CHAPTER 4

DISCUSSION AND CONCLUSION

Maspin is a tumor suppressor in human breast carcinomas and belongs to the serine proteinase inhibitor (Serpin) superfamily. Maspin has been shown to inhibit cancer cell migration, invasion, tumor growth and metastasis *in vitro* (4, 28, 29). Re-expression of maspin in cancer cells line causes several alter phenotypes and leads to inhibition of cell growth and metastasis *in vitro* and *in vivo* (9). Despite its similarity to inhibitory serpin members that lead to hypothesis that the tumor suppressor functions of maspin might be resulted from its ability to inhibit proteolysis. While the inhibition of the pericellular urokinase-type plasminogen activator (uPA) system by maspin has been reported in prostate tumor cells (26), others have not detected any inhibitory activity on several different proteases (11, 12). Thus, maspin as a noninhibitory serpin, may not directly exert its biological functions as a protease inhibitor. Numerous *in vivo* and in *vitro* studies have shown maspin's ability to reduce tumor cell motility and invasion. Maspin's tumor suppressing effect appears to depend in part on its ability to increase cell adhesion to ECM (55). Treatment of recombinant maspin in MDA-MB-435 cells (weakly invasive human breast carcinomas that lack maspin expression) resulted in increased level of integrin, especially α5- and α3- integrins of the cell. As a result, maspin increases adherence of the cancer cells to fibronectin (5). Previous studies using exogenously added recombinant proteins demonstrated that the RCL of maspin is essential for normal corneal stromal and cancer cell adhesion to type I collagen and fibronectin,

respectively (14). Nonetheless, this activity of RCL has yet to be demonstrated inside the cells. On the contrary, a study by Zhang in MCF-10A (normal mammary epithelial cells) suggested that maspin promotes cell adhesion independently of its RCL domain (37). The treatment of MCF-10A with anti-RCL antibody or with recombinant maspin RCL mutants did not abrogate the effect of maspin on cell-ECM adhesion. These differences may reflect different cell models and/or experimental approaches.

In this study, we elucidate the activity of maspin's RCL as functional moiety for these properties by transfecting the highly invasive human breast carcinoma MDA-MB-231 cells with plasmid ectopically expressing maspin wide-type, ovalbumin or maspin/ovalbumin RCL chimera proteins. Ovalbumin as a non-inhibitory serpin, was chosen as negative control because it shares a significant amino acid sequence similarity (31%) with maspin, but has no tumor suppressive activity (14). Here, we were able to demonstrate that only those transfected MDA-MB-231 cells expressing maspin RCL-containing proteins, namely, maspin and OMO, have reduced cell migration and invasion, but enhanced adhesion to fibronectin. These results suggested the RCL is indeed required and sufficient for anti-tumor properties of native maspin. Our findings are in agreement with the previous study in which yeast recombinant maspin/ovalbumin RCL chimera protein was exogenously added to carcinoma MDA-MB-231 culture (14). This mode of exogenously added maspin action relies on the presence of maspin/RCL receptor on the target cell surface. On the other hand, the approach described here for the first time sheds light on the role of RCL in the tumor suppressive properties of intracellularly expressed maspin. Maspin may exert both effects as it is present in the cytosol and also is secreted (56). Nonetheless, RCL is involved in such biological functions of maspin.

The ubiquitin-proteasome pathway in cancer cell 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). In the previous study, maspin expression in MDA-MB-435 cells decreased ubiquitin-proteasome activity via down-regulation of expression of 20S proteasome subunits. Maspin-expressing breast carcinoma cells decreased chymotrypsin-like activity of 20S proteasome compared with parental cells. Here, expression of maspin and OMO in transfected MDA-MB-231 cells resulted in increased accumulation of both free ubiquitin and ubiquitin-conjugates compared with mock and MOM- or OVA-expressing carcinoma cells. The results of our study further extend the knowledge that maspin RCL acts as the functional domain responsible for the inhibition of ubiquitin-proteasome pathway via reducing chymotrypsin-like activity of the 20S proteasome.

