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THE PROMISE AND CHALLENGE TOWARD THE CLINICAL APPLICATION OF MASPIN IN CANCER

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1. ABSTRACT

Since the identification of the human maspin gene, a decade of extensive research revealed the promise of maspin both as a valuable molecular marker for the diagnosis and prognosis of many types of cancers, and as a tumor suppressor at the level of tumor growth, invasion, angiogenesis, metastasis, and tumor sensitivity to drug-induced apoptosis. This review is intended to summarize the consensus of these findings, and provide an overview of the current challenges toward the clinical application of maspin. Specifically, this review discusses several likely molecular mechanisms underlying (1) the differential regulation of maspin expression, and (2) the biological activities of maspin in tumor progression.

2. INTRODUCTION

In a search for tumor suppressor genes by applying expression genetics (1), the maspin gene was identified by subtractive hybridization on the basis of its expression at the mRNA level in normal but not in tumor-derived human mammary epithelial cells (2). The cloned and sequenced cDNA consists of 2584 nucleotides, encoding a 42 kDa protein (376 amino acids) with the overall sequence homologies with serine protease inhibitors, or serpins (2). The name maspin was chosen for mammary homologue to serpins. Based on a protein sequence alignment, maspin has an Arginine residue at its P_1 site. The protein sequence of maspin is highly conserved among human, mouse and rat (3, 4). The human maspin gene has been mapped to a cluster of serpins at chromosome 18q21.3-q23 including PAI-2, SCCA-1 and -2, PI8, and PI10 (5-7), and is closely linked to several important cancer related genes including BCL-2, DCC, and DPC4 (8).

Maspin is expressed in several types of normal epithelial tissues including breast, prostate, placenta, testis, colon, small intestine, tongue, thymus, and epidermal keratinocytes (9, 10). Mouse maspin, mMaspin, is found to have a similar tissue expression pattern as human maspin (3). Maspin protein produced by cultured human breast and prostate epithelial cells is a 42 kDa monomer which is present as a secreted, a cytoplasmic, as well as a cell surface-associated protein (9-13).

A decade has passed since the discovery of the maspin gene. Significant strides have been made in a series of difficult investigations towards the insights into its biological activities, genetic and epigenetic regulations, and the underlying molecular mechanisms. In light of the potential clinical applications of maspin as a molecular marker and suppressor of human cancers, this review is intended to summarize progresses made in the following aspects: maspin as a molecular marker for cancer diagnosis and prognosis, masin and tumor suppression, potential maspin-based cancer interventions, and emerging new questions in maspin research.

3. MASPIN AS A MARKER OF CANCER DIAGNOSIS AND PROGNOSIS

The clinical relevance of maspin in human cancers is most extensively investigated in breast cancer. Maspin is highly expressed in normal breast epithelial cells, especially in myoepithelial cells (2, 14). In 1994, Zou, *et al.*, first reported a down-regulation of maspin expression in invasive and metastatic breast carcinoma cells (2). Subsequently, several groups reported consistent results. For example, using a nested reverse transcription polymerase chain (RT-PCR) reaction, Maass, *et al.* detected maspin expression in 45 primary breast cancer patients. No maspin expression was found in breast cancer specimens, whereas 64% of the normal breast tissues expressed detectable maspin mRNA. The expression of maspin was shown to correlate with recurrence free survival (15). An extensive immunohistochemical study

with a large series of breast tissue specimens showed that myoepithelium and epithelial cells in normal breast and fibrocystic change expressed the highest level of maspin, while a stepwise decrease in maspin expression was found in the sequence of ductal carcinoma *in situ*, invasive cancer and lymph node metastasis (16). Czerwenka *et al.* compared maspin with several other molecular markers in breast cancer specimens by two-dimensional SDS-PAGE and showed that breast cancer cells that had higher HER2 and HER3 expression expressed less maspin and keratin 8 (17). A recent tissue microarray study by Zhang, *et al.* demonstrated a consistently down-regulated expression of maspin during breast tumor progression, although there was no apparent correlation between the level of maspin expression and tumor grades. In addition, their data showed a significant inverse correlation between maspin and mutant p53 (18). Interestingly, Barsky and colleagues reviewed 200 cases of metastatic human breast cancer and found 21% of these cases showed features of reversion to a ductal carcinoma *in situ* (DCIS) growth pattern. These "revertants" tended to be ER-negative/EGFR positive and can be easily distinguished for the expression of maspin (19).

