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A Novel Serine Protease Target for Prevention of Breast Cancer by a Soy Bean-Derived Inhibitor

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This project established the interactions of a novel transmembrane, protease, matriptase, with the chemopreventive Bowman Birk Inhibitor (BBI), compared to its cognate, physiological inhibitor HAI-1. We first established the inhibitory interaction between matriptase and HAI-1, and between matriptase and BBI; we built a 3-D structure of the protease domain of matriptase, based on the homology modeling using the X-ray structure of human thrombin as template. This modeled matriptase structure was used in a structure-based screening of inhibitors. Screening the NCI small compounds database, allowed discovery of bis-benzimidines as potent matriptase inhibitors. We also identified natural trypsin inhibitor, SFTI, from sunflower seed, as a potent inhibitor of matriptase. We found that in non-transformed mammary epithelial cells, matriptase can be activated by lipid phosphates; the activated matriptase is then quickly binds to HAI-1, and is shed into media. Thus, engagement of membrane-bound matriptase with HAI-1 leads to its extracellular shedding. In addition, we identified hepatocyte growth factor and pro-UPA as likely physiological protein substrates of matriptase. Finally, we found that breast cancer cells constitutively activate matriptase, but that cells are not modulated in their proliferation or differentiation by matriptase inhibition in vitro, probably due to the lack of relevant matriptase substrates.

breast cancer

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INTRODUCTION

In 1999, we identified in human breast cancer a new, epithelial-derived, type 2 integral membrane, serine protease, matriptase, and its cognate inhibitor, the hepatocyte growth factor activator inhibitor 1 (HAI-1) \(^4\text{--}^6\). In our research plan for this grant, we proposed to study the inhibitory interactions between matriptase and HAI-1, and between matriptase and the Bowman-Birk Inhibitor (BBI). In the first year, we completed and reported parts of Aims 1 and 2. We reported the purification of matriptase and HAI-1 from human milk, characterization of the enzymatic specificity of matriptase, and characterization of a controllable system for matriptase activation in mammary epithelial cell culture. We also reported on an application of a molecular modeling approach for inhibitor screening. In our second year, we made further progress in this study, completing Aims 1 and 2 with the characterization of two classes of inhibitors of matriptase, and with the identification of sphingosine-1-phosphate (S-1-P) as a likely physiological activator of cellular matriptase. In year 3, we completed Aim 3 with a characterization of the constitutive activation of matrilipase in human breast cancer cells (compared to its S-1-P-regulated activation in non-transformed mammary epithelial cells); we also determined the effects of matriptase inhibitors in human breast cancer cells \textit{in vitro}.

STATEMENT OF WORK

\textit{Aim 1: We will first establish in detail the mechanism and kinetics in solution of inhibition of the 80-kDa protease by the BBI, compared to the Kunitz domain inhibitor (KSPI, HAI-1) we have previously identified. COMPLETED IN YEARS 1 AND 2.}
Aim 2: We will next examine whether the BBI, compared to KSPI (HAI-1) is capable of binding the membrane-bound 80-kDa protease and promoting its cellular internalization (and extracellular shedding). COMPLETED IN YEARS 1 AND 2.

Aim 3: We will test the ability of the BBI to influence proliferation and differentiation through the c-myc system. COMPLETED IN YEAR 3.

BODY
In the period of July, 2001-June, 2002, we completed studies for Aim 3; since this serves a final report, we will also summarize prior findings for Aims 1 and 2.

Aim 1: We will first establish in detail the mechanism and kinetics in solution of inhibition of the 80-kDa protease by the BBI compared to the Kunitz domain inhibitor (KSPI; HAI-1) we have previously identified.

We completed most of this aim and reported our findings in year 1. As described earlier, we utilized molecular modeling, instead of phage display, to optimize inhibitor discovery, based on BBI and HAI1 inhibitory domains. In the period of July 2000-June 2001, we completed these studies and published two manuscripts.

