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TITLE: Identification of Serine Proteinases Involved in Breast Cancer Progression

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Identification of Serine Proteinases Involved in Breast Cancer Progression

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The objective of the grant was to identify serine proteinases (a) upregulated in malignant breast epithelial T4-2 cells, and (b) for which specific inhibition by RNA interference (RNAi) results in suppression of the malignant phenotype, as assessed in three-dimensional culture assays. We successfully identified five serine proteinases significantly upregulated in T4-2 cells: mesotrypsin, prostasin, transmembrane protease serine 3 (TMPRSS3), neurotrypsin, and matriptase-1. Among these, inhibition of mesotrypsin expression in T4-2 cells by RNAi leads to a significant reduction in colony size in 3D culture, implicating mesotrypsin activity in the T4-2 cell malignant growth phenotype, and identifying mesotrypsin as an oncogenic serine proteinase and a potential therapeutic target.

serine proteinase, 3D cell culture model, breast cancer progression model

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Introduction

Extracellular proteinases are associated with nearly every aspect of breast cancer development – proliferation, invasion, angiogenesis, and metastasis – and have received attention as potential therapeutic targets. While most attention has focused on the matrix metalloproteinases (MMPs) as therapeutic targets, the serine proteinases play key roles in many of the same tumorigenic processes, and may pose more promising targets for treatment of breast cancer. Using a physiologically relevant, 3-dimensional culture system modeling early stages of human breast cancer progression, we discovered that treatment of HMT-3522 T4-2 breast cancer cells with the broad-spectrum serine proteinase inhibitor aprotinin could suppress the malignant phenotype. We hypothesized that specific inhibition of one or more secreted or membrane-associated serine proteinases blocked the malignant phenotype in our breast cancer model, and that this proteinase or group of proteinases might represent a novel and promising target for development of more highly selective molecularly targeted therapeutics for breast cancer treatment. We proposed to identify those serine proteinases for which upregulation correlated with malignancy in the HMT-3522 breast cancer progression series. We would then test the ability of specific inhibition of proteinase expression by RNA interference to attenuate the malignant growth phenotype of T4-2 cells in 3D culture, as a first step towards validating one or more serine proteinases as potential breast cancer drug targets.

Body

To identify specific serine proteinases that contribute to malignant growth in T4-2 cells, we proposed to employ two complementary approaches: (1) screening a panel of likely candidate proteinases for expression levels in malignant T4-2 cells relative to nonmalignant precursor S1 cells, both grown under physiologically relevant 3D conditions, and (2) an affinity-based proteomic approach to identify active proteinases secreted by T4-2 cells based on their affinity to the broad-spectrum serine proteinase inhibitor aprotinin. These two parallel approaches to candidate identification constituted Tasks 1 and 2 of our work plan. Subsequent testing of the effects of specific inhibition by RNAi of individual candidate proteinases, to evaluate contributions to the malignant growth phenotype and identify potential new drug targets for breast cancer, constituted Task 3.

Task 1. Identify by candidate screen approach serine proteinase(s) upregulated in HMT-3522 T4-2 cells relative to S1 cells. T4-2 cells belong to the HMT-3522 human breast cancer progression series(1-3); cell lines in this series, showing varying degrees of progression towards malignancy, originally derive from the normal breast epithelial S1 cell line, and share a common genetic background(4, 5). We hypothesized that the tumorigenic protease(s) of interest would have increased expression and/or activity with progression to malignancy in this cell series. Accordingly, we assessed selected candidate proteases for increased expression in T4-2 cells relative to nonmalignant precursor S1 cells, using real time quantitative RT/PCR techniques. The striking suppression of malignancy in T4-2 cells treated with aprotinin prompted us to begin defining the tumorigenic serine proteinase targets responsible for this effect by looking most closely at trypsin-like proteinases known or expected to be inhibited by aprotinin, as well as any serine proteinases previously reported in association with breast cancer. Aprotinin is known to most potently inhibit trypsins and plasmin(6), so these were initial candidates. Further candidates, gleaned from previous reports of upregulation or correlation with malignancy in breast cancer or relevant models, included urokinase plasminogen activator (uPA)(7), tissue plasminogen activator (tPA)(8), matriptase-1 (MT-SP1)(9, 10), matriptase-2(11), tryptase-γ1(11), and hepatocyte growth factor activator (HGFA)(9, 10). Among these, only two were significantly
upregulated in T4-2 cells relative to S1 cells: mesotrypsin (trypsin 4), and matriptase-1 (Figure 1, unpublished data). In addition to these initially selected candidates, we subsequently obtained several additional candidates through a microarray expression analysis of T4-2 cells vs. S1 cells, in which transmembrane protease serine 3 (TMPRSS3), prostasin, testisin, and neurotrypsin appeared to be upregulated with increasing malignancy. Of these, three gave reproducible confirmation by real time quantitative RT/PCR: TMPRSS3, prostasin, and neurotrypsin (Figure 1, unpublished data).

