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# MULTIPLE FUNCTIONS OF MASPIN IN TUMOR PROGRESSION AND MOUSE DEVELOPMENT

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## 1. ABSTRACT

Maspin is a unique serpin with diverse biological functions. Initially identified from human normal mammary epithelial cells, maspin expression is either reduced or completely silenced in breast cancers. Numerous studies have implicated maspin function in cancer progression and angiogenesis. Maspin has also been targeted for breast cancer gene therapy. Recently, transgenic and gene knockout mouse models have been used by our laboratory to identify the biological functions of maspin *in vivo.* In this review, we summarize the multiple functions of maspin in tumor progression and mouse development. These data demonstrate that maspin not only plays a role in tumor progression and metastasis but also is a key regulatory molecule for normal mammary gland and embryonic development.

## 2. INTRODUCTION

Serine protease inhibitors (Serpins) are comprised of a large family of molecules that play a variety of physiological roles *in vivo* (1, 2). They exist in almost every organism, from virus to mammals (3-5). Despite the fact that serpins have a very similar protein structure with a molecular weight about 400 amino acids, they differ greatly in their perspective functions. Some serpins are involved in cell migration and adhesion; others play active role in proteolysis and apoptosis. They can be divided into two categories: inhibitory and non-inhibitory serpins. Non-inhibitory serpins, typified by ovalbumin and PEDF, do not exhibit protease inhibitor activity, but rather function as a storage protein and neural differentiation factor, respectively (6-8). Inhibitory serpins ablate serine proteases through their functional domain-reactive site loop (RSL) (2, 9). Interestingly, some inhibitory serpins have evolved other regulatory functions. For example, plasminogen activator inhibitor 1 (PAI-1) not only specifically inhibits tPA and uPA (10, 11), but also regulates cell adhesion, which is independent of its protease inhibitor function, by blocking integrin $\alpha v\beta 3$ binding to vitronectin (12, 13). PEDF acts both as a neural differentiation factor and as a potent angiogenesis inhibitor (14, 15). This implies that not only serpins play diverse roles as a class, but also a single serpin molecule possesses multiple functions. As discussed in the following sections, maspin also plays pleiotropic roles.

Maspin was identified by differential expression screening from human normal mammary epithelial cells and breast tumors. Maspin is expressed at RNA level in normal human mammary epithelial cells but its expression is either reduced or completely silenced in breast cancers (16). Human maspin contains a cDNA of 2584 nucleotides which encodes a 42 kDa protein (375 amino acids) with the overall sequence homologies with serpins. Thus, the name maspin stands for **ma**mmary homologue to **s**er**pin**s. Since the discovery of maspin ten years ago, we have identified multiple biological functions. Earlier studies were focused on the biochemical and cellular properties of maspin using *in vitro* cell culture system. Recently, transgenic and gene knockout mouse models have been used by our laboratory to identify the biological functions of maspin *in vivo.* In this review, I summarize the analyses of maspin functions in tumor progression and mouse normal development.

## 3. ROLE OF MASPIN IN CANCERS

### 3.1. Maspin in breast cancer

The association of maspin down-regulation with breast tumor progression was first investigated by using a small panel of human breast cancer samples in 1994 (16). Since then, several clinical reports from different laboratories confirmed the initial observation. Maass *et al.* reported that and the level of maspin expression was reduced in breast cancer patients along with the progression from DCIS to more aggressive carcinoma and metastasis (17, 18). Myoepithelia were found to display strong maspin expression. A significant stepwise decrease in maspin expression occurred in the sequence DCIS-invasive cancer-lymph node metastasis (19). In other independent reports,

significant correlation between maspin expression was significantly correlated with lower grade of malignancy such as lower infiltration of the tumor into the surrounding tissue, a down regulation of c-erbB2 expression, and a decrease in tumor vessels (20, 21). These observations collectively suggested that maspin might act at the step of blocking tumor invasion and metastasis.

