กลไกการกระตุ้นการแสดงออกของเอนไซม์เอ็มเอ็มพี-9 โดยทีจีเอฟเบต้า1 ในมะเร็งบริเวณศีรษะและคอชนิดสแควมัสเซลล์คาร์ซิโนมา

นางสาว สรียา นุชนาฏพนิต

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THE MECHANISM OF TGF-β1-INDUCED-MMP-9 EXPRESSION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosohy Program in Oral Biology
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Thesis Title	THE MECHANISM OF TGF-BETA1-INDUCED-MMP-9 EXPRESSION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA
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สรียา นุชนาฏพนิต : กลไกการกระตุ้นการแสดงออกของเอนไซม์เอ็มเอ็มพี-9 โดยทีจีเอฟ-เบต้า1 ในมะเร็งบริเวณศีรษะและคอชนิดสแควมัสเซลล์คาร์ซิโนมา (THE MECHANISM OF TGF-BETA1-INDUCED-MMP-9 EXPRESSION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA) อ. ที่ปรึกษา : รศ.ทพ.ดร.ประสิทธิ์ ภวสันต์, อ.ที่ปรึกษาร่วม : ผศ.ทพ.ดร.อาทิพันธุ์ พิมพ์ขาวขำ, รศ.ทญ.ดร.นีรชา สารชวนะกิจ จำนวนหน้า 103 หน้า.

เอนไซม์เมตริกซ์เมทัลโลโปรตีนเนส-9 (เอ็มเอ็มพี-9) มีความสำคัญในการดำเนินโรคของมะเร็งโดยเฉพาะในการแพร่กระจายของมะเร็งที่ต้องการเอ็มเอ็มพี-9 ในการทำลายเนื้อเยื่อโดยรอบ ซึ่งในภาวะที่เป็นโรคพบว่ามีการสร้างสารโปรตีนมากมาย ที่สามารถกระตุ้นการสร้างเอ็มเอ็มพี-9 ได้ เช่น โปรตีนทรานส์ฟอร์มมิ่งโกรทแฟคเตอร์-เบต้า1 (ทีจีเอฟ-เบต้า1) ที่พบว่ามีปริมาณมากขึ้นในโรคมะเร็งเช่นกัน การศึกษานี้จึงมีวัตถุประสงค์เพื่ออธิบายกลไกการกระตุ้นเอนไซม์เอ็มเอ็มพี-9 โดยทีจีเอฟเบต้า1 ในเซลล์มะเร็งบริเวณศีรษะและคอชนิดสแควมัสเซลล์คาร์ซิโนมาโดยใช้วิธีเจลาตินไซโมกราฟฟี, อีไลซ่า, อาร์ที-พีซีอาร์ และเวสเทิร์นบลอท ในการวัดผลปริมาณเอ็มเอ็มพี-9 ที่เปลี่ยนแปลงภายหลังการกระตุ้นด้วยทีจีเอฟ-เบต้า โดยผลการทดลองแสดงให้เห็นว่า ทีจีเอฟ-เบต้า1 สามารถกระตุ้นการสร้างเอ็มเอ็มพี-9 ได้ตามปริมาณทีจีเอฟ-เบต้า1 ที่เพิ่มขึ้น ทั้งระดับการแสดงออกของยีน และ ระดับโปรตีน และ ภายหลังการใช้สารยับยั้งต่าง ๆ เพื่อค้นหากลไกการกระตุ้นเอ็มเอ็มพี-9โดยทีจีเอฟ-เบต้า1 พบว่าสารยับยั้งต่อตัวรับสัญญาณของระบบทีจีเอฟ-เบต้าชนิดที่1 สามารถลดการสร้างเอ็มเอ็มพี-9ได้เกือบทั้งหมด ทั้งในระดับการแสดงออกของยีนและโปรตีน ในขณะที่สารยับยั้งต่อระบบมัยโอซิน แสดงให้เห็นการลดของเอ็มเอ็มพี-9 ที่หลั่งออกมาภายนอกเซลล์เท่านั้น แต่ไม่สามารถลดระดับการแสดงออกของยีนเอ็มเอ็มพี-9นอกจากนี้ ในการวิเคราะห์โปรตีนพบว่าการยับยั้งตัวรับสัญญาณของระบบทีจีเอฟ-เบต้าชนิดที่1 สามารถลดการส่งสัญญาณผ่านระบบสแมดและมัยโอซินได้ ทีจีเอฟ-เบต้า1 ควบคุมการแสดงออกของเอ็มเอ็มพี-9 ผ่านทางโปรตีนสแมด และ มัยโอซิน

สาขาวิชาชีววิทยาช่องปาก
ปีการศึกษา2550

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
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4676125032 : MAJOR ORAL BIOLOGY
KEY WORD: TRANSFORMING GROWTH FACTOR-BETA1 / MATRIX METALLOPROTEINASE-9 / HEAD AND NECK SQUAMOUS CELL CARCINOMA

SARIYA NUCHANARDPANIT : THE MECHANISM OF TGF-BETA1-INDUCED-MMP-9 EXPRESSION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA THESIS ADVISOR : ASSOCIATE PROFESSOR PRASIT PAVASANT D.D.S Ph.D, THESIS COADVISOR : ASSISTANT PROFESSOR ATIPHAN PIMKHAOKHAM D.D.S Ph.D. B.A; ASSOCIATE PROFESSOR NEERACHA SANCHAVANAKIT D.D.S Ph.D, 103 pp.

Matrix metalloproteinase-9 (MMP-9) plays roles in cancer progression by degrading the extracellular matrix and basement membrane. Many growth factors including Transforming growth factor-beta1 (TGF-β1) could induce MMP-9 expression. We demonstrated that TGF-β1 induced MMP-9 mRNA and protein in human head and neck squamous cell carcinoma cell lines. Application of TGF-β receptor type I inhibitor (SB505124) reduced the MMP-9 expression markedly. Whilst, inhibitor of Myosin light chain kinase (MLCK) could reduce the level of secreted MMP-9 in both the supernatants and cell lysate but not the level of MMP-9 mRNA. These suggested that MLCK might regulate MMP-9 expression post-transcriptionally. Application of SB505124 and siRNA Smad2/3 reduced the phosphorylation of myosin light chain (MLC) suggested that MLC is downstream to TβRI/Smad2/3 signaling pathway. In conclusion, these results describe a novel mechanism for the potentiation of TGF-β1 signaling to induce MMP-9 expression via Smad and MLCK.

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LIST OF ABBREVIATIONS

ActR	activin receptor
ALK	activin-like receptor
AML	acute myeloid leukemia
AP-1	activator protein-1
ARC	activator recruited cofactor
ATF	activating transcription factor
α1-PI	alpha1 proteinase inhibitor
α2M	alpha-2-macroglobulin
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BMPR	bone morphogenic protein receptor
bZIP	basic leucine zipper domain
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CamKII	Ca^{2+}/calmodulin-dependent protein kinasesII or CaM kinasesII
CBP	c-AMP-response element binding (CREB)-binding protein
cDNA	complementary DNA
co-Smad	common-partner Smad
DI water	deionized water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxane
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
ETS	erythroblastosis twenty-six
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
GDF	growth and differentiation factor
GERD	gastroesophageal reflux disease
GF	human gingival fibroblast
GFCM	human gingival fibroblast conditioned medium
GTF	general transcription factor
GPI	glycosylphosphatidylinositol
HCl	hydrogen chloride
HGF	hepatocyte growth factor
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
ICAM	intercellular adhesion molecule
IFN-α	interferon-alpha

Ig	immunoglobulin
IL-1	interleukin-1
IL-2Rα	interleukin-2 receptor alpha
IL-6	interleukin-6
IL-8	interleukin-8
I-Smad	inhibitory Smad
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LAP	latency-associated protein
LTBP	latent TGF-beta-binding protein
MAPK	mitogen-activated protein kinase
MH	MAD homology
MLC	myosin light chain
MLCK	myosin light chain kinase
MMPs	matrix metalloproteinases
mRNA	messenger RNA
MSG	melanocyte specific gene
MT-MMPs	membrane-type- matrix metalloproteinases
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NaCl	sodium chloride
NF-κB	nuclear factor-kappa B
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEA-3	polyoma enhancer activator -3
PEX	hemopexin-like domain
PI3K	phosphatidyl inositol 3-kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
Pol II	RNA polymerase II
PVDF	polyvinylidene fluoride
rh-TGF-β1	recombinant human transforming growth factor-beta1
R-Smad	receptor-regulated Smad
RT-PCR	reverse transcription polymerase chain reaction
SARA	Smad achor for receptor activation
SBE	Smad binding element
SCC	squamous cell carcinoma
SD	standard deviation
SDS	sodium dodesylsulphate
SFM	serum free medium
siRNA	small interfering ribonucleic acid
SIP1	survivin interacting protein1
Smad	Mothers against decapentaplegic homolog (MAD)
SMIF	Smad4 interacting transcription factor
Smurf	Smad ubiquitin regulatory factor
SNIP	Smad nuclear interacting protein
Sp-1	stimulating protein-1

TAFs	TATA binding protein associated factors
TAK	Thylakoid-associated Kinase 1
TBE	Tris/Borate/EDTA buffer
TBP	TATA binding protein
TβR	transforming growth factor-beta receptor
TGF-β1	transforming growth factor-beta1
TGIF	transforming growth factor-beta-induced factor
TIE	transforming growth factor-beta inhibitory element
TIMPs	tissue inhibitor of matrix metalloproteinases
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TNF-α	tumor necrosis factor-alpha
TRIS	trishydroxymethylaminomethane
uPA	urokinase plasminogen activator
UV	ultraviolet
VEGF	vascular endothelial growth factor
Wnt	wingless-type MMTV integration site family
XBE	cognate DNA sequence
Zn^{2+}	Zinc ion

CHAPTER I

INTRODUCTION

Head and neck cancer is the seventh most common cancer worldwide with approximately 390,000 new cases annually and squamous cell carcinoma represents more than 90 percent of all head and neck cancers. In Thailand, squamous cell carcinoma of the head and neck comprises about 11 percent of all malignancies. This type of cancer is formed from reserve cells – cells that replaced injured or damaged cells in the epithelial cells. Five-year survival rates average about 50 percent. If the tumor is treated at an early stage before it has grown or spread significantly, survival rates are better – as high as 75 percent.

The necessity of having an effective therapeutic of cancer is rapidly growing alongside the implementation of medical technology. Head and neck squamous cell carcinoma can be treated through one or more of the following: **surgery, chemotherapy, radiation therapy,** as well as new investigative treatments such as immunotherapy and gene therapy. However, the conventional treatment is not sufficient to manage the severity of cancer patients. Finding an appropriate molecular target for these new treatments has become increasingly important today's cancer research. Recently, a new treatment that uses molecular biology to assist the conventional treatment has emerged. Among the molecular targets for developing the treatment, transforming growth factor-β1 (TGF-β1) and matrix metalloproteinase-9 (MMP-9) are very interesting according to their roles in cancer progression.

Over the last years, the relevance of MMP-9 or TGF-β1 in cancer research has grown considerably. MMP-9 was initially associated with the invasive properties of tumor cells, owing to its ability to degrade all major protein components of the

extracellular matrix (ECM) and basement membranes, while several studies have demonstrated the implication of TGF-β1 as a tumor promoter in later steps of tumor evolution, such as stimulation of cell migration and invasion which including up-regulating of MMP-9 expression. Conversely, TGF-β1 can be also activated by MMP-9. Thus, both TGF-β1 and MMP-9 may facilitate the cancer progression by this interaction.

To date, the signaling pathway of how TGF-β1 induces MMP-9 expression is still unclear. Therefore, a better understanding of the functional complexity of this mechanism will benefit the development of new approach for cancer treatment. For this reason, the mechanism of which TGF-β1 could induce MMP-9 expression will be necessary to clarify whether they could be targeted for the future therapies against cancer.

In this study, the model of head and neck squamous carcinoma cell lines were used to test the effect of TGF-β1 on MMP-9 expression and which mechanism was used in these cells by performing several molecular approaches including gelatin zymography, RT-PCR, western blotting, ELISA, EMSA and siRNA. The findings will be useful for further development of the gene therapy targeting both TGF-β1 and MMP-9, which will be an alternative treatment and provide a better outcome for the patients.

Problems

1 What is the effect of TGF-β1 on the MMP-9 expression in head and neck squamous cell carcinoma?

2 Which signaling pathway(s) is/are involved of TGF-β 1-induced-MMP-9 expressions in head and neck squamous cell carcinoma?

3 Is TGF-β1-induced-MMP-9 expressions in head and neck squamous cell carcinoma Smad-dependent?

Hypothesis

TGF-β1 could induce MMP-9 expression in head and neck squamous cell carcinoma and non-Smad signaling pathway(s) may participate in this mechanism.

Specific aims

The specific aims of this present study are

1. To study the expression pattern of MMP-9 induced by TGF-β in head and neck cancer cell line in each level

- transcriptional level
- protein synthesis level
- enzyme activity

2. To identify the possible non-Smad pathway(s) involved in TGF-β-induced MMP-9 expression in head and neck squamous cell carcinoma.

3. To identify the relevance of non-Smad pathway with Smad pathway, if any.

4. To clarify the importance of Smad2 and Smad3 in TGF-β-induced MMP-9 expression in head and neck squamous cell carcinoma.

5. To study the role of gene regulatory sequence, AP-1, in this mechanism.

Expected benefits

The findings will give a more understanding in the intracellular signaling of MMP-9 expression after TGF-β1 activation, which are the most complex and important factors that promote the invasive phenotype of cancer cell. Thus, these findings may be small jigsaw pieces that lead to the fulfilment of future treatment targeting TGF-β1/MMP-9 and improve the treatment outcome for the cancer patient.

CHAPTER II

REVIEW LITERATURES

Head and neck squamous cell carcinoma (HNSCC)

Head and neck cancer is the cancer that arises in the head or neck region, which are the nasal cavity, sinuses, lips, oral cavity, salivary glands, throat and larynx**.** Squamous cell carcinoma represents more than 90 percent of all head and neck cancers. In the United States, squamous cell carcinoma of the head and neck comprises about 4 percent of all malignancies. In Thailand, the estimated percent of oral cavity and pharynx cancer is about 6.8 and 4.8 percent of all cancers in male and female, respectively. In general, five-year survival rates average about 50 percent. If the tumor is treated at an early stage before it has grown or spread significantly, survival rates are better – as high as 75 percent (www.nci.go.th, www.cancer.gov).

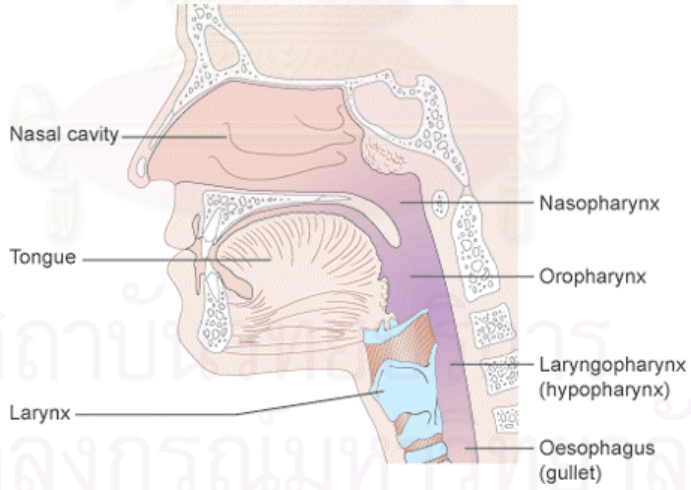


Figure 2.1. Head and neck squamous cell carcinoma (HNSCC) affected area. *HNSCCs make up the vast majority of head and neck cancers, and arise from mucosal surfaces throughout this anatomic region. These include tumors of the nasal cavities, paranasal sinuses, oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx. (www.cancerhelp.org.uk)*

Males have this type of cancer about twice as often as females. A Tobacco product, especially smokeless tobacco, is a primary cause. Females are more commonly experiencing this type of cancer as they use tobacco products. This type of cancer also is more common

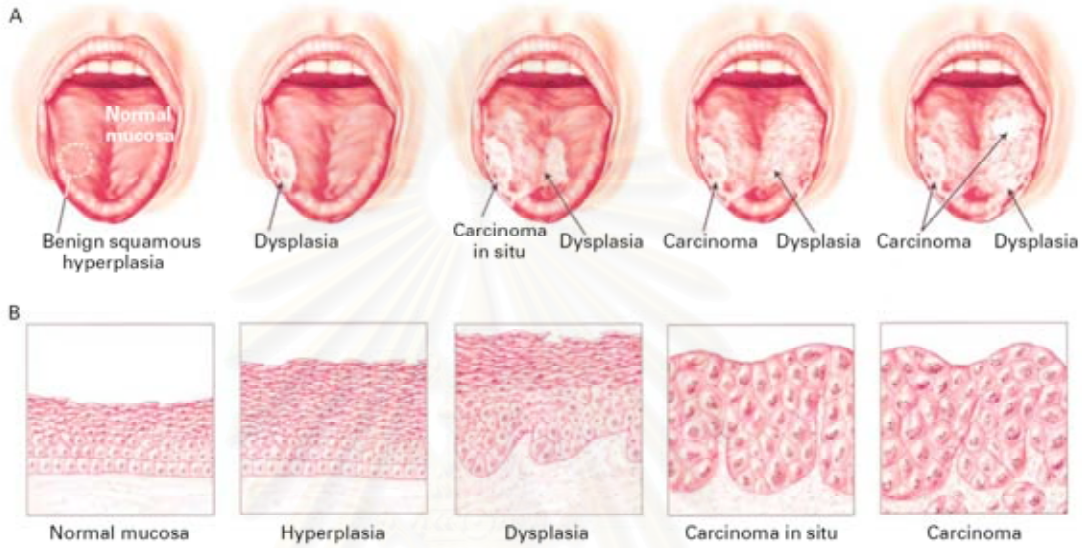


Figure.2.2. Illustraion of oral cancer to represent HNSCC progression. *(A) The typical clinical presentation of oral cancer. Benign squamous hyperplasia can often appear similar to normal mucosa. Novel molecular approaches have yielded considerable understanding of the field-cancerization hypothesis originally proposed by Slaughter and colleagues in 1953. In most patients, cellular repopulation in geographically distinct areas gives rise to multiple clinical lesions. Although these lesions may have different histopathological patterns, as shown, they are often clonally related, arising from the same cell. The progression from normal-appearing mucosa to invasive cancer is depicted in (B). Normal-appearing mucosa already harbors early genetic changes (Forastiere et al., 2001).*

among individuals in their 50s, 60s and older. Excessive alcohol use is also considered a risk factor in the development of squamous cell carcinoma, especially when used in conjunction with tobacco product use. In addition, Epstein-Barr virus (EBV); human papillomavirus (HPV) infection; gastroesophageal reflux disease (GERD); and exposure to paint fumes, plastic by products, wood dust, asbestos and gasoline fumes have been considered as possible risk factors. Irritation from poorly fitting dentures also has been implicated.

Early detection and treatment by multiple modalities is important for better prognosis in head and neck cancer. For all sites and stages in the head and neck region, 5-year survival rate is improved if the patient was early detected and underwent treatment before the spreading of cancer to the lymph node. Goals of treatment generally consist of removal of cancer load, maintenance of quality of life, and prevention of secondary cancer. However, conventional treatments that involved surgery usually cause morbidity in patients. The development of new modalities such as immune therapy or gene therapy that target molecular proteins may result in improved survival and quality of life.

