AD_____

Award Number: DAMD17-02-1-0294

TITLE: Identifying the Molecular Mechanism of Tumor Suppression by Maspin in Breast Cancer

PRINCIPAL INVESTIGATOR: Ming Zhang, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

REPORT DATE: May 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-05-2005	Final	15 Apr 2002 – 14 Apr 2005

4. TITLE AND SUBTITLE

Identifying the Molecular Mechanism of Tumor Suppression by Maspin in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0294

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ming Zhang, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Maspin is a unique member of the serpin family that shares extensive homology with monocyte-neutrophil elastase inhibitor, PAI-2 and other serpins. Initially identified as a class II tumor suppressor gene, maspin has been shown to inhibit invasion and motility of mammary tumors. When maspin gene was introduced into breast tumor cells, it was demonstrated that tumor transfectants expressing maspin exhibited significant decrease in breast tumor growth and metastasis in nude mice. Maspin gene expression is not detected in most breast tumors and loss of its expression is correlated with tumor invasiveness. Maspin is also found to be a potent angiogenesis inhibitor. Based on the previous work by my laboratory and other colleagues, I hypothesize that maspin possesses multiple functionality that requires its interaction with multiple target proteins. Extracellular maspin may bind to the cell surface and regulate cell adhesion through integrin signal pathway. Maspin could act intracellularly or be secreted to act in a paracrine fashion on adjacent cells. I propose to isolate maspin target(s) by combined genetic and biochemical approaches. Once the maspin targets are identified, deletion and mutagenesis studies will be carried out to identify the functional domain of maspin that is responsible for such protein-protein interaction.

15. SUBJECT TERMS
Breast Cancer: maspin, yeast two hybrid, target, receptor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	12	

Table of Contents

Introduction

Background: Maspin is a unique member of the serpin family that shares extensive homology with monocyte-neutrophil elastase inhibitor, PAI-2 and other serpins. Initially identified as a class II tumor suppressor gene, maspin has been shown to inhibit invasion and motility of mammary tumors. When maspin gene was introduced into breast tumor cells, it was demonstrated that tumor transfectants expressing maspin exhibited significant decrease in breast tumor growth and metastasis in nude mice. Maspin gene expression is not detected in most breast tumors and loss of its expression is correlated with tumor invasiveness. Maspin is also found to be a potent angiogenesis inhibitor. Other than its anti-tumor function, maspin clearly plays an important role in normal development. We have demonstrated that transgenic mice overexpressing maspin in mammary gland inhibit alveolar development and mammary cell differentiation, and maspin transgenic mice had a protective role against breast tumor growth and metastasis. Overall, maspin is a gene with an important function in both normal mammary development and breast tumorigenesis.

Objective/Hypothesis: Based on the previous work by my laboratory and other colleagues, I hypothesize that maspin possesses multiple functionality that requires its interaction with multiple target proteins. Maspin could act intracellularly or be secreted to act in a paracrine fashion on adjacent cells. I propose to isolate maspin target(s) by combined genetic and biochemical approaches. Once the target is identified, deletion and mutagenesis studies will be carried out to identify the functional domain of maspin that is responsible for such protein-protein interaction. Finally, such interaction will be verified in mammalian cells.

The specific aims for this three-year proposal are:
(1) Identification of maspin target protein by genetic and biochemical approaches, and (2) characterization of the functional domain of maspin responsible for protein-protein interaction.

Body

Materials and methods

Yeast library

A Hela cDNA library has been obtained from Dr. Roger Brent at Harvard Medical School. Yeast EGY48 cells were available at the PI's laboratory.

Yeast two-hyb assay

Yeast EGY48 cells will be transformed with LexA/maspin fusion plasmid and a LexA/lacZ reporter and selected on yeast Ura- and His- plates. The transformed yeast cells will be used to screen a Hela library consisting of B42/Hela cDNA fusions. The putative positive clones should be able to grow in the Ura-, His-, Trp- and Leu- plate containing galactose as the carbon resource and become blue in the presence of X-gal.

Antibodies
Antibody for HA tag of the fusion protein was purchased from Sigma. Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. All secondary antibodies were purchased from Zymed, Inc.

Western analysis
Total yeast proteins were isolated from cells. Protein extracts were prepared by lysing the cells in RIPA buffer. Total 100 ug protein extract will be loaded for electrophoresis. Western blot analysis will be carried out using HA and maspin antibodies to detect maspin and its interacting proteins.