To prove that maspin functions as a tumor suppressor *in vivo*, we have utilized several mouse models of breast cancer in the past few years. Firstly, we employed a maspin transgenic mouse model (WAP-maspin) to examine the effect of overexpression of maspin on tumor suppression. The WAP-maspin transgenic mice had targeted overexpression of maspin in mammary epithelial cells by a mammary specific promoter-WAP (22). We then crossed the WAP-maspin transgenic mice with a strain of oncogenic WAP-SV 40 T antigen (TAg) mice. WAP-TAg transgenic mice develop mammary tumors with high frequency by targeting the inactivation of both the p53 and the pRb related family of proteins (23, 24). We compared the tumor free time period and the rate of tumor growth of the WAP-TAg and the bitransgenic mice (25). The tumor free period in mice overexpressing maspin significantly decreased from 36.3 to 49.3 days. The tumor growth rate was also significantly decreased in maspin overexpression mice. Such inhibition resulted from the effect of maspin overexpression on microvessel density and apoptosis. In small mammary tumors (tumor size $\leq$ 0.6 cm in diameter), the microvessel density was significantly reduced ($p< 0.02$) while the apoptotic index was significantly increased in the presence of maspin overexpression ($p< 0.01$). We also examined the effect of maspin overexpression on lung tumor metastasis. The bitransgenic mice had a reduced rate of metastasis compared to that of the WAP-TAg single transgenic mice. Lung metastases developed in fifteen of the twenty-six (55.6%) WAP-TAg mice, while in bitransgenic mice the rate of metastasis was 37.5% (fifteen of the forty bitransgenic mice). Lung tissues were serially sectioned for microscopic analysis. The microscopic images of lung sections were captured to quantitate the difference in the number of tumor foci between these two mouse strains. The bitransgenic mice had decreased foci numbers (0.356 /$10^4$ pixels) compared to that of WAP-TAg mice (0.655 /$10^4$ pixels) (25).

One limitation in testing the tumor suppressing activity of maspin in the SV40 TAg mouse model is that the transgene was dependent on the WAP promoter which was activated strongly during pregnancy and very weakly activated in the estrous cycles (22). In addition, since endogenous maspin expression was controlled by the p53 transcription factor (26), the activation of TAg resulted in the inactivation of p53 which in turn decreased expression of endogenous maspin. These compounding effects favor the oncogenic process. In order to counteract such a potent oncogenic effect, the level of maspin expression must be increased in tumors by either systemic delivery of maspin or by placing the maspin transgene under the control of a constitutive promoter. We accomplished this goal in the following study by using a new breast tumor mouse model (27). We established stable clones in mammary tumor TM40D cells overexpressing maspin using the elongation factor promoter, which is constitutively active in mammalian cells. In the first experiment, two groups of paired mice of 8 weeks old were implanted in #4 mammary glands with either cells transfected with the maspin overexpression vector or the control vector. Implanted control TM40D cells developed palpable tumors with 100% frequency. In contrast, only 77.8% of mice implanted with maspin transfectants developed palpable tumors (27). The tumor growth rate was also significantly decreased in maspin expressing tumors. The mean time for tumor appearance TE50 (50% of tumor endpoint) in the control group was 24 days, while palpable tumors developed slower in the maspin transfectants within 36 days ($P<0.001$). The tumor histology was also drastically different in maspin expressing clones compared to the control tumors. Most sections from control TM40D tumors showed necrosis but lacked tumor encapsulation. In contrast, all maspin expressing tumors had a fibrous capsule surrounding the tumors but few had necrosis.

TM40D tumors developed in the mammary gland are highly invasive and can metastasize to several organs. Mice implanted with control TM40D cells had multiple tumors surrounding the intestines and some had tumors on the pleural surface, indicating that these tumors had invaded through the abdominal muscle from the #4 mammary gland. However, none of the maspin tumor-bearing mice had visible tumor formation on the intestine and pleural surfaces. We examined the lung metastasis by microscopic analysis. Our data showed that 33.3% of the control mice developed lung metastases while none of the maspin tumor-bearing mice had any lung metastases. Overall, we observed that 75% of the control tumor mice had either invasion or distant metastases. In addition, we observed local invasion into the muscle adjacent to the tumor and into blood vessels in the control TM40D tumor sections. However, no invasion was observed for maspin-overexpressing tumors. This study, together with the previous study using transgenic mice demonstrates that maspin indeed inhibits primary tumor growth and metastasis.

