN-22 cells. Cells were treated with 15 μg/ml of FAC for 24 hours. Cells were then extracted for RT-PCR. Levels of TIMP-1 mRNA were normalized to GAPDH. Histograms represent the average fold-increase of TIMP-1 expression relative to that of the untreated (0 μg/ml) FAC from three independent experiments which performed in triplicate. * Indicate significant difference from untreated FAC (p<0.05).

The effect of FAC on the expression level of Tf, TfR-1, and DMT-1

Transferrin, TfR-1, and DMT-1 are the main proteins involved in cellular iron uptake (Dunn et al., 2006). The expressions of Tf, TfR-1, and DMT-1 in HN-22 cells were determined by RT-PCR as shown in Figure 4.5. Treatment with 15 µg/ml of FAC increased Tf, and DMT-1 mRNA levels, but the expression of TfR-1 was decreased by FAC stimulation.

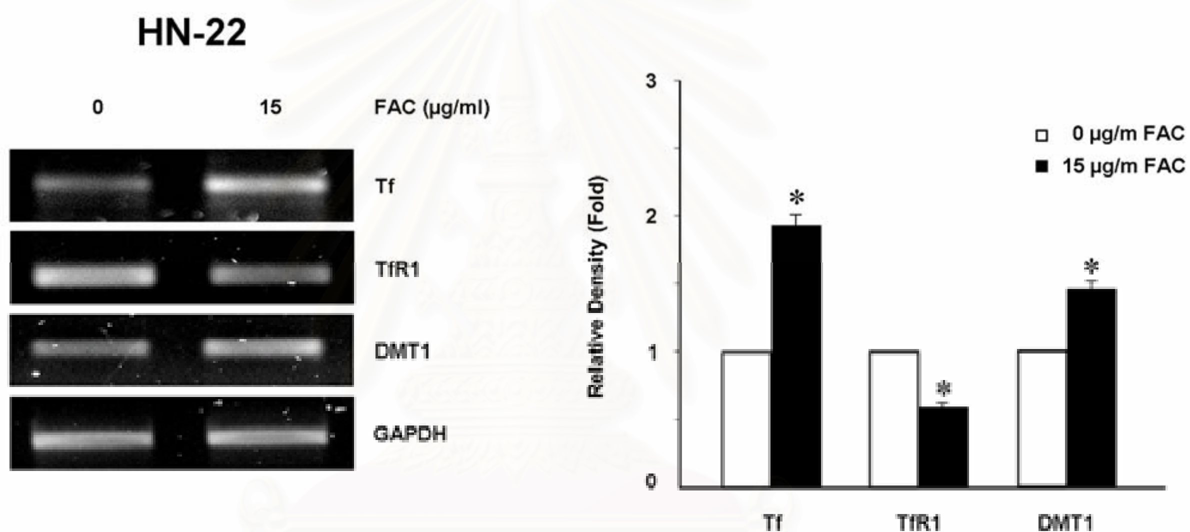

Figure 4.5 Effect of FAC on Tf, TfR-1 and DMT-1 expressions in HN-22 cells. Cells were treated with 15 µg/ml of FAC for 24 hours. Cells were then extracted for RT-PCR. Levels of Tf, TfR-1, and DMT-1 mRNA were normalized to GAPDH. Histograms represent the average fold-increases of TIMP-1, Tf, TfR-1, and DMT-1 expression relative to that of the untreated (0 µg/ml) FAC from three independent experiments which performed in triplicate. * Indicate significant difference from untreated FAC (p<0.05).

HN-22 cells responded to FAC using a non-transferrin dependent pathway

Uptake of iron by cells has been reported using two main different pathways; transferrin dependence and non-transferrin dependence (Hodgson et al., 1995). Transferrin is a class of related iron-binding glycoprotein for transporting iron to various organs and tissues in the body. There are two forms, holo-transferrin or iron-loaded transferrin and apo-transferrin or non iron-loaded transferrin, and both forms of transferrin can bind with transferrin receptor-1 (TfR-1) with higher affinity in holo-transferrin (Qian et al., 1997). Thus, holo-transferrin, apo-transferrin, and neutralizing antibody against human transferrin receptor (TfR Ab) were used to evaluate the iron uptake pathway. HN-22 cells were treated with holo-transferrin, apo-transferrin or neutralizing antibody against TfR-1 in the presence or absence of FAC for 24 hours. Treatment with holo-transferrin, apo-transferrin, and neutralizing antibody against TfR-1 had no toxic effect on cells at the ranging from 0 to 1000 μg/ml, 0 to 1000 μg/ml, and 0 to 40 μg/ml, respectively as determined by MTT assay (Figure 4.6A-B, and 4.7). Application of holo-transferrin and apo-transferrin at concentrations ranging from 10-1000 μg/ml (recommendation dose of both transferrins in cell culture are 25 μg/ml) alone could not affect on the level of MMP-9 expression in HN-22 cells as shown in Figure 4.8A and B. Moreover, application of apo-transferrin in the presence of FAC could not increase the level of MMP-9 expression compared to FAC treatment alone as shown in Figure 4.8B. Treatment of HN-22 with neutralizing antibody against TfR-1 up to 40 μg/ml (recommendation dose is 1-2 μg/ml) has not reduced FAC-induced MMP-9 expression

(Figure 4.9). These findings suggested that HN-22 cells responded to FAC using a non-transferrin dependent pathway for the regulation of MMP-9.

