Molecular mechanism of the regulation of *urokinase* (*uPA*) gene expression and its function in breast cancer

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Abstract

Urinary plasminogen activator (uPA), a member of the serine protease family, is implicated in the progression of various cancers including breast cancer due to its ability to provoke malignant cell invasion. uPA production is reported to be much higher in late stage, estrogen receptor (ER) negative breast cancer patients than those with benign hyperplasia. Since all existing evidence points to a role for uPA in breast cancer progression, exploring the mechanisms regulating its gene expression will be of immense value. Two human breast cancer cell lines were selected for this study. MDA-MB-231 represents late stage breast cancer. This cell line has high uPA expression and is highly invasive. In contrast, the MCF-7 cell line represents early stage breast cancer and fails to express detectable levels of uPA mRNA. I have demonstrated by methylation specific PCR (MSP) that the differential expression of the *uPA* gene in MDA-MB-231 and MCF-7 cells closely correlates with the amount of methylated cytosines present within the uPA promoter in these cells. The observation that the DNA methylation status of the uPA promoter directly affects the expression of the *uPA* gene was then confirmed using an *in vitro* luciferase reporter assay. Results suggest that the accessibility of the transcription factor Ets-1 is limited by DNA methylation. I further reported increased demethylase (DMase) activity with decreased maintenance activity of methyltransferases (DNMTs), which together favor the generation of a hypomethylated uPA promoter in these highly invasive MDA-MB-231 breast cancer cells. Given the pivotal role of uPA in breast cancer progression, I then disrupted the function of uPA and studied its effects on cancer progression. The effects of an 8-mer synthetic peptide (Å6) derived from the non-receptor-binding region of uPA were investigated. This peptide inhibits cancer cell invasion of both human (MDA-MB-231) and rat breast cancer cells (Mat B-III) using an *in vitro* cell invasion assay. The migration ability of human dermal microvascular endothelial cells (HDMVCs) was also impaired by this peptide in a dose dependent manner. When injected into mice with metastatic breast cancer, Å6 alone suppressed both tumor growth and metastasis significantly. This effect is even more pronounced when combined with tamoxifen (TAM). The inhibitory effects were attributed to the induction of tumor cell death and the inhibition of tumour angiogenesis.

Résumé

L'activateur urinaire du plasminogène (uPA) est un membre de la famille des sérines protéases qui peut provoquer l'envahissement des cellules malignes et qui joue un rôle dans l'évolution de divers cancers dont le cancer du sein. La production d'uPA semble être plus élevée chez les femmes atteintes d'un cancer à récepteurs d'estrogènes (ER) positifs que chez celles qui présentent une hyperplasie bénigne. Comme toutes les données recueillies à ce jour indiquent que l'uPA intervient dans l'évolution du cancer du sein, il sera fort utile d'examiner les mécanismes régissant l'expression du gène de cette enzyme. Aux fins de cette étude, nous avons sélectionné deux souches de cellules cancéreuses: la souche MDA-MB-231, qui représente un stade évolué du cancer du sein, affiche une forte expression du gène de l'uPA et est très envahissante, et la souche MCF-7, qui représente un stade peu évolué du cancer du sein et dont la production d'ARNm de l'uPA est négligeable. Grâce à la réaction en chaîne de la polymérase spécifique de la méthylation, nous avons démontré l'existence d'une corrélation étroite entre l'expression différentielle du gène de l'uPA dans les souches MDA-MB-231 et MCF-7 et la quantité de cytosines méthylées présentes dans le promoteur du gène de l'uPA de ces cellules. Nous avons ensuite confirmé l'incidence directe de l'état de méthylation de ce promoteur sur l'expression du gène de l'uPA au moyen d'une analyse *in vitro* par le gène rapporteur de la luciférase. Nos résultats semblent indiquer que l'accessibilité du facteur de transcription Ets-1 est limité par la méthylation de l'ADN. Nous avons également noté une augmentation de l'activité de la déméthylase (DMase) et une diminution de l'activité d'entretien des méthyltransférases (DNMTs), phénomènes dont la combinaison favorise un état d'hypométhylation du promoteur du gène de l'uPA dans les cellules de la souche MDA-MB-231. Étant donné le rôle clé de l'uPA dans l'évolution du cancer du sein, nous avons entravé son action et étudié les conséquences de cette intervention. Il s'agissait d'examiner les effets d'un peptide synthétique octamérique (Å6) issu de la région du gène de l'uPA non destinée à la fixation de récepteurs. Au moyen d'une analyse *in vitro* de l'envahissement cellulaire, nous avons observé que ce peptide inhibe l'envahissement des cellules du cancer du sein tant chez l'être humain (MDA-MB-231) que chez le rat (Mat B-III). Ce peptide

entraîne également une réduction proportionnelle à la dose de la capacité de migration des cellules endothéliales microvasculaires de derme humain (HDMVCs). Chez des souris présentant un cancer du sein métastatique, l'injection de Å6 seulement atténue considérablement la croissance des tumeurs et la dissémination métastatique. Cet effet est encore plus prononcé lorsque le Å6 est combiné à un traitement par le tamoxifène (TAM). Nous avons attribué ces effets inhibiteurs à l'induction de la morts des cellules tumorales et à l'inhibition de l'angiogénèse tumorale.

Ces résultats indiquent que la méthylation de l'ADN est l'un des principaux mécanismes de régulation de la production d'uPA dans le cancer du sein. Or, la modulation de l'expression du gène de l'uPA constitue une piste intéressante dans la recherche de nouveaux moyens pour freiner l'évolution du cancer du sein. L'administration de petits peptides non toxiques comme le Å6, seuls ou dans le cadre d'une hormonothérapie, représente une stratégie thérapeutique nouvelle qui pourrait améliorer le pronostic des femmes atteintes d'un cancer du sein envahissant.

Claim of originality

Urokinase (uPA) has been recognised as one of the major contributors to cancer progression. Overexpression of uPA is closely associated with tumor invasiveness and metastasis. Many antagonists have been designed based on the functional regions of uPA, and are currently undergoing further improvement to enhance their specificity and selectivity. A better understanding of the complex process of *uPA* gene transcription and regulation will therefore prove to be of immense value in developing novel therapies directed against the uPA system in cancer. With these goals in mind, we selected breast cancer as our study model due to its high incidence in the world and its high rate of morbidity and mortality as well as the invasive nature of this cancer.

My original contributions to the body of knowledge include the following findings:

1. I demonstrated that DNA methylation, an epigenetic change of DNA without altering its genetic code, closely correlates with the expression levels of the *uPA* gene in MDA-MB-231 and MCF-7 cell lines that represent early and late stage human breast cancer respectively.
2. I mapped for the first time methylated versus unmethylated cytosines in the CpG island of the uPA promoter in these breast cancer cell lines.
3. I demonstrated that the demethylated uPA promoter is sensitive to the actions of transcription factors such as Ets-1 in MDA-MB-231 cells. However, the inducible effects of Ets-1 on *uPA* gene transcription are totally abolished by the methylated uPA promoter.
4. I demonstrated for the first time that balanced methyltransferase and demethylase activity might determine the methylation status of the uPA promoter and its expression in these human breast cancer cells.
5. I generated a MDA-MB-231-GFP (green fluourescent protein) cell line for direct detection and quantitation of tumor micrometastases in a xenograph model of human breast cancer.
6. I demonstrated the ability of a novel peptide inhibitor Å6 derived from the non-receptor binding region of uPA to block MDA-MB-231 and Mat B-III

tumor growth and metastasis in syngeneic and xenograph models of breast cancer.

7. Combination therapy of Å6 with other conventional breast cancer treatment such as TAM showed additive effects in blocking tumor growth and metastasis compared to either reagent used alone due to their inhibitory effects on tumor angiognesis and the ability to induce tumor cell death.

8. I explored the potential mechanisms underlying the inhibitory effects of Å6 and TAM in a breast cancer model.

These studies have clearly demonstrated that uPA is a critical target for invasive cancer treatment. Transition of the uPA promoter from a methylated to demethylated status may play a pivotal role in defining the process of *uPA* gene transcription at different stages of cancer development. Addition of novel agents like Å6 that interfere with uPA/uPAR interaction to a standard hormone therapy (TAM) shows beneficial effects in our models of breast cancer where the uPA/uPAR system plays a key role in tumor progression.

Preface

This is a manuscript-based thesis. The following is reprinted from the "Guidelines for thesis preparation" of the Faculty of Graduate Studies and Research, McGill University:

"..., *the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:*

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In

addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

6. When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition."

In accordance with these guidelines, I have included the following manuscripts as part of this thesis:

1. **Yongjing Guo**, A. A-R Higazi, Ani Arakelian, Bruce S. Sachais, Douglas Cines Ronald H. Goldfarb, Terence R. Jones, H. Kwaan, Andrew P. Mazar and Shafaat A. Rabbani. **A Peptide Derived from the Non-Receptor Binding Region of Urokinase Plasminogen Activator (uPA) Inhibits Tumor Progression and Angiogenesis and Induces Tumor Cell Death *In Vivo***. *FASEB J.* 14(10):1400-1410, 2000.

This paper is adopted as chapter III of the thesis and reproduced with written copyright permission from the FASEB journal. I am responsible for most of the work, including invasion and proliferation assays of Å6 treated human breast cancer cells MDA-MB-231 and rat breast cancer cells Mat B-III as well as endothelial cell migration assay of Å6-treated HDMVCs. I was also responsible for all the *in vivo* work and tumor section analysis including H&E staining, factor VIII staining and *in situ* apoptosis assay. The contribution of co-authors as follows: Dr. Andrew P. Mazar and Dr. Terence R. Jones from Ångstrom Pharmaceuticals Inc. kindly provided Å6 peptide for all experiments conducted in chapter III and chapter IV. By using Surface Plasmon Resonance (SPR), Dr. Hagazi and his group tested the effects of Å6 on scuPA-suPAR interaction *in vitro*. Ani Arakelian helped to inject Å6 peptide to the first two sets of breast tumor bearing rats.

2. **Yongjing Guo.,** Andrew P. Mazar and Shafaat A. Rabbani. **An Anti-Angiogenic Urokinase Derived Peptide (Å6) Combined with Tamoxifen (TAM) Decreases Tumor Growth and Metastasis in a Syngeneic Model of Breast Cancer**. *Cancer Research* (Accepted).

This manuscript constitutes chapter IV. I am responsible for all of the experiments described in this paper.

3. **Yongjing Guo**., Pouya Pakneshan., Moshe Szyf and Shafaat A. Rabbani. **Regulation of DNA Methylation in Human Breast Cancer: Effect on Urokinase (uPA) Production and Tumor Invasion.** *Journal of Biological Chemistry* (Pending revision).

This manuscript comprises the entire chapter II. I did all of the work described in this paper. Dr. Moshe Szyf provided the protocols for methyltransferase and demethylase activity assays and critical advice related to these studies.

All work was supervised by Dr. Shafaat A. Rabbani and carried out in his laboratory located in the Royal Victoria Hospital, McGill University in Montreal.

List of publications contributing to this thesis

1. **Yongjing Guo**, A. A-R Higazi, Ani Arakelian, Bruce S. Sachais, Douglas Cines Ronald H. Goldfarb, Terence R. Jones, H. Kwaan, Andrew P. Mazar and Shafaat A. Rabbani. *A Peptide Derived from the Non-Receptor Binding Region of Urokinase Plasminogen Activator (uPA) Inhibits Tumor Progression and Angiogenesis and Induces Tumor Cell Death In Vivo.* ***FASEB J.*** **14(10): 1400-1410, 2000.**

2. **Yongjing Guo**, Andrew P. Mazar and Shafaat A. Rabbani. An Anti-Angiogenic *Urokinase Derived Peptide (Å6) Combined with Tamoxifen (TAM) Decreases Tumor Growth and Metastasis in a Syngeneic Model of Breast Cancer.* ***Cancer Research*** **(Accepted).**

3. **Yongjing Guo**, Pouya Pakneshan, Moshe Szyf and Shafaat A. Rabbani. *Regulation of DNA Methylation in Human Breast Cancer: Effect on Urokinase (uPA) Production and Tumor Invasion.* ***Journal of Biological Chemistry*** **(Pending revision).**

Acknowledgments

It has been my dream to pursue medical research since I began seeing cancer patients during my residency training in China. Believing that molecular-based medicine would one day become routine and *the* cure for cancer, I came to Canada to extend my basic medical science training several years ago, with the hope that one day I could explore novel therapeutic strategies for cancer patients. I have devoted the last several years to working towards my Ph.D. degree in the Division of Experimental Medicine, Department of Medicine of McGill University. I was delighted to work on uPA, one of the key players in cancer-related mortality. Through hard work and numerous sleepless nights, I have finally come to the conclusion of this project, which is of course the beginning of a number of new projects. Without the support of my supervisor, collaborators, thesis committee members, colleagues and family members, my job would have been impossible to accomplish. I would like to take this opportunity to express my grateful appreciation to all of them.

I am most appreciative for the supervision provided to me by my thesis supervisor, Dr. Shafaat A. Rabbani. Since he first gave me the opportunity to work with him, he has constantly impressed me by his knowledge, thoughtfulness and his dedication to science. What I have learned about science during my training with him has brought me closer to fulfill my dream.

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making the time I spent in the lab so memorable. Although some of them have moved on, my memories of their support, friendship and kindness will always keep me warm. I will pray for them all. I have been lucky to work with a group of outstanding and dedicated scientists: Drs. Huang, Miao and Panda, with whom I spent hours discussing scientific topics. I will also remember all the other members of the Calcium Laboratory who over the years became part of my extended family. Together they made my experience over the past few years unforgettable.

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Without the love and unconditional support both emotional and financial from my mother Xiaoling Wang and father Xiuyan Guo, my achievement today could not have been reached. I know they have waited a long time for their daughter to grow up and be successful. But they never thought of laying down the burden they were carrying. I would never have had the opportunity to study in Canada without the sacrifices my parents made for me. Since I decided to devote my future to the field of medical research, they have constantly encouraged me when I was low and reminded me that my hard work would pay off. This thesis is especially dedicated to them. I would also like to thank my grandpa Shaoyu Wang, mother-in-law, Wenshan and father-in-law, Rukun, who continue to love me.

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This thesis is dedicated to my family.

List of abbreviations

α2-MR:	α_2-macroglobulin receptor
AMLD:	amiloride
AP :	activator protein
APC:	adenomatous polyposis coli
AR:	androgen receptor
ATF :	amino terminal fragment
ATF-2:	activating transcription factor-2
5-azaCdR:	5-aza-2'-deoxycytidine
BAECs:	bovine aortic endothelial cells
BCSG1:	breast cancer specific gene 1
bp :	base pair
CCL4:	carbon tetrachloride
cDNA :	complementary deoxyribonucleic acid
CM:	conditioned medium
CML:	chronic myeloid leukaemia
COBRA:	combined bisulfite restriction analysis
COM:	co-operation mediator
CSF-1:	colony-stimulating factor
CSR:	cytoskeletal reorganization
C-terminal:	carboxyl-terminal
D1 (2, 3):	domain1 (2, 3)
DAG:	diacylglycerol
DEX:	dexamethasone
DFS:	disease free survival
OS:	overall survival
DMase:	demethylase
DNMT:	DNA methyltransferase enzyme (same as MTase)
DMAP1:	DNMT1 (MTase1) associated protein
E2:	17β-estradiol
EBS:	Ets binding sites

E-cad:	E-cadherin
ECM:	extracellular matrix
EFAs:	essential fatty acid
EGCG:	epigallocatechin-3-gallate
EGF:	epidermal growth factor
ER:	estrogen receptor
ERK:	extracellular signal-regulated kinase
ES:	embryonic stem
FAK:	focal adhesion kinase
FBS:	fetal bovine serum
FGF:	fibroblast growth factor
EPA:	eicosapentaenoic acid
FSDs:	free sulfhydryl donors
GFD:	growth factor domain
GFP:	green fluorescent protein
GLA:	gamma-linolenic acid
GM-CSF:	granulocyte-macrophage colony stimulating factor
GPI:	glycophosphotidylinositol
GR:	glucocorticoid receptor
Grb-2:	growth factor receptor binding protein-2
HBECs:	human breast epithelial cells
HCC:	hepatocellular carcinoma
HDACs:	histone deacetylases
HDMVC:	human dermal microvascular endothelial cells
H&E:	hematoxylin & eiosin
HGF:	hepatocyte growth factor
hK2:	kallikrein 2
HMECs:	human mammalian epithelial cells
HMW-uPA:	high molecular weight uPA
HRG1:	heregulin-β1
HRP:	horseradish peroxidase

HUVEC:	human umbilical vein endothelial cell
HVSMC:	human vascular smooth muscle cell
ICF:	immuno-deficiency, centromere instability and facial anomalies
IGF(R):	insulin-like growth factor (receptor)
IκB:	I kappa B
JAK:	Janus kinase
JNK:	c-Jun N-terminal kinase
KD:	kringle domain
KGF:	keratinocyte growth factor
LDL(R):	low-density lipoprotein (receptor)
IL:	interleukin
LMW-uPA:	low molecular weight uPA
LPA:	lysophosphatidic acid
LPS:	lipopolysaccharide
LRP:	low-density lipoprotein-receptor related protein
MAPK:	mitogen-activated protein kinase
MBD:	CpG methyl binding domain
MBDPs:	methylated DNA binding proteins
5-mC:	5-methylcytosine
MeCP:	methyl CpG binding protein
MEK:	mitogen-activated protein kinase
MEKK:	mitogen-activated protein kinase kinase
MeSABp50:	methylation-sensitive Alu binding protein
MMP:	matrix metalloprotease
MNNG:	monofunctional alkylating agent N-methyl-N-nitro-N-nitrosoguanidine
MSP:	methylation sensitive PCR
MT1-MMP:	membrane-type 1 MMP
NPI:	Nottingham Prognostic Index
N-terminal:	amino-terminal
O^6-MGMT:	O^6-guanosine methyltransferase

OA:	okadaic acid
OSM:	oncostatin M
PA:	plasminogen activator
PAI:	plasminogen activator inhibitor
PBS:	phosphate buffered saline
(r)PCI:	(recombinant) protein C inhibitor
PEA3:	polyomavirus enhancer A-binding protein-3
PGE2:	prostaglandin E2
PgR:	progesterone receptor
PI-3K:	phsphatidylinositol-3 kinase
PKC:	protein kinase C
PLC (D):	phospholipase C (D)
PMA:	phorbol myristate acetate
PCNA:	proliferating cell nuclear antigen
PSA:	prostate-specific antigen
RACK:	receptor for activated kinase C
RA(R):	retinoic acid (receptor)
(p)RB:	retinoblastoma (protein)
RCE:	retinoblastoma control element
RT:	room temperature
RTK:	receptor tyrosine kinase
RXRs:	retinoid X receptors
SAM:	S-adenosylmethionine
ScuPA:	single chain uPA
serpin:	**ser**ine **p**roteinase **in**hibitor
SF:	scatter factor
Sf9:	spodoptera frugiperda 9
SFM:	serum free medium
SPF:	S-phase fraction
STAT:	signal transducer and activator of transcription
suPAR:	soluble uPA receptor

TAM:	tamoxifen
Tat:	tyrosine aminotransferase
TdT:	terminal deoxynucleotidyl transferase
TF:	transcription factor
TFA:	trifluoroacetic acid
TGF-β:	transforming growth factor-beta
TIMP:	tissue inhibitor of metalloprotease
TK:	thymidine kinase
TNF-α:	tumor necrosis factor-alpha
TNF(R):	tumor necrosis factor (receptor)
TRD:	transcription repression domain
TSA:	trichostatin A
TSP-1:	thrombospondin-1
tPA:	tissue type plasminogen activator
TPA:	12-O-tetradecanoylphorbol-13 acetate
TSP-1:	thrombospondin-1
UEF:	urokinase enhancer factor
uPA(R):	urinary plasminogen activator or urokinase (receptor)
uPARAP:	urinary plasminogen activator receptor associated protein
UTI:	urinary trypsin inhibitor
3'UTR:	3'-untranslated region
VEGF(R):	vascular endothelial growth factor (receptor)
VHL:	Von-Hippel Lindau
VLDLR:	very low-density lipoprotein receptor
VN:	vitronectin

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Chapter I

General Introduction

1.1 Urinary plasminogen activator (uPA or urokinase)

The 55 kDa protein urokinase (uPA), which is the subject of this thesis, belongs to the serine protease family. The uPA/uPA receptor (uPAR) system includes at least four proteins: uPA, its membrane-bound glycophosphotidylinositol (GPI)-anchored receptor (uPAR) and two plasminogen activator inhibitors (PAI-1 and PAI-2) [1]. Initially isolated from human urine and fetal kidney cells, uPA was first reported to convert plasminogen to plasmin during clot lysis, a characteristic that has allowed it to be used therapeutically as a thrombolytic agent [2]. A number of studies later suggested that uPA also promotes tumor growth, invasion and metastasis [3, 4]. These uPA-mediated processes are critical for cancer biology since tumor metastasis to various organs continues to be the leading cause of cancer-related morbidity and mortality, despite recent advances in early detection and controlling the growth of primary tumors (e.g. breast cancer) [5]. In addition to uPA, a member of the serine protease family, proteases that belong to the metallo-, cysteine, and aspartyl proteinase families have now been implicated in cancer-related cell invasion and metastasis [6, 7]. Among them, uPA (serine proteinase) and MMP (matrix metalloprotease) family members are responsible for most proteolysis during physiological or pathological tissue remodeling [8, 9]. The cellular origins of the proteolytic machinery may vary depending on both tumor type and the surrounding stroma.

Pre-clinical and clinical studies strongly suggest the central role of the uPA/uPAR system in breast cancer progression. uPA contributes to breast cancer progression as a result of its overexpression in cancer [10, 11]. However, the molecular mechanisms underlying the upregulation of uPA were still not defined at the beginning of this project. This has therefore restricted our ability to block uPA at the transcriptional level in invasive cancers, a potentially effective approach to inhibit the early stages of cancer progression, and has led to the development of uPA enzyme inhibitors or antibodies as therapeutic agents. Due to multiple side effects and the high toxicity of existing inhibitory agents, few have been successful in clinical trials. It was therefore extremely important to develop a new class of inhibitors that would be both highly specific and have fewer side effects. In collaboration with Ångstrom

Inc., we developed and tested a novel peptide-based uPA inhibitor Å6, which can effectively inhibit breast cancer invasion and metastasis *in vitro* and *in vivo* (see Chapter III and IV). Apart from efforts to develop new types of uPA inhibitors, a better understanding of the regulatory mechanisms underlying *uPA* gene expression in cancer might provide new targets for pharmacological intervention to prevent breast cancer progression. My work has revealed that overexpression of uPA in late stage breast cancer is due to DNA hypomethylation, and epigenetic changes of the *uPA* gene might correlate with changes in the activities of several methylation-related enzymes (see Chapter II).

During the course of my graduate studies, I focused on the role of uPA and its expression and regulation in breast cancer progression as discussed in this thesis. In this chapter, I will review the current understanding of the role of uPA in malignancy.

1.1.1 *uPA* gene organization, distribution and regulation

Immunocytochemistry and immunohistochemistry staining revealed that physiological expression of the *uPA* gene is a property of cells of both fibroblast-like and epithelial-like origin [12]. Under stimulated conditions, expression of uPA is associated with processes such as fibrinolysis, tissue remodeling, cell migration, inflammation [2], wound healing [13], and neoplastic transformation *in vitro* and *in vivo* [14]. In the following paragraph, I will introduce the structure and the regulation of *uPA* gene expression in both physiological and pathological conditions.

1.1.2 *uPA* gene structure and its regulatory elements

The *uPA* gene, located on human chromosome 10, was cloned in 1984 [15]. This gene is 6.4kb long and is organized in 11 exons interrupted by 10 intervening introns, and encodes a 2.4kb mRNA. S1 nuclease mapping and primer extension has determined the 5' end of uPA mRNA and its transcription initiation site [16]. Exon I encodes most of the 5' untranslated region and exon II encodes the signal peptide. Exon III encodes the first 9 amino acid sequence of uPA. The growth factor domain (GFD), which is homologous to the receptor binding regions of epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), is mainly coded by exon IV. Exons V and VI encode a three-disulfide-bonded kringle domain (KD). Exons VII-XI encode part of the B chain of uPA, a catalytic domain of the uPA enzyme. The

residues that are essential for the catalytic activity of uPA are encoded by exons VII, VIII, XI [16].

The human *uPA* gene shares a high degree of homology with mouse and pig *uPA* genes throughout the 5'-flanking region and in its exon-intron organization [16, 17]. The 5'-flanking region of the *uPA* gene, containing sequence homology interrupted by repeat elements, was found to extend to -2.4kb in human, -4.6kb in pig and -6.6kb in mouse. However, only the conserved, 5' non-coding sequences that reside -2.0kb upstream of the transcriptional site and within 100 bp of the transcription start site of the *uPA* gene are required for maximal gene expression [17, 18]. Interestingly, densely populated CpG dinucleotides of the *uPA* gene are clustered within or closely around these regions, where the Alu sequences, an enhancer element (-2.0kb upstream of the transcription start site), and the uPA promoter (start from -802) that extends to the transcribed exon I (Fig. 1.1 Panel B&C) are located. Regulation of *uPA* gene expression may be dependent on the cytosine methylation status of the 5' CpG island of this gene [19], and can be identified using methylation sensitive enzymes such as HpaII and HhaI (Fig. 1.1. Panel B and Fig. 2.1).

Alu repeats are recombinogenic sequences that are subjected to methylation changes in different cancer cell lines. The correlation between methylation and expression of these repeats is probably due to the presence of methylation-related Alu binding protein [20]. An Alu sequence of uPA is present -2.0kb upstream from the 5' end of the *uPA* gene, whereas a dimeric Alu unit is located about 200 nucleotides downstream from the polyadenylation site. Both Alu sequences are in the same orientation as *uPA* gene transcription [16]. Whether the Alu sequences are involved in epigenetic changes of the *uPA* gene needs to be clarified. Downstream of the Alu sequence is an enhancer element (-2350 ~ -1824, Fig. 1.1. Panel B) that comprises two alternatively repeated octameric motifs, box A ($^{A}/_{G}$AAGAG $^{T}/_{G}{}^{T}/_{A}$) and box B (GAAGT$^{T}/_{G}$CT) which reside between -2133 ~ -2053. Immediately following box A and box B is another consensus sequence ($^{G}/_{A}$GGGG $^{A}/_{C}$G$^{G}/_{A}$A) repeated three times, which resembles the Ap2 binding site [18]. Direct interference of the binding of sequence specific TFs such as Ap2, E2F and NF-κB to methylated DNA has been reported [21]. Whether these Ap2 binding sites are subject to methylation

modification, and thus affect the expression of the *uPA* gene needs to be elucidated. Xing in our laboratory has reported that several cytosine methylation sites of the uPA promoter correlate with this gene expression [22]. Detailed information on epigenetic changes in the uPA promoter and regulation of its gene expression will be discussed in Chapter II. Further understanding of the regulation of the Alu repeats and the enhancer element of the *uPA* gene by DNA methylation will be explored (Fig. 1.1. Panel B). Epigenetic changes of these elements may alter the binding specificity of a number of TFs such as Ap2 to these regions (Fig. 1.1. Panel B), and thus induce aberrant expression of the *uPA* gene in cancer. A negative cis-acting sequence between -1824 ~ -1572 immediately following the enhancer region reduces the efficiency of the upstream enhancer rather than blocking downstream promoter activity.

The basic TATA box is an essential element of the uPA promoter since deletion of this part of the sequence totally abolishes the transcription capacity of the *uPA* gene [18]. A second silencing and enhancer region has been localized to -660 ~ -536 and -536 ~ -308 of the uPA promoter respectively. In addition to the 5' promoter, the 3' UTR (3' untranslated region) of the *uPA* gene contains multiple instability regulating sites for gene turnover [23]. These unique sequences have variable effects on *uPA* gene expression including transcriptional enhancement and tissue distribution [24].

1.1.3 Transcriptional regulation of the *uPA* gene

uPA expression is induced by various stimuli including oncogenic transformation [25], vascular injury and wound healing [26]. These induced expressions are growth and cell cycle related [27]. Growth affecting compounds, growth factors [28], hormones [29], retinoic acid and chemical stimuli (e.g. phorbol esters [30], TPA (12-O-tetradecanoylphorbol-13-acetate) [31], bombesin [32]) all affect *uPA* gene expression, so do the components of the extracellular matrix (ECM) such as fibronectin and vitronectin (VN) [33]. Most of these agents increase uPA expression, however, significantly decreased uPA mRNA levels and mRNA stability have been reported following treatment with dexamethasone (DEX; 10^{-7}M) [34].

In this section, the upregulation of *uPA* gene expression under various conditions is outlined. The reported stimuli (e.g. growth factors) not only increase uPA transcripts, but also facilitate the secretion of generated proteins. Growth factors such as hepatocyte growth factor (HGF) [35], insulin growth factor-1 (IGF-1) [28], EGF [36], macrophage colony-stimulating factor (CSF-1) [37], vascular endothelial growth factor (VEGF) [38], fibroblast growth factor (FGF) [39] and keratinocyte growth factor (KGF) [40] are well documented to induce uPA-mediated cell invasiveness by upregulation of its gene expression, which is mediated by growth factor activated receptor tyrosine kinases (RTKs), and a series of downstream signaling pathways, including rhoA, c-src, sos, ras, raf-1, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), phosphatidylinositol-3 kinase (PI-3K), MEK, mitogen-activated protein kinase (MAPK), cAMP and PKC isozymes [41-47]. In addition to RTK activity, the v-ras, v-src, v-yes, and v-ros as well as the oncogene products (e.g. p185, encoded by oncogene c-erbB-2) can directly increase cellular uPA transcripts [48, 49].

In addition, several growth factor- and growth factor receptor-associated molecules have also been shown to affect *uPA* gene expression. Heregulin-β1 (HRG1), which binds human EGFR 3 and 4, promotes motility and invasiveness of multiple tumor cells including breast cancer cells partly by the upregulation of uPA and uPAR in these cells [50, 51]. Amphiregulin, a heparin-binding EGF-related peptide, induced the accumulation of uPA and PAI-1 into culture media of breast cancer cells, but failed to exert similar effects in human mammalian epithelial cells (HMECs) [51]. Some of these stimuli (e.g. bombesin, prostaglandin E2 (PGE2), G-CSF and lysophosphatidic acid (LPA) [32, 52, 53]) can also induce the secretion of uPA in various cell types [54], which may involve the p38α MAPK signaling pathway [55].

Co-association of uPAR with fibronectin binding integrin α5β1 is coordinated with induced expression of uPA [56], and is essential for cancer growth. This association leads to greatly increased levels of active ERK [57], and suggests a possible autocrine loop for uPA production. However, binding of VN to integrin αvβ3 down-regulates the expression of uPA and uPAR and increases the levels of

their inhibitor PAI-1 in ovarian cancer cell lines [33]. These studies suggest the interactions among uPAR, VN and integrins on cancer cells may determine the invasive phenotypes of these cells by affecting the expression levels of uPA and uPAR.

These stimulus-mediated signaling pathways are ultimately transmitted to the nuclei of different cell types, which in turn induce the binding of their downstream TFs such as Ap1 (fos/Jun), Ets, E2F, Rel and Sp1 to the uPA promoter for its activation (Fig. 1.1. Panel A) [58, 59].

1.1.3.1 Ap1 (jun/fos) transcription factor

TF Ap1 is a complex of the Jun homodimer or Jun/fos heterodimer. Cells responding to cytoskeletal reorganization (CSR), tumor promoter okadaic acid (OA), growth factors (e.g. CSF-1) or middle-T antigen have induced uPA transcripts via binding of phosphorylated Ap1 to the uPA promoter [60-62]. This cascade can be initiated by activating FAK followed by src, sos, raf-1 and subsequently ras/Erk or PKC pathways [63, 64]. Several Ap1 binding sites have been identified. The polyomavirus enhancer A-binding protein-3 (PEA3, AGGAAATGAGGTCAT)/Ap1 enhancer element and an Ap1 element (GTGATTCACTTCCT) are located at -2.4 kb and -6.9 kb of the 5′ flanking region of the *uPA* gene respectively. Activating transcription factor-2 (ATF-2) and Jun bind to these response elements, and are required for TPA, interleukin-1 (IL-1) and FGF-2-mediated uPA induction [37, 65-68]. Cooperation of PEA3/Ap1 with other elements located within the uPA promoter has been reported to result in high uPA expression [65]. Cooperation Mediator (COM) element is such an example. It is required for the synergistic action of PEA3/Ap1 and Ap1 sites, and represents general enhancer functions for proteases, cytokines and chemokines. COM-binding protein urokinase enhancer factor (UEF) may be responsible for the induction of uPA [69].

Despite the inducible effects of Ap1 on uPA expression, a Ras-fos-heat shock factor 1-dependent reduction has been reported to attenuate uPA expression [70]. Lack of JunB expression deregulates *uPA* gene expression and results in a defective neovascularization of the decidua during mammalian placentation [71]. These studies

provide strong evidence for the important role of Ap1 in the regulation of uPA expression under physiological and pathological conditions [43, 62, 64, 72].

1.1.3.2 Sp1 and GC boxes

Pig and mouse uPA promoters each contain one hexanucleotide GGGCGG motif (GC box), a potential binding site for the TF Sp1 that resides close to the transcription initiation site. A CpG dinucleotide sits in the middle of the GC box and might be subject to DNA methylation. Similar structures were observed in several viral (herpes simplex virus) and eukaryotic promoters (rainbow trout protamine), which suggest a potential role for Sp1 in promoter activity [73, 74]. This same sequence is repeated three times at position -63, -48 and -37 between the CAAT and TATA boxes of the human uPA promoter [16]. Binding of Sp1 to its consensus GC box motif is important but not essential for the expression of human *uPA* gene since deletion of the Sp1 binding site reduced but not completely abolished its transcriptional activity [18]. However, Sp1 by itself can't induce uPA production, a finding that has been confirmed in our lab (unpublished observations). Sp1 might exert its role by forming functional transcriptional machinery via direct contact with other TFs as supported by several reports. Retinoic acid (RA) induces uPA expression through direct binding of Sp1 to its receptor (RAR) and the retinoid X receptors (RXRs) [75]. A discrete CTF/NF1 binding region between -54 and -42 within the human uPA promoter comprises a sequence centered around the Sp1 binding site that is essential for the formation of cAMP induced DNA-protein complexes [76]. Synergistic effects of RA with cAMP and PKC in the induction of uPA expression have been reported, which might involve Sp1 in these complexes [76]. A recently described novel kruppel-like factor (Zf9) has greatest affinity for the middle of 3 contiguous GC boxes of the uPA promoter, but not the mutated 'GC box' motifs, and thus transcriptionally activate uPA in bovine aortic endothelial cells (BAECs). This induction of uPA results in increased bioactive fibrogenetic cytokine transforming growth factor-beta (TGF-β) [77]. Whether Sp1 plays a role in Zf9-mediated inducible effects on uPA expression needs to be elucidated.

1.1.3.3 Ets family of transcription factors

Ets family members are expressed with a similar profile as various proteases including uPA in invasive cancers. All Ets family members recognize a core sequence GGAA/T within Ets binding sites (EBS) [78]. Ets-1 and Ets-2 are required for the expression of proteases such as uPA, MMP-9 [79], collagenase-1 (MMP-1) and stromelysin (MMP-3) that contribute to the invasive phenotypes of various cancers to render these tumors more aggressive [80, 81]. They have been reported to be associated with invasive glioma, astrocytic, squamous, gastric and ovarian cancers, but are rarely detectable in benign and non-invasive cancers [82-84]. A number of stimuli can induce the levels of Ets-1, Ets-2 and uPA simultaneously. Serum, FGF-2, EGF and HGF concomitantly induce the expression of Ets-1 and uPA in breast, oral squamous and non-small-cell lung cancers in which Ap1 may also play a role [39, 58, 79]. CSF-1 stimulates uPA production by activation of Ets-2 in macrophages [85]. Other Ets family members are also involved in cancer cell invasiveness. PU-1, a member of this family, increases uPA production at the post-transcriptional level [34]. In addition, high levels of paralogues of the Ets family members, Fli-1 and Elf-1, have been detected in the majority of adenocarcinomas overexpressing uPA [72].

Of these entire Ets family member, only Ets-1 has been reported to be associated with the overexpression of multiple invasiveness-related proteases [79]. Therefore, Ets-1 was selected as model molecule to study the accessibility of TF to the methylated versus unmethylated uPA promoter as reported in Chapter II. The presence of Sp1/Sp3 [86] or Ap1 [87] as cofactors of Ets family members in the regulation of the ECM genes might provide important clues for their roles in controlling *uPA* gene expression.

1.1.3.4 pRB/E2F transcription factor

Altered levels of pocket protein retinoblastoma (pRB)/E2F are associated with *uPA* gene expression. The working model of pRB/E2F has been well accepted. Generally, pRB in quiescent cells becomes dephosphorylated and binds to the transcriptional activation domain of E2F, thus actively suppressing E2F responsive genes. However, when cells are stimulated to divide, pRB is phosphorylated and is no longer associated with E2F. The dissociation of E2F leads to the activation of its

responsive genes, and cells progress into S-phase [88]. However, the effect of pRB/E2F on *uPA* gene expression was found to be opposite to what was predicted by this classical model. For the first time, E2F was reported to inhibit promoter activity and endogenous expression of uPA and uPAR in a pRB-independent manner, and may involve the Ap1 binding site regardless of the pRB control element (RCE) element of the uPA promoter [89]. Furthermore, the RCE-containing uPA promoter is activated during enforced expression of pRB [90]. These negative regulatory effects of pRB and E2F in the uPA/uPAR system are cell cycle-independent [89]. The molecular mechanism underlying this regulation has not yet been defined.

Direct interaction of the DNA methyltransferase 1 (DNMT1) with the A/B pocket region of pRB was reported [91]. As will be discussed later, DNMT1 is responsible for maintaining cytosine methylation and the histone structure of various genes (see 1.7.2.3.2). This induced effect of pRB might be due to its direct binding to DNMT1, and thus blocking the normal functions of DNMT1 on the *uPA* gene.

1.1.3.5 Rel and NF-κB transcription factors

The uPA promoter contains three potential Rel-like protein binding motifs: RRBE, 5'-NF-κB and 3'-NF-κB. Mutational disruption of 5'-NF-κB and 3'-NF-κB motifs resulted in approximately 40% and 20% reduction in uPA promoter activity respectively. Complete abolision of activity is observed in constructs that include mutated RRBE [92]. Phorbol myristate acetate (PMA), phorbol ester, IL-1 and tumor necrosis factor-alpha (TNF-α) all induce *uPA* gene transcription and require the presence of NF-κB and the decay of a short-lived I kappa B (IκB) [93]. IκB is an inhibitor of NF-κB that abolishes uPA overexpression via inhibition of the constitutive activity of RelA, a member of the Rel/NF-κB family [92, 94]. Rel might regulate *uPA* gene expression by forming Rel/p65 (a NF-κB subunit) heterodimer [95]. Cell-cell and cell-matrix interaction also regulate *uPA* gene expression via Rel. For example, integrin αvβ3-mediated cell adhesion to VN significantly reduces the activities of Rel and downregulates uPA and uPAR and upregulates PAI-1 in ovarian cancer cells [33].

In summary, the unique gene structure of *uPA* and a number of TFs as mentioned earlier determine levels of uPA transcripts and subsequent protein functions under various physiological and pathological conditions.

1.1.4 uPA-mediated plasminogen activation

uPA and tissue type plasminogen activator (tPA) are two serine proteases that share high sequence homology within their catalytic domains with the conserved active site triad of His_{204}-Asp_{255}-Ser_{356}. Receptor binding is required for activating their common substrate plasminogen, an inactive zymogen within the ECM [17, 96]. The resulting active plasmin contributes to uPA-mediated fibrinolytic functions in tumor biology, whereas tPA, is mainly involved in fibrinolysis in circulation [2].

1.1.4.1 Activation of inactive single chain uPA (scuPA)

uPA is synthesized and secreted as a single chain inactive zymogen named scuPA or pro-uPA. ScuPA has very low or no intrinsic enzyme activity. The binding of scuPA to its receptor is of high affinity (Kd=0.05~4nM) and is cell type- and species-dependent [97, 98]. Activation of scuPA is more rapid when it is receptor bound than its free form in fluid-phase [99]. This activation is catalyzed by plasmin, and was first demonstrated by Cubellis et al [100]. Plasmin catalyzes the proteolytic cleavage of a single peptide bond between Lys_{158}~Ile_{159} of scuPA, and generates a two-chain high molecular weight uPA (HMW-uPA or tcuPA) linked by one disulfide bond. HMW-uPA consists of an A-chain (a.a.1~158) at its N-terminal and a B-chain (a.a.159~411) at its C-terminal (Fig. 4.1) [100]. Proteases other than plasmin such as tumor associated trypsin, kallikrein, cathepsins, thermolysin, mast cell tryptase, T cell associated serine proteinase HuTSP-1 as well as prostate-specific antigen (PSA) have been shown to convert scuPA to HMW-uPA [101-105].

Further cleavage of HMW-uPA generates a 40KDa low molecular weight uPA (LMW-uPA, a.a.136~411) located at its carboxyl-terminal (C-terminal) and a 15KDa amino-terminal fragment (ATF, a.a.1~135 or a.a.1~143) at its N-terminal (Fig. 4.1). The conserved serine protease catalytic domain of LMW-uPA plays a critical role in plasminogen activation [96]. ATF is sub-divided into a GFD (growth factor domain, a.a.1~49) and a KD (kringle domain, a.a.50~131) [106]. Interestingly, two forms of ATF are present *in vivo*, which lead us to speculate that a short uPA

fragment (a.a.136~143) may be formed following activation of HMW-uPA [96, 106-109]. This potential peptide fragment sits within the mini-peptide region (a.a.132~158, other names are connecting peptide region or interdomain linker region) between ATF and LMW-uPA. However, its functional significance remains to be elucidated. In Chapter III and IV of this thesis, we report the role of a synthetic peptide Å6 (a.a.136~143) in breast cancer models *in vitro* and *in vivo*. The design of this peptide was based on the sequence of this potential uPA fragment, and therefore provides the basis for further characterization of the functions of this region.

Proteases (elastase and thrombin) that cleave scuPA at different positions to yield enzymatically inactive HMW-uPA have also been reported [96]. The physiological significance of this inactive form of HMW-uPA is unknown.

1.1.4.2 Plasminogen activation

Activation of scuPA to tcuPA upon binding to uPAR leads to the activation of plasminogen and generates plasmin that subsequently breaks down the components of the ECM. uPA-catalyzed plasminogen activation plays a fundamental role not only in a variety of physiological conditions such as tissue involution [110], but also in pathological conditions such as tumor progression [111], and requires co-accumulation of plasminogen on the cell surface. The concentration of plasminogen in plasma is about 2 μM, however, there is a large pool of extravascular plasminogen [112]. Administration of uPA to uPAR expressing cells dramatically increases the binding of plasminogen to the cell surface. Gangliosides is the site where plasminogen binds but with a low affinity (K_d = 0.3 ~ 2.8 μM) [113]. Both tPA and uPA can proteolytically cleave a single peptide bond (Arg_{561}-Val_{562}) within plasminogen, thus activating this enzyme. The plasmin generated has 10^4~10^6 higher fibrinolytic activity than plasminogen.

In addition, a plasmin cleavage-independent scuPA/suPAR (soluble uPAR) complex can activate plasminogen on the cell surface of monocytes and in clot lysis assays. This complex is resistant to inactivation by PAIs [114], thus prolonging its plasminogen activating effects, effects that may initiate activation of the uPA cascade under physiological conditions.

1.1.4.2.1 Targets of plasmin

Plasmin (M_r=90KDa) consists of an N-terminal A chain containing 5 kringle domains (KD1~5) and a C-terminal B chain containing the typical serine proteinase domain responsible for its catalytic function. Membrane-bound plasmin has a much higher activity (about 50-fold) than its free form in solution. Among all the ECM substrates of this enzyme (e.g. fibronectin, laminin and proteoglycans), the most favorable peri-cellular substrate is fibrin [6]. Plasmin catalyzes the hydrolysis of the peptide bonds of its substrates on the C-terminal side of Lys and Arg residues. In addition to its fibrinolytic activities, knockout studies (*uPA-/-*, *tPA-/-*, *plasminogen -/-*) confirmed the important role of plasmin in the activation of zymogens of MMPs (e.g. pro-MMP-3, pro-MMP-9 and pro-MMP-13) [115], which thus result in the indirect destruction of substrates such as collagen [6]. Furthermore, plasmin can activate latent growth factors and release ECM binding growth factors [112], and will be discussed later (see 1.4.1).

1.1.4.2.2 Angiostatin

Angiostatin, a fragment with KD 1~3 or 1~4 of plasminogen or plasmin, is mostly generated by plasmin-mediated autoproteolysis. *In vivo* conversion of plasminogen to angiostatin by uPA has been demonstrated in human pancreatic cancer cells [118]. It can also be catalyzed by pancreatic elastase, free sulfhydryl donors (FSDs) (N-acetyl-L-cysteine, D-penicillamine, captopril, L-cysteine), reduced glutathione and a variety of MMPs such as MMP-7 (matrilysin) and MMP-9 (gelatinase B) [116, 117]. Angiostatin is known as an angiogenesis inhibitor and has been shown to suppress Lewis lung carcinoma growth *in vivo* [119]. Therefore, angiostatin may play a critical role in uPA-mediated tumor angiogenesis *in vivo*.

In summary, activation of uPA is catalyzed by a number of enzymes (e.g. plasmin). It is solely dependent on the binding of uPA to its cell surface receptor (uPAR), thus initiating uPA-mediated plasminogen activation and the subsequent proteolytic cascade.