### 3.2. Maspin in prostate cancer

In addition to breast cancer, maspin has been shown to be associated with tumor progression in several other human cancers. The prostate is an organ whose development depends on androgenic hormones (28), and its tumorigenesis may result from similar molecular events as that in breast cancer. We began to study maspin in prostate by addressing whether maspin plays a similar tumor-suppressing role as that in the breast cancer. As a first step, we studied the mechanism underlying gene regulation of maspin in prostate cells. We have found that expression of maspin in normal and carcinoma-derived prostate epithelial cells is differentially regulated at the transcriptional level (29). We have identified two different kinds of cis elements, Ets and HRE, in the maspin promoter. The Ets element is active in regulating maspin expression in normal prostate epithelial cells but inactive in tumor cells. The HRE (hormone response element) site is a negative element recognized by the androgen receptor. We concluded that

loss of maspin expression during tumor progression resulted from both the absence of transactivation through the Ets element and the presence of transcription repression through the negative HRE element recognized by androgen receptor (29). Following our promoter study, Zou *et a.l* demonstrated that the mode of negative regulation by HRE and AR was indeed present in the *in vivo* context (30). They showed that maspin expression is significantly higher in tumor specimens (92%) of patients treated with neoadjuvant androgen ablation therapy before radical prostatectomy. LNCaP cells cultured in androgen-depleted medium showed induction of maspin promoter activity in a promoter luciferase reporter assay. Most importantly, castration or treatment of prostate cancer with anti-androgen reagent up-regulated maspin expression *in vivo* (30).

We have also used prostate tumor cells derived from the TRAMP prostate tumor model to study maspin's function in prostate cancer. The prostate tumor line C2N was derived from a primary prostate tumor in TRAMP mice (31). This cell line is highly tumorigenic and invasive (32). We introduced maspin gene into TRAMP C2N prostate tumor cells by a retrovirus approach. Several maspin overexpression clones were selected to compare their ability in growth and soft agar colony formation. Our data indicated that maspin-expressing clones were growth inhibited and such inhibition was maspin dose-dependent (33). To understand the molecular mechanism of maspin mediated tumor suppression, we examined the effect of maspin on cell-ECM adhesion. Extracellular matrix protein laminin and fibronectin are used in the cell adhesion assay. In the presence of both matrix components, maspin expressing cells were consistently more adherent to matrix. This result is in consistent with a previous report that breast tumor cells treated with maspin had increased integrin activity, which resulted in an increase of cell adhesion to fibronectin in MDA-MB-231 breast cancer cells (34). We believe that the increased adhesion in maspin-expressing C2N cells makes cells more attached to ECM, thus preventing the tumor cells from migrating freely through the extracellular matrix. In addition, using the Boyden Chamber system we evaluated maspin's effect on C2N cell invasion. The maspin-expressing clones were greatly inhibited in their ability to invade through the matrix membrane. Thus, our data demonstrated that maspin could inhibit C2N tumor invasion in a fashion similar to that observed in breast tumors (33).

In addition to these studies, recent clinical studies have linked maspin's tumor suppression function with better prognosis in prostate cancer (35). Machtens *et al.* showed that maspin protein expression is correlated with the recurrence-free survival of prostate cancer patients (35). In their study, tumor specimens obtained from 84 patients undergoing radical prostatectomy for localized prostate cancer were investigated for the expression of the maspin and p53 protein. Maspin protein level was correlated with tumor characteristics such as tumor stage, histologic grading, regional lymph node status, p53 protein expression and recurrence-free survival of the patients following radical prostatectomy. After a median follow-up of 64 months, 23 of 40 patients (58%) with a negative or decreased maspin expression developed local recurrence or systemic tumor progression in contrast to 8 of 44 patients (18%) with a retained expression of the Maspin protein (group 2) ($p = 0.02$). Additionally, loss of maspin protein expression was correlated to higher tumor stages ($p = 0.002$) and an increasing histologic dedifferentiation ($p = 0.03$). In another study, using microarray technology Chen *et al.* compared gene expression of maspin and hepsin in prostate cancers (36). They showed while hepsin as well as 7 of 22 previously reported up-regulated genes demonstrated a pattern of increasing expression with increasing malignant phenotype, the expression of maspin decreased with increasing malignancy of prostate cancers. They concluded that the increased ratio of hepsin-to-maspin might have an important role in prostate cancer progression and invasion.