Meanwhile, several other studies suggest that the correlation of maspin expression with breast cancer progression may not be so straight forward. In some cases, a higher level of maspin expression correlated with or predicted a poor prognosis of breast cancer. For example, studies by Umekita and colleagues showed positive maspin expression in a subpopulation of ductal carcinoma *in situ* and invasive carcinoma (20). This study also revealed a positive correlation of positive maspin expression with larger tumor size and higher histological grade. Furthermore, maspin expression appeared to be inversely correlated with relapse-free survival and overall survival (20, 21). Using real-time PCR, Bieche and colleagues showed a statistically significant correlation between low maspin mRNA levels and positive oestrogen status. In addition patients with maspin expressing tumors had significantly shorter relapse-free survival than patients who had maspin non-expressing or low-expressing tumors (22). When a sensitive technique such as RT-PCR is used, maspin may be even detected metastatic breast cancer cells or residual breast cancer cells. For instance, RT-PCR detected maspin in the blood and bone marrow specimens of breast cancer patients, and maspin mRNA positivity correlated with tumor size in patients with early stage breast cancer (23). In three independent studies, maspin was found in leukapheresis products or bone marrow from breast cancer patients, even though maspin was the least specific marker to detect breast cancer (24-26). However, results from Corradini and colleagues suggested that RT-PCR detection of maspin and mammaglobin in bone marrow and peripheral blood samples was both sensitive and specific for detecting residual breast cancer in patients who had undergone high-dose chemotherapy, their results showed that (27). Recently, attempts were also made to use maspin as a marker for detecting breast cancer cells in lymph node metastasis. However, maspin was not a useful molecular marker (28, 29). It is worth noting that different subcellular localization of maspin was reported by Mohsin *et al.* In this study which included more than a thousand clinical specimens, maspin was found to be compartmentalized either in cytoplasm or nuclei. While the maspin nuclear staining was significantly associated with good prognostic factors, the cytoplasmic staining of maspin was associated with poor prognostic markers (30).

In human prostate cancer, immunohistochemical detection of maspin showed that a decrease of maspin expression in patients who had undergone radical prostatectomy correlated with local recurrence or systemic tumor progression. The loss of maspin expression also correlated with a higher tumor stage and increasing histological dedifferentiation. Furthermore, decreased maspin expression correlated with an increased p53 expression. In contrast, patients who retained maspin expression had a significantly longer recurrence-free survival (31). Pierson *et al.* reported that benign basal cells consistently expressed maspin at a high level. The loss of basolateral maspin expression coincides with loss of the basal cell layer in human prostate cancer specimens. Maspin expression in secretory cells, on the other hand, appears to undergo a biphasic differential regulation, i.e., essentially absent in benign secretory cells, dramatically up-regulated in high-grade prostatic intraepithelial neoplasia (HGPIN), then progressively down-regulated through low-grade prostate carcinoma to high-grade prostate carcinoma. Maspin expression in prostate carcinoma is inversely correlated with tumor grade. Furthermore, maspin expression in HGPIN is inversely correlated with the Gleason's grade of the adjacent prostate cancer (32). Zou *et al.* subsequently reported that maspin protein was absent in a significant fraction of prostate specimens. However, when patients were treated with neoadjuvant androgen ablation therapy before radical prostatectomy, maspin expression was significantly higher. In parallel, androgen-depletion treatment of androgen-sensitive prostate cancer cells LNCaP resulted in increased maspin promoter activity. Consistently, castration induced maspin expression in LNCaP xenograft tumors in nude mice (33).

Studies on maspin expression in the progression of several other types of cancer also showed a correlation between down-regulation of maspin and tumor progression. Normal thyroid tissue, follicular adenomas, follicular carcinomas, poorly differentiated carcinomas and undifferentiated carcinomas of the thyroid do not express maspin, whereas papillary thyroid carcinomas express maspin (34). Interestingly, maspin expression in papillary thyroid carcinomas inversely correlated with mutant p53 expression, and correlated with longer recurrence-free survival (35). In the study of Song, *et al.*, a stepwise decrease of maspin expression inversely correlated with mutant p53 expression and microvessel densities, and positively correlated with the progression from adenoma to carcinoma of colon (36). In oral squamous cell carcinoma and in stage I and II oral tongue squamous carcinoma, high tumoral expression of maspin appeared to be associated

with improved survival (37, 38). In lung, except for the multipotent basal epithelial cells, most benign lung tissues did not express maspin. However, in lung cancer, maspin is one the most commonly expressed genes in preneoplastic bronchial lesions (39, 40). A nuclear staining of maspin correlated with increased survival and longer remission duration in respectable-staged patients with lung cancer (40).

Evidence from studies of several other types of cancers suggests a more complex involvement of maspin in tumor progression. For example, maspin expression was up-regulated in pancreatic cancer specimens (41), gastric adenocarcinoma, and gastric epithelial cells with intestinal metaplasia (42). Upon further investigation, maspin overexpression appeared to be associated with ductal adenocarcinomas, intraductal papillary mucinous tumors and mucinous cystic tumors, but not with acinar cell carcinoma, pancreatic endocrine tumors, solid-pseudopapillary tumors, and serous cystadenomas (43). Ohike *et al.* showed that overexpression of maspin in pancreatic ductal adenocarcinoma actually correlated with predominantly a low histological grade. Furthermore, maspin expression in pancreatic cancer lymph node metastases was decreased (44). In ovarian cancer, a subpopulation expressed maspin and more than 30% of the invasive ovarian cancer cells expressed an elevated level of maspin in the cytoplasm, while benign and low-malignant-potential tumors expressed maspin in the nuclei. Maspin overexpression correlated with high tumor grades, tumor metastasis and a shorter overall survival (45).

