# CHAPTER 1

# INTRODUCTION

## 1.1 STATEMENT AND SIGNIFICANCE OF PROBLEM

Cancer is one of the major public health problems worldwide, and now has a very high mortality rate in Thailand. Cancer cells are typically classified by uncontrolled cell growth and cell division leading to a mass of unwanted cells called a tumor. Besides, many cancer cells have an ability to relocalize to distant tissues and these distant settlements of cancer cells consequently account for the major cause of death in cancer. Carcinogenesis is a complicated multi-step process in which initiation and progression of cancer cells involve genetic/epigenetic aberrations of human genome. Such alterations in the genome lead to either over-activation (*e.g*. proto-oncogenes) or inactivation (*e.g* tumor suppressor genes) of specific groups of genes. Altered regulation of these genes in cancer cells eventually causes uncontrolled-cell proliferation, invasion, and metastasis.

Maspin (mammary serine protease inhibitor) is one of the class II tumor suppressor proteins whose genes are not mutated, but rather down-regulated upon tumorigenesis (1). Down-regulation of maspin in carcinoma tissues is correlated with progression of tumors (2, 3). Several studies have revealed the tumor suppressor role of maspin as an effective inhibitor of cancer cell invasion and metastasis (4-6). Re-expression of maspin in carcinoma cell lines modifies several phenotypes and leads to inhibition of cell invasion and metastasis both *in vitro* and *in vivo* (5-7). Maspin alters

integrin profile, especially α5- and α3- integrins leading to an increase in adherence of the cancer cells to fibronectin (5). Furthermore, maspin may regulate cell migration by regulating Rho GTPase signaling pathway since it decreases Rac1, but increases PI3K and ERK1/2 activities leading to an increase in focal adhesions and stress fibers (8). Shotgun proteomics analysis of maspin deficient and maspin expressing breast carcinoma cells revealed that the re-expression of maspin not only inhibits the invasiveness, but also has a major impact on the composition and function of the tumor cell proteome (9). Maspin elicits changes in the expression of proteins associated with the actin cytoskeleton that predicts a less motile and invasive phenotype (9). In addition, maspin expression reduces chymotrypsin-like activity of the 20S proteasome via down-regulation of β5 subunit of the proteasome that causes the accumulation of high molecular weight ubiquitin conjugates in maspin-transfected cells (9). These observations indicate that maspin's mechanism of anti-invasion may be mediated through the ubiquitin-proteasome pathway.

Maspin is a 42 kDa protein and belongs to the serine proteinase inhibitor (Serpin) superfamily including α1 anti-trypsin, plasminogen activator inhibitor (PAI), and ovalbumin (10). Despite its similarity to other serpin members, maspin is a noninhibitory serpin because it does not directly exert its biological functions as a protease inhibitor (11, 12). Earlier studies using recombinant protein have characterized the functional domain of maspin and showed that the reactive center loop (RCL) of maspin is necessary and sufficient for the inhibition of cancer cell invasion (13, 14). Deletion of maspin RCL or substitution with the ovalbumin RCL completely abolishes biological activities of the recombinant protein. The mode of maspin action appears to mediate RCL binding to a cell surface receptor since

addition of anti-RCL maspin antibody can block the effect of exogenously added maspin (14). However, the underlying mechanism by which the maspin RCL elicits its anti-invasive effects is not well established and still remains to be solved.

To identify potential molecular pathways for the tumor suppressive properties of maspin, ectopic expression of the maspin/ovalbumin RCL chimeric mutants in human breast carcinoma cell line will be characterized in this study. As the functional region of maspin, the RCL is proposed to be responsible for regulation of tumor cell proteome and phenotypes. Three specific aims are addressed herein 1) to determine whether maspin re-expressing MDA-MB-231 cells require the RCL for inhibition of cell invasion and stimulation of cell adhesion *in vitro*, 2) to determine whether the RCL of maspin is necessary for inhibition of chymotrypsin-like activity of the 20S proteasome, and 3) to investigate the effect of the maspin RCL on the proteome of MDA-MB-231 cells. The results of this study will lead to specific target proteins affected by the maspin RCL in the tumor cell, and further elucidate the mode of intracellular maspin's action via the RCL in order to gain insight into the molecular mechanisms of maspin's tumor suppressive function.

## 1.2 LITERAURE REVIEWS

### 1.2.1 Serpins: Structure and Function

Serpins (serine protease inhibitors) are a superfamily of protease inhibitors with similar protein structure (10). Serpins have the average size of protein 350–400 amino acids and the molecular weight of 40–50 kDa (15). Serpins share about 30-40 % amino acid sequence homology, but they adopt a conserved protein folding as shown in **Figure 1**. Typically, the serpin fold is comprised of three $\beta$-sheets (A, B, C) and nine alpha helices. Tethered between β-sheets A and C, the reactive center loop (RCL) is an exposed flexible stretch of ~17 residues with the most sequence heterogeneity.