### 3.3. Maspin in other cancers

Despite of these clinical reports correlating maspin with a protective role in breast and prostate cancers, several recent studies have indicated a negative role of maspin in certain other cancers (37-39). For examples, Maass *et al.* reported that maspin level was increased in pancreatic cancer while no or lower maspin was observed in normal and low graded pancreatic cancer (38). Maspin was expressed in 23 of 24 tumor specimens obtained from pancreatic cancer patients as well as all high-grade precancerous lesions (PanIN3 and intraductal carcinoma extension). In contrast, no expression was observed in normal and low-grade precancerous lesions. In another case, it was reported that maspin transcript was present in 40% of bone marrow samples from patients with hematological malignancy (37). Sood *et al.* showed that maspin was also overexpressed in ovarian cancers (40). Among the ovarian tumors examined, 57 (71%) were ranked positive for maspin. Thirty (37%) of the invasive tumors overexpressed maspin. Invasive cancers were more likely to have predominantly cytoplasmic staining compared with benign and low-malignant-potential tumors. Maspin overexpression was significantly associated with a high tumor grade ($P = 0.004$), the presence of ascites ($P = 0.02$), a lower likelihood of optimal surgical cytoreduction ($P = 0.04$), and a shorter duration of overall survival (median survival, 6.33 versus 2.67 years; $P = 0.003$). These data suggest that perhaps maspin play multiple roles in different cancers. The question why the same molecule exerts different functions *in vivo* is not understood.

### 3.4. Targeting maspin gene for cancer therapy

Maspin's tumor inhibitory effect and the clinical association of maspin expression with breast cancer progression prompted us to develop a cancer therapy, utilizing maspin gene as targeting molecule. Cancer gene therapy requires both a good animal model and an effective delivery system (41). In the past, many genetically engineered mouse tumor models are used for cancer therapy. One limitation of using these animal models in cancer therapy is that the time for tumor development in each animal varies in individual animal, depending on the activation of its transgene. This affects the evaluation of the treatment efficacy since all reagents are supposed to be

delivered to the group of animal at the same time. In addition, some of transgenic mice generally develop multiple sites of mammary tumors and tumors are metastatic to many organs in each animal, making both the measurement of primary tumors and the determination of metastasis rate in secondary sites a truly tedious task. To circumvent these problems, we established a syngeneic PyV-mT tumor transplantation model for gene delivery study. The PyV -mT cells were initially isolated from the mammary tumors in MMTV-PyV-mT transgenic mice (42). The PyV -mT cells were transplanted to syngeneic FVB female mammary fat pad bilaterally. Tumors developed 100% in transplanted sites and pathhistology showed a characteristic of solid adenocarcinoma. The unique feature of this transplantation model is that implanted tumors grow out uniformly and are only metastatic to the lung but not other organs. This property is particularly useful for gene therapy.

Cancer therapy requires an effective delivery system with low toxicity. We have chosen an improved DNA:liposome complexes for increased delivery and gene expression (43). To determine whether the system we chose could effectively deliver gene to target organs such as mammary gland, we injected a reporter DNA construct (CAT) with liposome to a group of wildtype mice through tail vein. Organ extracts were harvested 24 hr postinjection for CAT activity. We found that other than the organ of lung, mammary gland and heart represent two organs with highest delivery efficiency among more than 14 mouse organs surveyed (44). To test the therapeutic value of maspin against breast tumor progression, two groups of mice were implanted with PyV-mT tumor cells. After the tumors were developed in mammary gland orthotopically, mice were treated with maspin:liposome or with control plasmid DNA systemically. The maspin group received the DNA:liposome treatment for an average of 32.2± 1.8 days while the control group received 33.7±1.8 days (P>> 0.05, no significant difference). Primary mammary tumor growth was monitored by caliper measurement during the treatment and at the endpoint. Our data showed that both the tumor size and the overall tumor growth rate were significantly decreased for maspin treated tumors comparing to that in the controls (45).