Adhesion assay
LN5-rich matrix was prepared using MCF10A-deposited matrix, which deposited only laminin-5. Briefly, cells were grown until confluent in a 96 well dish. Wells were washed with PBS and treated with fresh 20mM NH_4OH for 5 min, or until cells are completely blown off. Wells were washed extensively with water and blocked with 10mg/ml of heat-inactivated BSA in PBS (80°C 15 min) for 1h at RT. Cells were harvested with enzyme-free cell dissociation solution (Cell & Mol. Technologies, Inc.) washed with serum-free DMEM/F12 medium and incubated with antibodies or recombinant proteins for 30 min at 37°C. 20,000 cells/well were plated and allowed to adhere for 30 min at 37°C. Wells were washed with PBS and adhered cells were fixed with 5% gluteraldehyde and stained with crystal violet. Dye was solubilized in 10% acetic acid and absorbance was determined at 590 nm.

Results and Discussion

Task 1. Identification of putative receptor of maspin
Months 1-36.

Identifying integrin β1 as a maspin interacting protein

We had carried out some receptor binding experiment. Maspin proteins made in E.coli were labeled by 125-I and used in the cell based binding assay, using normal mammary epithelial cells and MDA231 breast cancer cells. Although we did not characterize the binding kinetics (Kd and Bmax), we were able to shown that extracellular maspin could bind to the cell surface.

To investigate a role of maspin on cell-ECM interactions in normal mammary epithelial cells, MCF10A mammary cells which express very high level of endogenous maspin were treated with recombinant maspin or GST control at various dosages and cells were allowed to attach to the matrix for 30 minutes. Maspin significantly increased cell adhesion in a dose dependent manner (Fig.1A). We then treated MCF10A cells with anti-maspin serum or control serum and cells were allowed to attach to the matrix for 30 minutes. Confocal microscopy showed that MCF10A cells had high level of maspin protein on the cell surface. A significant decrease in cell adhesion was observed when cells were treated with a polyclonal anti-maspin antibody raised against the whole maspin protein but not an antibody raised specifically against the reactive site loop (RSL) domain of maspin. We then employed an RNA interference assay

to specifically down-regulate maspin expression in these cells. Maspin siRNA-expressing cells exhibited significantly less cell adhesion to their self-deposited matrix (data not shown). These data demonstrate that maspin positively modulates cell adhesion to the LN-5-rich ECM, and it does so in a rapid manner. We hypothesized that maspin might be associated with β1 integrin (for reasons not mentioned here). To test this hypothesis we examined β1 integrin and maspin co-immunoprecipitation. MCF10A whole cell extracts were immunoprecipitated with a maspin polyclonal antiserum and probed for β1 integrin by immunoblotting. We detected a major band at around 110 kDa, in agreement with the molecular weight of β1 integrin (Fig. 1A). In order to confirm the reciprocity of the interaction, β1 integrin was immunoprecipitated from two MCF10A extracts prepared independently. Immunoblotting analysis with an anti-maspin antibody revealed maspin co-immunoprecipitation in MCF10A extracts (Fig. 1A). β1 integrin immunoprecipitation was confirmed by reprobing the filter with anti-β1 integrin. This result indicates that maspin and β1 integrin are physically associated; suggesting that maspin may modulate cell adhesion by interfering with β1 integrin-dependent cell-ECM interaction. In addition, maspin-β1 integrin co-immunoprecipitation occurred in the presence of 1% Triton X-100, indicating that this interaction is very strong. We have also carried out immunostaining to co-localize maspin with β1 integrin in mammary tissue. Using frozen tissue sections, we were able to colocalize maspin with β1 integrin (Fig. 1B) in mammary ductal epithelial cells. These data are currently being summarized and a manuscript is in preparation. Not surprisingly, this study in mammary cells is supported by the findings from studies on the role of integrin β1 in embryogenesis. The deletion of integrin β1 completely duplicated the phenotypes observed in the maspin null embryos. For example, in β1-null embryos, endoderm morphogenesis was defective and the embryos die at E 5.5. Blastocyst outgrowths in the β1 null embryos were blocked due to an inner cell mass failure. However, trophoblast function which depends on different integrins in the β1 null embryos was largely normal; both the decidual reaction was induced and outgrowths on fibronectin coated substrates were observed. These similar phenotypes suggest again that the function of maspin is closely related to that of β1 integrin.

Task 2. Identification of maspin target by yeast two-hybrid system
Months 1-36.