The molecular mechanisms underlying maspin's diverse activities have not been clearly elucidated, and are now under intense investigation. A number of other studies have shown that maspin effects on tumor phenotype. Analysis by shotgun proteomics and multidimensional protein identification technology (MudPIT) revealed that re-expression of maspin in MDA-MB-435 cells lead to alterations in the expression level of ~27% of the detected proteome (9). Significantly, maspin elicits changes in the expression of proteins associated with the actin cytoskeleton, apoptosis and ubiquitin-proteasome pathway. The changes in these proteins are predicted to reduce in cell motility, invasion, apoptosis and degradation proteins activities. 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. In this study, we employed 2D-gel electrophoresis and GeLC-MS/MS analysis to compare the proteomes of

highly invasive human breast carcinoma MDA-MB-231 cells expressing maspin, MOM, OMO and ovalbumin. Due to limited sensitivity and resolution of the technique, 2D-gel electrophoresis approach could detect one protein spot in MDA-MB-231 cells expressing maspin and OMO, but absent in those expressing MOM and ovalbumin. This spot was subjected to protein database searching for proteins with similar pI and MW (pI ~4.1 and MW ~40 kDa). There are several proteins such as enolase, cell diversion proteins, and ketol-acid reductoisomerase; however, all of them have no functions associated with the tumor suppressor activities of maspin. Nevertheless, the preliminary result indicated the RCL specific pattern of proteome exists in MDA-MB-231 cell expressing maspin RCL-containing proteins.

Using an alternative highly sensitive GeLC-MS/MS method, a total of 3,122 proteins were significantly expressed differentially ($p<0.01$). Expression levels of many proteins in MDA-MB-231 cells are significantly altered by transfection with maspin and OMO, but not with MOM or ovalbumin. For example, connexin45 and sorting nexin-33 (SNX33) were >3 fold up-regulated as compared to mock cells. Connexins have been suggested to function as tumor suppressor proteins as re-expression of connexins causes reduced growth and tumorigenicity of cancer cells (57, 58). The promoter region of GLC1, encoding connexin45, is hypermethylated and transcriptionally silenced in colorectal cancer (58). SNX33 plays an important for cell cycle and cytoskeletal because overexpression of SNX33 in HeLa cells induces cell death with micronucleation via association with WASp and enhanced actin polymerization (59).

Conversely, several oncogenic proteins are significantly down-regulated, for example Rho GDP-dissociation inhibitor 2 and epithelial cell transforming 2 (ECT2). Both proteins are known to be frequently overexpressed in multiple types of human cancers and can regulate cancer progression, especially enhancing aggressive phenotypes such as cancer cell invasion and metastasis (60). ECT2, a guanine nucleotide exchange factor for Rho family GTPases, can selectively regulate Rac1 activity (36). Rac1 (acting through PAK1) regulates cell motility, and transfection of maspin in MDA-MB-231 causes suppression of Rac1/PAK activities leading to decreased cell motility (61). In our study, re-expression of maspin and OMO also caused down-regulation of ECT2 expression as shown by proteomic analysis and confirmed by RT-qPCR. The result provides an important new clue as to the effect of maspin on Rac1/PAK1 by which maspin (via its RCL) could decrease the expression of ECT2 that acts as a critical upstream effector of Rac1/PAK1 signaling pathway involved in cell motility.

In accordance with the result of proteomic analysis, total protein lysate of maspin- or OMO-expressing MDA-MB-231 cells contained less chymotrypsin-like activity of 20S proteasome compared with mock and MOM-expressing cells. This effect of maspin RCL may be due to a down-regulation of proteasome β3 subunit since the transcript level of gene expression was reduced in both maspin- or OMO-expressing MDA-MB-231 cells. In contrast, this result was different from the previous study in MDA-MB-435 showing only β5 subunit of proteasome was down-regulated in all maspin-transfected cells compared with the parent cells (9). Nonetheless, these observations indicated that subunit composition and activity of the proteasome are altered in maspin-expressing cells. Importantly, our study provides

the first evidence that the proteasome inhibitory effect might reside in the RCL of maspin.

For the future direction of this study, it remains to be explored a molecular mechanism by which maspin regulates expression of these genes in carcinoma cells. It is possible that maspin acts as transcription factor that can directly control gene expression since localization of maspin was observed in both nuclear and cytoplasm (4). Recently, Goulet et al. have demonstrated a direct association between decreased expression of colony-stimulating factor-1 (CSF1) and binding of nuclear maspin to its promoter (62). Using chromatin immunoprecipitation, nuclear maspin was enriched at the promoter of CSF-1 leading to down-regulation of CSF-1 mRNA. In addition, maspin binds the orphan nuclear receptor ESRRA promoter and that the mRNA levels encoding ERRα were downregulated in maspin–Flag–HA tumors. This study demonstrated that at least two genes crucial to progression of breast cancer are negatively regulated by nuclear maspin, supporting a nuclear role for its tumor suppressive activity. Therefore, maspin possibly regulates the expression of ECT2 and others via a similar mechanism involved nuclear association of maspin to the promoter region of target genes. It is also interesting to determine whether such interaction between nuclear maspin and promoter requires the presence of maspin RCL.