One manuscript “Structure-based approach for the discovery of bis-benzamidines as novel inhibitors of matriptase” was published in the Journal of Medicinal Chemistry. In this paper, the 3D structure of the protease domain of matriptase was first built, with homology modeling, using the X-ray structure of human thrombin as template. The modeled structure of the protease domain of matriptase, by analogy to thrombin, has a catalytic triad positioned on the surface, marked by Ser805, His656, and Asp711, corresponding to Ser195, His57 and Asp102 in
thrombin. Consistent with the observation that matriptase prefers substrates with an Arg or Lys as P1 residue, a negatively charged residue, Asp799, is located at the bottom of the S1 binding site. This modeled structure was then used to screen small compounds in the NCI database. Since the S1 site is considered to be the primary substrate binding site in serine proteases, it is likely to be a good target site for inhibitor design. Also, since the S1 site of matriptase is negatively charged, the potential inhibitor candidates that target this site should be positively charged in water under physiologic conditions. In addition, other two putative binding sites, the anionic site and the hydrophobic S1' site, were included in the docking site used for 3D-database searching with the program DOCK. One group of compounds that we have identified from this screening is bis-benzamidines. As shown in the paper, the 7 bis-benzamidines analogs available from the NCI database have Ki values ranged from 191 nM to greater than 10 μM against matriptase.

In addition to identification of small molecule inhibitors of matriptase, molecular modeling also allowed identification of a naturally occurring, small peptide inhibitor, termed sunflower trypsin inhibitor (SFTI). SFTI was initially purified as an actual product from sunflower seeds. It is a 14-aa backbone-cyclized peptide that is further stabilized by an intramolecular cystine disulfide bond. SFTI has been shown to be a potent and selective inhibitor of trypsin. By using synthetic SFTI peptides, we found that it inhibited matriptase with Ki of 0.92 nM, BBI. As we stated in previous report, the estimated Ki for HAI-1, purified from the human milk, is 1nM. Thus, SFTI appears to inhibit matriptase as efficiently as HAI-1, the cognate inhibitor of matriptase. In addition to its potency, SFTI is also selective. It has little inhibition to thrombin, and no inhibition to uPA. These observations were published in a manuscript entitled:
"Synthesis and evaluation of the sunflower derived trypsin inhibitor as a potent inhibitor of the type II transmembrane serin protease, matriptase", and published in *Bioorganic and Medicinal Chemistry Letters*.

**Aim 2**: We will next examine whether the BBI, compared to KSPI (HAI-1), is capable of binding the membrane-bound 80-kDa protease and promoting its cellular internalization. In year 1, we reported a controllable model for serum-dependent activation of matriptase on non-transformed mammary epithelial cells. During year 2, we identified sphingosine 1 phosphate (S-1-P) as the major blood-borne factor to induce matriptase activation in non-transformed mammary epithelial cells. In this controllable model, we discovered that activated matriptase is quickly bound to HAI-1, and the matriptase-HAI-1 complex is shed into media. These observations suggest that ectodomain shedding is the major way to remove the activated matriptase from cell surfaces. Although these discoveries could not fully exclude the possibility of internalization of matriptase/HAI-1 complex, removal of matriptase by ectodomain shedding appears to account for the fate of the most majority of the inhibitory bound protease, this process could prevent unwanted proteolysis on cell surfaces. Therefore, in this Aim, we emphasized study of the shedding of matriptase and matriptase/HAI-1 complexes, instead of the originally proposed internalization of matriptase (an approved change to our statement of Work). In the course of these studies, we observed that breast cancer cells have developed an autonomous way to activate matriptase and have escaped the control by S-1-P. Up to five-fold increased level of activated matriptase in complex with HAI-1 was shed into media by MDA MB-486 breast cancer cells, compared to non-transformed 184A1N4 mammary epithelial cells, grown in the absence of S-1-P. This discovery was further examined in Aim 3.
Aim 3: we will test the ability of the BBI to influence proliferation and differentiation through the c-Myc system.