To analyze maspin's effect on tumor metastasis, lung tissues from two groups were harvested from mice at the end point. Tissues were serially sectioned for histology. Histological analysis of these serial sections revealed a significant decrease of lung tumor metastasis in maspin treated group (44.5%) comparing to control (100%). Photo images of lung sections captured by digital camera were processed for the volume and the number of tumor foci. We found that the control treated samples had a total area of tumor foci of 88.62 pixels (per $10^4$ pixel of long area). However, this parameter was significantly decreased (253 %) in maspin treated samples (25.12 pixels/$10^4$ pixels lung area). The total numbers of tumor foci were also significantly reduced (185%) in maspin treated samples. Furthermore, we demonstrated that maspin:liposome treatment does not result in any toxic side effect after a long period of maspin treatment in wildtype mice (45). Thus, maspin:liposome treatment offers an effective therapy with low toxicity for breast cancer.

## 4. MASPIN IN ANGIOGENESIS

Tumor growth and metastasis requires neovascular formation, a process also termed angiogenesis (46, 47). Angiogenesis provides tumors with nutrients and aids in the removal of metabolic wastes (48, 49). Most solid tumors can not grow beyond a few millimeters without neovascular formation (50, 43). Very recently, several non-inhibitory serpin members have been discovered to regulate anti-angiogenesis properties. For example, PEDF, a serpin with known function of cell differentiation, is also a very potent anti-angiogenic factor (15). A cleaved product of anti-thrombin was shown to directly inhibit angiogenesis (51). Our study have shown that maspin acts as an angiogenesis inhibitor as well (52). In an initial study recombinant maspin was tested in a variety of angiogenesis assays. Maspin blocked endothelial cell migration induced by VEGF and bFGF in a dose dependent manner with an $ED_{50}$ of 0.2 μM-0.3 μM. *In vivo*, purified maspin effectively inhibited neovascularization. Rat corneas were surgically implanted with non-inflammatory slow release pellets containing maspin with bFGF and examined six or seven days later for ingrowth of vessels. Deletion and mutation analysis demonstrated that maspin's anti-angiogenesis property is not dependent on the RSL region. Recombinant maspin mutants in the RSL region retained the ability to inhibit endothelial cell migration and mitogenesis *in vitro*. These proteins also retained the ability to inhibit neovascularization in rat corneas *in vivo.*

To determine if the ability of maspin to inhibit angiogenesis plays a role in its well -documented anti-tumor activity, an athymic mouse xenograft model was utilized (52). LNCaP prostate tumor cells were implanted subcutaneously on the bidorsal back of nude mice and tumor growth and neovascularization were monitored following systemic treatment with exogenous maspin. We found that maspin-treated tumors contained significantly fewer vessels as determined by CD31 immunostaining than GST treated controls. To determine whether maspin effects on the tumor-induced vasculature were maintained during a more prolonged treatment, the above experiment was replicated with tumors harvested after 7 to 8 weeks. Thirty-two tumor sites were treated with maspin and thirty-seven with GST. When examined at week 8, the growth of 53% of the maspin-treated tumors had been completely inhibited. The remaining fifteen maspin-treated tumors were reduced in size by average 3.43 fold when compared to GST control treated tumors. The effect of maspin was reversible. To examine if the reduced size of maspin-treated tumors coincided with reduced neovascularization, twenty representative tumors from either maspin-treated (10 sites) or GST –treated tumors (10 sites) were used to quantify the density of microvessels after immunostaining with CD31 antibody. The density of vessels in maspin-treated tumors was reduced 2.6 fold in average to that in control tumors and this difference was highly significant. We also compared the treated and control tumors of similar size. A reduction of vessel density was also observed in the maspin treated samples. These data confirm

that maspin is an effective inhibitor of tumor angiogenesis and therefore could be developed into a potent anti-angiogenesis/anti-cancer therapy.

Recent study by Cher *et al.* (53) have provided further support regarding to maspin's effect on angiogenesis in cancer progression. They showed that maspin-expressing transfectant cells derived from prostate cancer cell line DU145 were inhibited in *in vitro* extracellular matrix and collagen degradation assays. They injected the maspin-transfected DU145 cells into human fetal bone fragments, which were previously implanted in immunodeficient mice. Their studies showed that maspin expression decreased tumor growth, reduced osteolysis, and decreased angiogenesis (53).