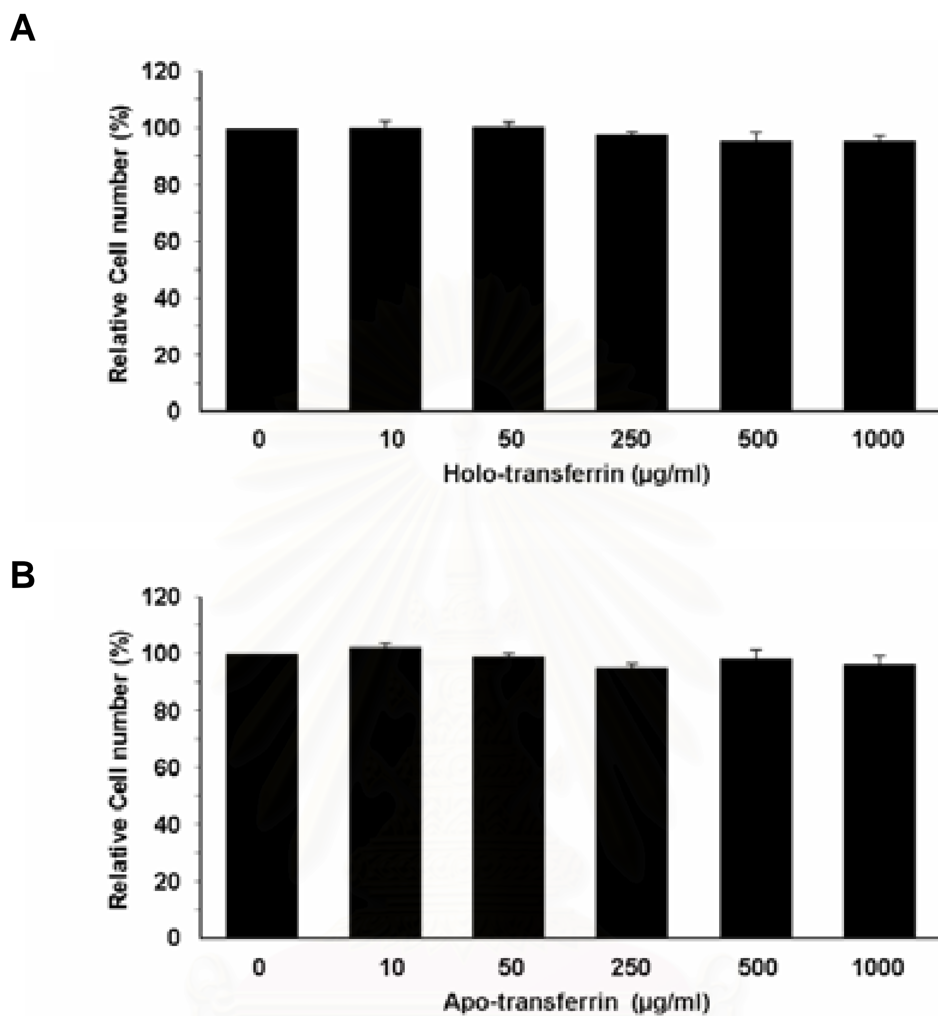

Figure 4.6 The effect of holo-transferrin and apo-transferrin on viability of HN-22 cells. Cells were treated with 0, 10, 50, 250, 500, 1000 μg/ml holo-transferrin (A) or apo-transferrin (B) for 24 hours and the viable cell number was determined by MTT assay. Histograms represent average percentage of cell number relative to that of the untreated (0 μg/ml) from three independent experiments which performed in triplicate.

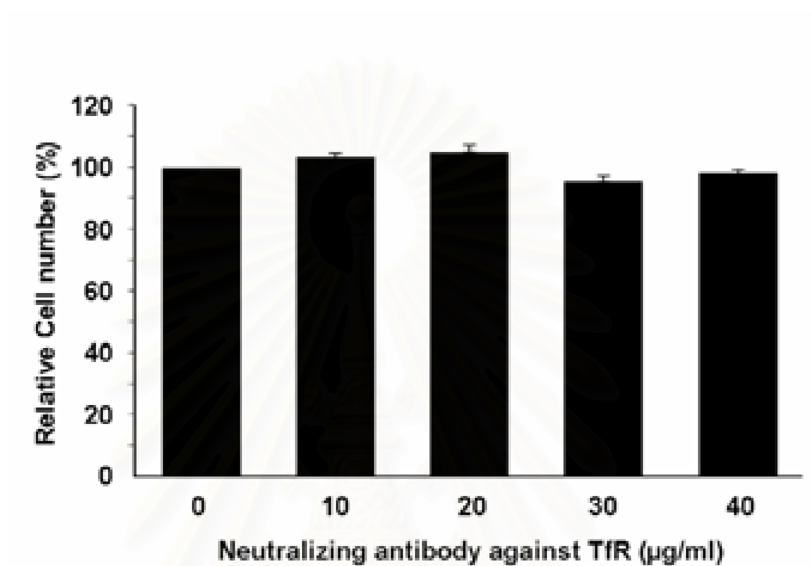

Figure 4.7 The effect of neutralizing antibody against human transferrin receptor (TfR) on viability of HN-22 cells. Cells were treated with 0-40 µg/ml neutralizing antibody against human transferrin receptor for 24 hours and the viable cell number was determined by MTT assay. Histograms represent average percentage of cell number relative to that of the untreated (0 µg/ml) FAC from three independent experiments which performed in triplicate.

Figure 4.8 HN-22 cells responded to FAC by using non-transferrin dependent pathway. HN-22 cells were treated with holo-transferrin (A) or apo-transferrin (B) in the presence (+) or absence (-) of FAC for 24 hours. Conditioned media were subjected to gelatin zymography and amount of protein loading was normalized to the cell number which determined by MTT assay.

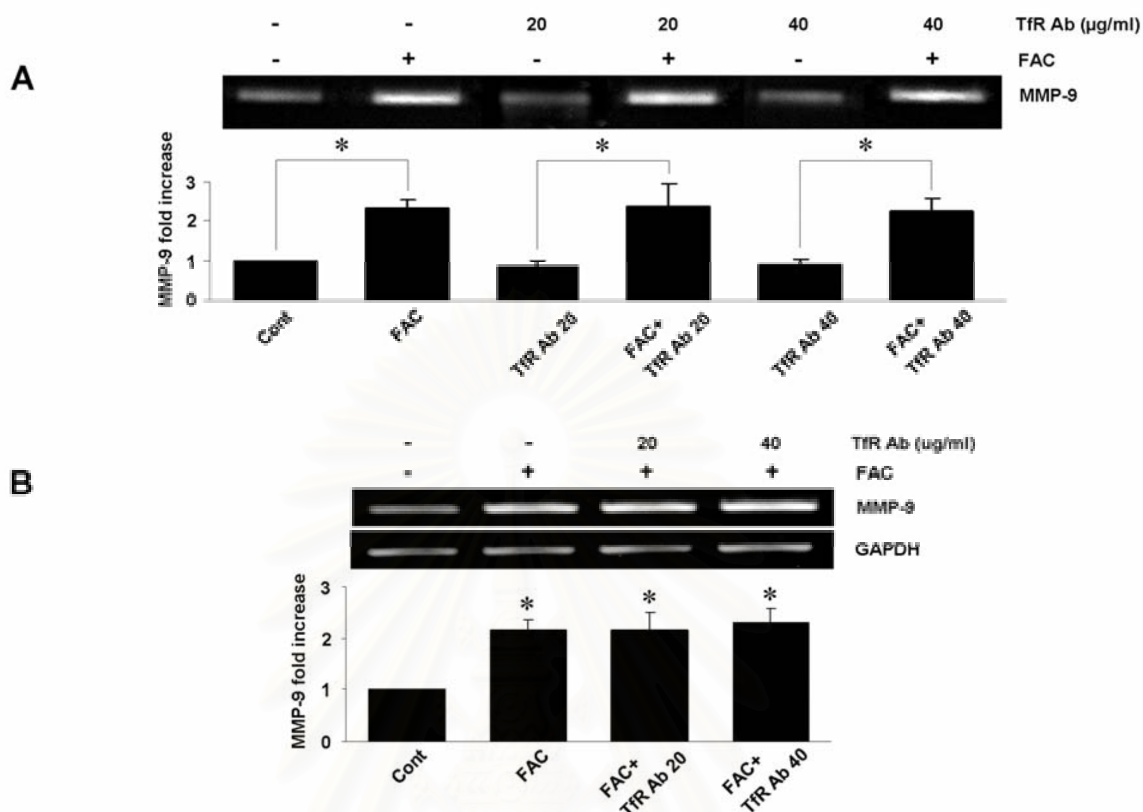

Figure 4.9 HN-22 cells responded to FAC by using non-transferrin dependent pathway. HN-22 cells were treated with neutralizing antibody against human transferrin receptor (TfR Ab) in the presence (+) or absence (-) of FAC for 24 hours. Conditioned media were subjected to gelatin zymography (A) and cells were extracted for RT-PCR (B). Amount of protein loading was normalized to the cell number which determined by MTT assay. Levels of MMP-9 mRNA were normalized to GAPDH. Histograms represent the average fold-increase of MMP-9 expression relative to that of the untreated FAC from three independent experiments which performed in triplicate. * Indicate significant difference from control ($p<0.05$).