Matrix metalloproteinases (MMPs)

1.The MMPs family

MMPs comprise a family of at least 28 secreted or transmembrane enzymes collectively capable of processing and degrading various Extracellular matrix proteins (ECM). Of these, at least 22 MMPs have so far been found in human tissues. MMPs share high protein sequence homology and have defined domain structures and thus, according to their structural properties, MMPs are classified either as secreted MMPs or membrane anchored MMPs, which are further divided into eight discrete

subgroups that are secreted MMPs include minimal-domain MMPs, simple hemopexin domain-containing MMPs, gelatin-binding MMPs, furin-activated secreted MMPs and vitronectin-like insert MMPs, while membrane bound MMPs include type I transmembrane MMPs, glycosyl-phosphatidyl inositol (GPI)-linked MMPs and type II transmembrane MMPs (Egeblad and Werb 2002.)

All MMPs are synthesized with a predomain containing a leader sequence, which targets the protein for secretion (Sternlicht and Werb, 2001). They are secreted as latent proforms, with a few exceptions of furin-processed proteinases, such as MMP-11 or MMP-28. The prodomain of MMPs has an egg-like shape, and contains a well-conserved cysteine switch motif for maintaining the pro-MMP latent (Springman et al., 1990, Van Wart and Birkedal-Hansen, 1990). Generally, the structures of all MMP catalytic domains are quite similar (Bode et al., 1999). The shape of the catalytic domain is spherical with a flat active site cleft, which extends horizontally across the domain to bind peptide substrates or inhibitors. The catalytic domain has the zinc-binding motif, which coordinates a zinc atom at the active site, and under the zinc, an ALMYP methionine-turn (Stöcker et al., 1995). The latency of the zymogen is maintained through cysteine-switch motif, in which the cysteine residue acts as a fourth zinc-binding ligand to maintain the enzyme inactive. In addition to the catalytic zinc, the catalytic domain also contains structural zinc and two to three calcium ions. C-terminal hemopexin or vitronectin-like domains affect substrate or inhibitor binding, membrane activation and some proteolytic activities. The hemopexin domain, very similar in structure among the MMPs, is an ellipsoidal disc, and is connected to the catalytic domain by a hinge region. The hinge region is flexible and rich in proline residues. It may also influence substrate specificity (Bode et al., 1999, Sternlicht and Werb 2001).

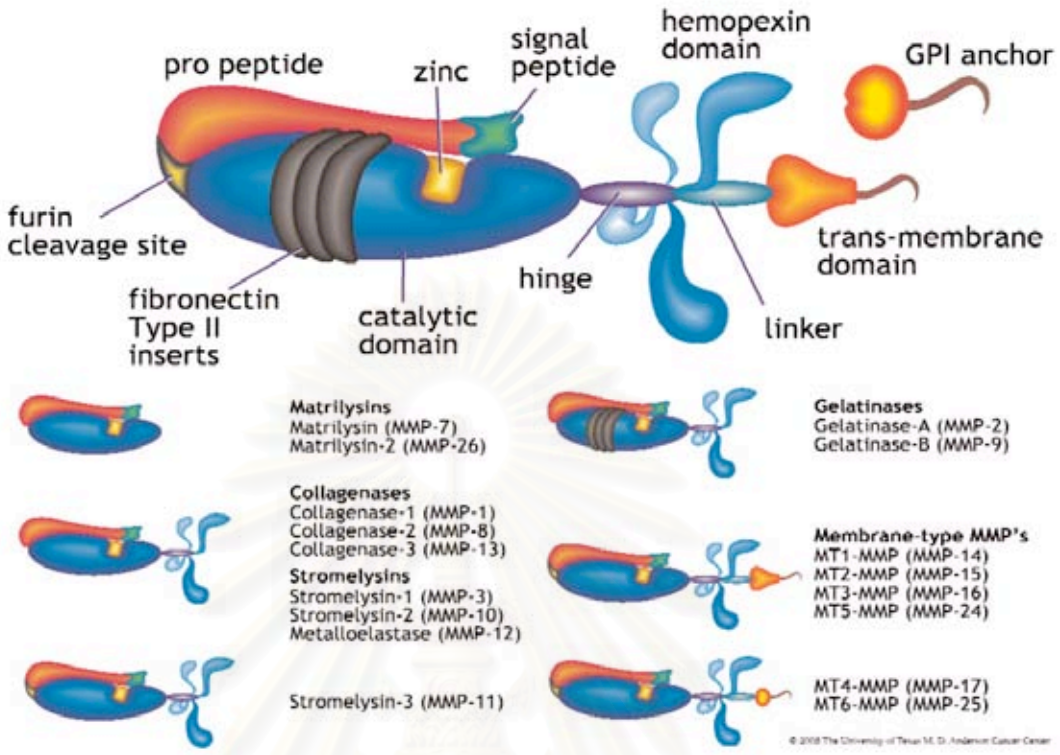


Figure 2.3. Basic structural domains of the MMPs. *The matrilysins contain the minimal domain structure onsisting of a signal peptide, a propeptide domain, and a catalytic domain with a highly conserved zinc-binding site. A conserved cysteine in the propeptide domain coordinates with the zinc in the active site to maintain latency of the proMMPs. The collagenases and stromelysins contain in addition to the minimal domain structure a hemopexin-like (PEX) domain in a four-bladed propeller-type structure connected to the catalytic domain via a hinge region. The gelatinases have three fibronectin type II repeats within their catalytic domains, which allow binding to denatured collagens (gelatin). The MT-MMPs are tethered to the cell surface via a transmembrane domain or via a glycosylphosphatidylinositol (GPI) membrane anchor. Some of the MMPs contain a furin-cleavage site between their propeptide and catalytic domains allowing activation by furin-type convertases (Rundhaug et al., 2005).*

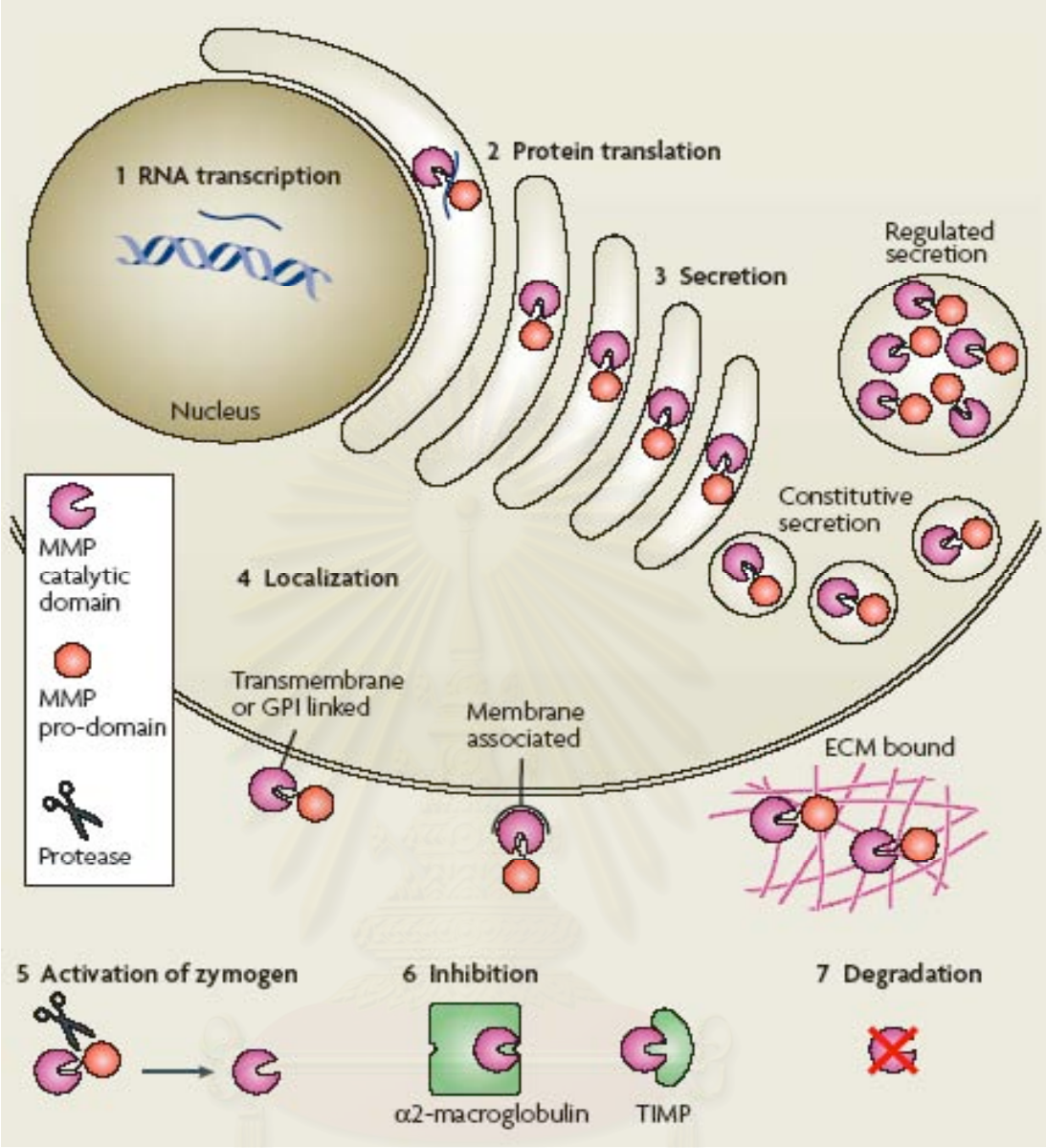


Figure 2.4. General mchanism of MMPs regulation. MMP function can be regulated at many levels. *In addition to (1) RNA transcription and (2) protein synthesis, MMP function can be regulated at the levels of (3) secretion, intracellular trafficking, (4) subcellular or extracellular localization, (5) activation of the zymogen form, (6) expression of their endogenous protein inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) and α2-macroglobulin, and (7) protease degradation. ECM,extracellular matrix; GPI, glycosylphosphatidylinositol. (Page-McCaw et al., 2007)*

1.1. The role of MMPs in cancer

The exact role of each individual MMP in each individual process is far from clear. Indirect evidence may support the coexistence of a specific MMP in a specific process, but this does not necessarily imply a causal relationship. Indeed, it may be that MMPs can replace each other in many processes. This assumption is supported by the fact that most MMP knockout mice do not have a sharply defined phenotype, and depletion of one specific MMP has not led to the death of an organism. Also, the specific substrates for each MMP are not clear. Because there are 100 known macromolecular components of the extracellular matrix, it will still take a huge amount of research to clarify precisely which component is a substrate for each specific MMP (Folgueras et al., 2004).

The proposed role of MMPs in cancer progression is based on in vitro and in vivo preclinical studies of clinical specimens. MMPs degrade the basement membrane and extracelllular matrix, thus facilitating the invasion of malignant cells through connectiv tissues and blood vessel walls and resulting in the establishment of metastases. In knockout mice lacking specific MMPs exhibit reduced tumorigenesis, angiogenesis and tumor progression (Wilson et al., 1997; Itoh et al., 1998; Masson et al., 1998). MMPs expression, although low or undetectable in most normal tissues, is substantially increased in the majority of malignant tumors. Numerous studies demonstrate overexpression of MMPs in malignant tissues in comparison to adjacent normal tissues (Kugler et al., 1998; Hashimoto et al., 1998; Sutinen et al., 1998). In addition, the plasma and urine levels of MMPs are elevated in patients with cancer compared with healthy subjects (Zucker et al., 1999). The MMPs in tumor tissues are produced not only by malignant tumors but also by stromal firoblast and inflammatory cells. These cells may produce cytokines and proteins that induce the

MMPs production. MMPs are also participated in the regulation of tumor growth by target and activate growth factors whose precursors are anchored to the cell surface or sequestered in the peritumor ECM (Yu and Stamenkovic, 2000). The ability of MMPs to target substrates that influence the apoptotic process is also relevance for cancer. Thus, MMP-3 has pro-apoptotic actions on the neighboring epithelial cells (Witty et al., 1995). Also in this regard, it is of interest that mice deficient in MMP-2, MMP-3 or MMP-9 have lower levels of apoptosis induced by TNF-α (Wielockx et al., 2001). MMPs activities have also been traditionally associated with a variety of escaping mechanisms that cancer cells develop to avoid host immune response (Coussens and Werb, 2002). Some MMPs, such a MMP-9 can suppress the proliferation of T-lymphocytes through disruption of the IL-2Rα signaling (Sheu et al., 2001). In addition, MMPs may modulate antitumor immune reactions through their ability to efficiently cleave several chemokines (Van den Steen et al., 2002). The role of MMPs in angiogenesis is also dual and complex. The relevance of these enzymes as positive regulators of tumor angiogenesis has been largely demonstrated. Thus, several pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or transforming growth factor-β (TGF-β) are induced or activated by these enzymes, triggering the angiogenic switch during carcinogenesis and facilitating vascular remodeling and neovascularization at distant sites (Belotti et al., 2003; Bergers et al., 2000; Sounni et al., 2002; Yu and Stamenkovic, 2000). An additional connection between angiogenic factors and MMPs derives from the recent finding that MMP-9 is induced in tumor macrophages and endothelial cells and promotes lung metastasis (Hiratsuka et al., 2002). Furthermore, host-derived MMP-9 contributes to the malignant behavior of ovarian carcinomas by promoting neovascularization (Huang et al., 2002).

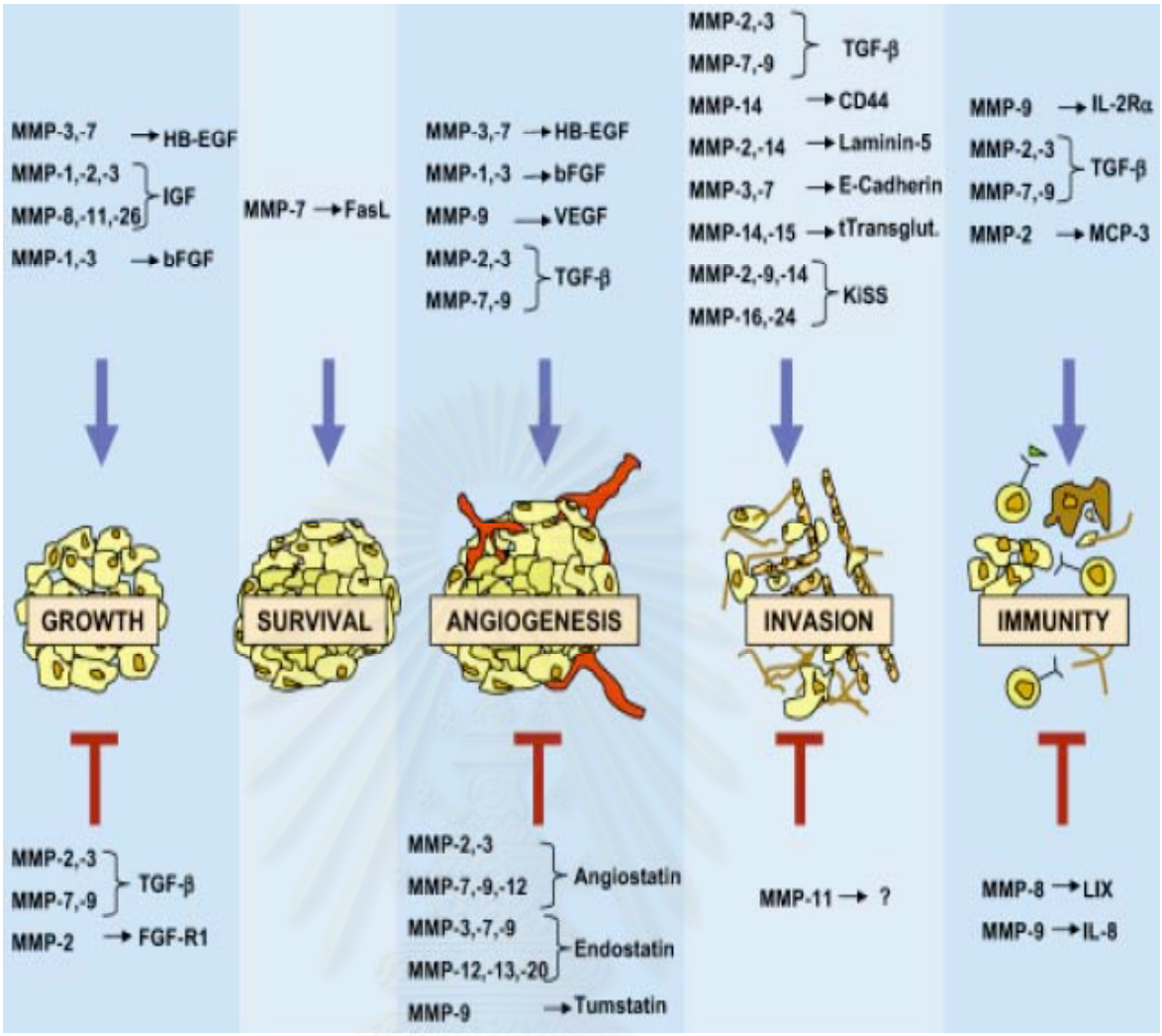


Figure 2.5. The functions of MMPs in tumor progression**.** *The opposite effects of bioactive molecule processing by MMPs on cancer development are shown (Folgueras et a., 2004).*

Taken together, these findings illustrate the diversity of MMP functions associated with cancer. Hence, it is critical to identify the physiological role of each individual MMP and its specific participation in the multiple stages of tumor evolution to better develop effective therapeutic interventions.

2. Matrix metalloproteinase-9

MMP-9 is belong to the gelatinases group (MMP-2 and MMP-9) in MMPs family, which are the main enzymes able to degrade the basement membrane, play a significant role in the ability of cancer to invade and metastasis. Because the disruption of basement membrane is a key step in malignant transformation, these MMPs are assumpted to play a key role during metastazing process. There were differences between MMP-2 and MMP-9. Many different types of tissue express continuously MMP-2 normally but MMP-9 only express in some specific tissue. Furthermore, MMP-9 is involved in many steps of cancer progression and more important for the invasive phenotype of cancer that MMP-2. This study will focus only on MMP-9, which is normally express in small number of cell type, but highly inducible in specific cells such as in tumor tissue.

2.1 Structure and functions of MMP-9

MMP-9 is another metalloproteinase capable of basement membrane degradation in vivo. Unlike MMP-2, which is constitutively expressed by many cells, MMP-9 expression normally only occurs in trophoblasts, osteoclasts, and leukocytes and their precursors (Borregaard et al., 1995, Harvey et al. 1995, Janowska-Wieczorek et al., 1999, Witty et al., 1996). While MMP-2 expression has only slight control at the transcriptional level, MMP-9 transcription can be highly induced by a wide range of agents. These agents include growth factors, cytokines, cell-to-cell adhesion and cell to extracellular matrix (ECM) adhesion molecules, and agents altering cell shape. (Dong et al., 2001, Martin et al., 2001) Along with the differences between the quantities of MMP-2 and MMP-9 synthesis induction, there also exist qualitative differences. For example, TGF-β1 strongly up-regulates MMP-9 mRNA

expression while simultaneously down-regulating MMP-2 expression (Thompson et al., 2001). These differences suggest that these two enzymes have different biological functions. Similarly to MMP-2, MMP-9 is also synthesized as a precursor with a molecular mass of 92 kDa, which is bound to TIMP-1 (Murphy et al., 1989; Moll et al., 1990). However, in cell cytosol, the enzyme can be stored in either a latent or an active form, which is in contrast to MMP-2, which can be stored only in a latent form (Nguyen et al., 2001). The activation of proMMP-9 is a complex process, which is regulated by interaction with TIMP and other MMPs (Kolkenbrock et al., 1995). Numerous enzymes have been suggested to be capable of proMMP-9 activation. These include MMP-2, leukocyte elastase, tissue kallikrein (Menashi et al., 1994, Ferry et al., 1997), stromelysin, collagenase-1 (Kolkenbrock et al., 1995), and trypsin (Bu and Pourmotabbed 1996). MMP-9 has several active metabolites with molecular weights of 82, 67, 49, 41.5 and 40 kDa. All TIMPs can inactivate MMP-9, but TIMP-1 seems to have the highest specific activity (Howard et al., 1991).