Most of Serpins are inhibitor of serine protease so called inhibitory serpins. Examples of inhibitory serpins include α1-antichymotrypsin (α1ACT), α1-antitrypsin (α1PI), antithrombin (AT), plasminogen activator inhibitor 1 and 2 (PAI1 and PAI2), etc. The RCL is important for the function of inhibitory serpins because it contains the protease recognition site. Inhibitory serpins use their RCL to trap the target protease and inhibit its activity (16). The mechanism of inhibition of serpin involves the covalent complex formation: initially serpin binds to a target protease through a noncovalent Michaelis-like complex by interactions with residues flanking the scissile bond (P1-P1′) of RCL (16). A covalent ester linkage between serine of the protease and the backbone carbonyl of the P1 residue results in the cleavage of the peptide bond. Next, RCL inserts into the $\beta$ sheet A and relocates the covalently bound protease with it. As a result, the protease is translocated and its active site gets distorted shown in **Figure 2** (16, 17). Distortion of the active site prevents the final

hydrolysis events and the result is an irreversible covalent serpin-enzyme complex. This conformational change termed as the Stressed to Relaxed (S to R) transition that increases in their conformation stability. Furthermore, the amino acids of the hinge region of RCL (P15-P9) have small side chain that allows loop flexibility necessary for complex formation (18).

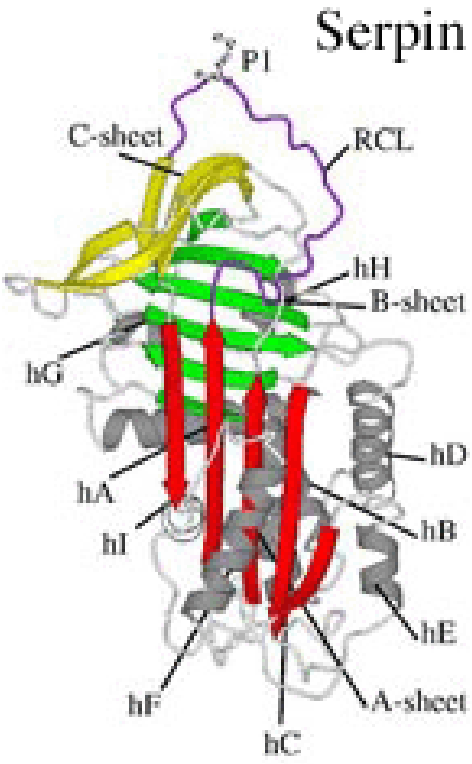


**Figure 1** Typical Structure of Serpins

(http://serpins.med.unc.edu/~fcc/)