FAC regulated MMP-9 expression through ERK1/2 and PI3K/Akt signaling pathways

Regulation of MMP-9 expression occurs through several signaling cascades including MAPKs and PI3K/Akt (Mook et al., 2004). Therefore, specific ERK1/2, JNK, p38 kinase, PI3K (LY294002) or Akt (SH-5) inhibitor was added to FAC stimulated HN-22 cells. The concentration of inhibitors and the duration of exposure used in the experiments had no effect on the viability of cells as determined by MTT assay. Treatment with ERK1/2 inhibitor, LY294002 and SH-5 significantly decreased the stimulatory effect of FAC on MMP-9 expression in both protein (Figure 4.10A and 4.11A-B) and mRNA (Figure 4.10B and 4.11C). In contrast, the inhibitors of JNK and p38 kinase had no effect on blockade of FAC increased MMP-9 expression (Figure 4.10A). The results suggested that FAC increased MMP-9 expression via ERK1/2 and PI3K/Akt signaling pathways in HN-22 cells.

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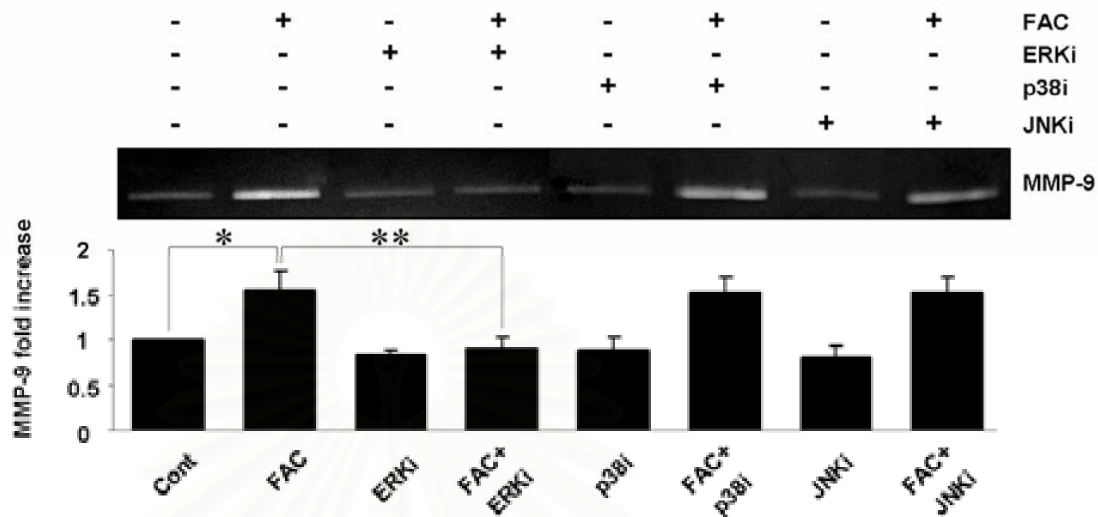
A
FAC
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p38i
JNKi
MMP-9
MMP-9 fold increase
Cont
FAC
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FAC+ ERKi
p38i
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JNKi
FAC+ JNKi

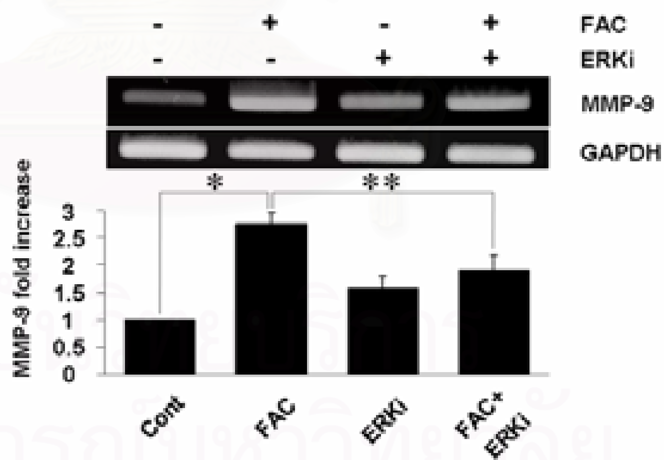
B
FAC
ERKi
MMP-9
GAPDH
MMP-9 fold increase
Cont
FAC
ERKi
FAC+ ERKi

Figure 4.10 FAC increased MMP-9 expression via ERK1/2 signaling pathway. HN-22 cells were stimulated with 15 µg/ml FAC for 24 hours (A and B, lane 2) or pretreated with ERK1/2 (A and B, lane 4), p38 kinase (A, lane 6) or JNK (A, lane 8) inhibitor. Treatment of the inhibitor alone indicated the effect of inhibitor on background expression of MMP-9 (A, lane 3, 5, 7, and B, lane 3). Conditioned media were subjected to gelatin zymography (A) and cells were extracted for RT-PCR (B). Amount of protein loading was normalized to the cell number which determined by MTT. Levels of MMP-9 mRNA were normalized to GAPDH. Histograms represent the average fold-increase of MMP-9 expression relative to that of the control (A and B, lane 1) from three independent experiments which performed in triplicate. * Indicate significant difference from control (p<0.05). ** Indicate significant difference from FAC treatment (p<0.05).

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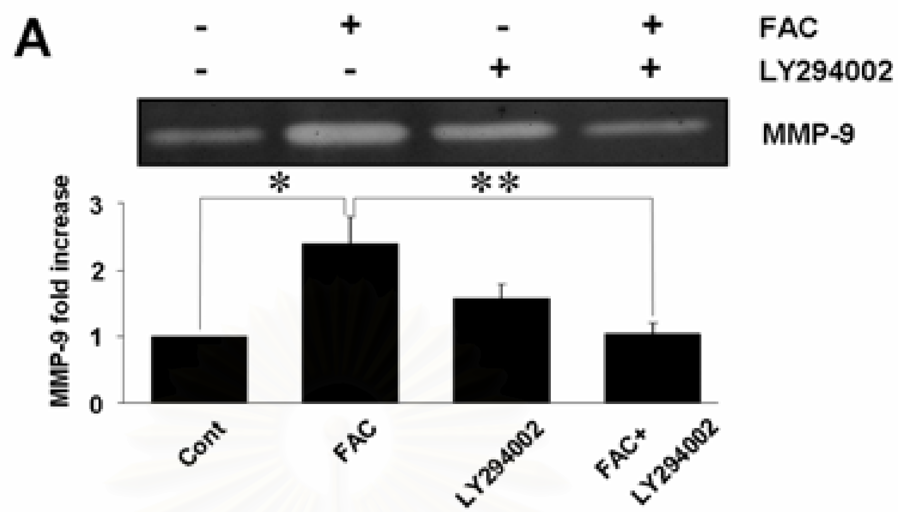