1.2 Characterization of uPAR

1.2.1 the *uPAR* gene and its regulation

The presence of uPAR (CD87) was first demonstrated by Vassalli et al about 16 years ago [120]. He observed that uPA was often associated with monocytes and monocyte-like cell surfaces. uPA has since been found to bind to many cells of neoplastic origin via uPAR. The *uPAR* gene is highly conserved between human and mice. Genomic uPAR is 21.23kb long and encodes a 1.4kb mRNA, and is organized into 7 exons interrupted by 6 introns [121]. The uPAR promoter was defined as a 188-bp fragment between bases -141 and +47 relative to the transcription start site. It lacks both TATA and CAAT boxes, but contains relatively GC-rich sequences that specifically bind Sp1 class members [122]. Binding of TFs (Sp1, NF-κB, Ap1 and Ap2) to the uPAR promoter activates its basal transcription and is seen especially in cancer cells as compared to benign cells [3, 123].

uPAR is normally present on the surface of circulating blood leukocytes, endothelial cells, vascular smooth muscle cells, fibroblasts, bone marrow cells and a variety of neoplastic cells, but is absent on quiescent cells [124]. Increased expression of uPAR strongly correlates with cell migration and invasive potential via a uPA-mediated, proteolytic-dependent or -independent cascade. Differential expression of the *uPAR* gene has been implicated in several physiological and pathological processes at transcriptional, translational and post-translational levels, as seen during angiogenesis, monocyte migration, trophoblast implantation, wound healing, cancer invasion and cancer metastasis [3, 125]. A number of tumor promoting factors are involved, including tumor promotion factors (PMA), growth factors (EGF and NGF), cytokines (TGF-β1), atherogenic lipoproteins as well as hypoxia [126, 127]. uPA itself also induces uPAR production independent of its receptor binding, which represents a novel pathway contributing to stromal remodeling in wound healing or neoplasia [128].

1.2.2 Different forms of uPAR

uPAR was purified from detergent extracts of PMA-stimulated human U937 monocyte-like cells. This purified receptor is a single-chain 313 a.a. glycoprotein and has a molecular weight of 55 to 60 KDa [129, 130]. uPAR belongs to the family of

GPI moiety anchored proteins [131]. Three forms of uPAR have been identified so far, GPI-anchored uPAR, suPAR and truncated uPAR.

GPI-anchored uPAR has three homologous, independently folded domains termed Domain 1, 2 and 3 (D1, D2 and D3). D1, at its N-terminal, is involved in uPA binding. The other two domains bind to VN, an integrin ligand located in the ECM. In addition, uPAR binds integrin directly, and this binding is distinguishable from its uPA and VN binding sites. The linker region connecting D1 and D2 is a proteinase-sensitive domain that has *in vivo* chemotactic activity, and can bind an unknown surface adapter [132].

In addition to the membrane bound, GPI-anchored uPAR, an anchorless variant named suPAR has been identified in conditioned medium (CM) from different types of cells [133] and in body fluids from cancer patients [134]. It may arise from differential splicing and lipolysis (e.g. phospholipase C (PLC) cleavage of the GPI anchored-uPAR) [135-137].

N-terminal truncated uPAR, containing D2 and D3 only, has been identified *in vivo* [134]. This truncated form might arise through catalysis of uPAR by uPA at Arg_{83}-Ala_{84} and Arg_{89}-Ser_{90} between D1 and D2, and in turn abolishes the binding of uPA to its receptor [138, 139]. The functional significance of this catalytic event is unknown, and might be involved in the uPAR turnover or degradation.

1.2.3 Other potential uPA binding proteins

uPA also weakly binds to C4.4A, a molecule with a low degree of homology to uPAR. C4.4A encodes a 1.6 kb mRNA and codes for a 352 a.a. GPI-anchored molecule, whose molecular weight varies in different cells (94~98 kDa) depending on the extent of N- and O-glycosylation. Under physiological conditions, C4.4A is expressed only in the gravid uterus and epithelial tissue in the upper gastrointestinal tract. C4.4A adheres to laminin and allows normally non-invasive cells to penetrate the matrix and metastasize. This process can be completely prevented by addition of C4.4A antibody. Therefore, C4.4A may display functional activities similar to uPAR [140].

In summary, interactions between uPA and its associated receptors or binding proteins lead to a wide variety of functions relating to cell motility, invasion and

proliferation. However, the uPA/GPI-anchored uPAR complex is internalized and quickly degraded once its inhibitor PAI-1 is bound. In the following section, the role of several inhibitors of the uPA/uPAR system, especially PAI-1, will be introduced.

1.3 Serine proteinase inhibitors:

PAIs (PAI-1 and PAI-2), maspin, α_2-antiplasmin and α_2-macroglobulin are the classical members of the serpin (**ser**ine **p**roteinase **in**hibitor) superfamily. Both PAI-1 and PAI-2 inhibit the proteolytic activities of uPA and tPA especially when they are in fluid phase [141]. A newly defined serpin, maspin, shows similar inhibitory effects as compared to PAIs [142]. α_2-antiplasmin and α_2-macroglobulin are mostly involved in inhibiting plasmin instead of uPA [113, 143].

1.3.1 PAI-1

PAI-1 (M_r=50KDa), the main inhibitor of plasminogen activators (uPA and tPA) in plasma and in the peritoneum indirectly impairs plasmin formation. It binds and inhibits the activity of both free and receptor-bound tcuPA at the same rate [144]. However, binding of scuPA to its receptor is relatively resistant to PAI-1 [145].

PAI-1 inhibition shortens the half-life of uPA by inducing its internalization and degradation via binding of uPAR to endocytosis receptors of the low-density lipoprotein receptor (LDLR) family including α_2-macroglobulin receptor (α2-MR) / LDLR related protein (LRP) [146], gp330/megalin, or very low-density lipoprotein receptor (VLDLR) [147, 148]. Once the quaternary uPAR-uPA-PAI-1-LRP complex is internalized, uPA-PAI-1 is degraded in the lysosome. Free uPAR is recycled back to the cell surface for a second round of adhesion and chemotaxis where it binds to newly released uPA [149]. Decreasing levels of LRP using antisense technology promotes uPA accumulation in the medium of an astrocytic tumor cell line [150]. A uPAR-associated protein (uPARAP/endo180) has recently been identified and acts as an internalisation receptor that might play a similar role in mediating local turnover of the uPA/uPAR complex [151].

Various factors including some members of the serpin superfamily have been reported to affect the expression levels and inhibitory enzymatic activities of PAI-1. TGF-β [152], thrombospondin-1 (TSP-1) [153], oestradiol, progestin [154] and

hypoxia [155] increase PAI-1 antigen levels and activities in a time- and concentration-dependent manner. In addition, serp-1, a secreted myxoma virus glycoprotein serpin, binds and inhibits plasminogen activators and increases PAI-1 transcripts and activity to block angiogenesis [156]. Orthopoxvirus serpin SPI-3 has similar inhibitory constants for plasmin, uPA, and tPA as compared to serp-1 [157]. Furthermore, a newly identified 60 KDa regulatory protein binds to the 3'-untranslated region of PAI-1 mRNA, and appears to be involved in the post-transcriptional regulation of *PAI-1* gene expression by human lung carcinoma cells *in vitro* [158].

Inactivation of PAI-1 by human kallikrein 2 (hK2) has been demonstrated. HK2 is a serine protease predominantly expressed in the prostate that has 80% homology to PSA. Apart from its inhibitory effects on PAI-1, it can also activate pro-PSA, pro-hK2 and scuPA [159].

1.3.2 PAI-2

Secreted PAI-2 (M_r=50KDa) is a less efficient inhibitor of uPA compared to PAI-1, but displayed similar inhibitory characteristics as PAI-1 [141]. It is known to bind and inhibit tPA as well [160]. The distribution of PAI-2 is less well defined, but is normally not found in plasma.

1.3.3 α_2-Antiplasmin and α_2-Macroglobulin

α_2-antiplasmin is the primary physiological inhibitor of plasmin and ensures its short half-life in tissues and blood. Plasmin generated by receptor-bound uPA is completely protected from α_2-antiplasmin while it is cell surface bound. However, this binding is of low affinity and its dissociation from the cell surface results in rapid inhibition [113].

α_2-macroglobulin is another major plasma protease inhibitor of the fibrinolytic system. It not only inhibits fibrin bound plasmin, but also regulates growth of many cell types and gene expression in these cells by binding and neutralizing TGF-β [143].

1.3.4 Maspin

Maspin is a novel member of the serpin family with potential to suppress tumor invasion and metastasis in breast and prostate cancer. Maspin blocks tumor

invasion, at least in part, by inhibiting cell motility. Although maspin does not inhibit tPA and uPA in cell-free solutions, purified recombinant maspin (rmaspin) can inhibit cell surface bound uPA at a Ki value comparable to PAIs [142].

1.4 uPA/uPAR complex and its associated functions

The uPA/uPAR complex is required for tumor cell invasion and metastasis as determined using *in vitro* basement membrane invasion assays and *in vivo* tumor progression models [161]. Binding to uPAR also protects uPA from plasmin-mediated cleavage between the GFD and KD and subsequent degradation [162]. Both scuPA and tcuPA bind to uPAR [98, 163]. However, receptor bound scuPA has an activity about 250 fold less than that of tcuPA [164]. Residues 12~30 within the GFD of uPA are required for receptor binding. Specifically, residues 13~19 provide the proper conformation to the adjacent binding region, whereas residues 20~30 confer receptor-binding specificity [163]. All three domains of uPAR are required for uPA binding, whereas D1 and D3 are more directly involved [165]. Two sub-regions (residues 2~10 and 47~53) within D1 are critical for uPA binding [166]. Using chemical modification and photo-affinity labeling of human uPA and uPAR, the residues of uPAR that are in close contact with uPA (Arg_{53}, Tyr_{57} and Leu_{66} in D1 and His_{251} in D3) have been identified. Furthermore, Tyr_{24} in GFD of uPA and Tyr_{57} in the D1 of uPAR are intimately engaged in the receptor-ligand interaction [167].

uPA bound uPAR normally accumulates at the leading edge of cell movement [168], and this binding complex often leads to degradation of adhesion receptors and their ECM ligands via plasmin-mediated proteolysis. The uPA/plasminogen system is now appreciated to be far more complex than originally believed. Proteolytic-independent functions related to uPAR have been identified. These include uPAR-mediated endocytosis of members of the LDLR family (see 1.3.1), interaction of uPAR with cell surface molecules such as PAI-1 and VN (see 1.3.1 and 1.4.2.2), and uPA/uPAR-mediated signal transduction (see 1.4.3). Furthermore, the uPA/uPAR complex often accumulates at the cell-substratum and focal contacts of various cell types especially with fibroblastic origin, where it can interact with clustered integrins

or actin filaments of other cells to promote adhesion and migration of these cells (see 1.4.4) [6].

1.4.1 Interaction of uPA/uPAR with growth factors within the ECM

The ECM imposes important regulation on cell shape, migration, growth and differentiation through the interaction between its multiple components and cell surface. uPA/uPAR interaction is a tightly regulated process, and is influenced by a number of growth factors and proteases within the ECM in a plasminogen-dependent or -independent manner. As mentioned earlier (see 1.1.4.2.1), plasminogen activation directly regulates stroma remodeling via plasmin and plasmin-activated MMPs, which results in the release of angiogenesis associated growth factors such as FGF-2, VEGF and TGF-β from the cells or the surrounding matrix [169]. Plasmin can also convert the inactive single chain macrophage-stimulating protein and HGF/scatter factor (SF) to their active forms with tyrosine kinase activities. Plasminogen-independent processing of these growth factors has been demonstrated as well. uPA itself can process HGF [170] and upregulates the expression of the VEGF receptor KDR/flk-1 [171]. These direct or indirect increased levels and activities of VEGF, HGF and FGF-2 by uPA further induce the expression of both uPA and uPAR in endothelial cells. Moreover, generated uPA can activate TGF-β via plasmin, which in turn limits uPA synthesis and its activity, and opposes the activation of FGF-2 [172].

1.4.2 uPAR and adhesion molecules

Following the observation some 20 years ago that various tumor cells have different patterns of cell adhesion [173], cell adhesion has been a major focus in cell biology research. Cell adhesion is regulated through interaction with the surrounding stroma and matrix remodeling that ultimately leads to cell movement. The uPA/uPAR system interferes with co-localized adhesion molecules and matrix deposits to facilitate cell migration. Among these molecules, members of the integrin family and VN are major players. uPAR interacts with integrin and VN to accumulate and stabilize these molecules at its expression site, thus uPAR itself can act as an adhesion molecule [174].

1.4.2.1 uPAR and integrin

uPAR mediates cell adhesion by interacting with integrin and extracellular substrata in a cell type-specific manner. It selectively colocalizes with different integrin subunits such as β1, β2, β3, α3, α5, α6 and αV based on the specific substrata on which the cells are cultured [175, 176, 178, 179]. For example, uPAR and integrin associate in normal, resting human leukocytes but dissociate after cell polarization and migration [177]. These findings indicate that uPAR confers the specific binding affinity of integrin to adhesion molecules on adjacent cells and different components of substrata in the ECM (e.g. VN), and the interaction may be essential for cell adhesion and migration. Cell context including dynamic reorganization of the actin cytoskeleton as well as the adhesion and remodeling of the ECM all play critical roles in these processes.

1.4.2.2 uPA/uPAR system and vitronectin (VN)

VN (M_r=78KDa), known as a ligand for integrin, is present in many forms in blood plasma and ECM. An RGD (Arg-Gly-Asp, a.a.45~47) sequence of VN has direct contact with several forms of integrin such as αVβ1, αVβ3, αVβ5, αIIbβ3, and α8β1 [180, 181]. Cells overexpressing uPAR adhere to and spread on VN in an RGD-independent manner. This is due to the direct and favorable binding of uPAR to VN rather than integrin in malignant tumors [182]. The association of VN to uPAR localises the uPA/uPAR-complex to cell surfaces that are in direct contact with the ECM, where it accumulates the plasminogen activator activity required for cell adhesion, migration and tissue remodelling. Direct contact of uPAR and VN is abolished by inter-domain cleavage of uPAR, which is reversible following the addition of exogenous suPAR [178], suggesting that all three domains of uPAR are required for binding to VN. Phosphorylation of serine residues (a.a. 138/303) of scuPA inhibits uPAR-dependent adhesion of myelomonocytic cells to VN [187], thus establish the critical role of these residues in uPAR-mediated cell adhesion on VN. Antibodies or antisense oligonucleotides targeting to uPAR reduced uPAR-mediated adhesion of monocytes to VN [188]. Under certain circumstances, scuPA, uPA, ATF and the uPA-PAI-1 complex all stimulate the association of uPAR and VN in a dose-

dependent manner to promote cell-substratum adhesion [183-185], while the addition of PAI-1 blocks these stimulatory effects [186].

The predominant inhibitory form of PAI-1 in plasma is the complex formed by high affinity binding to VN. PAI-1 directly interacts with an N-terminal somatomedin-like domain that lies close to the integrin as well as the uPAR-binding site of VN [191]. It thus competes for the binding of VN to integrin or uPAR in addition to its serpin activity, and prevents VN-mediated cell adhesion and migration [191, 192]. Complex formation also protects VN from uPA-mediated degradation on the cell surface in a time- and concentration-dependent manner [189]. Since VN is the site where PAI-1 attaches to the ECM, other protein substrates surrounding VN can thus be protected from degradation [190].

In summary, interactions between members of the uPA/uPAR system and other cell surface molecules mostly take place at the leading edge of cell movement where uPAR recruits and localizes a number of associated ligand and binding proteins to promote cell adhesion and migration.

1.4.3 Downstream signaling of uPA/uPAR interaction

Binding of uPA (or ATF) to cell surface uPAR activates intracellular signal transduction pathways that are involved in cell adhesion, chemotaxis, migration, invasion and mitogenic responses [193]. Proteolytic cleavage of the linker peptide located between D1 and D2 of uPAR induces a similar signaling cascade in human monocytic THP-1 cells [132]. Therefore, uPA/uPAR interaction or endogenous proteinase cleavage of uPAR may cause a conformational change of uPAR to expose an identical epitope, and thus initiating the signal transduction cascade [194]. Most of these signalings are mediated by GPI-anchored uPAR. However, suPAR that is not cell-surface bound can also play a role. SuPAR is mostly observed in cancer, the interaction of suPAR monomer with scuPA detected in an *in vitro* system suggests a novel mechanism to explain uPA-mediated signal transduction in cancer [195].

uPAR is often observed to be co-immunoprecipitated and /or co-localized with signal transmitters including hck, lck, fgr, fyn, lyn, Jak1 and tyk2 [26, 197-199]. This is due to its accumulation with caveolins (cholesterol binding scaffolding protein), integrins and signal transmitters in caveolae, the invaginations of lipid rafts

with a high content of cholesterol and glycosphingolipids [196]. Tyrosine phosphorylation of hck, focal adhesion kinase (FAK), Erk, MAPK, paxillin, $p130^{cas}$ facilitates the binding of TFs such as Stat1 to its downstream targeted DNA [203, 204]. Some examples of how the signals are transmitted following binding of uPA to its receptor are given here. Binding of uPA to uPAR mediates human vascular smooth muscle cell (HVSMC) migration by activating uPAR-associated tyk2 and PI-3K pathways, which eventually activates TFs Stat1 (signal transducer and activator of transcription 1), Stat2, and Stat4 [200, 201]. Binding of ATF/uPA to its receptor in MCF-7 cells activates Erk-1 and Erk-2 and results in increased cellular motility [202]. Early response oncogenes *c-myc*, *c-Jun* and *c-fos* can be induced following the binding of ATF to uPAR in human osteoblast derived osteosarcoma cells that are specifically involved in the mitogenic responses [205]. In addition to tyrosine kinase receptors, G protein-coupled receptors can be activated by uPAR-mediated signaling pathways [206]. The activation of a G protein-coupled receptor, reportedly following uPAR activation, involves molecules such as protein kinase Cγ (PKCγ) [207], cAMP [107] and diacylglycerol (DAG) [208] However, the relative order of the signal transduction pathway has not been clarified, and the cell type specifically involved in each pathway has yet to be identified.

Taken together, uPA and uPAR are multifunctional proteins that influence a great variety of signal transduction pathways and ultimately contribute to cell adhesion, chemotaxis, cell migration, cell invasion, gene expression as well as cell proliferation. These uPA- and uPAR-mediated actions act in concert with integrins, VN, G proteins, or with yet unidentified co-receptors or adapter molecules.

1.4.4 The uPA/uPAR complex-mediated functions

1.4.4.1 Cell migration

One of the major effects of uPA/uPAR interaction is cell migration, a process of locomotion of cells over an ECM substratum [6, 178]. Cell migration is a continuous process that is accomplished simultaneously by moving forward the leading cell edge and releasing the trailing cell edge, which requires both proteolytic-dependent and -independent activities of uPA. The uPA-mediated, plasmin-independent process is promoted by stimulating uPAR/VN binding [178], initiating

signal transduction pathways at the leading edge of cell movement [202] and regulating integrin-binding activity [178] at both the leading and trailing edges of cells. uPA/plasmin-mediated degradation of the ECM proteins and adhesion receptors release the trailing cell edge [6]. Cell migration on VN can be disrupted by suPAR or antibodies against uPA or αvβ5 integrin in a human pancreatic carcinoma cell line [209], which indicates the functional coordination of VN and uPA/uPAR with integrin to promote cell migration.

PAI-1 inhibited integrin-dependent migration of rabbit vascular smooth muscle cells, human epithelial cells, and bovine aortic endothelial cells [191, 210, 211]. However, some studies reported stimulatory rather than inhibitory effects of PAI-1 on integrin-dependent migration of human melanoma cells and bovine aortic smooth muscle cells [210, 212]. The net effects of PAI-1 depend on the relative importance of integrin-VN interactions at the leading and trailing edge of each cell line. Endocytosis of uPA/uPAR/PAI-1 complexes via receptors of the LDLR family might facilitate this polarized endocytic cycle [213]. For example, PAI-1 inhibits cell migration via plasmin-mediated degradation of adhesion molecules and inhibition of integrin and VN binding [211]. Angiostatin, an autoproteolytic fragment of plasminogen or plasmin, has an anti-migratory effect on endothelial cells, and thus provides negative feedback on plasmin-dependent cell migration [214]. uPA may stimulate cell migration by counteracting the effects of PAI-1.

Therefore, a model of uPA-stimulated cell migration can be proposed. Once scuPA is converted to its active form at the ventral surface of the cells, proteolytic mechanisms are localized at the trailing edge of the cells, while non-proteolytic mechanisms are dominant at the leading edge to provoke cell movement. Most importantly, the net effects of uPA (both active and inactive forms)/PAI-1 would be solely dependent on: 1) the level and location of uPAR, 2) how uPAR interacts with other cell surface molecules (such as integrins and endocytosis receptors), 3) interaction of uPAR with the components of the ECM [215-217].

1.4.4.2 Cell invasion

Cell invasion includes not only cell locomotion, but also the active penetration of cells through the ECM facilitated by the plasmin-mediated degradation of its

constituents. Both uPA and uPAR are key mediators of extracellular proteolysis. They participate in ECM remodeling, activate cells and enable them to migrate and invade through multiple tissue layers. Therefore, the uPA/uPAR system is considered a rate-limiting factor in cell invasion [6].

The role of PAI-1 in cell invasion is extremely complicated. PAI-1, an inhibitor of uPA, suppresses uPA/uPAR-mediated cell invasion in certain cell types, but promotes cell invasiveness in others [218]. The relative amount of PAI-1 and other cell components such as integrins, endocytosis receptors, and the composition of the ECM may be important in determining its fate as either a stimulator or an inhibitor of cell invasion. At high concentrations, PAI-1 protects the ECM against plasmin-mediated proteolysis, and invasion is inhibited. However, at low concentrations, protection of ECM proteins for traction dominates [219], which may also generate excessive plasminogen activation by facilitating the turnover of uPAR. Unlike PAI-1, PAI-2 is consistently found to inhibit uPA-mediated cell invasion [6].

1.4.4.3 Chemotaxis

Chemotaxis is a process whereby cells sense and move along a concentration gradient of soluble molecules. Binding of these soluble molecules to their cell surface receptors activates intracellular signaling pathways and remodels the cytoskeleton through the activation or inhibition of various actin-binding proteins. Direct or indirect exposure of the chemotactic epitope of uPAR transforms it into a cell-surface chemokine that is able to act on the same, or neighboring cells, which in turn initiates signaling cascades for cell movement [132, 187, 194]. The chemotactic epitope of uPAR resides in the linker region between D1 and D2. A linear peptide corresponding to residues within this linker region has chemotactic potency in the 0.1pM range. Cleavage of the suPAR fragment between D1 and D2 by chymotrypsin also results in chemotactic activity [138]. Binding of uPA, its ATF fragment or scuPA to uPAR induces chemotactic effects in various cell lines including activated blood leukocytes, endothelial cells, macrophages, fibroblasts, vascular smooth muscle and cancer cells [99, 187, 194, 215]. uPA-induced chemotaxis or proteolytic cleavage of uPAR requires the transient activation of uPAR associated $p56/p58^{hck}$ tyrosine kinase in monocytic cells [132].

1.4.4.4 Cell proliferation

uPA-mediated cell proliferation has been reported in many primary cell culture systems derived from human smooth muscle cells, endothelial cells, epithelial cells, keratinocytes and T lymphocytes and damaged tissues via binding to its receptor [220-224]. The mitogenic effects of uPA on cancer cells have also been observed including breast, prostate, renal, hepatocellular, ovarian, head and neck carcinoma cells, epidermal tumor cells and melanoma cells [193, 225-227]. These effects are cell cycle related and might correlate with the expression of proliferating cell nuclear antigen (PCNA) in tumor cells [228]. A unique signaling complex that contains uPAR and multiple tyrosine kinase receptor-associated signals such as MAPK, JNK and casein kinase 2 and subsequent downstream signals including Stat1, Stat2, Stat4, fos, myc and Jun are responsible for the mitogenic effects of uPA [26, 203-205, 229]. uPA indirectly stimulates cell proliferation and intimal thickening of vessels via increasing DNA synthesis or activation of plasmin mobilized growth factors (e.g. FGF-2) within the ECM [221, 230].

ATF is required for the binding of uPA to its receptor. ATF alone stimulates osteoblast-derived osteosarcoma [231] and ovarian carcinoma [232] cell growth in culture. However, autocrine ATF may act as an antagonist of uPA-uPAR interaction and thus efficiently inhibit invasion and metastasis without altering the proliferation rate of lung cancer cells [233]. ATF binding to uPAR was recently found to suppress the release of viral particles from HIV-1 infected cell lines without affecting host cell proliferation [234]. Therefore, the effects of ATF on cell proliferation may be a function of the cell system tested.

Although the mitogenic effects of uPA have been well documented *in vitro*, its *in vivo* functions are less clear. PAI-1 and PAI-2 have been reported to inhibit tumor cell proliferation, possibly as a direct result of their inhibitory effects on the release and action of uPA or ATF [223, 226, 232].

1.5 Role of the uPA/uPAR system in cancer

Carcinogen exposure, UV irradiation and other stimuli (described in section 1.5.1) play major roles in accelerating cancer progression. They increase the

production and accumulation of uPA, uPAR and PAI-1 to promote cell invasion, migration, proliferation and selective adhesion, and strongly correlate with a malignant tumor cell phenotype during the multi-step processes of metastasis [235, 236].

1.5.1 The uPA/uPAR system and cancer progression

Upregulation of uPA/uPAR/PAIs in progressive stages of invasive cancers such as breast [10], prostate [237], lung [158], ovarian [232], endometrial [238], head and neck [239], gastric [48], pancreatic [240], colorectal [241] and hepatocellular carcinoma (HCC) [242] has been demonstrated, and is consistent with their roles in determining invasive potential and aggressiveness of the cancer cells expressing them. The importance of stromal in addition to tumor cells in cancer progression is directly supported by the observation of delayed primary tumor growth and decreased metastases formation in $uPA^{-/-}$ mice that lack of stromal cell derived uPA as compared to wild type mice [8].

Oncogenic, environmental or chemical stimuli may render the transformed cells tumorigenic and metastatic *in vivo*. They activate Ras-Erk, PKC-Erk, PKA-cAMP, Rac-JNK, PLD-PKC, MKK-MAPK1/2 or p38 MAPKs-JNK/SAPK in a constitutive fashion, which leads to increased mitogenesis in these cells and elevated uPA and uPAR levels in tumor samples [46, 55, 243-250]. Therefore, levels of these internal signals resolve the dominant invasive phenotype in transformed cells [25]. uPA production can be further manipulated by increasing copy numbers of the *uPA* gene, thus mimicking the amplification as observed in hormone-refractory prostate carcinomas [251]. Several mutations have been revealed in the *uPA*, *uPAR* and *PAI-1* genes. However, the polymorphisms observed in these genes are similar in cancer patients and their normal counterparts, suggesting that these mutations play a minor role in uPA/uPAR system-mediated cancer development [252].

Members of the uPA/uPAR system are hallmarks of metastasis as suggested by many studies [253]. Metastatic cells represent 1~2% of the total primary tumor cell population, which not only escape from the primary tumor but also possess the correct phenotype to adhere and proliferate at a secondary site. Disemination of tumor cells does not necessarily lead to metastatic disease. In order to metastasize, tumor

cells attach to and interact with components of the basement membrane and the ECM, perform local proteolysis, degrade the ECM, intravasate, survive and migrate within the vessels, extravasate, then grow. During the early steps of this process, cells expressing uPA/uPAR are mostly involved in invasion and intravasation. However, at the late stages, these cells are primarily involved in extravasation and colonization. Invasion of cancer cells to the extravascular fibrin deposit is required for spreading, and provides a matrix for further angiogenesis and desmoplasia (see 1.5.1.1) [254, 255]. uPA-catalyzed plasmin generation is an important rate-limiting factor for cancer metastasis in different animal models. $uPA^{-/-}$ mice developed less local invasion of cancer cells and reduced progression of highly metastatic cancer [256].

Expression of members of the uPA/uPAR system is usually significantly higher in primary tumors compared to their normal counterparts and correlates with a shorter disease-free interval and lower overall survival rate [257]. Therefore, they are also proposed to be prognostic factors in various cancers [258]. However, the levels of these proteins may vary in a specified tumor type in each individual patient [6, 259]. In one study of breast cancer patients, the only parameters as suggested by multivariate Cox analysis that independently predicted survival were total PAI-1 level, lymph node status and hormone receptor status for recurrence-free survival and overall survival [260]. Other studies suggested that the flow cytometric S-phase fraction (SPF), a reference for cell-kinetic analysis, thymidine kinase (TK) and uPA are the better independent markers for distant recurrences than the Nottingham Prognostic Index (NPI), tumor size and histologic grade [261, 262]. In addition, high levels of suPAR in body fluids and blood plasma were correlated with poor patient prognosis [263]. Moreover, uPAR and VEGF might contribute synergistically to the liver metastasis of colorectal cancer [264]. Therefore, expression profiles of multiple factors such as uPA/uPAR system members and their related molecules may provide extremely important prognostic information for clinic diagnosis.

1.5.1.1 The uPA/uPAR system and tumor angiogenesis

The uPA/uPAR system plays an essential role in tumor biology not only in neoplastic transformation, cancer cell proliferation, migration and invasion, but also in cancer cell-mediated tissue remodeling including angiogenesis and probably

desmoplasia [4, 265, 266]. These processes contribute to tumor growth, invasion and metastasis at different stages of tumor development. Angiogenesis is a tightly regulated process that occurs mainly during reproduction, development, wound healing and cancer, and is known as neovascularization. It provides nutrients for tumor cell survival and assists hematogenous disemination and metastasis in cancer [267]. Desmoplasia involves the stimulation of fibroblast and myofibroblast cell proliferation and ECM protein synthesis once the basement membrane is broken down by proteases [268, 269]. Ever since Dr. Folkman predicted a link between tumor angiogenesis and metastasis in 1974, many anti-cancer approaches that target the process of angiogenesis have been tested by inhibiting key factors involved, such as VEGF, FGF-2, PDGF, EGF, TGF-β and MMPs [169, 171, 172, 270, 271]. Studies using *in vitro* and *in vivo* angiogenesis models strongly suggest a role for the uPA/uPAR system in angiogenesis, thus providing a novel anti-angiogenic target [272-274]. A uPA directed-proteolytic cascade in tissue and vascular remodeling usually goes through two stages during the multiple processes in angiogenesis: 1) degradation of ECM in conjunction with desmoplasia, 2) activation of TGF-β as well as the release of matrix-bound growth factors such as FGF-2 and HGF [275]. The accumulated activated growth factors or cytokines will in turn stimulate uPA production by tumor and stromal cells that contribute to the chemotactic and mitogenic responses of these cells through both a plasmin-dependent (proteolysis) and -independent mechanism (uPAR-mediated signaling pathway) [236, 271].

Immunohistochemical staining provides key information on the distribution of uPA and uPAR, which are often observed on tumor-associated endothelial cells and stromal cells (e.g. macrophages) that contribute to tumor angiogenesis in addition to tumor cells themselves [276-279]. A positive correlation of uPA with microvessel density, vascular invasion, proliferation rate and macrophage content in breast cancer strongly suggests a role for uPA in tumor-related angiogenesis [276]. The direct action of uPA on vascular cells has been reported. Increased levels of uPA enhance the invasive capacity of endothelial and smooth muscle cells, as well as the proliferation rate of smooth muscle cells [280, 281]. Binding of uPA to cell surface uPAR on endothelial cells stimulates several pathways that lead to endothelial cell

differentiation and capillary tube formation on Matrigel by both the catalytic-dependent and -independent pathways [284]. uPAR is relevant to the vasculature of many types of tumors of which melanoma is a good example. Antagonists of uPAR inhibit primary growth and angiogenesis of B16 murine melanoma in syngeneic mice [283]. Interestingly, the detection of uPAR only on tumor-associated vessels but not on pre-existing vessels in normal tissue suggests that uPAR represents a marker of tumor-specific angiogenesis. uPAR is also involved in non-neoplastic pathological angiogenesis, e.g. diabetic retinopathy [282].

The specific inhibitor PAI-1 is well known to block uPA-mediated proteolysis. Paradoxically, uPA coordinated expression of PAI-1 plays critical roles in tumor invasion and angiogenesis. Absence of PAI-1 prevents cancer cell invasion and vascularization in PAI-1 deficient mice transplanted with malignant keratinocytes, however, tumor-associated angiogenesis was restored with administration of PAI-1 [285]. This unresolved mechanism might be due to the inhibitory effects of PAI-1 on uPA-mediated proteolysis to prevent the destruction of newly formed vessels and ECM proteins. As a result, excessive fibrin deposition and tumor desmoplasia recruit and localise circulating tumor cells. Integrins, expressed by endothelial cells, bind to surrounding ECM proteins including VN or fibronectin, and thus promote the motility of these cells in the process of angiogenesis. PAI-1 promotes endothelial cell migration by interfering with VN and integrin binding, and towards fibronectin-rich tumor tissues [286, 287]. During these processes, plasminogen, the substrate of uPA, does not seem to be an essential mediator for angiogenesis, since plasminogen$^{-/-}$ mice inoculated with Lewis lung carcinoma have similar levels of neovascularization as compared to control animals [288].

Hypoxia suppresses vascular perfusion and increases macrophage infiltration, and is a well-known factor initiating angiogenesis within tumors. It induces angiogenesis by increasing the antigen content of tumor-associated uPA, uPAR, growth factors (VEGF, FGF-2 and PDGF), MMPs and endothelin-1 related vasoconstriction in cancer. It also decreases integrin- and uPAR-mediated cell adhesion to substratum [183, 292-295]. In addition, hypoxia alters the metabolic balance by recruiting macrophages to tumor sites and inducing the release of

angiogenic molecules from these cells [296], which can also be detected in non-neoplastic inflammatory conditions. Inflammatory cytokine oncostatin M (OSM) is produced by activated macrophages and T-lymphocytes, and modulates different phases of angiogenesis through both increased levels and stability of uPA and uPAR mRNA [236]. Macrophages also release significant amounts of PAI-1 under conditions of low oxygen tension that is related to angiogenesis, and play important roles in ischemia-induced thrombosis by suppressing vascular fibrinolysis [155].

Endostatin, an endogenous inhibitor of tumor angiogenesis and an inducer of tumor apoptosis, down-regulates the uPA/uPAR system, dissociates focal adhesion and reorganizes cytoskeleton structure in endothelial cells [289]. Several studies also suggest a potential role for the uPA/uPAR system in apoptosis although the mechanisms are unresolved. The binding of TNF-α to its receptor, TNFR, induces c-myc-dependent apoptosis and anoikis in an ovarian cancer cell line. This pro-apoptotic effect is partly achieved through the interference with interactions between cells and basment membranes via uPA [290]. Administration of uPA to mice suffering from fas-mediated massive hepatocyte apoptosis reverses this phenomenon and facilitates hepatic regeneration through stimulation of HGF maturation [291]. Taken together, the uPA/uPAR system-associated tumor angiogenesis is critical for cancer biology. In the following section, I will briefly introduce the role of the uPA/uPAR system involved in breast cancer progression.

1.5.1.2 The uPA/uPAR system and breast cancer progression

Breast cancer is an epithelial-derived malignancy. Breast cancer becomes life threatening when the malignant cells diseminate to distant sites via invasion and angiogenesis. The identification of patients at high risk of relapse is currently one of the most important issues in breast cancer research. Histologic grade, NPI, ER or progesterone receptor (PgR) status, EGFR, HER-2/neu contents and tumor size have previously been shown to be important prognostic indicators for distant recurrences of breast cancer [5]. Most recently, studies have suggested that high levels of expressed uPA, uPAR or PAI-1 significantly predicted shorter survival and early relapse in breast cancer and other malignancies. One study shows that the expression of uPA, uPAR, and PAI-1 in fibroblasts rather than tumor cells has a key impact on

the clinical behavior of breast cancer [10], suggesting stromal proteases are critical for interactions between tumor cells and the surrounding stroma. The new information may provide additional parameters in multivariate analysis [277, 297]. Intriguingly, the finding of diseminated cancer cells with high expression levels of uPA and uPAR in the plasma or bone marrow of breast cancer patients suggests a feasible screening protocol for the follow-up monitoring of these patients [298, 299]. Although overexpression of suPAR effectively eliminates uPA and impairs proteolysis, growth and the metastatic potential of breast carcinoma cells *in vivo* [300], high levels of suPAR in body fluids and blood plasma were correlated with poor patient prognosis [263]. The mechanisms are poorly understood and need to be elucidated.

Among the myriad growth factors that alter uPA expression and activity, TGF-β plays a controversial role in breast cancer remodeling. It inhibits cell proliferation and uPA activity in normal human breast epithelial cells (HBECs), however, TGF-β released from breast cancer cells up-regulates uPA and PAI-1 antigen at both transcriptional and translational levels in tumor-associated macrophages, and only a modest increase was observed in macrophages in the surrounding normal tissue. This may be due to differences in the density of the TGF-β receptor on different types of cells [271, 301]. In a potential positive feedback loop, uPA-activated TGF-β within the ECM can further increase the expression and activity of uPA, and markedly enhance the invasive capacity of human breast cancer cell lines [302]. However, the involvement of growth factors or cytokines that up-regulate uPA production in coordination with TGF-β, and the presence of other TGF-β induced proteolytic enzymes could not be excluded [4, 28, 303]. Exceptions were seen in osteosarcoma and uveal melanoma cells in which TGF-β decreased basal uPA secretion and its enzyme activity [304, 305].

Epidemiological evidence strongly supports the theory that endogenous steroid hormones such as estrogen play crucial roles in breast cancer etiology and progression. 17β-estradiol (E2) and some estrogen receptor antagonists with similar agonistic profiles as E2 are associated with increased *uPA* gene expression in ER positive breast cancer cells [306, 307]. Similar effects following steroid hormone

treatment are seen in androgen treated androgen receptor (AR) positive prostate cancer cells [308]. However, androgen and estrogen decrease uPA production in hormone receptor transfected human breast and prostate cancer cells *in vitro* and *in vivo* [237, 309], which suggests that hormonal regulation of uPA expression is associated with the stage of cancer rather than having a direct impact on its expression. In addition, cells expressing high levels of uPA and other members of the uPA family are resistant to hormonal therapies. Therefore, the levels of the uPA/uPAR system members might be useful in predicting the overall response of metastatic disease to hormonal therapies [310].

A highly useful treatment for most breast cancer patients with ER positive tumors is hormonal therapy, which blocks breast cancer cell growth by directly interfering with the binding of estrogen to its receptor. TAM is the most prescribed drug for the treatment of ER positive breast cancers and has demonstrated efficacy in the chemoprevention of breast cancer in women at high-risk [311]. ER-independent mechanisms of TAM are likely due to its ability to alter the secretion of a number of growth factors by endothelial cells thus affecting subsequent growth factor-mediated signaling pathways [311, 312]. Similar observations are shown in the combination therapy of TAM with a uPA antagonist in a syngeneic breast cancer model as described in Chapter IV. Resistance to hormonal treatment is an invariable outcome in patients with ER positive breast cancer after extended treatment. Many mechanisms have been proposed and include the identification of point mutations in the hormone-binding domain of the ER [313] and alterations in ER mRNA splicing leading to constitutively active forms of the receptor [314]. Crosstalk between ER and growth factor tyrosine kinase receptors or G-protein-coupled receptors favors the agonistic effects of TAM [315-318] by either phosphorylating ER [316], or affecting the levels of ER co-activators and co-repressors [319]. TAM metabolites have been found to contain estrogenic activity [312]. Since the uPA/uPAR system plays a key role in patients with advanced breast cancers that contain a mixed population of ER positive and negative breast cancer cells, the use of anti-proteolytic agents in addition to TAM may favor the disease outcome (also demonstrated in Chapter IV) [11].

1.5.1.3 Other proteases involved in cancer progression

Cancer invasion and metastasis is a process requiring a coordinated series of anti-adhesive, migratory, and pericellular proteolytic events and involves a number of protease families. A large and expanding literature has emerged over the last decade describing different types of tumor-associated proteases, their inhibitors and receptors involved in tumor invasion and metastasis. Four classes of proteases have been reported: 1) Matrix metalloproteases (MMPs), e.g. collagenases, gelatinases and stromelysins. 2) Cysteine proteases; e.g. Cathepsins B and L. 3) Aspartyl protease; e.g. Cathepsin D. 4) Serine proteases; e.g. uPA, tPA and plasmin [7].

Proteases form a complex network that contributes to the malignant phenotype of different solid tumors (Fig. 1.2). Members of the uPA/uPAR system and MMPs are both required for the processes of tumor growth, invasion, angiogenesis and metastasis in a number of cancers [320]. MMPs, a family of zinc-dependent endopeptidases, are secreted as inactive pro-MMPs. The active MMPs degrade the basement membrane and interstitial stroma and are frequently overexpressed in malignant tumors [321]. Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are well studied in cancer biology. These two MMPs along with uPA and plasminogen are all associated with the cell surface. Activation and degradation of pro-MMP-2 and pro-MMP-9 occur in a dose- and time-dependent manner in the presence of physiological concentrations of plasminogen and uPA. In the absence of tissue inhibitors of MMPs (TIMPs), plasmin in the soluble phase or generated from plasminogen by uPA catalysis can directly activate pro-MMP-9 (as confirmed in our lab, unpublished observations) [322]. uPA activates pro-MMP-2 indirectly via membrane-type-1 MMP (MT1-MMP) [323] (Fig. 1.2). Thus, the uPA-plasmin system may represent a physiological or possibly a pathological mechanism for the control of gelatinase activities [324]. In addition, growth factors can concomitantly induce the expression of uPA and MMP-9, thus contributing to their roles as promoters of cell invasion [79].

In addition, lysosomal proteinase cathepsins were identified as important prognostic factors in breast cancer as well as other malignancies [325]. It has been suggested that both the proforms and active forms of Cathepsin B and D participate in

tumour invasion and metastasis [326, 327]. Local acidification or proteolytic cleavage catalyzed by other proteinases such as elastase mediates pro-Cathepsin processing [328]. Among all these lysosomal cysteine proteases, Cathepsin B is mostly associated with the metastatic phenotype of tumor cells. It is primarily secreted from tumor epithelial cells and surrounding endothelial cells. Increased levels and activity of this protease and its plasma membrane association confer the invasiveness of the cells expressing it [329]. A series of inhibitors of Cathepsin B such as cystatin C as well as stefin A are present in the cytoplasm and extracellular millieu, but are present in lower amounts in breast cancer tissues [330, 331]. Furthermore, Cathepsin B can activate scuPA and thus initiate an extracellular proteolytic cascade through uPA/uPAR/plasminogen → plasmin → MMPs cascade as shown in Fig. 1.2 [332]. Of all the lysosome proteinases, the most abundant one is aspartic proteinase Cathepsin D. Although Cathepsin D is characterised as a protease, it contributes to tumor progression by increasing the number of tumor cells rather than its proteolytic activity [333]. Cathepsin D is an estrogen-regulated protease. Overexpression of Cathepsin D appears primarily in tumor cells but not in its surrounding stromal cells, which might be dependent on the estrogen response element located within its promoter region [334]. Cathepsin D has also been reported to prevent tumor growth and angiogenesis via its ability to generate angiostatin [335]. Cathepsins B and D therefore play different roles during cancer progression and provide significant, statistically independent prognostic information for DFS and OS in a number of cancers including breast cancer [325-327, 329].

In summary, high levels of serine proteases (uPA) with Cathepsins or MMPs are better prognostic markers in cancer as compared to the examination of individual proteases [336-338]. The increased levels of proteases and their inhibitors ultimately deregulate the functions and behaviours of tumors to favour their growth and metastasis, and may reflect the emergence of the drug resistant phenotype during cancer treatment [339].

1.5.2 uPA and uPAR as therapeutic targets for various cancers

Given our understanding of the central role of the uPA/uPAR system in tumor growth, invasion, angiogenesis and metastasis, the design of new strategies to inhibit one or all of these processes is now feasible.

1.5.2.1 Direct inhibitors of the uPA/uPAR system

1.5.2.1.1 Synthetic inhibitors

In tumor cells, uPA and uPAR expression may be constitutively activated as a result of oncogenic transformation [247]. The design of most classical inhibitors of uPA was based on the catalytic domain of uPA and was intended to block its enzyme activities and thus tumor cell growth and invasion. P-aminobenzamidine (a low molecular weight uPA inhibitor) and amiloride were among the first to be recognized and used [340]. The diuretic drug amiloride (AMLD) suppresses *uPA* gene expression and enzyme activity in a number of *in vitro* and *in vivo* cancer models [341, 342]. The prostate cancer cell line PC-3, which overexpresses uPA, was more sensitive to amiloride treatment than mock transfected cells with low uPA expression [251]. B-428 and B-623 are derivatives of amiloride with higher efficacy. Similar to amiloride, they inhibit tumor cell-associated uPA secretion and enzyme activity, and thus block uPA-mediated degradation of the ECM *in vitro* [343]. However, both compounds have minor effects on angiogenesis and spontaneous metastasis in experimental animal models *in vivo* [344]. This suggests that domains other than the catalytic region of uPA are required for angiogenesis-mediated functions, and these may serve as novel targets for putative inhibitors.

1.5.2.1.2 Peptides

uPA and tPA share high sequence homology in their catalytic domains. Therefore, inhibitors directed against the catalytic domain of one enzyme might also affect the other [345]. Several peptides designed to inhibit the binding of uPA to its receptor result in enhanced binding of uPAR to VN and decreased tumor growth *in vivo* [346, 347]. An inhibitor of uPA-uPAR interaction was shown to inhibit FGF-2 and VEGF stimulated endothelial cell tube formation in a fibrin matrix [283, 348]. Moreover, as discussed in chapter III, a synthetic peptide Å6 was designed based on the non-receptor binding region of uPA. It inhibits tumor and endothelial cell

migration, invasion and vasoconstriction *in vitro*, and blocks tumor growth, angiogenesis and metastasis *in vivo* [272, 273].