To date, the consensus that maspin expression predicts a better prognosis still holds for prostate cancer, colon cancer, thyroid cancer, lung cancer, and oral squamous cancer, but it is not as clear cut for breast cancer, gastric cancer, pancreatic cancer and ovarian cancer. Several factors may contribute to the complex outcome of those clinical studies. First, the transcriptional regulation of maspin during tumor progression may be dependent on the genetic background and etiology in specific types of cancer. Second, it is yet to be clearly defined for each type of cancer where and when a dysregulation of maspin expression occurs. The evidence with prostate cancer and pancreatic cancer is intriguing in that maspin expression appears to undergo a biphasic differential regulation, i.e., up in carcinoma precursor (32) or low grade cancer cells (44), but down-regulated in invasive (32) and metastatic cancer cells (44). The correlation between maspin re-expression in metastatic breast cancer revertants (19) further suggests that the differential regulation of maspin during tumor progression may be a dynamic process that is sensitive to changes of tissue microenvironment. Third, the biological function of maspin may be further regulated at post-transcriptional level. It is important to find out: (i) why the subcellular localization of maspin in breast cancer (30), lung cancer (40), and ovarian cancer (45) cells could be cytoplasmic or nuclear, and (ii) why the cytoplasmic localization of maspin correlates with a more invasive phenotype. Finally, the biological significance of maspin differential expression may be specific for each type of cancer. To this end, mammary myoepithelial tumors that are associated with high expression of maspin along with several other protease inhibitors are intriguing examples of low invasive neoplasm (46-48). The low invasiveness may attribute to the fact these protease inhibitors help preserve the integrity of the extracellular matrix (ECM). In fact, mammary myoepithelial tumors are known to accumulate an abundant ECM (48).

4. MASPIN AND TUMOR SUPPRESSION

4.1. Maspin blocks tumor invasion, angiogenesis and metastasis

In 1994 Zou and colleagues first reported that re-expression of maspin by stable transfection of mammary carcinoma cells MDA-MB-435 significantly inhibited tumor cell invasion *in vitro* and metastasis in nude mice (2). This finding is supported by several *in vivo* experiments using genetically modified mouse models. For example, in WAP-TAg/WAP-maspin bitransgenic mice, maspin overexpression reduced angiogenesis and pulmonary metastases (49). Using the syngeneic mammary tumor model, Shi *et al.*, showed that maspin overexpression in TM40D mammary tumor cells blocked tumor local invasiveness and metastasis (50, 51). Similar studies were reported with *in vivo* models for other types of cancers. Sternlicht and colleagues showed that human myoepithelial xenografts accumulated an abundant extracellular matrix (named Humatrix) which contained sequestered proteinase inhibitors such as maspin. Humatrix inhibited tumor cell invasion *in vitro* in a maspin-dependent manner. Humatrix also inhibited tumor invasion and metastasis in SCID mice (52). Transferring chromosome 18, where the maspin gene is located, into several human pancreatic cancer cell lines restored maspin expression. Mice inoculated with these hybrid cells were associated with significantly less vascular density and fewer numbers of micrometastasis (53). Recently, using a novel intraosseous SCID-Hu model that reproduces the organ- and species-specific prostate-bone interaction (54), Cher *et al.* showed that maspin overexpression in prostate carcinoma cells DU145 decreased tumor growth, tumor-induced osteolysis, and tumor angiogenesis (55).

The experimental observation that maspin suppresses tumor-induced angiogenesis is consistent with a couple of correlative clinical studies. Hojo *et al.* showed a significant correlation of maspin expression with decreased microvessel staining in human breast cancer specimens (56). A similar finding was reported by Song *et al.* in human colon cancer (36). The effect of maspin on angiogenesis was further investigated by Zhang *et al.* In their study published on Nature Medicine in 2000, maspin protein blocked endothelial cell migration toward basic fibroblast growth factor and vascular endothelial growth factor, and inhibited endothelial tube formation *in vitro*. Furthermore, maspin protein blocked neovascularization in the rat cornea pocket model. Interestingly, a mutant form of maspin at the serpin reactive site loop (RSL) region remained active in inhibiting angiogenesis both *in vitro* and *in vivo* (57). The inhibitory effect of maspin on tumor angiogenesis was also confirmed in WAP-Tag/WAP-maspin bitransgenic mice that were derived from crossing

WAP-maspin transgenic mice with the WAP-Tag mice. In this model, maspin overexpression resulted in reduced angiogenesis and increased apoptosis (49, 58). More recently, Cher and colleagues showed that maspin expression inhibited tumor induced angiogenesis in human bone microenvironment (55), suggesting the maspin effect on angiogenesis is not limited to primary tumor.