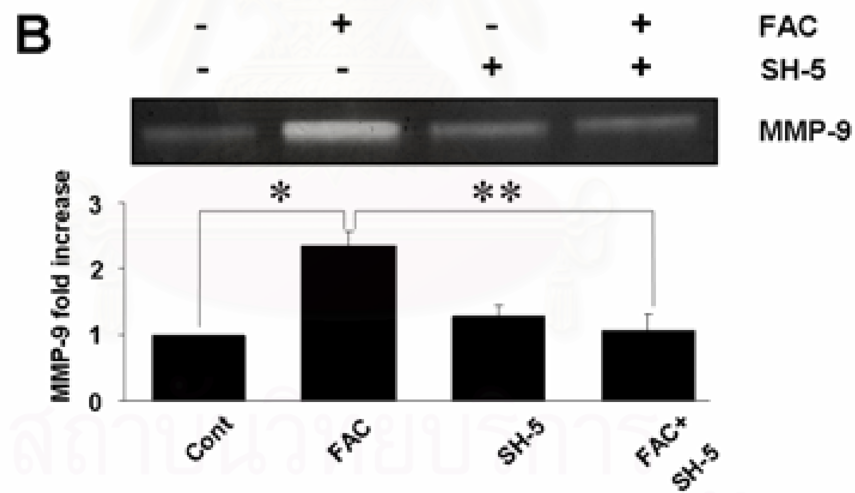

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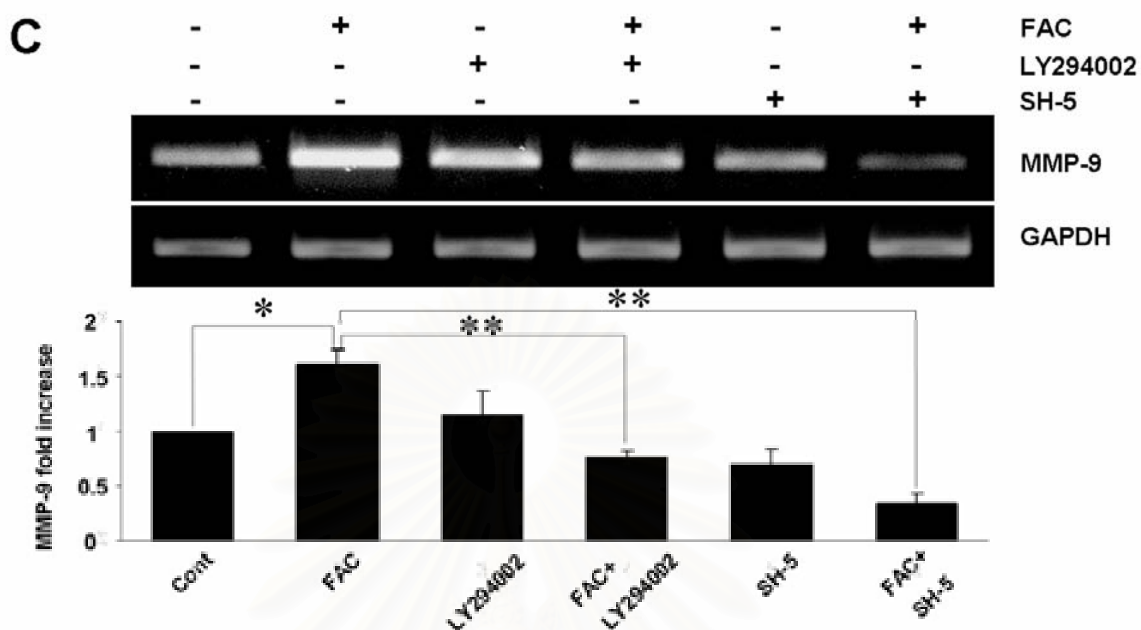
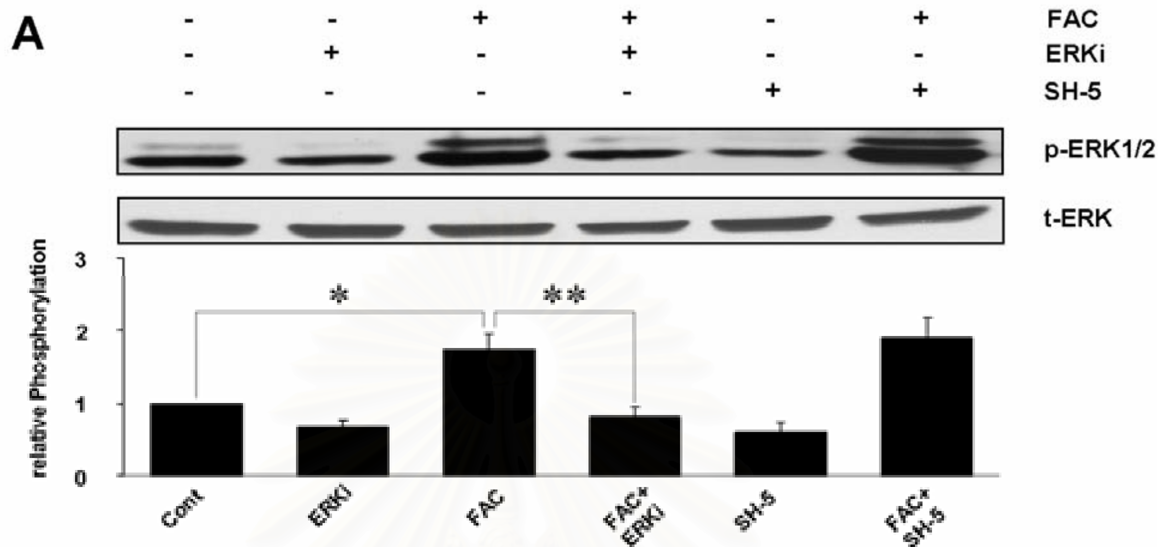


Figure 4.11 FAC increased MMP-9 expression via PI3K/Akt signaling pathway. HN-22 cells were stimulated with 15 μg/ml FAC for 24 hours (A, B and C, lane 2), or pretreated with LY294002; PI3K inhibitor (A and C, lane 4) or SH-5; Akt inhibitor (B, lane 4 and C, lane 6) for 1 hour. Treatment of the inhibitor alone indicated the effect of inhibitor on background expression of MMP-9 (A and B, lane 3; C, lane 3 and 5). Conditioned media were subjected to gelatin zymography (A and B) and cells were extracted for RT-PCR (C). Amount of protein loading was normalized to the cell number which determined by MTT. Levels of MMP-9 mRNA were normalized to GAPDH. Histograms represent the average fold-increase of MMP-9 expression relative to that of the control (A, B and C, lane 1) from three independent experiments which performed in triplicate. * Indicate significant difference from control (p<0.05). ** Indicate significant difference from FAC treatment (p<0.05).