In conclusion, we have clearly demonstrated that the tumor suppressor activities of maspin (inhibition of cell migration and invasion, enhancement of cell adhesion to fibronectin, alteration of carcinoma proteome, and derangement of ubiquitin-proteasome system) indeed require the presence of cognate RCL (**Figure 26**). We can

establish a new finding of maspin effect on cancer cell migration signaling pathway in which maspin, (via its RCL) down-regulates epithelial cell transforming 2 leading to decreased activity of Rac. In addition, maspin RCL play an essential functional domain in regulation of ubiquitin-proteasome pathway via decreased expression of proteasome subunit beta type-3. Finally, these data support the notion that up-regulation of maspin should be considered as a possible strategy in the control of cancer metastasis.

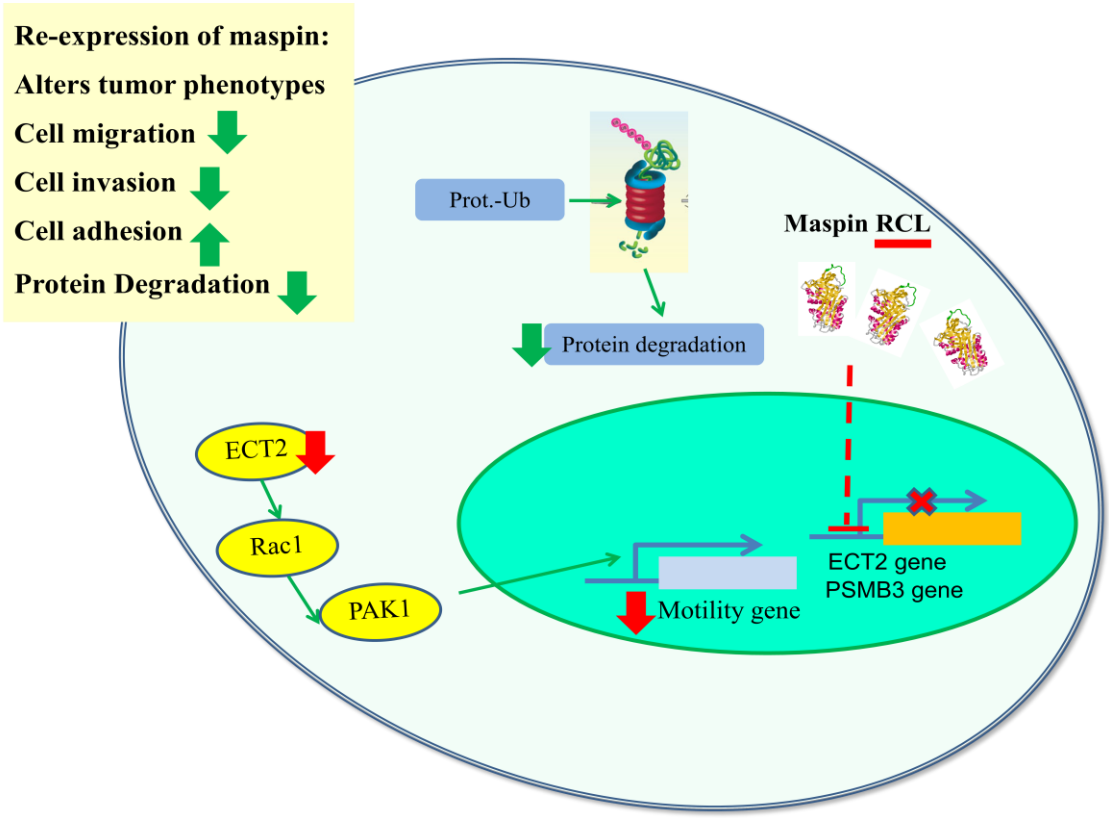

Figure 26 Modes of Maspin RCL Actions