The Zn^{2+}binding domain of MMP-9 contains the conserved sequence AHEXGHXXGXXH, in which the three histidines are responsible for the coordination of the catalytic Zn^{2+}-binding domain, forms the active site and is essential for the enzymatic activity. In the human proenzyme, the fourth ligand of the $Zn^{2+}$is $cysteine_{86}$ of the conserved sequence PRCGXPD in the prodomain. This prodomain is removed by various types of proteolysis or is distorted by substrate binding (Bannikov et al., 2002) to yield the active enzyme through the cysteine-switch mechanism (Van Wart and Birkedal-Hansen, 1990). The function of the hemopexin-domain is less clear. It was shown that it is important for the binding of the TIMPs. The fibronectin type II repeats in MMP-9 is responsible for binding to gelatin, laminin, and collagen type I and type IV. The activation status of MMP-9 is

also important, because pro-MMP-9 bind with higher affinity to collagen type I and to gelatin, and with lower affinity to collagen type IV compared with active MMP-9 (Van den Steen et al., 2002).

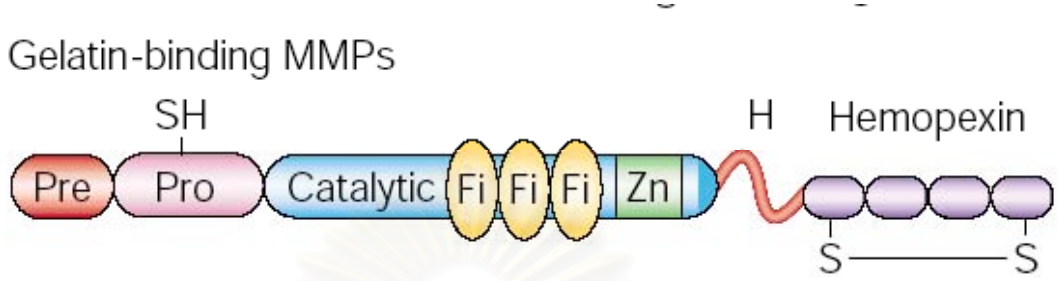


Figure 2.6. The protein structure of MMP-9. *MMP-9 contain an amino-terminal signal sequence (Pre) that directs them to the endoplasmic reticulum, a propeptide (Pro) with a zinc-interacting thiol (SH) group that maintains them as inactive zymogens and a catalytic domain with a zinc-binding site (Zn). In addition to the domains that are found in the minimal domain MMPs, the simple hemopexindomain-containing MMPs have a hemopexin-like domain —that is connected to the catalytic domain by a hinge (H) —which mediates interactions with tissue inhibitors of metalloproteinases, cell-surface molecules and proteolytic substrates. The first and the last of the four repeats in thehemopexin-like domain are linked by a disulphide bond (S–S). The gelatin-binding MMPs contain inserts that resemble collagen-binding type II repeats of fibronectin (Fi) (Egeblad and Werb, 2002).*

The substrate-specificity of MMP-9 depends on the primary sequence of the substrate, because, in general, endopeptidases posses a clear preference for peptide sequences that can bind in the groove of the catalytic site. However, the three-dimensional conformation and accessibility of the cleavage site in a substrate is also important. There are numerous reports demonstrating the ability of MMP-9 to cleave type IV collagen in vitro. The in vivo situation, however, is not equally clear. In

addition to type IV collagen, MMP-9 is able to cleave the type V and XI collagens (Pourmotabbed et al., 1994). To a lesser degree, it also has activity against aggrecan (Fosang et al., 1992) and elastin (Senior et al., 1991), but not against type I collagen (Murphy et al., 1982). Physiologically, MMP-9 participates in trophoblast implantation, bone development, wound healing, and inflammatory processes, probably by enabling inflammatory cells to invade into the inflammatory focus and by participating in the regulation of inflammatory responses (Borregaard et al., 1995, Harvey et al., 1995, Janowska-Wieczorek et al., 1999, Goetzl et al., 1996,Witty et al., 1996, Sheu et al., 2001). Although there are physiologically only a few cell types expressing MMP-9, there are wide ranges of tumors showing MMP-9 expression either in the tumor cells or in the normal cells surrounding the tumor (Pyke et al., 1992, Canete-Soler et al., 1994, Soini et al., 1994, Ashida et al., 1996, Iwata et al., 1996).

	ECM	**Other proteins**
MMP-9 substrates	Agrecan Collagen IV, V, Xi, XIV Decorin Elastin Fibrillin Gelatin Laminin Link protein Osteonectin Vitronectin	α2M α1PI Casein C1q Fibrin, Fibrinogen, Plasminogen IL-1β Pro-TGF-β TNF-α Substance-P

Table 2.1. List of MMP-9 substrates.

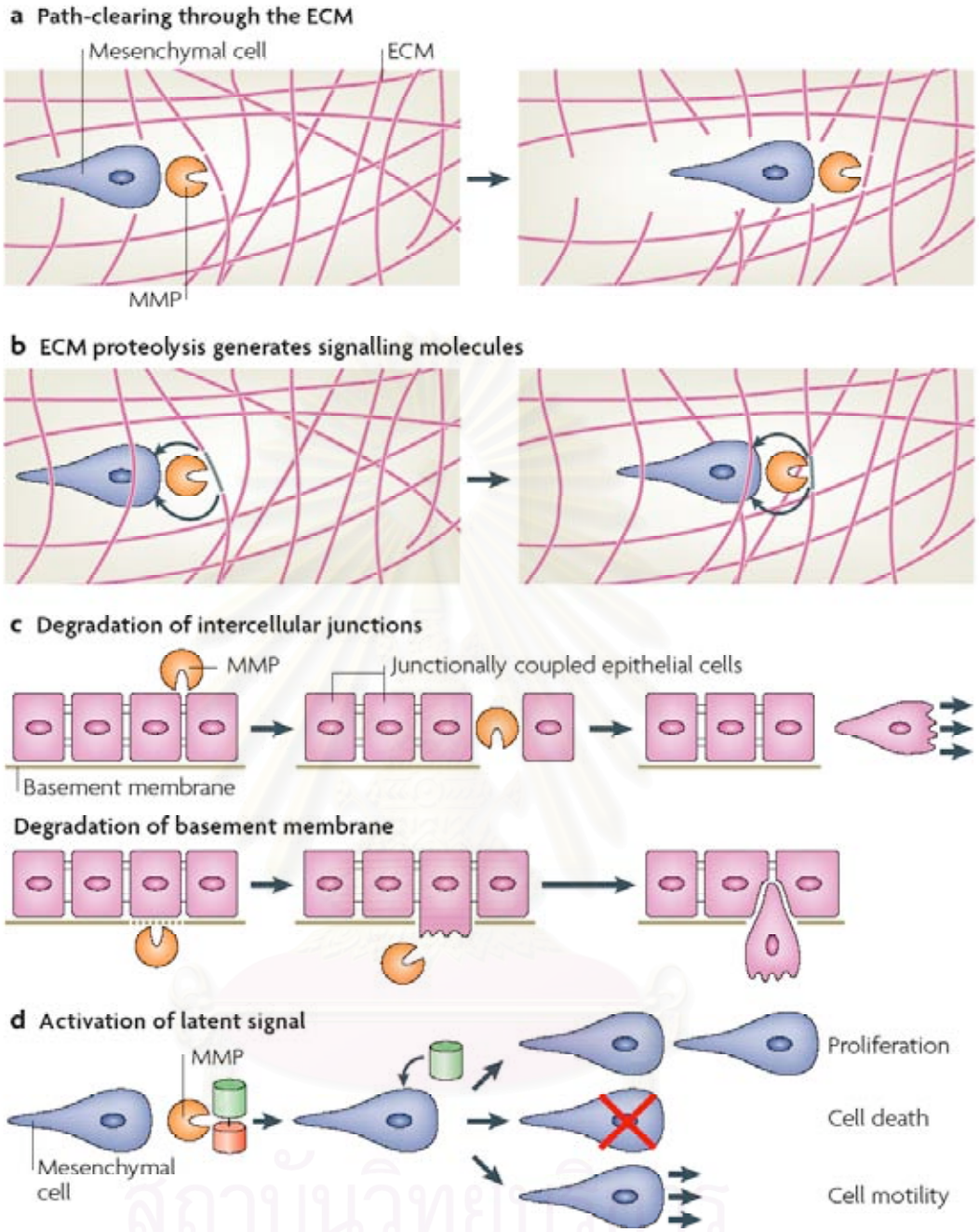


Figure 2.7. MMP-9 functions. *(a) Cleavage component of ECM. (b) Alternatively, MMP-9 proteolysis can generate specific cleavage product that then signal in an autocrine or paracrine manner. (c) Regulate tissue architecture by cleavage intercellular junction or basement membrane. (d) Activate or modify latent signaling molecules (modified from Page-McCaw et al., 2007).*

2.2 The regulation of MMP-9 activity

In general, MMP-9 is low in expression, but highly inducible by several cytokines, growth factors and oncogenes such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) (Björklund M and Koivunen, 2005). The regulation of MMP-9 is proven to be complex and controversial because multiple pathways are involved. Unlike the oncogenes, most MMPs are not up-regulated by gene amplification or activating mutation, therefore, the increased of MMP-9 expression is probably due to transcriptional changes rather than genetic alterations (Egeblad and Werb, 2002). Previous studies concluded that the regulation of MMP-9 could be at the transcriptional level, post-transcriptional level, secretion, zymogen activation and inhibition of proteolytic activity by its inhibitors such as TIMP-1 (Chakraborti et al., 2003).

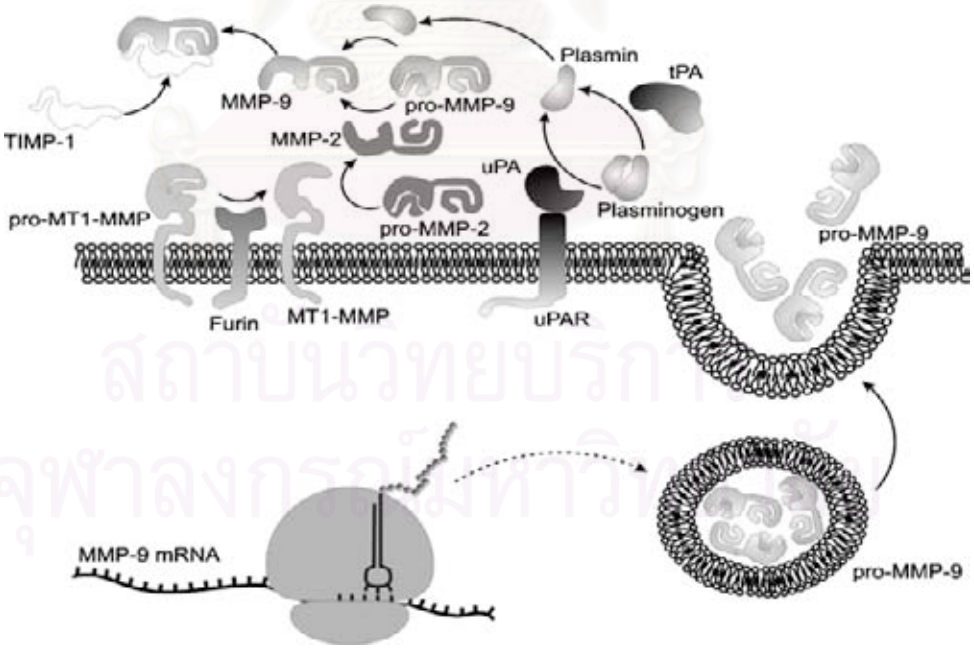


Figure 2.8. Regulation of MMP-9 activity. *Like other MMPs, MMP-9 can be regulated at transcriptional level, protein synthesis level, secretion, activation and inhibition.*

The MMP-9 gene (chromosome 20q13.2) is transcribed into a 2.5 kb mRNA species (Huhtala et al., 1991). Several binding sites for transcription factors have been described. At position -29 a TATA motif-like sequence is located and a consensus sequence for nuclear stimulating protein-1 (Sp-1), also named GC box is present at -563 bp relative to the transcriptional start site. More proximally, at position -54 bp, a retinoblastoma binding element (RBE) or GT box is located and is also recognized by Sp-1. A consensus of TGF-β-inhibitory element (TIE) is located at -472 bp. Furthermore, the promoter contains at least four 12-*O*- tetradecanoyl-phorbol-13-acetate (TPA)-responsive elements (TRE) or activating protein-1 (AP-1) binding sites. Several sequences with homology to the polyomavirus enhancer A-binding protein-3 (PEA-3), which are recognized by Ets-1 and Ets-2 proto-oncogenes also found in MMP-9 promoter. Moreover, the MMP-9 promoter also contains a nuclear factor-kappa B (NF-κB) motifs and a microsatellite segment of alternating CA residues (Sato and Seiki, 1993; Himelstein et al., 1997; Gum et al., 1997; Huhtala et al., 1991; Van den Steen et al., 2002).

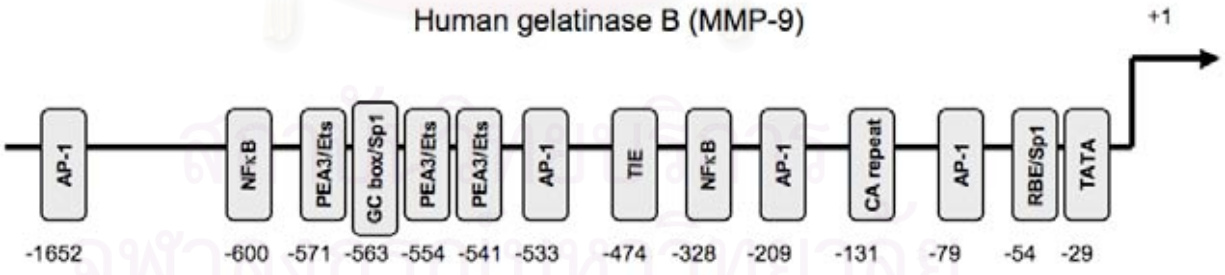


Figure 2.9. Diagrammatic representation of the transcription factor binding sites in the MMP-9 promoter. Transcription factor binding sites are represented as boxed areas (Sato and Seiki, 1993; Himelstein et al., 1997; Gum et al., 1997; Huhtala et al., 1991; Van den Steen et al., 2002).

The 5’ flanking region of the gene contains binding sites for AP-1, NF-κ B, and Sp1, which synergistically mediate the induction of MMP-9 gene expression by TPA or TNF-α, and TGF-β inhibitor element (TIE) (Huhtala et al., 1991, Sato & Seiki 1993). The GT box located downstream of the AP-1 site is essential for the induction of gene transcription by v-Src, which is also able to mediate promoter activation via the AP-1 site (Sato et al., 1993). Ets and Sp-1 are essential for activation of MMP-9 gene expression in fibroblasts (Himelstein et al., 1998). NF-κ B is necessary for the upregulation of MMP-9 gene by inflammatory cytokines, IL-1α or TNF-a, but not by bFGF or PDGF. AP-1 slightly mediates the gene transcription by bFGF, PDGF, IL-1α or TNF-α (Bond et al., 1998). Functional polymorphism in the promoter of the MMP-9 gene results in variation in its expression at the transcriptional level (Peters et al., 1999). However, the expressions of mechanism of MMP-9 regulated by these mediators are still not clear and may depend on the context of the cell type and staging. The intracellular signaling pathways that contribute to MMP-9 gene transcription is vary depend on the cell-type and the inducers. These well-documented signaling pathways are MAPK, STAT, PI3K/Akt, Smad and PKC (Van den Steen et al., 2002).

2.3 The role of MMP-9 in cancer

As mentioned earlier, MMP-9 is involved in many steps of cancer progression, which are regulation of tumor growth, tumor metastasis, tumor neovascularization and suppression of the host immune response to tumor. In head and neck cancer, numerous studies indicated the important roles of MMP-9 in both in vitro and in vivo. MMP-9 participates in the invasion of cells through matrix barriers and collagenolysis during invasion and tumor progression by degrading the matrix macromolecules.

Many animal studies suggest that MMP-9 (along with MMP-2) has a critical role in tumor invasion (Sier et al., 1996). For example, the human osteosarcoma cell line up-regulates MMP-9 expression in response to TNF-α and becomes more invasive in vitro. Treatment of these cells with TNF-α prior to injection into nude mice results in an increased number of lung metastases in a dose-dependent manner (Kawashima et al., 1994). In ICAM-deficient nude mice, lymphomas are not able to disseminate before they attain the capability of continuous MMP-9 expression (Lalancette et al., 2000). In vivo study showed the loss of ability to metastasis and reduced angiogenesis in the Mmp-9 null mice (Itoh et al., 1998, 1999). Hence, studies of the mechanisms that regulate expression of MMP-9 are important for understanding the process of cancer progression.

MMP-9 is expressed in head and neck carcinoma cells and may take part in the progression and invasion of tumors (O-Charoenrat et al., 2000). An association between MMP-9 mRNA, protein or enzyme activity to invasion or to lymph node metastasis in head and neck cancers had been suggested (de Vincente et al., 2005). Overexpression of MMP-9 mRNA was found associated with progression of oral dysplasia to cancer (Jordan et al., 2004). In addition, highly expression of MMP-9 was reported to be associated with survival rate of head and neck squamous cell carcinoma patients (Ruokolainen et al., 2004).

Transforming growth factor-beta1 (TGF-β1)

1. The TGF-β family

The transforming growth factor-β family comprises a large number of structurally related polypeptide growth factors, each capable of regulating a fascinating array of cellular processes including cell proliferation, lineage

determination, differentiation, motility, adhesion, and death. Expressed in complex temporal and tissue-specific patterns, TGF-β and related factors play a prominent role in the development, homeostasis and repair of virtually all tissues in organisms. Collectively, these factors account for a substantial portion in the intracellular signals governing cell fate (Derynck et al., 1998).

TGF-β and related factors are multifunctional agonists whose effects depend on the state of responsiveness of the target cell as much as on the factors themselves. To date, there are about 9 subfamilies in TGF-β family which are Bone morphogenic protein 2 (BMP-2) subfamily, BMP-5 subfamily, Growth and differentiation factor 5 (GDF-5) subfamily, Vg1 subfamily, BMP-3 subfamily, Activin subfamily, TGF-β subfamily, Intermediate members such as Nodal and several distantly related members. TGF-β is the prototype of this family and three subfamiles are well characterized in vertebrate as listed in Table 2.2.