ERK1/2 activation is up-stream regulation of Akt in FAC increased MMP-9 expression

To examine directly whether FAC regulated MMP-9 expression through ERK1/2 and Akt signaling pathway, Western analyses of phosphorylated and total ERK1/2 and Akt were performed. Phosphorylations of ERK1/2 and Akt were significantly increased by FAC (Figure 4.12A and B lane 3) and decreased when treated with specific inhibitors of ERK1/2 and Akt (Figure 4.12A and B lane 4). Total ERK1/2 and Akt were unchanged through the experiment indicating no effect of FAC on ERK1/2 and Akt expression. These results clearly demonstrated that the activations of ERK1/2 and Akt were required for MMP-9 expression in response to FAC in HN-22 cells. We next determined whether activations of ERK1/2 and Akt were in the same cascade of signaling pathway. The results in Figure 4.12B, lane 6, demonstrated that phosphorylation of Akt, which induced by FAC was decreased in response to ERK1/2 inhibitor, but level of phosphorylated ERK1/2, which induced by FAC was unaffected in the presence of Akt inhibitor (Figure 4.12A, lane 6). These findings indicated that activation of ERK1/2 was up-stream regulation of Akt in FAC mediated MMP-9 expression.


A
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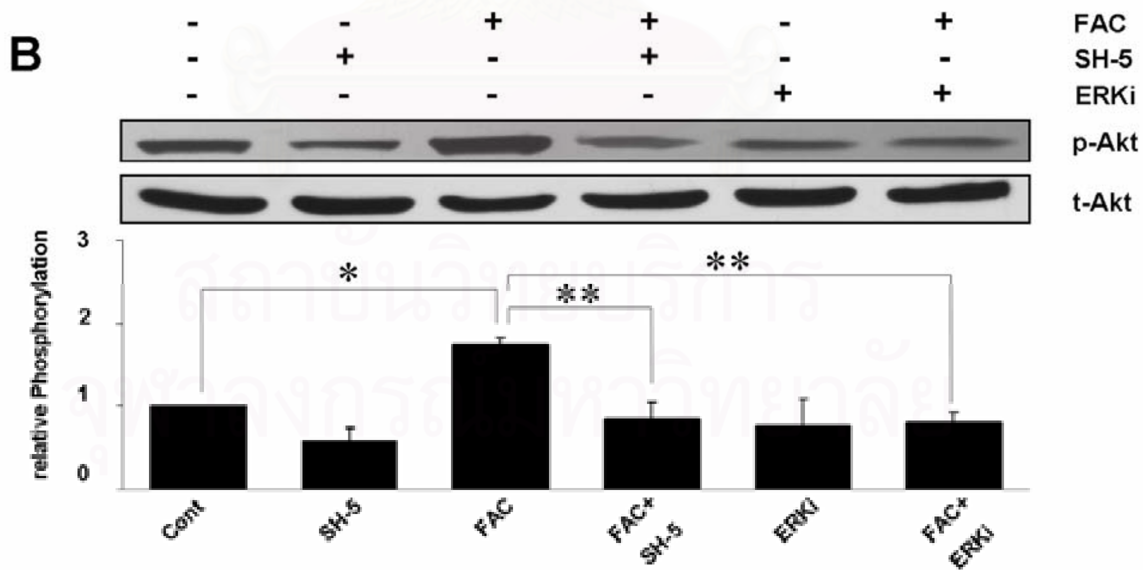
B
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FAC+ SH-5
ERKi
FAC+ ERKi

Figure 4.12 Activation of ERK1/2 and Akt on MMP-9 expression by FAC. HN-22 cells were stimulated with FAC alone (A and B, lane 3) or pretreated with ERK1/2 inhibitor (A, lane 4; B, lane 6) or SH-5 (A, lane 6; B, lane 4). Treatment of the inhibitor alone indicated the effect of inhibitor on background expression of MMP-9 (A and B, lane 2 and 5). Cell lysates were analysed by Western analysis for phosphorylated (p) and total (t) ERK and Akt. Histograms represent average fold-increase of the phosphorylated ERK1/2 (A) or Akt (B) relative to that of the control (A and B, lane 1) from three independent experiments which performed in triplicate. * Indicate significant difference from control (p<0.05). ** Indicate significant difference from FAC treatment (p<0.05).

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FAC regulated AP-1 DNA binding activity

It has been shown that AP-1 and NF-κB binding site are the major regulatory elements on MMP-9 promoter (Sato and Seiki, 1993; Mook et al., 2004). We examined whether FAC up-regulates MMP-9 expression through an increase in AP-1 or NF-κB binding activities. An EMSA assay using oligonucleotide probes corresponding to the AP-1 or NF-κB binding sequences in the MMP-9 promoter were performed. Stimulation by FAC in HN-22 cells clearly increased only AP-1 (Figure 4.14A and B, lane 4), but not NF-κB (Figure 4.13) DNA binding activity in the nuclear extract. This data indicated that AP-1 activation was induced by FAC. Treatment of either ERK1/2 inhibitor (Fig. 4.14A, lane 6) or Akt inhibitor (Fig. 4.14B, lane 6) reduced AP-1 DNA binding activity to the background level (Figure 4.14A and B, lane 2). This result further demonstrated and confirmed that FAC up-regulates MMP-9 expression by the activation of AP-1 via ERK1/2 and Akt. Specificity of the reaction was confirmed by a competition assay using a 100-fold molar excess of unlabeled DNA probe (Figure 4.13, lane 4 and 5, Figure 4.14A and B, lane 3, 5 and 7).

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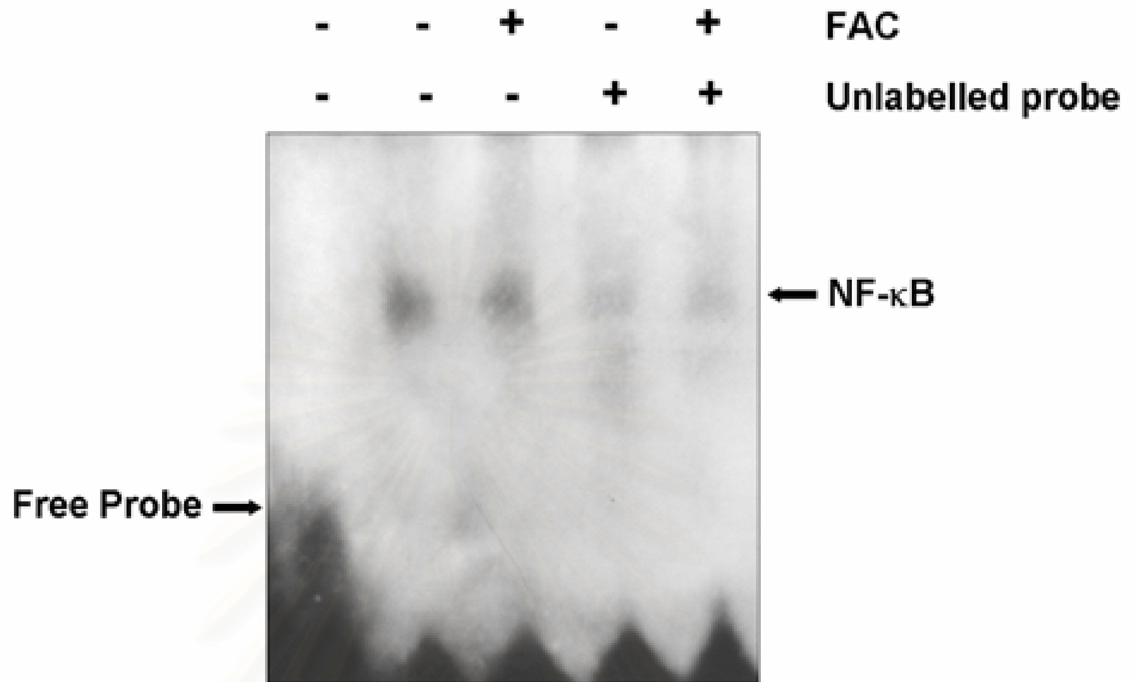
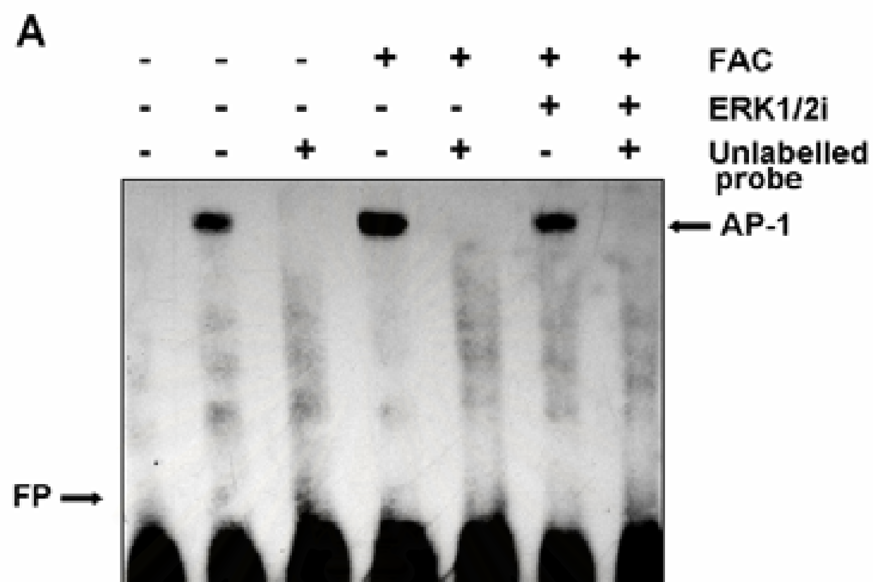