1.5.2.1.3 Immunotherapy

Antibody-based techniques seem promising in treating cancers especially breast cancer and lymphoma [349]. Several approaches using uPA, uPAR, and even PAI-1 antibodies had remarkable outcomes. uPA antibody has been tested in both non-neoplastic and cancer models, and has been shown to block fusion and differentiation of myoblast cells *in vitro* [65]. uPA derived from prostate tumors has mitogenic effects on prostate cancer cells and osteoblasts, and increases cell invasiveness [231, 350, 351]. Antibodies against uPA reverse these effects by suppressing the proliferation and differentiation of prostate osteoblast cells [350]. uPAR monoclonal antibodies completely inhibited FGF-2 and TNF-α mediated tubular formation in a fibrin matrix [352]. The antibody to uPAR that blocks tumor growth *in vivo* has also been demonstrated in our laboratory (submitted to Cancer Research). PAI-1 antibody has been shown to suppress pulmonary metastases of human fibrosarcoma by inhibiting tumor cell lodgment in vessels *in vivo* [353]. A genetically engineered uPA-IgG fusion protein (uPA-IgG) antagonizes uPAR and inhibits the initiation of primary tumors and micrometastasis [354, 355].

1.5.2.1.4 Potential applications for gene therapy

Gene therapies using adenovirus-conjugated or antisense oligodeoxynucleotides-based technologies have been designed to specifically target the biosynthesis or activities of uPA and uPAR [3, 356-359]. ATF, the non-catalytic region of uPA, interferes with uPA-uPAR interaction and blocks uPA enzyme activity [233]. This discovery not only defines ATF as an inhibitor of the uPA/uPAR system, but also provides a potential targeting site of the uPA/uPAR system. AdmATF is a recombinant adenovirus encoding a secreted ATF of murine uPA. The administration of AdmATF dramatically reduced primary tumor volume and subsequent metastasis [358]. Adenovirus-mediated antisense *uPAR* gene transfer or antisense uPAR mRNA reduces tumor cell spread *in vitro* and *in vivo* [3, 356]. Inhibition of colon cancer metastasis by both 5'- and 3'-end antisense uPAR was observed in a nude mouse model [360]. Administration of antisense oligos of TF NF-κB-RelA suppresses

synthesis of uPA in ovarian cancer cells [359]. Antisense oligos that limited the availability of uPAR also showed promising inhibitory effects on endothelial cell proliferation, migration and invasion by blocking uPAR-mediated signaling pathways [357].

1.5.2.1.5 Other inhibitors

Urinary trypsin inhibitor (UTI), a urinary trypsin inhibitor and a negative modulator of invasive ovarian tumor cells, efficiently inhibits soluble and tumor cell-associated plasmin activity and subsequently inhibits tumor cell invasion and metastasis by down-regulation of uPA through a PKC and MEK/ERK/c-Jun-dependent pathway [41]. A method to conjugate the UTI domain II (HI-8) to the receptor-binding ATF of uPA has been developed in order to reduce cell-associated proteolytic activity located close to the uPAR-expressing tumor cell surface. This strategy may effectively inhibit tumor cell invasion and metastasis [361]. Tumor cell selective cytotoxins such as uPA-targeted mutated anthrax toxin-protective antigen, activated selectively on the surface of uPAR-expressing tumor cells in the presence of scuPA and plasminogen, killed uPAR-expressing tumor cells [362] and may provide new therapeutic agents for cancer treatment.

Others have studied the potential inhibitory effects of various herbal products. PSK, a protein-bound polysaccharide, is widely used in Japan as an immuno-potentiating biological response modifier for cancer patients. PSK significantly decreases the invasiveness of pancreatic and gastric cancer cells by inhibiting uPA, TGF-β1, MMP-2, and MMP-9 at both mRNA and protein levels [363]. Epigallocatechin-3-gallate (EGCG), the essential component of crude green tea extract, also has inhibitory effects on tumor cell invasiveness [364]. Dietary fiber may protect against invasive colon cancer as well since short chain fatty acids such as butyrate inhibit human colon cancer cell invasion by reducing uPA activity and stimulating TIMP-1 and TIMP-2 activities [365]. Sodium butyrate inhibited both normal and breast cancer cell proliferation and uPA activity [301]. Certain essential fatty acids (EFAs), such as gamma-linolenic acid (GLA) and eicosapentaenoic acid (EPA), inhibit uPA activity and may affect the invasion and metastasis of cancer cells [366].

Using a computer-derived molecular model of uPA, inhibitors have been designed and synthesized. Although not yet characterized in an *in vivo* model, they may provide the basis for the future development of new drugs. N-(1-adamantyl)-N'-(4-guanidinobenzyl) urea is a highly selective non-peptidic uPA inhibitor. The x-ray crystal structure of the uPA B-chain complexed with this inhibitor has been revealed [367].

1.5.2.2 Indirect inhibition

Multiple malignancy-related changes are involved in the complex interactions of tumor cells and the surrounding stroma (endothelial cells, stromal fibroblast, inflammatory macrophages and T cells) via altered signaling networks, and thus in favor of the growth and motility of cancer cells [368]. The development of specific inhibitors of signaling mediators is now a focus of new therapeutic regimes in cancer treatment. The PKC pathway is involved in the TNF-mediated induction of uPA expression and secretion as well as subsequent matrix remodeling, the formation of tube-like structures in neovascularization, wound healing, and leukocyte extravasation [369, 370]. Bryostatin-1, an inhibitor that specifically blocks TPA-induced uPA expression through suppressing the PKC pathway is currently in early clinical trials [246]. Other members of the complex network of receptor tyrosine kinase-mediated plasmin activation, cell motility and invasion can serve as important targets [244]. Thus, inhibitors or antagonists of the uPA/uPAR system provide important therapeutic potential for cancers. The anti-proteolytic antagonists may serve as useful adjuvant agents in combination with existing cancer chemotherapy.

1.5.2.3 Combination therapy with anti-proteolytic agents

For decades, early detection of cancer remains the major factor that determines DFS. Research is still focusing on combining existing cancer treatments such as hormonal therapy or radiotherapy with second and third generation chemotherapeutic agents (cytotoxic agents). Systemic treatment of breast cancer by adjuvant therapy is largely based on the diagnosis of auxiliary metastases, and the successful treatment of advanced metastatic solid tumors has remained an elusive goal.

Traditional combination therapy for breast cancer consists of chemotherapy, cytoreductive therapy and radiotherapy. Several studies have suggested that certain cytotoxic agents can improve the response of breast tumors to the treatment of anti-estrogen agent TAM [372]. Such agents include cyclophosphamide, methotrexate, and fluorouracil. A study conducted earlier in our laboratory has demonstrated the beneficial effects of the combination of hormonal and anti-proteolytic therapies in breast cancer, suggesting the use of proteinase inhibitors of uPA and MMPs may improve disease outcomes [371]. TAM in combination with B428, a chemical inhibitor of uPA enzyme activity, showed additive effects in blocking the proliferative and invasive capacity of rat breast cancer cells *in vitro* and *in vivo* [11]. These effects are a result of the partial suppression of *uPAR* gene transcription. Measurement of uPA levels in primary breast tumors may also be useful in predicting the overall response of metastatic disease to TAM therapy [373]. Other studies have shown that small multifunctional inhibitors represent promising agents for the combination therapy of solid tumors. Novel types of double-headed inhibitors directed to different tumor-associated proteolytic systems were generated for cysteine lysosomal protease inhibition (cystatins) and both inhibit the activity of Cathepsins and interfere with uPA/uPAR interaction [374].

1.6 Physiological and non-neoplastic pathological roles of uPA/uPAR

1.6.1 Physiological roles of uPA: indications from genetically engineered mice

uPA may play a role in early human embryo implantation and development. Its levels are highest at the follicular phase, and are lowest at the ovulatory phase. uPA production is detected in human pre-implantation embryos that might assist trophoblast cells invade the endometrium [375]. Endometrial stromal cells increase the secretion of uPA during decidualization, a tissue remodelling process during embryo implantation. PGE2 can regulate the levels of uPA by increasing and/or stabilizing its transcripts in these cells [54]. In addition, uPA activity can be detected in mouse embryos as early as day 6 of pregnancy, and is present through the blastocyst stage [376].

Insight into the physiological functions of the uPA/uPAR system has mostly come from studies of genetically engineered mice. In mice, disrupting the genes for *uPA*, *tPA* [377], *plasminogen* [378], *uPAR* [379], *PAI-1* [380] and *VN* [381] results in animals with a short life span and multiple tissue dysfunctions. This may be a result of functional overlap, or because this system has been restricted to certain processes such as invasion and migration in inflammation under physiological conditions. The animals eventually develop to adulthood and are fertile, suggesting that the uPA/uPAR system, though not essential to life, is necessary for normal development.

Mice lacking uPA ($uPA^{-/-}$) have a clear immunodeficient phenotype with increased susceptibility to bacterial infection (*Cryptococcus neoformans* and *staphylococcal botryomycosis, pleuritis*) due to a lack of T cells and macrophage recruitment, as well as effacement of lymphoid follicles [224, 382]. Similar phenomena are seen in $plasminogen^{-/-}$ and $uPAR^{-/-}$ mice, which have difficulty in recruiting inflammatory cell populations (such as leukocytes, neutrophils and eosinophils) to the inflammatory sites [175, 383]. Moreover, $uPA^{-/-}$, $tPA^{-/-}$ and $plasminogen^{-/-}$ mice developed disseminated intra- and extravascular fibrin deposition [378]. $uPA^{-/-}$, $tPA^{-/-}$ and $uPAR^{-/-}/tPA^{-/-}$ mice have less abnormal fibrin deposition as compared to $plasminogen^{-/-}$ and $uPA^{-/-}/tPA^{-/-}$ mice, which implies that uPA may substitute for tPA in both fibrinolysis and its proteolytic functions. It also suggests that uPAR is not necessarily involved in uPA-mediated fibrinolysis [377, 384]. $Fibrinogen^{-/-}$ mice , having depleted the substrate of uPA do not show any fibrin deposition even in $uPA^{-/-}$, $tPA^{-/-}$ and $uPAR^{-/-}/tPA^{-/-}$ mice [385]. $PAI\text{-}1^{-/-}$ mice display a mild hyperfibrinolytic state and are resistant to thrombosis [380].

Dysregulated proteolysis in mice overexpressing uPA may have developmental consequences such as enamel defects [386] and impaired learning capacity due to neural disorder [387]. Therefore, the use of multi-organ or tissue designated engineering of uPA or its related proteins may shed light on the physiological roles of uPA in various organs and the underlying mechanisms involved in its regulation.

In vitro studies have also revealed many aspects of uPA's role in tissue-related physiological functions. Differential expression of uPA has been shown in

myogenesis. uPA is induced in myoblast proliferation but is repressed in myoblast differentiation. A number of growth factors and cytokines may contribute to the overexpression of uPA and increased proteolytic activity required during myoblast migration and fusion, as well as in muscle regeneration [388].

1.6.2 Role of uPA in pathogenesis of non-neoplastic diseases and clinical implications

Elevated plasminogen activation may play a crucial role in the pathogenesis of diseases that involve inflammatory, smooth muscle and endothelial cell replication, accumulation and migration [389]. Several pathological processes involving the uPA-uPAR complex include skin ulcers [390], venous leg ulceration [391], aneurysm [392], human muscular dystrophy [393] and chronic hypoxia-related right heart failure [394].

Elevated levels of uPA contribute to wound repair and healing [395], assist reorganization of the liver lobule after liver injury [396], facilitate cellular infiltration into multiple sclerotic lesions and middle cerebral artery occlusion [397], and hastening recovery by regenerating peripheral nerves [398]. On the other hand, thrombolytic serpins, including serp-1 and PAI-1, are central regulatory agents in vascular wound-healing responses by providing a balance of uPA [156]. Administration of uPA as a thrombolytic agent is standard in patients who have uPA deficiency-related diseases such as portal vein thrombosis [399]. Gene therapy by local infection of adenovirus-mediated, cell surface-directed plasmin inhibitor ATF.BPTI, provides a novel approach for its specific delivery and targeting [400]. Combination therapy of uPA with other agents may have a significant advantage in treating certain diseases. For example, uPA is capable of enhancing the neuro-protection effects of citicoline in treating focal ischemia [401].

In summary, the physiological and pathological role of uPA has been well established. Overexpression of uPA usually contributes to cancer cell invasiveness that ultimately leads to cancer metastasis. However, the mechanisms of this upregulation in cancer are still poorly understood. As introduced in section 1.1.2., the 5'-CpG island of the human *uPA* gene begins in its non-transcribed 5'-flanking region, and extends to the second intron, in which a high G+C content and a high frequency

of CpG dinucleotides relative to the bulk genome were observed [19]. An earlier study has shown uPA expression is consistent with the methylation status of several methylation sensitive enzyme recognition sites within the uPA promoter [22]. In Chapter II of this thesis, we report that the differential expression of uPA in early and late stage breast cancer is determined by the cytosine DNA methylation of its promoter. In the next section, I will briefly review how DNA methylation regulates gene expression in cancer.

1.7 Transcriptional Regulation of Genes by DNA Methylation

1.7.1 Introduction of DNA methylation

DNA methylation is one of the many processes that can influence gene expression without altering the genetic code. Both adenine and cytosine can be methylated during DNA replication and rearrangement, however, only cytosine methylation occurs in humans [402]. As first reported in 1948, about 3~5% of the cytosine residues in mammalian genomic DNA are present as 5-methylcytosine (5-mC) [403]. The methylation patterns of genes are inheritable. Differential methylation patterns of tissue specific genes are seen in mammals during embryonic development and cancers [22, 404]. A high density of 5-mC residing at the promoter, enhancer, or some particular sequence can dramatically suppress gene transcription [405, 406]. Diverse regions of the human genome have been shown to have cancer-associated methylation alterations as determined by CpG methylation-sensitive restriction endonuclease (e.g., HpaII and HhaI) digestion, followed by Southern blot hybridization [22] (Fig. 1.1. Panel B&C and Fig. 2.1) as well as combined bisulfite restriction analysis (COBRA) [407]. Methylation sensitive PCR (MSP) [408] provides more detailed information and can differentiate methylated cytosines from unmethylated ones.

Cytosine DNA methylation occurs on either hemimethylated (maintenance methylation) or symmetrically unmethylated (*de novo* methylation) CpG dinucleotides (hereafter referred to CpG, where p denotes the phosphate group) (Fig. 1.3). *De novo* methylation is an active process that establishes new methylation patterns of genes during gametogenesis, early post-implantation development and

cancer development. Following DNA replication, maintenance methylation converts hemimethylated DNA to its complete methylated form. Both initiation and maintenance of DNA methylation are catalyzed by a family of enzymes called DNA methyltransferases (DNMTs), which can transfer a methyl group from the methyl donor S-adenosylmethionine (SAM) to the 5 position of the pyrimidine ring of cytosines [409]. While DNMTs promote DNA methylation, another activity conferred by demethylase (DMase) can actively remove the methyl group from the cytosines to restore unmethylated residues required for expression. Although DNA hypomethylation is proposed to be mainly mediated by DMase, the absence of methylation maintenance activity during DNA replication can not be excluded [410-413]. An overview of the working model of DNA methylation is shown in Fig. 1.3.

1.7.2 Regulation of the methylation status in cancer

Lapeyre and Becker's pioneer work demonstrated a link between altered methylated cytosine levels and cancer in 1979 [414]. Derangement in DNA methylation plays roles in the alterations in normal DNA methylation pathways or the activation of DNA demethylation. With technical advances, more and more genes that are critical to cancer biology are shown to have methylation-dependent expression. One such example is uPA, the main theme of this thesis [22].

DNA methylation has both a direct and indirect impact on human carcinogenesis. First, methylation of tumor suppressor genes is of major importance in inactivating these genes in cancer. Second, genome-wide hypomethylation of DNA is detected in various cancers. Third, methylation of cytosines markedly increases the possibility of C to T mutation at some hot spots in both germ line and somatic cells. Fourth, altered expression and activity of DNMTs and DMases have been documented during cancer progression.

1.7.2.1 CpG island hypermethylation

Hypermethylation of genes can suppress their expression in a number of cells. In many cases, they are tumor suppressor genes involved in cancer [415]. *Calcitonin* was the first demonstrated hypermethylated gene that was tumor-associated [416, 417]. Increased methylation of *calcitonin* may be related to the expansion of abnormal blast cell populations in chronic myeloid leukaemia (CML) [418]. Similar

findings were observed in other familial cancer related genes or potential tumor suppressor genes such as $p16^{INK4a}$, E-cadherin (E-cad), Von-Hippel Lindau (VHL), O^6-methyl guanosine methyltransferase (O^6-MGMT), TIMP-3 and $p15^{INK4a}$ [419-422]. Given these reports, a pattern of DNA hypermethylation during cancer formation is emerging. Densely methylated cytosines in the genes that suppress tumorigenesis and progression can suppress their expression, thus favoring the development of cancer. However, genome-wide DNA hypomethylation is more characteristics of the epigenetic changes seen in cancer [423].

1.7.2.2 Genome-wide hypomethylation (or demethylation)

Genome-wide hypomethylation in cancer was first reported in the early 1980's [424]. In primary human tumors, hypomethylation of a number of tumor-promoting genes (e.g. proto-oncogenes and uPA) [22, 425] and some retro-elements (e.g. intracisternal A particles) closely correlate with their high expression levels [426]. CpG islands appear to be protected from dense methylation in these genes, even though flanking regions are heavily methylated. Hypomethylation of these genes is probably due to: 1) methionine auxotrophy, which reduces the levels the methyl group donor, SAM 2) insufficient DNA methylation machinery to maintain the levels of methylated cytosines after DNA replication [427]. 3) An oncogenic stimulus such as Ras may induce aberrant expression and activities of DNA DNMTs and DMases that favor the methylation changes of various genes in tumors [428, 429].

1.7.2.3 Regulation of DNA methylation by DNMTs and DMases

1.7.2.3.1 Discovery of DNMT1 and DMase

Two types of methylation related enzymes, DNMTs (e.g. DNMT1, DNMT2 and a series of DNMT3) [430-432] and DMases have been cloned and identified [410]. The 190 kDa DNMT1 is a maintenance methylase, and has 5~30 fold higher affinity for hemi-methylated DNA as compared to un-methylated DNA [433]. Sequence analysis revealed that this enzyme shares high inter-species homology in human, mice, chickens and sea urchins [434-437]. Ramchandani and co-workers later cloned a CpG methyl-binding domain 2b (MBD2b) that confers demethylase activity, which is the only identified mammalian DMase to date [438, 439].

1.7.2.3.2 Functions of DNMT1

Approximately 80% of cytosines are methylated in CpG sites, and are maintained in a cell- or tissue-specific manner by maintenance DNMT1 despite cell replication. The C-terminal domain of DNMT1 is similar to the catalytic domain of bacterial methylases. The N-terminus contains multiple domains and provides functions that are specialized to eukaryotes, such as nuclei translocation and co-ordination of replication and methylation during S-phase, and are probably required for the maintenance activity of this enzyme [409]. Following semi-conservative replication, two hemimethylated daughter strands derived from the methylated parental DNA are formed. DNMT1 has a marked preference for this hemimethylated DNA [434, 440]. DNMT1 then catalyzes the addition of methyl groups to the newly incorporated unmethylated cytosine in the CpG sites of the daughter stands. Although known as a maintenance methylase, DNMT1 also retains low levels of *de novo* enzyme activity, and catalyzes *de novo* methylation in embryonic stem (ES) cells during gametogenesis and early embryonic development. Overexpression of DNMT1 induces hypermethylation of selected promoters in fibroblast cells [441].

DNMT1 is of great importance in various physiological and pathological processes. ES cells that contain mutated DNMT1 grow normally with severely demethylated genomes, but undergo cell-autonomous apoptosis when induced to differentiate [442]. Overexpression of DNMT1 can transform fibroblast NIH 3T3 cells [443] via Ras-fos signaling pathway [444, 445]. In addition, increased DNMT1 levels have been detected in multiple neoplastic cell types and are cell cycle-dependent [446]. These are probably due to the interactions of DNMT1 with a number of related proteins (p21, p23, annexin V, pRB, MBD3, DMAP1 and histone deacetylases (HDACs)) [449]. However, functional significance of these interactions is still unclear.

1.7.2.3.2.1 Methylation and cell cycle regulation

DNMT1 contains a cysteine rich region at the N-terminus that is capable of binding zinc, and targets the replication foci of DNA in S-phase nuclei, where it is involved in maintenance methylation and histone modification. DNMT1 is expressed constitutively in proliferating cells with higher levels observed in fast growing

tumors, however, significant down-regulation of DNMT1 was observed during cell arrest [447]. DNMT1 interacts with proliferating cell nuclear antigen (PCNA) and interferes with the binding of PCNA to p21 (WAF1). This dissociation of PCNA from p21 initiates DNA replication and cell cycle progression [448]. Interestingly, expression profiles of uPA is identical to the DNMT1, and is cell cycle-dependent [89]. $P16^{INK4a}$ and pRB are classical cell cycle regulators that are controlled by the Cyclin D-pRB pathway. A genetic change or hypermethylation of the promoters of these genes completely abrogates the suppression effect of these genes in tumors [421]. Malfunctions of these cell cycle-related proteins confer the malignant phenotypes of variant tumors.

1.7.2.3.2.2 Methyl CpG binding proteins and chromatin remodeling

DNA is assembled into nucleosomes that contain differentially modified histones. Hypomethylated promoters are generally enriched in hyperacetylated histones and lack histone H1, and correlate with active chromatin structures accessible to TFs [450]. Hypermethylated promoters become enriched in deacetylated core histones by recruiting histone deacetylation machinery [451], and are packed into condensed chromatin structures to prevent the loading of TFs [452]. HDACs (HDAC1 and HDAC2) catalyse these histone modifications through association with DNMT1, and thus directly mediates transcriptional repression of chromatin during DNA replication in an inheritable manner [453, 454]. Recent reports have suggested Methyl-CpG binding proteins (MeCPs) and methylated DNA binding proteins (MBDPs) are also involved in the chromatin remodelling machinery in addition to HDACs [455, 456]. These proteins recognize methylated DNA via a related amino acid motif within CpG methyl binding domain (MBD) [457].

MeCPs (MeCP1 and MeCP2) recognize the symmetrical CpG dinucleotides by a MBD that makes contact with the major groove of the double helix, and function through a transcription repression domain (TRD) that interacts with several other regulatory proteins [458]. MeCPs mediate gene inactivation via the recruitment of MBDPs and their associated HDACs to methylated DNA, and favours local deacetylation of core histone tails [456]. MeCP2 is the best-characterized methyl-CpG binding protein so far. It recruits a co-repressor complex containing the

transcriptional repressor Sin3A and HDAC activity to methylated DNA [459, 460], which can explain why MeCP2 co-localizes with non-randomly distributed 5-mC at pericentromeric satellite DNA on chromosomes 1.9.16 and the long arm of the Y chromosome [461, 462].

MBDPs bind directly to methylated DNA, and sterically block the access of other TFs. A family of MBD-containing proteins was cloned and includes MBD1, MBD2, MBD3 and MBD4. Co-localization of MBD2 and MBD3 with DNMT1 is observed in the late S-phase of cells, which indicates the involvement of a large complex of DNMT1 in the regulation of gene methylation/demethylation and histone modification during DNA replication. Although MBD3 was the only MBDP that does not bind methylated DNA [457], heterodimers of MBD2 and MBD3 were reported to have high affinity for hemimethylated DNA [463]. These observations suggest that the MBD2 and MBD3 complex may be required specifically during the process of DNA demethylation, since a truncated form of MBD2 contains a demethylase activity *in vitro* [456]. An increasing body of evidence links defects in chromatin remodelling machinery to cancer. The resulting co-suppression of tumor suppressor genes by methylation and deacetylation may be due to blocked access of the regulatory factors to DNA. Although the functional significance of these protein-DNA interactions is still unknown, several interesting scenarios have been proposed. MeCPs or MBDPs recruit the deacetylase complexes with DNMTs (or possibly DMase) to the pre-existing methylated DNA, and maintain the epigenetic profiles of chromatin. During cancer development, the binding specificity of these proteins is altered, thus in association with a deacetylase complex, they bind to parasitic elements and different compartments of methylated DNA. In addition, other functional proteins in association with DNMT1 have been identified. An example is DMAP1, which represses transcription of replicate forks as well as copies the methylation patterns of the parental strands. A selective co-localisation of HDACs, DNMT1 and DMAP1 during late S phase may be critical for the maintenance of the epigenetic changes of genomes [454].

Trichostatin A (TSA), a specific inhibitor of cellular HDAC activity, has been shown to reactivate a number of methylated genes. TSA can be used to analyse the

correlation between hypermethylated uPA promoter and its chromatin structure as described in Chapter II. In certain cases, its effects are more dramatic following the treatment of a methylated gene with a low dose of demethylation agent, 5-aza-2'-deoxycytidine (5-azaCdR) [464].

1.7.2.3.3 Other DNMTs

Mice with the homozygous deletion of DNMT1 were embryonic lethal [442]. However, low levels of DNA methylation persist during the passaging of the cultured cells derived from these mice embryos [465, 466], suggesting the existence of other DNMTs. Several DNMTs or proteins with conserved DNMT motifs have been identified, including DNMT2, the DNMT3 family (DNMT3A, DNMT3B and DNMT3L) and double stranded RNA (dsRNA) adenosine deaminase [430, 431, 467, 468].

Disruption of DNMT2 had no obvious effect on global genomic methylation patterns in ES cells or newly integrated retroviral DNA [469, 470]. The biological role of DNMT2, DNMT3L and dsRNA adenosine deaminase in DNA methylation remains to be examined. A significant capacity for *de novo* DNMT enzyme activity is associated with DNMT3a and DNMT3b after embryonic implantation. These enzymes initiate *de novo* methylation of a number of genes, but with much lower activities as compared to DNMT1 [471-473]. DNMT3s also act as maintenance enzymes with an equal preference for either hemimethylated or unmethylated substrates, and may compensate for the maintenance activity of DNMT1 in certain situations [474, 475].

1.7.2.3.4 DMase

Demethylation and transcriptional activation of genes or viral elements were seen in 5-azaCdR treated cells or DNA DNMT knockout embryos [22, 476, 477]. No distinct demethylation process has been identified so far, and may involve a passive or an active mechanism. Passive global demethylation might be associated with the loss of maintenance activity in ES cells through the inhibition of DNMT1, and be replication-dependent [476]. Active removal of the methyl group from full- or hemimethylated CpGs independent of DNA synthesis has been observed [411, 478]. 5-mC N-glycosylase contains both essential protein and RNA components, and

cleaves an N-glycoside bond to release the 5-mC base from the deoxyribose group. This might be the basis of demethylation in pre-implanted chicken embryos, with hemimethylated CpGs as its preferential templates [438, 439]. A potential mammalian DMase, MBD2b, can cleave the methyl moiety directly without altering the phosphodiester backbone, and is responsible for demethylation *in vitro*. Whether this enzyme has *in vivo* functions needs to be clarified. Forced expression of v-Ha-ras in mouse embryonic P19 cells results in genome-wide demethylation that accompanies increased DNA demethylation activity, suggesting that the oncogene *ras* is a potential upstream controller of DNA DMase [428]. In addition, ras also upregulates the expression and enzyme activity of DNMT1 in an adrenocortical tumor cell line [429]. Thus, oncogene initiated cellular transformation may create a methylation balance disorder in a number of genes that provokes tumor progression.

1.7.3 DNA methylation based clinical implications in cancer

1.7.3.1 Methylation modification of the critical genes in breast cancer

Altered methylation status determines the loss or gain of expression of a variety of critical genes in breast cancer, including cell cycle related proteins ($p16K^{INK4a}$), steroid hormone receptors (ER and PR), tumor susceptibility (BRCA1), carcinogen detoxification (GSTP1), cell adhesion (E-cad), proteases (uPA) and their inhibitors (TIMP-3) as shown in Table 1.1 [479-483].

The best example of these breast cancer related genes is ER (Table 1.1, left column), which is methylated in ~50% of unselected primary breast cancers and most ER negative breast cancer cells (e.g. MDA-MB-231). 5-azaCdR and TSA treatment results in a dramatic demethylation and hyperacetylation of this gene and thus restores partial functions of the ER in these cells [481, 484]. Several reports have suggested the induced expression and activity of DNMT1 can directly generate a methylated ER via *de novo* methylation. However, more complex changes may occur to allow these ER negative cells to escape normal cell cycle-dependent controls via DNMT1-mediated dissociation of p21 from PCNA [485]. Interestingly, hypermethylation of the *BRCA1* gene is only found in breast and ovarian cancer [482], which suggests the essential role of sex steroid hormones such as estrogen in the regulation of this gene in cancer.

Overexpression of a number of tumor promoting factors (e.g. breast cancer specific gene 1 (BCSG1) and uPA) in breast cancer have been demonstrated (Table 1.1, right column) [22, 486]. Demethylation of the cis-regulatory elements of these factors alters the binding specificity of a number of transcription activators and repressors, and thus induces the aberrant expression of these genes in breast carcinomas.

1.7.3.2 DNA methylation based therapies in cancer

How global demethylation occurs during carcinogenesis and tumor development remains to be elucidated, especially in view of the high DNA DNMTs levels in tumors [479]. The non-random distribution of CpG island methylation indicates that the selective targeting of some but not all CpG sites by DNMTs and DMase is possible [415].

Many tumor suppressor genes and tumor-related genes are hypermethylated and inactivated in cancer. Methylation changes in a specific gene can be detected in different tumor types [487] or are unique to a specific tumor type [488] at the early stage of tumor progression. They are sometimes even detectable in the pre-malignant stage. Therefore, altered methylation patterns or multiple loci of a gene could serve as a promising biomarker for early tumor detection, monitoring prognosis or establishing a prevention strategy based on cancer risk status [412, 421, 487]. MSP is designed to selectively amplify either methylated or unmethylated DNA sequences using discriminating primers, and is one of the most sensitive and relatively convenient biochemical assays for detecting aberrant methylation of a gene derived from different sources, even paraffin embedded tissues.

Because the role of DNA methylation in cancer progression is not yet fully understood, methylation-based cancer therapies are currently unavailable. Understanding the epigenetic changes occurring in DNA can lead to new approaches to cancer therapy, which might improve cancer treatment outcomes. Present research is still focusing on the use of possible therapeutic regimes to reactivate methylated tumor suppressor genes. HDAC inhibitor (FR091228), antisense DNMT oligos or DNMT inhibitors (e.g. 5-azaCdR) have anti-transformation and anti-tumor effects [22, 445, 489]. 5-azaCdR inhibits DNMTs by forming covalent adducts with DNMTs

that ultimately lead to the damage of DNA [490]. Due to its high cytotoxicity, 5-azaCdR has only been routinely administrated in acute myeloid leukemia and myelodysplasia to induce cellular differentiation rather than demethylation in clinical trials [491, 492]. Therefore, agents with therapeutic properties but with lower toxicity have to be explored. In general, using inhibitors of HDACs such as phenylbutyrate [493] together with a demethylation agent (5-azaCdR or FR091228) may induce the expression of suppressed genes by DNA methylation in cancer. Meanwhile, reduced doses may decrease the toxicity of each chemical. Secondly, researchers should develop small molecules or peptide inhibitors of DNMT as well as DMase to block their expression and/or activities without incorporating into DNA, thus avoiding cytotoxicity. Third, the use of oncogene antagonists to prevent or even reverse the high expression levels and activities of DNMT and DMase, thus preventing a number of disorders related to oncogenic transformation and aberrant DNA methylation [428, 429].

uPA is inducible by a number of oncogenes that are known to play key roles in cellular transformation as well as tumor-related malfunctions [48]. These same oncogenes, such as r*as*, have also been reported to control the expression and enzyme activities of DNMT and DMase, and thus may affect the methylation status of their targeted genes e.g. *uPA* in cancer. Having established the epigenetic changes and the transcriptional regulation of the *uPA* gene during cancer progression, inhibitory approaches including blocking oncogenic signals such as ras and methylation related enzymes (DNMTs and DMases) will become promising therapeutic strategies in invasive cancers.

1.8 Hypothesis for the study

Having determined the critical role of uPA during cancer progression, the strategies aimed at this protease either at the gene expression levels or the functional levels could be useful for cancer diagnosis and treatment. Although several *uPA* gene polymorphisms have been reported, they however, do not result in the overexpression of *uPA* gene. By far, no deletion or translocation of this gene has been observed in cancer, suggesting the likelihood of epigenetic changes. CpG dinucleotides are the

predominant sites for DNA methylation modification. Since uPA contains multiple CpG dinucleotides at its promoter region and extends to the exon 1, indicating that DNA methylation may regulate uPA production at the transcriptional or posttranscriptional stages. However, there is no effective approach available at this moment to interfere this regulation even though if this hypothesis was solid, and leads to the targeting of uPA at the protein level still the major focus. Classical uPA antagonists were mostly synthesized based on its catalytic region to further block its proteolytic activities. In addition, a number of reports also have suggested the critical roles of the connecting peptide region in the uPA associated tumor biology, we then synthesized a number of peptides based on the connecting peptide region of uPA, and tested them in the *in vitro* and *in vivo* model to check whether they have beneficial effects for invasive breast cancer models. Detailed proposal will be included in the preface of each chapter.

1.9 Objective of this thesis

The objectives of this thesis are to investigate the molecular mechanisms underlying the differential regulation of *uPA* gene expression during breast cancer progression, and to study the reversal of breast cancer using a novel peptide inhibitor of uPA. We focus on the following specific goals:

1. Using an *in vitro* breast cancer progression model, we will investigate the mechanisms that determine the overexpression of uPA in late stage breast cancer. Information from this study will further our understanding of the mechanisms that regulate aberrant uPA expression in cancer. Upstream modulators that control uPA expression, once identified, may be potential targets for blocking uPA action.
2. To investigate the effects of the peptide Å6 derived from the non-receptor-binding domain of uPA on breast cancer progression.
3. To investigate the possible synergistic effects of Å6 and tamoxifen on breast cancer progression, and to explore the molecular mechanisms underlying this suppression.

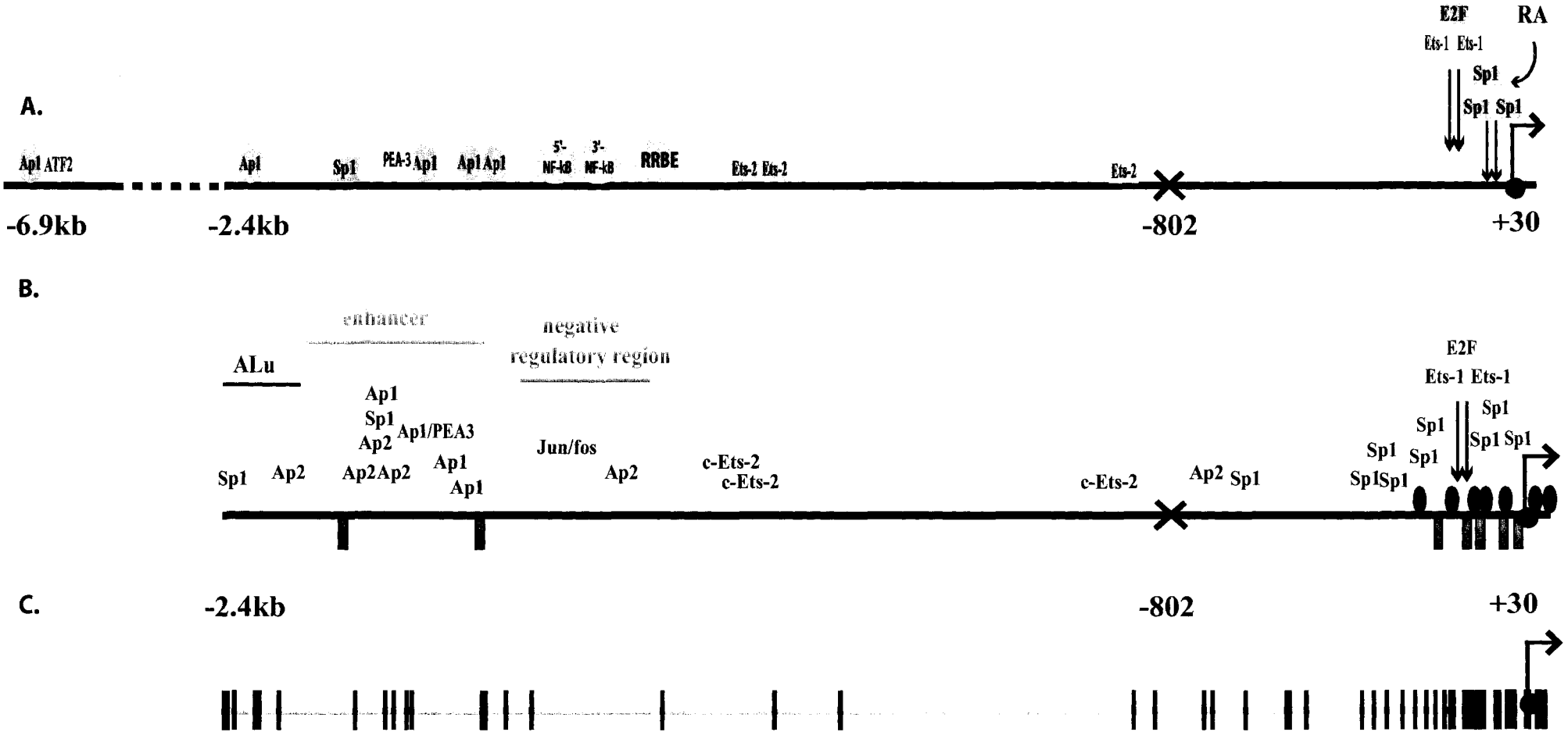
A.
Ap1 ATF2
Ap1
Sp1
PEA-3 Ap1
Ap1 Ap1
5'- NF-kB
3'- NF-kB
RRBE
Ets-2 Ets-2
Ets-2
E2F
Ets-1 Ets-1
RA
Sp1
Sp1 Sp1
-6.9kb
-2.4kb
-802
+30
B.
enhancer
negative regulatory region
ALu
Ap1
Sp1
Ap2
Ap1/PEA3
Sp1
Ap2
Ap2Ap2
Ap1
Ap1
Jun/fos
Ap2
c-Ets-2
c-Ets-2
c-Ets-2
Ap2 Sp1
Sp1
Sp1
Sp1
Sp1Sp1
E2F
Ets-1 Ets-1
Sp1
Sp1 Sp1
C.
-2.4kb
-802
+30
× : promoter starting site
: transcriptional initiation site
| : CpG dinucleotides
● : HpaII recognizing site
■ : HhaI recognizing site

Fig. 1.1. Schematic representation of the 5'-flanking region of the *uPA* gene.

Panel A. Potential transcription factor binding sites within the 5'-flanking region of the *uPA* gene. **Panel B.** Alu repeats, enhancer elements and a negative regulatory region of the *uPA* gene are located approximately -2.0kb upstream of its transcription initiation site (↷) as shown in the top panel. X indicates the uPA promoter start site. Using TESS (Transcription Element Search System) analysis, several transcription factor (e.g. Ets-1) binding sites containing CpG dinucleotides are identified and indicated. Methylation sensitive enzyme [HpaII (ovals) and HhaI (square)] recognition sites are outlined. **Panel C.** Locations of CpG dinucleotides are shown as " | " .

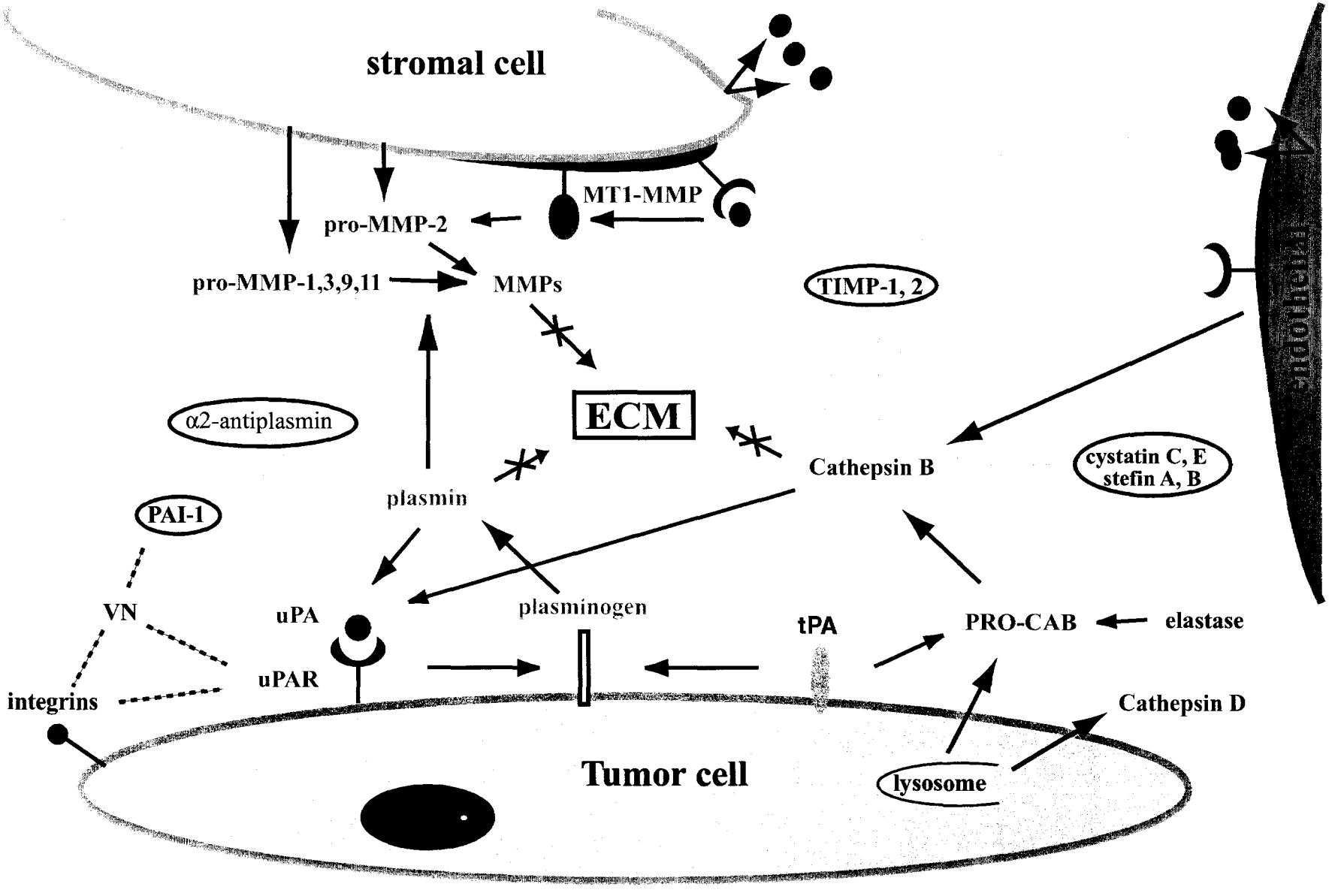
stromal cell
MT1-MMP
pro-MMP-2
pro-MMP-1,3,9,11
MMPs
TIMP-1, 2
α2-antiplasmin
ECM
Cathepsin B
cystatin C, E
stefin A, B
plasmin
PAI-1
VN
uPA
plasminogen
tPA
PRO-CAB
elastase
uPAR
integrins
Cathepsin D
Tumor cell
lysosome

Fig. 1.2. Interactions of different proteases in promoting cancer growth and invasion.

A simplified overview of proteases and their extensive networks of interaction is provided in this figure. Proteases such as uPA (burgundy dots), MMPs and Cathepsins are secreted as inactive pro-forms. Tumor, stromal and tumor-associated endothelial cells with increased and activated proteases are actively involved in cancer metastasis via the degradation of the ECM (X). An important feature of protease systems in cancer is that activation of one protease may affect the activities of others as indicated by the red arrows. This in turn promotes the malignancy of these tumor cells. Cathepsin B is one of the key players in promoting tumor metastasis following its activation by elastase and tPA. uPA is activated by proteases such as biologically active Cathepsin B and plasmin following binding to its receptor, which then activates an extracellular proteolytic cascade by converting plasminogen to biologically active plasmin. Plasmin is critical for the degradation of the ECM and can subsequently activate a number of pro-MMPs (e.g. pro-MMP-1, 3, 9 and 11). Indirect activation of pro-MMP-2 by uPA is through the catalyzing of MT1-MMP. Furthermore, endogenous inhibitors of these proteases (e.g. TIMP-1,2, Cystatin C & E, Stefin A& B, PAI-1 and α_2-antiplasmin, shown inside the blue oval circle) can block proteolytic activities as marked by the yellow lines (e.g. PAI-1 for the uPA family, α2-antiplasmin for plasmin, TIMPs for MMPs and cystatins as well as stefins for the Cathepsin family).