TGF-β family members, their receptors and signaling molecules (Heldin et al., 1997)			
Subfamily	TGF-β	Activin	BMP
Ligands	TGF-β1 TGF-β2 TGF-β3	Activin A	BMP-2 BMP-4 BMP-7
Type II receptor	TβRII	ActRII ActRIIB	BMPRII ActRII ActRIIB
Type I receptors	TβRI	ActRI ActRIB	BMPRIA BMPRIB ActRI
Pathway-restricted Smads	Smad2 Smad3	Smad2 Smad3	Smad1 Smad5 Smad9
Common-partner Smad	Smad4	Smad4	Smad4
Inhibitory Smads	Smad6 Smad7	Smad6 Smad7	Smad6 Smad7
Responses	Regulation of mitogenicity Induction of ECM	Induction of dorsal mesoderm Induction of erythroid differentiation Induction of follicle-stimulating hormone release	Induction of ventral mesoderm Induction of cartilage and bone Induction of apoptosis

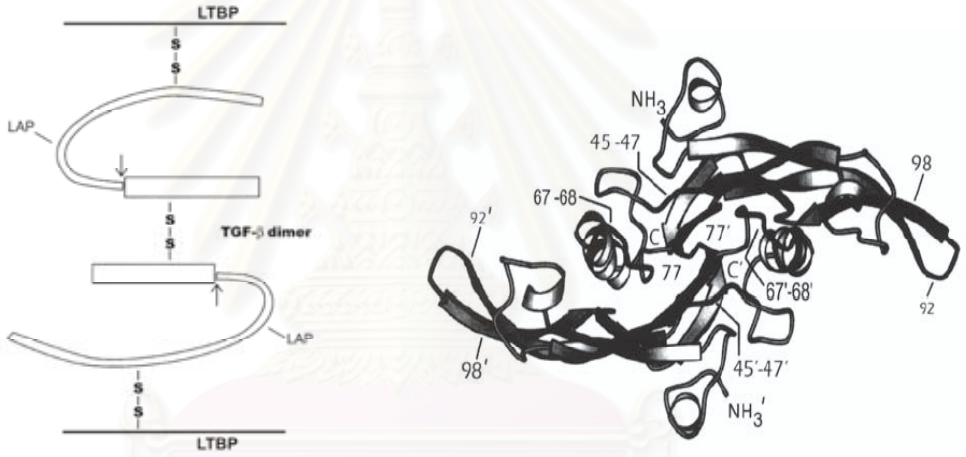
Table 2.2. TGF-β family member, their receptors and signaling molecules (Heldin et al., 1997)

2. The TGF-β1 protein

Transforming growth factor beta (TGF-β) is a protein that comes in three isoforms called TGF-β1, TGF-β2 and TGF-β3. Recently, two more isoforms have been discovered, TGF-β4(Tabibzadeh et al., 1998) and TGF-β5(Chimal-Monroy and Diaz de Leon, 1999).Their amino acid sequences display homologies on the order of 70-80 %. TGF-β1 is the prevalent form and is found almost ubiquitously while the other isoforms are expressed in a more limited spectrum of cells and tissues. The biologically active forms of all isoforms are disulfide-linked homodimers. Disulfide-linked heterodimers of TGF isoforms have been reported also. The heat- and acid-stable monomeric subunits have a length of 112 amino acids. TGF-β4 contains two additional amino acids in the vicinity of the aminoterminal end. The isoforms of TGF-β arise by proteolytic cleavage of longer precursors (TGF-β1: 390 amino acids, TGF-β2 : 412 amino acids, TGF-β3 : 412 amino acids, TGF-β4 : 304 amino acids, TGF-β5 : 382 amino acids). The isoforms are derived from the carboxyterminal ends of these precursors.It was also the original name for TGF-β1, which was the founding member of this family. Many cells synthesize TGF-β1 and almost all of them have specific receptors for this peptide. TGF-β1 controls proliferation, cellular differentiation, and other functions in most cell types. It can also act as a negative autocrine growth factor.

TGF- β is stored in the ECM as a large latent complex composed of TGF-β, its propeptide TGF-β latency-associated protein (LAP), and a latent TGF-β-binding protein (LTBP) (Taipale et al., 1994). However, different inactive TGF-β forms may exist, since osteoblast-like cells produce small latent TGF-β complex lacking the LTBP (Dallas et al., 1994). The matrix association and release of TGF-β form a finely regulated network for the maintenance of ECM. The mechanisms of

TGF-β activation are not known in detail. The main fraction of the factor in the serum is covalently attached to one of the Acute phase proteins, Alpha-2-Macroglobulin (α2M) the synthesis of which is known to be induced several hundred-fold by IL-6. α2M/TGF-β complexes are believed to represent TGF-β molecules released by platelets after tissue injuries and destined to degradation. Multiple proteases such as serine proteases or MMPs are able to release TGF-β from ECM, and further proteolytic activation of the inactive TGF-β by MMPs or by acid treatment, enable its signalling through type I and type II serine/threonine kinase receptors (Taipale et al., 1992, Wrana et al., 1994, Yu and Stamenkovic 2000, Maeda et al., 2002).



(a) (b)

Figure 2.10. Sructure of TGF-β1. *(a) TGF-β latency complex.The structure of the inactive TGF-β complex is shown with the TGF-β dimer interacting with the latency associated peptide (LAP) and the latent TGF-β binding protein (LTBP). Arrow indicates cleavage site. (b) Structure of TGF-β. The crystal structure of TGF-β is shown. Amino acids important in regulating binding of TGF-β to receptors and binding proteins have been highlighted (www.wikipedia.org).*

3. Smad proteins and TGF- β1 signaling

Members of the TGF-β family exert their effect by binding to heteromeric complexes of two different kinds of serine/threonine kinase receptors denoted type I and type II (Massagué et al., 1998; Heldin et al., 1997). Seven different type I receptors (activin receptor-like kinase (ALK)-1 to 7) (ten Dijke et al., 1994) and four different type II receptors have been identified to date. The ligand specificity of these receptors has been determined primarily by their ability to bind a given ligand and activate specific downstream genes. After ligand stimulation, the activated type I receptor transduces the signal by phosphorylating a member of a family of proteins known as Smads. Eight Smad proteins have been identified in mammals so far and have been divided into three classes based on their structure and function: receptor-regulated Smads (R-Smads), common-partner Smads (co-Smads) and inhibitory Smads (I-Smads). These Smad proteins play a significant role in TGF-β signaling pathway.

The Smad proteins, consisting of about 400-500 amino acids each, have conservative N-terminal (40-94% sequence identity) and C-terminal (38-90% sequence identity) domains known as MH1 and MH2 domains (Mad homology domains) linked by a proline-rich linker region differing in sequence and length.

The R-Smads can be further divided in 2 subtypes: those activated by TGF-β and activin receptors (Smad2, Smad3), and those activated by BMP receptors (Smad1, Smad5 and Smad8), although data is accumulating which suggests that Smad1, 5, 8 might also act promiscuously with TGF-β receptors (Macias-Silva et al., 1998; Lux et al., 1999). One co-Smad (Smad4) has been described so far in mammals, but others might exist; two co-Smads were found in Xenopus laevis (Howell et al., 1999; Masuyama et al., 1999).

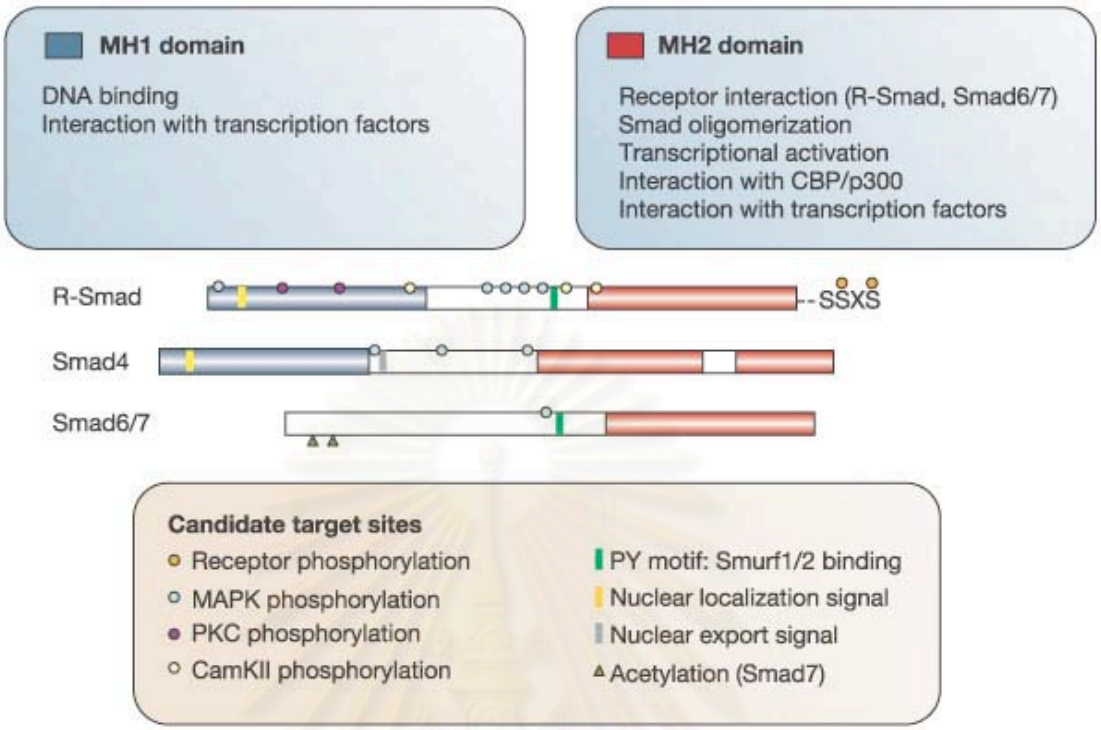


Figure 2.11. Structural organization and role of the domains of Smads, and candidate target sites for kinase pathways. *Such pathways include Erk MAPK and JNK, as well as CamKII and PKC. The significance of candidate MAPK phosphorylation sites in Smad4 and Smad6/Smad7 is not known (www.nature.com).*

The R-Smads and Smad4 are expressed in most, if not all, cell types. The Co-Smad, Smad-4, forms hetero-oligomers with the pathway-restricted Smads and is a common mediator of TGF-β, activin and BMP signaling (Lagna et al., 1996; Zhang et al., 1997). Although ubiquitously involved in Smad-mediated transcription, Smad4 is not essential for TGF-β response because some TGF-β responses occur in the absence of Smad4 and some Smad4-deficient cell lines have limited responsiveness to TGF-β (Sirard et al., 2000).

To date, two I-Smads have been identified in mammals, Smad-6 and Smad-7 (Imamura et al., 1997; Nakao et al., 1997; Topper et al., 1997). These Smads have

been identified as inhibitors of TGF-β, activin and BMP signalling and might function in negative feedback loops since TGF-β, activin and BMPs are all able to induce their expression.

Upon ligand binding, the constitutively phosphorylated type II receptor kinase trans-phosphorylates and activates the type I receptor to initiates downstream signaling (Wrana et al., 1994). The first intracellular step in the TGF-β/Smad pathway, the recruitment of Smad2 and Smad3 to the TGF-β receptor complex, is controlled by amembrane-associated FYVE-domain-containing protein, termed Smad anchor for receptor activation (SARA)(Tsukazaki et al., 1998). R-Smads interact directly with activated type I receptor and the receptors will then internalized in endosomes. Upon phosphorylation of Smad2 and Smad3 by activated type I receptors, R-Smads and SARA dissociate from the TGF-β receptor complex. Phosphorylation of R-Smads relieves the auto-inhibitory MH1-MH2 interaction and allows R-Smads to form complexes with Smad4 through their MH2 domains and translocation into nucleus and regulate transcription of the target genes. The released SARA is capable of recruiting other non-activated Smad2 or Smad3 for receptor presentation (ten Dijke et al., 2000).

In a non-activated state, R-Smads exist as monomers and upon receptor-mediated phosphorylation, they form homo-dimers and hetero-dimers with each other, as well as hetero-dimers or hetero-trimers with Smad4 (Kawabata et al., 1998; Wu et al., 2001). Without ligand stimulation, R-Smads localize in the cytoplasm, whereas Smad4 is distributed in the nucleus and the cytoplasm (Inman and Hill, 2002).

I-Smads interact stably with activated type I receptors and prevent phosphorylation of the R-Smad by these receptors. Smad-7 interacts with all activated type I receptors (Souchelnytskyi et al., 1998) and is a general inhibitor of TGF-β

superfamily induced responses, whereas Smad6 is thought to inhibit preferentially the phosphorylation of BMP Smads (Itoh et al., 1998) although this is controversial (Imamura et al., 1997). While Smad-7 mRNA expression is rapidly upregulated by R-Smads, Smad-6 mRNA is induced after several hours and is maintained for 48 hours or more (Miyazono et al., 1999). This again suggests different mechanisms for the action of the two I-Smads.

Ubiquitin-proteasome-mediated degradation controls the levels of Smds post-transcriptionally. The HECT (homologous to the E6-AP carboxy terminus) family E3 ubiquitin ligases, Smurf1 (Smad-ubiquitin-regulatory factor 1) and Smurf 2, antogonize TGF-β family signaling by interacting with R-Smads and target them for degradation (Derynck and Zhang, 2003). Proteasome degradation also regulates the R-Smad levels after translocation into the nucleus. However, only a small fraction of Smad2 and Smad3, in the absence or presence of TGF-β, is ubiquitinated, and, upon TGF-β signaling, phosphorylated Smad2 or Smad3 is not target for degradation, but dephosphorylated and relocated to the cytoplasm (Inman and Hill, 2002).

In contrast, Smad4 is not subjected to ubiquitin-mediated degradation. Instead, sumoylation of Smad4 enhances its stability (Lee et al., 2003). However, some tumor-associated mutations allow ubiquitination and/or decrease the stability of Smad4 (Xu et al., 2000).

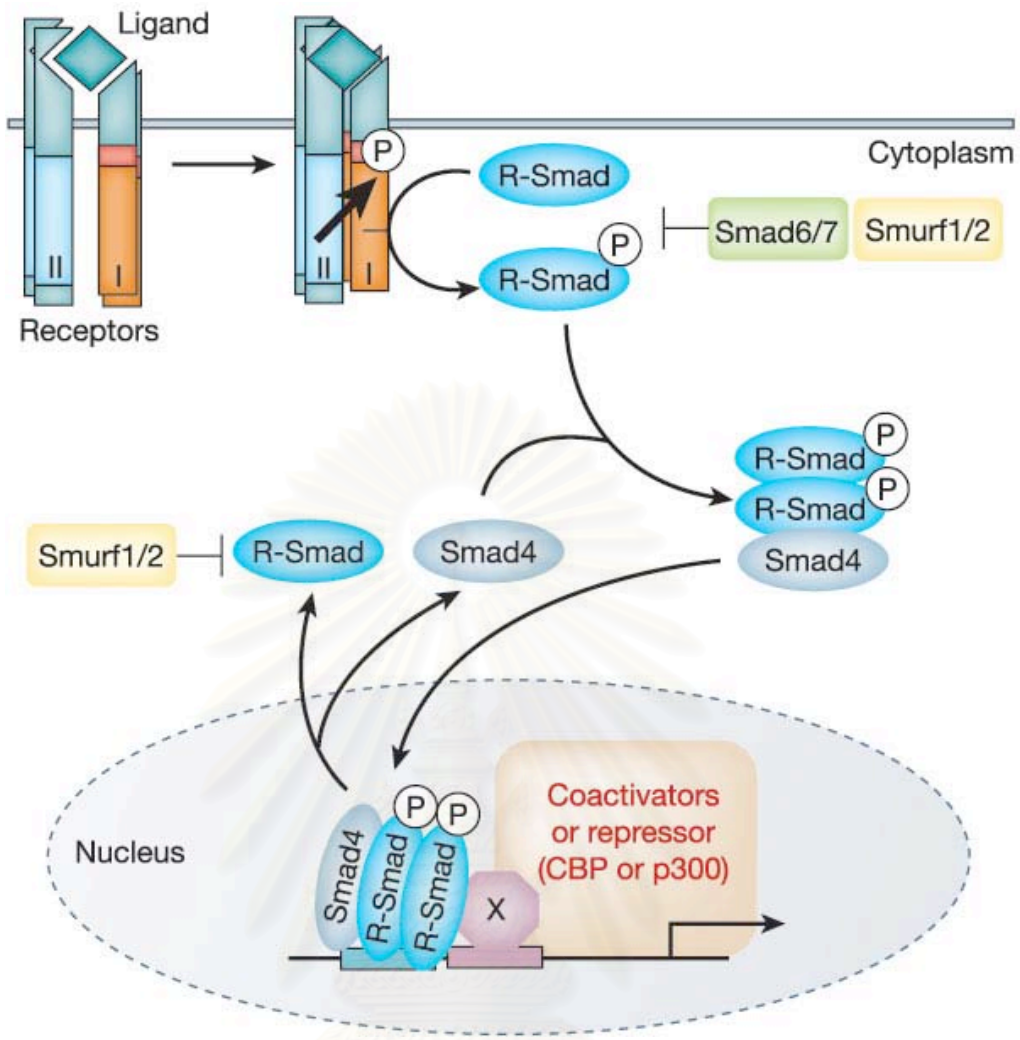


Figure 2.12. General mechanism of TGF-β receptor and Smad activation. *At the cell surface, the ligand binds a complex of receptor types I and II. The consequently activated type I receptors phosphorylate selected receptor-activated Smads (R-Smads) and then form a complex with a common Smad4. Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes, through physical interaction and functional cooperation with DNA binding transcription factors (X) and CBP or p300 coactivators. Activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7. R-Smads and Smad4 shuttle between nucleus and cytoplasm. The E3 ubiquitin ligases Smurf1 and Smurf2 mediate ubiquitination and consequent degradation of R-Smads and type I receptor(www.nture.com).*

After the nuclear localization of Smad-4 complex, they bind to DNA and affect gene transcription. TGF-β may induce c-Fos and c-Jun proto-oncogenes, which heterodimerize to form an AP-1 complex (Risse et al., 1989, Subramaniam et al., 1995). For many TGF-β regulated genes, Smads co-operate with the AP-1 complex at the AP-1 binding site, although Smads may also independently bind to AP-1 sequence, to regulate transcription of genes such as MMP-1 or MMP-13 (Zhang et al., 1998, Yuan & Varga 2001, Tardif et al., 2001). Inhibitory Smads act as negative regulators of signaling by the TGF-β s or BMPs (Nakao et al., 1997a, Imamura et al., 1997).

