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Natalie Mitchell

Tyson DeAngelis

Trygve O. Tollefsbol

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Natalie Mitchell[1], J. Tyson DeAngelis[1] and Trygve O. Tollefsbol[1,2,3,4]
[1] Department of Biology [2] Center for Aging [3] Comprehensive Cancer Center [4] Nutrition Obesity Research Center
University of Alabama at Birmingham

Abstract
Maspin (SERBINB5), a novel protease inhibitor, is a tumor-suppressor shown to inhibit tumor cell motility, invasion in cell culture, tumor growth and metastasis. High maspin expression is present in normal breast epithelial cells but is down-regulated during tumorigenesis in metastatic breast cancers, potentially leading to an increase in invasive potential and spread of metastatic disease. We examined the mRNA and protein expression of maspin as well as analyzed DNA methylation of the maspin promoter region in the genetically-related MCF10 cell lines, representing normal breast epithelial cells (MCF10A), pre-malignant breast disease (MCF10AT) and two separate metastatic carcinomas (MCF10CAa.1 and MCF10CAd.1α). Maspin expression was progressively down-regulated over the course of tumorigenesis in these cell lines. There was no evidence of overall DNA methylation in the maspin promoter in any of the cell lines studied. Thus, loss of maspin expression in these cell lines is not associated with aberrant cytosine methylation in the promoter region of the gene.

Introduction

Breast cancer is currently the second leading cause of cancer deaths in women today. One in eight women will be diagnosed with this disease in their lifetime (1). While evidence suggests that tumorigenesis is a distinctive multistep process involving the transformation of a normal human cell into a malignant cell, the mechanisms of tumor formation as well as acquisition of metastatic characteristics are not entirely known (2, 3).

Epigenetic modifications have been implicated in playing a role in the development of breast cancer. In particular, aberrant cytosine methylation at CpG dinucleotides in the promoters of tumor-suppressor genes is often associated with transcriptional silencing in carcinogenesis and metastasis (4, 5). DNA methylation changes are likely to occur during the transition from a non-metastatic neoplasia to a metastatic carcinoma.

The MCF10 isogenic breast cancer progression model mimics the multistep progression of breast cancer in humans and permits the analysis of specific gene expression at each distinctive stage of the disease (6-8). The MCF10 cell line was initiated as a mortal cell line from which the two immortal non-tumorigenic derivatives, MCF10A (attached cells) and MCF10F (floating cells), were derived. MCF10A cells were transformed with the addition of *c-Ha-Ras* to yield the tumorigenic, pre-metastatic MCF10AT cell line (9). The MCF10AT cells were injected via the tail vein into severe combined immunocompromised mice to generate two metastatic clones, MCF10CAa.1 and MCF10CAd.1α. The MCF10CAa.1 and MCF10CAd.1α cell lines are two separate variants that display all characteristics of a fully malignant breast cell type (10).

Maspin (*SERPINB5*) is a 42-kDA protein first identified in a screen for potential tumor suppressors that are lost in human breast cancer cells (11). Maspin is related to the serpin family of intracellular and extracellular protease inhibitors; however, maspin does not undergo the conformational change that is typical of members of the serpin family (12, 13). Down-regulation of maspin is an early event in breast tumorigenesis, making maspin expression a well-established diagnostic and prognostic indicator of cancer progression (14). Reintroduction of maspin into tumor cells inhibits growth, cell migration and invasion and angiogenesis. Loss of maspin expression typically leads to an increase in invasive potential and spread of metastatic disease (11). Recent studies suggest that maspin is restricted to an intracellular and possibly nuclear role in which it indirectly influences cell-matrix interactions (13). Thus, while the precise functions as well as cellular location of maspin have not been fully elucidated by researchers, its implications in breast cancer make it a target gene of interest.

Here, we report a study of gene expression and DNA methylation patterns in the maspin promoter of premalignant MCF10AT cells compared to the metastatic clones, MCF10CAa.1 and MCF10CAd.1α. This model allows us to identify discrete epigenetic modifications at each stage of breast tumorigenesis from premalignant breast cell to fully invasive breast cancer cell and attribute these epigenetic changes to phenotypic changes.