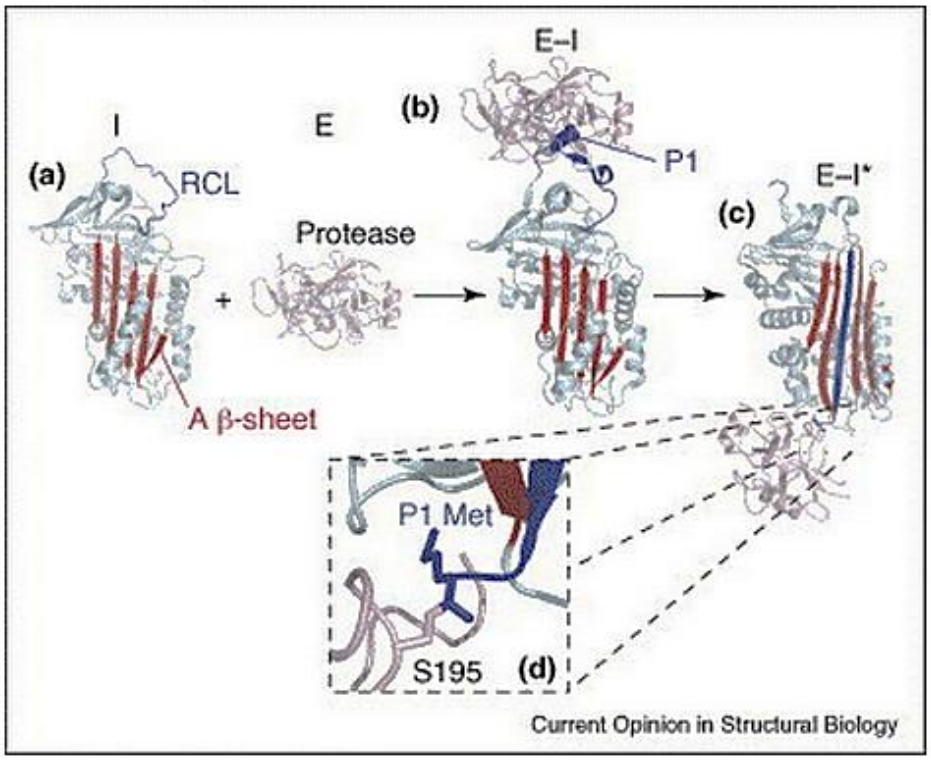


**Figure 2** Mechanism of Inhibitory Serpins (19)

**a.** The structure of native serpin and protease

**b.** The Michaelis-like complex between serpin and protease

**C.** The final serpin enzyme complex

Biological functions of human serpins are shown in **Figure 3**. Inhibitory serpins (red) involve many key biological processes such as coagulation and fibrinolysis, apoptosis, inflammation, etc. Several members of serpins do not require protease inhibition so called "noninhibitory serpins". They have shorter $NH_2$ and COOH termini, and also lack the classic serpin secretory signal peptide (20). Human noninhibitory serpins (green) can perform other roles such as hormone transport (thyroid-binding globulin (TBG), corticosteroid binding globulin (CBG)), blood pressure regulation (angiotensinogen (AGT)), and tumor suppressor (maspin).