We initially hypothesized that matriptase could modulate cell proliferation and differentiation through cleavage of specific substrates. We then identified HGF and pro-uPA as the substrates of matriptase. We showed in our paper “Activation of hepatocyte growth factor and urokinase type plasminogen activator by matriptase, an epithelial membrane serine protease”, published in J. Biol. Chem., that HGF is cleaved into two chains after incubation with active matriptase. This cleaved HGF can stimulate scattering of Madin-Darby canine kidney epithelial cell line and tyrosine phosphorylation of the receptor c-Met in A549 human lung carcinoma cell. Also shown in this publication is the fact that matripatase can proteolytically convert pro-uPA into the active, two-chain form of uPA, that is able to cleave its specific peptide substrate. Matriptase, however, does not cleave plasminogen, despite the high amino acid sequence homology between HGF and plasminogen. Matriptase does not appear to directly cleave other extracellular matrix proteins such as collagens, fibronectin, and laminin (data not shown). However, it is likely that matriptase serves to indirectly cleave these proteins, through its activation of pro-uPA and possibly other proteases.

Based on these observations, we examined the effects of inhibitors of matriptase, such as BBI, on cell proliferation and differentiation. Some prior literature had suggested that BBI could exert such effects in certain non-mammary cells, but in the absence of any information about the expression of matriptase or its substrates. As noted in Aim 2, we found that human breast cancer cells constitutively shed matriptase/HAI-1 complexes into their media, in contrast to immortalized mammary epithelial cells, that required serum (or S-I-P, contained in its
lipoproteins) for this shedding. This led us to demonstrate in our paper "Deregulated activation of matriptase in breast cancer", in press, Clin and Expt'l Met., 2002, that matriptase activation itself was independent of the serum factor in human breast cancer cells, we examined the effect of BBI and other inhibitors of matriptase in several human breast cancer cell lines. Results, not shown, failed to detect any effect of these inhibitors (at matripase-inhibitory concentrations) on cell proliferation or differentiated appearance. To begin to further explore the reason for these negative results in vitro, we examined expression of uPA and HGF/c-Met (matriptase substrates) in our breast cancer cell lines. We found no expression of any of these matriptase substrates, possibly explaining the failure of BBI to exert significant effects on our breast cancer cell lines. We have widened our search for other breast cancer cells that might co-express matriptase with its substrates, but future experiments with BBI and other matriptase inhibitors will prioritize in vivo breast cancer models to ensure the availability of a more complete system for matriptase inhibitor testing.
KEY RESEARCH ACCOMPLISHMENTS:

Over the course of this project, we have:

- Built a modeled structure of protease domain of matriptase, and found it to be suitable in structure-based screening of inhibitors. The model was recently verified by X-ray structure results\textsuperscript{1}.

- Identified a group of bis-benzamidines as potent small compound inhibitors of matriptase.

- Shown that iodo' and bromo' substituents on the benzamidine phenyl rings of these inhibitor compounds can improve their selectivity.

- Developed two bis-benzamidine derivatives that can be used in cell culture systems and possibly \textit{in vivo} systems.

- Shown that SFTI as well as HAI-1 inhibits matriptase activity.

- Shown that SFTI has little to no inhibition of thrombin and uPA.

- Shown that activation of matriptase in non-transformed mammary epithelial cells can be stimulated by S-1-P, resulting in extracellular shedding, rather than intracellular internalization.

- Found that matriptase in breast cancer cells is present primarily in its activated forms. These cells do not respond to S-1-P stimulation, and cells constitutively release the matriptase complexes into their extracellular media.

- Identified HGF and pro-uPA as biological substrates of matriptase.
• Found that BBI has little effect \textit{in vitro} on proliferation and differentiation of human breast cancer cells, which express activated matriptase, but not the uPA, HGF/c-Met matrisplase substrate systems.