We have started to use the Hela cDNA library for yeast two hybrid screening. Briefly, yeast EGY48 cells were transformed with LexA/maspin fusion plasmid and a LexA/lacZ reporter and selected on yeast Ura- and His- plates. The transformed yeast cells were then transformed with Hela library consisting of B42/Hela cDNA fusions. In the last eight months, we have obtained over 200 candidate clones that were able to grow in the Ura-, His-, Trp- and Leu- plate containing galactose as the carbon resource and became blue in the presence of X-gal. Plasmids were isolated from these clones and retransformed to yeast cells to confirm the ability of these plasmid to confer x-gal inducibility. Over the 200 primary clones, 30 or so secondary clones were identified. Many plasmid sequenced contained nonsense coding sequences and were further eliminated. Seven clones contained cDNA sequences that are in-frame with LexA fusion. B-gal activity was analyzed for these clones (Fig.2). These clones were then retested in yeast two-hyb assay as well as in other in vitro and in vivo assays. Currently, we have focused on the CCT7 gene that is an actin-binding protein. In vitro translated CCT7 protein can be immunoprecipitated with maspin by maspin antibody. We are currently studying the maspin-CCT7 interaction in normal mammary epithelial MCF10A cells.

Task 3. Identification of maspin target by biochemical approach
Month 6-36

Since we have identified B1 integrin as a maspin binding protein on cell surface, we begin to identify the functional domain of maspin that mediated cell surface function by a biochemical approach. We made several deletion and point mutation mutants of maspin for this study. These mutants include: Mas (1-139aa), Mas (1-228aa), Mas (229-375aa), Mas (140-375aa), Mas (140-340aa), Mas (1-340aa), and a point mutation of Arg to Gln at amin acid 340. These mutants were tested in cell adhesion assays to delineate the domains required for maspin-mediated cell adhesion. We treated MCF10A cells with recombinant proteins (including mutant maspins) in an adhesion assay. The result showed that a region between 140-225 aa of maspin was responsible for maspin mediated cell adhesion through integrin B1 (Fig.3). This study together with the datafrom the study of task 1 has been summarized for the preparation of a manuscript.

Key research accomplishments

We have identified integrin B1 as a maspin interacting protein on the cell surface and maspin acts to increase cell adhesion. The functional domain of maspin involved in this action has been identified. The yeast two hybrid screening has identified seven candidate clones (Fig.2). Among them, the CCT7 gene has been characterized to interacts with maspin by IP-western assay and functional study in normal mammary epithelial cells is currently being carried out to further delineate the effect of such intracellular interaction on cell proliferation and migration.

Reportable outcome

A manuscript is being prepared for the study of maspin-integrin interaction.

Conclusion

Maspin is located both intracellularly and extracellularly and therefore has multiple target proteins. Through this study, we have identified on cell surface maspin binding partner and at least one intracellular maspin binding protein CCT7. These discoveries laid the foundation for identifying the molecular mechanism of maspin mediate tumor suppression. Further studies may help us develop new therapeutic tools for cancer treatment.

Reference

None

Appendices

The title and abstract pages of one submitted manuscript are provided in page 11-12.