Extensive *in vitro* experiments helped further define the mode of action of maspin in inhibiting tumor invasion. These *in vitro* experiments can be generally divided based on the form of maspin used. Following the initial reports that maspin re-expression in breast cancer cells via stable transfection inhibits tumor invasion (2, 59), maspin re-expression was achieved in several human cancer cell lines as well as a mouse cell line by stable transfection or retrovirus infection. Expression of maspin in mammary carcinoma cells MDA-MB-435 and prostate carcinoma cells DU145 was shown to inhibit tumor cell invasion, at least in part, by inhibiting cell motility (2, 11, 60). A recent study by Abraham *et al.* showed that maspin expression in retroviral infected mouse prostate tumor cells TRAMP C2N enhanced cell adhesion specifically to fibronectin (61). These results suggest that maspin plays an important role in regulating cell interaction with ECM. It is logical to hypothesize that the maspin effect on cell adhesion, motility and invasion is associated with changes of specific signaling pathways. Indeed, using maspin transfected cells derived from MDA-MB-235 cell line, Odero-Marah *et al.* showed that the inhibitory effect of maspin on cell motility correlated with a decrease in Rac 1 effector PAK1, and an increase of PI3K and ERK1/2 activities (62).

Several forms of recombinant maspin protein have been produced and purified. In the first report by Sheng *et al.*, three forms of recombinant maspin proteins were produced and purified from *E. coli*, yeast and baculo virus-infected insect cells, respetively (63). The recombinant maspins produced in yeast and baculo virus-infected insect cells were wild type full-length maspin, while the recombinant maspin produced in E. coli was fused N-terminal to glutathione-S-transferase. These three forms of maspin dose-dependently inhibited the invasion of a series of breast cancer cell lines in *in vitro*. In parallel, the N-terminal domain of maspin immediately upstream of its putative P_1'site in the RSL region, resulting from limited cleavage by trypsin, had no effect on tumor cell invasion. Consistently, the effect of maspin on tumor invasion was specifically neutralized by a maspin RSL peptide-derived antibody. Subsequently, recombinant maspin protein produced in baculoviral infected insect cells was shown to inhibit the motility of both breast and prostate carcinoma cells *in vitro*. In these experiments, the effect of exogenously added recombinant maspin was comparable to maspin endogenously expressed in stably transfected cells, and was specifically blocked by maspin-neutralizing antibody. Furthermore, when maspin was preincubated with cells, the activity of maspin on cell motility was no longer inhibited by subsequent treatment with the maspin-neutralizing antibody. This data indicates that the biological effect of maspin on tumor cell invasion was not in pericellular space (11). In a subsequent study, Seftor *et al.* reported that recombinant maspin purified from baculoviral infected insect cells induced a higher cell surface level of $\alpha 5$ and $\alpha 3$ integrin in MDA-MB-435 cells. In accordance, maspin-treated MDA-MB-435 cells exhibited an increased adhesion to fibronectin and a more epithelial-like phenotype (64).

The biological effect of recombinant maspin on cell adhesion, motility and invasion is not likely limited to breast and prostate cancer cells. The study of Dokras and colleagues showed that maspin significantly decreased cytotrophoblasts invasion *in vitro* (65). In 2001, Ngamkitidechakul *et al*. showed the adhesion of late-passage corneal stromal cells to several ECM proteins was inhibited by purified His-tagged maspin *in vitro* (66). This result suggests an important role of secreted maspin in regulating the biological behavior of stromal cells.

Mouse maspin (mMaspin) is approximately 90% homologous to human maspin, and seems to have a similar biological activity. Zhang at el. overexpressed GST-mMaspin fusion protein in *E. coli*. Purified GST-mMaspin inhibited two mouse mammary carcinoma cell lines in *in vitro* invasion and motility assays. As with human maspin, GST-mMaspin also inhibited mouse mammary tumor motility. Deletion in the putative mMaspin RSL region resulted in the loss of the inhibitory effect on both tumor invasion and motility (3).

The underlying molecular mechanism of maspin in regulating cell adhesion, motility and invasion has been a subject of debate. It is important to point out that the evidence that the RSL sequence of maspin in critical in its inhibitory effect on tumor cell motility and invasion is consistent with the hypothesis that maspin acts as an inhibitory serpin towards a serine protease target. The data of McGowen and colleagues demonstrated that maspin specifically inhibited surface-associated urokinase-type plasminogen activator (uPA) with a K_i value of 20 nM. The proteolytic inhibitory effect of maspin was quantitatively consistent with its inhibitory effect on the motility of DU145 cells *in vitro* (13). On the other hand, the study of Bass *et al.* reported that despite the inhibitory effect of maspin on the migration of tumor cells and vascular smooth muscle cells, maspin did not inhibit cell-associated uPA (67). Interestingly, an elegant study by Ngamkitidechakul *et al*. showed that the specific binding of maspin protein to the surface of breast carcinoma cells MDA-MB-231 depended on maspin RSL sequence. The maspin RSL peptide inhibited maspin binding to cell surface, presumably by a competitive inhibitory mechanism. Replacement of the RSL sequence of maspin with that of ovalbumin, or a point mutation at the P_1' site of maspin, resulted in the loss of the stimulatory effect of maspin on the adhesion of MDA-MB-231 cells and corneal stromal cells and to ECM. Conversely, substitution of the RSL of ovalbumin with that of maspin converted inactive ovalbumin into a fully active molecule in parallel adhesion assay (68). The central issue of whether maspin acts as an inhibitory serine protease inhibitor will be further discussed

in the section on *Maspin in Extracellular Matrix Degradation*.