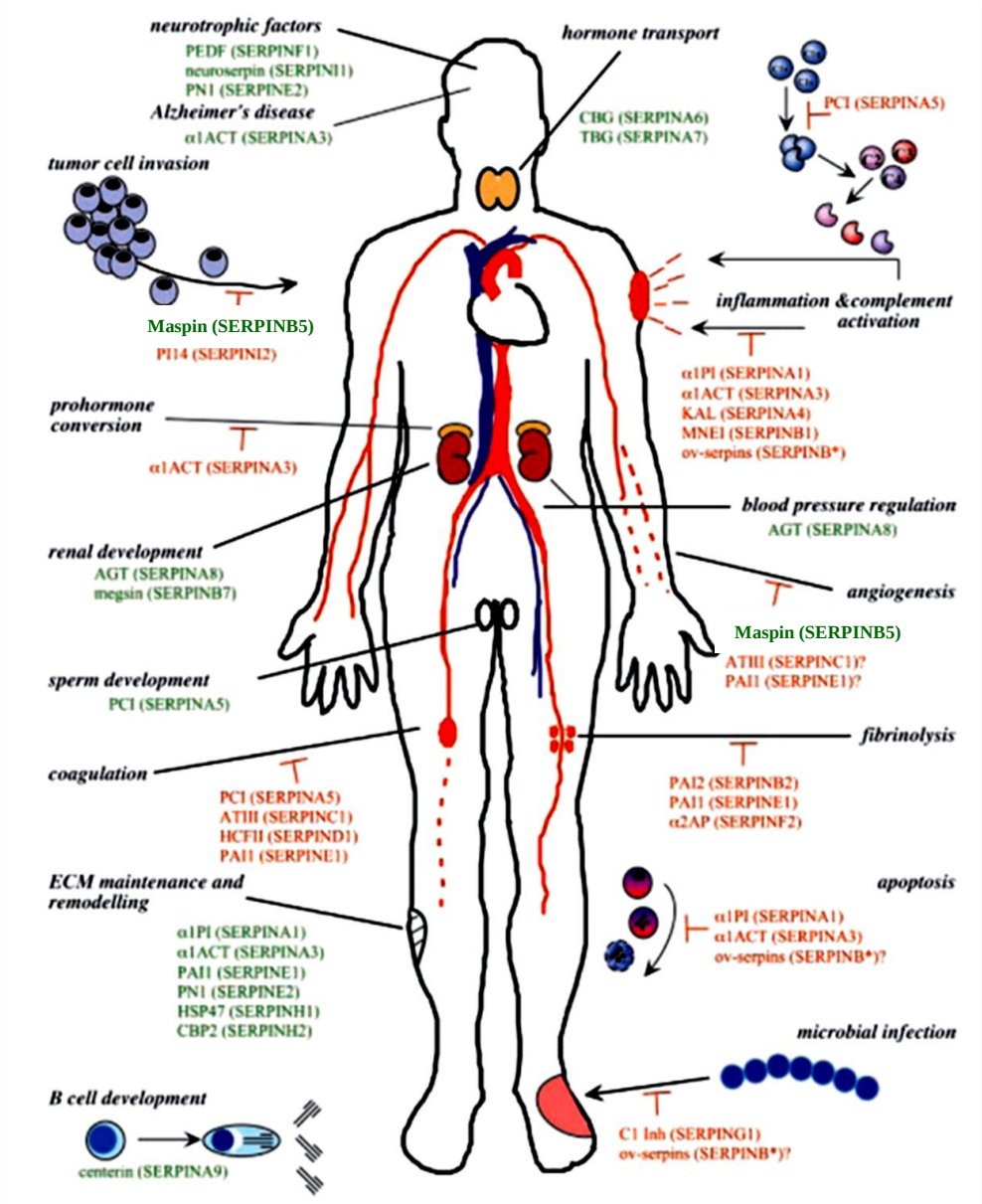


**Figure 3** Biological Functions of Human Serpins (17)

### 1.2.2 Maspin

Maspin (mammary serine protease inhibitor) was originally discovered as a human mammary tumor suppressor in 1994 (6) and has been shown to exert tumor suppressing activity against breast tumor growth and metastasis (21). Maspin is present at high concentration in normal mammary myoepithelial cells, but its expression is down-regulated in primary breast cancer cell lines and lost in invasive mammary carcinoma (6, 22, 23). Down-regulation of maspin was critical transition from noninvasive to invasive carcinomas (24).

Maspin (or SERPIN B5) is a 42 kDa protein closely related to noninhibitory clade B serpins as it exhibits a significant sequence similarity (31%) to noninhibitory serpin ovalbumin (**Figure 4**). The primary structure of maspin is also highly similar to some inhibitory serpins such as plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2), and α1-antitrypsin (16). This homology leads to the initial hypothesis that its tumor suppressor function might result from its ability to inhibit proteolysis. A few studies have attempted to demonstrate maspin as an inhibitor of tissue-type plasminogen activator (tPA) (25) and urokinase-type plasminogen activator (uPA) (26). Unfortunately, in those studies, maspin acts as a competitive substrate rather than inhibitor, so its inhibitory effect on the protease activities seems to be indirect (13).

Several structural features support maspin's classification as a noninhibitory serpin. First, the RCL of maspin is not conserved and is shorter than most RCLs of the serpin superfamily. It has been hypothesized that the short RCL decreases the stability of the molecule, rendering maspin extremely sensitive to limited proteolysis

(27). Second, maspin does not inhibit a number of serine proteases *in vitro*. This could also contribute to maspin's inability to undergo the ''stressed to relaxed'' transition, a step required by serpins for protease inhibition (10). However, the RCL of maspin is clearly important for its functional; studies using synthetic maspin reactive center loop peptides and recombinant maspin/ovalbumin chimeric proteins revealing that this region is important for its function.