## 5. MASPIN IN MOUSE DEVELOPMENT

One of the views that we believe strongly is that a better understanding of maspin in mouse normal development will aid in our elucidation of maspin function in tumor progression. This philosophy has guided the research in my laboratory in the past few years. Two approaches have been employed to study role of maspin in mouse development. The first is the use of maspin transgenic mice to study the effect of maspin overexpression on mouse mammary gland development. The mouse mammary gland undergoes a dramatic series of cyclical changes during development (54). Mammary specific promoter such as WAP and MMTV are widely used to target gene overexpression in mammary gland during pregnancy and lactation. At these stages, alveolar epithelial cells rapidly proliferate and differentiate under different regulatory mechanisms. Alveolar cells grow from the ductal skeleton, and appear as lobulo-alveolar units by the end of pregnancy. These alveoli are the functional unit of milk production at lactation. Following lactation, the mammary gland undergoes massive remodeling and apoptosis, resulting in involution of the gland and a return to the ductal structure similar to the non-pregnant state. We utilized a transgenic mouse system to examine the effect of overexpression of maspin under control of the whey acidic protein (WAP) promoter (55). Whole mount and histology of the mammary glands from wild-type and transgenic mice were analyzed. Ductal elongation and branching appeared to be normal in wild-type and transgenic virgin mice. No difference in alveolar structures was present between the transgenic and wild type animals up to day 10 of pregnancy. However, significant differences became noticeable at day 15 of pregnancy following the activation of the WAP promoter-driven transgene. These mammary glands exhibited decreased alveolar densities, which was further reduced as compared to controls at day 19 and resembled the morphology of the midpregnant wildtype controls. The mammary glands from transgenic mice contained not only fewer lobular-alveoli structures, but also the reduced size of each alveolar structure. The defect in alveolar structures in the WAP-maspin mice during late pregnancy severely hampered the ability of the mother to successfully nurse her entire litter. The production of milk proteins such as WAP and β-casein were reduced in pregnant transgenic mice. This observed decrease was due to the effect of reduced number of alveolar cells and closed lumens in the late pregnant transgenic mice.

Further analyses showed that the luminal epithelial cells had increased apoptosis compared to non-transgenic mammary cells. We hypothesized that overexpression of maspin perturbs the adhesion of alveolar cells to the ECM as does the chimeric β1 transgene and, thus inhibits the motility of alveolar cells at a stage when invasion into the fat pad is critical. Indeed, expression of a dominant negative β1 integrin in the mammary gland, which disrupted the function of β1 and its associated integrins, resulted in a phenotype similar to that is observed in the WAP-maspin transgenic mice (56). Both maspin and chimeric β1 transgene expression caused underdevelopment of the mammary gland in midpregnancy and early lactation, which was accompanied by an increase in apoptosis. Recent studies by my laboratory have linked the function of maspin with β1 integrin in cell-ECM adhesion in mammary epithelial cells (Cella *et al.*, unpublished data).

A second approach is to study the loss of maspin in mouse development using maspin knockout mice (57). We have cloned mouse maspin genomic DNAs and designed a vector to selectively knock out the maspin gene in mouse. The heterozygous Mp+/- mice progeny appeared to be normal morphologically after birth. However, when they were crossed, no homozygous maspin deletion progeny were obtained at birth. Among the 571 live-born offspring, 343 were heterozygotes and 228 were wild type mice. This indicates that the homozygous maspin null mice are lethal during embryonic development. We then proceeded to determine when the homozygous lethality happened during embryonic development. We first examined the pre-implantation stage of embryonic development. Mp+/- mice were intercrossed; embryos at the 2-cell, 4-cell, and blastocyst stages were compared. Both Mp-/- and Mp+/+ embryos were observed by the late blastocyst stage, indicating that the Mp-/- embryos do not die before implantation. To further determine the time of embryonic death after implantation, *in utero* embryos ranging from 4.5 dpc to late gestation were microdissected and genotyped by either PCR or by immunostaining. Our immunostaining data indicated that Mp-/- embryos died before 5.5 dpc. At 4.5 dpc, maspin was not expressed in the inner cell mass, the primitive endoderm, or in the uterine cells surrounding the embryo. However, maspin protein appeared in the visceral endoderm (VE) at 5.5 dpc. This expression was restricted to both the embryonic and the extraembryonic visceral endoderm at 6.5 dpc and 7.5 dpc. No maspin expression was observed in the parietal endoderm, the ectoplacental cone, the chorion, and the amnion. As an early event of embryonic development, the generation of the visceral endoderm provides the embryo with nutritional and hematopoietic functions (58, 59). Since maspin is specifically expressed in the visceral endoderm cells after implantation, its deletion seems to be destructive to the formation of the endodermal cell layer and for the morphogenesis of the epiblast, thus preventing further embryo development.