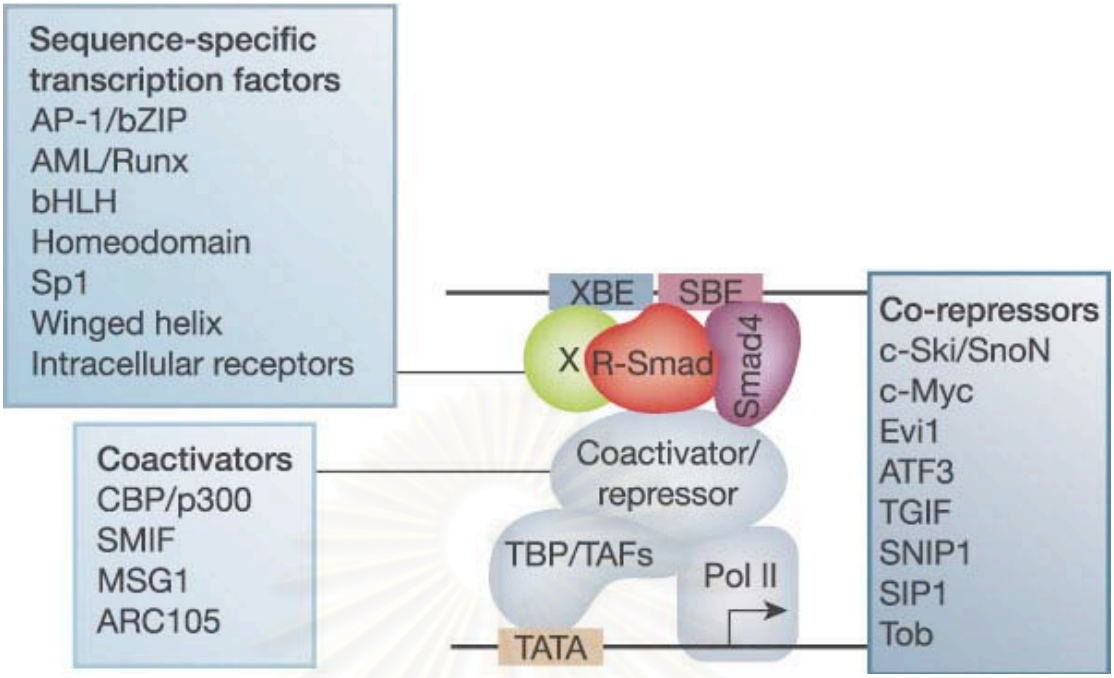


Figure 2.13. The R-Smad–Smad4 complex cooperates with sequence-specific transcription factors (X). *The complex bind with high affinity to a cognate DNA sequence (XBE), yet also binds with lower affinity to a Smad-binding DNA element (SBE). R-Smads interact directly with the essential CBP or p300 coactivator, and Smad4 serves as coactivator for R-Smads by stabilizing the R-Smad interaction with CBP/p300. Other Smad-interacting coactivators further define the level of Smad-mediated transcription activation Smad interacting co-repressors downregulate Smad-mediated transactivation. Several of these are proto-oncogenes, for example, c-Ski and the related SnoN, c-Myc99, 100 and Evi1—linking malignant transformation to repression of TGF-β /Smad-induced transcription. Other Smad co-repressors, for example, the homeodomain proteins TGIF (TGF-β-induced factor) and SNIP1 (Smad nuclear interacting protein) repress not only TGF-β/Smad-mediated transcriptional activation, but also Smad-independent transcription. Interaction of Tob with BMP-activated Smads represses BMP-activated gene expression, whereas its interaction with Smad2 represses interleukin-2 expression in T cells (www.nature.com).*

3.1. The possible networks of signaling induced by TGF-β

TGF-β response are not solely the result of the activation of Smad cascade, but are highly cell-type specific and dependent upon interactions of Smad signaling with a variety of other intracellular signaling mechanism initiated or not by TGF-β that may either potentiated, synergize or antagonize the linear TGF-β pathway. Nowadays, many studies showed the possibilities of network of crosstalks with other signaling pathways including the mitogen-activated protein kinase (MAPK), the NF-kB or phosphatidylinositol-3-OH kinase (PI3K/Akt) pathway, that largely contribute to modify the Smad signals and allow the pleiotropic activities of TGF-β and these pathways may be called as the Smad-4 independent pathway.

The identification of Smad dependent and independent genes causally involved in these TGF-β-mediated tumor promoting effects requires further research. Of note, Hocevar et al., recently reported c-Jun N-terminal kinase (JNK) dependent TGF-β-induced fibronectin expression in cell lines lacking the Smad4. The Smad-4 independent pathways, especially MAPK pathways, that can be activated by TGF-β have been described in many studies, but their biological significance remain largely unknown in carcinogenesis. Ras signaling has been proposed to inhibit TGF-β signaling via the ERK pathway by blocking the nuclear translocation of Smad1, 2, and 3 (Kretzschmar et al., 1999) that may explain why some cells with hyperactive Ras signaling do not respond to TGF-β (Calonge and Massague, 1999 ; Kretzschmar et al.,1999) While the activation of MAPK pathway may have positive or negative regulatory effects on R-Smads depending on the nature of MAPK activation. These could be concluded that there is a particularly complicated and intimate inter-relationship between the TGF-β system and Ras / MAPK pathway in

carcinogenesis. The AP-1 transcriptional complex is a primary target of a number of MAPK pathways and it has been shown that AP-1 components can interact directly with Smad3 (Zhang et al., 1998; Peron et al., 2001; Verrecchia et al., 2001) suggesting that AP-1 may be central to cross-talk between Smad and MAPK pathways.

Recent studies identified NF-kB transcription factor as another key modulator of TGF-β-induced epithelial-mesenchymal transition (EMT) in mammary epithelial cells overexpressing Ras oncogene (Huber et al., 2004). Inhibition of NF-kB blocked EMT in these cells, while its ectopic activation induced mesenchymal phenotypes independently of TGF-β and its inhibition in mesenchymal cells restored the epithelial phenotype. Thus, a cooperation of TGF-β, Ras and NF-kB is critical for epithelial plasticity manifested by EMT (Zhou et al., 2004; Bachelder et al., 2005).

TGF-β activates PI3K in a RhoA-dependent manner, and PI3K/Akt signaling is required for migration of breast cancer cells (Bakin et al., 2000). Interestingly, some of the features of malignancy tumors such as cell motility may overlap with PI3K-dependent cell scattering induced by HGF (Royal and Park, 1995; Day et al., 1999).

In prostate cancer cell line, Murine myeloma cell line, M1, and the human hepatoma cell line, Hep3B, the activation of p38 and JNK contribute in the TGF-β-induced apoptosis (Edlund et al., 2003; Sanchez-Capelo et al., 2005) and it has been proposed that delayed p38 activation by TGF-β rather than rapid Smad-independent p38 activation, participates in the induction of apoptosis by TGF-β (Yoo et al., 2003).

Moreover, a number of studies have shown an involvement of p38 kinase activity in TGF-β induce several MMPs biosynthesis in fibroblast, breast epithelial cell or in transformed keratinocytes (Ravanti et al., 1999; Johansson et al., 2000; Kim

et al., 2002, 2003). Overexpression of endogenous TGF-β in Smad-4 deficiency human oral keratinocytes lead to growth inhibition in vivo and tumor suppression in vitro by mechanism that are independent of Smad-4 expression and TGF-β induced G1 arrest, a particularly intriguing finding because loss of Smad-4 thought to be a key factor in driving tumor progression. (Paterson et al., 2002).

Furthermore, the fact that replacement of Smad-4 restores TGF-β responsiveness in Smad-4 defective cells (De Winter et al., 1997) is compelling evidence that Smad-4 has the capacity to act as a tumor suppressor. More recently, however, alternative pathways have been identified and it is now known that TGF-β1 can activate fibronectin (Hocevar et al., 1999), the 3TP-Lux reporter (Fink et al., 2001) and PAI-1 (Sirard et al., 2000) in Smad-4 deficient cells. These studies demonstrate that TGF-β1 can elicit transcriptional responses in the absence of Smad-4 but they do not explore the functional significance of Smad-4-independent pathways.

TGF-β also induces activation of Ras, RhoB and RhoA, as well as of TAK1 and protein phosphatase 2A, which leads to the activation of several MAP kinase pathways and the downregulation of S6 kinase activity. The mechanisms of activation of these non-Smad signaling events and how they connect to the heteromeric TGF-β receptor complex remain to be characterized.

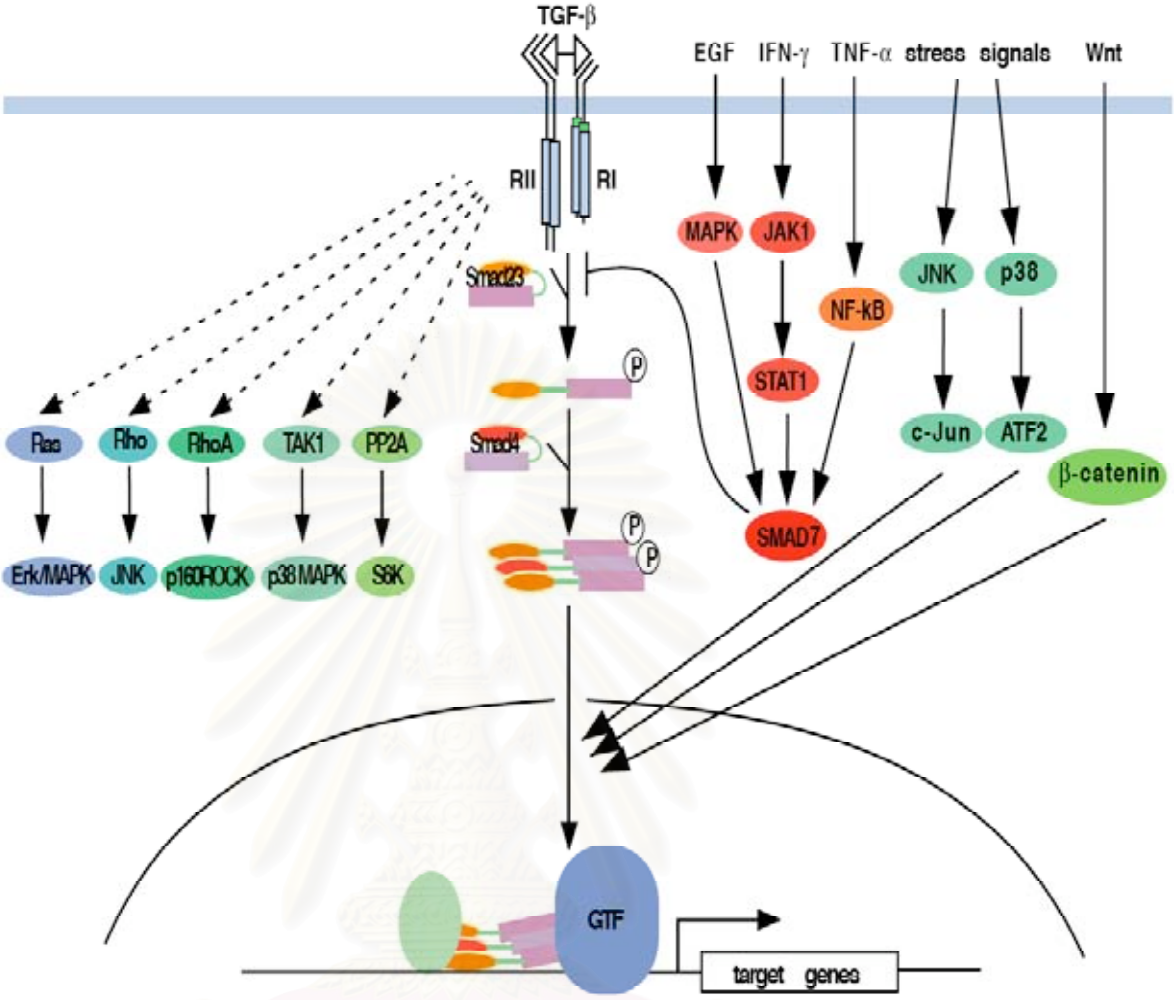


Figure 2.14. TGF-β-induced signaling through Smads, and several non-Smad signaling mechanisms. *Several other signaling pathways also regulate both signaling by Smads and Smad-mediated gene expression, as exemplified here by the activation of JNK and p38 MAP kinase signaling in response to various stress signals, and β-catenin signaling in response to Wnt proteins (www.ucsf.edu/derynck).*

4. The role of TGF-β1 in cancer

In carcinogenesis, changes in the response to growth factors such as TGF-β are likely to be critical steps to successful metastasis. TGF-β is believed to participate in the acquisition of invasion/metastasis abilities of cancer cells (Sehgal et al., 1996).

In mammal cells, there are three well-documented TGF-βs, TGF-β1, TGF-β2 and TGFβ-3, which are encoded by different genes and which all function through the same receptor system. Of these, TGF-β1 is most frequently upregulated in tumor cells and is the focus of most studies on the role of TGF-β1 in carcinogenesis. In the mouse multistage model of skin carcinogenesis, for example, TGF-β1 is not detectable in chemically-induced papillomas with a high frequency of malignant progression (Glick et al., 1993), loss of autocrine production of TGF-β1 facilitates tumor progression in keratinocytes with a targeted deletion of the TGF-β1 gene and in p53 null mice (Cui et al., 1994; Glick et al., 1994) and targeted expression of a dominant negative type II TGF-β receptor (DN-TβR-II) in mouse skin leads to an increase in carcinoma incidence and a decrease in tumor latency (Amendt et al., 1998).

TGF-β1 plays a dual role in carcinogenesis. In early stage, this cytokine display tumor suppressor activities by its anti-proliferative activity, its ability to induce apoptosis and to promote genomic stability, while in advance stage TGF-β acts as a promoter of tumor metastasis, stimulating the EMT, angiogenesis and also MMPs expression especially MMP-2 and MMP-9 (Javelaud and Mauviel, 2005). The observation that TGF-β1 signaling is rarely completely lost in tumors led to the suggestion that retention of some TGF-β1 responses may actually be advantageous for tumor cells. The biologic activities of TGF-β1 that could promote tumor progression include its ability to enhance tumor cell invasiveness and migration, and to inhibit immune surveillance.

As with a variety of other cancers, Smad-4 genes (Kim et al., 1996) have been reported in human head and neck cancer and current thinking suggests that such anomalies provide tumor cells with a selective growth advantage. Defects of Smad-2 are uncommon and abnormalities of Smad-3 have not been reported in human tumors.

The functional significance of defects in Smad gene expression is under intense scrutiny at the present time. To date, studies have focused on the role of Smad-4 in neoplasia not least because mutations of Smad-4 genes are common in pancreatic and colorectal cancers (Hahn et al., 1996; Schutte et al., 1996; Riggins et al., 1997), haploid insufficiency of Smad-4 is considered to be sufficient for tumor initiation (Takaku et al., 1999; Xu et al., 2000) and bi-allelic loss of Smad-4 is thought to play a key role in tumor progression (Kinzler and Vogelstein, 1997).

The involvement of MMP-9 and TGF-β1

Regulation of MMPs by TGF-β1 has been reported in many studies. TGF-β1 display the roles in regulation many MMPs such as MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9. TGF-β1 up-regulates MMP-9 expression in odontoblasts, osteoblasts, normal equine chondrocytes and oral mucosal keratinocytes (Salo et al., 1991, Tjäderhane et al., 1998, Festuccia et al., 2000, Thompson et al., 2001) but not significantly in gingival fibroblasts (Salo et al. 1991). There is also evidence that TGF-β increases MT-MMP-1 and MMP-9 expression in metastatic melanoma (Janji et al., 1999). On the other hand, TGF-β1 suppresses TNF-α induced MMP-9 secretion in monocytes (Vaday et al., 2001).

In cancer cells, TGF-β1 seems to activate the expression of MMP-9. In a study using oral squamous carcinoma cells found TGF-β1 co-operated together with Integrin αVβ6 to relay the signal intracellularly and then up-regulated the expression of pro-MMP-9 (Thomas et al., 2002). Co-stimulation of prostate cancer cell line with TGF-β1 together with Actinomycin-D, the mRNA synthesis inhibitor, indicated that TGF-β1 does not stimulate transcription of MMP-9 but appear to induce through increased mRNA stability, while cycloheximide could inhibit the production of

MMP-9 by TGF-β1 observed by Northern blot suggested the newly synthesized protein are required for TGF-β1 stimulation of MMP-9 mRNA (Sehgal and Thompson, 1999) which contrast to the stimulation of MMP-9 by TNF-α, oncogene ras, jun, v-src and phorbol ester that increased transcription of MMP-9 (Gum et al.,1996) . Moreover, TGF-β1 found to upregulate MMP-9 in invasive type of mouse prostate cancer cells but not in non-invasive type. (Sehgal et al., 1996), thus indicates the different signaling pathway might use in response to TGF-β1.

CHAPTER III

MATERIALS AND METHODS

1. Cell culture

HSC-5 was a gift from Professor Teruo Amagasa, Tokyo Medical and Dental University, Tokyo, Japan, and WSU-HN-22 and WSU-HN-31 were a gift from Professor Silvio J. Gutkind, NIDCR, National Institute of Health; NIH). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100 U/ml penicillin, 100 ug/ml streptomycin and 5 ug/ml amphotericin B, all reagents were purchased from Gibco-BRL (Carlsbad, CA, USA) and cells were grown at 37^{o}C in humidified atmosphere of 95%, 5%CO_2. Cells were grown until 70-80% confluent before starting any treatment.

2. Reagents

Recombinant human TGF-β1 (rhTGF-β1), ERK inhibitor peptide II (ERKi), SB203580 (p38 inhibitor), JNK inhibitor II (JNKi), In solution^{TM} Rho kinase inhibitor , Akt inhibitor and Cytochalasin B were obtained from Calbiochem (EMD Chemicals, Inc., Gibbstown, NJ, USA). SB505124, a TβRI inhibitor, and curcumin, an AP-1 inhibitor, were purchased from Sigma (Sigma-Aldrich Chemical, St.Louis, MO, USA). MLCK inhibitor (MLCKi) was from Tocris Bioscience (Bristol, UK). Integrin beta-1 blocking antibody was from Chemicon (Chemicon International, Inc., Temecula, CA, USA). Antibody against phospho-Smad3 (pSmad3), total Smad2/3, phospho-MLC (pMLC), total MLC and MMP-9 were from Cell signaling Technology (Beverly, MA, USA). The biotinylated anti-rabbit antibody, biotinylated anti-mouse

antibody and streptavidin horseradish peroxidase antibody were from Zymed (Zymed laboratories, South Sanfrancisco, CA, USA). The phospho-ERK1/2 (pERK1/2) and total-ERK were from R&D systems (R&D, Minnepolis, MN, USA).

3. Cell proliferation assay

Cell proliferation was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of the detergent solution containing 1:9 of DMSO and glycine buffer (0.1M glycine / 0.1M sodium chloride pH10) results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be measured spectromically used the absorbance at 570 nM (Genesys UV scanning, Thermospectronic, Roche, NY, USA). All measurements were done in triplicate.

4. Gelatin zymography

The presence of MMP-9 in cancer cells and fibroblasts conditioned media was analyzed by zymography in 12% polyacrylamide gel containing 1mg/ml gelatin (Sigma). Samples were mixed with Laemmli sample buffer without reducing agent or heating and were subjected to SDS-PAGE. The gels were incubated for 30 minutes at room temperature in renaturing buffer (2.5% TritonX-100), and then incubated in developing buffer (50 mM Tris buffer pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$) for 48

hours at 37^{o}C. The gels were stained with 0.2% Comassie Blue in a solution of and then destained. Individual bands were quantified using Scion Image software (Scion, Frederick, Maryland, USA).

5. Enzyme linked immunosorbent assays (ELISA)

Cells were cultured and treated as indicated in DMEM without phenol red (Gibco-BRL, Carlsbad, CA, USA). Supernatants were collected and assayed to quantify concentrations of MMP-9 by ELISA kit (R&D, Minnepolis, MN, USA) according to the manufacture instruction. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human total MMP-9 has been pre-coated onto a microplate. Briefly, supernatants were diluted with the calibrator diluent and then the assay diluent of 100 ul was added into the microplate followed by adding the samples and cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm. Aspirate each well and wash, repeating the process three times for a total of four washes with wash Buffer. After the last wash, add the MMP-9 conjugate and incubate for 1 hour at room temperature on the shaker. Then, repeat the wash as described before. Add the substrate solution to each well and incubate at room temperature on the benchtop and protect from light. After incubation for 30 minutes, add the stop solution to each well. The color in the wells should change from blue to yellow. Determine the optical density by the spectrophotometer set to 450 nm within 30 minutes.

6. RNA isolation and RT-PCR

Total RNA from cell cultures were extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to munufacturer's instruction. The concentration of puified RNA was determined by measuring the absorption at 260/280 nm using a spectrophotometer (Genesys UV scanning, Thermospectronic, Roche, NY, USA). One microgram of total RNA for each sample was used to generate cDNA by using Reverse transcription kit (Promega, Madison, WI, USA). Then, a polymerase chain reaction were performed using PCR-kit (Quigen, Hilden, Germany) by a thermocycler (Tpersonal, Whatman Biometra, Goettingen, Germany) to detect MMP-9 (30 cycles, 60^{o}C). Glyceraldehyde-3-dehydrogenase (GAPDH) was used as an internal control (22 cycles, 60^{o}C). The PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by Ethidium bromide fluorostaining. The band intensity was quantified using Scion Image software (Scion, Frederick, Maryland, USA).