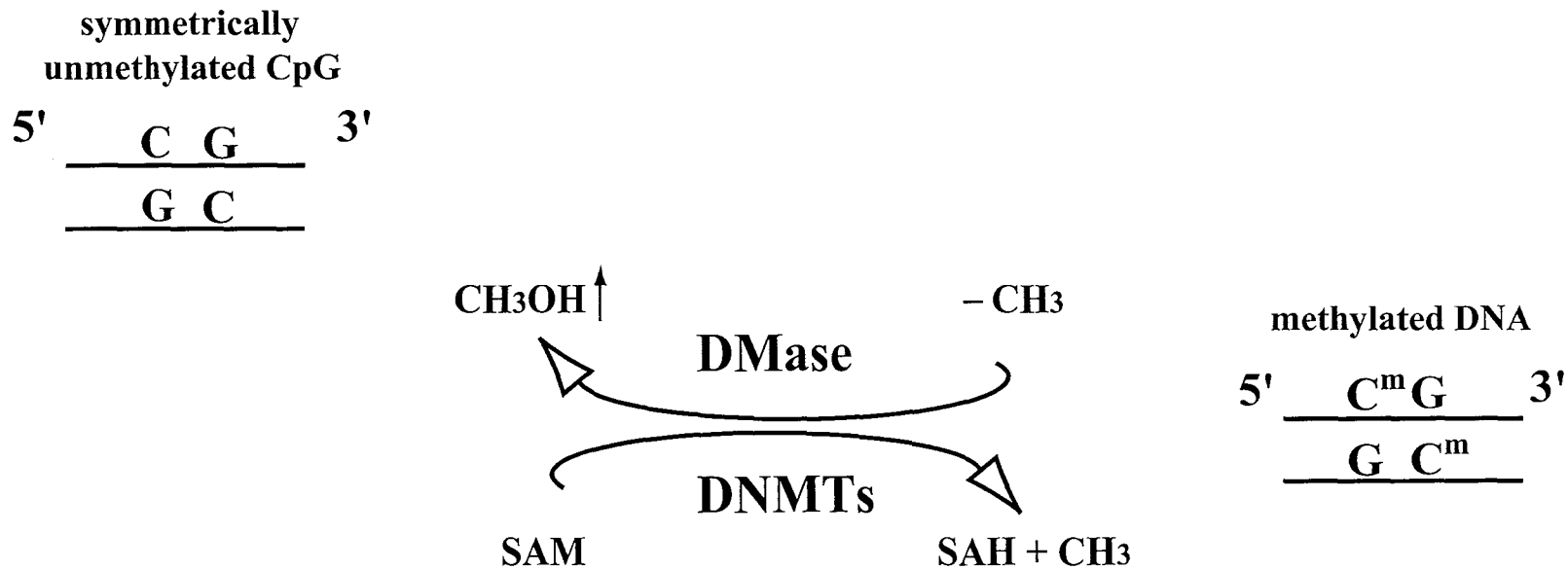
symmetrically
unmethylated CpG
5'
C G
3'
G C
CH3OH
– CH3
DMase
DNMTs
SAM
SAH + CH3
methylated DNA
5'
CmG
3'
G Cm
hemimethylated DNA
5'
CmG
3'
G C

Fig. 1.3. The dynamics of DNA methylation patterns.

DNA methylation patterns are generated via dynamic processes of methylation and demethylation. DNMTs catalyze cytosine methylation (C^m) within both unmethylated and hemimethylated DNA by transferring methyl groups from the methyl donor SAM to unmethylated cytosines. The reverse reactions are catalyzed by DMase by actively remove the methyl groups from C^m, resulting in the release of a volatile methyl residue (methanol).

Table 1.1 Genes with altered DNA methylation patterns in human tumors

Gene activated by hypomethylation	Gene silenced by hypermethylation
BCSG1	***WT1***
uPA	***GSTP1***
pS2	***ER***
Ras	***PR***
Fos	***BRCA-1***
Myc	***E-cadherin***
Xmrk	***RAR-β***
IGF-2	***14-3-3s***
Growth hormone	***TIMP-3***
Thyroid hormone	***APC***
Gonadatropin	***p16 INK4A***
γ-globin	*p15 INK4A*
γ-crystallin	*TSP-1*
a-chorionic	*p53*
Ornithine decarboxylase	*p73*
MAGE-1	*ARF/INK4A*
LIM-HOX	*VHL*
PTH-rP	*pRB*
	O^6-MGMT
	AR
	Calcitonin
	hMLH2

Genes in bold: breast cancer related genes with altered methylation patterns

Table 1.1. Genes with altered DNA methylation patterns in human tumors.

Altered methylation status of a variety of genes determines the up- or downregulation of their expressions in tumors. Genes activated by DNA hypomethylation are listed in the left column, whereas inactivated genes due to DNA hypermethylation are listed in the right column. Genes in bold are known to be associated with breast cancers.

Chapter II

Differential regulation of the *urokinase* (*uPA*) gene expression by DNA methylation in human breast cancer cells.

2.1 Preface

uPA plays an important role in promoting breast cancer progression [4, 10, 96, 274]. However, the molecular mechanisms underlying its upregulation in late stage breast cancer are not fully understood. Multiple CpG dinucleotides in the uPA promoter suggest that its transcription may be regulated by the methylation status of these cytosines. This hypothesis was supported by reports from Xing and co-workers in our laboratory, which for the first time, showed evidence for the role of DNA methylation in the regulation of uPA expression [22]. However, detailed information hasn't been provided. In addition, a direct regulation model has not been established. The aim of this study is therefore to investigate this regulation.

In this chapter, I determined the methylation status of the uPA promoter in cells derived from two breast cancer cell lines, MCF-7 and MDA-MB-231, representing early and late stage breast cancer respectively. The growth of MCF-7 cells is under the control of estrogen. These cells are barely invasive and express undetectable levels of uPA mRNA [22]. MDA-MB-231 cells represent ER-independent late stage breast cancer. They express high levels of uPA mRNA and are highly invasive [22]. As discussed in this chapter, CpG dinucleotides are heavily methylated in MCF-7 cells, whereas in MDA-MB-231 cells, hypomethylation was noted. Using methylation specific PCR and sequencing, the methylation status of individual cytosines in the uPA promoter was determined. Interestingly, epigenetic changes of the uPA promoter due to DNA methylation may affect its transcription and the accessibility of transcription factors (TFs) that are essential for its expression, e.g. Ets-1. In this study, the role of two novel regulators of DNA methylation, DNMTs and DMase, were explored.

This work advances our understanding of the differential expression of uPA in breast cancers since: 1) it determines the methylation status of the individual cytosines within CpG in the uPA promoter during breast cancer progression; 2) provides the first direct evidence that the regulation of uPA can be altered by DNA methylation status; 3) discusses the potential roles of DNMT and DMase in this regulation.

This chapter has been written as a manuscript entitled "Differential regulation of the *urokinase* (*uPA*) gene expression by DNA methylation in human breast cancer cells.", which is in pending revision in *Journal of Biological Chemistry*.

2.2 Abstract

Urokinase-Type Plasminogen Activator is a member of the serine protease family, and can break down various components of the extracellular matrix to promote invasion, growth and metastasis of several malignancies including breast cancer. In the current study we examined the role that the DNA methylation machinery might be playing in regulating differential uPA gene expression in breast cancer cell lines. uPA mRNA is expressed in the highly invasive, hormone insensitive human breast cancer cell line MDA-MB-231, but not in hormone responsive cell line MCF-7. We show using methylation sensitive PCR that 90% of CpG dinucleotides in the uPA promoter are methylated in MCF-7 cells, whereas fully demethylated CpGs were detected in MDA-MB-231 cells. uPA promoter activity, which is directly regulated by the Ets-1 transcription factor, is inhibited by methylation as determined by uPA promoter-luciferase reporter assays. We then test whether the state of expression and methylation of the uPA promoter correlates with the global level of DNA methyltransferase and demethylase activities in the respective cell lines. We show that maintenance DNA methyltransferase activity is significantly higher in MCF-7 cells than in MDA-MB-231 cells, whereas demethylase activity is higher in MDA-MB-231. We suggest that the combination of increased DNA methyltransferase activity with reduced demethylase activity contributes to the methylation and silencing of uPA expression in MCF-7 cells. The converse is true in MDA-MB-231 cells, which represents a late stage highly invasive breast cancer. The histone deacetylase inhibitor, Trichostatin A induces the expression of the uPA gene in MDA-MB-231 cells but not in MCF-7 cells. This supports the hypothesis that DNA methylation is the dominant mechanism involved in the silencing of uPA gene expression. Taken together, these results provide insight into the mechanism regulating the transcription of the uPA gene in the complex multi-step process of breast cancer progression.

2.3 Introduction

The breakdown of the extracellular matrix (ECM) involves a variety of growth factors and proteases, and is an important step in the process of tumor invasion and metastasis [4, 494]. Urokinase type plasminogen activator (uPA) and its cell surface glycophosphotidyl inositol (GPI)-linked receptor (uPAR) play important roles in several malignancies. uPA produced by tumor cells and the surrounding stroma is intimately involved in tumor cell invasion, migration and proliferation. The uPAR localizes the proteolytic effects of uPA within the tumor cell environment. Additionally, uPA enhances neovascularization of tumors thus further contributing to the process of tumor progression [4]. Numerous data demonstrate the causal role of uPA in tumor growth and metastasis. First, expression of uPA or uPAR was shown to enhance tumor growth and metastasis [8, 299]. Second, increased uPA gene expression in various malignancies including breast cancer is closely related with disease stage [4, 320]. Third, inoculation of human breast cancer cells in mice lacking the uPA gene results in tumors of significantly smaller volume than tumors implanted in wild type mice [8]. Fourth, we have previously demonstrated that a peptide derived from the non-receptor binding domain of uPA (Å6) decreases breast cancer invasion, growth and metastasis due to its pro-apoptotic and anti-angiogenic effects [495]. Fifth, the active site inhibitor of uPA (B-428) alone or in combination with the anti-estrogen tamoxifen blocks the growth and metastasis of prostate and breast cancers [11, 344]. Sixth, antibodies directed against uPA or uPAR are able to decrease tumor growth [65, 354, 355].

DNA methylation marks inactive genes and can suppress gene expression either directly by interfering with the binding of transcription factors, or by attracting methylated DNA binding factors that recruit histone deacetylases and precipitate an inactive chromatin structure [454, 496]. Aberrant DNA methylation patterns are commonly observed in cancer [424]. Neoplastic cells have the ability to simultaneously harbor widespread hypomethylation and regional hypermethylation that contribute to tumor progression [424, 496]. Whereas hypermethylation and silencing of tumor suppressor genes has attracted much attention recently, the molecular mechanisms underlying hypomethylation of tumor progression factors such

as RAS, MYC, HOX11 and XMRK that are upregulated during cancer development are poorly described [412, 474, 496], suggesting hypomethylation might also play an important role in regulating gene expression during tumorigenesis similar to that of hypermethylation [425, 426, 497, 498].

In the current study, we tested the hypothesis that the expression of uPA, a well-defined marker of highly invasive tumor cells and activated at the late stages of breast cancer, can be regulated via the changes in the methylation status of its promoter region. As a follow-up to our previous studies where we demonstrated that the uPA gene is transcriptionally suppressed by DNA methylation [22], we have now focused on the examination of the role of DNA demethylation and DNA methylating enzymes such as DNA methyltransferase (DNMT) and demethylase (DMase) in regulating uPA expression during breast cancer progression.

2.4 Materials and Methods

Cells and cell culture

All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The human breast adenocarcinoma cell line MDA-MB-231 was maintained in L-15 medium (Gibco BRL, Life Technology, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml of penicillin-streptomycin sulphate and 0.2% gentamycin. The human breast cancer cell line MCF-7 was maintained in culture in MEM medium (Gibco BRL) supplemented with 10% FBS, 2 mM L-glutamine, 25 mM 4-(2-hydroxyethy)-1-piperazineethanesulfonic acid, 26 mM sodium bicarbonate, 100 units/ml of penicillin-streptomycin sulphate (Gibco BRL), 0.2% gentamycin (Sigma Chemicals, St. Louis, MO, USA) and 0.01 mg/ml bovine insulin. Cells were incubated at 37°C in 5% CO_2.

Northern Blot analysis

MCF-7 cells were treated with or without 5-aza-2'-deoxycytidine (5-azaCdR) (Sigma Chemicals, St. Louis, MO, USA) at a concentration of 50ng/ml for 3 days and were cultured in the absence of the drug for additional 24 hrs. MDA-MB-231 and MCF-7 cells were treated with Trichostatin A (TSA) (Sigma Chemicals) at a concentration of 100ng/ml for 2 days. RNA from these cells was isolated using the

TRIZOL method following the manufacturer's instructions (Gibco BRL, Burlington, ON, Canada), and was fractionated on a 1.5% agarose gel in MOPS buffer and transferred onto nylon filters (Nytran, Amersham, Oakville, ON, Canada). The filters were hybridized with a ^{32}P-labelled human uPA cDNA at 65°C for 24hrs, which were then stripped of the probe and re-hybridized with an 18S rRNA probe to normalize for the amount of RNA loaded in each lane. Autoradiography of the filters was carried out at -80°C using XAR film (Eastern Kodak Co., Rochester, NY, USA). The intensity of hybridization to uPA mRNA and 18S rRNA was quantified by densitometric scanning. The normalized uPA mRNA expression was determined by dividing the intensity of the uPA mRNA signal by the intensity of the 18s signal per each lane.

Boyden chamber matrigel invasion assay

The invasive capacity of MDA-MB-231, MCF-7 and MCF-7 cells treated with 50ng/ml 5-azaCdR was determined utilizing two compartment Boyden chamber Matrigel invasion assay as previously described (Transwell, Costar, USA) [11, 495]. The 8 μm pore polycarbonate filters at the bottom of the upper chambers were coated with basement membrane Matrigel (50 μg/filter). 5 x 10^4 cells treated with or without 5-azaCdR in 0.1 ml of medium were added to the upper chamber, and placed on top of a lower chamber pre-filled with 0.8 ml of serum-free medium supplemented with 25 μg/ml fibronectin (Sigma, Oakville, ON, Canada), and then incubated at 37°C for 24 hrs. After the incubation, medium was removed, and the polycarbonate filters with invaded cells were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde (Sigma) in 0.1M phosphate buffer, pH 7.4 at room temperature for 30 min, and were stained with 1.5% Toluidine blue, which were then removed and mounted onto glass slides. Number of cell invaded was examined under a light microscope. Ten fields under 400 X magnification were randomly selected and the mean cell number was calculated.

Southern Blot Analysis

Cellular genomic DNA was isolated using DNAZOL (Gibco BRL) for analyzing the methylation status of the CpG island of the uPA promoter by methylation-sensitive restriction enzyme digestion and Southern blotting. Briefly, 10 μg of genomic DNA was extracted from MDA-MB-231, MCF-7 and MCF-7 treated

with 50ng/ml 5-azaCdR as recommended by the manufacturer. Extracted DNA was first digested with EcoRI or Hind III and was further digested with Pst I, Pst I/HpaII or PstI/HhaI (8 units/μg of total DNA) respectively for 18h at 37°C. The digests were fractionated on a 0.8% agarose gel and transferred to a nylon membrane (Nytran, Amersham) by capillary blotting. The filters were hybridized with a ^{32}P-labelled 778 bp long SmaI-AvrII fragment of the uPA promoter derived from the uPA-Cat construct (gift from Dr. F. Blasi, Milan, Italy) at 42°C for 24hrs. They were then successively washed in 1X SSC (150mM NaCl, 50 mM sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS) once for 15 min at room temperature, 0.5 X SSC, 0.58% SDS once for 15 min, 0.1 X SSC, 0.1% SDS twice for 15 min at room temperature, and at last once for 30 min at 55°C. Autoradiography of the filters was carried out at -80°C using XAR film (Eastern Kodak Co.).

Methylation specific PCR

Genomic DNA (20 μg) from MDA-MB-231 and MCF-7 cells was digested with restriction enzyme DraI, and denatured in 0.3 M NaOH for 20 min at 37°C in a volume of 100 μl. The purified genomic DNA (2 μg) was treated with sodium bisulfite (2.2M $Na_2S_2O_5$ and 1mM hydroquinone (pH 5.0) at 55°C for 14 hrs, which was then purified using a Qiagen PCR purification column, followed by an additional denaturation in 0.3 M NaOH for 20 min at 37°C, and neutralization with 3M ammonium acetate (pH 7.0). After ethanol purification, an aliquot of treated DNA was amplified with modified primers (5'-outer: (-532) ~TATAGAGGGAGTTTTT ATAGG~(-512); 3'-outer: (+287)~ATAACCAAACTCCCCAACTA~(+306); 5'-inner: (-421)~ TTTATAGTTTTATTTAGTTG ~(-402); 3'-inner: (+35)~ ACAAAAA CAAATAAACCCTA~(+54)) for 35 cycles at the amplification condition that consists of 1-min denaturation at 95°C, 45s-annealing at 56°C (outer primers)/or 58°C (inner primers) followed by 1-min extension at 72°C. A 10-min extension step at 72°C was added at the end of the PCR cycles. All reactions were carried out in 100 μl total reaction buffer containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 20 pmol primers, and 2.5 units Taq DNA polymerase (MBI, Flamborough, ON, Canada) with GeneAmp PCR system 9600 (Perkin Elmer Inc., Shelton, Connecticut). The PCR products were run on a 1.2% agarose gel, and was extracted and purified

from the gel. They were then subcloned into Topo-PCR TA cloning vectors according to the manufacturer's instruction (Clonetics) for sequencing (S&T Biolab, Montreal, QC, Canada).

Luciferase Reporter Assay

The uPA promoter region (-745 ~ +30) was cut from uPA-Cat reporter vector (gift from Dr. F Blasi, Milan, Italy) at AvrII and SmaI sites, and then inserted into a luciferase reporter vector pGL-3 basic (Promega, Madison, WI, USA) digested with NheI and SmaI to generate uPA-luc plasmid. Unmethylated uPA promoter construct was obtained by treating uPA-luc plasmid with the methyl donor S-adenosylmethionine (SAM) and in the absence of methyltransferases. The uPA-luc plasmid was methylated at different CpG sites *in vitro* with bacterial CpG methyltransferases (mSssI, mHpaII and mHhaI) and using the methyl donor as recommended by the manufacturer (New England Biolabs, Mississauga, ON, Canada). Complete methylation was confirmed by resistance to HpaII and HhaI restriction enzymes. PGL-3 basic was used as a negative control. The different treated plasmids were transiently transfected into MDA-MB-231 cells using Lipofectamine as a carrier according to the manufacturer's protocol (Gibco BRL, Burlington, ON, Canada). pEVRF0 or pEVRF-Ets-1 were gifts form Dr Graves B.J at University of Utah School of Medicine, Salt Lake City, UT, USA. PSV-β-gal (Promega, Madison, WI, USA) containing the β-galactosidase gene under the control of the constitutively active SV40 promoter and enhancer was co-transfected with the different plasmids in each transfection experiment at a concentration of 0.5 μg/sample to normalize for transfection efficiency. 48 hrs after transfection, cells were scraped in 1X reporter lysis buffer (Promega) followed by 14,000rpm centrifugation. Luciferase activities in the supernatants were then analyzed by mixing 45 μl cell lysate, 5 μl luciferase assay reagent (30 mM ATP, 150 mM KH_2PO_4, 300 mM $MgCl_2$) and 100 μl of substrate luciferin (250 μM) for 20s in a luminometer (Monolight 2010). For β-galactosidase activity, 50 μl lysates were mixed with 200 μl of β-galactosidase assay buffer (24 μM Na_2HPO_4, 16 μM NaH_2PO_4, 4 μM KCl, 400 nM $MgCl_2$, 20 μM β-mercaptoethanal and 0.3 mg ONPG per sample) in a 96 well plate and incubated overnight at 37°C. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_2 and the absorbance was

measured at 420 nm in a V_{max} plate reader (Molecular Devices). Activity was determined by comparison to a standard curve. Luciferase reporter activity in relative luminescence units was normalized to β-galactosidase activity as described [499].

DNA methyltransferase activity assay

A total nuclear extract was obtained by lysing cells in 500 µl of buffer A (10 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 5 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5% NP40) followed by centrifugation at 2000 rpm for 10 min. The precipitated nuclear pellets were resuspended gently in 30 µl buffer B (20 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM DTT, 0.4 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF) and centrifuged at 13,000 rpm for 30 min. 5 µg of the nuclear extract obtained were incubated with the methyl donor ^{3}H-SAM (S-adenosyl-L-[methyl-^{3}H] methionine) and hemimethylated double stranded oligos (poly (mdC-dG; dC-dG), Sheldon biotechnology center, Montreal, QC, Canada) to measure maintenance methylase activity, or unmethylated double stranded oligos (poly (dI-dC: dI-dC), Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Canada) to measure *de novo* methylase activity. After incubating for 3 hrs at 37°C, the level of incorporation of methyl groups into DNA was determined by 10%TCA precipitation and filtration through GF/C filters (Whatman Ltd, Maidston, England), followed by liquid scintillation counting. Reaction mixtures containing only substrate oligos but no nuclear extract were used as negative controls. Total radioactivity retained by GF/C filters from reaction mixtures of respective cell lines in the absence of substrate oligos but with nuclear extract was subtracted to exclude DNA-independent methylation activities in the extracts. Results are expressed as the mean disintegration/min of ^{3}H-CH_3 incorporated into substrate oligonucleotides per microgram of nuclear protein ± SD of triplicate cell extracts. Each nuclear extract was assayed in triplicate.

DNA demethylase activity assay

Total nuclear extracts (6 mg) obtained from MDA-MB-231 and MCF-7 cells were loaded onto DEAE-Sephadex A 50 columns. Following elution with a continuously increasing gradient salt buffer (from 0.2N-5N NaCl), eluted fractions (500 µl X 10) from the total nuclear extract were collected. A 20 µl sample of each eluted fraction was incubated with ^{3}H-methyl-DNA substrate in an open microfuge

placed in a sealed scintillation vial containing 2 ml of scintillation cocktail for two nights at 37°C. Demethylation of DNA results in release of the methyl moiety as a volatile methyl residue (methanol) that is trapped in the scintillation cocktail. To determine the level of released methyl groups, the vials were counted in a scintillation counter. Results are expressed as the mean disintegration per minute per sample ± SD per the peak fraction of each of the cell extracts, which were assayed in triplicate.

2.5 Results

Evaluation of uPA mRNA expression and its effect on tumor cell invasion

To test the hypothesis that uPA might play a role in breast cancer cell invasion, we first examined the expression of uPA mRNA in the estrogen receptor (ER) positive, hormone sensitive human breast cancer cell line MCF-7, and in highly invasive ER negative hormone insensitive MDA-MB-231 cells using Northern blot analysis. As seen in Fig. 2.1A, uPA mRNA is only detected in MDA-MB-231 cells. In order to examine the effect of uPA on the invasive capacity of these cells, we carried out a modified Boyden chamber Matrigel invasion assay. These studies show that MDA-MB-231 cells expressing high levels of uPA are able to invade through Matrigel whereas MCF-7 cells that do not express uPA are unable to invade (Fig. 2.1B). These results are consistent with previous studies by us and others showing that uPA expression is directly related to the tumor cell's invasive capacity [500].

Analysis of *uPA* gene methylation in breast cancer

We then determined whether this switch in expression of uPA is epigenetically controlled. The change in uPA gene methylation status was examined as a potential molecular mechanism regulating the differential expression of uPA in tumor cells representing early (MCF-7) and late (MDA-MB-231) stage human breast cancer. Genomic DNA was isolated from these cell lines and digested first with the methylation insensitive endonuclease PstI (Fig. 1C, lane a) and then with the methylation sensitive (HpaII, HhaI) endonucleases (Fig. 1C, lanes b & c). These samples were subjected to a Southern blot analysis and hybridized with a probe recognizing the uPA promoter region. Since this region of the gene bears multiple

CCGG and CGCG sites, it can be extensively cleaved by HpaII and HhaI restriction enzymes when it is unmethylated, as is the case with DNA prepared from MDA-MB-231 cells expressing abundant amounts of uPA (Fig. 2.1C, b1 & c1). In contrast, in MCF-7 cells, both HpaII and HhaI failed to cleave this region resulting in an identical pattern to the one observed after PstI digestion alone (Fig. 2.1C, b2 & c2). In order to further confirm that this differential digestion with HpaII and HhaI is due to DNA methylation, MCF-7 cells were treated with the DNA methylation inhibitor 5-azaCdR and subjected to a similar Southern blot analysis. The pattern of cleavage of the uPA promoter was identical to the one observed in MDA-MB-231 cells (Fig. 2.1C, b3 & c3). A Matrigel invasion assay showed that following 5-azaCdR treatment of MCF-7 cells, there was an increase in the number of tumor cells invading through Matrigel (Fig. 2.1B). This increase in tumor cell invasive capacity following demethylation correlated with induction of uPA mRNA (unpublished observations).

Methylation status of CpG dinucleotides within the uPA promoter region as determined by methylation sensitive PCR

Complete characterization of methylation status of the uPA promoter region in MCF-7 and MDA-MB-231 cells was carried out by methylation specific PCR (MSP). For these studies we selected PCR primers that could amplify a 558 bp DNA fragment of the promoter and part of exon 1 within the uPA gene that are rich in CpG dinucleotides (Fig. 2.2A), and can serve as sites for methylation modification. The analysis of this region was also carried out by TESS (Transcription Element Search System) DNA analysis program showing the presence of several important DNA transcription factor binding sites in this region, which may play a significant role in regulating uPA expression (Fig. 2.2A). MSP analysis of DNA from MDA-MB-231 and MCF-7 cells revealed that greater than 90% cytosines of CpG dinecleotides are methylated in MCF-7 cells which do not express uPA. In contrast, in uPA expressing MDA-MB-231 cells, the uPA promoter was overall unmethylated (Fig. 2.2B). These results are in agreement with methylation of the uPA gene in MCF-7 cells resulting in silencing of uPA gene expression.

Analysis of the impact of methylation on *uPA* gene promoter activity in breast cancer cells

Following our establishment of a correlation between promoter methylation and uPA gene transcription, we examined the effect of changes in promoter methylation on gene transcription. For these studies, the uPA promoter reporter construct (-745 ~ + 30 bp) uPA-luc was methylated by either mHpaII , mHhaI or mSssI methyltransferases or mock treated *in vitro*, and was transiently transfected into the human breast cancer cells MDA-MB-231. Luciferase reporter activity was determined 48 hrs after transfection. As a control we used a promoter-less luciferase construct pGL-3 basic. The mock treated unmethylated uPA-luc plasmid exhibited at least 6 fold higher luciferase activity as compared to cells transfected with the promoter-less control plasmid as expected. Methylation of the different plasmids with different methyltransferases reduced the promoter activity significantly (Fig. 2.3A).

Transcription factor Ets-1 activates uPA promoter and induces its gene expression. As shown in Fig. 2.3B, Ets-1 binding site coincides with methylation sites and is therefore a candidate to be affected by DNA methylation. We then determined whether the inducible effect of Ets-1 could be blocked by methylation. Methylated or unmethylated uPA-luc was co-transfected with either pEVRF0 (empty vector for pEVRF-Ets-1, lane 3) or pEVRF-Ets-1 (lane 4) encoding Ets-1 as shown in Fig. 2.3B. Although pEVRF-Ets-1 activates unmethylated uPA-luc more than 5 fold, it fails to stimulate the transcription activity of all methylated uPA-luc plamides (Fig. 2.3B). This marked suppression is consistent with methylation regulating the uPA promoter. Although there are several methylated cytosines in regions surrounding Sp1 binding sequences, transfection of Sp1 plasmid failed to cause any significant change in the uPA-mediated luciferase activity, as was observed following Ets-1 plasmid transfection (unpublished observations).

Maintenance and *de novo* methyltransferase (DNMT) enzyme activities in human breast cancer cells

We then examined whether differences in the state of methylation of the uPA promoter between MDA-MB-231 and MCF-7 cells reflect a global change in the DNA methylation machinery. We first examined the levels of *de novo* and maintenance DNA DNMT activities in nuclear extracts prepared from the two cell lines by quantitation of the total amount of ^{3}H-methyl group that has been catalyzed

by nuclear extracts prepared from these cells onto either hemimethylated (maintenance DNMT activity) or unmethylated DNA substrates (*de novo* DNMT activity). As shown in Fig. 2.4A, the overall maintenance DNA DNMT activity observed was 3 fold higher in MCF-7 cells (lane 5) which bear a methylated uPA promoter as compared with MDA-MB-231 cells which bear an unmethylated uPA promoter (lane 3). In contrast, as shown in Fig. 2.4B, endogenous *de novo* DNMT activity was found to be significantly higher in MDA-MB-231 cells (lane 3) as compared to MCF-7 cells (lane 5). Thus, the maintenance but not the *de novo* DNMT activity correlates with the state of methylation of the uPA gene.

Determination of demethylase activity in human breast cancer cells

We then determined whether there are differences in global DNA DMase activity in MCF-7 cells in comparison with MDA-MB-231 cells. It has been previously suggested that tumor cells bear high levels of DMase activity that might be responsible in part for the hypomethylation observed in these cells. Active removal of the $^{3}H\text{-}CH_3$ moiety from methylated cytosines in *in vitro* methylated DNA has been previously shown to result in a release of a volatile residue that was identified as methanol. Using a volatile assay we quantitated DMase activity in these breast cancer cells. DMase activity was 3~4 fold higher in MDA-MB-231 cells, in which the uPA promoter is hypomethylated as compared to MCF-7 cells where the promoter is methylated (Fig. 2.5). Thus, global DMase activity correlates with the state of methylation of the uPA promoter in breast cancer cells.

TSA treatment increases uPA expression in MDA-MB-231 but not in MCF-7 cells

Gene silencing is frequently associated with DNA methylation and histone deacetylation, whereas gene expression is associated with DNA demethylation and histone acetylation. Together, these two regulatory mechanisms play a critical role in the regulation of gene transcription during tissue development, tumor transformation and tumor progression. It has been shown that DNMT1, and several methyl-CpG binding proteins such as MeCP2, MBD2, and MBD3 are associated with histone deacetylases that are intimately involved in gene silencing [456, 461, 501]. These observations have provided key links between DNA methylation (demethylation) and

histone deacetylation (acetylation). TSA is a chemical deacetylase inhibitor that is well described to induce DNA acetylation resulting in gene activation. In order to examine the role of histone acetylation in uPA gene transcription, MDA-MB-231 and MCF-7 cells were treated with TSA for 2 days followed by determination of uPA mRNA expression by Northern blot analysis. These studies showed that uPA mRNA was markedly increased following treatment of MDA-MB-231 cells with TSA. In contrast, MCF-7 cells treated with TSA failed to exhibit any significant change in uPA mRNA expression. uPA mRNA continued to be undetectable in these breast cancer cells which are merely invasive and show hypermethylation of the uPA promoter (Fig. 2.6). These results demonstrate that DNA methylation and not histone deacetylation is the dominant mechanism suppressing uPA expression in MCF-7 cells.

2.6 Discussion

uPA is now believed to play an important role in several cancers where increased uPA production is associated with late stages of cancer [4, 6]. It is therefore important to understand the mechanisms responsible for regulation of uPA expression during tumor progression. In this paper, we tested the hypothesis that DNA methylation is involved in the differential regulation of uPA during tumor progression. We used two breast cancer lines in this study. MCF-7 is a low invasive cell line, and represents early stage human breast cancer. Animals inoculated with MDA-MB-231, a hormone insensitive cell line, develop large tumors that can metastasize to several sites *in vivo* [495]. Thus the highly invasive MDA-MB-231 cell line represents late stage breast cancer. uPA expression correlates with tumor cell invasion and provides a link between uPA expression and tumor stage [297, 320]. Using this model we examined whether the methylation state of the uPA gene plays a role in regulating the differential expression this gene through tumor progression. Our data shows that the methylation status of the uPA promoter correlates with its state of expression as demonstrated by methylation sensitive endonuclease Southern blot analysis and sodium bisulfate mapping. DNA methylation plays a causal role in controlling the uPA expression since the DNMT inhibitor 5-azaCdR induces the

expression of uPA in MCF-7 cells and increases their invasive capacity. Further support for the causal role of DNA methylation in regulating uPA gene expression is drawn from experiments showing that *in vitro* methylation inhibits uPA promoter activity and its transactivation by the transcription factor Ets-1. Ets-1 is required for the expression of uPA in a number of tissues [79, 80, 82] and interacts with sequences that close to CpG dinucleotide as shown in Fig. 2.2. Methylation might inhibit uPA promoter activity by either inhibiting the interaction of Ets-1 with methylated CpGs in its recognition sequence or by recruiting methylated DNA binding proteins that precipitate an inactive chromatin structure [21, 454, 502]. Further experiments are required to determine the exact mechanism involved.

Aberrations in the DNA methylation machinery are well documented in cancer that exhibits global hypomethylation and regional hypermethylation. It has been previously suggested that induction of both DNMT and DMase activities might play a role in the complex changes in DNA methylation observed in cancer cells [474]. However, most of the attention in the field has been however directed to the hypermethylation of tumor suppressor genes in cancer cells and its potential role in tumorigenesis [479]. A large number of studies have demonstrated that inhibition of DNMT reverses tumor growth and antisense DNMT1 inhibitors are currently in clinical trials [445, 503, 504]. Our data suggests however that the involvement of methylation in tumorigenesis is more complex and that hypomethylation of certain genes might play a critical role in tumor progression as well. Therefore, activities that are responsible for demethylating genes required for tumor invasion might be important anticancer targets.

Our data is consistent with a model in which DNMT and DMase activities are differentially expressed, and play distinct roles at different stages of tumor progression (Fig. 2.7). Early stage cancer cells such as MCF-7 show higher maintenance DNMT activity that is possibly required for maintaining the transformed state and involved in the silencing of tumor suppressor genes. However, later in tumor progression, increased DMase activity is vital to induce the expression of genes that are silenced by DNA methylation but are critical for tumor invasion such as uPA. In accordance with this hypothesis we show that in later stage MDA-MB-231 cells,

maintenance DNMT activity is reduced whereas DMase activity is increased. It has previously been shown that ectopic expression of the ras oncogene can lead to increased DMase activity and an active DMase was recently purified from human lung carcinoma cells. It is not clear yet which is the specific DMase induced in MCF-7 cells, and further experiments are required to characterize this DMase. Nevertheless our results demonstrate that DNA methylation activities do undergo distinct changes during tumor progression and might play different roles at specific stages.

2.7 Acknowledgements

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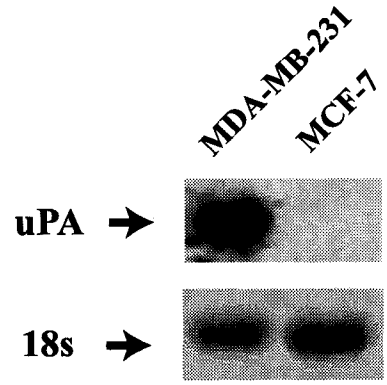
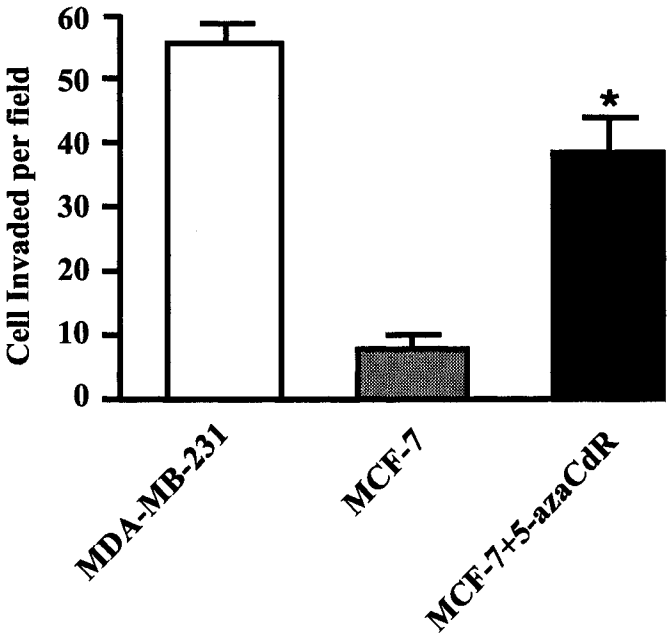
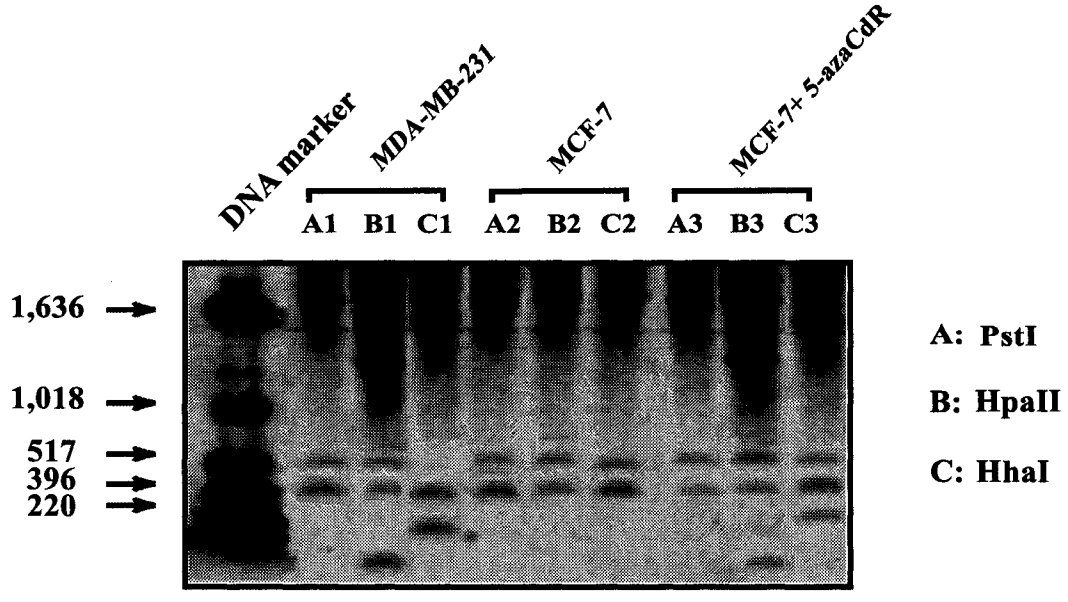
A


MDA-MB-231
MCF-7
uPA
18s

B


Cell Invaded per field
60
50
40
30
20
10
0
*
MDA-MB-231
MCF-7
MCF-7+5-azaCdR

C


DNA marker
MDA-MB-231
MCF-7
MCF-7+ 5-azaCdR
A1 B1 C1 A2 B2 C2 A3 B3 C3
1,636
1,018
517
396
220
A: PstI
B: HpaII
C: HhaI

2.8 Figures

Fig. 2.1. Evaluation of uPA expression, tumor cell invasion and methylation status in human breast cancer cells.

20 μg of total RNA was isolated from human breast cancer cells MCF-7 and MDA-MB-231 and electrophoresed on 1.1% agarose formaldehyde gel and blotted to nylon membrane. All blots were probed with a ^{32}P labeled human uPA and 18S cDNA, as described in "Materials and Methods" (panel A). The invasive capacity of MCF-7 and MDA-MB-231 cells and MCF-7 cells treated with 5-azaCdR was assessed by Boyden chamber invasion assay (panel B). Southern blot analysis of MDA-MB-231, MCF-7 and MCF-7 cells treated with 5-azaCdR is shown in panel C. 10 μg of genomic DNA isolated from these cells were digested with non-methylation sensitive enzyme PstI (lanes a1, b1, and c1) or with methylation sensitive enzymes HpaII (lanes a2, b2, and c2) and HhaI (lanes a3, b3, and c3). DNA digests were resolved on 0.8% agarose gel and all blots were probed with a 778 bp human uPA promoter fragment (panel C). Results are representative of at least 3 different experiments. Data of invasion assay (panel B) is expressed as mean ± SD of values from three independent experiments. Significant difference in the number of invading from control is represented by asterisk ($P<0.05$).

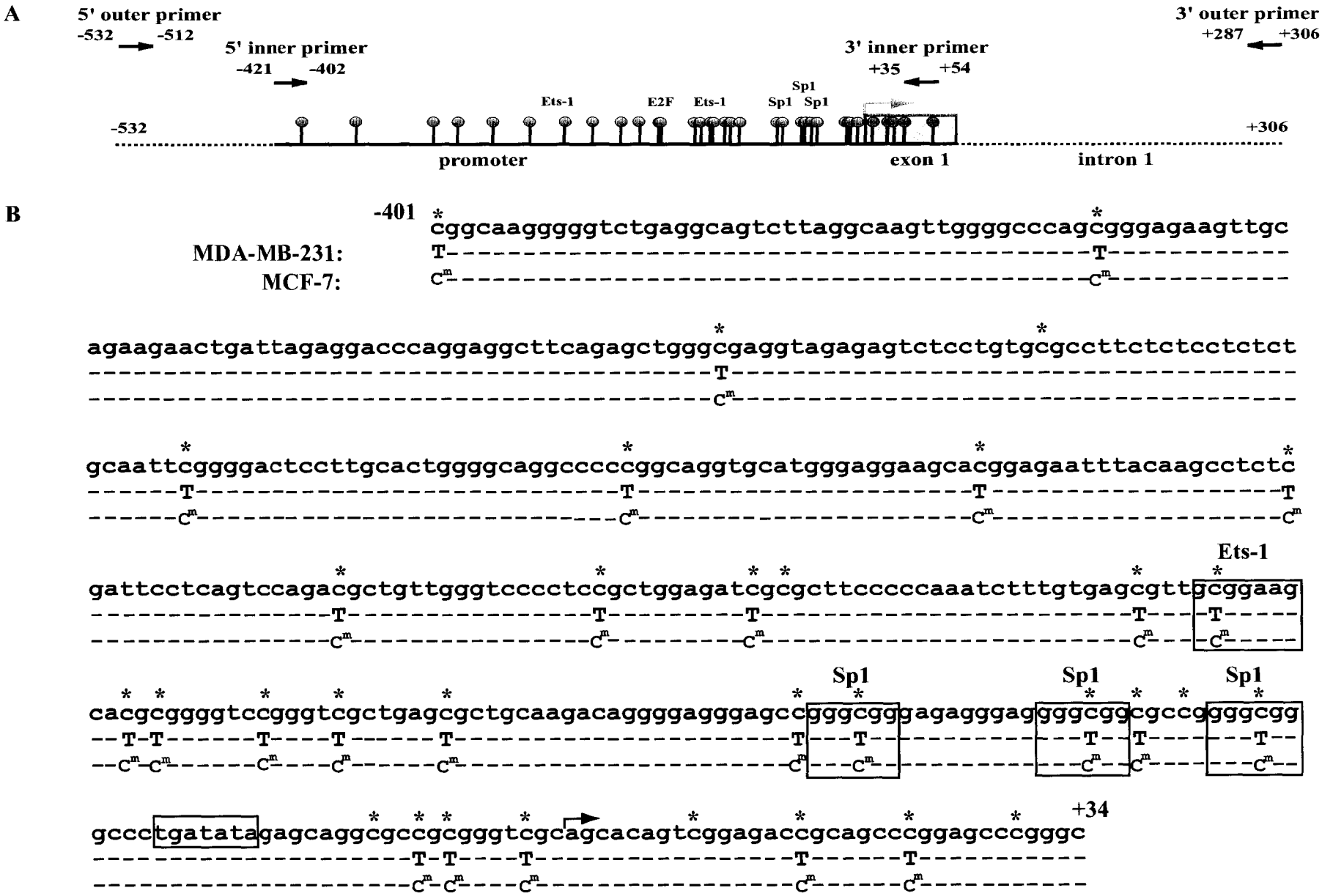

A
5' outer primer
-532
-512
5' inner primer
-421
-402
3' outer primer
+287
+306
3' inner primer
+35
+54
Sp1
Ets-1
E2F
Ets-1
Sp1
Sp1
-532
+306
promoter
exon 1
intron 1
B
-401
cggcaaggggtctgaggcagtcttaggcaagttggggcccagcgggagaagttgc
MDA-MB-231:
MCF-7:
agaagaactgattagaggacccaggaggcttcagagctgggcgaggtagagagtctcctgtgcgccttctctcctctct
gcaattcggggactccttgcactggggcaggcccccggcaggtgcatgggaggaagcacggagaatttacaagcctctc
Ets-1
gattcctcagtccagacgctgttgggtcccctccgctggagatcgcgcttcccccaaatctttgtgagcgttgcggaag
Sp1
Sp1
Sp1
cacgcggggtccgggtcgctgagcgctgcaagacaggggagggagccgggcgggagagggaggggcggcgccggggcgg
+34
gccctgatatagagcaggcgccgcgggtcgcagcacagtcggagaccgcagcccggagcccgggc

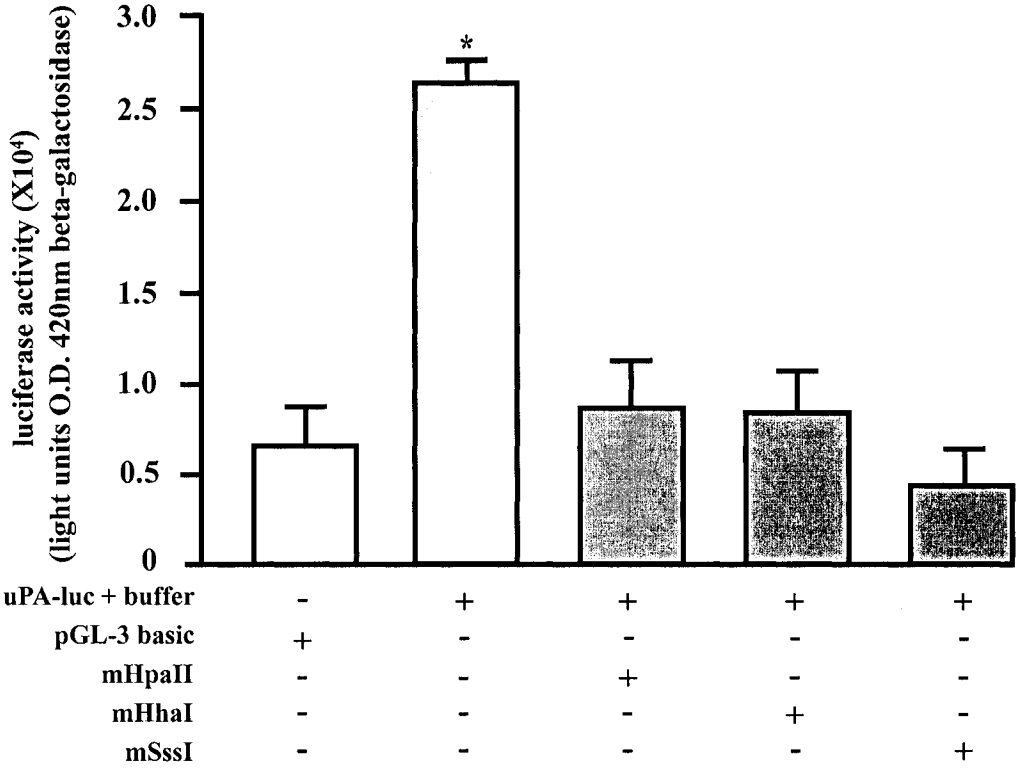
Fig. 2.2. Analysis of the methylation status of CpG dinucleotides within the *uPA* gene promoter by methylation specific PCR.