To further delineate maspin function in early

embryonic development, we used two *in vitro* approaches to mimic the embryonic development in the uterus. The first is to culture blastocysts derived from Mp+/- matings. During the outgrowth process, blastocysts undergo both cell growth and differentiation. We noticed that the inner cell mass (ICM) of Mp-/- blastocysts failed to grow out appropriately, although trophoblasts from Mp-/- embryos differentiated and expanded without any noticeable defects. The second approach is to grow embryonic stem cells *in vitro* and to induce ES cells differentiation into embryoid bodies (EB). We found that most of the Mp+/+ ES cells could be differentiated into embryoid bodies with a large lumen in the center and an well-organized layer of visceral endoderm. In contrast, Mp-/- ES cells formed EBs of smaller size consisting of disorganized cell masses. Furthermore, we stained EBs with antibody against GATA-4, an embryonic ectoderm marker. In Mp+/+ EBs, the embryonic ectoderm cells, which are positive for Oct4, lied closely adjacent to the basement membrane surrounded by the VE layer. However, almost no cells in Mp-/-EBs were positive for Oct4. This result indicates that the development of embryonic ectoderm or epiblast is defective in the absence of maspin, likely resulting from the lack of the presence of a continuous layer of VE cells lining the outside of the ectoderm. When we introduced maspin to Mp-/- EB by adenovirus infection, the re-expression of maspin partially rescued the defect of Mp -/- EBs, as evident by the appearance of ectoderm cells and a layer of endodermal cells surrounding the ectoderm. In addition, a maspin antibody specifically blocked normal EB formation, indicating maspin controls the process through a cell surface event. This was proved to be the case since we showed that maspin directly increased endodermal cell adhesion to laminin matrix, and Mp+/- endodermal cells grew significantly slower than Mp+/+ endodermal cells on laminin substrate. We conclude that maspin affects VE attachment to laminin, a key component of extracellular matrix thereby controlling further embryonic development.

Since the discovery of maspin as a putative tumor suppressor gene, a series of animal experiments have been carried out demonstrating the inhibitory function of maspin against breast tumor metastasis. However, the mechanism of metastasis inhibition is not fully understood. The study of maspin in early embryonic development provides a definitive answer to one of the mechanisms of maspin action. Tumor metastasis requires the detachment of tumor cells from the extracellular matrix as well as extensive invasion through the basement membrane and stroma (60, 61). The increased cell adhesion caused by maspin could hinder such a process and thereby prevent tumor metastasis. Further experiments on the role of maspin in endoderm differentiation and cell-cell interactions during embryonic development will likely shed more light on our understanding of its role in tumor invasion and metastasis.

## 6. CONCLUSION

Maspin is a complex and versatile protein with a multitude of effects on cells and tissues at an assortment of stages of development. Beginning with embryo development, maspin is required for the appropriate cell-matrix interactions to occur so that a healthy and viable embryo develops. In adult mouse, maspin is present in the epithelial cells of most tissues. Our laboratory has begun to unveil the role of maspin in the development of mammary gland and several other organs. With regard to its role in tumorigenesis, emerging evidence indicate that maspin has played both inhibitory and promoting functions in different cancers. Such diverse functions demand for further investigation about the mechanism of maspin action. Another new frontier is the study of gene regulation of maspin during mouse tissue development and tumorigenesis. A key question is why maspin gene expression is activated or silenced. Understanding the mechanism of gene regulation may provide invaluable information for therapeutic intervention of certain cancers.

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