**REPORTABLE OUTCOMES:**

**ABSTRACTS:**

Oberst, M., Johnson, M., Anders. J., Dickson, R. B., and Lin, C.-Y., 2000, Full length cloning of the serine protease matriptase and characterization of its expression in breast cancer primary tumors, metastasis, and cell lines. AARC, 91\textsuperscript{st} Annual Meeting. San Francisco, CA

Lee, S.-L., Dickson, R. B., and Lin, C.-Y., 2000, Biological function of matriptase, a novel trypsin-like protease. FACS/The Salk Institute for biological studies. 16\textsuperscript{th} Annual Meeting on Oncogenes and tumor suppressor. La Jolla, CA


**PUBLICATIONS:**


CONCLUSION:

In summary, we conducted a molecular modeling approach, building a 3-D structure model of the protease domain of matriptase, based on the homology modeling with the X-ray structure of human thrombin as template. Using the modeled matriptase structure, we developed bis-benzamidines, as potent and selective matriptase inhibitors. Using the same molecular modeling approach, we also found that the BBI-related, natural trypsin inhibitor, SFTI, from sunflower, as well as its endogenous inhibitor HAI-1 were likely to interact tightly with the catalytic site of matriptase. Subsequent biochemical experiments confirmed that all 3 compounds inhibited matriptase, each with Ki values in the sub-nanomolar range. These results emphasized that molecular modeling is capable of being used in searching for an inhibitor of a protein, without an established x-ray structure; the model was recently validated by the published X-ray structure⁴花瓣。
From our studies, this approach resulted in identification of potent inhibitors that have the potential to be used with in vivo breast cancer models, and possibly in clinical application. We showed that matriptase can be activated in normal breast cells by S-1-P; but matriptase is constitutively activated in breast cancer cells, independent of exogenous S-1-P. We have found that matriptase is a potent activator of HGF and pro-uPA, but not of plasminogen; matriptase does not appear to directly cleave extracellular matrix proteins. These results suggest that matriptase may be an important upstream activator in cancer cell pericellular proteolysis and migration. These studies may result in a new therapeutic approach to breast cancer. However, we found that most breast cancer cells in culture do not express matriptase substrates, and therefore do not respond to matriptase inhibitors, such as BBI. Future studies with appropriate in vivo models will be required to pursue matriptase as a target of breast cancer therapy.
REFERENCES


APPENDICES


Bicyclic Peptide Inhibitors of an Epithelial Cell-Derived Transmembrane Protease, Matriptase

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Introduction
Matriptase is a recently identified type II transmembrane protease that is found on the surface of epithelial cells [1,2]. It is overexpressed in most cancer cells, including human breast cancer cells. It is a multidomain protein with a C-terminal extracellular region containing the protease domain. It activates urokinase-type of plasminogen activator (uPA), and the protease activated receptor (Par-2). It has been demonstrated that matriptase can proteolytically activate hepatocyte growth factor (HGF/Scatter Factor) to its active form, and thus it may function in epithelial cell migration, cancer invasion and metastasis [2]. Developing inhibitors of matriptase provides for a therapeutic approach to metastatic diseases, and in particular, cancer.

We have chosen the recently isolated natural product, SFTI-1 (Sunflower Trypsin Inhibitor-1) [3], as a prototype inhibitor of matriptase (Figure 1). The structure of SFTI-1 was elucidated using X-ray crystallography. It is a 14 amino acid long backbone cyclized peptide, bisected with a single cystine disulfide bridge. It was reported to possess very potent inhibitory activity to serine proteases [3]. We report here the synthesis and further characterization of this inhibitor for its inhibitory effectiveness on matriptase.

Results and Discussion
SFTI-1 was synthesized using Fmoc chemistry based protocols. The linear peptide was assembled on a Rink acid resin. Gly is attached as the first amino acid on the resin, in order to avoid racemization during the synthesis. The side chain protected peptide was cleaved from the resin with 2% TFA, and the peptide was cyclized with HATU/HOAt/DIEA/DMF. The protective groups were removed with TES–TFA–H₂O. The bicyclic peptide, SFTI-1, was generated by air oxidation of the two cysteines in weakly basic medium. This approach also provided us the methodology to prepare a variety of analogs for SAR studies. Enzyme inhibitory assays were carried out with

![Fig. 1. Structure of SFTI-1.](image-url)
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matriptase proteolysis assay using the fluorescent substrate, N-t-Boc-Gln-Ala-Arg-AMC [2]. \(K_i\) values were determined using Dixon plots (Table 1).