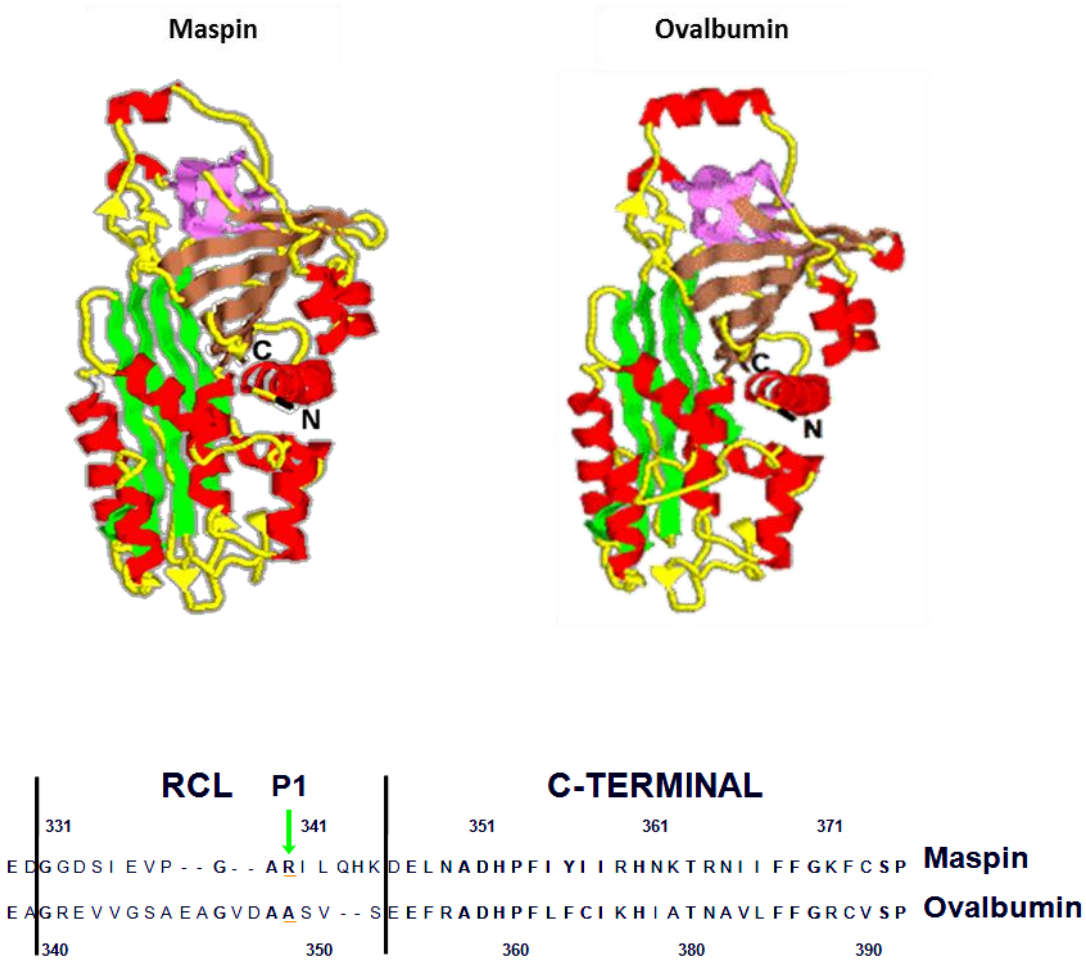


**Figure 4** Three Dimensional Structure of Maspin vs Ovalbumin (top)

Amino Acid Sequence Alignment of RCL toward C-terminal Region (bottom)

### 1.2.3 Biological Functions of Maspin on Cancer Cell

Maspin has been consistently shown to suppress the aggressive tumor phenotypes by inhibiting cell invasion and mobility *in vitro* and inhibiting tumor growth and metastasis in experimental animal models (4, 28, 29). Importantly, metastatic lesions account for nearly all cancer-related deaths, implicating metastasis as a key step in cancer progression. Maspin's ability to inhibit the invasive and migratory potential(s) of cancer cells has significant implications when considering the multi-step progression of the metastatic cascade. The metastasis process involves cell migration, invasion through the lamina propria, and growth in a foreign microenvironment. Reducing the ability of tumor cells to invade local stroma and intravasate/extravasate holds significant potential to inhibit the process of metastasis. Extensive studies have been undertaken to determine the underlying mechanisms employed by maspin to produce its anti-metastatic effects. Summary of validated effects of maspin in cancer is shown in **Figure 5**.

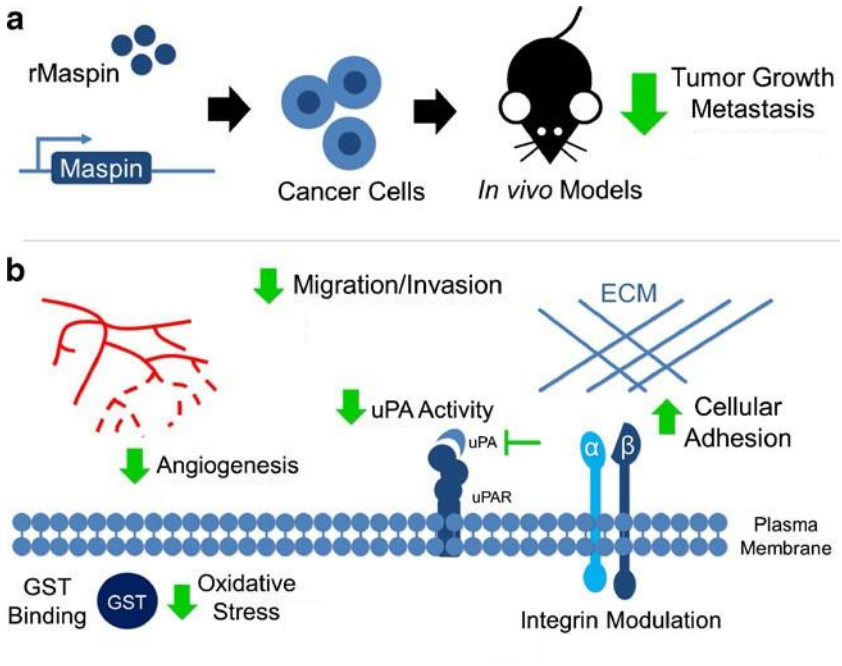


**Figure 5** Summary of Validated Effects of Maspin in Cancer Biology (30)

a) Re-expression of maspin or treatment with recombinant maspin decreases tumor growth and metastasis both *in vitro* and *in vivo* models.

b) Biological effects of maspin in cancer cells

### 1.2.4 Maspin Inhibits Cancer Cell Motility and Invasion

One of the most common activities of maspin on cancer cells, whether endogenously expressed or exogenously applied, is the inhibition of cancer cell motility and invasion. Mammary carcinoma cells transfected with maspin were significantly less invasive *in vitro* and less metastatic in nude mice (6). Addition of recombinant maspin to human breast and prostate cancer cells acts at the cell membrane to inhibit invasion and motility. Using time-lapse video microscopy, the migration of maspin transfected cells in two-dimensional cultures was also reduced (31), further supporting a role for maspin on the suppression of cell motility. Transfection of maspin-null MDA-MB-435 breast carcinoma cells with a maspin-expressing plasmid caused a significant inhibition of these transfectants's invasion through Matrigel (6). Interestingly, addition of maspin neutralizing antibodies to the culture conditions reversed the ability of transfected cells to invade, suggesting that maspin may act at the cell surface, and mediate signaling that affects cytoskeletal networks. The anti-invasive property of maspin was confirmed by other studies using both human breast and prostate cancer cells (31, 32). Similarly, the mouse ortholog of maspin can reduce both the motility and invasiveness of murine mammary tumor cells (13). TM40D mammary tumor cells were highly invasive into surrounding tissue after implanted into the mammary fat pads of BALB/c mice. These cells also metastasize to intestine and lung. Expression of maspin in TM40D cells blocked the tumors' invasiveness with no signs of metastasis (33).