4.2. Maspin in tumor growth, differentiation, and apoptosis

Since the discovery of the maspin gene, it has long been noted that overexpression of maspin in cancer cells always results in growth inhibition in various *in vivo* tumor models (2, 49-51, 53, 55, 57, 59). Of particular significance, in a recent study by Cher *et al.* overexpression of maspin in stably transfected DU145 cells led to redifferentiation of these cells into glandular structures in the human bone microenvironment (55). This phenomenon has not been reported in other tumor models where maspin expressing tumor cells (either via stable transfection or viral infection) were implanted subcutaneously or orthotopically. Although *in vitro* biological studies have been fruitful to study the effect of maspin on cell adhesion, motility, and invasion (see the section on *Maspin Blocks Tumor Invasion, Angiogenesis and Metastasis*), neither recombinant maspin nor endogenous re-expression of maspin directly inhibited tumor cell growth *in vitro* (11, 60, 63, 69). The difference between the *in vivo* and *in vitro* observations may reflect the differences in tumor microenvironments.

Accumulated evidence suggests that the *in vivo* inhibitory effect of maspin on tumor growth is, at least in part, due to an increased apoptosis (49, 50, 58). A conceivable difference between the *in vitro* cell culture and *in vivo* tumor is the level of stress, such as oxidative stress. Several *in vitro* studies have shown that maspin expression may be induced by oxidative stress (see section on *The Transcriptional Regulation of Maspin Expression*). Maspin may inturn further regulate cellular response to changes in the redox homeostasis. It has been reported peroxisome proliferator-activated receptor-gamma (PPARγ)-induced maspin expression correlated with a more differentiated phenotype in both breast carcinoma cells (70) and colon cancer cells (71). Further evidence by Khalkhali-Ellis and colleagues suggests that maspin enhances nitric oxide-induced apoptosis of MCF-7 cells (72). Another conceivable difference between the *in vitro* cell culture and *in vivo* tumor may be cytotoxic cytokines that are mostly absent *in vitro* but are secreted by stromal or immune cells in the tumor microenvironment. The study by Jiang and colleagues showed although maspin protein does not induce spontaneous cell death, endogenous maspin (but not exogenously added recombinant maspin) significantly sensitized mammary carcinoma cells MDA-MB-435 to drug-induced apoptosis (69). A subsequent study by Liu *et al.* revealed that maspin expression in DU145 cells led to increased Bax expression. Furthermore, the effect of maspin in sensitizing cells to induced apoptosis depends on the Bax-mediated mitochondrial pathway. This finding is consistent with the evidence that maspin sensitizes the apoptotic response of breast and prostate carcinoma cells to various drugs, ranging from death ligands to endoplasmic reticulum stress (73).

The evidence that maspin re-expression leads to tumor cell redifferentiation *in vivo* should encourage efforts to develop maspin-based differentiation therapies, while the link of maspin with the elevated Bax-mediated cellular sensitivity to apoptosis further suggests that maspin may be used as a modifier for apoptosis-based cancer therapy. Maspin is the only proapoptotic serpin amongst all serpins so far implicated in apoptosis regulation.

5. TOWARD MASPIN-BASED CANCER INTERVENTION

5.1. The Transcriptional Regulation of Maspin Expression

Biological studies suggest that re-expression of maspin may lead to tumor suppression. To achieve this goal in cancer patients, it is crucial to understand the molecular mechanism(s) that governs the regulation of maspin expression. Although lost of heterozygosity of 18q is observed in human malignancies such as head and neck cancer (74, 75), the differential expression of maspin in tumor progression occurs, by far, at the epigenetic level. In particular, transcription factors and methylation may both contribute to the regulation of maspin expression at the transcriptional level.

In an extensive study, Futscher and colleagues showed that normal cell type specific expression of maspin is controlled, in part, by cytosine methylation of the maspin gene promoter. In maspin-expressing normal cells, the maspin promoter is unmethylated, whereas in normal cells that do not express maspin, maspin promoter is completely methylated (76). In 2002, Maass *et al.* first reported a link of hypermethylation and maspin silencing in human breast cancer. In this study, treatment of 5-aza-2'-deoxycytidine, trichostatin A or a combination of both led to the re-expression of maspin in a series of maspin-negative breast cancer cell lines (77). Consistent results subsequently published by Primeau *et al.* showed that treatment with 5-aza-2'-deoxycytidine and depsipeptide led to a significant activation of maspin and gelsolin expression, which correlated with a dramatic antineoplastic effect against MDA-MB-231 and MDA-MB-435 cells in culture (78). Akiyama *et al.* showed that in a maspin-negative gastric cancer cell line GCIY, maspin expression could be reactivated after by 5-aza-2'-deoxycytidine treatment. Upon further analyses, the maspin promoter region of all normal gastric epithelial cells was found to be hypermethylated on both alleles, whereas in gastric cancers, frequent demethylation of the maspin promoter that extends to both alleles was found (79). In thyroid, a larger fraction of the papillary thyroid carcinomas had a methylated maspin promoter (80). An oligonucleotide microarray screen identified the maspin and S100P genes as hypomethylation targets in pancreatic cancer (81). Using bisulfite genomic sequencing and chromatin immunoprecipitation methods, Fitzgerald *et al.* showed that maspin-negative pancreatic cells have a methylated maspin promoter which is associated hypoacetylated H3 and H4 histones. Pancreatic carcinoma cell lines that express maspin displayed demethylated promoters and hyperacetylated H3 and H4 histones. Furthermore, combined treatment with 5-aza-2'-deoxycytidie and trichostatin A

synergistically re-activated the expression of maspin in pancreatic cells where maspin promoter was methylated (81, 82).