In Panel A, the transcription start site (↱) and clusters of CpG dinucleotides (❡) in the promoter region and exon 1 (open box) of uPA gene are indicated (Panel A). Using TESS (Transcription Element Search System), the presence of consensus sequences for the binding of various transcription factors such as Ets-1 and Sp1, and DNA sequence corresponding to MSP primers are shown.

In panel B, asterisks mark all potential sites subjected to DNA methylation within -401 to +34 of the uPA gene. Total cellular genomic DNA was extracted from MDA-MB-231 and MCF-7 cells, followed by MSP as described in "Materials and Methods". Unmethylated cytosines of CpG dinucleotides were converted to T following MSP analysis, and were observed mostly in MDA-MB-231 cells. However, methylated cytosine remained as a C, and was indicated as C^{m} in MCF-7 cells.

Results represent MSP and DNA sequence analysis of three different experiments.

A:


luciferase activity (X10⁴)
(light units O.D. 420nm beta-galactosidase)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
uPA-luc + buffer - + + + +
pGL-3 basic + - - - -
mHpaII - - + - -
mHhaI - - - + -
mSssI - - - - +

B:

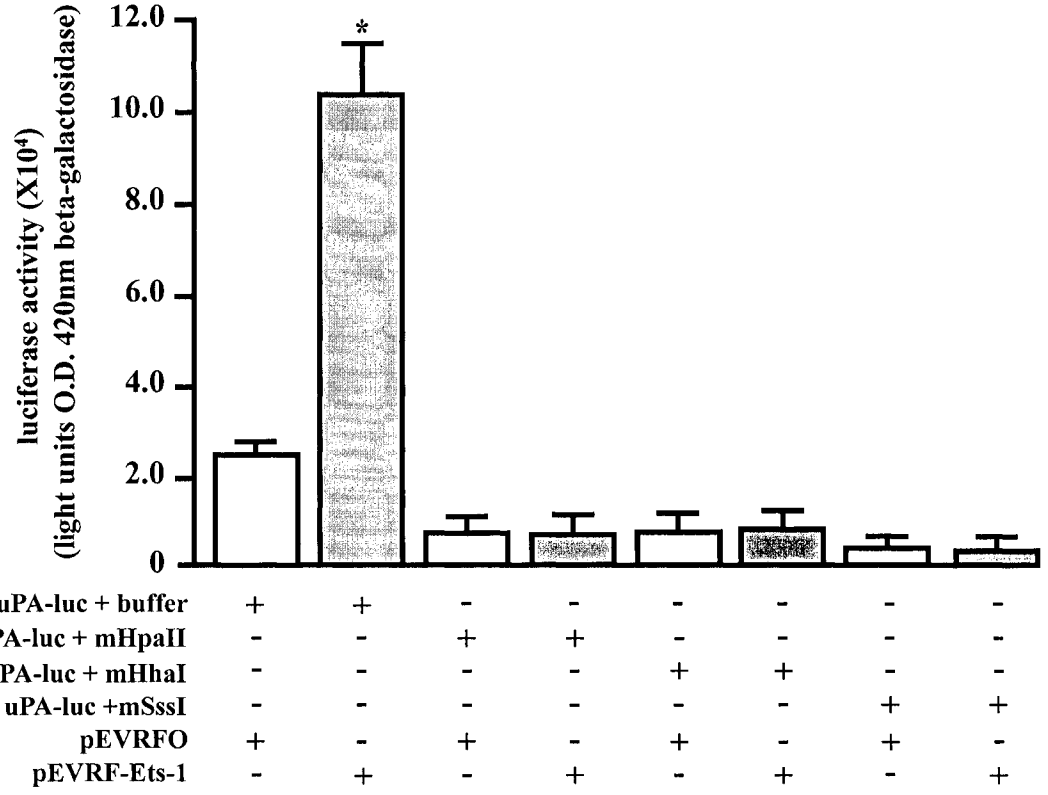
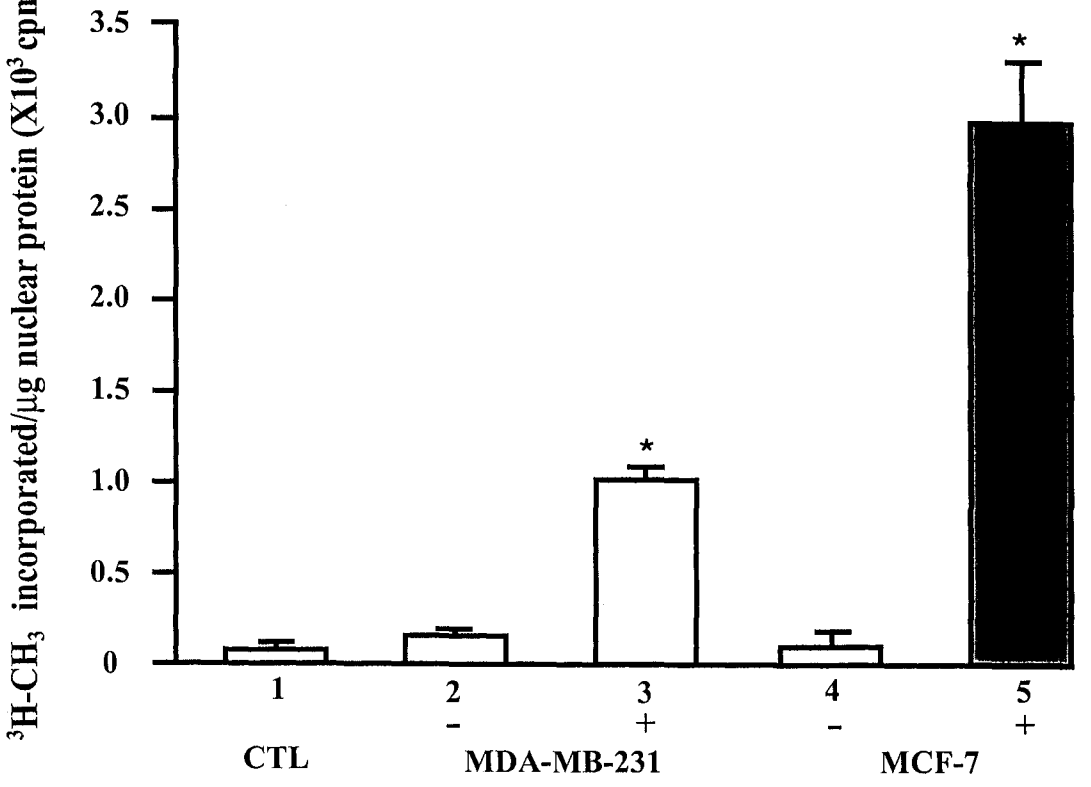

luciferase activity (X10⁴)
(light units O.D. 420nm beta-galactosidase)
12.0
10.0
8.0
6.0
4.0
2.0
0
*
uPA-luc + buffer + + - - - - - -
uPA-luc + mHpaII - - + + - - - -
uPA-luc + mHhaI - - - - + + - -
uPA-luc +mSssI - - - - - - + +
pEVRFO + - + - + - + -
pEVRF-Ets-1 - + - + - + - +

Fig. 2.3. Analysis of the transcriptional activity of methylated and unmethylated uPA promoter.

Methylation of uPA-luc at different sites was generated *in vitro* by incubating this promoter constructs with bacterial CpG methylase (mHpaII, mHhaI and mSssI) and the methyl-donor SAM. The pGL-3 basic vector was used as controls. The mock treated unmethylated uPA-luc was also used. These purified methylated or unmethylated uPA promoter reporter constructs and pGL-3 basic were transiently transfected into MDA-MB-231 cells (Panel A). Same methylase treated or untreated constructs were also transiently cotransfected with pEVRF0 or pEVRF-Ets-1 into MDA-MB-231 cells. Luciferase activities were then analyzed in cell lysates 48 hrs after transfection as described "Materials and Methods" (Panel B). Results are expressed as mean ± SD of values from three different experiments and tested by analysis of variance. Significant differences in induction of transcription activity of the uPA promoter was determined by quantitating luciferase activity as compared to the pGL-3 basic control vector and are represented by asterisks ($P<0.05$).

A


^{3}H-CH$_3$ incorporated/µg nuclear protein (X10^3 cpm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
1
2
3
4
5
-
+
-
+
CTL
MDA-MB-231
MCF-7

B

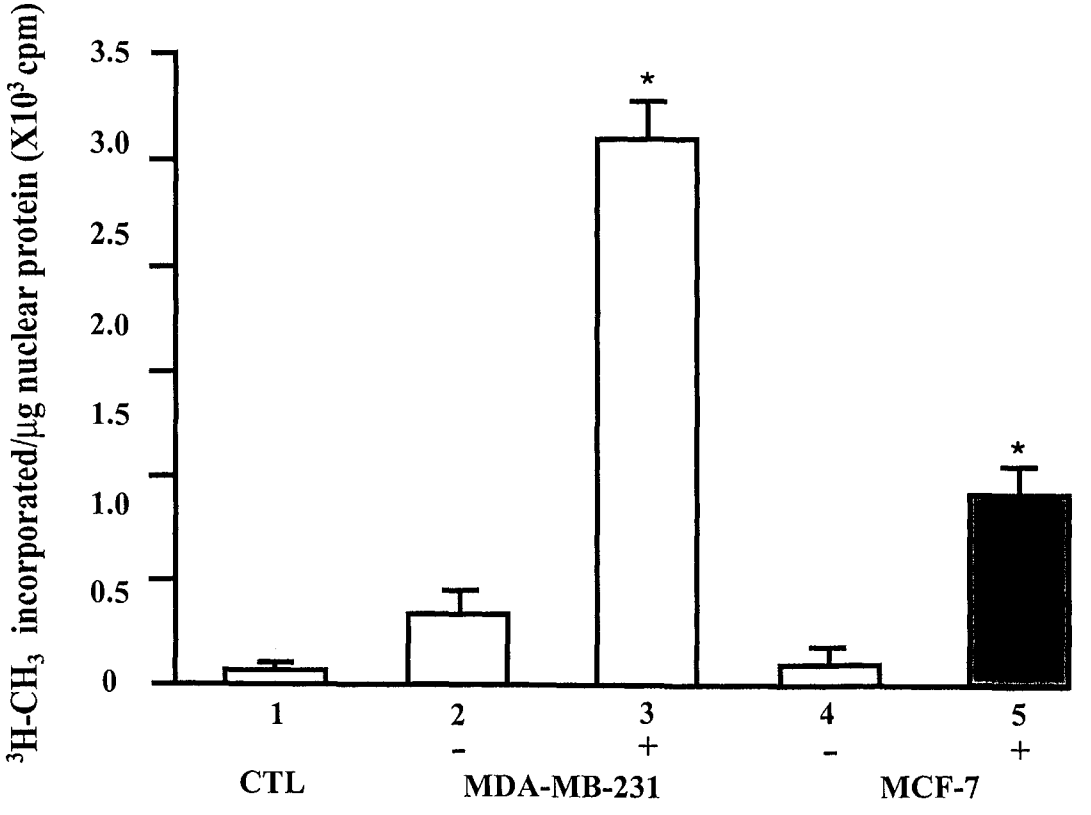

^{3}H-CH$_3$ incorporated/µg nuclear protein (X10^3 cpm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
1
2
3
4
5
-
+
-
+
CTL
MDA-MB-231
MCF-7

Fig. 2.4. Maintenance and *de novo* methyltransferase (DNMT) enzyme activities in human breast cancer cells.

Total nuclear extracts (5 μg) obtained from MDA-MB-231 and MCF-7 breast cancer cells were incubated with the methyl donor ^{3}H-SAM and substrate [hemimethylated oligos poly(mdC-dG; dC-dG) or unmethylated oligos poly(dI-dC; dI-dC)]. After incubating for 3 hrs at 37°C, the mixtures were passed through GF/C filters and precipitated with TCA. Total counts of mean disintegration/min of ^{3}H-CH_3 incorporation retained from the GF/C filters represent the level of maintenance activity (Panel A) or *de novo* (Panel B) methylase activity of DNMT in these breast cancer cell lines, as marked by (+) signs respectively (lanes 3, and 5 in Panel A/B). CTL represents the reaction mixture containing only substrate oligos but no nuclear extract. Total radioactivity retained by GF/C filters from reaction mixtures of respective cell lines in the absence of substrate oligos are shown by (-) signs (lanes 2 and 4 in panel A/B). Results are expressed as the mean disintegration/min of ^{3}H-CH_3 incorporated into substrate oligonucleotide per microgram of nuclear protein ± SD of triplicate cell extracts, each of which was assayed in triplicate.

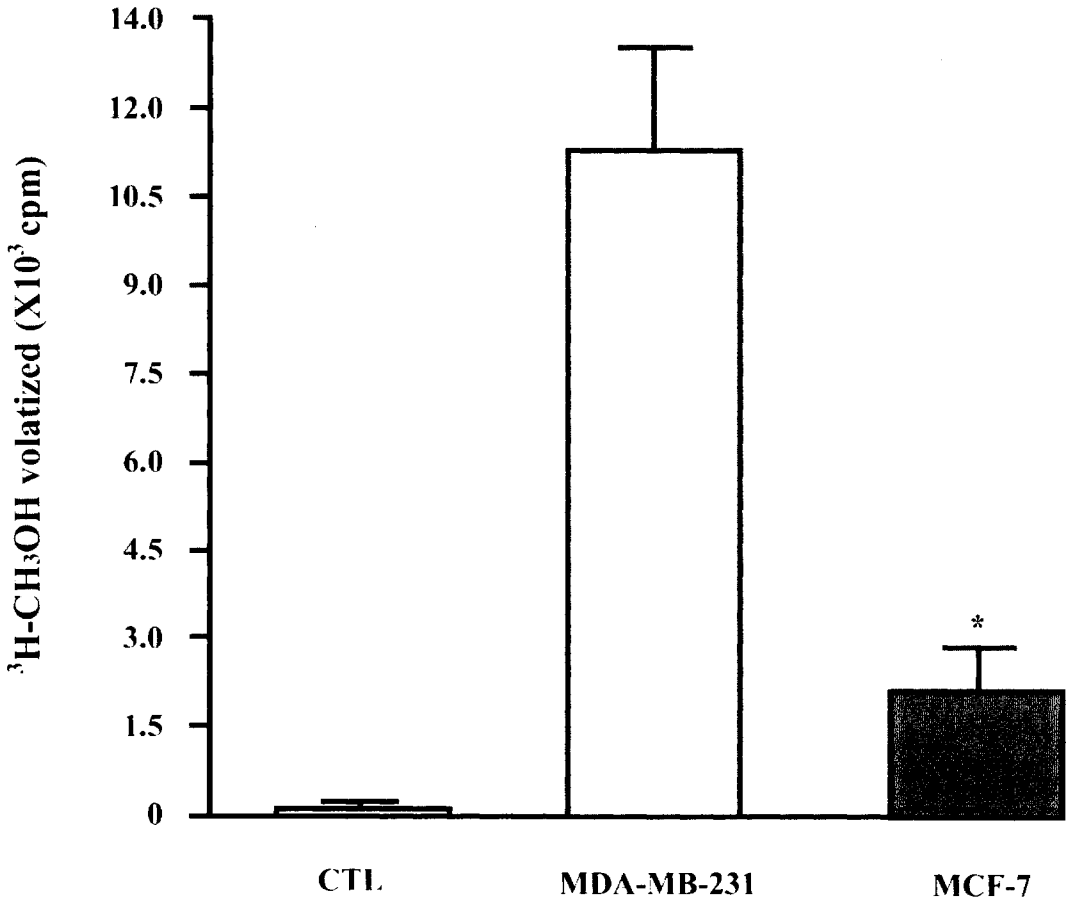
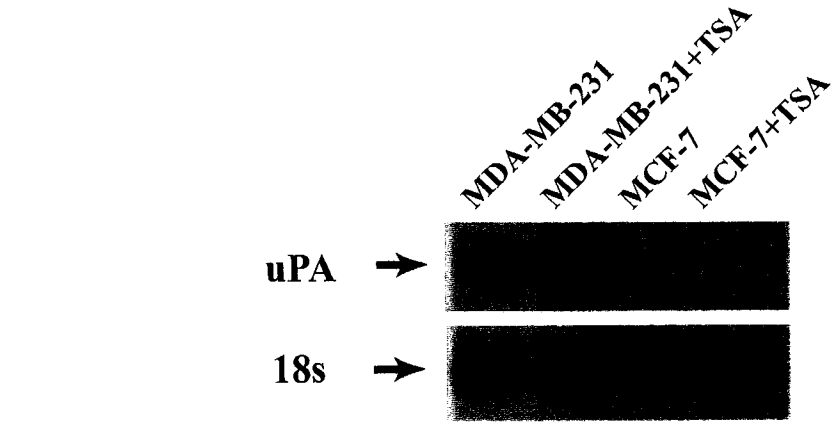

14.0
12.0
10.5
9.0
7.5
6.0
4.5
3.0
1.5
0
^{3}H-CH$_3$OH volatized (X10^3 cpm)
*
CTL
MDA-MB-231
MCF-7

Fig. 2.5. Demethylase activity in human breast cancer cells.

Total nuclear extracts (6 mg) obtained from MDA-MB-231 and MCF-7 cells were loaded onto DEAE-Sephadex A 50 columns. Following elution with a continuously increasing gradient salt buffer, eluted fractions (500 μl x 10) from the total nuclear extract were collected. A 20 μl sample of each eluted fraction was incubated with ^{3}H-methyl-DNA substrate overnight at 37°C. Amount of generated volatized $^{3}H\text{-}CH_3OH$ was counted for each cell line. Results are expressed as the mean disintegration per min per sample ± SD of cell extracts, each of which was assayed in triplicate.


MDA-MB-231
MDA-MB-231+TSA
MCF-7
MCF-7+TSA
uPA
18s

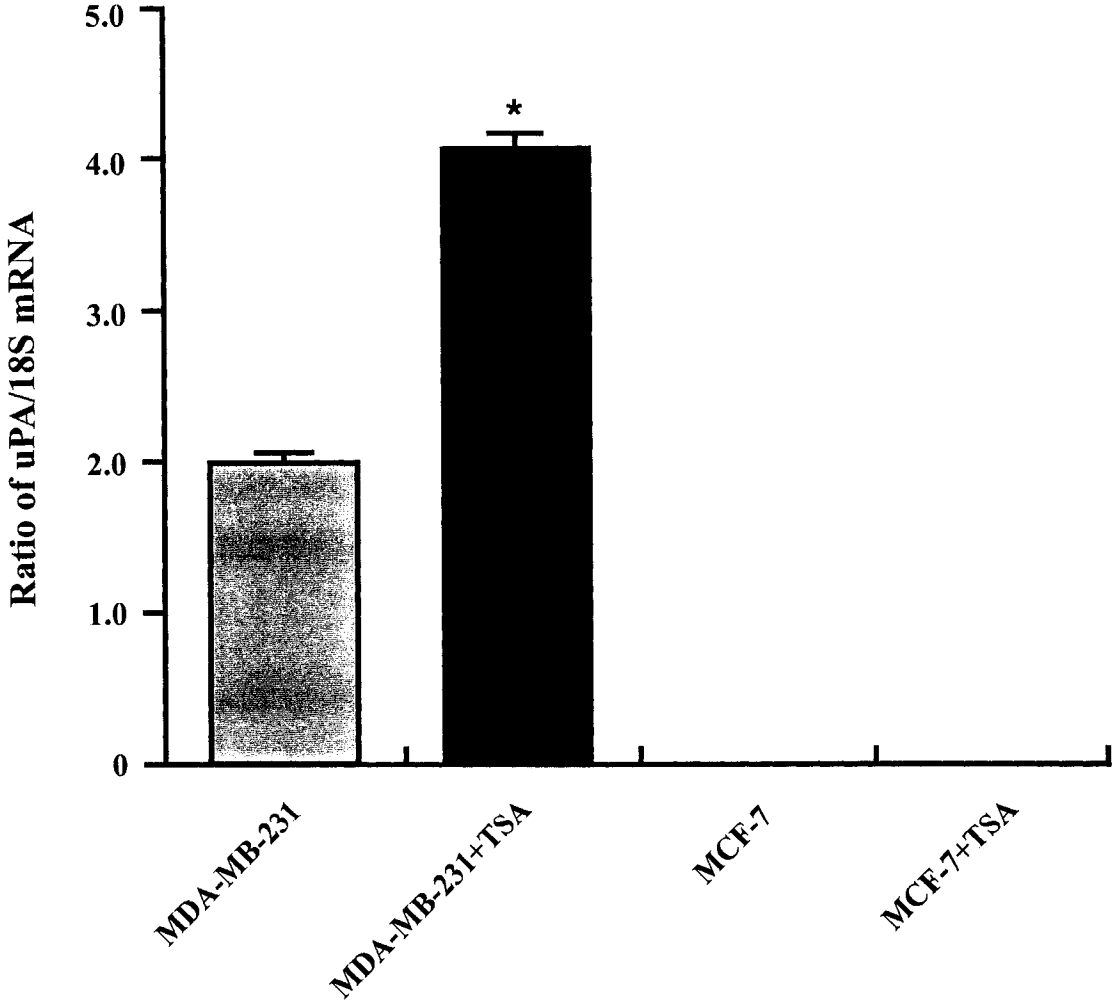

Ratio of uPA/18S mRNA
5.0
4.0
3.0
2.0
1.0
0
*
MDA-MB-231
MDA-MB-231+TSA
MCF-7
MCF-7+TSA

Fig. 2.6. Expression of *uPA* gene with TSA treatment in human breast cancer cells.

Total cellular RNA was extracted from MDA-MB-231 and MCF-7 cells treated with or without 100 ng/ml TSA for 4 days. 15 μg of total cellular RNA from each cell line was analyzed by Northern blot analysis to monitor the level of uPA mRNA expression. All blots were probed with ^{32}P-labeled uPA or 18S cDNA, which were then scanned by laser densimetric scanning. Changes in uPA mRNA expression as determined by plotting the ratio of uPA/18S mRNA are shown. Results represent ± SD of 4 different experiments. Significant difference from control is represented by asterisks ($P<0.05$).

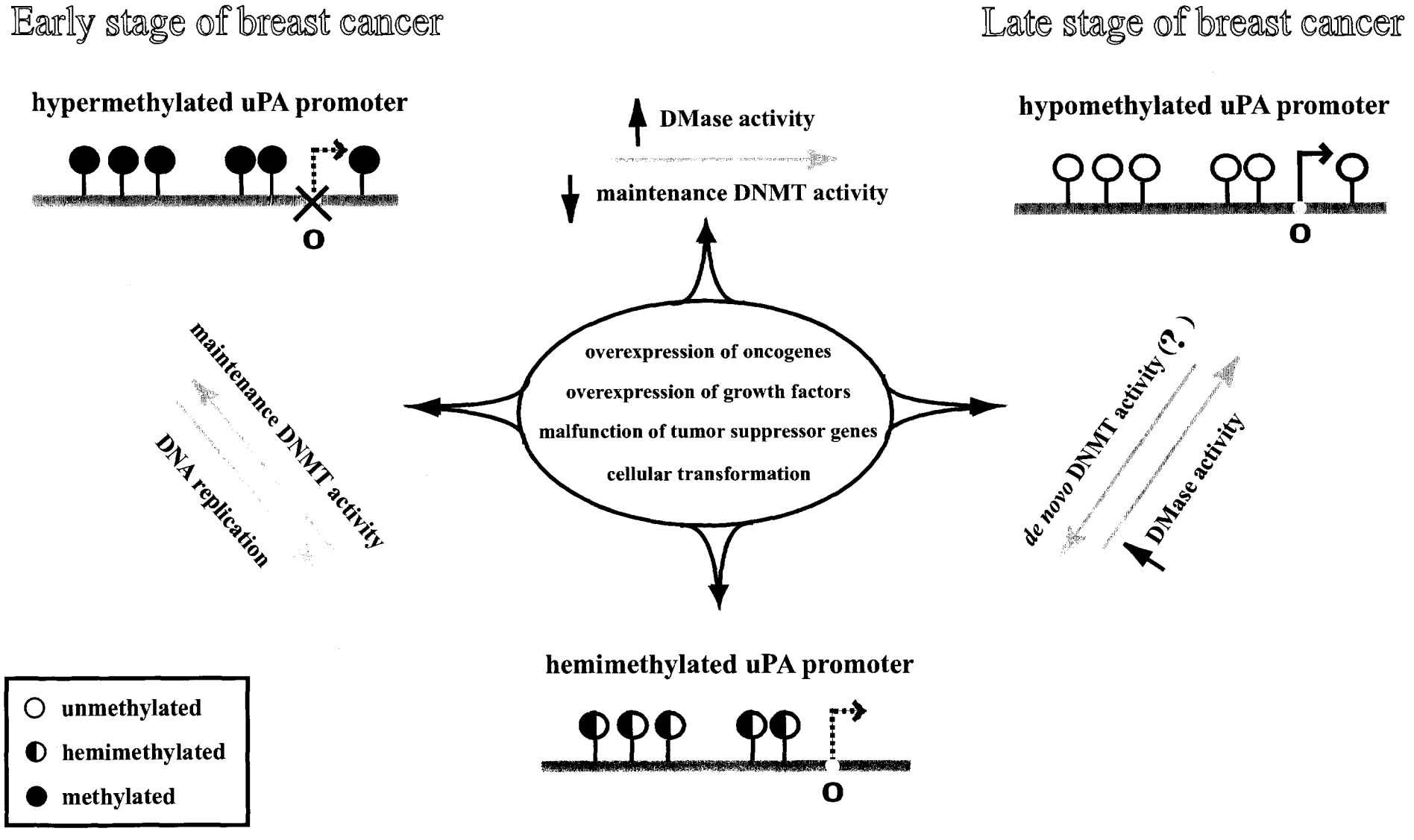

Early stage of breast cancer
Late stage of breast cancer
hypermethylated uPA promoter
hypomethylated uPA promoter
DMase activity
maintenance DNMT activity
maintenance DNMT activity
DNA replication
overexpression of oncogenes
overexpression of growth factors
malfunction of tumor suppressor genes
cellular transformation
de novo DNMT activity (?)
DMase activity
hemimethylated uPA promoter
unmethylated
hemimethylated
methylated

Fig. 2.7. Schematic representation of the potential mechanisms that control *uPA* gene expression through DNA methylation during breast cancer progression.

During early stage breast cancer, uPA expression is undetectable, and the uPA promoter is hypermethylated and maintained in this hypermethylated form after DNA replication. A high level of maintenance DNA DNMT activity catalyzes hypermethylation. During cancer progression, hypermethylation of the uPA promoter is switched to hypomethylation due to increased DMase activity in coordination with gradually decreased maintenance DNA DNMT activity. This is achieved either directly (via demethylation) or indirectly (via hemi-methylation) during DNA replication. Overexpression of various tumor progression factors in coordination with the suppression of tumor suppressor genes are able to break the endogenous balance of DNMTs and DMases to promote hypomethylation of the uPA gene at late stage breast cancer progression.

Chapter III

A Peptide Derived from the Non-Receptor Binding Region of Urokinase Plasminogen Activator (uPA) Inhibits Tumor Progression and Angiogenesis and Induces Tumor Cell Death *In Vivo.*

3.1 Preface

Patients with highly invasive breast cancer have a higher incidence of morbidity and mortality. Numerous studies have suggested that the overexpression of uPA plays a key role in the progression of this malignancy [4, 10, 274]. In the previous chapter, I explored the role of altered DNA methylation in the upregulation of uPA in late stage breast cancer cells (Chapter II), which is critical for cancer cell invasion and metastasis. Given this correlation, we studied whether blocking uPA can prevent breast cancer progression. In this chapter, I investigate the inhibitory effects of a uPA-derived peptide, Å6, on breast cancer progression *in vitro* and *in vivo*.

Amino acids 136~158 of uPA were designated as a "connecting peptide" that connects ATF and the catalytic domain of uPA [96, 108, 109]. An 8 amino acid fragment (KPSSPPEE) corresponding to residues 136~143 of uPA resides within this region. Interestingly, this region might be generated physically by scuPA activation as discussed in the Introduction (section 1.1.4.1). Ser138 was shown to be essential for the chemotactic effects of scuPA [187]. No evidence has been presented to date suggesting this region may be involved in any protein-protein interaction. In this chapter, I report the development of a synthetic peptide (Å6) corresponding to a.a. 136~143 of uPA, as well as the effects of this peptide on the biological activities of uPA. The 8-mer Å6 peptide was assayed for its ability to block the invasion of breast cancer cells in an *in vitro* invasion assay. The presence of Å6 can also inhibit the migration of endothelial cells, a cellular behavior that is essential for angiogenesis. When injected into rodents carrying breast cancers, Å6 successfully suppresses the growth of the primary tumor as well as tumor metastases, achieved via the induction of tumor cell death and the inhibition of tumor angiogenesis. Although still elusive, data presented in this chapter suggest that this region might be engaged in protein-protein interaction between uPA and potential binding proteins other than uPAR.

3.2 Abstract

Urokinase plasminogen activator (uPA) plays an important role in the progression of several malignancies including breast cancer. We have identified a non-competitive antagonist of uPA-uPAR interaction derived from a non-receptor binding region of uPA (amino acids 136-143). This 8-mer capped peptide (Å6) inhibited breast cancer cell invasion and endothelial cell migration in a dose-dependent manner *in vitro* without altering cell-doubling time. Intraperitoneal administration of Å6 resulted in a significant inhibition of tumor growth and suppressed the development of lymph node metastases in several models of breast cancer cell growth and metastasis. Large areas of tumor necrosis and extensive positive staining by TUNEL were observed upon histological and immunohistochemical analysis of experimental tumor sections derived from Å6-treated animals. Å6 treatment also resulted in a decrease in factor VIII-positive tumor microvessel hot-spots. These results identify a new epitope in uPA that is involved in uPA-uPAR interaction and indicate that an antagonist based on this epitope is able to inhibit tumor progression by modulating the tumor microenvironment in the absence of direct cytotoxic effects *in vivo*.

3.3 Introduction (urokinase, breast cancer, angiogenesis, apoptosis)

The urokinase plasminogen activator (uPA) system has been implicated in the progression, metastasis and angiogenesis of numerous solid tumors [6, 112, 215, 505]. Expression of the components of the uPA system and its specific cell-surface receptor (uPAR) often increases with disease progression and is correlated with poor prognosis and outcome in patients [259, 506]. The expression of uPA and uPAR is not restricted to tumor cells alone since other tumor-associated cells, such as angiogenic endothelial cells, macrophages and fibroblasts have been demonstrated to express one or both components of this system [507-509]. Moreover, the pattern of expression and the cells responsible for this expression may differ depending on the type and stage. Expression of uPA and uPAR is associated with tumor

progression and is often localized to the leading, invasive edge of a tumor [217, 510].

The uPA system has pleiotropic functions in tumor progression whereby several pathways may be temporally activated when uPA binds to uPAR. Various intracellular signalling pathways are initiated when uPA binds to uPAR including the up-regulation of oncogene expression, stimulation of cell adhesion, regulation of chemotaxis, and activation of the MAP kinase pathway [187, 202, 203, 205, 511]. However, the mechanism of signalling via uPAR, a glycolipid-anchored receptor that lacks a transmembrane signalling domain, and the identity of the adapter molecule(s) hypothesised to couple ligand binding to intracellular signalling remain elusive. Receptor binding also results in the activation of scuPA, the single chain zymogen form of uPA, and initiates an extracellular proteolytic cascade that leads to the downstream activation of plasminogen and matrix metalloproteases [324, 512]. These enzymes remodel extracellular matrix (ECM) and the basal lamina associated with endothelial cells, and also release or activate various growth factors sequestered within the ECM such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and transforming growth factor β (TGF-β) [169, 513, 514]. The net result of this proteolytic flux combined with uPA-dependent intracellular signalling is acceleration of tumor cell invasion and tumor-associated angiogenesis.

The primary interaction of uPA with uPAR is mediated through the growth factor domain, amino acids (a.a.) 1-48 of uPA. However, we have identified a second site in uPA that interacts with uPAR. This region, termed the connecting peptide, is comprised of a.a. 136-143. Here we present data that a small capped peptide derived from this region, Ac-KPSSPPEE-Am (Å6), inhibits the interaction of uPA with uPAR in a non-competitive manner. Administration of this peptide to animals bearing experimental breast cancer tumors results in inhibition of tumor growth and metastasis in the absence of direct cytotoxic or anti-proliferative effects.

3.4 Materials and Methods

Peptide synthesis.

Å6 was synthesized by standard solid phase methodology using *p*-methyl-benzhydrylamine resin and L-amino acids protected with the *t*-butyloxycarbonyl (BOC) group. Removal of the BOC group was with 50% trifluoroacetic acid (TFA) in dichloromethane. Coupling was achieved with 1-hydroxybenzotriazole and dicyclohexylcarbodiimide. Side-chain protection was 2-chlorobenzyloxycarbonyl for lysine, benzyl for serine, and cyclohexyl for glutamic acid. The N-terminal lysine was capped by treatment with acetic anhydride. Deprotection and detachment of the completed peptide from the resin was accomplished by treatment with anhydrous hydrofluoric acid in the presence of anisole. HPLC on a Waters C18 preparative column using a 0-40% linear gradient of 1.0% aqueous triethylamine phosphate into CH_3CN gave fractions containing pure material that were reapplied to the column. The column was washed with 3 column volumes of 1.0 % aqueous acetic acid and then eluted with a 0-50% linear gradient of 1.0 % aqueous acetic acid into CH_3CN. Lyophilization afforded the >99% pure product as colorless, hygroscopic crystals easily soluble in water to >500 mM. Å7 (Ac-KPSSPPE-Am), Å8 (Ac-PSSPPEE-Am), Å10 (Ac-KPSSPPEELK-Am) and Å14 (Ac-KPSSPPEEL-Am) were prepared using similar methodology. For Å9 (NH_2-KPSSPPEELK-COOH) and Å13 (NH_2-KPSSPPEE-COOH) the first amino acid was attached to the solid phase carrier through an ester linkage and the N-terminal capping step was omitted.

Determination of Å6 dose for *in vivo* studies.

Single administrations of Å6 up to 1500 $mg \cdot kg^{-1}$ did not demonstrate any evidence of toxicity. An LC/MS assay was developed to detect Å6 in plasma. Pharmacokinetic experiments demonstrated a short plasma half-life for Å6 (0.2 hr) in mice. We initially attempted to achieve a plasma level at steady-state (C_{SS}) that was at least equivalent to the *in vitro* IC_{50} for tumor cell invasion (5~10 μM). This objective was accomplished by delivering Å6 via continuous infusion using osmotic mini-pumps at a dose of 75 $mg \cdot kg^{-1} \cdot day^{-1}$. We then compared administration of this dose by continuous infusion or by twice daily (*bid*) injections. Both protocols yielded identical anti-tumor results indicating that the pharmacokinetics did not correlate with the anti-tumor activity of Å6 and that a steady-state plasma level of Å6 was not

required for anti-tumor activity. For simplicity, the IP protocol was used for all subsequent studies.

Vehicle (phosphate-buffered saline, or PBS) was used as the control for *in vivo* studies since it is relevant to the therapeutic setting. Although scrambled peptides are useful for establishing specificity *in vitro*, they are completely different chemical entities and most likely have different behavior *in vivo*. Thus they were not considered as controls for the animal studies.

Cells and cell culture.

The rat mammary adenocarcinoma cell line Mat B-III was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in culture *in vitro* in McCoy's 5A modified medium (Gibco BRL, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 25 mM 4-(2-hydroxyethy)-1-piperazineethanesulfonic acid, 26 mM sodium bicarbonate, 100 units/ml of penicillin-streptomycin sulphate (Gibco BRL) and 0.2% gentamycin (Sigma, St. Louis, MO).

Human breast adenocarcinoma cell line MDA-MB-231 was obtained from ATCC and maintained in L-15 medium (Gibco BRL) supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin sulphate and 0.2% gentamycin. MDA-MB-231-GFP (green fluorescent protein) cells were generated by transfecting the expression vector, which contained the codon optimized human GFP-S65T gene (Clontech Laboratories, Inc., Palo Alto, CA) using lipofectin reagent (Gibco BRL). Cells with stably integrated plasmids were selected for neomycin resistant gene with geneticin (G418) (Gibco BRL).

Surface Plasmon Resonance.

Binding kinetics of suPAR in the presence and absence of Å6 was measured using a BIA 3000 optical Biosensor (Biacore, AB, Sweden) [515]. This method detects binding interactions in real time by measuring changes in the refractive index (RI) at a biospecific surface, and enables association and dissociation rate constants to be calculated. For these studies, recombinant scuPA (a kind gift of Dr. Jack Henkin, Abbott Laboratories) and recombinant soluble urokinase receptor (suPAR) were

coupled to a B1-research grade sensor chip flow cell (Biacore, AB, Sweden) via standard amine coupling procedures [283] using N-hydroxysuccinimide/N-ethyl-N-[3-(dimethylamino)propyl]carbodiimine hydro- chloride (Pierce, Rockford, IL) at a level of 1000RU each. Sensor surfaces were coated with ligands (10 μg/ml) in 10 mM NaOAc buffer, pH 5.0. Following immobilization, unreacted groups were blocked with 1M ethanolamine, pH 8.5. Binding buffer was phosphate buffered saline (PBS), pH 7.4, 0.005% TWEEN-20. Binding of suPAR (± Å6) was measured at 25°C at a flow rate of 100 μl/min for 1 minute, with 2 minutes of dissociation examined. The bulk shift due to changes in RI was measured using the suPAR surface, and was subtracted from the binding signal at each condition to correct for non-specific signals. Surfaces were regenerated with 2 X 30s pulses of 1M NaCl, pH 3.3, followed by an injection of binding buffer for 1 min to remove this high salt solution. All injections were performed in a random fashion using the RANDOM command in the automated method. Binding of suPAR was performed at 100 nM, 33.3 nM, 11.1 nM, 3.7 nM and 1.24 nM, in the absence or presence of 60 μM, 20 μM, 6.6 μM, 2.2 μM, 0.74 μM or 0.24 μM Å6. Data were fit using a 1:1 Langmuir reaction mechanism using BIA evaluation 3.0 software (Biacore, AB, Sweden). Dissociation and association rates were calculated separately to examine the effect of Å6 on the affinity of suPAR to scuPA. Clot lysis assay was performed as described previously [114].

Boyden chamber matrigel invasion assay.

The effect of Å6 on treated Mat B-III and MDA-MB-231-GFP cells was determined by two compartment Boyden chambers (Transwell, Costar, USA) and basement membrane Matrigel invasion assay as previously described [500]. The 8 μM pore polycarbonate filters were coated with basement membrane Matrigel (50 μg/filter). Matrigel was then reconstructed by adding 0.1 ml serum-free culture medium to the upper chamber and incubated for 90 min. After removal of medium, cells (5×10^4) in 0.1 ml of medium with or without Å6 were added to the upper chamber and placed in a lower chamber pre-filled with 0.8 ml of serum-free medium supplemented with 25 μg/ml fibronectin (Sigma, Oakville, ON, Canada) and

incubated at 37°C for 24 hrs. At the end of incubation, medium was removed, filters were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde (Sigma) in 0.1 M phosphated buffer, pH 7.4 at room temperature for 30 min. After washing with PBS, all filters were stained with 1.5% toluidine blue and filters were mounted onto glass slides. Cells were examined under a light microscope. Ten fields under 400 X magnification were randomly selected and the mean cell number was calculated.

Endothelial cell migration assay.

Transwell (Costar, 8.0 μM pore size) were coated with type I collagen (50 μg/ml) by adding 200 μl of the collagen solution per transwell, then incubating overnight at 37°C. The transwells were then assembled in a 24-well plate and bFGF (1 ng/ml) was added to the bottom chamber in a total volume of 0.8 ml M199 containing 2% FBS. Human dermal microvascular endothelial cells (HDMVC) were detached from monolayer culture using Verseen, centrifuged and reconstituted in M199 containing 2% FBS (1×10^6 cells/ml). 0.2 ml of this cell suspension was added to the upper chamber of each Transwell. Inhibitors to be tested were added to both the upper and lower chambers, and the migration was allowed to proceed for 5 hrs under 5% CO_2 in a humidified atmosphere at 37°C. The transwells were then removed from the plate and the upper chamber wiped clean with a cotton swab. Giemsa stain was used to fix and stain the cells and the numbers of cells that had migrated to the bottom aspect of the membrane were counted. Data is presented as the average number of migrated cells per 10 fields.

Animal protocols.

Inbred female Fischer 344 rats weighing 200-220g were obtained from Charles River Inc. (St. Constant, QC, Canada). Before inoculation, Mat B-III tumor cells grown in serum-containing medium were washed with Hank's balanced buffer and trypsinized for 5 mins. Cells were then collected in Hank's balanced buffer and centrifuged at 1500 rpm for 5 mins. Cell pellets (1×10^6 cells) were resuspended in 0.2 ml saline and injected using one ml insulin syringes into the mammary fat pad of rats anesthetized with ethanol/Somnotal (MTC Pharmaceuticals, Cambridge, ON, Canada). Control and experimental animals were injected intraperitoneally (i.p) with

PBS and Å6 (75 $mg \cdot kg^{-1} \cdot day^{-1}$) respectively twice a day for 16 days. All animals were numbered, kept separately and were examined for the development of tumors daily for up to 17 days. The tumor mass of control and experimental animals was measured in two dimensions by callipers and the tumor volume was calculated. Control and experimental animals were sacrificed at the end of this study (day 17) and were examined and scored for the development of macroscopic tumor metastases in various tissues. Primary tumor tissues were also removed from control and experimental animals for histologic examination.

For xenograft studies, twenty-four 5-week-old BALB/c (*nu/nu*) female mice were obtained from Charles River Inc. Prior to inoculation, MDA-MB-231-GFP cells grown in serum containing culture medium were washed with Hank's balanced buffer and centrifuged at 1500 rpm for 5 mins. Cell pellets (5×10^5 cells/mice) were resuspended in 100 µl of Matrigel (Becton Dickinson Labware, Mississauga, ON, Canada) and saline mixture (20% Matrigel) and injected into the mammary fat pads of the mice. All animals were numbered and kept separately in a temperature-controlled room on a 12h/12h light/dark schedule with food and water *ad libitum*. Tumors were allowed to grow to the size of 15~25 mm^3 prior to drug administration. At this time animals were randomized into two groups. Å6 (75 $mg \cdot kg^{-1} \cdot day^{-1}$) and sterile PBS were injected i.p twice a day into the 2 groups of mice, respectively. The tumor mass was measured in two dimensions with callipers twice a week. At the end of xenograft study, the mice were sacrificed and their lungs, liver, spleen and other organs were removed. These fresh tissues from control and experimental mice were sliced at approximately 1 mm thickness and observed directly under a fluorescence microscope. Numbers of tumor cells from ten random sites were counted per field of examination and photographed.

Histology and Immunohistochemistry.

Primary tumors were fixed in 4% paraformaldehyde overnight, dehydrated and embedded in paraffin (Fisher Scientific, Montreal, QC, Canada) the next day. Tumor sections (4 µm) were deparaffinized then rehydrated and stained with hematoxylin/eiosin (H&E). For TUNEL assay paraffin embedded tissue sections were

soaked in Toluene to deparaffinize, then rehydrated in graded alcohol series (100% to 70%). For enzyme predigestion of formalin-fixed tissue, the sections were incubated for 30 min at room temperature (RT) in 15 μg/ml of proteinase K/10 mM Tris/HCl. The primary antibody, terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim, QC, Canada) diluted in TUNEL reaction mixture was added to the slides and incubated at 37°C for 60 min. After rinsing with PBS, the sections were coated with 50 μl of secondary antibody conjugated with converter-POD (horseradish peroxidase) at 37°C for 30 min. For factor VIII staining enzyme predigestion of formalin-fixed tumors was done by incubation at 37°C in 0.1 g pronase type 14/100 ml PBS followed by washing in 10 mM PBS (pH 7.6). Anti-human endothelial cell antibody against factor VIII (DAKO Diagnostica Inc., Mississauga, ON) was used as primary antibody diluted in serum/PBS (1:60). Tumor sections were incubated for 60 min at room temperature followed by further incubation for 30 min with biotinylated anti-mouse link antibody (Zymed Laboratories Inc., CA). Sections were rinsed with PBS followed by coating with streptavidin conjugated to horseradish peroxidase (HRP) for 10 min. The substrate DAB was then incubated for 10 min at room temperature (Sigma, ON, Canada). Finally, the sections were counter-stained with hematoxylin and mounted. All histologic examinations were carried out by light and fluorescence microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattlevoro, VT). For quantitation of TUNEL positive cells three sections from each tumor were analyzed using NIH image version 1.61 and expressed as integrated density per field of examination. In control and Å6 treated tumors, microvessels were counted and expressed as angiogenesis density representing the mean of at least three areas with high vascularization in three different sections from each tumor. All slides were interpretated by two independent investigators [516].

Statistical analysis.

Results are expressed as the mean ± SD of at least triplicate determinations and statistical comparisons are based on Student's *t* test or analysis of variance. A probability value of <0.05 was considered to be significant.