Figure 4.13 FAC was not mediated MMP-9 expression by enhancing NF-κB binding in HN-22 cells. Free probe without nuclear extract is on lane 1 (FP) and baseline level of NF-κB binding in unstimulated HN-22 cells is on lane 2. HN-22 cells were stimulated with FAC (lane 3). Nuclear extract was isolated from each of above conditions and incubated with biotin-labeled oligonucleotides corresponding to NF-κB binding site of MMP-9 promoter. Competition was performed using unlabeled oligonucleotide binding sequence of NF-κB (lane 4 and 5).


A
FAC
ERK1/2i
Unlabelled probe
AP-1
FP

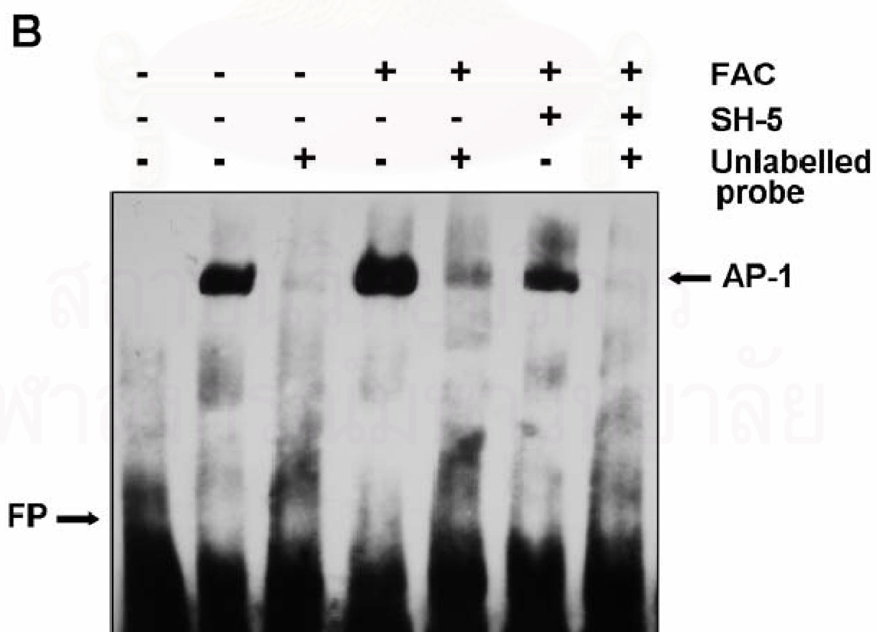

B
FAC
SH-5
Unlabelled probe
AP-1
FP

Figure 4.14 Enhanced AP-1-binding-mediated MMP-9 expression by FAC in HN-22 cells. Free probe without nuclear extract is on lane 1, A and B (FP) and baseline level of AP-1 binding in unstimulated HN-22 cells is on lane 2, A and B. HN-22 cells were stimulated with FAC alone (A and B, lane 4 and 5) or pretreated with ERK1/2 inhibitor (A, lane 6 and 7) or SH-5 (B, lane 6 and 7). Nuclear extract was isolated from each of above conditions and incubated with biotin-labeled oligonucleotides corresponding to AP-1 binding site of MMP-9 promoter. Competition was performed using unlabeled oligonucleotide binding sequence of AP-1 (A and B, lane 3, 5 and 7).

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CHAPTER V
DISCUSSION AND CONCLUSION

Metastasis occurs when cancer cells invade beyond the boundaries of the primary site and establish new tumors in distant organs. Because metastasis is responsible for most cancer deaths, attention has focused on the mechanisms by which cancer cells acquire invasive metastatic properties. MMP-9 is one of MMPs that plays an important role in tumor invasion and metastasis. In this study, we showed for the first time that iron increased MMP-9 expression in HNSCC cell line through increasing AP-1 binding via ERK1/2 and Akt activation. Our study suggests that, in patients with iron overload, iron may increase MMP-9 expression level in the tumors.

In human, iron overload, apart from hereditary hemochromatosis, usually occurs in thalassemia, sideroblastic anemia, chronic hemolytic anemia, dietary iron overload, chronic liver disease or other conditions requiring frequent blood transfusion. Excess iron manifests in almost all tissues, but the majority is deposited in reticuloendothelial macrophages in spleen, liver, and bone marrow, and parenchyma cells of the liver, pancreas, thyroid, parathyroid, adrenal, and pituitary gland, including heart, brain, joints and skin. This leads to skin hyperpigmentation and clinical complications such as liver fibrosis, cirrhosis, hepatocellular cancer, arthritis, and cardiac failure (Papanikolaou and Pantopoulou, 2005; Siah et al., 2006; Youssry et al., 2007).

There is an emerging body of evidence implicating iron in the malignant progression of epithelial cancers such as breast, liver, and colon (Huang, 2003; Brookes et al., 2006).

The correlation between iron and cancer initiation and promotion has received more attention. It has been shown that tumors grew better in an iron-rich environment, and proliferation of cancer cells was inhibited by iron chelator, desferrioxamine (Kicic et al., 2002). Interestingly, iron overload from dietary intake caused high cutaneous iron deposition and brought about the rapid progression of skin papilloma to squamous cell carcinoma in mice (Bhasin et al., 2004).