7. Protein extraction and Western blotting

WSU-HN-31 cultures treated in serum free medium were pretreatment with indicated inhibitors in the presence and absence of 1 ng/ml of TGF-ß1. Cold Phosphosafe was added into cells and left at room temperature for 5 minutes, then cells were scraped and transferred to 1.5 ml microcentrifuge tube and spin at 14,000g, 4^{o}C, for 5 minutes. Supernatant was transferred into new tubes and assay immediately. The concentration of protein was quantified utilizing BCA Protein Assay reagent (PIERCE, Rockford, IL, USA) and measured at the absorption of 560 nm. Equivalent of protein extracts were mixed with 3X Laemmli buffer (50mM Tris-HCl, pH 6.8, 100 mM DTT, 10% glycerol) and denatured by boiling for 10 minutes then separated by 12.5% SDS-PAGE and transferred to nitrocellulose membrane at 25V for 1h.

Membrane were blocked with 5% skim milk in DI water with 0.1% Tween-20 for 1 h at room temperature and probed with primary antibody diluted in 5% skim milk in DI water with 0.1% Tween-20 overnight at 4^{o}C. The membranes were then washed six times for 5 minutes with PBS and incubated with biotin conjugated secondary antibody at room temperature for 30 minutes then rewashed six times for 5 minutes with PBS. Finally, the membranes were incubated with streptavidin horseradish peroxidase-conjugatede antibody for 30 minutes at room temperature and rewashed six times for 5 minutes with PBS. Immunoreactive bands were visualized by chemiluminescence (PIERCE).

8. Electromobility shift assay (EMSA)

To determine the gene regulation and the transcription factor-DNA interaction of MMP-9 gene, EMSA was performed. Cells were treated as indicated and collected the nuclear protein by the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (PIERCE) and assayed with LightShift® Chemiluminescent EMSA Kit (PIERCE). Double-stranded oligonucleotides containing consensus recognition sites for AP-1 and NF-kB transcription factors were labelled with biotin. Oligonucleotide probes were: AP-1 5'-CgC TTg ATg AgT CAg CCg gAA-3'; NF-kB 5'-AgT TgA ggg gAC TTT CCC Agg C -3'. EMSAs were performed by incubation of nuclear protein extracts (10 ug) in EMSA buffer with 20 fmol of labelled or 4 pmol unlabelled oligonucleotides for 20 minutes at room temperature. The electrophoresis was performed in a pre-run non-denaturing 6% polyacrylamide gel in 10x TBE buffer at 100 V for 1 h. Gels were transferred to the nylon membrane at 100 V for 30 minutes at 4^{o}C. Then, the transferred DNA were crosslinked to the membrane by facing down the membrane onto the UV transilluminator for 15 minutes. Detection the biotin-

labeled DNA by chemilumiscence reaction using the reagents provided in the kit and exposed the membrane by placing in the film cassette.

9. siRNA transfection

Cells (2x105 cell / well) growing in 6-well plates (70-80% confluent) were added with the mixed solution of siRNA oligonucleotides specific to Smad2/3 or Smad4 (Santa Cruz). Cells were grown for 6 hours before diluted in 2x of supplemented growth medium (DMEM) and incubated for another 12 hours before treatment and collected the RNA or the protein for assayed.

10. Cell invasion assays (Boyden-chamber assay)

Cancer cells invasiveness was studied in modified Boyden chambers containing chemotaxis membranes of 13 mm diameter with 12 μm pore size (Nucleopore), which were coated with 1mg/ml of the reconstituted basement membrane Matrigel (Beckton Dickinson), which were kindly provided by Associate Professor Dr. Erik W. Thompson (St. Vincent institute of medical research, Melbourn, Australia). Cells were detached with 0.5%EDTA/PBS, counted by using hemocytometer, centrifuged and then resuspended in serum-free media to the concentration of 10^6 cells/ml. Then cells were added to the upper compartment of Boyden chamber. Serum-free media with or without additional factor and chemoattractant (human gingival fibroblast conditioned media and/or neutralizing TGF-β antibody) was placed in the lower compartment. After incubation at 37°C for 18 hours, filters were fixed and stained with H&E dye and the cells attached to the bottom side of the membrane were counted visually under microscope. The data are expressed as the total number of cells counted per ten microscopic fields.

11. Statistics

All experiments were performed three times with reproducible results. Data was presented by mean ± SD. The statistical significance of data was analyzed using a Student's *t*-test and a value of $P < 0.05$ was considered significant.

CHAPTER IV

RESULTS

TGF-β1 DERIVED BY GINGIVAL FIBROBLAST INDUCED MMP-9 EXPRESSION IN HNSCC

: A PILOT STUDY

In this chapter, the study aimed to examine the interaction between stromal cell and cancer cell. Since it has been well documented that the cell-cell interaction plays a role in cancer metastasis which, the increased of MMP-9 expression is one of the consequences of this interaction. Hence, we hypothesized that the stromal cell might provide TGF-β1 for cancer cell and then TGF-β1 activates the cancer cell to induce MMP-9 expression. Please note that the study in Chapter IV and Chapter V were done in parallel.

The human gingival tissue taken from patients underwent third molar surgery for orthodontic reason, was explanted and the subculture cells were used in this study. All patients were informed consent. Gingival fibroblasts at passage3-4 were cultured until 80% confluent, then replaced the medium with Serum free medium (SFM) and cultured for further 24 h. The gingival fibroblast conditioned medium (GFCM) was collected and used immediately or stored in tight sealed tube at -80^{o}c and use within 2 weeks. GFCM used in the present study was prior assessed for the presence of MMP-9 by zymography and ELISA to confirm that no detectable level of MMP-9 was found in GFCM.

Gingival fibroblast conditioned medium (GFCM) induced MMP-9 expression in HNSCC cell lines.

HNSCC cell lines, HSC-5 derived from palate and WSU-HN-22 derived from esophagus were used in this study. Cancer cells were treated with mixture of GFCM and SFM (1:1) for 24 h., then conditioned medium and RNA were collected and measured for MMP-9 expression by gelatin zymography, ELISA and RT-PCR. The results of gelatin zymography (Fig4.1a), ELISA (Fig.4.1b) and RT-PCR (Fig.4.1c) demonstrated that GFCM could increase the level of MMP-9 in both HNSCC cell lines. Therefore, it is possible that GFCM might contain soluble factor(s), which could induce MMP-9 expression in HNSCC.

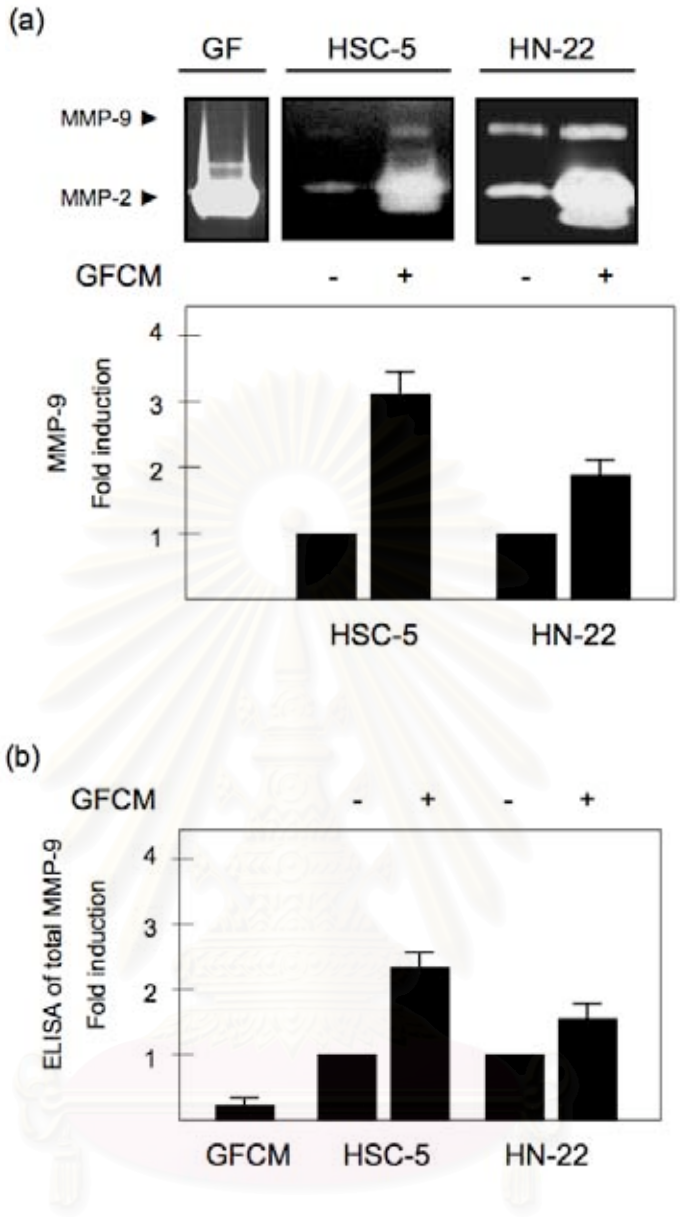


Fig.4.1 Induction of MMP-9 expression in cancer cell lines by GFCM. HSC-5, WSU-HN-22 and BT-549 were treated with GFCM. Conditioned medium from cultures were analyzed for MMP-9 expression by (a) gelatin zymography, the graph represents the intensity band of gelatin zymography analyzed by Scion image software. (b) ELISA of total human MMP-9, MMP-9 level in each HNSCC cell in the absence of GFCM was use as a control and normalized to 1. All data represented as a fold-increased compared to the control.

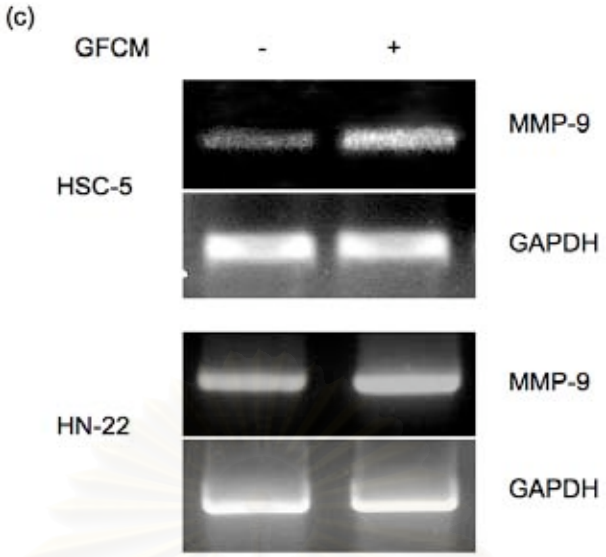


Fig.4.1 (cont.) Induction of MMP-9 expression in HSC-5 and HN-22 by GFCM. (c) RT-PCR, showed MMP-9 mRNA level in the absence and presence of GFCM and GAPDH was used as an internal control.

GFCM induced TGF-β1 expression in HSC-5.

In Chapter V, it has demonstrated that TGF-β1 could induce MMP-9 expression. In this pilot study, from figure 4.1 showed that GFCM could also induce MMP-9 expression in HNSCC cells. Thus, we hypothesized that GFCM might contain TGF-β1 or induce TGF-β1 expression in HNSCC cells. We investigated the effect of GFCM in TGF-β1 expression in HSC-5 by ELISA. The result presented that GFCM could also induce TGF-β1 expression in HSC-5 to 2-fold induction (Fig.4.2a). To confirm our hypothesis that the induction of MMP-9 belongs to TGF-β1 in GFCM, the specific neutralizing antibody to TGF-β1 was used and found that the GFCM-induced MMP-9 expression in HSC-5 was markedly inhibited. The induction of MMP-9 by GFCM was not influenced by an unspecific antibody used as an isotope control (Fig.4.2b). However, ELISA showed a small number of TGF-β1 presented in GFCM. We therefore suggest that administering of GFCM in HSC-5 culture resulted in the increased of TGF-β1 expression in our system and it is sufficient for MMP-9 induction in HSC-5.

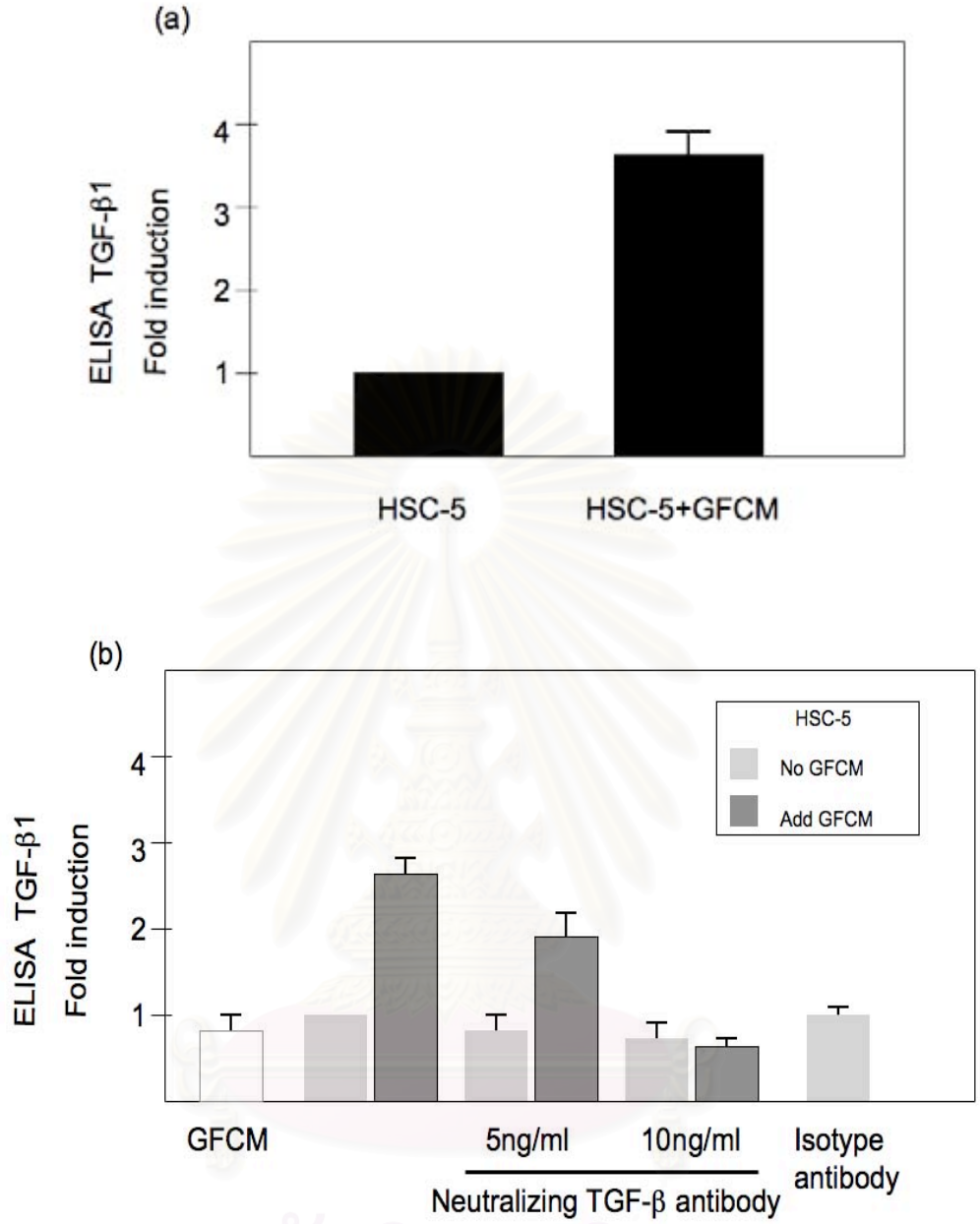


Fig.4.2. Induction of TGF-β1 by GFCM increased MMP-9 expression in HSC-5. (a) HSC-5 was treated with GFCM and analyzed for TGF-β1 concentration by ELISA. (b) To determine the effect of TGF-β1 in MMP-9 induction, HSC-5 was treated with GFCM in the presence and absence of neutralizing TGF-β1 antibody and then quantified the MMP-9 concentration by ELISA. TGF-β1 level in HSC-5 was used as a control and normalized to 1.

TGF-β1 derived from GFCM may involve in HSC-5 invasion across matrigel-coated membrane.

We used Boyden-chamber assay with matrigel-coated membrane to assess the capacity of GFCM to promote HSC-5 invasion through basement membrane (Fig4.3). GFCM significantly stimulated HSC-5 to invade across matrigel-coated membrane in 24 h. compared with HSC-5 in serum-free medium (SFM), which used as a control group. Adding the neutralizing TGF-β1 antibody in GFCM markedly inhibited the invasion of HSC-5, while adding isotype antibody in GFCM could not inhibit HSC-5 invasion. The invasive ability of the control group and the group that treated with neutralizing TGF-β1 antibody alone showed no difference. This result affirmed us that TGF-β1 in GFCM plays an important role in cell migration, which promotes the malignant phenotype of HNSCC.

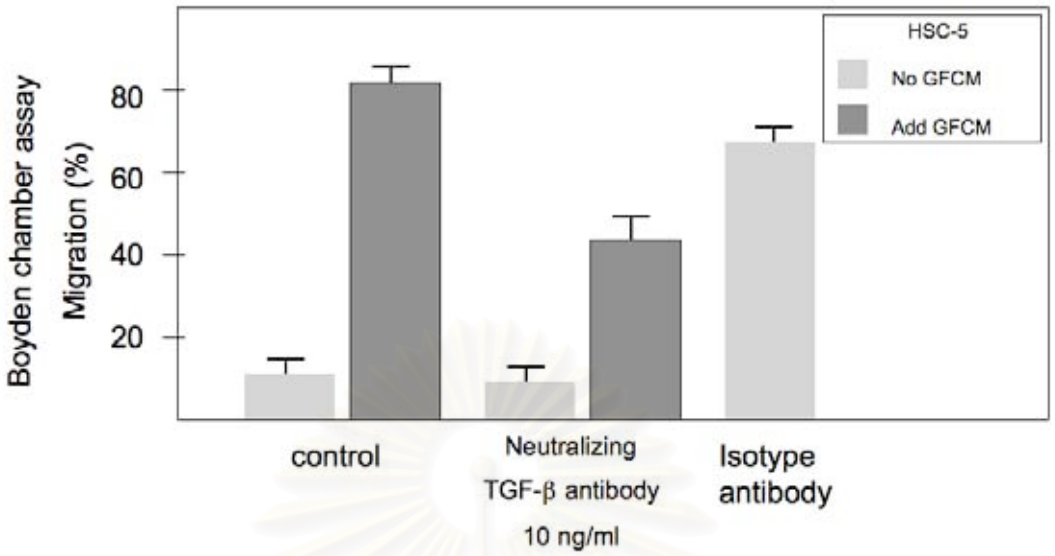


Fig.4.3 Stimulation of cancer cell invasion through synthetic basement membrane by GFCM. HSC-5 was stimulated by GFCM (50% v/v), neutralizing TGF-β1 antibody (10ng/ml) and normal goat IgG (as an isotype control) to invade across matrigel-coated membrane in boyden-chamber assay for 24 h. The bar represented cell invasion (%) compared with HSC-5 in serum-free medium (control group) (n=8 / group).

Basal levels of TGF-β1 expression in gingival tissues and conditioned medium derived from cancer cell-mediated stimulus of TGF-β1 expression in human gingival fibroblast (GF) culture.