3.5 Results

Å6 inhibits scuPA-suPAR interaction.

Binding of suPAR (soluble uPAR) to immobilized scuPA (single-chain uPA) was examined using a BIA 3000 optical biosensor in the absence or presence of Å6. Å6 inhibited the binding of 100 nM suPAR to scuPA in a dose-dependent manner at concentrations of Å6 as low as 0.25 μM (Fig. 3.1A). Similar results were observed at 33.3 nM and 11.1 nM suPAR (data not shown). The specificity of this inhibition was demonstrated by the lack of effect of Å29, an analog of Å6 in which the first serine is replaced by a glutamic acid (data not shown). This amino acid substitution was chosen because it mimics a phosphorylated serine at this position. Franco et al [517] have described that high molecular weight uPA (HMW-uPA) can be phosphorylated at this serine and that phosphorylated uPA is unable to mediate monocyte chemotaxis or adhesion, in contrast to unmodified uPA [515]. Association and dissociation rate constants were calculated for suPAR binding to scuPA in the absence and presence of Å6 at the three highest concentrations of suPAR. The k_d and the k_a were calculated separately for each of 21 conditions and the data was fitted to a 1:1 Langmuir model. Langmuir isotherms in the absence and presence of Å6 were not parallel, suggesting that Å6 did not inhibit the suPAR-scuPA interaction in a competitive manner (data not shown). The K_d of suPAR was 1 nM and 1.7 nM in the absence and presence of Å6, respectively, whereas the R_{max} decreased in a dose-dependent manner (Fig. 3.1B, 100 nM suPAR). The maximal inhibition observed was 50%, indicating that a new steady state was established in the presence of Å6. Increasing concentrations of Å6 did not significantly alter the K_d of suPAR for scuPA and therefore, it was not necessary to carry out a Schild regression as it was evident that the inhibition was not competitive. Overall, the kinetic and equilibrium binding constants were not altered by Å6 [$k_d = (1.0\text{x}10^{-3} \pm 1.1\text{x}10^{-4})$ s^{-1}, $k_a = (7.5\text{x}10^{5} \pm 7.0\text{x}10^{4})$ $M^{-1}s^{-1}$, and the $K_d = (1.3\text{x}10^{-9} \pm 2.9\text{x}10^{-10})$ M]. In contrast, 10 nM soluble scuPA was able to completely inhibit the binding of equimolar concentrations of suPAR. Soluble scuPA (10 nM) decreased the apparent affinity of suPAR to immobilized scuPA (100 nM suPAR, $K_d = 2.6\text{x}10^{-9}$ M, 33.3 nM suPAR, $K_d = 2.6\text{x}10^{-7}$ M), as would be expected for a

competitive inhibitor. These data are consistent with Å6 inhibiting the suPAR-scuPA interaction in a non-competitive, allosteric manner.

Additional evidence for the allosteric inhibition of the scuPA-suPAR interaction by Å6 was derived from the finding that Å6 inhibits plasminogen activation by the scuPA-suPAR complex in a clot lysis assay [295]. The plasminogen activating activity of this complex has been hypothesised to result from a conformational change in the complex that leads to the formation of an active site in the absence of conversion to two-chain uPA (tcuPA) [295]. Å6 did not inhibit clot lysis initiated by tcuPA (data not shown). However, Å6 almost completely abrogated clot lysis mediated by the scuPA-suPAR complex, whereas the control peptide Å29 had no such effect (Fig. 3.1C).

Å6 inhibits breast cancer cell invasion and endothelial cell migration *in vitro*.

Tumor cell invasion and endothelial cell migration during angiogenesis are key events that contribute to tumor progression. The uPA-uPAR system has been demonstrated to play a major role in both processes. Since Å6 inhibited the interaction of suPAR with scuPA, we assessed its ability to inhibit cell invasion and migration *in vitro*. Peptides of different length based on the connecting peptide region of uPA (a.a. 136-158) were prepared and tested for their ability to inhibit MDA-MB-231 tumor cell invasion *in vitro*. Of all the peptides tested, only Å6 was able to inhibit tumor cell invasion in this assay (Fig. 3.2A). Uncapped versions of Å6 also had no activity indicating that Å6 represented the minimal active epitope of the connecting peptide, at least from the standpoint of invasion (Fig. 3.2A). The dose dependence of Å6 inhibition of rat and human breast cancer cell invasion was evaluated in a Boyden chamber invasion assay. Å6 decreased the number of rat and human breast cancer cells invading through Matrigel with an IC_{50} between 5~25 μM for both cell lines tested (Fig. 3.2B and 3.2C). Invasion was not inhibited further at Å6 concentrations greater than 50 μM. In contrast to molecules that inhibit the binding of the GFD (a.a. 1~48) of uPA to uPAR [283], the ability of Å6 to inhibit invasion did not appear to be species specific. Finally, Å6 also inhibited the migration of human dermal microvascular endothelial cells (HDMVC) on Type I collagen (Fig. 3.2D). The IC_{50}

for endothelial cell migration (25~50 μM) was slightly higher than that observed in the tumor cell invasion assay.

Å6 blocks rat breast cancer growth and metastasis *in vivo*.

Since the uPA-uPAR system contributes to the invasion and motility of multiple cell types associated with tumor progression (e.g. tumor cells, endothelial cells), we hypothesized that the inhibition of the uPA-uPAR interaction using Å6 would have significant anti-tumor effects *in vivo*. We expected that a capped peptide would be less susceptible to exoprotease degradation in the plasma (although this was not specifically tested) and would therefore have a half-life sufficient to provide efficacy *in vivo*. Rat Mat B-III breast cancer cells (1 x 10^6 cells) were inoculated into the mammary fat pads of female Fischer rats. Å6 treatment (75 $mg \cdot kg^{-1} \cdot day^{-1}$) was initiated on the same day as the inoculation and continued for 16 days. Tumor volumes were determined daily and compared to the control group, which received vehicle alone. Infusion of Å6 resulted in a marked decrease (55%) in tumor volume throughout the course of this study (Fig. 3.3A). Control and Å6-treated animals were killed on day 17 and evaluated for the presence of macroscopic tumor metastases. Control animals, receiving vehicle alone, developed macroscopic tumor metastases to lungs, retroperitoneal and auxillary lymph nodes. In contrast, all Å6-treated animals exhibited significantly fewer or no macroscopic tumor metastases at these sites (Fig. 3.3B).

Å6 blocks human breast cancer growth and metastasis *in vivo*.

We then examined the anti-tumor effects of Å6 in a xenograft model of human breast cancer. To facilitate the detection of metastatic tumor cells, we first transfected human breast cancer cells (MDA-MB-231) with the cDNA for green fluorescent protein (MDA-MB-231-GFP). MDA-MB-231 and MDA-MB-231-GFP cells exhibited no significant difference in their invasive capacity in a Matrigel invasion assay (data not shown). For *in vivo* studies, MDA-MB-231-GFP cells (5 x 10^5) were co-inoculated into the mammary fat pads of female BALB/c (*nu/nu*) mice with Matrigel. Tumors were allowed to grow until palpable (15~25 mm^3, ~ 4 weeks after inoculation) at which time Å6 administration was initiated. Animals received Å6

(75 $mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle alone for 6 wk and tumor volumes were determined at weekly intervals. Administration was discontinued after 6 wk and the animals killed and evaluated at the end of week 7. Å6 administration resulted in a significant (~90%) decrease in the primary tumor volume (Fig. 3.3C), which was clearly evident upon visual inspection of killed animals (Fig. 3.3D). Necropsy examination revealed that control animals receiving vehicle alone routinely developed a large number of macroscopic tumor metastases in the lungs and auxillary lymph nodes. In contrast, Å6 treatment resulted in significantly fewer and smaller metastases to lymph nodes and a trend to decreased macroscopic metastases to lungs (Fig. 3.3E). We also evaluated the number of microscopic tumor foci present in other organs (liver, lungs, and spleen). Microscopic dissemination was observed only in these organs and the number of metastatic tumor cells was significantly lower in the liver and lungs of Å6-treated animals when compared to vehicle controls (Fig. 3.3F). No significant difference in the number of disseminated tumor cells was observed in the spleens of control and Å6 treated group of animals.

Å6 treatment leads to extensive tumor necrosis.

Necrotic cores are often observed in rapidly growing experimental tumors, and we observed this in both the syngeneic (Mat B-III) and the xenograft (MDA-MB-231-GFP) models. Although the core necrosis (~ 20% of the total tumor volume) might be expected to occur as a result of hypoxia, this is not the case at the periphery of the tumors. The periphery of primary Mat B-III control tumors appeared predominantly viable when assessed by H&E staining (Fig. 3.4A). In contrast, H&E staining of sections from Å6-treated tumors revealed large areas of haemorrhage necrosis (in this context, necrosis is used to describe tumor cell death, regardless of the mechanism or pathway involved in that process), which comprised greater than 75% of the peripheral tumor area (Fig. 3.4B). TUNEL staining of tumor sections revealed very few TUNEL-positive cells in sections obtained from control Mat B-III tumors (Fig. 3.4C). However, sections obtained from Å6-treated tumors revealed extensive positive staining by TUNEL (Fig. 3.4D). TUNEL-positive foci were significantly more numerous in Å6 treated animals and were observed in both

necrotic and non-necrotic regions suggesting that one mechanism that could be responsible for cell death was apoptosis (Fig. 3.4, bottom). Å6-treatment of tumor cells did not inhibit proliferation or directly lead to apoptosis *in vitro* (data not shown). Similar results were also observed in sections obtained from MDA-MB-231-GFP xenograft tumors (data not shown).

Å6 treatment results in decreased tumor vessel hot spots.

Based on the ability of Å6 to inhibit endothelial cell migration *in vitro*, we decided to evaluate whether Å6 had any effect on tumor vessel formation *in vivo*. We used anti-Factor VIII to stain both rat and mouse blood vessels in our respective models. Sections from Å6-treated tumors had significantly less Factor VIII-positive hot spots than sections from control Mat B-III tumors (Fig. 3.5). Similar results were observed in sections obtained from the MDA-MB-231-GFP xenograft model (data not shown).

3.6 Discussion

The urokinase plasminogen activator system has pleiotropic roles in tumor growth, angiogenesis and metastasis. Initial studies on the role of this system in tumor progression focused on the proteolytic cascades initiated by uPA, mediated by the catalytic B-chain, which resulted in matrix remodeling and allowed for the invasion of tumor cells and the migration of endothelial cells. More recently, signaling cascades mediated by the binding of uPA to uPAR via the GFD have been described although their role in tumor progression is thus far poorly understood.

In this report, we describe the activity of a peptide, Å6, derived from the connecting peptide region of uPA that is capable of inhibiting both of these cascades simultaneously through its inhibition of the uPA-uPAR interaction. Since Å6 is derived from the connecting peptide (a.a. 136-143) of uPA, it suggests that this region of uPA may represent a novel epitope involved in the uPA-uPAR interaction. Franco et al [517] have recently demonstrated that Ser 138 (corresponding to Ser 3 in the Å6 sequence) is phosphorylated in HMW uPA and that this phosphorylation abrogates the ability of phosphorylated HMW uPA to mediate monocyte chemotaxis. From a

drug development standpoint, Å6 is especially intriguing since it apparently inhibits the uPA-uPAR interaction in an allosteric manner, perhaps by altering or stabilizing a particular conformation of one or both of these proteins. Å6 does not inhibit the growth of any cell line tested thus far *in vitro* including tumor and endothelial cells. However, despite this lack of direct cytotoxic or anti-proliferative activity, Å6 treatment leads to a striking suppression of tumor growth and metastasis in several animal models using breast cancer cell lines.

Å6 inhibits the tumor growth of the MDA-MB-231 cells to a greater extent than the Mat B-III cells *in vivo*. Mat B-III tumors grow much faster than the MDA-MB-231 tumors *in vivo* and may overcome the inhibitory effects of Å6. Our data suggests that Å6 maintains tumor dormancy and a rapidly proliferating tumor such as the Mat B-III might overcome this suppressive effect much more rapidly than a more indolent tumor such as the MDA-MB-231. This seems to be an emerging paradigm for anti-angiogenic agents when they are used as monotherapy in pre-clinical models, as no single anti-angiogenic agent (including angiostatin and endostatin) is able to reduce tumor burden once tumors have reached 100 mm^3 [518]. The MDA-MB-231 model may be more relevant to human disease as most human solid tumors (including breast) are not highly proliferative. We also present *in vitro* data on the ability of Å6 to inhibit tumor cell invasion and human endothelial cell migration and the differences observed with Å6 treatment *in vivo* could reflect species specificity. The high-affinity binding of uPA to uPAR (via its growth factor domain) is species specific and the affinities of the uPA for uPAR across species (for example, human uPA binding to rat uPAR) differ by at least two orders of magnitude. However Å6 may represent a secondary, weaker affinity interaction between uPA and uPAR that modulates conformation rather than direct binding and it is not known whether this interaction is species specific. In addition, our data on the effects of Å6 on the uPA-uPAR interaction have only been obtained using pure proteins and have not been extended to cell systems. Therefore, the activity of Å6 against tumor cells or endothelial cells could occur via some non-uPAR-dependent mechanism as well.

Å6 is derived from the human uPA sequence and the corresponding sequence from rat and mouse are KPSSTVDQ and KPSSSVDQ. Since the KPSS is conserved in all species, it is tempting to speculate that this may represent the biologically active sequence of the peptide. We demonstrate in Figure 2A that deletion of the N-terminal lysine abolished the anti-migratory effect of Å6 indicating that the correct N-terminal amino acid is important for the activity of Å6. However, similar results were also observed when the C-terminal amino acid was removed. The C-terminal amino acid is semi-conserved (Gln in rat and mouse and Glu in human) and perhaps this is sufficient to maintain activity of the peptide. However, it is difficult to make conclusions regarding species specificity based on the data presented in this report without knowing the contribution of each amino acid to activity.

Å6 may inhibit metastasis in several interdependent ways. Inhibition of tumor growth may directly lead to decreased metastasis simply through a reduction in tumor burden. Smaller tumors may be less vascularized, affording less opportunity for hematogenous dissemination. Å6 may also directly suppress the outgrowth of disseminated tumor cells once they have seeded. Disseminated tumor foci also depend on angiogenesis to survive and proliferate. The anti-angiogenic effects of Å6, described below, could suppress the ability of small tumor foci to recruit blood vessels necessary for survival. The inhibition of metastasis by Å6 could also result from direct anti-invasive effects on the tumor cells themselves. The ability of tumor cells to invade depends on multiple cellular activities such as mobility, adhesion and cytoskeletal reorganization occurring in a temporally organized manner. Since Å6 is able to inhibit the invasion of tumor cells *in vitro*, it is possible that it interferes with one or more of these activities *in vivo* as well.

Our data also demonstrates that Å6 also has anti-angiogenic activity resulting in substantially less Factor VIII-positive hot spots in sections obtained from Å6-treated animals. The effects of Å6 on vascular contractility and signaling have also recently been demonstrated by Higazi et al (see companion manuscript), suggesting another possible hypothesis to explain the activity of Å6 [273]. The inhibitory effects of Å6 on vascular contractility may impede blood flow to the tumor in addition to its

effects on angiogenesis. Tumor neovessels, which are prone to collapse and occlusion, may be especially susceptible to an agent, such as Å6, that dysregulates vascular tone. Aggressive, rapidly growing tumors contain large zones of hypoxic cells. Low oxygen tension results in the up-regulation of both vasoconstricting (e.g. endothelin-1) as well as angiogenic (VEGF, PDGF) growth factor expression by endothelial cells [295]. Hypoxia has been implicated as a catalyst for the initiation of tumor angiogenesis [519] and may mediate both new vessel formations (long term effect) as well as local vascular contractility within a tumor. The fact that Å6 antagonizes the activity of vasoconstricting agents such as phenylephrine and endothelin-1 suggests that part of its anti-tumor effects may result from a suppression of hypoxia-dependent pathways of angiogenesis in addition to the inhibition of vessel formation. Further, Å6 does not seem to affect non-stimulated vessels as no effects on vasoconstriction were observed by Higazi et al [273] in the absence of vasoconstricting stimuli. Experiments are currently underway to examine the effects of Å6 on the expression of hypoxia-related genes and the initiation of angiogenesis in tumors (angiogenic switch). This hypothesis is consistent with the data observed in tumor models in which Å6 treatment must be started before a tumor reaches a certain critical size, after which time angiogenesis has already initiated and Å6 treatment alone is no longer sufficient to inhibit tumor progression.

The inhibition of vessel formation through either an anti-angiogenic or an anti-vascular mechanism of action could also lead to increased tumor cell death through apoptosis or related mechanisms. Clinical agents (e.g. Combretastatin) that disrupt tumor blood flow have demonstrated potent anti-tumor effects leading to tumor cell death [520]. Despite extensive TUNEL staining in sections derived from Å6-treated tumors, we cannot conclude that this represents apoptosis since any process that leads to DNA fragmentation would also result in TUNEL-positive staining. Nevertheless, the fact that tumor cell death (histological examination of tissues obtained from Å6-treated animals revealed no evidence of toxicity or non-tumor necrosis) occurs in the absence of any direct cytotoxic effects points to

modulation of the tumor microenvironment by Å6 as one likely aspect of its mechanism of action.

We are currently in the process of identifying more potent analogues of Å6. These will be used to further elucidate the molecular basis of the anti-tumor effects described in this manuscript. The effect of an allosteric inhibitor such as Å6 cannot be competed away by ligand, making such a compound especially useful for inhibiting receptor-ligand interactions such as the binding of uPA to uPAR.

3.7 Acknowledgements

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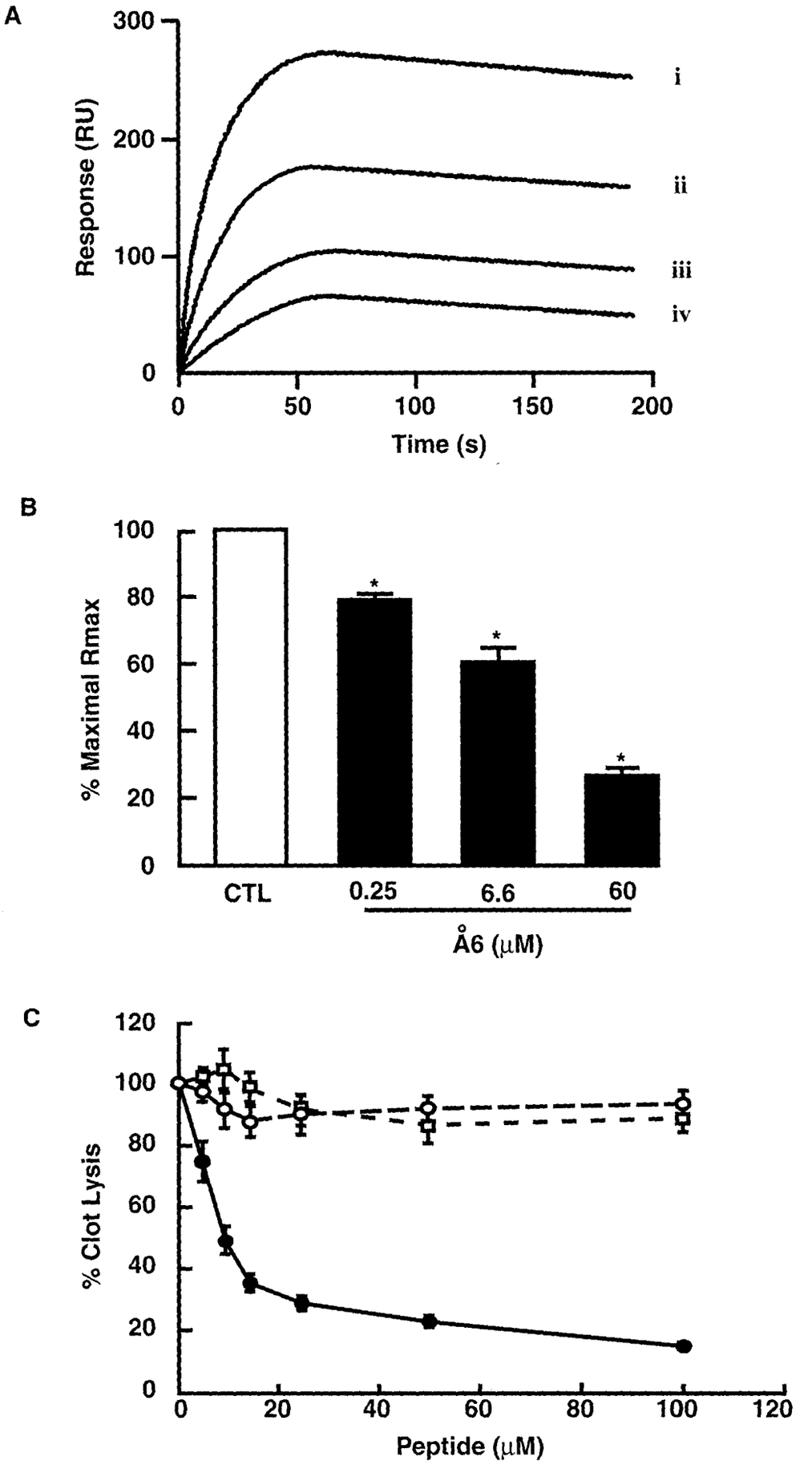

A
300
200
100
0
Response (RU)
i
ii
iii
iv
0
50
100
150
200
Time (s)
B
100
80
60
40
20
0
% Maximal Rmax
*
*
*
CTL
0.25
6.6
60
Å6 (μM)
C
120
100
80
60
40
20
0
% Clot Lysis
0
20
40
60
80
100
120
Peptide (μM)

3.8 Figures

Fig. 3.1. Inhibition of uPA/uPAR interaction by Å6.

A) Sensorgrams of 100 nM suPAR binding to immobilized scuPA in the absence (i) and presence (ii, iii, iv) of Å6. Concentrations of Å6 shown are 0.25 μM (ii), 6.6 μM (iii) and 60 μM (iv). B) Plot of maximal binding from panel A, demonstrating that Å6 decreases the B_{max} in a dose-dependent manner. The clear bar denotes the absence of Å6 (CTL). C) Effect of increasing concentrations of Å6 on clot lysis in the presence of scuPA-suPAR complex (●----●), tcuPA (O----O), scuPA-suPAR+Å29 (□----□). Results represent the mean ± SEM of four different experiments. Significant difference from control is denoted by asterisks ($P<0.05$).

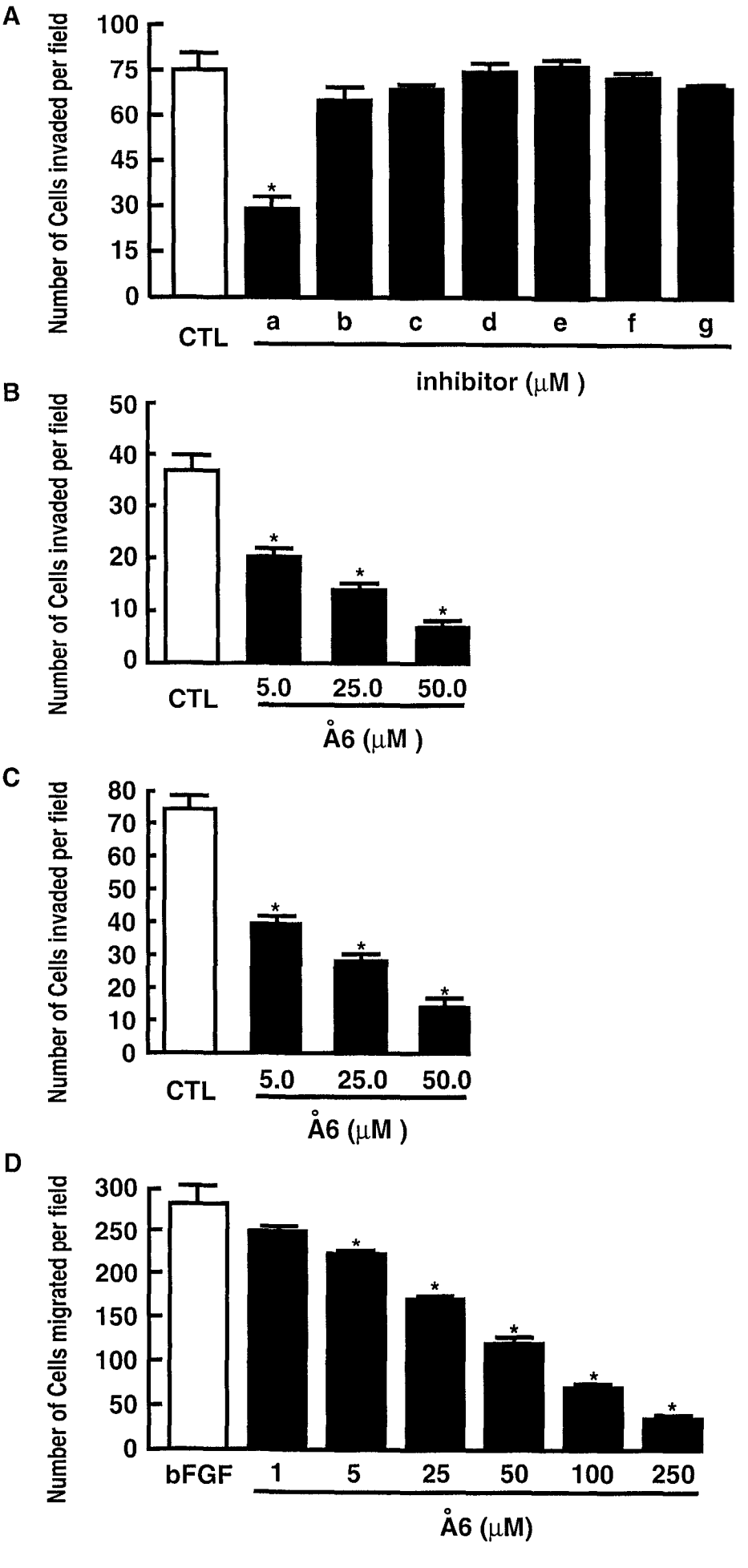
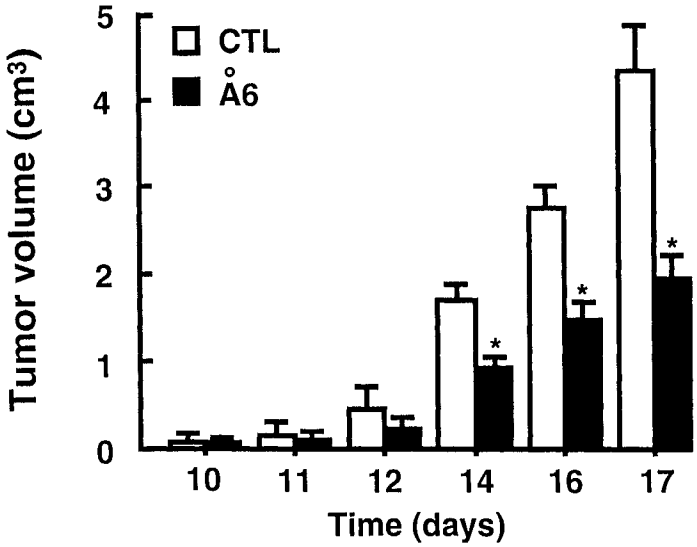

A
Number of Cells invaded per field
100
75
60
45
30
15
0
CTL
a
b
c
d
e
f
g
inhibitor (μM)
B
Number of Cells invaded per field
50
40
30
20
10
0
CTL
5.0
25.0
50.0
Å6 (μM)
C
Number of Cells invaded per field
80
70
60
50
40
30
20
10
0
CTL
5.0
25.0
50.0
Å6 (μM)
D
Number of Cells migrated per field
300
250
200
150
100
50
0
bFGF
1
5
25
50
100
250
Å6 (μM)

Fig. 3.2. Effect of Å6 on breast cancer cell invasion and an endothelial cell migration.

5 x 10^4 human (MDA-MB-231-GFP) or rat (Mat B-III) breast cancer cells were added to the upper compartment of the Boyden chambers. Fibronectin was added to the lower chamber as a chemoattractant. MDA-MB-231-GFP cells were tested in the presence of Å6 (a) Å7 (b) Å8 (c) Å9 (d) Å10 (e) Å13 (f) Å14 (g), or vehicle alone (CTL) (*A*). Mat B-III and MDA-MB-231-GFP cells were also tested in the presence of different concentrations of Å6 or vehicle alone. Total number of rat (*B*) and human (*C*) breast cancer cells migrating to the lower aspect of Boyden chamber filters was counted. Number of cells invading in the presence of vehicle alone was used as control (CTL). HDMVC cells were tested in the presence of bFGF (1.0 ng/ml) or bFGF plus different concentrations of Å6. The number of cells migrating per field were counted and compared with that of bFGF (*D*) Results represent the mean ± SEM of four different experiments. Significant difference in inhibition by Å6 from control is denoted by asterisks ($P<0.05$).

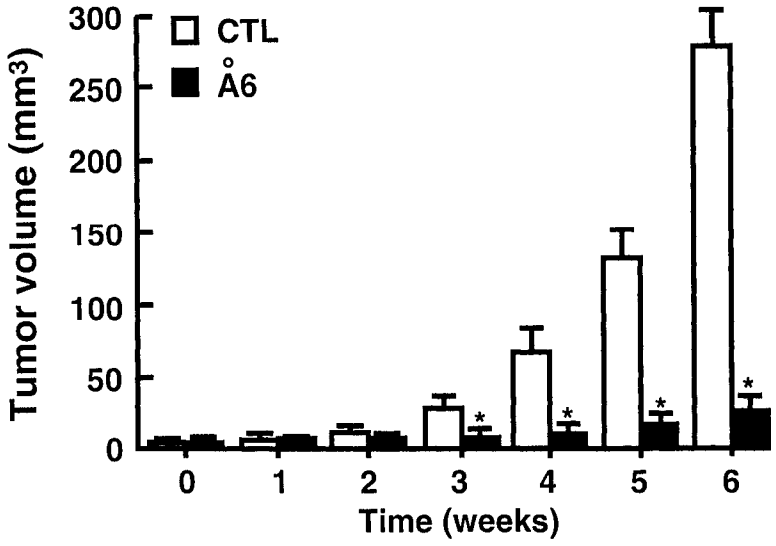
A



B

TISSUE	CONTROL	Å6
LUNG	5 ± 1	2 ± 1
R.P. LYMPH NODES	4 ± 2	1 ± 1
AX. LYMPH NODES	2 ± 1	1 ± 0

C



D

E

	TISSUE	CONTROL	Å6
Macroscopically (number of tumor foci)	LUNG	6 ± 2	3 ± 1
	AX. LYMPH NODES	5 ± 2	1 ± 1
Microscopically (tumor cells per field)	LUNG	4.23 ± 0.18	2.09 ± 0.30
	LIVER	18.83± 0.36	3.11 ± 0.14
	SPLEEN	35.67 ± 0.82	28.30 ± 2.80

F

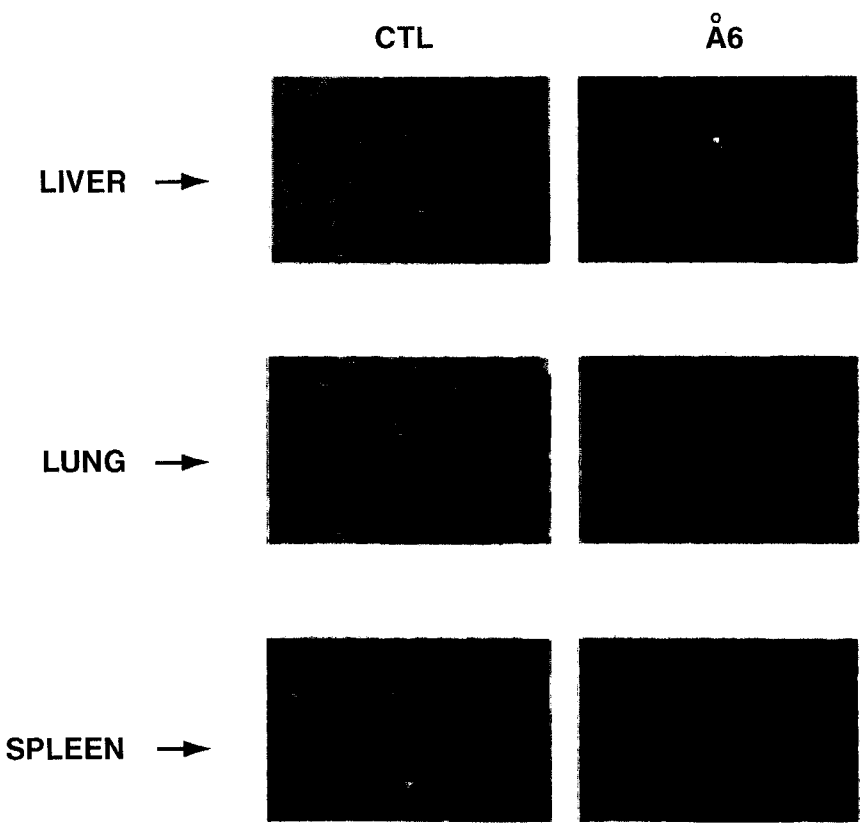
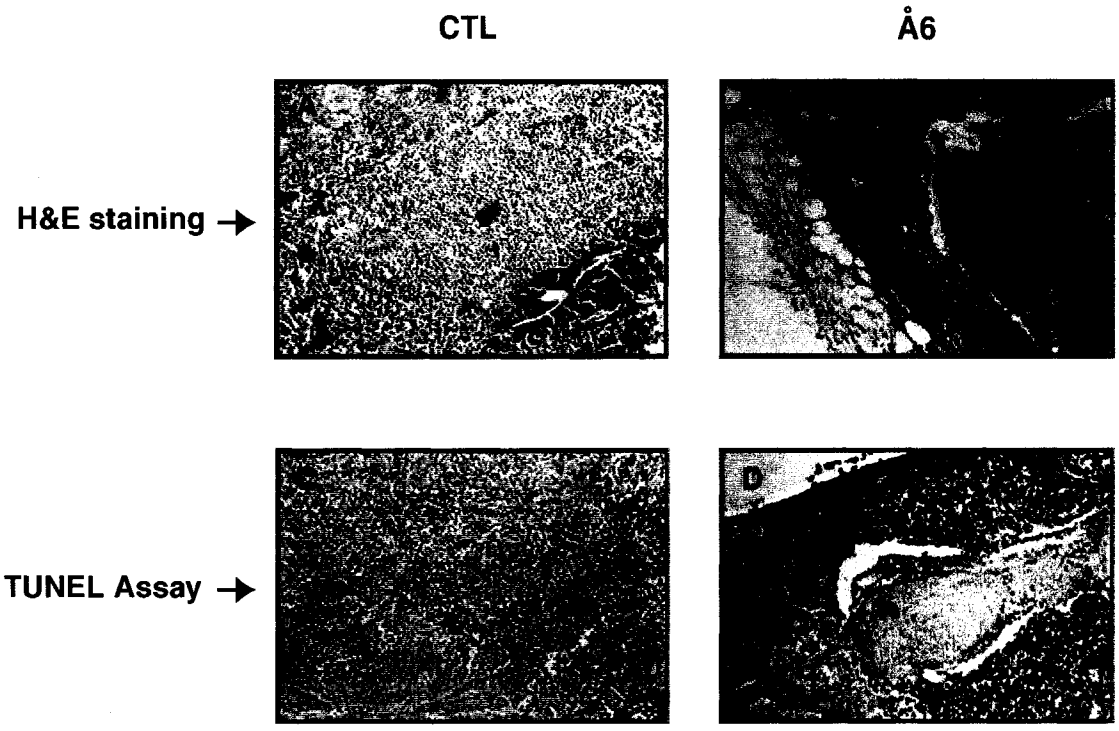


Fig. 3.3. Effect of Å6 on tumor growth and metastases.

Mat B-III tumor bearing female Fischer rats were injected i.p with Å6 or vehicle alone for 16 days. Tumor volume in control (CTL) and experimental (Å6) animals was determined at timed intervals (*A*). At day 17 post tumor inoculation, animals were sacrificed and the total number of macroscopic metastatic foci were counted (*B*). MDA-MB-231-GFP tumor bearing BALB/c (*nu/nu*) mice were injected i.p with Å6 or vehicle alone (CTL) and tumor volume determined at weekly intervals (*C*). Following 6 weeks of treatment, gross tumor mass was compared in control (CTL) and Å6 treated animals (*D*). (*E*) At the end of this study, control and experimental mice were sacrificed to count the number of macroscopic tumor foci (*E*). Fluorescent tumor cells in control and Å6 tissues were also counted under a microscope (*E*). A representative photomicrograph of each organ from control (CTL) and Å6 treated animals (Å6) from three such experiments is shown (*F*). Results represent the mean ± SEM of 6 starting animals in each group in 4 different experiments. Significant difference from control tumor bearing animals after treatment with Å6 is denoted by asterisks ($P<0.05$).


CTL
Å6
H&E staining
TUNEL Assay

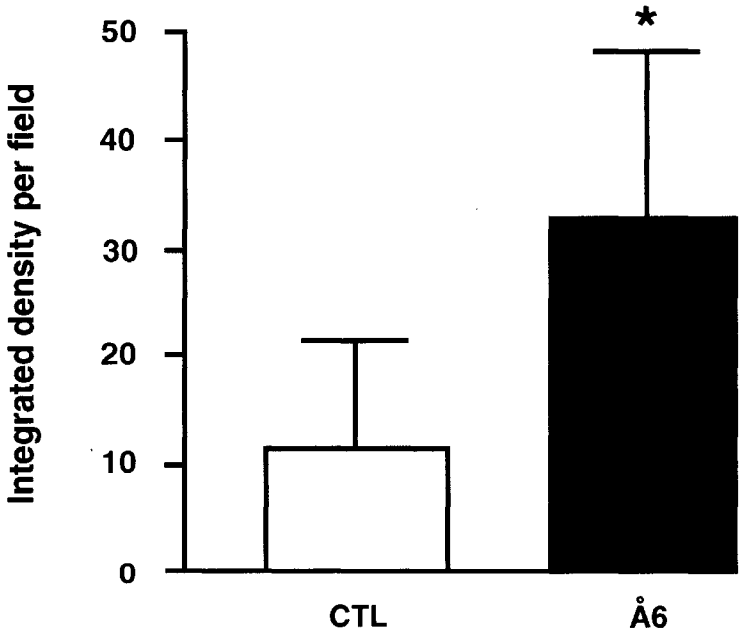
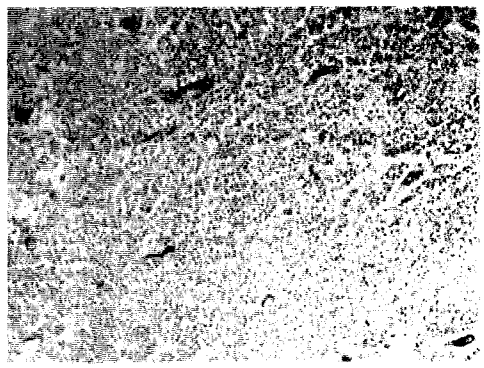

Integrated density per field
50
40
30
20
10
0
*
CTL
Å6

Fig. 3.4. Histological examination of Å6 treated Mat B-III tumors.

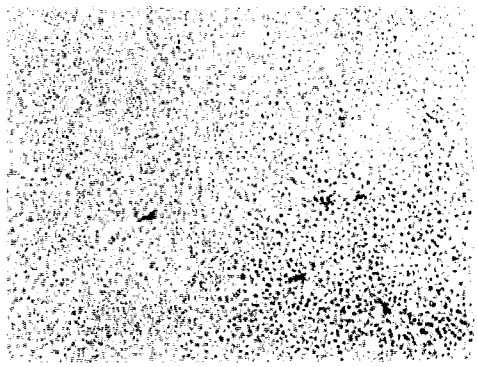
Top: Control (*a, c*) and experimental animals (*b, d*) were sacrificed and their tumors were removed. All tumors were formalin-fixed and paraffin embedded. Sections (4 μm) were prepared and analyzed by H & E staining (*a, b*) and TUNEL assay (*c, d*) (100 X magnification). Bottom: TUNEL-positive cells were quantitated as described in Material and Methods. Results represent the mean ± SEM of three determinations in each tumor. Significant difference from control (CTL) is denoted by asterisks ($P<0.05$).

A)

CTL



Å6



B)

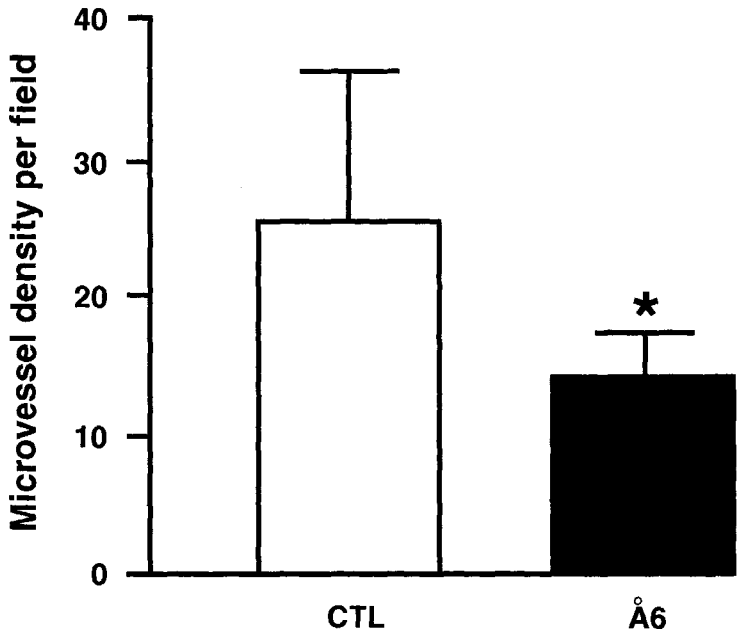


Fig. 3.5. Immunohistochemical analysis of Å6 treated Mat B-III tumors.

All primary tumors were removed, formalin fixed and paraffin embedded. Sections (4 μm) were prepared and stained with anti-factor VIII antibody. Representative photomicrograph from control (CTL) and Å6 treated animals (Å6) is shown (100 X magnification). *B*) Areas of high vascularization in control and Å6 treated tumors were counted for microvessel density as described in Materials and Methods. Results represent the mean ± SEM of three determinations on each tumor. Significant difference from control (CTL) is denoted by asterisks ($P<0.05$).

Chapter IV

An Anti-Angiogenic Urokinase Derived Peptide (Å6) Combined with Tamoxifen (TAM) Decreases Tumor Growth and Metastasis in a Syngeneic Model of Breast Cancer

4.1 Preface

Accumulating evidence has indicated that the uPA/uPAR system is related to prognosis in primary as well as advanced breast cancer. The efficacy of the response of this cancer to hormonal therapy such as tamoxifen (TAM) in recurrent disease is reduced following treatment. In the previous chapter, I reported the development of an 8-mer peptide, Å6, derived from the non-connecting peptide region of uPA. Intraperitoneal injection of Å6 results in the suppression of primary breast tumor growth in nude mice as well as the metastases formation. These inhibitory effects have been attributed to the inhibition of tumor cell invasion, tumor angiogenesis and the induction of tumor cell apoptosis (see section 3.5). Å6 is currently under evaluation in Phase I clinical trials. Having characterized Å6 as an effective agent blocking the progression of breast cancer, I further evaluated the ability of Å6 to block breast cancer progression when used in conjunction with other chemotherapeutic agents, such as TAM.

TAM was initially developed as an agent that antagonizes the effects of estrogen via its receptor. It shares structure homology with the receptor-binding site of estrogen, but is not able to elicit the functions of ER. Because the role of ER is important in the progression of breast cancer, TAM has been shown to be effective in impairing the progression of ER-positive breast cancer [317, 521]. The major tumor inhibitory effects of TAM are elicited through the competition of ER with estrogen, TAM is not effective in late stage breast cancer that is ER-independent, meanwhile, even TAM-responsive tumors eventually develop resistance to TAM [312], and the mechanism is poorly defined. Therefore, there is a pressing need to find an effective chemotherapy regimen that is effective in blocking breast cancer progression even at its late stage.

My data presented in Chapter III showed that Å6 is effective in blocking breast cancer progression regardless of stage, suggesting that Å6 is a universal chemotherapeutic agent for breast cancer. However, its effects may be greatly enhanced if used in combination with other agents. Combination therapy has beneficial effects that may improve the outcomes and survival rate of breast cancer patients [372], and prolong the disease-free period. When used together, drug

interactions may elicit additive or synergistic effects and may allow the dosage of each individual drug to be decreased, thus further limiting the probability of side effects.

In this chapter, we explore the effects of Å6 on a rat breast cancer model in combination with TAM. As discussed in the following section, this combination exerts an additive effect in the inhibition of breast cancer progression as compared to that of either agent used alone, suggesting a potential clinical application for a combination therapy using these two agents.

The material discussed in this chapter has been written as a manuscript entitled "An Anti-Angiogenic Urokinase Derived Peptide (Å6) Combined with Tamoxifen (TAM) Decreases Tumor Growth and Metastasis in a Syngeneic Model of Breast Cancer", which has been submitted to review in *Cancer Research*.