Our results in the present study may have clinical significance, because patients with head and neck cancer may be chronically exposed to supraphysiological levels of non-transferrin-bound iron (NTBI). For example, NTBI at micromolar concentration can be demonstrated in the serum of cancer patients undergoing treatment with chemotherapy (Harrison et al., 1994; Bradley et al., 1997), and has been detected in the serum of long-term survivors of acute leukemia and bone marrow transplantation (Harrison et al., 1996). Elevated level of NTBI has also been observed in serum after blood transfusion (Grosse et al., 2005) or intravenous iron transfusions (Breuer et al., 2000; Scheiber-Mojdehkar et al., 2004), both of which are common treatments for anemic cancer patients.

In this study FAC were used as a source of iron to simulate an iron overload condition in cell culture. In our experiments, treatment with FAC at concentration higher than 50 μg/ml was cytotoxic to HNSCC cell lines as determined by MTT assay. However, FAC can be used in *in vitro* studies between the ranges of 0.01-100 μg/ml depending on cell types (Scheiber-Mojdehker et al., 2004; Juang, 2004; Baldys et al., 2007). Normally, serum iron level is 60-180 μg/dl, whereas exceeds 180 μg/dl is marked iron overload such

as in hemochromatosis (Mercola, 2002). Under physiological circumstances iron is bound to transferrin. However, in some pathological conditions including iron overload diseases, iron can also be found in the transferrin-free form at concentrations up to 0.8-2.5 μg/ml (Richardson and Ponka, 1997). Therefore 15 μg/ml of FAC used in this study could simulate an iron overload condition without any toxic to the cells.

The effects of iron on MMPs have previously been demonstrated. In normal fibroblasts, iron has been shown to increase MMP-1, 2, 3 and 9 expressions in culture via JNK2 activity (Brenneisen et al., 1998, Campo et al., 2006). Iron could also stimulate MMP-2 activity in rat hepatic stellate cells leading to an increase in matrix degradation (Gardi et al., 2002). Furthermore, an increase of iron in serum of patients with chronic venous disease caused an over-expression of MMP-9 and increased tissue destruction (Lee et al., 2003; Zamboni et al., 2005). The present study provided new evidence that iron could also up-regulate MMP-9 expression in HNSCC cell lines.

A higher level of MMP-9 expression as well as an increase in MMP-9 activation has been found in malignant tissues as compared to adjacent normal tissues (Patel et al., 2005). Several studies have shown a correlation between an increased expression of MMP-9 by either tumor cells or surrounding stromal cells to a more aggressive stage of cancer and a poor survival rate of head and neck cancer patients (Riedal et al., 2000; O-Charoenrat et al., 2001). Since MMP-9 plays an important role in the modulation and remodeling of the extracellular matrix, increased level of MMP-9 expression can promote cancer cell proliferation and migration at each stage of tumor invasion and metastasis (O-Charoenrat et

al., 2001; Ondruschka et al., 2002; Baker et al., 2006).

MMP-9, like other MMPs, is expressed in a latent form, which requires activation to function. In this study, we did not observe the active MMP-9 (82 kDa) after iron stimulation. Iron could only increase the synthesis of MMP-9, but was insufficient to induce MMP-9 activation *in vitro*. However, several factors in an *in vivo* microenvironment have been reported to activate pro-MMP-9. A number of activated proteases such as MMP-2, 3, 13, trypsin and plasmin/ plasminogen activator system have been demonstrated to activate MMP-9 (Van den Steen et al., 2002). In addition, binding to some ECM components such as type I collagen and fibronectin could also lead to MMP-9 activation (Mook et al., 2004). Therefore, increased latent MMP-9 by iron may still contribute to ECM degradation *in vivo*. The balance between MMP-9 and TIMP-1, MMP-9 specific inhibitor, is another main regulator of MMP-9 function (Goldberg et al., 1992). Our results demonstrated no change of the expression level of TIMP-1 by RT-PCR in FAC treated HNSCC cells. This result suggested that none of MMP-9 activation was not resulted from the elevation of TIMP-1.

Iron uptake by cells has been reported to occur via two main pathways; transferrin dependent and non-transferrin dependent (Conrad et al, 1994; Abboud and Haile, 2000; Dunn et al., 2006). Since iron enhances tumor growth, tumor cells are much more likely to express a large number of TfR, produce their own Tf or Tf-like proteins and obtain iron from non-Tf sources for their proliferation than normal cells (Richardson and Ponka, 1997). In our study, we found that Tf and TfR-1 constitutively expressed in a number of HNSCC cell lines. Interestingly, those constitutive expressions of TfR-1 corresponded with the levels of their

MMP-9 expression (data not shown). Previous studies have shown that TfR-1 was expressed more abundantly in malignant tissues than their normal counterparts assuming its roles in cell growth and division (Aisen, 2004). Moreover, levels of TfR were associated with the metastatic potential of rat and human mammary carcinoma cell lines (Inoue et al., 1993). However, in the present study using neutralizing antibody against human TfR demonstrated that HN-22 cells used a non-transferrin dependent pathway for iron uptake in the regulation of MMP-9 expression. However, we haven't checked the neutralizing ability of this antibody, more experiment may be required for definite conclusion. Therefore, changes in the expressions of Tf, TfR-1, and DMT-1 mRNA after FAC stimulation may result in some other non-identified cell responses, but not MMP-9 expression. A non-transferrin dependent pathway of iron uptake has been reported in human cervical cancer cells (Sturrock et al., 1990), leukemia (Inman and Wessling-Resnick, 1993), melanoma (Richardson and Baker, 1991), and hepatocellular cancer (Parkes et al., 1995). There was a report demonstrating iron uptake via a non-transferrin dependent pathway in human prostate cancer cell line, PC-3, which increased the expression of uPA (Ornstein and Zacharki, 2007). Although our data suggests the presence of a non-transferrin dependent iron transport pathway in HN-22 cells, additional investigations are required to reveal the detail of mechanism.

In case of iron overload level caused toxicity to the cells, the biological mechanism was believed that iron disrupted the redox balance of the cells and generated ROS, which modified the expressions of a number of genes and signal transduction pathways (Benhar

et al., 2002; Thannickal et al., 2000). Dysregulation of signal cascades stimulated by metal ions via ROS regulated by two distinct protein families, the MAPKs and the redox sensitive signaling factors such as thioredoxin leading to activation of several redox-regulated transcription factors; AP-1, NF-κB, and Egr-1 and resulting in modulation of gene expressions, cell proliferation, motility, and apoptosis (Valko et al., 2006; Waris and Ahsan, 2006). In the present study, we did not determine the intracellular ROS production, since we conducted the experiment under the non-toxic dose of FAC.

The expression of MMP-9 gene is under the control of several growth factors and cytokines. By using specific inhibitors, several studies have revealed the involvement of various signaling pathways in MMP-9 regulation depending on cell types and nature of the stimuli. The signaling pathways via MAPKs, NF-κB and PI3K/Akt have been reported (Mook et al., 2004). Treatment with TNF-α or IL-1 up- regulated MMP-9 expression via NF-κB signaling molecule (Esteve et al., 2002), whereas fibronectin induced MMP-9 secretion in ovarian cancer cells via MAPK/MEK1 and PI3K/Akt pathways (Thant et al., 2000). The increase of MMP-9 in LPS-activated human monocytes involved PI3K/Akt and NF-κB pathways (Lu et al., 2005), while the stimulation of MMP-9 transcription by IL-1β in Balb 3T3, a normal mouse fibroblast cell line, occured through both Akt and ERK signaling pathways (Ruhul Amin et al., 2003).