![Figure 1. Upregulated expression of five serine proteases in T4-2 cells relative to S1 cells (unpublished data).](image)

**Task 2.** Identify by affinity-based proteomic approach secreted and cell surface associated serine proteinase(s) overrepresented in HMT-3522 T4-2 cells relative to S1 cells. We had proposed to employ an affinity selection of protein lysates using aprotinin coupled to a solid support; however, our initial efforts to generate this affinity resin were unsuccessful. Strategies to couple aprotinin to activated resins through primary amine groups resulted in coupling primarily through a lysine side chain located within the proteinase-binding region of this small protein inhibitor; the resultant resin retained very little affinity to bovine trypsin in preliminary method validation experiments. We sought to eliminate this unproductive coupling and favor production of an aprotinin-coupled resin retaining proteinase affinity by producing recombinant aprotinin in which the lysine residue within the proteinase binding region was conservatively mutated to arginine. We obtained a construct for recombinant bacterial expression of aprotinin in inclusion bodies from Dr. Pierre Neuenschwander (University of Texas, Tyler) and successfully introduced the desired Lys-to-Arg mutation. However, while we could successfully express small quantities of the protein in *E. coli*, the oxidative refolding reaction resulted in substantial losses, and we were unable to generate the milligram quantities of pure protein that would be required to produce an affinity column. Recently, we have obtained a new expression construct from Dr. Peter Walsh (Temple University, Philadelphia), for production of the Lys-to-Arg mutant aprotinin in the yeast *Pichia pastoris*, as a correctly-folded, secreted protein. Initial expression studies have been very promising, and although we do not yet have pure protein from a large scale expression, it appears as though this approach may finally lead to a resin that will be useful for our proteomic screen. Our efforts in this direction are ongoing.

**Task 3.** For candidates identified in Task 1 or Task 2, determine whether specific inhibition by RNAi knockdown in the HMT-3522 T4-2/3D culture assay results in phenotypic reversion. Of the five proteinases identified and confirmed to be upregulated in T4-2 cells relative to S1 cells by real time quantitative RT/PCR, mesotrypsin and prostasin showed the greatest expression increases in malignant breast cells (Figure 1). Paradoxically, existing studies on prostasin suggest that this proteinase is actually an inhibitor of invasive growth in prostate and breast cancers(12, 13). Conversely, while relatively little is known about the role of mesotrypsin in
cancer progression, it is implicated in increased cancer invasiveness and poorer survival in non-small cell lung cancer (NSCLC)(14). Selecting mesotrypsin as a prime candidate for further analysis in our breast cancer culture model, we directly tested the ability of specific mesotrypsin inhibition to attenuate the malignant phenotype of T4-2 cells by RNAi knockdown using stable transfection with lentiviral shRNA constructs. The results indicated that shRNA knockdown of mesotrypsin expression in T4-2 cells (Figure 2a) led to a statistically significant reduction in colony size (Figure 2b-e), further implicating mesotrypsin activity in the T4-2 cell malignant growth phenotype (unpublished data).

We have subsequently embarked on similar evaluation of the 3D growth effects on T4-2 cells of shRNA knockdown of prostasin, TMPRSS3, neurotrypsin, and matriptase-1. For each of these genes, we have screened 4-5 targeted shRNA constructs, and identified viruses that reproducibly achieve greater than 80% reduction in expression levels in T4-2 cells, as assessed by real time quantitative RT/PCR. Studies are underway to evaluate the 3D growth phenotypes of knockdown strains, as compared with T4-2 cells transfected with non-target control virus.

**Key Research Accomplishments**

- Identification of mesotrypsin, prostasin, TMPRSS3, neurotrypsin, and matriptase-1 as serine proteinases significantly upregulated in malignant HMT-3522 T4-2 cells relative to nonmalignant precursor S1 cells.
- Validation of mesotrypsin as an active contributing factor in promoting malignancy in the HMT-3522 T4-2 3D cell culture model of breast cancer.
- Confirmation that specific inhibition of mesotrypsin in this model attenuates the malignant phenotype, suggesting mesotrypsin as a potential drug target for development of new breast cancer therapeutics.
**Reportable Outcomes**

- **Funding applied for** based on work supported by this award:
  - DOD CDMRP BCRP Idea Award BC061719 – Identification of new serine protease targets and serine protease inhibitors for breast cancer chemoprevention
  - NIH NCI 1R03-CA128005-01 – New serine protease targets and inhibitors for breast cancer chemoprevention
  - Susan G. Komen Breast Cancer Foundation BCTR0706989 – New approaches to breast cancer therapy using miniprotein serine protease inhibitors (SPIs)
  - NIH NCI 1R01-CA130834-01 – Biochemistry of mesotrypsin, an oncogenic, inhibitor-resistant serine protease
  - Florida Department of Health Bankhead-Coley Cancer Research Program New Investigator Research Grant 07BN-07 - Structural and Mechanistic Studies of Mesotrypsin, an Oncogenic, Inhibitor-resistant Serine Protease

- **Funding awarded** based on work supported by this award:
  - Florida Department of Health Bankhead-Coley Cancer Research Program New Investigator Research Grant 07BN-07 - Structural and Mechanistic Studies of Mesotrypsin, an Oncogenic, Inhibitor-resistant Serine Protease

**Conclusion**

With the resources provided by the BCRP Concept Award, we have identified five candidate serine proteinases for which significant upregulation correlates with malignancy in a physiologically relevant, three-dimensional cell culture model of breast cancer progression. For one of these proteinases, mesotrypsin, we have established a functional role in promoting malignant growth, and have determined that specific inhibition of the expression of this enzyme attenuates the 3D malignant growth phenotype in our breast cancer model. Our findings lay the groundwork for future studies to define the physiological mechanism(s) by which mesotrypsin promotes malignant growth, and to extend the scope of the research to encompass animal models of breast cancer and clinical populations. Mesotrypsin has not previously been associated with breast cancer, but based on the present findings, this proteinase now presents a novel candidate for the development of new molecularly targeted therapies for breast cancer.

**References**

12. L. M. Chen et al., Prostate 48, 93 (Jul 1, 2001).