4.2 Abstract

Expression of urokinase (uPA) and its receptor (uPAR) are associated with increased tumor cell invasion and metastasis in several malignancies including breast cancer. An 8-mer peptide derived from the non-receptor binding domain of uPA (Å6) has been shown to have anti-angiogenic and pro-apoptotic effects to block breast cancer progression *in vivo*. In the current study we have evaluated the effect of Å6 and the anti-estrogen tamoxifen (TAM) alone and in combination on estrogen receptor positive Mat B-III rat breast cancer cells *in vitro* and *in vivo*. Treatment of Mat B-III cells with Å6 and TAM resulted in a dose-dependent decrease in tumor cell invasion through Matrigel, these effects were more marked when Å6 and TAM were tested in combination. In addition, treatment of Mat B-III cells with Å6 or TAM resulted in a significant reduction of vascular endothelial growth factor receptor (flk-1) expression and TGF-β activity, effects which were significantly higher following combined treatment with Å6 and TAM. For *in vivo* studies, female Fischer rats were inoculated with Mat B-III cells (1×10^6) into the mammary fat pad. These orthotopic tumors were staged to 30-40 mm^3 in volume at which time treatment was initiated with Å6 (75 mg/kg/day), TAM (3 mg/kg/day) alone or in combination. Both Å6 and TAM caused a significant reduction in tumor volume, however these anti-tumor

effects were significantly greater in animals receiving both Å6 and TAM where a 75% reduction in tumor growth as compared to control animals was observed. The number of macroscopic tumor foci was significantly reduced in Å6 treated animals, while TAM failed to exhibit any anti-metastatic effects. Histological analysis of primary tumors from different groups showed decreased new blood vessel density and increased tumor cell death in Å6 and TAM treated animals and these effects were greater in experimental animals receiving Å6 and TAM in combination. Collectively, these studies demonstrate that the addition of novel anti-angiogenic/anti-metastatic agents like Å6 to hormone therapy can enhance the anti-tumor effects of hormone therapy through increased inhibition of angiogenesis and induction of tumor cell death.

4.3 Introduction

Urokinase (uPA) plays a key role in degrading extracellular matrix (ECM) and basement membrane in various cancers (such as breast and prostate cancers) and therefore promotes metastasis and angiogenesis [219, 266, 522]. uPA is secreted as a single chain zymogen (scuPA) that exhibits very low or no intrinsic enzyme activity [523]. After enzymatic cleavage by plasmin, the scuPA is converted into an active, disulfide bond-linked two-chain high molecular weight uPA (HMW-uPA). This HMW-uPA comprised of an A-chain (a.a. 1~158), and a low molecular weight uPA (LMW-uPA) (a.a. 159~411) that contains the catalytic activity for ECM degradation [524]. Two sub-domains are located within the A-chain; the growth factor domain (GFD) is the site that mediates binding of uPA to its cell surface receptor, uPAR, and a single kringle domain [163, 525]. In addition to the uPAR, uPA binds with low affinity to an unidentified membrane associated protein to trigger uPA-induced signal transduction [526]. In addition to the GFD and kringle, the A-chain contains a connecting peptide (a.a. 136~158). Phosphorylation of uPA at Ser138 within this region has been demonstrated to abolish the adhesion of chemotaxis of myelomonocytic cells [187]. An 8-mer capped peptide Å6 (a.a. 136-143), derived from this region was previously demonstrated to inhibit cell motility and contractility including endothelial cell migration and tumor cell invasion (Fig. 3.1) [495].

Breast cancer is one of the leading malignancies affecting women, and results in a high incidence of morbidity and mortality [527]. In hormone-dependent breast cancer, the steroid hormone estrogen stimulates tumor cell proliferation [521, 528]. The use of anti-estrogen agents such as tamoxifen (TAM) is thus a standard therapeutic regimen for patients with estrogen receptor (ER) positive tumors [521, 529]. In addition to steroid hormones, the expression of proteolytic enzymes, such as uPA, promotes breast cancer progression by enhancing angiogenesis and tumor cell invasion [274, 495].

A series of basic and clinical studies have demonstrated a correlation between uPA production and tumor invasiveness and disease stage in several malignancies including breast cancer [258, 259, 530]. Analysis of uPA production in a variety of human breast cancer cell lines has revealed high levels of uPA expression in highly invasive human breast cancer cells MDA-MB-231. In contrast, uPA mRNA was undetectable in low invasive MCF-7 cells. This lack of uPA expression in MCF-7 cells was due to hypermethylation of the uPA promoter region resulting in silencing of *uPA* gene transcription (see Chapter II) [22]. Previous reports by others and us have demonstrated the species specificity of uPA actions where human uPA fails to interact with rat uPAR [108, 135]. These studies underscore the significance of the uPA/uPAR interaction in an allogeneic or syngeneic system that can allow complete interaction between uPA/uPAR produced by tumor cells with host proteins produced by tumor surrounding stromal cells to fully elicit its effects on tumor angiogenesis and tumor cell death [495].

In the current study, we have used the ER positive rat breast cancer cell line Mat B-III, which produces high levels of uPA and serves as a useful model for the study of breast cancer progression. Using this syngeneic *in vivo* model of breast cancer, we have examined the ability of Å6 alone and in combination with TAM to decrease Mat B-III tumor invasion and metastasis *in vivo*, and explored the underlying mechanisms of the inhibitory effects.

4.4 Materials and Methods

Cells and cell culture

The rat mammary adenocarcinoma cell line Mat B-III was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in culture *in vitro* in McCoy's 5A modified medium (Gibco BRL, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 25 mM 4-(2-hydroxyethy)-1-piperazineethanesulfonic acid, 26 mM sodium bicarbonate, 100 units/ml of penicillin-streptomycin sulphate (Gibco BRL) and 0.2% gentamycin (Sigma, St. Louis, MO).

***In Vitro* assays**

The effect of Å6 and TAM on Mat B-III cell invasion was determined by two compartment Boyden chambers (Transwell, Costar, Cambridge, MA) and basement membrane Matrigel invasion assay as previously described [11, 495]. The 8 μm pore polycarbonate filters were coated with basement membrane Matrigel (50 μg/filter). Matrigel was then reconstructed by adding 0.1 ml serum-free culture medium to the upper chamber and incubated for 90 min. After removal of medium, cells (5×10^4) in 0.1 ml of medium with or without Å6 or TAM were added to the upper chamber and placed in a lower chamber prefilled with 0.8 ml of serum-free medium supplemented with 25 μg/ml fibronectin (Sigma, Oakville, ON, Canada) and incubated at 37°C for 24 hr. At the end of incubation, medium was removed and filters were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde (Sigma) in 0.1 M phosphate buffer, pH 7.4 at room temperature (RT) for 30 min. After washing with PBS, all filters were stained with 1.5% toluidine blue and filters were mounted onto glass slides. Cells were examined under a light microscope. Ten fields under 400x magnification were randomly selected and the mean cell number was calculated.

In the dose-response studies the effect of Å6 and TAM on Mat B-III cell proliferation was determined by measuring the reduction of Alamar blue (AB) as described by the manufacturer (Serotec, Kidlington, U.K.) with modifications. The assay is based on metabolic reduction of the AB dye into a fluorescent species, which can easily be detected after excitation of the reduced AB dye at 560 nm and subsequent emission at 580 nm. The assay was adapted to 96-well fluorescent plates with clear-bottomed wells, which allowed direct reading of the plates without sample transfer. AB reduction was measured every second day, by removal of the growth

medium and substituting it with AB-containing medium. Reduction of AB was determined at 2 hr after addition of the AB substrate, using a total volume of 300 µl of a 1:20 diluted AB stock solution per well. Fluorescence was measured at a slit width of 5 nm for both excitation and emission using a Perkin-Elmer luminescence spectrophotometer LS 50B, equipped with a microtiter plate reader [531]. After reading the plates, the AB-containing medium was removed, the cells were rinsed twice with PBS, and fresh medium containing the test compounds was added to the plates. The proliferative activity after 4 days of treatment is presented as absorbence at 580 nm.

In order to evaluate the effect of Å6 and TAM on TGF-β activity, Mat B-III cells were seeded at a density of 2×10^5 per 6-well plate. Cells were transfected 24 hr after with 5 µg of the reporter plasmid p3TP-Lux (kindly provided by Dr. J.J. Lebrun, McGill University), together with pSV-β-galactosidase Control Vector (1 µg), using Lipofectamine [532]. 15 hr later, the medium was changed to McCoy's 5A modified medium containing 4% FCS, and the cells were incubated for an additional 9 hr followed by treatment with Å6 (5.0 µM), TAM (100.0 nM) or Å6 and TAM for 48 hours. Cells were lysed, and the luciferase activity was measured and normalised to the relative β-galactosidase activity as described [532].

For Western blot analysis to determine the level of flk-1 production, Mat B-III cells treated with vehicle, Å6 (5.0 µM), TAM (100.0 nM), or Å6 + TAM were homogenised in RIPA buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 8 µM aprotinin, 2 mM phenylmethylsulfonlfluoride, 10 mM Leupeptin and 2 mM sodium orthovanadate). The supernatant was removed and total protein concentration was determined by Bio-rad protein assay (Bio-rad Laboratories, CA). 50 µg of total protein from various groups of Mat B-III cells was separated by SDS-PAGE in 7.5% gels under reducing conditions. Proteins in the gel were electrophoretically transferred to nitrocellulose membranes and reacted with antibodies at a dilution of 1:50 with 0.5% skimmed milk in buffered saline (pH 7.5). All membranes were soaked in buffer containing flk-1 or peroxidase conjugated anti-mouse rabbit immunoglobulin for flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were analyzed by developing with ECL Kit (Amersham). Level of flk-1

production was quantified by densitometric scanning and plotted as intensity per mm^2 as previously described [533].

For PAI-1 ELISA, Mat B-III cells were treated with vehicle alone, Å6 (5.0 μM), TAM (100.0 nM), Å6 and TAM for 72 hr. Cells conditioned culture medium from control and experimental cells was collected and tested at different concentrations for PAI-1 production using a rat PAI-1 ELISA kit (American Diagnostica Inc., Greenwich, CT) according to manufacturer's instructions.

Animal Protocols

Inbred female Fischer 344 rats weighing 200-220 g were obtained from Charles River Inc. (St. Constant, QC, Canada). Before inoculation, Mat B-III tumor cells grown in serum-containing medium were washed with Hank's balanced buffer and trypsinized for 5 min. Cells were then collected in Hank's balanced buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (1×10^6 cells) were resuspended in 0.2 ml saline and injected using 1 ml insulin syringes into the mammary fat pad of rats anaesthetised with ethanol/Somnotal (MTC Pharmaceuticals, Cambridge, ON, Canada). Control and experimental animals were injected intraperitoneally (i.p.) with PBS, Å6 (75 mg/kg/day), TAM (3 mg/kg/day) or Å6 and TAM in combination twice a day for 17 days. All animals were numbered, kept separate and examined for the development of tumors daily for up to 18 days. The tumor mass of control and experimental animals was measured in two dimensions by callipers and the tumor volume was calculated. Control and experimental animals were sacrificed at the end of this study (day 18) and examined and scored for the development of macroscopic tumor metastases in various tissues [11, 108, 495]. Primary tumor tissues were also removed from control and experimental animals for histologic examination.

Histology and Immunohistochemistry

Primary tumors were fixed in 4% paraformaldehyde overnight, dehydrated and embedded in paraffin (Fisher Scientific, Montreal, QC, Canada) the next day. Tumor sections (4 μm) were deparaffinized, then rehydrated and stained with hematoxylin/eiosin (H&E). For TUNEL assay, paraffin-embedded tissue sections were soaked in toluene to deparaffinize, then rehydrated in graded alcohol series (100% to 70%). For enzyme predigestion of formalin-fixed tissue, the sections were

incubated for 30 min at RT in 15 μg/ml of proteinase K/10 mM Tris/HCl. The terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim, QC, Canada) diluted in TUNEL reaction mixture, was added to the slides and incubated at 37°C for 60 min. After rinsing with PBS, the sections were coated with 50 μl of anti-fluorescein antibody conjugated with converter-POD (horseradish peroxidase) at 37°C for 30 min. For factor VIII staining enzyme predigestion of formalin-fixed tumors was done by incubation at 37°C in 0.1 g Pronase type 14/100 ml PBS, followed by washing in 10 mM PBS (pH 7.6). Anti-human endothelial cell antibody against factor VIII-related antigen (Von Willebrand factor) (DAKO Diagnostica Inc., Mississauga, ON, Canada) was used as primary antibody diluted in PBS (1:600). Tumor sections were incubated for 60 min at RT followed by further incubation for 30 min with biotinylated anti-rabbit link antibody (Zymed Laboratories Inc., San Francisco, CA). Sections were rinsed with PBS, followed by coating with streptavidin conjugated to horseradish peroxidase for 10 min. The substrate DAB was then incubated with factor VIII-related antigen sections for 10 min at RT (Sigma, Oakville, ON, Canada). Finally, the sections were counterstained with hematoxylin and mounted. All histologic examinations were carried out by light and fluorescence microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattlevoro, VT). For quantitation of TUNEL-positive cells, three sections from each tumor were analyzed using NIH image version 1.61 and expressed as integrated density per field of examination. In control and experimental tumors, microvessels were counted and expressed as angiogenesis density representing the mean of at least three areas with high vascularization in three different sections from each tumor. All slides were interpreted by two independent investigators [495].

Statistical Analysis

Results are expressed as the mean ± SD of at least triplicate determinations and statistical comparisons are based on Student's *t* test or analysis of variance. A probability value of <0.05 was considered to be significant.

4.5 Results

Effect of Å6 and TAM on Mat B-III Cell Invasion and Proliferation *in vitro*

The effect of different concentrations of Å6 (1.0~5.0 μM) and TAM (10.0-100.0 nM) on the invasive capacity of Mat B-III was determined using a modified two compartment Boyden chamber Matrigel invasion assay. Treatment with both Å6 and TAM caused a significant dose-dependent decrease in the number of cells invading through Matrigel as compared to vehicle treated control. Both Å6 and TAM demonstrated similar anti-invasive effects as stand alone agents (30~50%) for Å6 and 40~60% for TAM over the range of concentrations tested (Fig. 4.2A and B). An additive inhibitory effect (45~80%) was observed when cells were treated with the combination of Å6 and TAM (Fig. 4.2C).

The effect of Å6 (5.0 μM) or TAM (100.0 nM) alone and in combination on Mat B-III cell proliferation was evaluated using the Alamar blue assay. Following four days of treatment of Mat B-III cells, Å6 had no significant effect on cell doubling time while treatment with TAM caused a small but statistically significant decrease in cell proliferation. Combination treatment with Å6 and TAM exhibited a similar effect on cell growth as observed by TAM alone (Fig. 4.3). Throughout the course of these studies, no noticeable effect on cell morphology or viability as determined by Trypan blue assay underscoring the non-cytotoxic nature of these agents (data not shown).

Effect of Å6 and TAM on Tumor Growth *in vivo*

Mat B-III cells were inoculated into the mammary fat pads of female Fischer rats. Tumors were staged to 30-40 mm^3 at which time animals were randomized and injected daily with vehicle alone, Å6 (75 mg/kg/day), TAM (3 mg/kg/day) and the combination of Å6 and TAM for 17 days. Tumor volume was determined every other day beginning on day 16 post-tumor cell inoculation. Animals receiving either Å6 or TAM exhibited a significant decrease in the tumor growth rate of ~50%. This decrease in tumor volume was 50~60% at the end of this study on day 18 as compared to control animals. The decrease in tumor growth was significantly greater (75%) in animals receiving both Å6 and TAM (Fig. 4.4). Animal weight did not

change throughout the study (data not shown) indicating no overt toxicities and good tolerance of this regimen by the animals.

Effect of Å6 and TAM on Tumor Metastases

All control and experimental animals were euthanized on day 18 and evaluated for the presence of macroscopic tumor metastases. Control animals inoculated with Mat B-III tumor cells routinely showed the presence of macroscopic tumor metastases to the lungs, liver, auxiliary and retroperitoneal lymph nodes. A significant decrease in the number of macroscopic tumor metastases foci was seen in animals receiving Å6, while no change in tumor metastases was observed following treatment with TAM (Table 4.1). Treatment with both Å6 and TAM exhibited a decrease in tumor metastases that was similar to that observed following infusion of Å6 alone. Collectively, these results showed that while both Å6 and TAM were able to reduce the growth of primary tumor, only Å6 decreased the incidence of tumor metastases in this syngeneic *in vivo* model of breast cancer (Table 4.1).

Effect of Å6 and TAM on Tumor Angiogenesis and on Tumor Cell Death

Rapidly growing tumors are highly dependent on the formation of neovessels that fuel tumor growth. In order to evaluate the effect of Å6 and TAM on tumor neovascularization, control and experimental tumors were examined for new blood vessel formation by immunohistochemical analysis using anti-factor VIII related antigen. Sections of primary tumors from both Å6 and TAM treated animals exhibited a decrease in the number of factor VIII hot spots as compared to control tumors (Fig. 4.5). This decrease in hot spots was more pronounced in tumor sections from animals receiving both Å6 and TAM (Fig. 4.5).

In previous studies, we have routinely observed that Mat B-III tumors exhibit a significant necrotic area, which is a direct result of tumor cell death via several mechanisms. In our studies, significantly greater areas of tumor necrosis were observed in primary tumors from all experimental tumors as evaluated by H & E staining of histological sections (data not shown). In order to define the mechanism of this tumor cell death, histological sections of control and experimental tumors were evaluated for apoptotic effects of these agents by TUNEL assay. Control sections exhibited a limited number of TUNEL positive cells, the number of which was

significantly greater in tumors from Å6 or TAM treated animals. These effects were found to be further increased in animals receiving Å6 and TAM in combination as determined by integrated density per field of examination of TUNEL positive cells (Fig. 4.6).

Molecular Mechanism of Å6- and TAM- Mediated Effects *In Vitro* and *In Vivo*

In order to understand the mechanisms of Å6- and TAM-mediated effects in Mat B-III invasion, growth and metastasis, we examined the ability of these agents to activate latent TGF-β, which is known to play an important role in tumor progression. For these studies, we used plasminogen activator inhibitor PAI-1 promoter luciferase construct (p3TP-Lux) as an indicator to measure the amount of generated active TGF-β in treated- or untreated-cells, which can be monitored as an increase in luciferase activity. TGF-β activity was assessed in Mat B-III cells transfected with p3TP-Lux plasmid and treated with Å6 (5.0 μM) or TAM (100.0 nM) alone or in combination. In this assay, both Å6 and TAM reduced the activity of TGF-β as seen by the reduction in relative luciferase activity. However, this reduction in TGF-β activity was further reduced to 60% following treatment with both Å6 and TAM (Fig. 4.7).

Mat B-III cells treated with vehicle, Å6, TAM, and Å6 and TAM in combination for 48 hrs were isolated and a total protein extract was analyzed by Western blot analysis for the level of production of flk-1, a VEGF receptor. Both Å6 and TAM decreased the production of flk-1 as compared to control cells, however this decrease in flk-1 levels was found to be more pronounced in cells treated with both Å6 and TAM (Fig. 4.8).

Mat B-III cell conditioned culture medium from vehicle treated or Å6 (5.0 μM), TAM (100.0 nM), and Å6 and TAM treated cells for 72 hr was removed and evaluated for the level of PAI-1 production by ELISA in different concentrations. These studies showed that while both Å6 and TAM were able to decrease PAI-1 production, these effects were significantly greater in Mat B-III cells treated with Å6 and TAM in combination (Fig. 4.9). The ability of Å6 and TAM to decrease PAI-1 production was time-dependent with increased reduction following 48 hr of treatment with these agents (unpublished observations).

4.6 Discussion

In this study, we explored the inhibitory effects of Å6 on experimental breast cancer progression when combined with TAM as well as the underlying mechanisms responsible for this inhibition. We and others had previously demonstrated that Å6 alone or in combination with cisplatin could significantly inhibit tumor angiogenesis leading to decreased rates of tumor growth as well as a survival benefit in a xenograft model of glioma [272, 273]. Similar effects were observed in the current study where Å6 and TAM alone each significantly inhibited tumor cell invasion *in vitro*, and angiogenesis and tumor growth *in vivo*. However, the combination of the two agents had super-additive effects, resulting in an additional ~50% decrease in microvessel density and a 2.5-fold increase in the number of TUNEL-positive foci observed in tumor sections as compared to either agent alone. Further, we observed a significant decrease in TGF-β activity using a PAI-1 promoter luciferase reporter gene construct. We then identified several other molecules whose regulation could explain the observed decreases in microvessel density and increased TUNEL-positive foci in response to Å6 and TAM treatment through the inhibition of TGF-β activity.

The effect of TGF-β on tumor growth is bi-phasic: carcinogenesis and early tumor growth are suppressed by TGF-β whereas this growth factor apparently accelerates tumor progression in more advanced aggressive tumors [534, 535]. This is probably due to its role to suppress tumor angiogenesis and induce tumor cell apoptosis. TGF-β is known to regulate the expression of the serpin inhibitor of uPA catalytic activity, PAI-1. PAI-1 also behaves in a bi-phasic manner, in some models it has been shown to decrease tumor growth and angiogenesis whereas in other systems, PAI-1 has been shown to promote angiogenesis and tumor growth [286, 287, 536]. High PAI-1 levels in cancer patients are almost always associated with poor prognosis but this paradox may be explained by a concentration effect, whereas at physiological levels PAI-1 may inhibit angiogenesis by inhibiting uPA-dependent remodelling of basement membrane as well as adhesion of cells to VN. At higher PAI-1 concentrations, however, the inhibition of excess proteolytic activity and induction of migration towards fibronectin may act to promote angiogenesis [277, 286, 287, 537]. In this study, we have demonstrated that both TAM and Å6 inhibit the

expression of PAI-1 and that the combination of agents inhibits PAI-1 expression by ~50% in cell culture *in vitro*. We are currently investigating whether the decrease in PAI-1 expression by tumor cells treated with TAM and Å6 correlates with the inhibition of angiogenesis *in vivo*.

Recent data has also demonstrated the autocrine regulation of tumor cell invasion and signaling by VEGF in prostate and breast cancer cells [538, 539]. In this study, we observed the expression of the rodent VEGF receptor, flk-1, in Mat B-III cells (homologous to human KDR) and this expression could be attenuated by >50% in the presence of Å6 alone. This attenuation was further enhanced in the presence of TAM to about 33% of control. Recently, a correlation has been demonstrated between VEGF expression and PAI-1 expression in human colorectal tumors [540]. The expression of VEGF and PAI-1 correlated with stage, with much higher expression levels being associated with advanced disease. Finally, it has also been demonstrated that blocking PAI-1 expression inhibits the expression of VEGF in smooth muscle cell raising the possibility that a similar axis of regulation may exist in tumors [541]. The existence of a possible autocrine VEGF loop in tumor cells implies that VEGF receptors like e.g. flk-1 might also be regulated. Unfortunately, it has been difficult to evaluate flk-1 expression directly in tumor sections due to the lack of a suitable, commercially available antibody against flk-1 that is useful for immunohistochemistry, necessitating the use of alternative approaches. Experiments are currently ongoing to determine whether a PAI-1-dependent autocrine loop regulated through TGF-β signaling and leading to flk-1 expression is responsible for the invasiveness and tumorigenicity of Mat B-III cells *in vitro* and *in vivo*.

Collectively, this study demonstrates the combination of an anti-estrogen (TAM) and an anti-angiogenic and anti-invasive peptide derived from uPA (Å6) in reducing the growth rate of rat breast tumors through the inhibition of angiogenesis and invasiveness with a concomitant increase in cell death as measured by TUNEL staining. We present data for the first time describing aspects of the molecular mechanism underlying the anti-angiogenic and anti-invasive activity of Å6, which has previously been demonstrated to inhibit angiogenesis leading to the inhibition of tumor growth, and the implication of a VEGF receptor and PAI-1 as part of this

mechanism. We also present data suggesting the utility of combining an anti-estrogen with non-cytotoxic anti-angiogenic therapy as a potential therapeutic approach to suppress breast cancer progression, an approach that may improve the efficacy of current first-line therapy.

4.7 Acknowledgements

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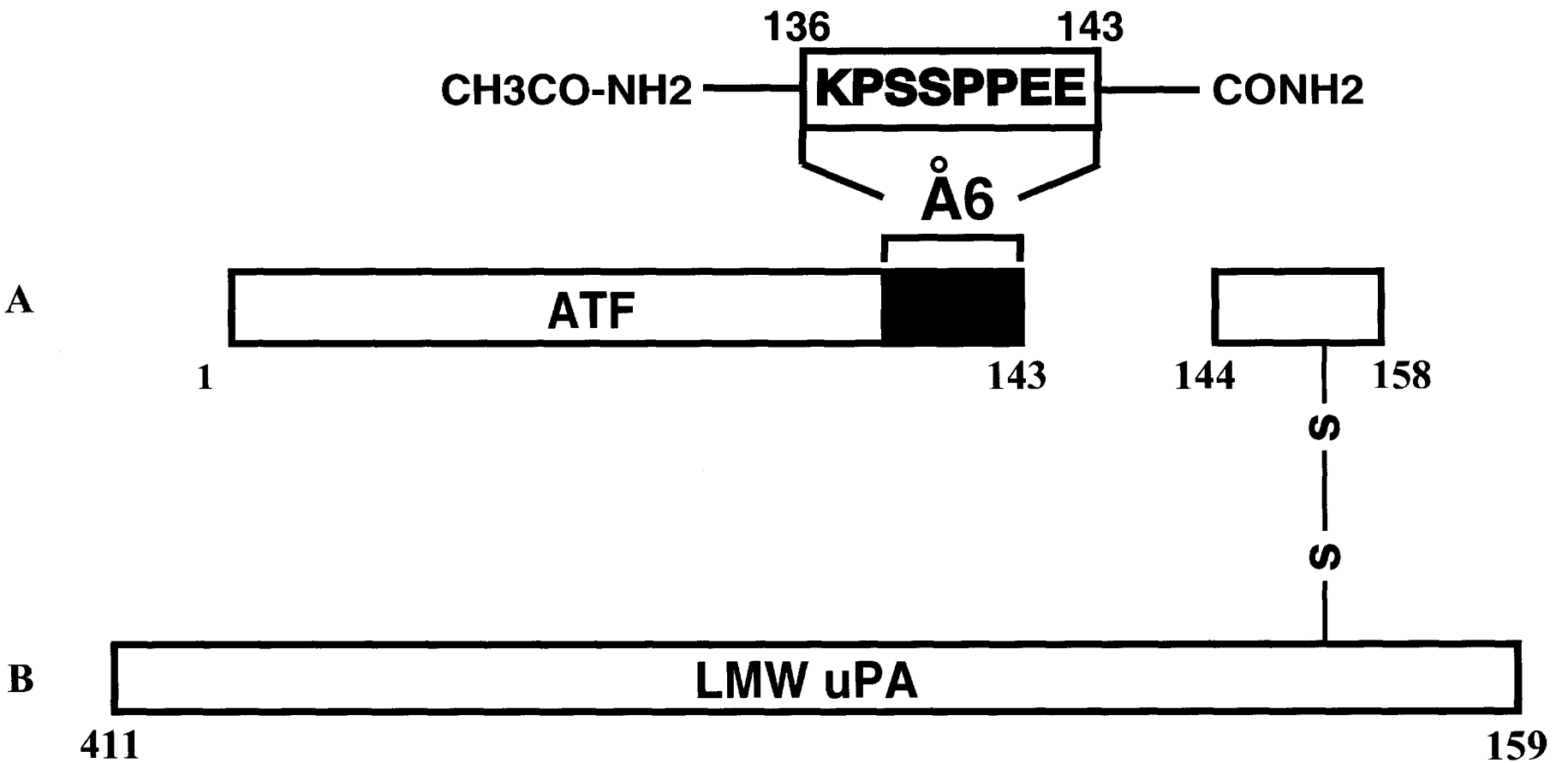

136
143
CH3CO-NH2
KPSSPPEE
CONH2
Å6
A
ATF
1
143
144
158
S
S
B
LMW uPA
411
159

4.8 Figures & Table:

Fig. 4.1. Structure of uPA and Å6.

Following its proteolytic processing, high molecular weight uPA (HMW-uPA) is converted to an amino terminal fragment (ATF) and proteolytically active low molecular weight uPA (LMW-uPA). From the carboxy terminus of ATF, an 8-mer peptide corresponding to amino acid 136-143 was selected and termed Å6 that was used throughout the course of this study.

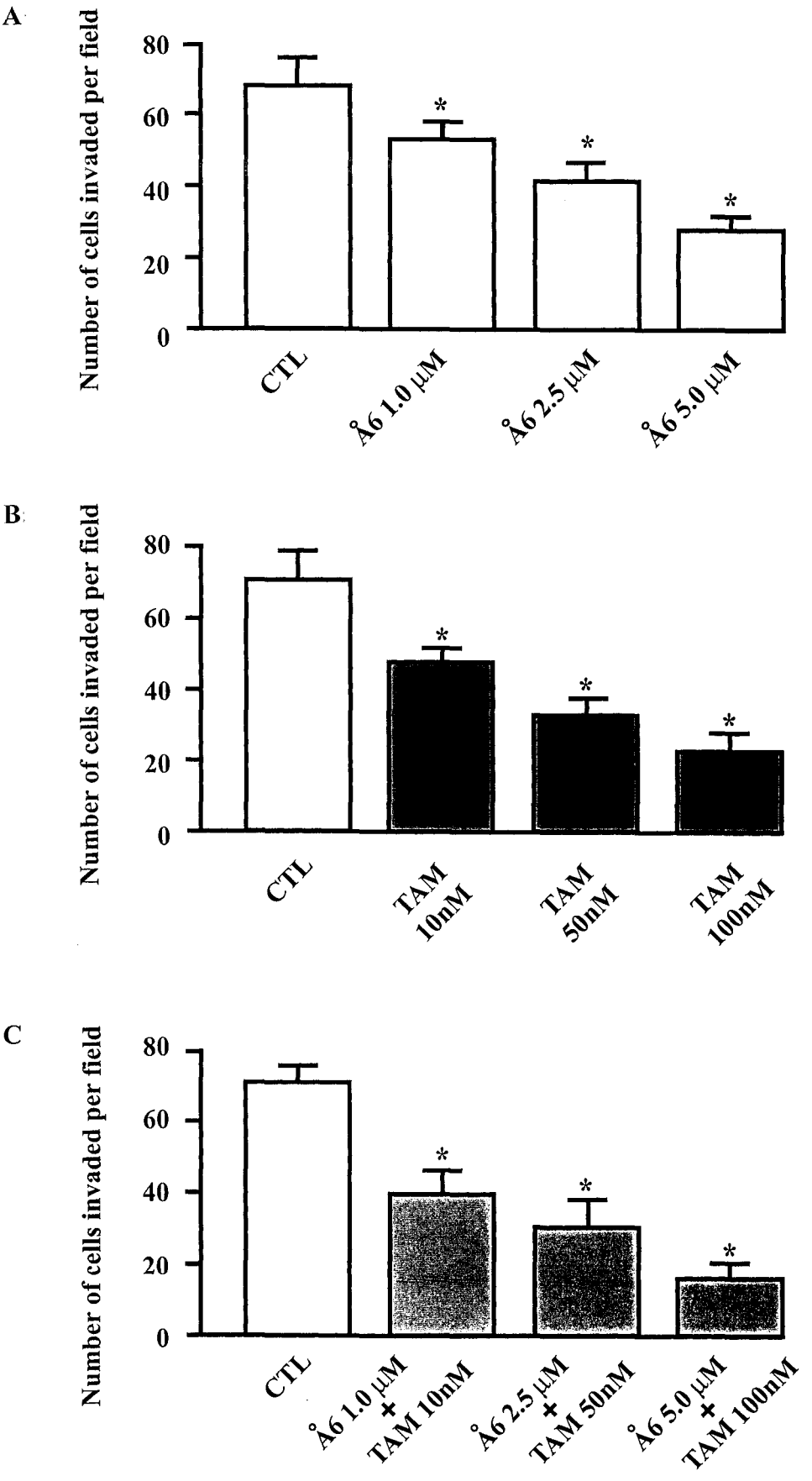

A
Number of cells invaded per field
80
60
40
20
0
*
*
*
CTL
Å6 1.0 μM
Å6 2.5 μM
Å6 5.0 μM
B
Number of cells invaded per field
80
60
40
20
0
*
*
*
CTL
TAM 10nM
TAM 50nM
TAM 100nM
C
Number of cells invaded per field
80
60
40
20
0
*
*
*
CTL
Å6 1.0 μM + TAM 10nM
Å6 2.5 μM + TAM 50nM
Å6 5.0 μM + TAM 100nM

Fig. 4.2. Effect of Å6 and TAM alone or in combination on Mat B-III cell invasion.

Mat B-III rat breast cancer cells were grown in culture as described in "Materials and Methods". The number of tumor cells migrating to the lower aspect of Boyden chamber filter following treatment with different doses of Å6 (panel A), TAM (panel B) or combination of Å6 and TAM (panel C) were counted. Comparison was made with the number of cells invading following treatment with vehicle alone (CTL). Results represent ± SD of three different experiments. Significant difference in the number of cells invading as compared to control groups.

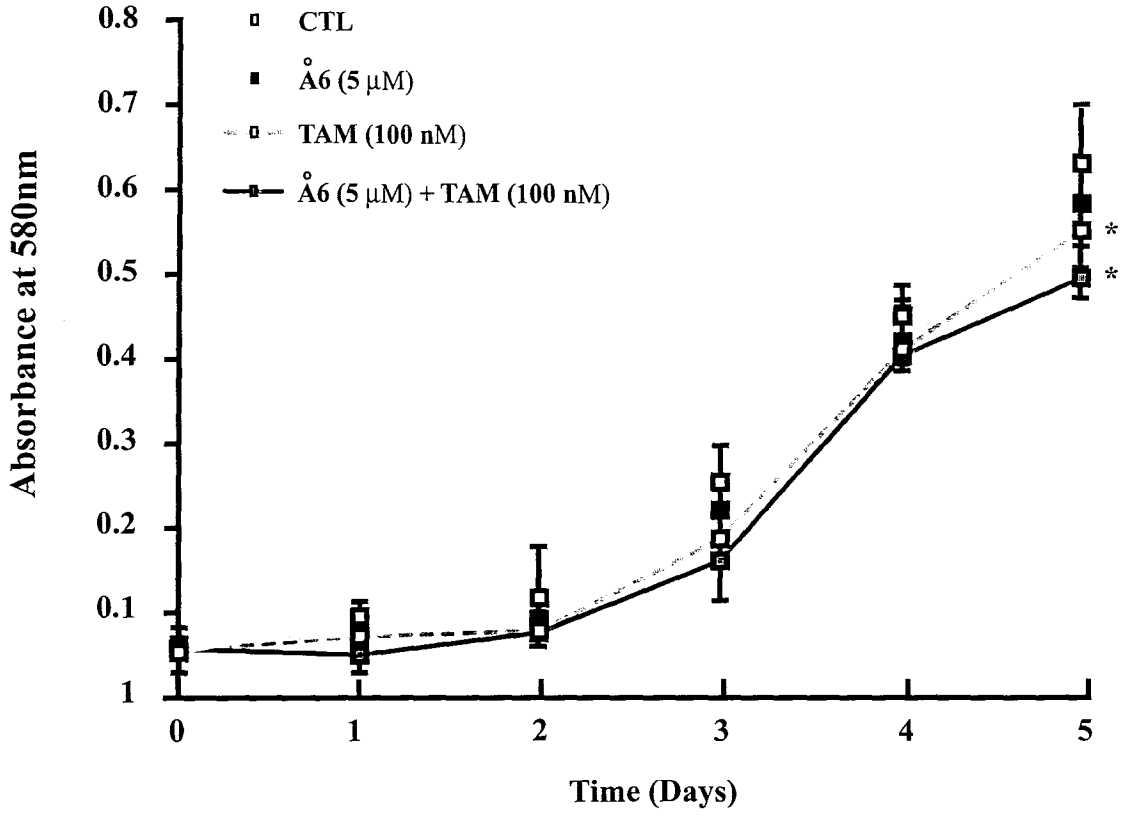

CTL
Å6 (5 μM)
TAM (100 nM)
Å6 (5 μM) + TAM (100 nM)
Absorbance at 580nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
0
1
2
3
4
5
Time (Days)
*
*

Fig. 4.3. Effect of Å6 and TAM on Mat B-III cell growth.

Mat B-III cells were grown in culture alone and in the presence of Å6 (5.0 μM), TAM (100.0 nM) alone and Å6 and TAM in combination for 4 days. Cell numbers in triplicate petri dishes were quantitated daily by Alamar blue assay as described in "Materials and Methods". Total absorbence at 580 nm following 4 days of treatment with these agents is shown. Results represent ± SD of three different experiments. Significant difference in the number of cells as compared to control groups was represented by asterisks ($P<0.05$).

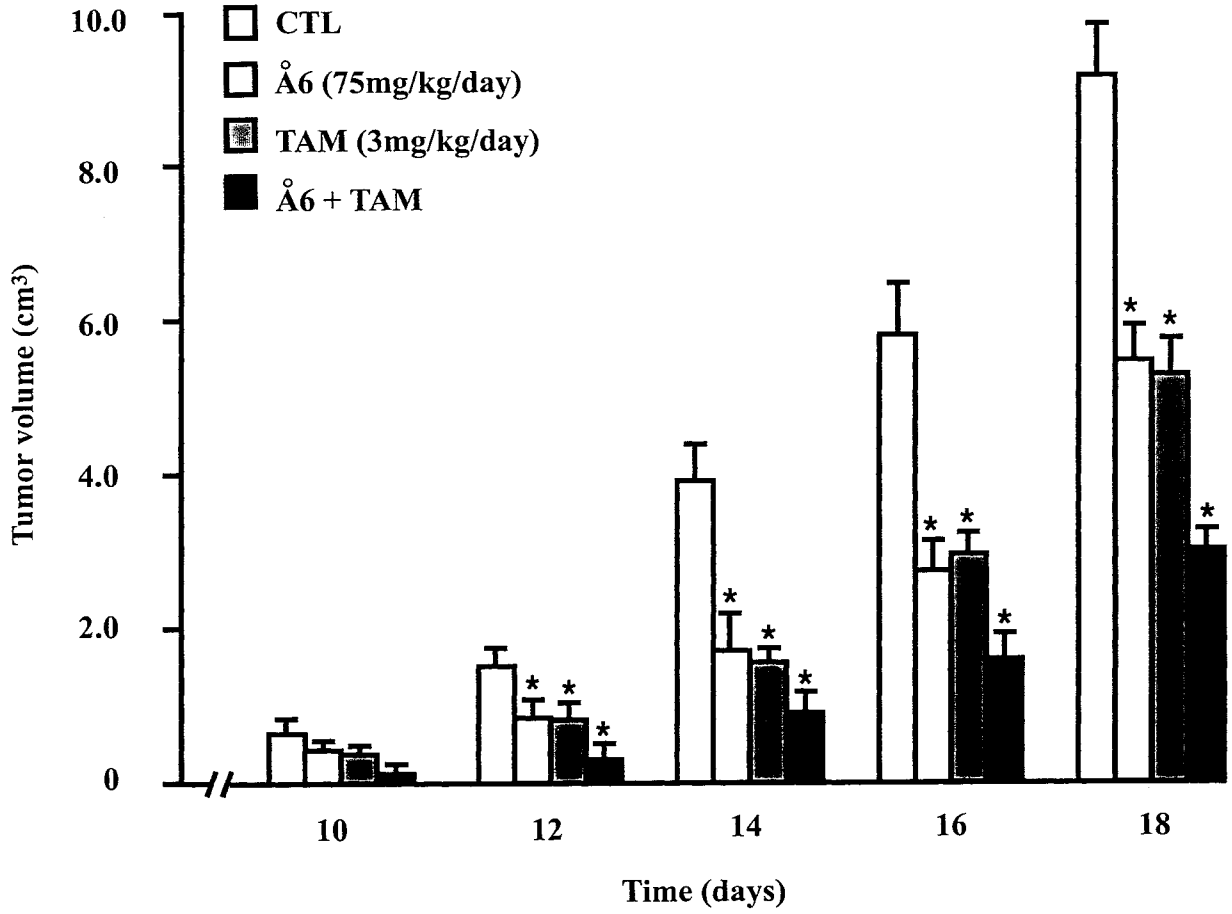

CTL
Å6 (75mg/kg/day)
TAM (3mg/kg/day)
Å6 + TAM
Tumor volume (cm3)
10.0
8.0
6.0
4.0
2.0
0
10
12
14
16
18
Time (days)

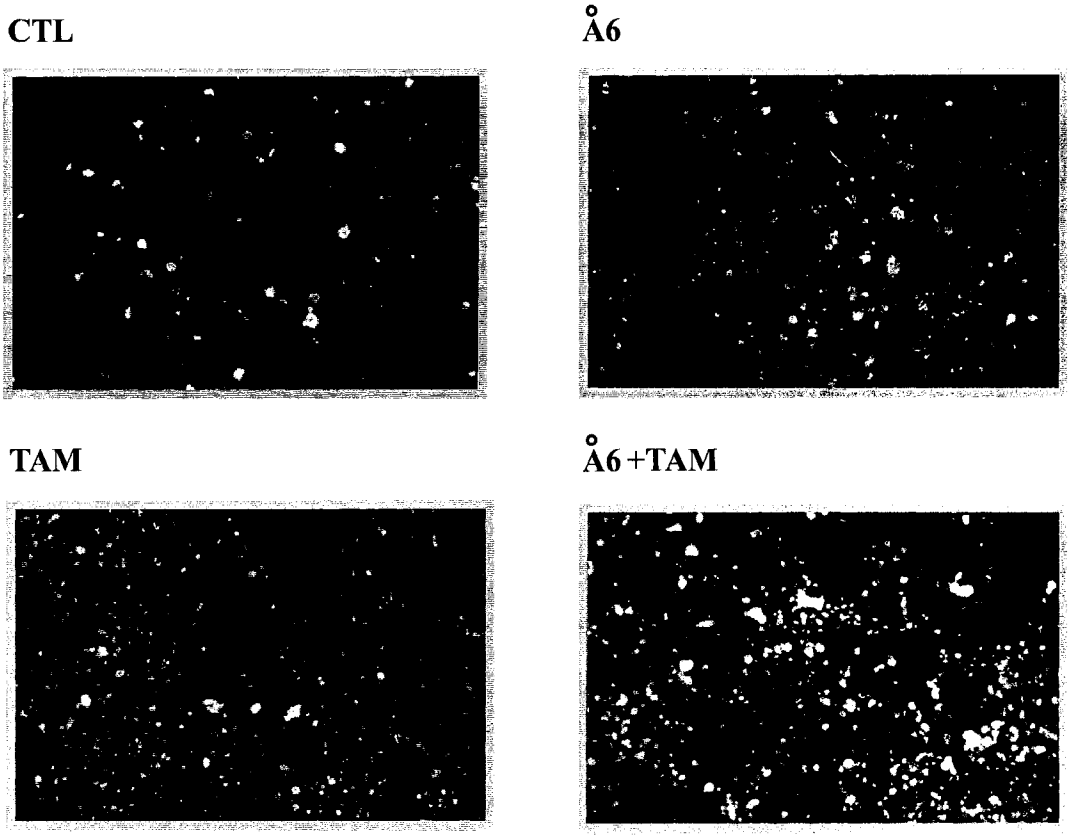
Fig. 4.4. Effect of Å6 and TAM on tumor volume.

Tumor volume of animals inoculated with Mat B-III tumor cells was determined following treatment with vehicle alone (CTL), Å6, TAM and Å6 and TAM at timed intervals as described in "Materials and Methods". Results represent ± SD of six starting animals in each group in three different experiments. Significant difference from control is shown by asterisks. ($p<0.05$)

	CTL	Å6	TAM	Å6 + TAM
LUNG	5 ± 2	2 ± 1	5 ± 1	2 ± 1
LIVER	3 ± 1	1 ± 0	3 ± 2	1 ± 0
AX. LYMPH NODES	4 ± 2	1 ± 1	4 ± 1	1 ± 1
R.P. LYMPH NODES	3 ± 1	1 ± 1	3 ± 1	1 ± 1

Table 4.1. Effect of Å6 and TAM on Tumor Metastases.

Female Fischer rats were inoculated with Mat B-III breast cancer cells and treated with either vehicle (CTL), Å6 (75 mg/kg/day), TAM (3 mg/kg/day) alone, and in combination as described in "Materials and Methods". All control and experimental animals were sacrificed at day 18 post-tumor cell inoculation and evaluated for the presence of macroscopic tumor metastases. Total number of metastatic foci in various organs were counted and compared. Results represent at least four animals in each group in three different experiments.