In recent year, the PI3K pathway has been suggested to be highly involved in tumor cell invasion. Among the downstream kinases of PI3K, Akt has been identified as the main signal transmitter. Akt is one of the most frequently activated protein kinases in human

cancer. The activation of Akt has been identified in many cancers such as breast, ovarian, and pancreatic cancer. Akt signaling appears to play a prominent role in several processes considered hallmarks of cancer. In addition, Akt activation contributed to tumor invasion and metastasis by stimulating secretion of MMP-2 and -9 (Furuya et al., 2006; Han et al., 2006). Study using iron chelator has shown inhibition of signaling through JNK- regulated cell cycle arrest in prostate cancer (Antosiewicz et al., 2006). Iron was indicated to induce IL-6 expression through ERKs and p38 kinase pathway in rat hepatocytes (Dai et al., 2004). Iron also increased TNF-α expression by activation of NF-κB in Kuffer cells (She et al., 2002). Iron increased the expression of uPA in human prostate cancer cells by which iron may stimulate uPA production through the generation of intracellular ROS and activation of NF-κB (Ornstein and Zacharki, 2007). In this study, the results indicated that, iron induced MMP-9 expression in HN-22 involving ERK1/2 and PI3K/Akt pathway in which ERK1/2 was Akt-up-stream regulation.

The promoter of MMP-9 is generally regulated by the binding of multiple transcription factors; AP-1, PEA-3, NF-κB, and SP-1, to their responsive elements (Sato and Seiki, 1993). A report published recently indicated that some metals are able to affect the activity of NF-κB transcription factor. Similarly, induction of AP-1 by certain metals and other stressors in cancer process is mediated mainly by JNK and p38 kinase cascades as shown in the several experiments (Pinkus et al., 1996; Benhar et al., 2002). Activation of MAPKs is important for AP-1 DNA binding activity, however activation of NF-κB requires Akt or p38 kinase depending on cell types and stimuli (Van den Steen et al., 2002). It has

recently been proposed that iron can induce early signaling pathways that may modulate activities of several oxidative-responsive transcription factors, such as AP-1 and NF-κB. Using AP-1 luciferase reporter stably transfected mouse epidermal JB6 cells was found that iron compounds activated AP-1 luciferase activity. Interestingly, iron containing bioavailability induced phosphorylation of ERKs and p38 kinase, but not JNKs, either in mouse epidermal JB6 cells, primary rat hepatocytes or human lung epithelial A549 cells (Dai et al., 2004). To determine whether iron-induced ERK1/2 and Akt activations regulated MMP-9 through interaction with specific transcription factors via AP-1 and NF-κB binding sites, we assessed the binding of AP-1 and NF-κB oligonucleotides with consensus sequences found in the promoters of MMP-9 to nuclear extracts from iron treated HN-22 cells. In the present study, inhibition of either the ERK1/2 or Akt activation induced by iron resulted in suppression of only AP-1 but not NF-κB binding activity, indicating that iron-induced MMP-9 was regulated through ERK1/2/Akt and subsequently through AP-1. AP-1 transcription factors are leucine zipper proteins that bind to a consensus DNA sequence as a dimeric complex (Karin et al., 1997). AP-1 complex are heterodimers of a family of Jun and Fos that include different Jun proteins (c-Jun, JunB, and JunD) and Fos proteins (c-Fos, FosB, Fra-1, Fra-2, and FosB2). Different AP-1 dimers bind DNA with different affinities, which are partially responsible for the diverse biological effects. To assess the subunit composition of AP-1, specific antibodies against Jun/Fos will be use for supershift analysis.

In conclusion, our studies indicate that iron stimulates MMP-9 expression in HNSCC through increasing AP-1 binding via ERK1/2/Akt signaling pathway (Figure 5.1).

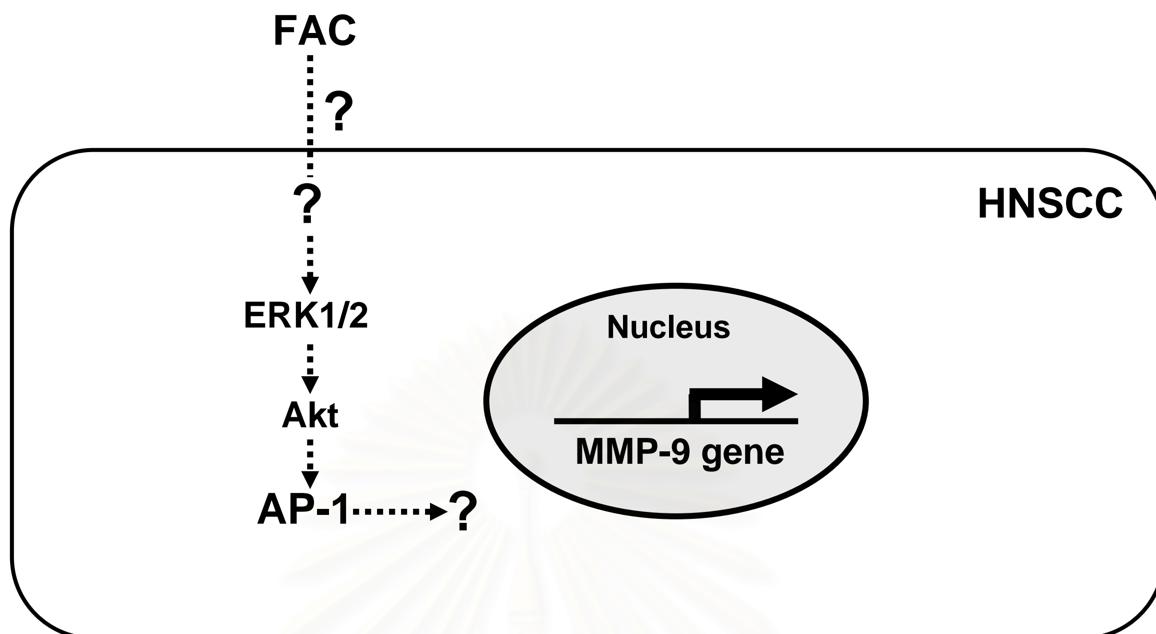

Figure 5.1 Schematic representation of signal pathway in HNSCC in response to FAC

This study reveals a novel molecular mechanism by which iron modulates MMP-9 expression in HNSCC. This basic knowledge will provide a new viewpoint to understand the mechanism underlying the biological function of iron-induced MMP-9 expression in HNSCC that may contribute to the cancer progression. Moreover our finding may start researching in the iron and cancer metastasis in both clinical and cell biological fields.

Future studies

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

1. To determine which the regulatory element in the promoter area regulates MMP-9 expression induced by iron using the promoter mutagenesis.

2. To measure the intracellular ROS generation which possibly increase after iron stimulation, since ROS can activate the expression of a number of genes and signal transduction pathways, including MMP-9.

3. To explore the effect of iron on the functional behaviors of cancer cells in culture and animal models.

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