Molecular investigation of maspin effects on migration was early focused on the ability of maspin to inhibit proteolysis. Maspin indirectly inhibits cell surface-bound

urokinase plasminogen activator (uPA)/ uPA receptor (uPAR) complex in prostate cancer cells (31). The uPA/uPAR complex is involved in the conversion of plasminogen to plasmin, an active protease which is able to cleave a number of extracellular matrix proteins such as fibronectin, fibrin, and laminin. Although maspin binds the pro-uPA zymogen and suppresses the uPA activity (34), such effect was not a direct inhibition (35) due to the characteristic of noninhibitory serpin.

More extensive mechanistic data related to maspin inhibition of cancer cell migration and invasion showed that the addition of recombinant maspin to the culture media decreased downstream signaling of Rac1 and Cdc42 through JNK activity. Rac1 and Cdc42 are GTPases that are involved in cytoskeletal rearrangement and migration of MDA-MB-231 cells (36). These results provide a more mechanistic insight to the regulation of cellular motility by maspin protein.

### 1.2.5 Maspin Affects Cancer Cell Adhesion to Extracellular Matrix

Despite an unclear mechanism of maspin's biological function, *in vitro* and *in vivo* studies have supported the maspin's role in stimulation of cancer cell adhesion to extracellular matrix (ECM), especially fibronectin. Addition of recombinant maspin in MDA-MB-435 breast cancer cells that lack maspin expression resulted in increased levels of several integrins, especially α5- and α3- integrins and an overall decrease in β1-containing integrins. As a result, maspin increases adherence of the cancer cells to fibronectin, leading to a reduction in invasion through a fibronectin/gelatin matrix (5). Besides, addition of an RGD peptide (known to block integrin function) could reverse the effects of exogenously added recombinant maspin. Maspin-treated breast carcinoma cells also had an increase in the distribution of the α5β1 integrin in the

cells. Interestingly, recombinant maspin could alter the intracellular and extracellular levels of matrix metalloproteinase-2 (MMP-2) probably via the α5β1 integrin signaling pathway (5).

The underlying mechanisms by which maspin regulates cancer cell-ECM adhesion has been shown in some reports. Using exogenously added recombinant maspin and mutants, the presence of maspin's RCL is required for promoting the adhesion of breast carcinoma cells MDA-MB-231 to fibronectin and human corneal stromal cell to type I collagen. The RCL of maspin is also sufficient since addition of the RCL peptide or the ovalbumin mutant containing the RCL of maspin can efficiently induce increased the ECM adhesion of these cells (14). Yet, the mode of RCL's action is currently unclear. Treatment of MDA-MB-231 cells with recombinant maspin or re-expression of maspin in MDA-MB-435 cells could alter signaling pathways involved in cell adhesion (8). Maspin increases PI3K and ERK1/2 activities leading to increased focal adhesions and stress fibers. This study suggests that maspin may regulate cell adhesion via PI3K/ERK pathways.

Additional work using the nontransformed, immortalized human mammary epithelial cell line, MCF-10A (which expresses high levels of endogenous maspin), showed that maspin physically and functionally associates with a "β1 integrin" where it co-localizes on the cell surface (37). Maspin was also associated with cytoskeletal elements suggesting that it may form part of a supramolecular structure of adhesion plaques. However, this study showed that treating MCF-10A cells with recombinant maspin increased their adhesiveness without involving the RCL which contradict to previous studies in tumor and corneal stomal cells (14). The increased adhesion of

MCF-10A cells in response to maspin treatment was shown to be facilitated by amino acids 139–225 in the molecule of maspin. More recent work has extended these studies with MCF- 10A cells and demonstrated that maspin can form a bridge between uPA and its receptor uPAR with a "β1 integrin" to form a mega-complex that regulates mammary epithelial cell adhesion (38). Using competition peptides and mutation analyses, two regions of maspin (amino acid residues 190–202 and 260–275) facilitate the increase in the adhesion of MCF-10A cell. Furthermore, this study demonstrated that the uPA–uPAR complex is required for the localization and adhesion function of maspin in MCF-10A cells.