It was found that DNA-damaging agents and cytotoxic drugs induced endogenous maspin expression in cells containing the wild type p53. In contrast, maspin expression was refractory to the DNA-damaging agents in cells containing mutant p53 (83). In fact, in an *in vitro* promoter activity study, p53 was shown to directly activate maspin transcription in both breast cancer (MCF-7) and prostate cancer (LNCaP, DU145 and PC3) cells (83). Using a high-density, membrane-based hybridization arrays, Martin and colleagues examined the mRNA expression patterns of known differentially regulated genes in breast cancer cell lines as well as breast tissues. Cluster analysis linked p53 and maspin in a group that is strongly associated with estrogen receptor status (84). It is worth noting that the transcriptional regulation of maspin is likely to be regulated by different mechanisms in different types of cells. Even in the same cells, multiple pathways may cooperate to regulate maspin expression. For example, Oshiro and colleagues showed that despite aberrant DNA methylation of maspin promoter in some cells, p53 could partially restore maspin expression. It appears that binding of wild type p53 to the maspin promoter stimulates histone acetylation and enhances chromatin accessibility of their promoters. This, in turn, may help to overcome, at least partially, the repressive barrier of DNA methylation (85).

Several other factors have been shown to regulate the transcription of the maspin gene. It has been suggested that maspin expression in prostate epithelial cells may be regulated by a positive Ets element (86). Indeed, three recent studies identified maspin as a target of prostate-derived Ets factor (PDEF) in normal breast epithelial cells as well as in breast cancer cells (87-89). Maspin transcription in tumor progression is likely to be regulated by hormonal factors based on previous correlative clinical studies (see the section on *Maspin as a Marker for Cancer Diagnosis and Prognosis*). In addition, maspin expression is differentially regulated in the development of mammary gland and placenta (3, 65). An earlier *in vitro* study showed that maspin expression in prostate epithelial cells may be regulated by a negative hormone responsive cis-element (86). Confirmative evidence was recently reported by Zou at al. that neoadjuvant androgen ablation therapy before radical prostatectomy significantly enhanced maspin expression. Consistently, maspin expression in androgen-sensitive LNCaP cells was significantly induced either by androgen-deprivation *in vitro*, or by castration of the nude mouse hosts in a xenograft model (33). In an independent study, γ-Linolenic acid, a potent inhibitor of 5 α-reductase (inhibiting the conversion of testosterone to 5 α-dihydrotestosterone) was shown to induce maspin expression in mammary carcinoma cells MDA-MB-231 (90).

The cellular redox system seems to play a role in regulating maspin expression. Peroxisome proliferator-activated receptor-gamma (PPARγ) (a nuclear receptor that stimulates the terminal differentiation of adipocyte precursors when activated by ligands such as eicosanoids) induced the expression of differentiation-associated genes including maspin in breast tumor cells MDA-MB-231. This effect correlated with a differentiated phenotype, a reduced growth rate, a reduced colony formation property of breast tumor cells (70). Recently, PPARγ was also shown to induce caveolin-1 and maspin expression in human breast cancer MCF-7 cells and induce a more differentiated phenotype (71). Nitric oxide (NO), a water and lipid soluble free radical, induced maspin expression in MCF-7 cells. The effect of NO on maspin expression was associated with decreased cell motility and invasiveness, and increased apoptotic index (72). Overexpression of manganese superoxide dismutase (MnSOD) in a series of breast and prostate cancer cells induced maspin expression (91). Interestingly, the effect of MnSOD did not appear to depend on either p53 or the demethylation mechanism. Instead, based on maspin promoter activity analyses, transcription run-on and mRNA stability assays, MnSOD overexpression correlates with increased stability of maspin mRNA (92). This data suggests that the regulation of maspin expression may not be limited to the step of gene transcription.

Despite the aforementioned significant progresses, the exact signaling pathways and transcription factors that govern the differential expression of maspin at each step of the progression of each type of cancer remain elusive. Appropriate animal models may prove useful for detailed *in vivo* investigations. To this end, Reddy *et al.* showed that MMTV/TGF-α transgenic mice spontaneously developed hyperproliferation, hyperplasia, and carcinoma in mammary gland. In this model maspin expression was lost at the critical transition from carcinoma *in situ* to invasive carcinoma. These data are consistent with the maspin expression profile found in human cancer and suggests that the MMTV/TGF-α transgenic mouse model is advantageous for *in vivo* evaluation of both the expression and the biological function of maspin during the multi-stage progression of mammary tumor (93).