Results show that the synthetic SFTI-1 inhibited matriptase at 1 nM concentration, and it was equally inhibitory to trypsin. It was much less potent against the serum protein, thrombin, and essentially non-inhibitory to uPA [4].

**Table 1. Protease inhibitory properties of SFTI-1.**

<table>
<thead>
<tr>
<th>Protease</th>
<th>(K_i) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matriptase</td>
<td>0.92</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1.06</td>
</tr>
<tr>
<td>Thrombin</td>
<td>5 050</td>
</tr>
<tr>
<td>uPA</td>
<td>500 000</td>
</tr>
</tbody>
</table>

In order to establish a rational basis for selective inhibitor design, we carried out homology modeling to build the 3D structure of matriptase complexed with SFTI-1. Matriptase has a 34% amino acid identity and 53% similarity to thrombin. In addition, the X-ray structure of SFTI-1 complexed with trypsin was available for the initial homology modeling [3]. This structural model suggests that the two basic amino acids, Lys\(^5\) and Arg\(^2\) of SFTI-1 provide important interactions, within the catalytic cavity of matriptase. Lys\(^5\) of SFTI-1 binds to the S1 pocket of the enzyme. Arg\(^2\) forms an H-bond with the main chain carbonyl of Phe706, and interacts with Phe708 and Phe706 sidechains of matriptase through \(\pi\)-cation interactions. Since the homology modeling based distance geometries are taken only as approximate, SAR studies will be performed to optimize the effectiveness of Lys\(^5\) and Arg\(^2\) sidechains, in particular. Efforts are also focused on replacing the cystine disulfide linkage of the peptide with redox stable thioether and olefinic bisecting bridges.

SFTI-1 is a rigid molecule with an extensive network of intramolecular hydrogen bonds. It is proteolytically stable on account of its rigid architecture, and this makes it well suited for evaluation in cells for inhibitory effectiveness on the activation of the latent form of HGF. SFTI-1 and its analogs may well be suitable for inhibitory studies of metastatic tumors in animal models also.

**Acknowledgments**

We thank for the support by a Department of Defense Fellowship DAMD 17-00-1-0269, to S.-L. Lee.

**References**

DEREGULATED ACTIVATION OF MATRIP'TASE IN BREAST CANCER CELLS

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Running head: Activation of matriptase in breast cancer cells

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Key words: actin filament, breast cancer cells, EGF, HAI-1, mammary epithelial cells, matriptase, shingosine 1-phosphate
Abstract:

Matriptase is an epithelial-derived, cell surface serine protease. This protease activates hepatocyte growth factor (HGF) and urokinase plasminogen activator (uPA), two proteins thought to be involved in the growth and motility of cancer cells, particularly carcinomas, and in the vascularization of tumors. Thus, matriptase may play an important role in the progression of carcinomas, such as breast cancer. We examined the regulation of activation of matriptase in human breast cancer cells, in comparison to non-transformed mammary epithelial cells 184A1N4 and MCF-10A. Results clearly indicated that unlike non-transformed mammary epithelial cells, breast cancer cells do not respond to the known activators of matriptase, serum and sphingosine 1-phosphate (S1P). Similar levels of activated matriptase were detected in breast cancer cells, grown in the presence or absence of S1P. However, up to 5-fold higher levels of activated matriptase were detected in the conditioned media from the cancer cells grown in the absence of serum and S1P, when compared to non-transformed mammary epithelial cells. S1P also induces formation of cortical actin structures in non-transformed cells, but not in breast cancer cells. These results show that in non-transformed cells, S1P induces a rearrangement of the actin cytoskeleton and stimulates proteolytic activity on cell surfaces. In contrast, S1P treatment of breast cancer cells does not activate matriptase, and instead these cells constitutively activate the protease. In addition, breast cancer cells respond differently to S1P in terms of the regulation of actin cytoskeletal structures. Matriptase and its cognate inhibitor, HGF activator inhibitor 1 (HAI-1) colocalize on the cell periphery of breast cancer cells and form stable complexes in the extracellular milieu, suggesting that the inhibitor serves to prevent undesired proteolysis in these cells.
Finally, we demonstrate that treatment of T-47D cells with epidermal growth factor (EGF), which promotes cell ruffling, stimulates increased accumulation of activated matriptase at the sites of membrane ruffling, suggesting a possible functional role at these sites.
Abbreviations