### 1.2.6 Maspin Alters the Carcinoma Proteome

Re-expression of maspin in cancer cells has widespread effects on the cellular proteome. Shotgun proteomics analysis of maspin-deficient and maspin-expressing MDA-MB-435 cells revealed that maspin leads to alterations in the expression level of ~27% of the detected proteome (9). In most cases, the protein expression is affected without changes in mRNA levels, indicating that maspin has a significant influence on posttranscriptional regulation of protein levels. Significantly, maspin elicits changes in the expression of proteins associated with the actin cytoskeleton. The changes in these cytoskeletal proteins are predicted to result in a reduction in cell motility, invasion, and metastatic phenotype. Moreover, differential proteomic analysis showed that proteins known to promote apoptosis are up-regulated while anti-apoptotic proteins are down-regulated in maspin-expressing cells (9). Lastly, maspin appears to have an effect on the ubiquitin-proteasome pathway, indicating that the proteasome could be a key downstream regulator of tumor progression. Although

these observations revealed the effects of maspin on multiple protein networks, the mode of RCL's action on tumor cell proteome is currently unclear.

### 1.2.7 Maspin Affects the Ubquitin-Proteasome System

In cancer, the ubiquitin-proteasome pathway plays a number of important roles including the regulation of tumor growth through multiple targets impacting cell cycle progression and apoptosis, cell adhesion, invasion, and metastasis (39). Since the ubiquitin-proteasome pathway manipulates multiple biological processes through protein regulation, it is possibly that dysregulation of proteasome function is necessary for tumor cells to escape regulated cellular functions as the tumors advance toward malignancy.

#### 1.2.7.1 The Ubiquitin-Proteasome System

Ubiquitin is an abundant and essential cellular 9 kDa protein. Ubiquitin is normally used by cells as a covalent modifier of other proteins both to activate their function and to target them for degradation, depending on the degree of ubiquitin ligation (40). Addition of long polyubiquitin chains targets proteins for degradation by the 26S proteasome (40, 41). Because ubiquitin has a broad range of biochemical interactions and effects, the control of ubiquitination is complicated and is regulated by a wide array of ubiquitin ligase enzymatic complexes whose assembly is triggered by diverse signals in the cell (42). The ubiquitin-proteasome protein degradation pathway is comprised of ubiquitin, a three-enzyme ubiquitination complex, the intracellular protein ubiquitination targets, and the proteasome that is a specialized organelle of protein degradation **(Figure 6)** (42).

The ubiquitin-activating enzyme, E1, initiates ubiquitin ligation by adenylating ubiquitin (43). The ubiquitin molecule is transferred to the ubiquitin conjugating enzyme E2, which transiently carries ubiquitin. E2 works in conjunction with the ubiquitin ligase E3, which is responsible for conferring substrate specificity on the reaction. E3 mediates the transfer of ubiquitin to an internal lysine of the target protein. The polyubiquitinated target protein undergoes proteolytic cleavage by the proteasome, which is a large, cylindrical multi-subunit complex (42).

The 26S proteasome is a large multi-catalytic protease that degrades polyubiquitinated protein to small peptides. It is composed of two major subunits that can assemble in an ATP dependent manner (44, 45). The 20S catalytic component contains multiple proteolytic sites, and the 19S regulatory component contains multiple ATPases and a binding site for ubiquitin concatemers (**Figure 7**) (46).