Methods

Generation of the MCF10 Progression Model and Cell Culture:

The cell lines studied in this experiment were acquired as a kind gift from Dr. Danny Welch (University of Alabama at Birmingham). Cell lines were generated as described by Miller (9, 15) and Santner (10) and were cultured as described by Welch (16).

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RNA, DNA and Protein Extraction:
RNA, DNA and protein were precipitated as per the manufacturer's protocol from MCF10AT, MCF10CAa.1 and MCF10CAd.1α cell pellets using *Trizol Reagent* (*Invitrogen; Carlsbad, CA*). A stepwise procedure was used to sequentially precipitate RNA, DNA and protein through a series of separatory layers (17). For each sample, four biological replicates were generated. RNA pellets were suspended in 100% deionized formamide and stored at -80°C. Following the final ethanol wash, DNA and protein pellets were stored in 100% ethanol at -20°C.

Quantitative-Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR):
qRT-PCR was used to assess the mRNA expression of maspin. RNA suspended in formamide from the MCF10AT, MCF10CAa.1 and MCF10CAd.1α cell lines was removed from -80°C and repurified using the RNA Cleanup Protocol in the RNeasy Kit (*Qiagen; Valencia, CA*) and then resuspended in RNase-free water. cDNA was generated using cDNA First Strand Synthesis Kit (*Invitrogen; Carlsbad, CA*) from 4 μg of RNA. cDNA was then purified using QIAprep PCR Reaction Cleanup (*Qiagen; Valenica, CA*). Real-Time PCR was carried out on 10 ng of cDNA. mRNA expression of maspin (Hs00985283_m1) was performed using Taqman Assays (*Applied Biosystems; Carlsbad, CA*) on a MiniOpticon Real-time Thermocycler (*Bio-Rad; Hercules, CA*). Specific assay ID is in parentheses. *GAPDH* served as the endogenous control. Expression values for MCF10CAa.1 and MCF10CAd.1α were calculated using the ΔΔCt relative to MCF10AT expression. The efficiency of target amplification was compared between the maspin expression in the MCF10CAa.1 and MCF10AT cells. This was compared to the efficiency of target amplification between the *GAPDH* expression in the MCF10CAa.1 and MCF10AT cells. The following formula was used:

$$\frac{E_{maspin}^{C_{t(MCF10AT)}-C_{t(MCF10CAa.1)}}}{E_{gapdh}^{C_{t(MCF10AT)}-C_{t(MCF10CAa.1)}}}$$

E=Efficiency C_t=Cycles to Threshold

This was also used to analyze the efficiency of target amplification in the MCF10CAd.1α and MCF10AT cells (18).

Western Blot:
Western blotting of protein extracts was used to determine protein expression of maspin. Protein concentration was determined with the 2D-DIGE Quant Kit (*Amersham Biosciences; San Francisco, CA*). Total protein extract (20 μg) was loaded onto a 4-15% Tris-HCl gel and separated by electrophoresis at 100 V until the dye ran off of the gel. The separated proteins were transferred to a nitrocellulose membrane and transferred at 100 V at 4°C. Membranes were blocked in 5% dry milk in Tris buffered saline solution with 0.1% Tween (TBST) overnight. Primary antibody incubations were completed overnight at 4°C using mouse monoclonal antibodies specific to maspin (sc-166260) and actin (sc-1616). The antibodies used in immunoblotting were obtained from Santa Cruz Biotechnology (*Santa Cruz, CA*).

Bisulfite Treatment and DNA Methylation Analysis:
Genomic DNA was prepared as described above using Trizol Reagent for the MCF10AT, MCF10CAd.1α and MCF10CAa.1 cell lines. Two μg of purified genomic DNA were bisulfite treated using the QIAgen Epitect Bisulfite Treatment Kit (*Qiagen; Valencia, CA*). Primers specific for the maspin gene region were used to amplify bisulfite-modified DNA via nested PCR (first round F: 5'-AAAAGAATGGAGATTAGAGTATTTTTT-GTG-3' and R: 5'-CCTAAAATCACAATTATCCTAAAAAA-TA-3') and (second round F: 5'-GAAATTTGTAGTGT-TATTATTATTATA-3' and R: 5'-AAAAACACAAAAAC-CTAAATATAAAAA-3'). PCR products were loaded on a 1.5% agarose gel; bands were purified and extracted using QIAquick Gel Extraction Kit (*Qiagen; Valencia; CA*). Purified promoter DNA generated as described above were cloned into a TOPO-TA Vector (*Invitrogen; Carlsbad, CA*). Plasmids were transformed into TOP10 Chemically Competent Cells (*Invitrogen; Carlsbad, CA*) and plated onto Luria-Bertani agar plates containing 50 μg/mL kanamyocin. Seven individual colonies were selected and used to inoculate 3 mL cultures of LB. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (*Qiagen; Valencia, CA*). Plasmids containing the PCR products were sequenced using a T7 primer and a 3730 DNA Sequencer (*Applied Biosystems; Carlsbad, CA*).