CTL
Å6
TAM
Å6 +TAM

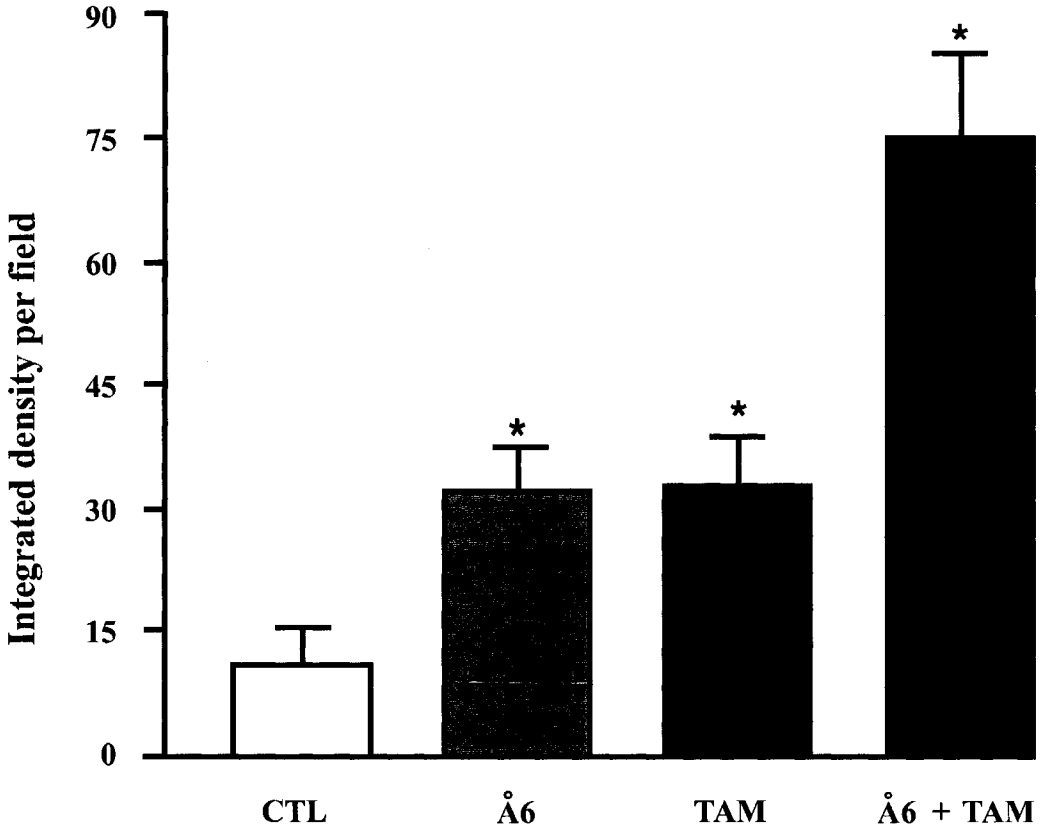
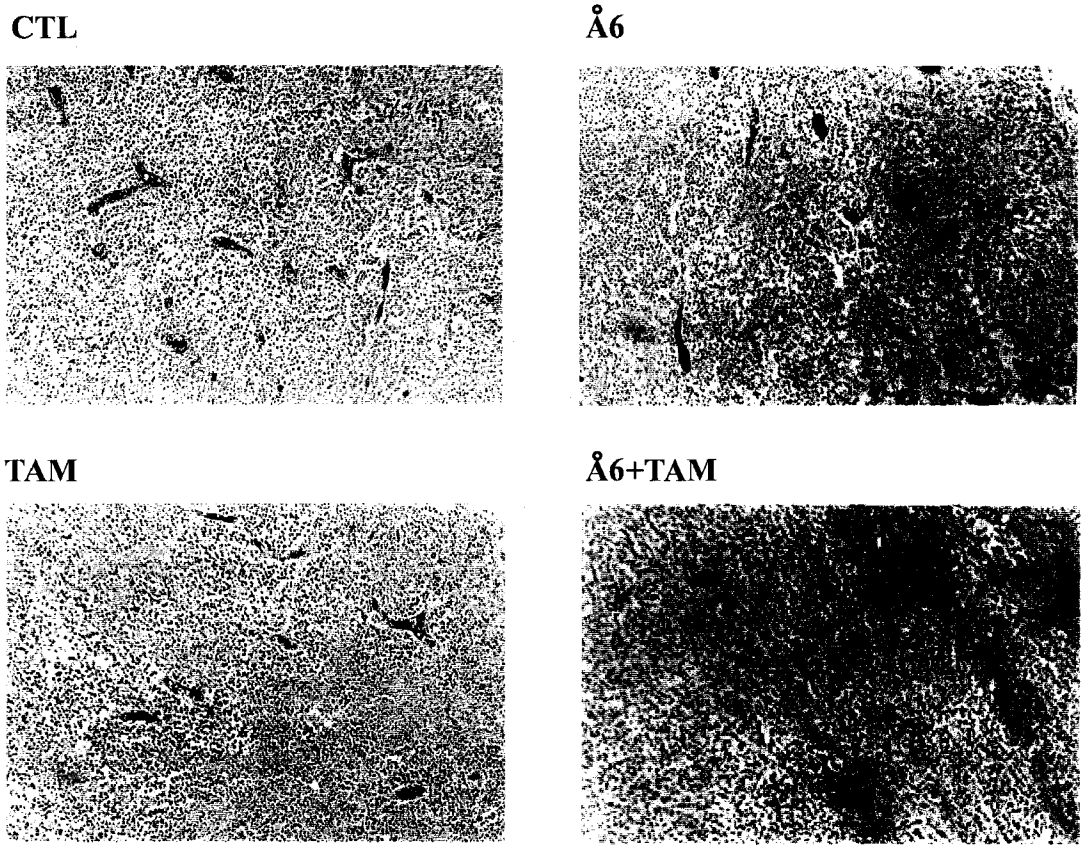

Integrated density per field
90
75
60
45
30
15
0
*
*
*
CTL
Å6
TAM
Å6 + TAM

Fig. 4.5. Immunohistochemical analysis of tumors from animals treated with Å6 and TAM.

Primary tumors from all control and experimental animals were removed, formalin fixed, and paraffin embedded. 4 μm sections were prepared and stained with anti-factor-VIII related antibody. Representative photomicrographs from vehicle treated (CTL), Å6, TAM and Å6 and TAM in combination are shown (upper panel) (100X magnification). Areas of high vascularization in all groups were counted for microvessel density as described in "Materials and Methods" and plotted (lower panel). Results represent ± SD of three determinations in each section from all groups. Significant difference in microvessel density per field of examination from vehicle treated control (CTL) is shown by asterisks. ($p<0.05$)


CTL
Å6
TAM
Å6+TAM

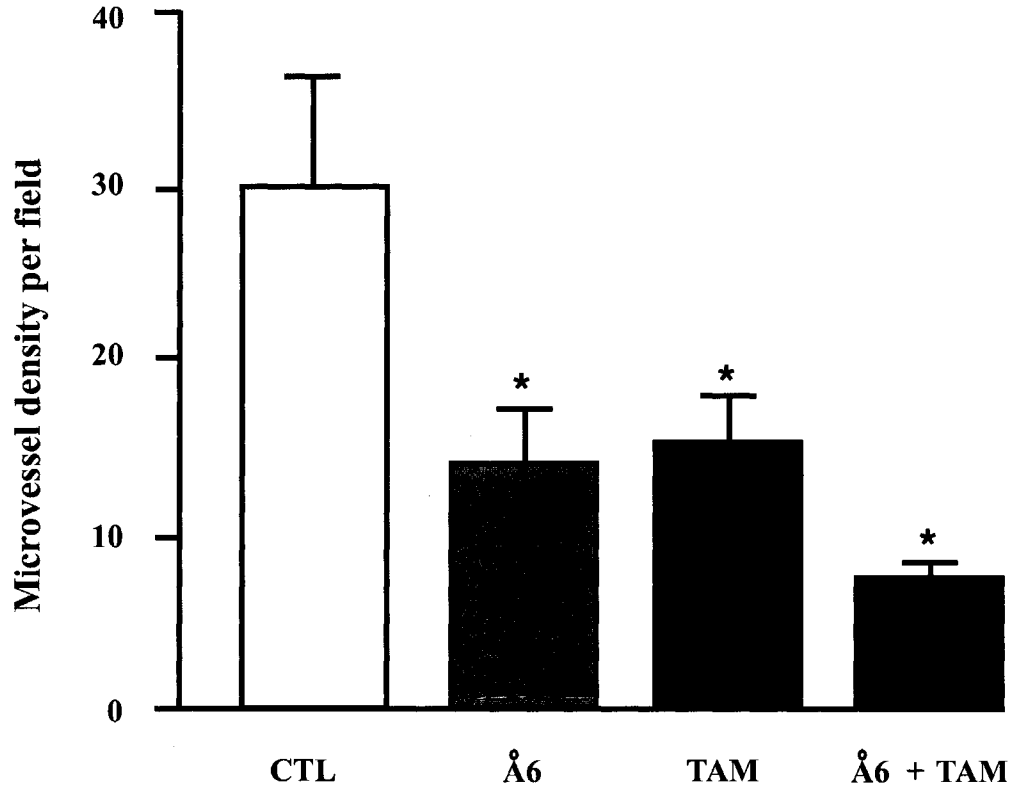

Microvessel density per field
40
30
20
10
0
*
*
*
CTL
Å6
TAM
Å6 + TAM

Fig. 4.6. Histological analysis of Å6 and TAM treated Mat B-III tumors.

Animals inoculated with Mat B-III tumors and receiving vehicle alone (CTL), Å6, TAM and Å6 and TAM were sacrificed on day 18 post-tumor cell inoculation. Primary tumors from all animals were removed, formalin fixed, and paraffin embedded. 4 μm sections of tumors were cut and subjected to TUNEL assay and quantified, as described in "Materials and Methods". Representative photomicrograph from each group is shown (400X magnification). Results represent ± SD of three different determinations in each tumor. Significant difference from control (CTL) is represented by asterisks. ($p<0.05$)

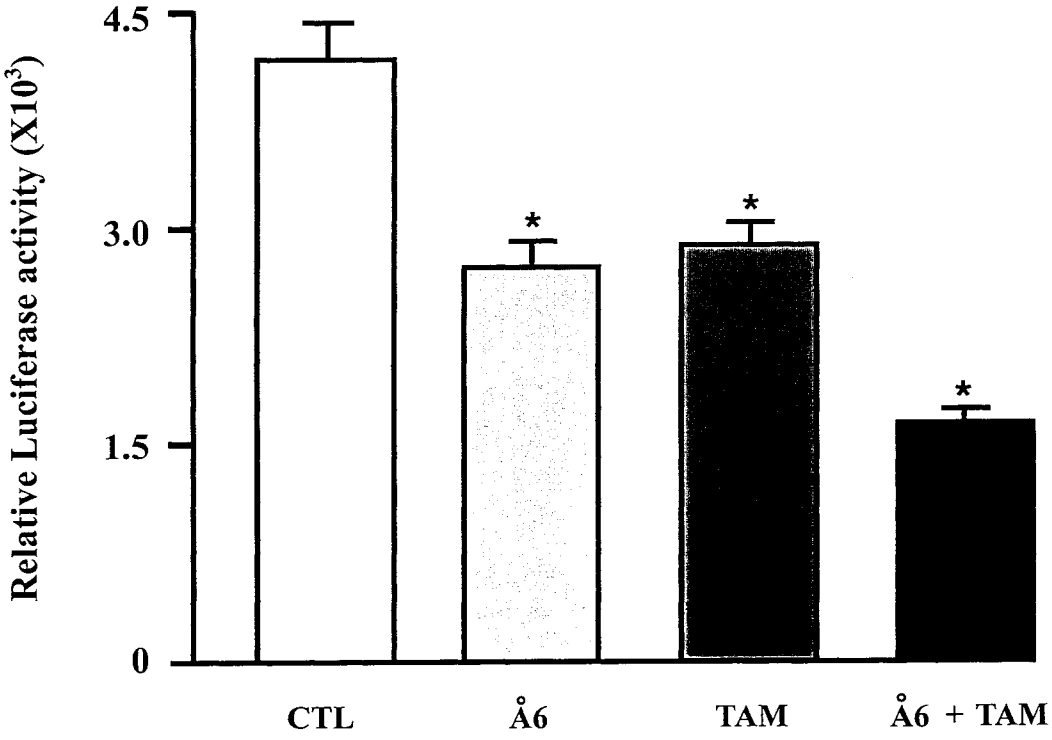
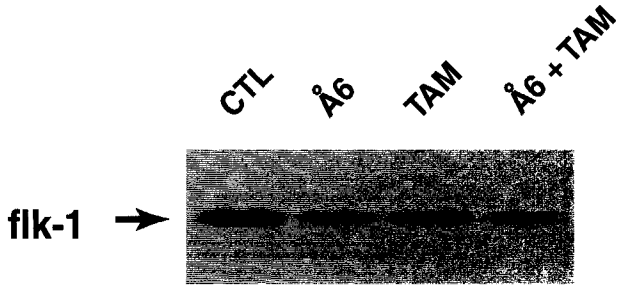

Relative Luciferase activity (X10³)
4.5
3.0
1.5
0
*
*
*
CTL
Å6
TAM
Å6 + TAM

Fig. 4.7. Effect of Å6 and TAM on TGF-β activity.

Mat B-III cells were transfected with PAI-1-luciferase plasmid and following 20 hr of incubation cells were treated with Å6 (5.0 μM), TAM (100.0 nM), and Å6 and TAM in combination. Induction of PAI-1 mediated relative luciferase activity was determined as described in "Materials and Methods". Results represent ± SD of three different experiments. Significant difference in luciferase expression from vehicle treated control (CTL) is represented by asterisks. ($p<0.05$)


CTL
Å6
TAM
Å6 + TAM
flk-1

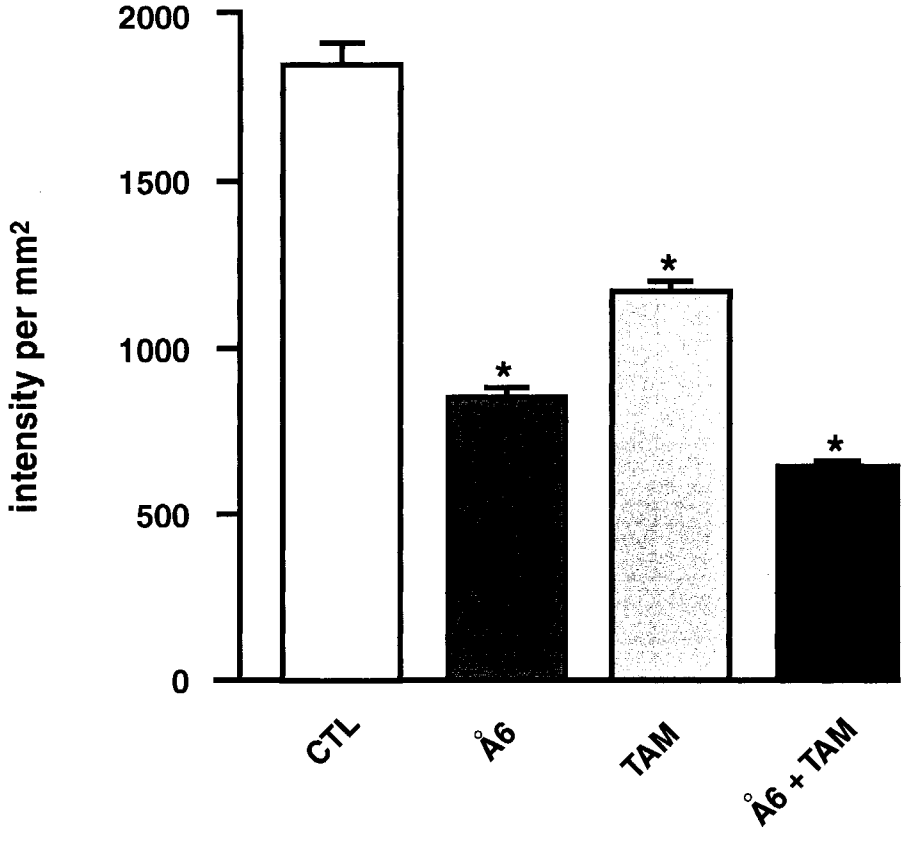

2000
1500
1000
500
0
intensity per mm²
*
*
*
CTL
Å6
TAM
Å6 + TAM

Fig. 4.8. Effect of Å6 and TAM on flk-1 production.

Mat B-III cells were treated with vehicle alone (CTL), Å6 (5.0 μM), TAM (100.0 nM) and Å6 and TAM for 48 hr. Level of flk-1 production was assessed by Western blot analysis as described in "Materials and Methods". Results represent ± SD of three different experiments. Significant difference from control is represented by asterisks. ($p<0.05$)

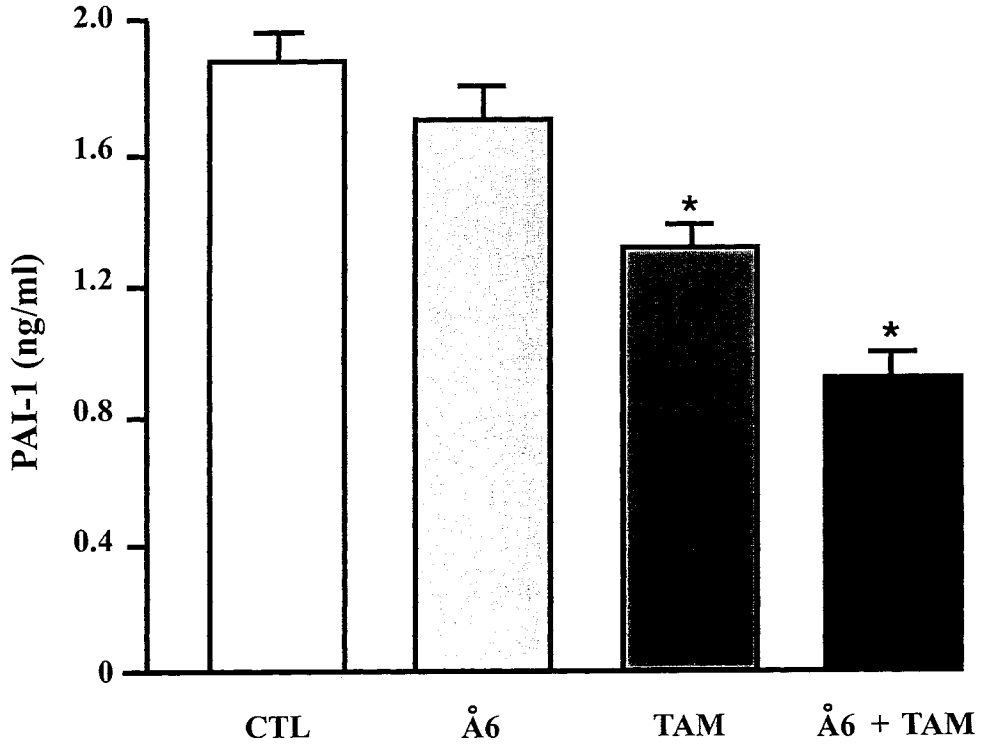

2.0
1.6
1.2
0.8
0.4
0
PAI-1 (ng/ml)
*
*
CTL
Å6
TAM
Å6 + TAM

Fig. 4.9. Effect of Å6 and TAM on PAI-1 production.

Mat B-III cells were treated with Å6 (5.0 μM), TAM (100.0 nM) and Å6 and TAM for 48 h. Cells conditioned culture medium was removed and tested for the level of PAI-1 production by ELISA as described in "Materials and Methods". Results represent ± SD of three different experiments. Significant difference in PAI-1 production as compared to vehicle treated control (CTL) Mat B-III cells is represented by asterisks. ($p<0.05$).

Chapter V

General discussion

Breast cancer is a common disease that adversely affects the lives of many women worldwide. Breast cancer emerges through a multi-step process that results in cellular transformation and metastasis [311, 542]. The molecular mechanisms underlying carcinogenesis, its progression and the formation of metastases have been the major focus of cancer research. It is hoped that progress in these areas will lead to the development of effective treatments. Under the influence of multiple factors including environmental factors, hormonal factors such as high levels of estrogen, genetic factors, overexpression of oncogenes, the cellular transformation of normal breast epithelium is initiated. In most cases, breast cancer does not stop *in situ*. Instead, the overproduction of multiple proteases and pro-angiogenic factors facilitate cell invasion and promote cancer metastasis [311]. Given the importance of uPA in breast cancer progression, the main focus of this study was an investigation of the molecular mechanisms driving the overexpression of uPA in late stage breast cancer, and how to reverse the progression of breast cancer by disrupting the functions of uPA.

uPA is a serine protease that is synthesized as a single chain inactive zymogen. It is processed to a two chain HMW-uPA once it is uPAR bound, which can be further cleaved to an ATF that contains a receptor binding GFD and a proteolytically active LMW-uPA. Among the many proteases secreted by highly metastatic breast cancer cells, uPA plays a key role in provoking cancer cell invasion. In the early stage of breast cancer, *uPA* gene is expressed at a low level, but its expression is upregulated in late stage, highly metastatic breast cancer cells. This expression profile was observed in our *in vitro* breast cancer cell model. We chose two cell lines that represent an *in vitro* model for breast cancer progression. MCF-7 was developed from an early stage human mammary adenocarcinoma. This low invasive cell line expresses ER, and its growth is hormone-dependent. When examining total mRNA from MCF-7 cells, we failed to detect uPA transcripts (Fig. 2.1A), correlating to its low cell invasiveness using an *in vitro* Boyden chamber invasion assay (Fig. 2.1B). Another cell line MDA-MB-231 that expresses high levels of uPA (Fig. 2.1A) was chosen to represent late stage breast cancer, and is highly invasive (Fig. 2.1B). Although the mechanisms underlying uPA-enhanced breast

cancer cell invasion have been extensively investigated, relatively little is known about how the expression of *uPA* gene is upregulated in late stage breast cancer. Xing and coworkers in our laboratory were the first to report that uPA levels may be correlated with genome-wide DNA methylation status [22], suggesting a possible regulatory mechanism for *uPA* gene expression by DNA methylation. However, it was not known at the beginning of this project whether *uPA* gene expression is directly related to the methylation status of its promoter. If so, what are the upstream regulators that control the methylation status of the *uPA* gene during cancer progression? My Ph.D. project was intended to answer these questions, and to understand the molecular mechanisms involved. As discussed in chapter II, I mapped detailed cytosine methylation status of CpG island in the uPA promoter from both early and late stage breast cancer cell lines (Fig. 2.2). In MCF-7 cells, more than 90% of the cytosines of CpG dinucleotides within the uPA promoter are methylated. In contrast, only unmethylated cytosines are detected in MDA-MB-231 cells (Fig. 2.2). Given that the methylation pattern of the uPA promoter is negatively correlated with breast cancer invasiveness, and is consistent with genome-wide hypomethylation in cancer development, I further determined the molecular mechanisms underlying how DNA methylation alteration of the uPA promoter affects its expression.

Initially proposed in 1982, DNA methylation has been suggested as an epigenetic change that alters gene expression and was reviewed in chapter I, section 1.7 [543]. The proposed working model suggested that genomic DNA, especially the regulatory segments, confer different conformations before and after cytosine methylation. This change alters the accessibility of TFs that determine gene expression [544]. Various TFs are involved in the transcriptional regulation of *uPA* gene expression (as discussed in chapter I). Among them, the Ets family of TFs plays a pivotal role. Overexpression of Ets-1 has been correlated with the overproduction of uPA and metastasis of cancers with different origins including breast, lung and prostate cancers [82]. The role of the Ets family in the regulation of the uPA promoter has been characterized previously. It is generally believed that the binding of the Ets family of TFs to the promoter region of uPA is essential for its expression. Several Ets-1 binding sites have been revealed in the uPA promoter. As shown in Fig. 2.2,

both an Ets-1 binding site and its adjacent Sp1 binding motifs contain methylated CpG dinucleotides in MCF-7 cells but not in MDA-MB-231 cells. Methylation changes in CpG dinucleotide within the Ets-1 binding site may be the key to alter the binding ability of Ets-1 to the uPA promoter and thus affect its expression. Either direct blocking the interaction of Ets-1 with its response element, or indirect interfering with access of the TF complex of Ets-1 to its binding site may be responsible for these effects. Using *in vitro* methylation, DNA transfection and promoter luciferase assay, I directly demonstrated that the overexpression of Ets-1 resulted in dramatically enhanced promoter activity of uPA when it is unmethylated. However, this induction was abolished when the uPA promoter is methylated. These data provide direct evidence showing that methylation status determines the transcriptional capacity of the uPA promoter and therefore the accessibility of Ets-1. Given that Ets-1 is crucial to the expression of the *uPA* gene when it is unmethylated [39, 79, 80, 82], my work shows for the first time that the induction effect of TFs on the *uPA* gene is partially dependent on the cytosine methylation status of this gene. Both CpG dinucleotides residing within and away from the core binding sites of TFs may affect the accessibility of these factors to their binding motifs.

Although the theory of DNA methylation was first proposed more than a decade ago, only recently were two enzyme families, the DNMTs and DMases, identified [432, 438]. How these two enzyme families regulate the methylation status of DNA remains unknown. It has been suggested that DNMTs possess *de novo* methylase activity that converts cytosine within a CpG dinucleotide from an unmethylated to methylated nucleotide [474]. However, the methyl group on cytosine remains unstable and is constantly and actively removed by DNA DMase [474]. Therefore, maintenance activity of DNMTs is needed to preserve methylated DNA, especially following DNA replication [434, 440].

Several techniques have been developed to discern these different activities within cells, namely *de novo* methylase activity, maintenance activity and demethylase activity. *De novo* activity of DNMT catalyzes the transfer of methyl-group from its ^{3}H-labeled methyl-donor SAM to its unmethylated DNA substrate, generating a methylated DNA from a previously unmethylated one. Maintenance

enzyme activity preserves the same methylation pattern of parental DNA after DNA replication. Once a methylated DNA undergoes replication, the two daughter strands of DNA will become hemimethylated, made up of one strand from parental methylated DNA, and another non-methylated newly synthesized strand. Therefore, maintenance DNMT activity plays a critical role for maintaining an inheritable methylation pattern of DNA. It is achieved by catalyzing the transfer of a methyl group from ^{3}H-SAM to the hemimethylated daughter strand, and generates fully methylated DNA. Several methylase and demethylase enzyme activity assay systems have been designed based on these theories. Two sets of substrates were used in the methylase activity assays, hemimethylated oligos (^{m}dC-dG) to measure maintenance methylase activity, or unmethylated oligos (dC-dG) to measure *de novo* methylase activity. Following the incubation of a cell nuclear extract and a methyl donor with specific unmethylated substrates, total counts of ^{3}H-CH_3 incorporation into substrates were quantitated, representing the level of *de novo* methylase activity. When hemimethylated substrates were chosen, the incorporation of ^{3}H-CH_3 reflects the maintenance activity of DNMTs. DMase activity can also be measured using methylated or hemimethylated DNA, and results in the generation of unmethylated DNA and ^{3}H-labeled volatile methyl residue methanol (^{3}H-CH_3OH). In this assay, total cell nuclear extract was incubated with methylated DNA substrate (^{3}H-methyl-DNA), and the amount of volatized ^{3}H-CH_3OH reflects DMase enzyme activity within each cell line.

We observed that maintenance and *de novo* methylase activities are inversely in MCF-7 and MDA-MB-231 cells (Fig. 2.4). These differences may reflect the dominance or the balance of different DNMTs in these cells. Our results showed that maintenance methylase activity is much higher in MCF-7 cells, where the *uPA* gene is hypermethylated (Fig. 2.4A). However, *de novo* methylase activity is significantly higher in MDA-MB-231 cells that contain the hypomethylated *uPA* gene (Fig. 2.4B). MDA-MB-231 cells with high *de novo* methylase activity and low maintenance activity fail to maintain the *uPA* gene in a hypermethylated state, suggesting that methylated DNA requires sufficient maintenance rather than *de novo* methylase activity to retain its proper methylation pattern. It has been proposed that the level of

maintenance methylase activity could be a result of a balanced DMase and *de novo* methylase activity, and is dependent on the dominant activity present in the cells. Consistent with this hypothesis, DMase activity is much higher in MDA-MB-231 cells but low in MCF-7 cells (Fig. 2.5), and thus favors the demethylation status of the uPA promoter in MDA-MB-231 cells. Furthermore, a parallel increase of overall genomic hypomethylation and *de novo* enzyme activity has been reported in several tumors. Therefore, the high level of *de novo* activity in MDA-MB-231 cells is probably due to a negative feedback mechanism caused by decreased level of maintenance activity in this cell line. However, the relative orders of these feedback loops are poorly understood. In addition, the presence of the specific substrates of *de novo* and maintenance DNMTs can not be ruled out.

Most ER negative breast cancer cells such as MDA-MB-231 are also PR negative. About 40% of PR is hypermethylated in PR negative breast cancers. *PR* is an inducible gene of ER, and acts as an indicator for functional ER. ER mediated chromatin remodeling is necessary for 5-azaCdR induced demethylation of the *PR* gene, whereas 5-azaCdR by itself fails to induce the expression of PR in the absence of ER. Furthermore, ER can activate *PR* gene expression even it is hypermethylated [545]. These observations suggest that ER activates gene expression regardless of the methylation status of its response element, and may even initiate the demethylation of methylated genes containing the ER response element. If it is true, one must ask if estrogen confers the global genomic demethylation including the *uPA* gene in breast cancer progression. Recently, glucocorticoid receptor (GR), another nuclear receptor family member, has been reported to elicit local DNA demethylation of the *tyrosine aminotransferase* (*Tat*) gene at its -2.5kb enhancer region [546]. This provides a model to study the mechanisms of *uPA* gene demethylation, especially after long-term exposure to estrogen during breast cancer development. We hypothesize that estrogen is an early player in the carcinogenesis of breast cancer. A high level of estrogen during cancer development may cause changes in the chromatin of the *uPA* gene via an ER responsive element that resides -1.6kb upstream of its transcription initiation site, thus allowing access to complex demethylation machinery for its expression. An ER positive cell line such as MCF-7 can be used to characterize

whether estrogen has long-term effects on the demethylation and overexpression of the *uPA* gene.

Methylation is one of the epigenetic changes of DNA that is tightly related to histone deacetylation. Both methylation and histone deacetylation are associated with transcriptionally inactive chromatin compartments [501]. Inhibition of HDACs by TSA could modify chromatin structure, and has been suggested to reverse methylation-dependent transcriptional silencing [460, 461]. We showed here that blocking HDAC using TSA differentially regulated uPA expression in early stage MCF-7 and late stage MDA-MB-231 breast cancer cells (Fig. 2.6). In the hypomethylated MDA-MB-231 cells with high uPA levels, TSA treatment further increased uPA expression (Fig. 2.6). However, in the hypermethylated MCF-7 cells, TSA failed to induce detectable uPA expression (Fig. 2.6), but demethylation agent such as 5-azaCdR can induce its gene expression. These results suggested that histone acetylation-induced uPA expression is hypomethylation-dependent. We argue that the demethylation of the *uPA* gene in combination with DNA acetylation results a more open structure in the uPA promoter, thus allowing easier access of TFs.

In breast cancer, overexpression of the *uPA* gene has also been attributed to a response to a number of growth factors, such as EGF, IGF-1, HGF and their tyrosine kinase receptors and oncogenic stimulation [28, 35, 39]. In the IGF-stimulated breast cancer cells, several intracellular signal transduction pathways have been proposed to play important roles in the up-regulation of uPA expression, including p44/42 MAP kinase, p38 stress kinase and PI-3K/PKB pathways [28]. Chemical inhibitors specifically targeting these pathways abolished growth factor or growth factor receptor induced *uPA* gene expression. The activation of these pathways enhances breast cancer progression and was closely associated with an invasive phenotype of these cells [28]. This evidence clearly argues for a role for these classical signaling pathways in the regulation of uPA overexpression and breast cancer metastasis. Interestingly, the activation of these pathways has been reported to increase the expression of the Ets family of TFs. For example, expression of Ets-2 has been shown to be upregulated by the activation of p44/42 MAP kinase, p38 kinase and PI3-K/PKB [85, 547], although the status of other members of this family such as Ets-1

remains unknown. Furthermore, the same signalings could be activated by a group of oncogenic stimuli that also control the levels of DNA methylation related enzymes as well as uPA overexpression as introduced in Chapter I. Using this information, a working model of how uPA is upregulated during breast cancer progression has now emerged and is depicted in Fig. 5.1 (model illustrate). The upregulation of uPA can be achieved through the binding of TFs such as Ets to the uPA promoter, which depends on two parameters: 1) availability and abundance of the TFs 2) chromatin structure of the uPA promoter, whether it favors the binding of these TFs. During breast cancer progression, it appears that the activation of p44/42 MAP kinase, p38 kinase and PI-3 K/PKB pathways, which are generally superactive in cancerous states, can coordinately fulfill these two requirements to ensure the overexpression of uPA either directly or indirectly.

The overexpression of uPA has been observed at the tumor cell front in highly invasive cancers such as breast cancer, prostate cancer and gastric cancer [10, 48, 237]. In breast cancer, elevated uPA levels is clearly associated with the invasiveness and poor prognosis of this malignancy [96]. Given this correlation, blocking uPA function appears to be an effective approach to reverse breast cancer metastasis. Many *in vitro* and *in vivo* studies have shown that targeting the uPA/uPAR system is sufficient to block cancer cell invasion and tumor metastasis [11]. Several approaches used so far, including the development of synthetic compounds, introduction of neutralizing antibodies against uPA and uPA antisense cDNA transfection [350, 358], showed the value of targeting the uPA system in cancer treatment. However, problems persist in the application of gene therapy to the treatment of cancer, and other approaches need be explored. Although effective in cell culture, the delivery of antisense uPA cDNA as a strategy to block uPA expression has not moved beyond the laboratory. Neutralizing antibodies did abolish cancer progression, but the levels of antibodies required generally exceeded the capacity to produce them. Synthetic compounds easily block uPA function *in vitro* and *in vivo*, for example, our laboratory previously tested B-428, a compound that targets the active site of uPA, and efficiently blocks cancer progression. However, solubility problems have made them unusable in patients. Efforts are now underway to develop highly selective

soluble uPA inhibitors that can be delivered effectively to cancer patients. Another promising approach is the development of synthetic peptides, which are generally soluble, have few side effects, and are amenable to large-scale synthesis.

In collaboration with Ångstrom Inc., our laboratory studied the effects of the synthetic peptide drug Å6 in blocking the uPA/uPAR system. Å6 is one of the many peptides that were developed based on the connecting peptide region of uPA. Of which, Å6 possesses a unique activity to block tumor cell invasion and endothelial cell migration (Fig. 3.2). It was therefore chosen for further study. As discussed in chapter III, Å6 inhibits primary tumor growth and metastasis in two breast cancer animal models (Fig. 3.3). uPA can stimulate the proliferation of some mammalian cells in culture [163, 225, 231] through its GFD. However, when tested in cell culture, Å6 failed to affect the rate of breast cancer cell proliferation (Fig. 4.3), contrary to its inhibitory effects on the growth of primary tumors *in vivo* (Fig. 3.3 & Fig. 4.4). As introduced in Section 1.2&1.4, uPAR not only localizes the proteolytic functions of uPA at the leading edge of cell movement, but also acts as an adhesion molecule. The overall effects of uPAR are to increase the turnover rate of uPA as well as the enhancement of its activity. Since Å6 was designed based on the sequence of the connecting peptide region of uPA, which does not fall into the classical domain of uPA that interacts with uPAR [96, 108, 109], the ability of Å6 to block tumor cell invasion *in vitro* suggests the existence of other potential mechanisms (Fig. 3.2). Å6 inhibits the interaction between scuPA and suPAR in *vitro* (Fig. 3.1), suggesting that part of the biological activity of Å6 is mediated through the inhibition of uPA/uPAR interaction at a second, unidentified binding site. uPAR is also associated with PAI-1 and a number of adhesion molecules such as integrins and VN [174, 189]. The involvement of other molecules such as PAI-1, VN and integrins *in vivo* could be responsible for the inhibitory effect of Å6. However, the possibility that Å6 interferes with the interaction between uPA and other yet to be identified binding proteins cannot be excluded. As discussed in Section 1.2.3 of Chapter I, uPA has been reported to bind to the protein C4.4A, which shares low sequence homology with uPAR, although the exact interaction site is yet to be defined. Given that C4.4A is involved in cell invasion [140], the effects of Å6 on uPA/C4.4A interaction is a

promising candidate mechanism. It will be interesting to use this information to investigate potential partners that interact with the connecting peptide region of uPA. Studies on other molecules have provided useful references for this investigation. For example, PKC-ε is a member of serine-threonine kinase family of protein kinase C [548]. Johnson and co-workers reported an 8-residue peptide corresponding to the V1 domain of this kinase blocked the activation of PKC-ε [549], suggesting that the V1 domain of PKC-ε may interact with potential regulatory proteins. Using the V1 domain as bait, a protein called RACK (receptor for activated kinase C) was identified that regulates the activity of PKC-ε [550]. Our data from Å6 suggest a novel epitope in uPA with potential uPA binding proteins. Using a similar approach to that used in the identification of RACK, one might successfully identify these potential uPA-binding proteins. This study constitutes an independent project that is beyond the scope of this thesis.

Based on these data, it appears that Å6, but not other related peptides, can inhibit endothelial cell migration and tubular formation (unpublished observations), an effect corresponding to the inhibitory effects of Å6 on *in vivo* tumor metastasis and angiogenesis. This is probably achieved through the inhibition of tumor angiogenesis (Fig. 3.5 & 4.5) and the induction of tumor cell death by blocking expression of the VEGF receptor flk-1 and PAI-1 as well as the activation of TGF-β (Fig. 4.7, 4.8 & 4.9). In terms of the inhibitory effects on breast cancer cells by Å6, we observed a discrepancy between *in vitro* and *in vivo* results. Following administration of Å6 to breast tumor bearing mice, the growth of the primary tumors was dramatically inhibited. However, Å6 failed to suppress the proliferation of breast cancer cells in culture with a concentration up to 100 μM (unpublished observations). Since Å6 inhibits tumor angiogenesis, we suggest that Å6 suppresses the growth of primary tumors partly by reducing the supply of blood and nutrients, which might further lead to DNA fragmentation (Fig. 3.4 & Fig. 4.6).

Tumorigenesis is a multi-step process that affects numerous cellular events. All tumors share some essential traits that characterize their malignant nature: 1) cell invasion and metastasis; 2) uncontrolled tumor growth due to various activated mitogenic stimuli or inhibited tumor cell apoptosis and 3) tumor angiogenesis [235,

236, 551]. These events are mutually dependent. Successful establishment of tumor angiogenesis requires a sufficient nutrient and oxygen supply to promote tumor growth [4, 265, 266]. Many drugs have been developed to target these steps in order to reverse the progression of cancer. For example, the anti-estrogen TAM was developed to antagonize the proliferative effects of estrogen on breast cancer cells, which also has anti-angiogenic effect [311]. Angiostatin and endostatin are used as anti-angiogenic agents to induce cancer regression [119, 289]. These agents are effective in blocking cancer progression at different stages. However, the clinical achievements of these drugs are less than satisfactory since they affect only one specific aspect of cancer progression. Compared to these drugs, Å6 is of particular interest since it has been observed to affect multiple aspects of breast cancer behavior *in vitro*. Following its injection into animals, Å6 inhibits the growth of primary tumor by inducing cancer cell death (Fig. 3.4 & 4.6) and blocking tumor angiogenesis (Fig. 3.5 & 4.5) and suppresses overall breast cancer metastasis regardless of the hormone receptor status of the breast tumors (Fig. 3.3 & Table 4.1).

Although early detection and improved treatment have begun to affect rates of breast cancer mortality, research must continue to focus on the treatment of this malignancy. Treatment generally requires multiple approaches, including mastectomy, radiation therapy, chemotherapy, endotherapy and the combination of these modalities [372]. TAM is an ER antagonist and its anti-breast cancer effects can be achieved through ER-dependent or -independent mechanisms. By binding to nuclear ER, TAM blocks the interaction between estrogen and ER, and therefore abrogates estrogen-induced gene transcription and estrogen-induced cell proliferation [11]. TAM has other anti-cancer effects that are ER-independent. For example, the effects of TAM include induction of antibody synthesis [552], inhibition of angiogenesis [553], induction of immune reaction [554] and a decrease in the production of many autocrine mitogens or protease receptors for cancer cells including EGF, IGF-1, TGFs and uPAR [11, 316]. Combination therapies using TAM and other chemotherapy agents have been reported to reduce the odds of recurrence and death in breast cancer patients [11, 542]. Breast cancer is generally a mixture of cells with multiple genetic backgrounds and consists of both high and low malignant

cancer cells [555]. The primary targets of TAM will be those low-grade cancer cells that generally express ER. During tumor progression, there is an increased population of tumor cells that have lost their sensitivity to TAM treatment, and have increased expression levels of uPA. The addition of Å6 will bridge this gap by targeting those malignant cells with high levels of uPA expression, regardless of their hormone receptor status. Therefore it is an ideal drug to block tumor progression, especially those tumors with a high invasive capacity.

In summary, DNA methylation plays an important role in the regulation of *uPA* gene expression. The lowed maintenance DNMT and increased DMase activities favor the demethylation status of the *uPA* gene, which result in the upregulation of uPA expression at late stage breast cancer. To block breast cancer progression, we developed a synthetic peptide, Å6. Its effects on breast cancer in animal models have been documented and include the inhibition of primary tumor growth, suppression of cancer metastasis, induction of tumor cell death and inhibition of cancer angiogenesis. The latter is probably due to the suppression of flk-1 and PAI-1 levels as well as the activity of TGF-β. When administered together, Å6 and TAM displayed synergistic effects on the suppression of breast cancer progression, rendering it a promising combination regimen for breast cancer therapy. The hypothesis that DNA methylation regulates *uPA* gene expression was tested in two cell lines representing different stages of breast cancer progression at this stage, in the future study, more cell lines should be tested to confirm the universal application of this observation. Eventually, *in vivo* study using either animal model or human cancer samples should be conducted to validate this hypothesis. Since all cancers share similarities although the nature of the biology and the genotype of each cancer vary, the development and biological characterization of Å6, may also be applied to other cancer types. Further studies are needed to fully understand the mechanisms underlying the actions of this peptide.

As a result of this study, I conclude that Å6, a synthetic peptide, is an ideal breast cancer chemotherapy agent. uPA promoter methylation status is a determinant for its expression during breast cancer progression and, will provide a new direction for cancer targeting.

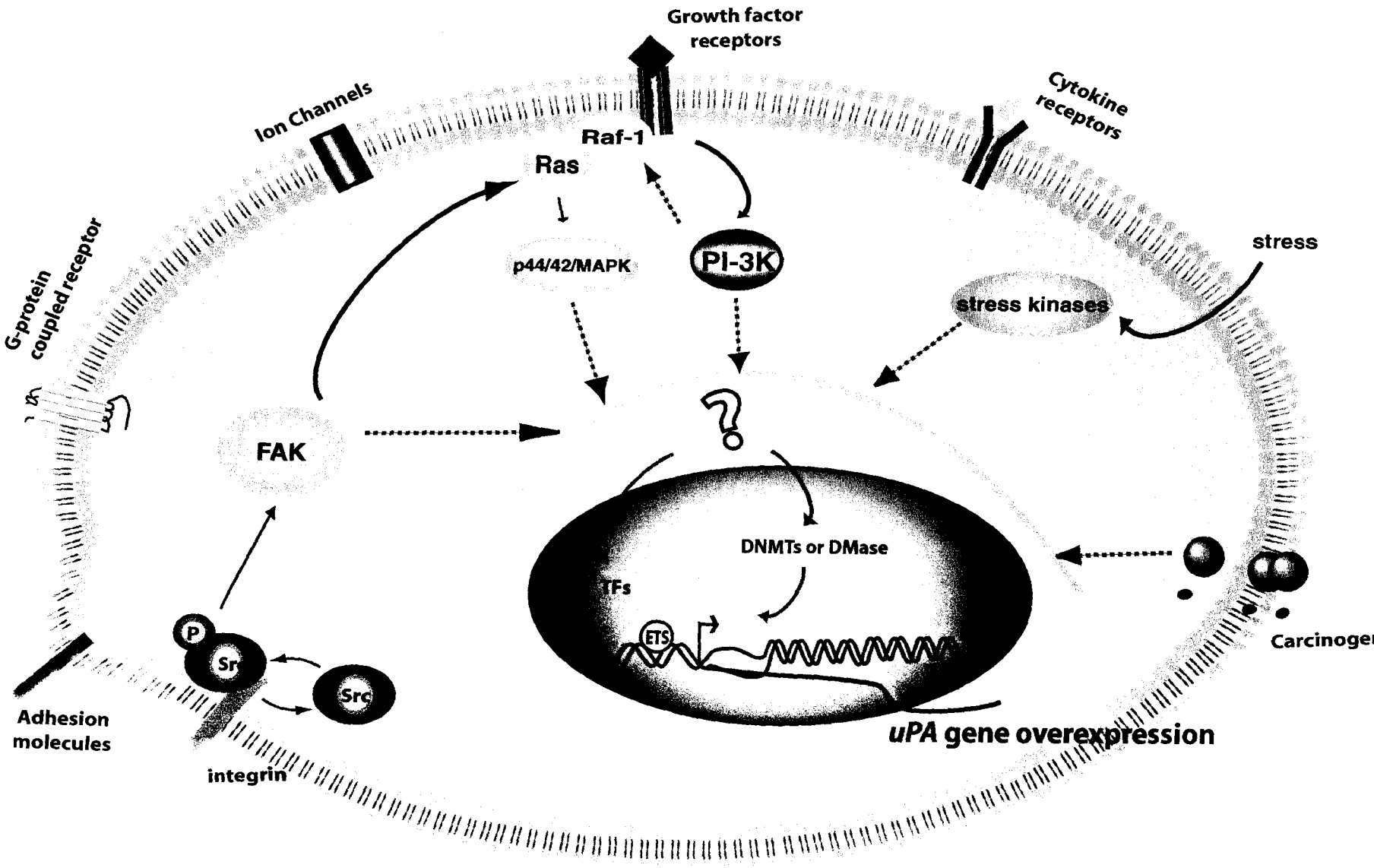

Growth factor receptors
Ion Channels
Cytokine receptors
Raf-1
Ras
p44/42/MAPK
PI-3K
stress
stress kinases
G-protein coupled receptor
FAK
DNMTs or DMase
TFs
ETS
P
Src
Src
Carcinogen
Adhesion molecules
integrin
uPA gene overexpression

Fig. 5.1. Simplified model of *uPA* gene expression regulation in cancer.

Expression of the *uPA* gene depends on two parameters as shown in this figure: 1) Availability and abundance of transcription factors (TFs) such as Ets for the uPA promoter. Various external stimuli (e.g. growth factors, cytokines, oncogenes, adhesion molecules, stress and carcinogen exposure etc.) have been reported to affect this parameter by activating multiple signaling pathways including p44/42 MAP kinase and PI3-K/PKB pathways. 2) Altered *uPA* gene structure due to DNA methylation and histone modification, which in turn determines the accessibility of the required transcription factors. Balanced activities between DNMTs (or MTases) and DMase might regulate these epigenetic changes in the *uPA* gene in cancer. Activation of certain signaling pathways or oncogenic transformation may alter the levels or activities of these methylation-related enzymes, and therefore determine *uPA* gene expression directly or indirectly.

Chapter IV

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