5.2. Maspin in Extracellular Matrix Degradation

The biological activity of maspin in inhibiting cell invasion and motility has been localized on the cell surface, and requires the intact maspin RSL (2, 3, 11, 13, 60, 63, 68). In light of the important role of proteases and protease inhibitors in the regulation of tumor invasion and metastasis, it would be consistent if maspin is an inhibitory serpin that inhibits serine protease-mediated ECM degradation. However, to date, the key issue of whether maspin acts as a typical inhibitor of serine proteases is not yet resolved.

Structural considerations seem to favor the notion that maspin is a nonclassical serpin. Maspin protein has a unique RSL sequence, which is shorter than that of classical inhibitory serpins (2). In addition, the hinge sequence, located 9-15 residues NH_2-terminal to the P_1-P_1' peptide bond of maspin, deviates somewhat from the conserved sequence of inhibitory serpins (94, 95). According to the current paradigm, the conformational change of a serpin in which RSL is inserted into the β-

pleated sheets (as strand s4A) is a hallmark for the inhibitory interaction between a serpin and its serine protease target in solution (96). The RSL of an inhibitory serpin needs to have not only an appropriate sequence, but also the correct length (97). By this criterion, the atypical RSL sequence of maspin may not allow efficient insertion into the β-pleated sheets, thus rendering maspin non-inhibitory. Pemberton *et al.* showed that the RSL of purified maspin was in an exposed conformation, and did not undergo the stressed-relaxed transition, typical of proteinase-inhibitory serpins. (98). Using circular dichroism and intrinsic tryptophan fluorescence to monitor the conformational changes of maspin under urea denaturing conditions, Liu and colleagues showed that the unfolding and self-association of maspin involved three states: monomer form, unfolding intermediate, and dimmer form (99). Fitzpatrick *et al.* generated a computer model for the tertiary structure of maspin using the crystal structure of noninhibitory serpin ovalbumin as a prototype. This theoretical structure of maspin suggests the absence of disulfide bonds in the molecule and the presence of an unstable RSL that adopts a distorted helical structure.

Some experimental evidence supports the hypothesis that maspin is a non-inhibitory serpin. For example, purified recombinant maspin is sensitive to limited proteolysis (63, 95). In solution-based biochemical studies, recombinant maspin did not to inhibit several purified proteases including tissue-type plasminogen activator (tPA), urokinase-plasminogen activator (uPA), trypsin, chymotrypsin, elastase, plasmin, thrombin (98, 100). Bass *et al.* reported that maspin inhibits the motility of tumor cells and vascular smooth muscle cells *in vitro* without inhibiting tPA, uPAR-bound uPA or cell surface associated uPA (67). Interestingly, the study of Ngamkitidechakul *et al*. showed that maspin RSL sequence is sufficient to stimulate the adhesion of corneal stromal cells to type I collagen, fibronectin, and laminin, and to stimulate the adhesion of breast cancer cells MDA-MB-231 to fibronectin. Chimeric maspin/ovalbumin, which has maspin RSL sequence replaced by that of ovalbumin, did not have any activity in parallel assays. In contrast, the substitution of the RSL of ovalbumin with that of maspin converted inactive ovalbumin into a fully active molecule (68). These data argue against the notion that maspin acts as a typical inhibitory serpin. The same data, however, would also argue against the suggestion that maspin acts like ovalbumin. It is worth noting that since only full-length maspin protein, but not maspin RLS peptide, has been detected in cells or biological samples so far, how the functionality of the maspin RSL can be totally independent of the general framework of maspin *in vivo* is not clear.

It has been noted that purified recombinant maspin has poor stability (9, 100, 101) and tends to undergo spontaneous three-state unfolding and polymerization under cell-free conditions (99). In contrast, endogenous maspin is always found in an intact monomeric form. It is likely that the *in vivo* microenvironment of maspin is more complex, possibly involving complexes with other associated molecules. Some serpins are known to be activated by co-factors such as low molecular weight heparin (102). Maspin binds to heparin affinity column with a low affinity (63). However, heparin and several other potential serpin co-factors failed to confer an inhibitory activity on purified maspin in solution-based assays (98). Interestingly, several recent studies showed that when tPA and uPA were associated with fibrinogen and tumor cell surface, respectively, their activity in converting plasminogen to plasmin was inhibited by recombinant maspin (13, 100). A direct protein interaction was implicated by the evidence that single-chain tPA (sctPA) in tumor cell conditioned media was specifically pulled down by a maspin RSL peptide affinity column (100). The effect of maspin on cell surface-associated uPA was similar to that of a uPA-neutralizing antibody and was reversed by maspin RSL peptide-derived antibody. The proteolytic inhibitory effect of maspin was quantitatively consistent with its inhibitory effect on the motility of DU145 cells *in vitro* (13).