Results

Maspin is down-regulated over the course of metastasis

Protein expression of maspin was qualitatively analyzed in the MCF10A, MCF10AT, MCF10CAa.1 and MCF10CAd.1α cell lines with a Western Blot (Figure 1). Maspin expression was less in the MCF10AT cell line compared to the MCF10A cell line. Maspin expression was undetectable in the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α.

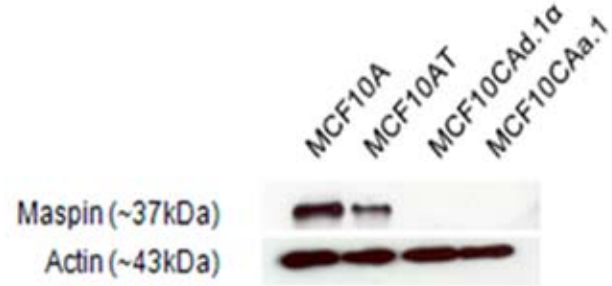

Figure 1. Western blot of maspin protein expression. Maspin expression was less in premalignant MCF10AT cells compared to immortalized MCF10A cells. Maspin expression was undetectable in the metastatic carcinomas (MCF10CAa.1 and MCF10CAd.1α cells). Actin served as the endogenous control.

In order to further validate the results generated from the Western Blot, we qualitatively assessed mRNA levels in the MCF10AT cell lines and the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α (Figure 2). It was especially necessary to quantify the maspin mRNA expression of the metastatic carcinomas due to the lack of maspin protein band in each of those cell lines. Expression ratios for the MCF10CAa.1 and MCF10CAd.1a cells were calculated using the ΔΔCt method relative to the expression of MCF10AT cells. Maspin expression was nearly half in the metastatic carcinomas compared to the premalignant MCF10AT cells.

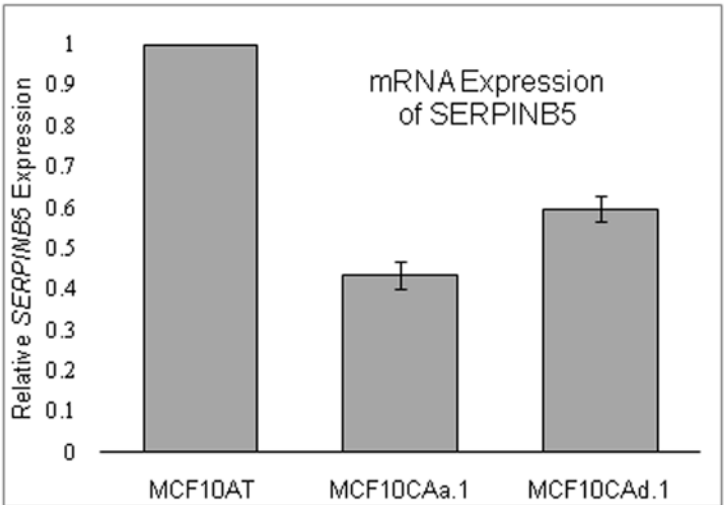

Figure 2. Real-Time PCR analysis of mRNA expression of maspin (SERPINB5) in metastatic progression model. Expression ratios for the MCF10CAa.1 cells and MCF10CAd.1α cells were calculated using the ΔΔCt method relative to the expression of MCF10AT cells (set to 1.0). Error bars represent SEM. GAPDH served as the endogenous control.

The down-regulation of maspin in both the Western Blot and real-time PCR indicate its association with a metastatic phenotype.