Fig.1

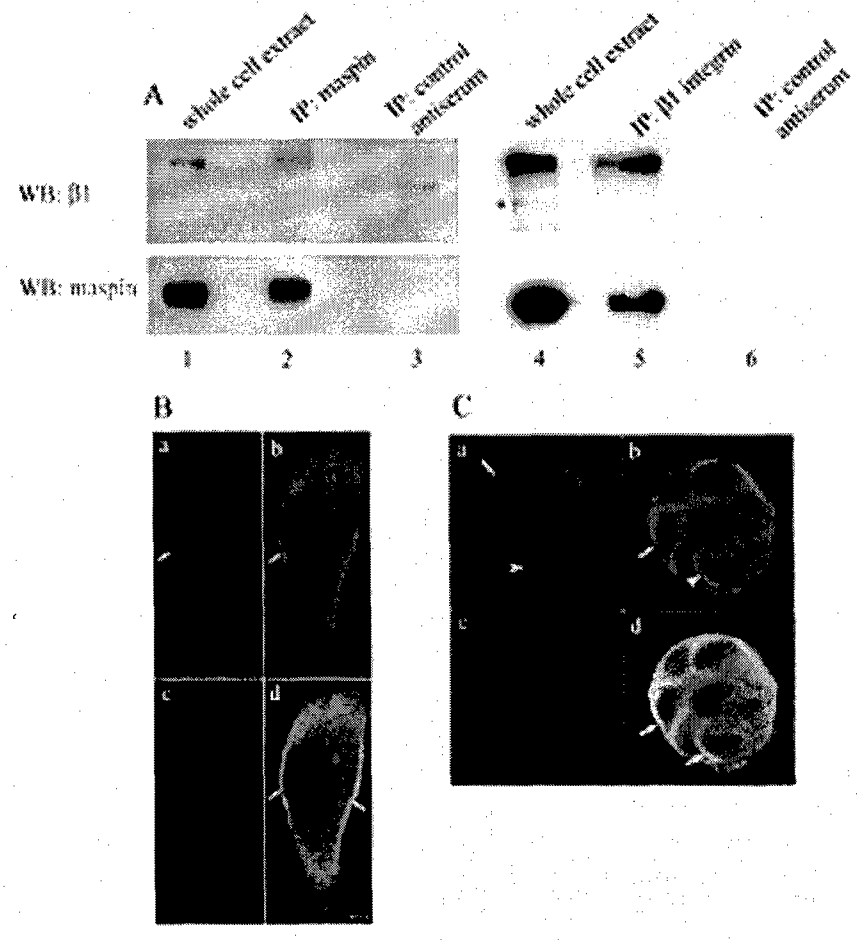


Figure 1. . Maspin and β1 integrin co-immunoprecipitate and co-localize in MCF-10A cells grown in monolayers and in 3D culture. (A) MCF-10A cells were chemically cross-linked, lysed and 500 μg of protein extracts were immunoprecipitated with the indicated antibodies. An irrelevant rabbit anti-serum was used as a negative control (lanes 3 and 6). Immunoprecipitates were separated in SDS-PAGE gels as detailed in Material and Methods and analyzed by Western blot as indicated. * pre-β1 integrin. (B) MCF-10A cells were plated on coverslips, fixed, permeabilized, and stained for maspin (a) and β1 integrin (b). Nuclei are shown in blue (c) and merged image is shown in (d). Arrows indicate that maspin and β1 integrin are located in the periphery of the cell (panels a and b, respectively) and are co-localized (panel d); Bar 10 μm. (C) MCF-10A cells were embedded in the Matrigel and allowed to form acini for 15 days. Cryosections (8 μm) were prepared and stained for maspin (a) and β1 integrin (b). Nuclei are shown in blue (c) and merged image is shown in (d). Arrow and arrowhead in (a) and (b) indicate localization on the basal membrane and in sites of cell-cell contact, respectively. Arrows in panel (d) indicate sites of maspin and β1 integrin co-localization; Bar 20 μm.

Fig.2 β-gal activity of seven maspin-interacting clones comparing to the control yMZ4

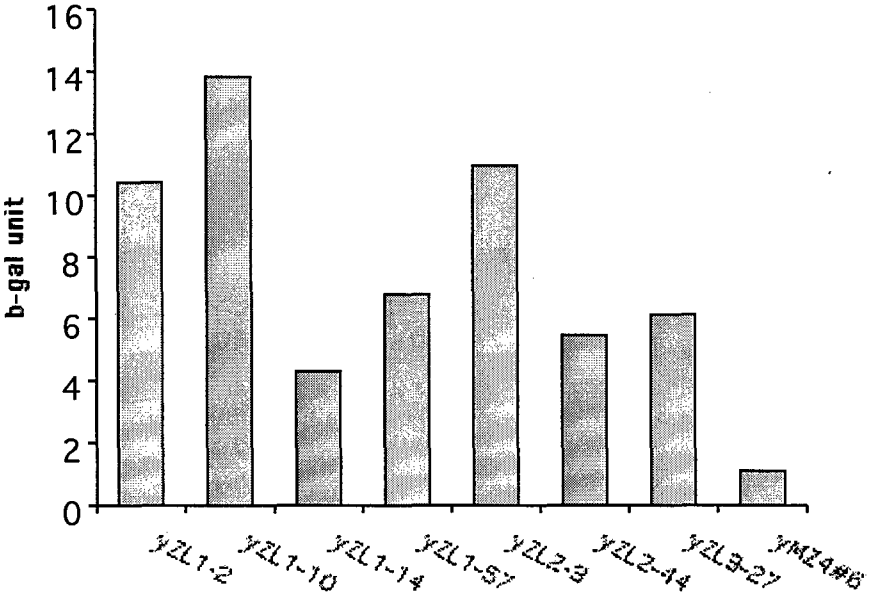
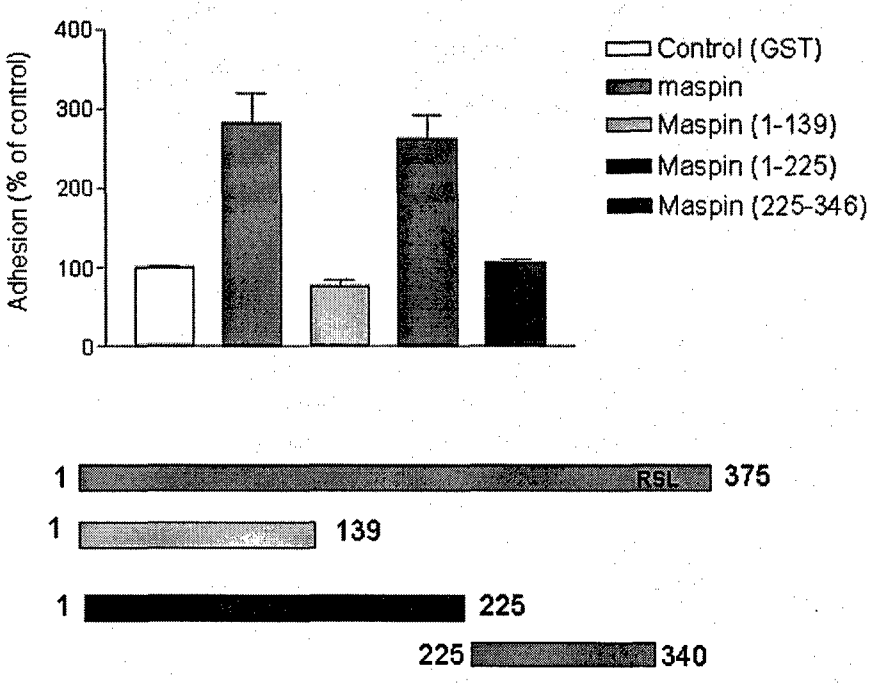