Subsequently, Biliran and colleagues showed that overexpression of maspin in DU145 cells led to a dramatic reduction in the release of active uPA, both high and low molecular weight, into the conditioned culture medium. Consistently, the conditioned medium of maspin transfectant clones had a significantly lower activity in converting plasminogen to plasmin (60). Of particular importance, maspin expression led to a significantly reduced level of cell surface-bound uPA and uPA receptor (uPAR) proteins. It has been reported that the uPAR/uPA complex may interact with LRP localized in cell surface caveolae lipid raft (103). Treatment with receptor-associated protein (RAP), a specific inhibitor of low-density lipoprotein receptor-related protein (LRP), led to a significantly increased level of secreted uPA and cell surface uPAR in maspin transfectants but not in the mock control cells (60). These data suggest that maspin may specifically interact with uPAR-bound uPA in the caveolae microenvironment and trigger the LRP-mediated internalization of uPA and uPAR. The recent study of Cher *et al.* provided the first evidence that maspin expression in stably transfected DU145 cells inhibits tumor-mediated ECM and collagen degradation (55). Cher and colleagues subsequently tested maspin transfected DU145 cells in a novel SCID-Hu model for prostate cancer bone metastasis and showed that expression of maspin correlated with decreased tumor growth, reduced osteolysis, and decreased angiogenesis. Furthermore, the maspin-expressing tumors are associated with a significantly reduced level of uPA and a dramatic increase in fibrosis (55).

In light of the underlying molecular mechanism for the tumor suppressive activity of maspin, tPA and uPA are reasonable targets for maspin because they initiate a powerful proteolytic cascade by converting plasminogen to plasmin. Plasmin generated by plasminogen activation, in turn, plays a key role in degrading ECM components, activating metalloproteinases, and activating growth factors (see review (104, 105)). In particular, the localized pericellular uPA activity is facilitated by cell surface-anchored uPAR (106-109). Overwhelming evidence in the literature demonstrates that the cell surface-associated uPA/uPAR complex is causatively involved in tumor

invasion and metastasis of many types of cancers by exerting multifaceted functions via direct or indirect interactions with integrins, endocytosis receptors, and growth factors (see review (110)). The dependence of the inhibitory activity of maspin on the coexistence of an allosteric activating protein (fibrinogen for tPA) or the intact cell surface (for uPA) raises the possibility that the proteolytic inhibitory potency of maspin may be sensitive to changes of the microenvironment that dictates the biochemical presentation of the target protease.

6. EMERGING QUESTIONS

The maspin gene is among several ovalbumin type serine proteases found in the same cluster on chromosome 18q21. These genes appeared to be evolutionarily related (111) and may be regulated epigenetically as a gene cluster (112). Despite a closer sequence homology to ovalbumin, most serpins in this gene cluster such as PAI-2, PI6, PI10, SCCA1 and SCCA2, etc., have been shown to inhibit serine proteases (5, 6, 113-116). Interestingly, SCCA1 and SCCA2 also cross-inhibit cysteine proteases cathepsin S, K, L, and papain (117). Thus, the current search for the extracellular target of maspin may not be limited to serine proteases. Given its novel sequence and structural features, it is equally, if not more important, to keep in mind the possibility that maspin may interact with non-proteolytic proteins. To this end, Blacque *et al.* published the results of a yeast-two-hybrid screen using a truncated form of maspin. In this study, α-2 chain of type I collagen and a translation initiation factor were identified as candidate maspin-associated molecules (118). These results are yet to be independently confirmed.

Endogenous maspin is secreted, cell-surface-associated, and cytoplasmic (2, 10-13, 60, 69). A couple of recent studies even suggest a link of maspin nuclear localization with tumor progression in breast cancer, lung cancer, thyroid cancer and ovarian cancer (30, 34, 40, 45). Although purified recombinant maspin appeared to have similar biochemical and biological activities as endogenously expressed maspin, only endogenous maspin sensitizes cells to induced apoptosis. In addition, endogenously expressed maspin has an exclusively inhibitory effect on cell surface-bound in multiple stably transfected clones (60), whereas purified maspin exhibits a biphasic effect on both fibrinogen associated tPA and cell surface-associated uPA (i.e., inhibitory at low concentrations and stimulatory at higher concentrations) (13, 100). Thus, the biological function of maspin as well as its underlying mechanism may be further regulated by subcellular compartmentalization. On the other hand, since maspin is secreted, the initial evidence by Ngamkitidechakul *et al.* that purified maspin stimulated the adhesion of corneal stromal cells to ECM (66) encourages further investigation on whether maspin generally regulates the biological behavior of stromal cells.

Since maspin does not have a typical signal sequence for extracellular secretion or a nuclear localization sequence for nuclear translocation, the mechanisms by which maspin protein is partitioned among different subcellular compartments are not clear. The study of Pemberton *et al.* showed that intracellular maspin is associated with trafficking vesicles and such association requires the first 50 amino acids at the N-terminus of maspin (9). As suggested by Biliran *et al.* secreted maspin may be subsequently internalized (60). The consequence of maspin internalization adds further to the complexity of maspin biology. Consistent with an earlier finding (60), a recent study of Odero-Marah *et al.* suggested that internalized maspin remains stable (62). Furthermore, the internalized maspin appears to be functionally active in augmenting the PI3K and ERK1/2 signaling pathways, in accordance to the its effect on cell motility and adhesion (62).

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