No overall DNA methylation in the maspin promoter

DNA from the MCF10AT cell lines and the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α was bisulfite treated and DNA methylation was analyzed in the maspin promoter at CpG dinucleotides (Figure 3). Bisulfite sequencing indicated an overall lack of methylation in the maspin promoter of each of the cell lines, indicating that maspin down-regulation in the metastatic progression model is not attributed to DNA methylation.

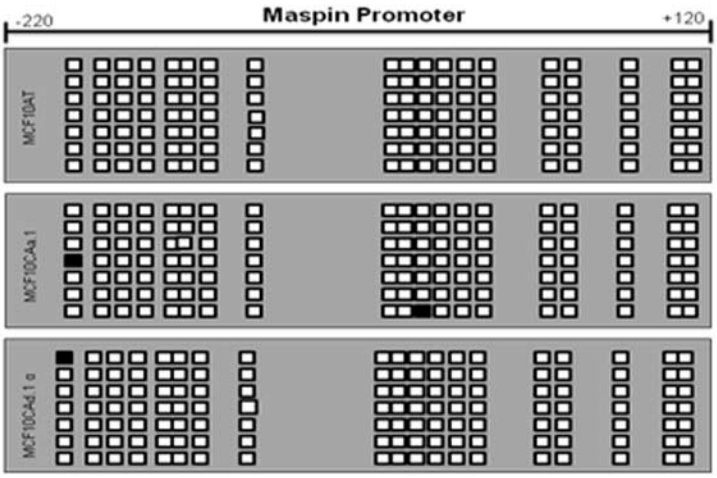

Figure 3. DNA methylation analysis of the promoter region of the maspin gene. Each box represents a CpG dinucleotide. Each column represents a CpG island in the maspin promoter. Individual rows correspond to the methylation pattern of a single colony.

Discussion

Maspin has been shown to inhibit tumor cell motility, invasion in cell culture, tumor growth and metastasis (11). High maspin expression is apparent in normal breast cells but is strongly down-regulated and lost in metastatic cells, indicating maspin's role as a tumor suppressor (21). Loss of maspin expression typically leads to an increase in invasive potential and spread of metastatic disease. Although maspin expression is silenced over the course of tumorigenesis, maspin gene deletions and mutations have not yet been found (11). Thus, a logical hypothesis is that the maspin gene may be silenced by epigenetic modifications, such as aberrant DNA methylation at CpG dinucleotides of the maspin promoter.

After confirming the down-regulation of maspin over the course of metastasis, we analyzed the cytosine methylation status of the 19 CpGs present in the maspin promoter in the MCF10AT cell lines and the metastatic carcinomas, MCF10CAa.1 and MCF10CAd.1α. For each cell line, 7 bacterial clones were bisulfite sequenced. Overall aberrant cytosine methylation was not detected in any of the cell lines studied. No CpGs were methylated in the MCF10AT cells while the MCF10CAd.1α and MCF10CAa.1 had two and one clone with a single methylated site, respectively. These findings indicate that the loss of maspin expression during the course of tumor progression and metastasis in the cell lines studied is not a result of aberrant cytosine methylation in the promoter region of the gene. It is interesting to note that DNA hypomethylation has been demonstrated to play a causal role in tumor formation, possibly by promoting chromosomal instability (22, 23). While this does not provide enough evidence to link the lack of methylation at the maspin promoter to breast cancer metastasis, it certainly provides a solid hypothetical foundation.

It is important to consider that loss of maspin expression has been shown to be closely linked with aberrant DNA methylation in the maspin promoter in other breast cell lines not used in this experiment (14). However, our findings are the first to indicate a lack of DNA methylation and the down-regulation of maspin expression in the MCF10 Breast Cancer Progression Model.

To fully understand other epigenetic modifications that may be associated with maspin gene silencing in breast cancer, it is also possible to assess chromatin accessibility of the maspin promoter of the cell lines in the metastatic progression model. However, the down-regulation of mRNA expression coupled with no protein expression and lack of DNA methylation changes suggests that RNA interference (RNAi) may be controlling the gene activity. Analyzing RNAi in the maspin promoter of the cell lines of the metastatic progression model is a more likely future research direction. In addition, due to complexity of metastasis, further experimentation is necessary to identify other gene targets involved in the malignant progression of breast cancer.

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