To investigate the basal level of TGF-β1 mRNA in normal stromal tissue, we used three individual normal human gingival tissues from patients underwent third molar surgery for orthodontic reason. RT-PCR was performed and the result showed that all of three gingival tissues expressed TGF-β1 (Fig.4.4a). In culture, ELISA analysis using conditioned medium derived from human gingival fibroblasts (GFCM) revealed a low level of TGF-β1 concentration, but, interestingly, conditioned medium derived from HNSCC cell line, HSC-5 (5CM), stimulated a 2 to 4 fold induction of TGF-β1 concentration in GF (Fig.4.4b).

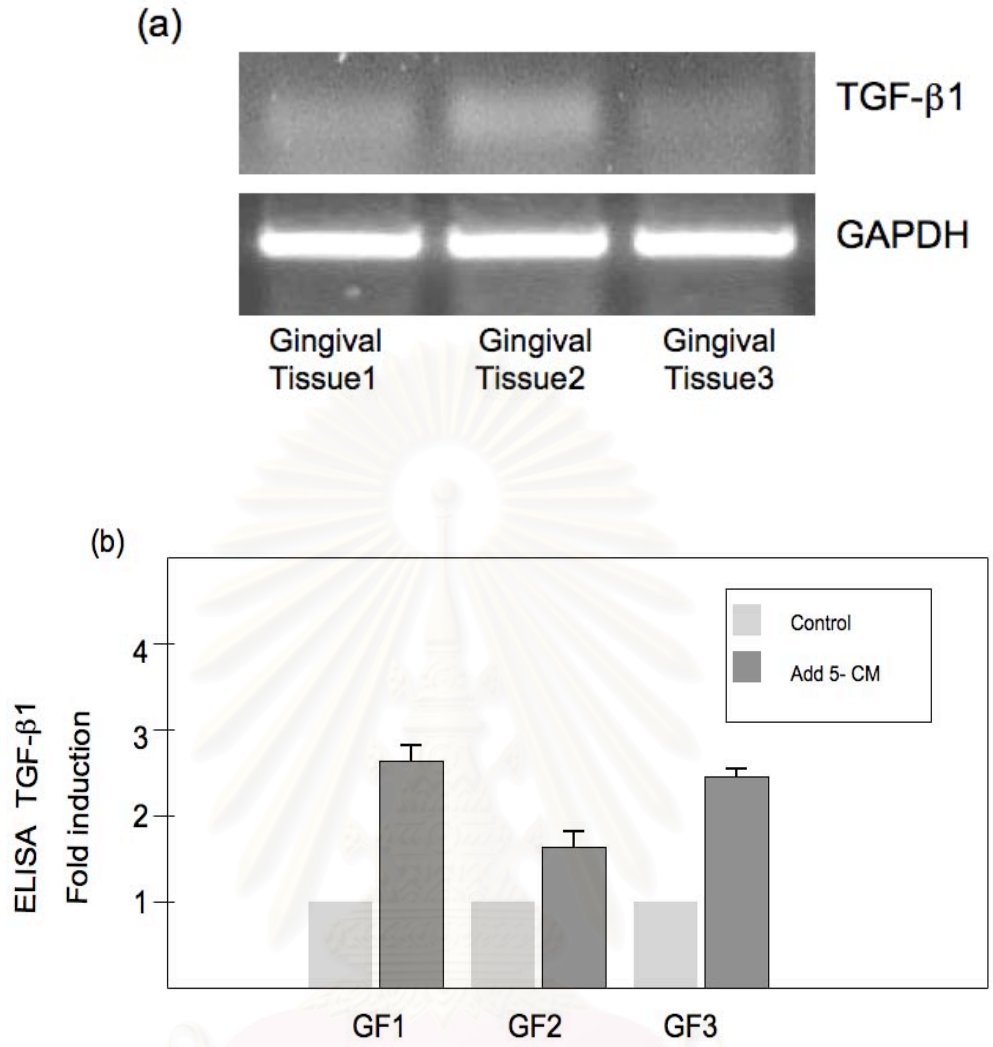


Fig.4.4. TGF-β1 expression in three difference human gingival tissues and human gingival fibroblast cultures (GF1, 2, 3). (a) RT-PCR was performed using samples from three individual gingival tissues to investigate the level of TGF-β1 mRNA *in vivo*. (b) Induction of TGF-β1 expression in three individual GFs that treated with conditioned medium derived from HSC-5 (5-CM) for 24 h. Conditioned medium samples of each culture were analyzed for TGF-β1 concentration by ELISA. Each control was normalized to 1.

CHAPTER V

RESULTS

TGF-β1-INDUCED-MMP-9 EXPRESSION IN HNSCC VIA SMAD AND MLCK SIGNALING PATHWAY.

TGF-β1 induced MMP-9 mRNA expression and protein synthesis in HNSCC cell lines.

Three HNSCC cell lines, HSC-5, HN-22 and HN-31, were used to determine the effect of TGF-β1 in MMP-9 expression. Cells were treated with rh-TGF-β1 (0-10 ng/ml) for 24 h. in a serum-free condition. The supernatants and RNA were collected to determine the level of MMP-9 secretion and mRNA expression, respectively. MMP-9 protein and activity were increased dose-dependently in all cell lines particularly HN-31 as shown by gelatin zymography (Fig.5.1a) and ELISA (Fig.5.1b). However, activated band of MMP-9 was not observed. Similarly, MMP-9 mRNA of these cell lines increased in a dose-dependent manner (Fig.5.1c).

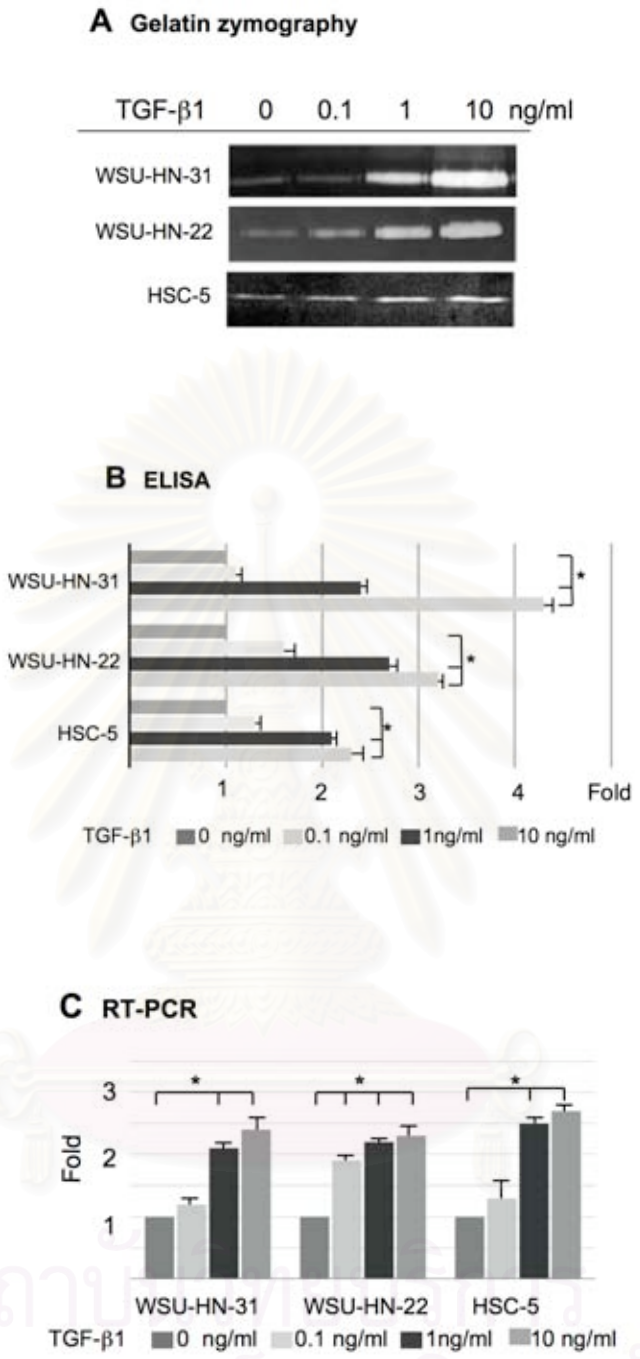


Fig.5.1. Determination of MMP-9 level after administering TGF-β1 (0-10 ng/ml) in HNSCC cell lines. MMP-9 protein level was determined by gelatin zymography (a) , ELISA (b) and RT-PCR (c). Data are mean ± SD from three separate experiments (* P< 0.05). MMP-9 mRNA level was shown by RT-PCR and each cell line used GAPDH as an internal control.

MLCK and Smad pathways were responsible for the TGF-β1 induced MMP-9 expression.

To elucidate the signaling pathway responsible for the TGF-β1-induced-MMP-9 expression in HNSCC, several inhibitors specific to the candidate signaling proteins according to previous reports were used. The result from gelatin zymography demonstrated that inhibitors belong MLCK and TβRI/Smad pathway could markedly inhibit the inductive effect of TGF-β1 on MMP-9 expression (Fig.5.2a, b) whereas inhibitors of ERK partially reduced the effect of TGF-β1 and the inhibitors belong to the other pathways had no effect (PI3K/Akt, Integrin beta1, Rho kinase, p38/MAPK, JNK/MAPK, NF-κB) (data not shown). The decrease of MMP-9 mRNA was also observed when cells were treated with SB505124, concomitant with the result shown by gelatin zymography (Fig.5.3a). In contrast, MLCK inhibitor inhibited MMP-9 expression only in the protein level as demonstrated by zymography but not the mRNA (Fig.5.3a). Furthermore, gelatin zymography assay using sample from both supernatant and cell lysates of the corresponding experiment was also performed. The reduction of MMP-9 after treated with TGF-β1 was observed in both the supernatant and in the cell lysates (Fig.5.3b).

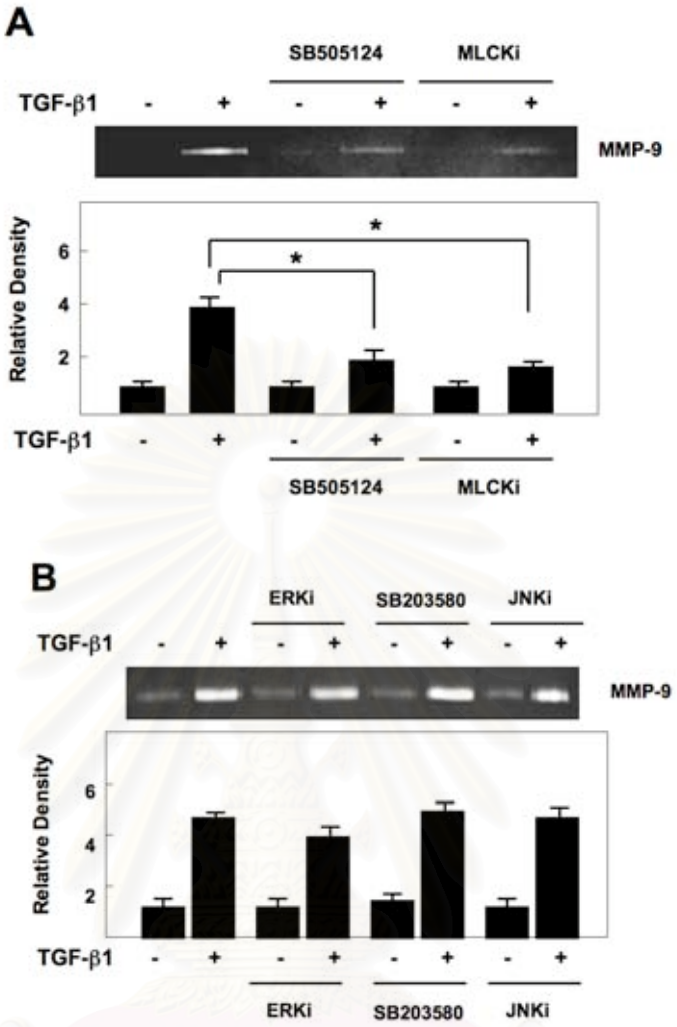


Fig.5.2. Effect of inhibitors of MAPK (ERK/ERKi, p38/SB203580, JNK/JNKi, TβRI (SB505124) and MLCK (MLCKi) on TGF-β1-induced MMP-9 expression in HN-31 cells. (a, b) MMP-9 level determined by gelatin zymography. HN-31 with no treatment was used as a control and normalized to 1. Data from three separate experiments were shown by graph as mean ± SD (* P< 0.05) as a fold-induction compared to the control.

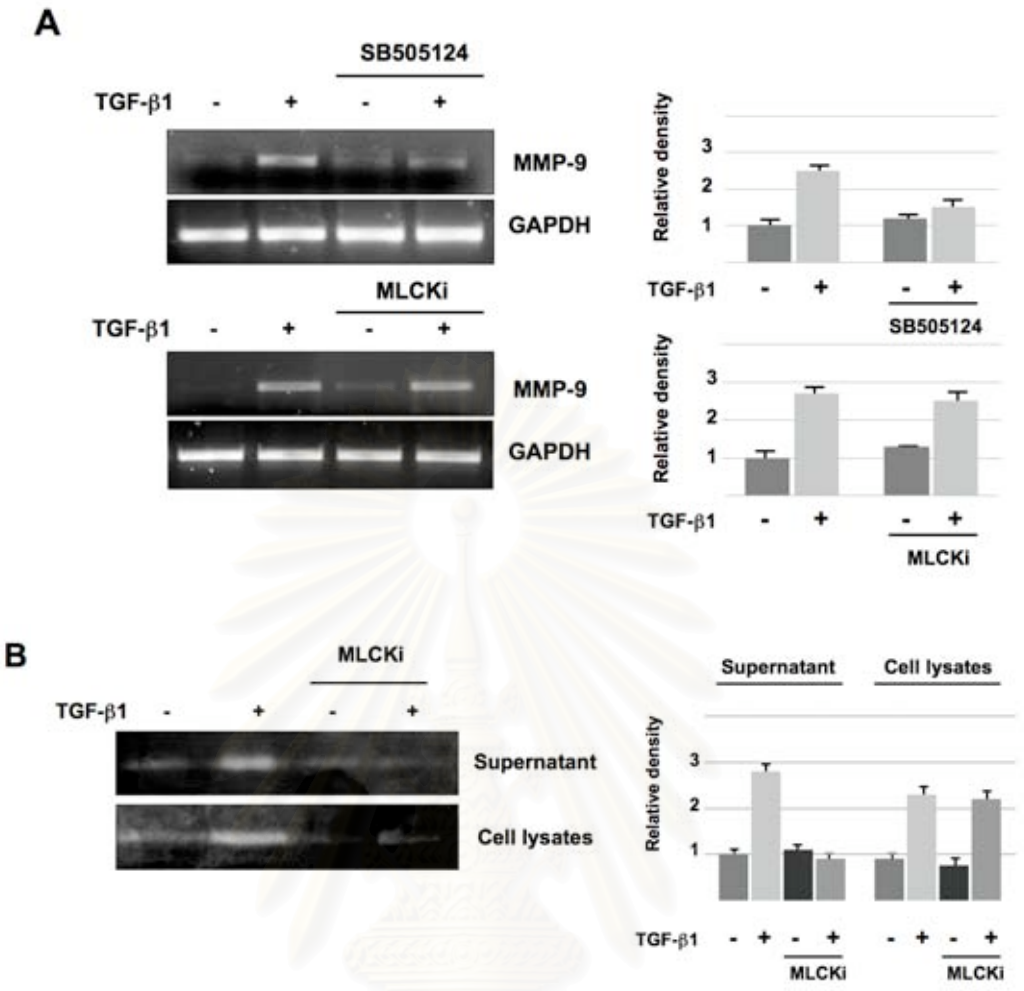


Figure 5.3. (A) RT-PCR analysis of MMP-9 mRNA level. (B) MMP-9 level from cells lysate and supernatant analyzed by gelatin zymography.

Smad3 and MLCK functioned as a regulator in TGF-β1-induced-MMP-9 expression.

Western analysis of active MLC and Smad3 were performed in order to examine the molecular pathway of TGF-β1-induced MMP-9 expression. As expected, application of TGF-β1 increased the activation of Smad3 and MLC. To confirm the role of Smad2/3 in TGF-β1-induced MMP-9 expression, siRNA of Smad 2/3 was introduced into HN31. The results showed that siRNA of Smad2/3 inhibited the inductive effect of TGF-β1 on MMP-9 synthesis (Fig5.4b). The decreased level of pMLC was also observed without any effect on total MLC.

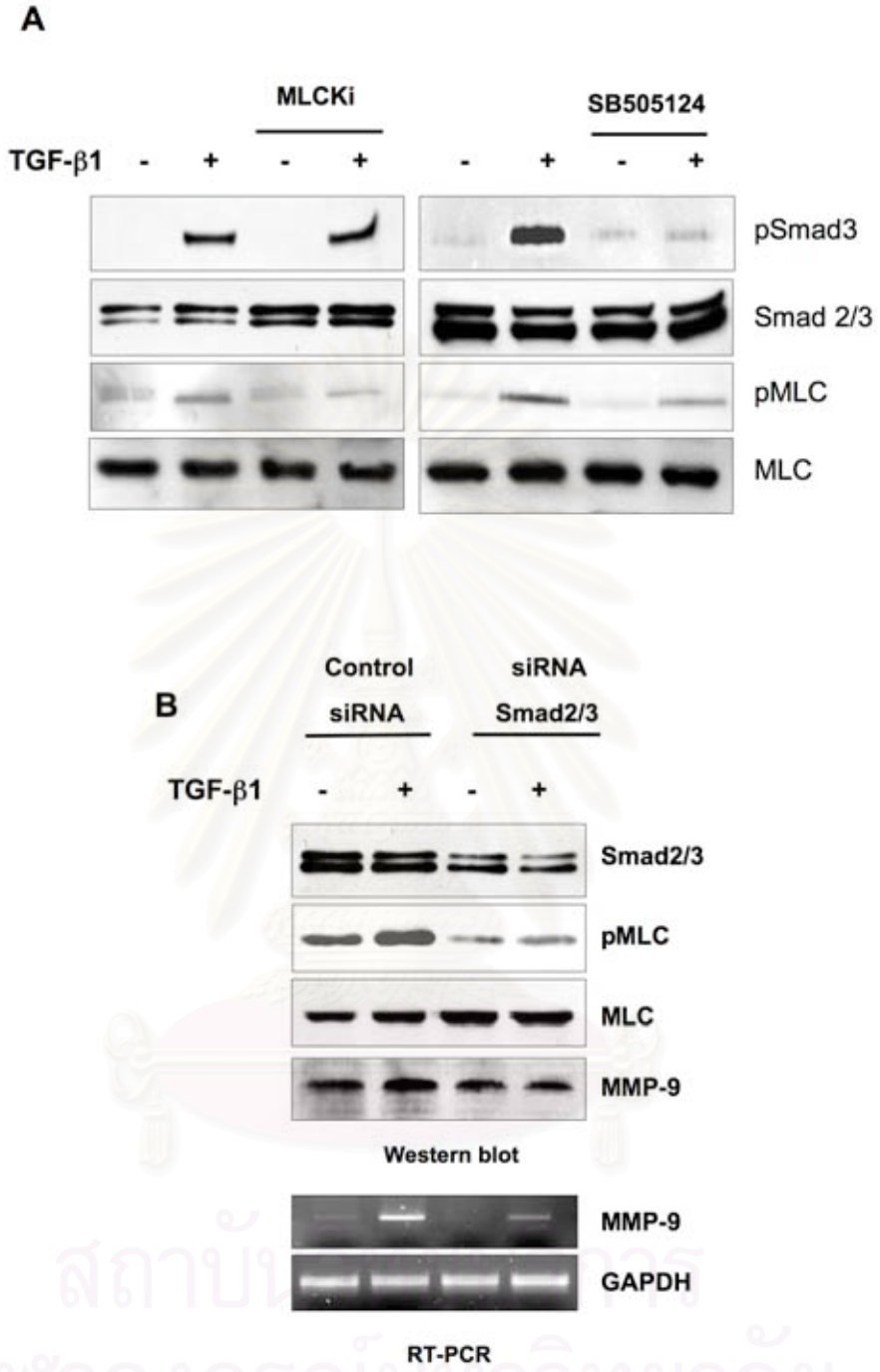


Fig.5.4. Western blot analysis of the effect of TGF-β1 signaling on Smad and MLCK signaling protein in HN-31 cells. (a) the effect of TβRI-inhibitor (SB505124) and MLCKi on Smad3 and MLCK activity. (b) The upper panel showed the Western blot analysis of Smad2/3, pMLC and MMP-9 exprssion after treatement with TGF-β1 in

cells transiently transfected with siRNA Smad2/3. The lower panel showed the result from gelatin zymography of HN-31 after transfected with siRNA Smad2/3.