Table 1. Putative maspin target proteins identified from yeast two-hybrid

1.yZL1-2	chaperonin containing TCP1 subunit 7
2.yZL1-10	ferritin
3.yZL1-14	triosephosphate isomerase 1
4.yZL1-57	elongation translation factor 1
5.yZL2-3	phosphoglycerate (PGK1)
6.yZL2-44	CRM1 protein
7.yZL3-27	nucleolar protein ANKT

Fig.3. Effect of maspin deletion mutants on cell adhesion in MCF10A cells.



Maspin is physically and functionally associated with integrins regulating cell adhesion in mammary epithelial cells

Nathalie Cella, Alejandro Contreras, Khatri Latha, Jeffrey M. Rosen, and Ming Zhang*

Baylor College of Medicine
Department of Molecular & Cellular Biology
One Baylor Plaza, Houston, TX 77030
Key words: maspin, cell adhesion, integrins, RSL, ECM

*Corresponding author
Dr. Ming Zhang
Baylor College of Medicine
Department of Molecular & Cellular Biology
ALKEK Building, Room N630
One Baylor Plaza, Houston, TX 77030
Tel: 713-798-3817
Fax: 713-798-3896
Email: mzhang@bcm.tmc.edu

Running head: Maspin is physically and functionally linked to integrins

Abbreviations: ECM, extracellular matrix; FN, fibronectin; RSL, reactive site loop; siRNA, small interfering RNA; 3D, three dimensional

Abstract

Maspin is a tumor suppressor serpin (serine protease inhibitor) which inhibits cell invasion and migration. Here we analyzed maspin function in cell adhesion in non-transformed mammary epithelial cells and investigated the underlying mechanism involved in this process. We report that maspin acts on the cell surface to increase cell adhesion. Addition of recombinant maspin rapidly increased MCF-10A cell adhesion to the endogenously deposited matrix, and conversely both an anti-maspin antibody and maspin knockdown by RNA interference resulted in decreased cell adhesion. Mutation analyses revealed that a region of 86 amino acids located between aa139 and aa225 was responsible for maspin effect on adhesion. Function-blocking antibodies against β1, β4 and α2 integrins prevented maspin-dependent increase in cell adhesion, and co-immunoprecipitation assay demonstrated the association of maspin with β1 integrin, indicating that maspin is functionally and physically associated with integrins. In addition, we show that maspin increases cell avidity for substrata, and maspin is also associated with detergent-insoluble cortical cytoskeleton elements. Collectively, these results suggest that maspin is part of the supramolecular structure of the adhesion plaque and it modulates cell adhesion via a physical and functional link with integrins.