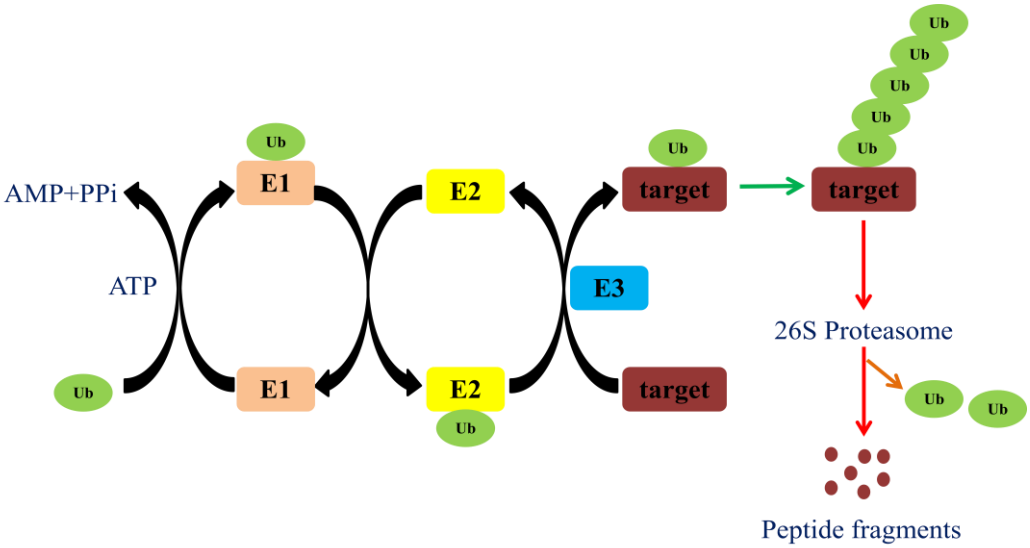


**Figure 6** The Ubiquitin-Proteasome System

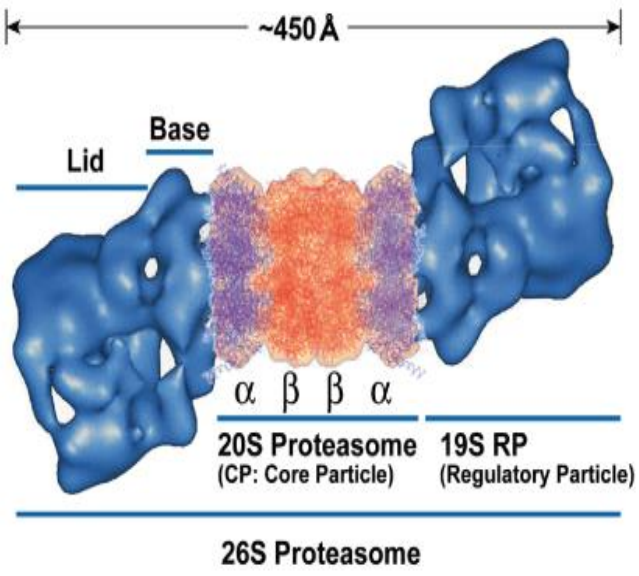


**Figure 7** Composition of the 26S Proteasome

(http://www.tanpaku.org/e_icsg2008/07_01.php)

The 20S subunit is the core of the 26S proteasome and is made up of four heptameric protein rings stacked like four doughnuts. The 20S catalytic β-subunit has three major proteolytic activities: a chymotrypsin-like, a trypsin-like, and a peptidylglutamyl peptide hydrolyzing activity (PGPH) (47). The two alpha rings sandwich the beta rings. The amino terminus of the β-subunits blocks access to the proteolytic chamber. Thus, the inner cavity of the proteasome is only accessed through the narrow pores on either end of the cylinder (48).

The 19S regulatory components assemble at each pore of the 20S subunit to form the 26S proteasome. The 19S regulatory subunit acts as a gate agent to limit entry to the proteasome to targeted proteins. The 19S subunit is also essential for proteolytic activity because the 20S subunit alone is inactive (49). The 19S regulatory particle is composed of two substructures, a lid and base, and is involved in substrate selection, preparation, and protein translocation into the catalytic 20S chamber for degradation. The outer-lid subcomplex of the 19S component is involved in the recognition and ubiquitin chain processing before substrate translocation and degradation (50).

According to the previous study on cancer cell proteome, maspin also appears to have an effect on the ubiquitin-proteasome pathway, indicating that the proteasome could be a key downstream regulator of tumor progression. Re-expression of maspin causes reduced chymotrypsin-like activity of 20S proteasome via the down-regulation of the β5 subunit leading to the accumulating of the high molecular weight ubiquitin conjugates in all maspin-transfected cells (9). These results also suggest an inverse correlation between the expression of maspin and proteasome activity. Thus, a new

hypothesis of maspin function is established based on the regulation of proteasome function. However, it is still not known whether the maspin RCL is required for reduction of the proteasome activity in tumor cells.

## 1.3 HYPOTHESIS

Although previous studies showed that the RCL of maspin is necessary for maspin's tumor suppressive functions in cancer cells, the molecular mechanism of maspin's actions is still inconclusive, and remains to be further explored. The hypothesis of this study is the RCL as a functional region of maspin could affect the cellular protein expression and the proteasome activity of breast carcinoma MDA-MB-231 cells, leading to regulation of the carcinoma phenotypes including cell adhesion, migration, and invasion. This study employed re-expression of maspin and mutant proteins in MDA-MB-231 by transfecting with plasmids expressing wild type maspin, ovalbumin (a highly homologous noninhibitory serpin with no anti-tumor properties), or maspin/ovalbumin RCL chimeric mutants in which maspin RCL is replaced by ovalbumin (MOM) and vice versa (OMO). These transfectants cells were further studied using biological and biochemical assays as well as proteomic analysis approach.

## 1.4 OBJECTIVES OF THE STUDY

Using the maspin/ovalbumin chimeric mutants, this study is aimed:

1. To determine whether maspin re-expressing MDA-MB-231 cells require the RCL for inhibition of cell migration, invasion and stimulation of cell adhesion *in vitro*.

2. To determine whether the RCL of maspin is necessary for inhibition of chymotrypsin-like activity of the 20S proteasome.

3. To investigate the effect of the maspin RCL on the proteome of MDA-MB-231 cells.