CHAPTER VI
DISCUSSION

TGF-β1 is abundantly expressed in various tumor of epithelial origin (Derynck et al., 1985; Keski-Oja et al., 1987) and can exacerbate the malignant phenotype at later stages of carcinogenesis in which TGF-β1 can suppress immune surveillance, foster cancer invasion and promote the development of metastasis (Teicher et al., 2007; Biswas et al., 2007; Welch et al., 1990). In this study, we showed that TGF-β1 significantly increased both MMP-9 mRNA and protein expressions in HNSCC cell lines, suggesting the potential role of TGF-β1 in regulating MMP-9 expression and in cancer progression.

Besides the transcriptional regulation, function of MMP-9 can also be regulated by its endogenous inhibitor, TIMP-1 or tissue inhibitor of matrix metalloproteinase-1 (La Fleur et al., 1996). The balance between expression of MMP-9 and TIMP-1 is important for MMP-9 activity. In this study, the level of TIMP-1, which reversibly inhibits MMP-9 in a 1:1 stoichiometric fashion, was not altered in response to TGF-β1 treatment.

The TGF-β signaling is a linear signaling pathway from the type II to type I receptor kinase to Smad activation. Binding of TGF-β1 to type II receptor dimer triggers the phosphorylation of type I receptor, which then activate the R-Smad, Smad2 and 3. Type II receptor signaling in the absence of type I receptor has never been reported. The present study showed that application of TGF-β type I receptor (TβRI) inhibitor, SB505124, could significantly reduce the MMP-9 expression induced by TGF-β1 indicating the involvement of TβRI-dependent pathway. In addition, increased pSmad3 but not pSmad2 after TGF-β1 treatment was observed

corresponded with the report showing that Smad3 and Smad4 mediated most TGF-β-induced transcription (Inman and Hill, 2002; Yang et al., 2003). Furthermore, application of SB505124 and siRNA of Smad2/3, which inhibit the expression of Smad3, could attenuate the inductive effect of activation of TGF-β on MMP-9 expression. These findings indicated that the induction might occur via the TβRI-Smad3 pathway.

There were studies showing that TGF-β1 could induce MMP-9 through MAPK signaling pathway including ERK1/2, p38 and JNK1/2 in a cell-type specific manner (Kim et al., 2005, Santibanez et al., 2002; Kim et al., 2004). Our results revealed that ERK inhibitors could slightly reduce TGF-β1 induced MMP-9 expression, however, p38 and JNK showed no effect in this mechanism.

Interestingly, to the best of our knowledge, this is the first study demonstrated that TGF-β1 induced MMP-9 through myosin light chain kinase activation (MLCK). TGF-β is well described to induce MLCK or MLC in myogenic differentiation (Meyer-ter-Vehn et al., 2006). However, there were few studies in cancer cells showed the correlation between TGF-β1 and MLCK (Hisataki et al., 2004; Yamamoto-Yamaguchi et al., 1996). MLCK, a Ca^{2+}-calmodulin dependent multi-functional enzyme, plays a critical role in the regulation of smooth muscle contraction and cellular migration. It regulates the contractile interaction between actin microfilaments and myosin by phosphorylating the myosin light chain (MLC) during non-muscle cell contraction, cytokinesis, stress fiber formation, and motility (Matsumura et al., 2001). MLCK also play a role in cancer cell migration. Inhibition of MLCK or MLC could reduce cell migration in breast and pancreatic cancer cells as well as fibrosarcoma cell line (Betapudi et al., 2006; Niggli et al., 2006; Kaneko et al., 2002). Moreover, MLCK could retard the growth of prostate cancer cells and breast

cancer cells (Gu et al., 2006). Clinical study of non-small cell lung cancer patients found a significant positive correlation between expression levels of MLCK and likelihood of disease recurrence and metastasis (Minamiya et al., 2005).

In this study, inhibition of MLCK reduced MMP-9 secretion but not the transcription after treated with TGF-β. This result suggests the role of MLCK in post-transcriptional regulation of MMP-9. The result from gelatin zymography showed that the amount of MMP-9 decreased in the supernatant but not in the cells, suggesting the role of MLCK in protein transportation. Our hypothesis is supported by the evidences that myosin can play a role in regulating microtubule dynamics (Even-Ram et al., 2007) which participate in the protein transportation. However, application of several inhibitors including neutralizing antibody to integrin-β1, cytochalasinB and Rho kinase, which are involved in actin cytoskeletal rearrangement, could not inhibit the TGF-β1-induced MMP-9 expression (data not shown). The exact role of cytoskeleton in MMP-9 expression induced by TGF-β requires further investigation.

Application of TβRI inhibitor could inhibit the activation of both Smad3 and MLC suggested that signal from TβRI activate both molecules. However, application of MLCK inhibitor could decrease only the activation of MLC but not the phosphorylation of smad3 indicated that MLC is the downstream target of Smad3 in the regulation of MMP-9.

In conclusion, our results showed that TGF-β1 induced MMP-9 expression in head and neck cancer cell lines through TβRI/Smad3. The phosphorylated Smad3 relayed the signals downstream to activate MLCK, which regulated the MMP-9 protein expression. These suggest the combinatorial interaction of both Smad and non-Smad signaling in MMP-9 regulation by TGF-β1.

The source of TGF-β1 in the in vivo conditioned still unclear. We hypothesized that the interaction between HNSCC and stromal cells may participate in the induction of TGF-β1. We used gingival fibroblasts as the representative of stromal cells in this study. The results from the pilot study demonstrated that gingival cells stimulate TGF-β1 secretion and enhanced MMP-9 expression in HNSCC cells. These results suggest the importance of cell-cell interaction in MMP-9 induction and synthesis.

Over the past decade, it has become increasingly apparent that the complex interplay between different cell types, e.g. epithelial cells, and the microenvironment are critical for maintaining normal balanced tissue homeostasis. Comparison of the connective tissue of normal organs, which is able to maintain normal tissue homeostasis, with the tumor stroma revealed a disrupted balance in the epithelial stromal interactions in both composition of the ECM and the functional state of the stromal cells (Coussens and Werb, 2001; Tlsty, 2001). These alterations seem, indeed, to be crucial for tumor growth, invasion, and metastasis (Fidler, 1990). Growth-promoting effects of activated stromal cells on tumor cells have been reported (Gregoire et al., 1995), indicating persistent functional alterations in tumor fibroblasts (Turner et al., 1997; Olumi et al., 1999).

Induction of several growth factors including IL-1, IL-6, IL-8, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), TNF-α and TGF-β were also reported in tumor-stromal interaction (Zigrino et al., 2005). In this study, the application of neutralizing antibody in the GFCM mixture could abolish the MMP-9 expression in HNSCC cell. This data revealed that the growth factor induced in our model is TGF-β1.

In addition, co-cultivation of the malignant tumor cells with stromal

fibroblasts induced the expression of MMP-1 and MMP–9 which did not occur with the benign tumor cells (Borchers et al., 1997). The expression and secretion of proMMP-2 and proMMP-9 in fibroblasts have been reported to be increased due to cell–cell contact between fibroblast and human ovarian carcinoma cells (Westerlund et al., 1997) or metastatic-transformed rat embryonic cells (Himelstein et al., 1998). Bair et al. also reported that the expression of proMMP-7 is augmented by the co-culture of oral squamous cell carcinoma SCC-25 cells and human foreskin fibroblasts. Furthermore, previous study demonstrated that human squamous carcinoma A431 cells augment MT1-MMP on the cell surface of tumor cells by interacting with normal human dermal fibroblasts (Sato et al., 1999). Therefore, the cell–cell contact between tumor cells and surrounding normal stromal cells brings about an augmentation of tumor invasiveness by increasing MMP-mediated pericellular proteolysis.

The result from the pilot study indicated that TGF-β1 induction by GF increased MMP-9 secretion and enhanced the invasion of HSC-5 in the chemotaxis assay. This results support the previous findings that interaction between cancer and stromal cells play an important role in cancer metastasis. Increasing level of TGF-β1 was found in oral tissue with chronic inflammation. The increasing of TGF-β1 could be one of the etiologies of HNSCC or enhance the invasiveness of HNSCC. All these reports stress the importance of the stromal compartment in malignant tumors and strongly indicate that continuous interactions between the carcinoma and stromal cells. *In vitro* models developed so far are not able to faithfully mimic the complex interactions that occur between tumor and stromal cells *in vivo*. On the other hand, *in vivo* studies on the functional role of the stromal compartment in established neoplasms, either autochthonous or transplanted, are often difficult to interpret due to

the intermingled close association of carcinoma and stroma elements. The lack of models encompassing different tumor stages, corresponding environmental conditions, as well as the dynamic pattern of the carcinoma-stroma interaction are further limitations. More understanding of the signaling pathway involved in the regulation of TGF-β1 in tumor-stroma interaction may provide the insight of cancer prediction, prevention and treatment in the future.

Future studies

1. Further investigation the role of myosin in MMP-9 transportation and MMP-9 secretion.
2. Further investigation the transcriptional regulation of MMP-9 gene expression activated by TGF-β1.
3. Investigate the underlying mechanism of MMP-9 expression activated by GFCM.

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APPENDICES

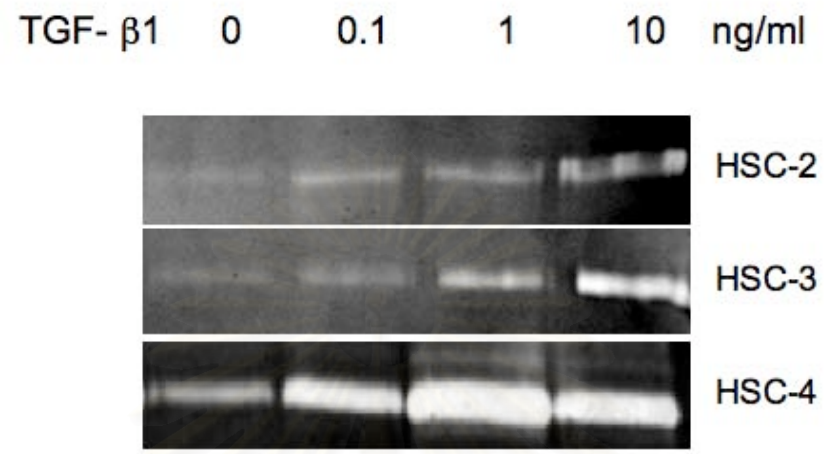

Figure 1. Gelatin zymography of three additional HNSCC cell lines in response to TGF-β1 treatment.

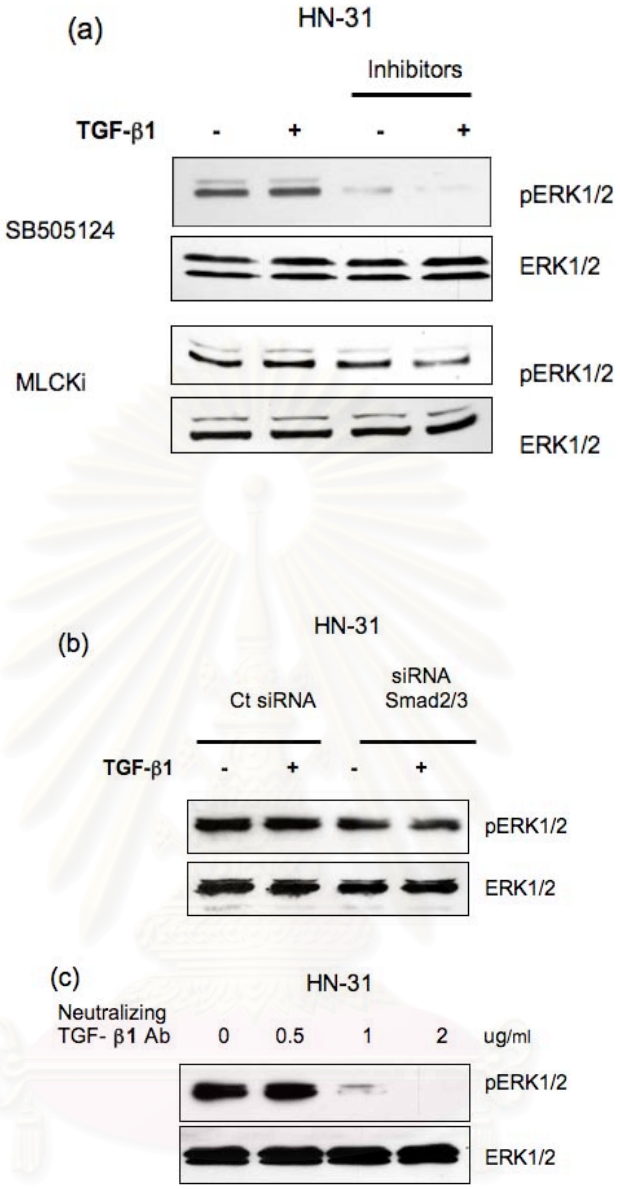

Figure 2. The interaction between ERK and Smad/MLCK pathway in TGF-β1-induced-MMP-9 expression in HN-31 cell. Western blotting showed the level of phosphorylated ERK1/2 and total ERK1/2 after application with (a) SB505124 and MLCKI, (b) siRNA Smad2/3 and (c) Neutralizing TGF-β1 antibody.

The results suggested that ERK might involve in TGF-β1-induced MMP-9 expression. Further study is required to clarify this hypothesis.

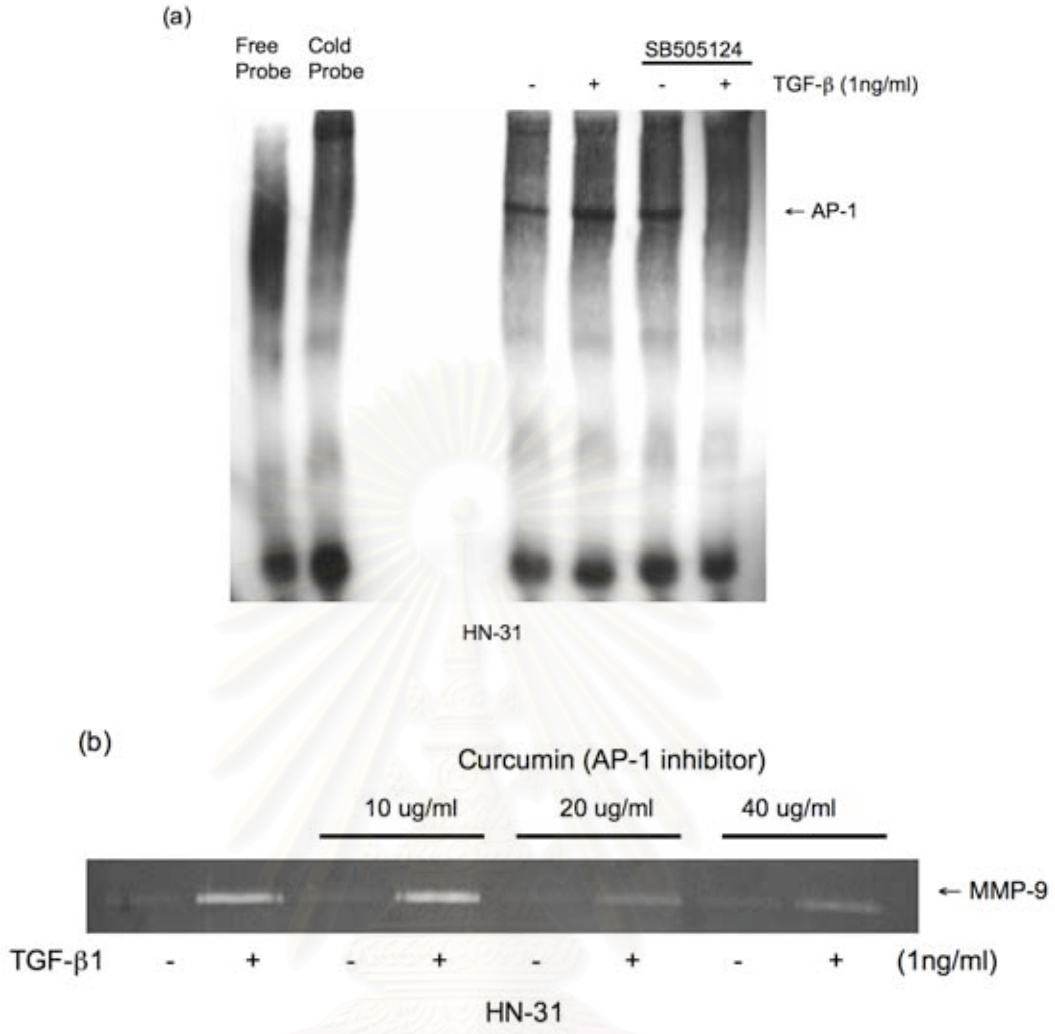

Fig.3. The role of AP-1 in TGF-β1-induced MMP-9 expression in HN-31 cell. (a) AP-1 oligonucleotides and nuclear protein interaction from HN-31 was analyzed by EMSA. Nuclear protein extracts were prepared as described in materials and methods from HN-31 in the presence and absence of TGF-β1 and SB505124. Double-stranded oligonucleotides (20 fmol) or a 200-fold excess molar of unlabeled cold probe (4 pmol) were incubated with nuclear protein extracts (10 μg). (b) Gelatin zymography showed that AP-1 inhibitor could inhibit MMP-9 expression in HN-31 cells.

The results suggested that TGF-β1-induced MMP-9 expression may involved AP-1 regulatory region.

BIOGRAPHY

I, Sariya Nuchanardpanit was born on June 16, 1977 in Bangkok. Inspired by His Royal Highness Prince Mahidol of Songkhla's works, I decided to study in the medical field after high school graduation. In 2000, I received the doctor of dental surgery degree from the faculty of Dentistry, Chulalongkorn University. Afterwards, I worked as an instructor in the department of dental public health at the Sirindhorn College of Public Health in Trang province for one year. Later, I returned to the faculty of Dentistry, Chulalongkorn University to start my graduate study in Certificate of clinical science in Oral Medicine. In 2003, I entered the program of Doctor of Philosophy in Oral Biology and I received Chulalongkorn University graduate scholarship to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej from Graduate school of Chulalongkorn University, the Royal Golden Jubilee scholarship from the Thailand Research Fund and my work was also supported by a Ratchadaphisek Somphot Endowment for the Research Unit of mineral tissue, Faculty of Dentistry, Chulalongkorn University, Thailand. Currently, I work as a private dentist at Launpruksa Dental Clinic in Samutprakan.