The abbreviations used are: EGF, epidermal growth factor; FBS, fetal bovine serum; HAI-1, hepatocyte growth factor activator inhibitor-1; HGF, hepatocyte growth factor; IMEM, Iscove’s minimal essential medium; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; uPA, urokinase plasminogen activator.
Introduction:

Matriptase [1] (also called MT-SP1 [2], and TADG-15 [3] in humans and epithin in mice [4]) is a serine protease, which belongs to the type II integral membrane, serine protease (TTSP) family [5]. Like other TTSP family members, matriptase is composed of multiple domains. The protease contains a putative transmembrane domain at the amino terminus, a serine protease domain at the carboxyl terminus, and two tandem CUB domains and four tandem LDL receptor A domains in between. Expression of matriptase is mainly associated with epithelial cells and epithelial-derived cancer cells, both in vitro and in vivo [6]. Matriptase could play an important role in cellular invasion by serving as an activator, on the surfaces of epithelial and cancer cells, of a stromal-derived, ECM-degrading protease, the urokinase plasminogen activator (uPA), and hepatocyte growth factor (HGF)/scatter factor (SF), a cell growth/motility factor [7;8].

The proteolytic activity of matriptase, on the surfaces of non-malignant epithelial cells, is tightly regulated. Matriptase is rapidly and transiently activated on the surface of these cells by exposure to serum [9], or to the serum-derived sphingosine 1-phosphate (S1P)[10]. In addition, S1P simultaneously causes a rapid rearrangement of actin filaments in 184 A1N4 mammary epithelial cells, to form peripheral, cortical actin structures. Activated matriptase subsequently binds to its cognate inhibitor, the hepatocyte growth factor activator inhibitor 1 (HAI-1), to form a 120-kDa matriptase/HAI-1 complex on the surface of cells [9;10]. Formation of the matriptase/HAI-1 complex may serve an important role in preventing excessive and/or unwanted proteolytic activity. Removal of the matriptase/HAI-complex from cell
surfaces occurs by ectodomain shedding, resulting in the appearance of 110- and 95-kDa matriptase/HAI-complexes in cell culture media [11]. This shedding also results in a reduction in the concentration of total matriptase on cell surfaces, during the activation stimulus. Matriptase is then resynthesized and replenished on the cell surface to its initial level, upon cessation of the activation process [9]. Therefore, in non-transformed mammary epithelial cells, the activity of matriptase is under tight control. Matriptase is rapidly activated in response to a physiological stimulus, such as exposure to serum. After depletion of the stimulus, the proteolytic activity is rapidly terminated by binding to HAI-1, and activated matriptase is removed from the cell surface by shedding of the protease/inhibitor complex. This activation and removal process also apparently occurs in vivo, particularly in the lactating mammary gland, since the activated form of matriptase, complexed with HAI-1, is present in human milk [12].

Although western blotting demonstrated a much higher tissue concentration of matriptase in primary human breast tumors than in surrounding tissues, this observation undoubtedly reflects the higher cellularity in breast tumors than in surrounding tissues [6]. In fact, by immunohistochemistry, matriptase protein levels were similar, comparing normal ductal breast cells and nearby breast cancer cells. Therefore, it does not appear that breast cancer cells significantly increase their expression of matriptase per se to promote malignant behavior. Interestingly, in immortalized mammary epithelial cells, activation and shedding of matriptase mainly depends on the presence of activators, such as S1P [9;10]. In contrast, we have observed activated matriptase in serum-free conditioned media from breast cancer cells, grown in the absence of S1P or serum [11;13]. Thus, we propose that cancer cells deregulate matriptase, downstream of its
expression, at the level of activation of the protease. In the current study, we compared mammary epithelial cells to breast cancer cells in terms of their response to S1P. Our results indicate that breast cancer cells constitutively activate matriptase, regardless of the presence of S1P, and they differentially respond to S1P in terms of actin cytoskeletal rearrangement, compared to immortalized mammary epithelial cells. In addition, treatment of breast cancer cells with EGF, a major regulator of actin filaments, causes accumulation of activated matriptase on cell ruffles.
Materials and methods:

Materials—Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) were purchased from Avanti (Alabaster, AL). EGF was from BD Biosciences (Bedford, MA). Fetal bovine serum (FBS) and modified Iscove’s minimal essential medium (IMEM) were from Invitrogen, Gibco (Rockville, MD).

Tissue culture and western blotting- Immortalized human mammary epithelial cells, 184 A1N4 (provided by Martha Stampfer, Berkeley, CA) and MCF-10A were maintained as previously described [9]. The human cancer cells MCF-7, T47D, SkBr3 and MDA MB-468 were grown in IMEM containing 5% FBS. Cells were maintained for 72 hrs in 2% FBS, and then incubated for the indicated times with either IMEM or IMEM containing S1P.

For the detection of matriptase in whole cell lysates, cells were lysed in 1% Triton X-100/PBS. Protein concentration was measured with the BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of whole cell lysates were resolved by SDS-PAGE, under nonboiled and nonreduced conditions. Activated matriptase and total matriptase were detected by western blotting, using monoclonal antibodies M69 (activated matriptase) and M32 (total matriptase) [9], followed by a goat anti-mouse HRP linked secondary antibody (Biorad, Hercules, CA).

For detection of matriptase in the conditioned medium, 5x10^6 cells for each cell line were seeded in 150 mm dishes overnight. Cells were then washed with PBS and maintained in serum- free IMEM for 8 hrs. The media were removed, and replaced by 15 ml of new serum- free IMEM. The conditioned media were collected after 24 hrs, and concentrated to 0.5 ml. Equal volumes of concentrated condition media were resolved by SDS-PAGE. Alternatively, cells present in each plate were lysed in 1% Triton X-100 in PBS, and the total protein
concentration determined (as above). The volume of conditioned media loaded for western blotting was then normalized to the total amount of protein from the corresponding plate. The western blot results were analyzed by densitometry (ChemiImager 5500, Alpha Innotech Corporation).

*Labeling mAbs with fluorescent dyes*—To label mAbs with fluorescent dyes, 1 mg of each mAb, including M32 and M19, were solubilized in 0.1 M sodium bicarbonate, and then labeled with Alexa Fluor® 488 or Alexa Fluor® 594 (Molecular Probes, Eugene OR), based on the manufacture’s instructions. The unbound dyes were removed by dialysis against PBS. The ratios of mole dye per mole IgG were determined to be 2.5-3.5 for Alexa Fluor® 488 and 5-6 for Alexa Fluor® 594.

*Immunofluorescence*—Cells were fixed in 2% paraformaldehyde in PBS for 10 min at room temperature. Activated matriptase was detected, using monoclonal antibody M69, followed by a goat anti-mouse FITC labeled secondary antibody (Jackson Immunoresearch, West Grove PA). For double labeling of matriptase and HAI-1, cells were fixed in 2% paraformaldehyde in PBS, and matriptase was visualized with Alexa Fluor 488-labeled mAb M32, and HAI-1 was visualized with Alexa Fluor 594-labeled mAb M19. For double labeling of matriptase and actin, cells were fixed in 2% paraformaldehyde, and permeabilized with 0.05% Triton X-100 in PBS; matriptase was visualized with Alexa Fluor 488-labeled mAb M32, and actin was visualized with Texas red-conjugated phalloidin (Molecular Probes, Eugene, OR). Cells were mounted, using Prolong Antifade (Molecular Probes), and observed on an Olympus IX70 confocal microscope or a Nikon Eclipse E600 digital microscope. The images were captured
using the Olympus Fluoview system for the confocal microscope and the Metavue software package for the Nikon digital microscope.
Results:

Deregulation of matriptase activation in human breast cancer cell lines

Matriptase, and its cognate inhibitor, HAI-1, are expressed both in the non-tumorigenic, immortalized human mammary epithelial cell lines, 184 A1N4 and MCF-10A, and in the breast cancer cell lines MCF-7, T47D, SkBr3, and MDA MB-468 [6]. Using an unique monoclonal antibody (M69), that specifically recognizes an epitope present only in the activated, two-chain form of matriptase, we have recently shown that the activation of matriptase is tightly regulated in non-transformed human mammary epithelial cells [9]. Only very low levels of the activated form of matriptase can be detected in serum-deprived 184 A1N4 cells. Treatment of cells with serum or S1P, induces the rapid, transient activation of matriptase [9;10]. We thus set out to examine whether the activation of matriptase is similarly regulated in breast cancer cell lines.

Consistent with our previous report on 184 A1N4 cells [10] (Fig. 1), the independently derived, immortalized, non-tumorigenic MCF-10A cell line also displayed very low levels of activated matriptase under serum starved conditions (Fig. 1) or in lipid-depleted, charcoal stripped serum (data not shown). Furthermore, treatment of both 184A1N4 and MCF-10A with S1P induced a strong activation of matriptase (Fig. 1). In contrast, a panel of breast cancer cell lines, expressing matriptase (MCF-7, T-47D, and MDA MB-468 cells), all maintained a constant level of activated matriptase under the different culture conditions (Fig. 1). Serum starvation did not lead to a decrease in the level of activated matriptase in whole cell lysates. Furthermore, neither S1P treatment (Fig. 1 and 2), nor LPA nor serum stimulation (data not shown), induced an increase in levels of activated matriptase. In both immortalized mammary epithelial cells and breast
cancer cells, the activated form of matriptase was present in its non-complexed 70 kDa form, and in a 120 kDa complex with the intact 55 kDa membrane-bound form of its cognate inhibitor, HAI-1 (Fig.1) [9;10;12]. The ratio between the HAI-1-complexed and non-complexed forms of activated matriptase varied from experiment to experiment, for immortalized mammary epithelial cells. The basis for this variation requires further study. Although the ratio between the HAI-1-complexed and non-complexed forms of activated matriptase also varied in breast cancer cells, we consistently observed a predominance of activated matriptase in its inhibitor-free form, as represented in Figure 1. These results indicate that, in contrast to the non-tumorigenic cell lines, where the activation of matriptase is under tight regulation, human breast cancer cell lines lose the S1P-dependent regulation of matriptase activation. Instead, they constitutively express activated matriptase, even under serum-free conditions.

**Increased shedding of activated matriptase by breast cancer cells**

In 184A1N4 cells, the activity of matriptase is regulated by a tightly controlled mechanism, whereby matriptase activity is switched on upon the presence of S1P and is then switched off by the binding of its cognate inhibitor and the removal of the protease/inhibitor complex from the surfaces of cells [9]. Therefore, the amount of activated matriptase, detected in cell lysates, may only reflect a portion of the total amount of matriptase which has been activated. Since breast cancer cells displayed constitutive expression of activated matriptase, we speculated that the tumor cells may also continuously activate and release activated matriptase into their culture medium. Thus, the levels of activated matriptase, detected in total cell lysates, may be an underestimation of the actual level of activated matriptase produced by the tumor cells.
We have further examined and compared the levels of activated matriptase in the conditioned media among immortalized breast epithelial cells and breast cancer cells (Fig. 2). As expected, only low levels of matriptase were released from 184 A1N4 cells under serum-free conditions. S1P treatment of 184 A1N4 cells significantly increased the amount of matriptase detected in the cell-conditioned media (Fig. 2A). However, when compared to 184A1N4 cells, breast cancer cells shed higher levels of total matriptase into the conditioned media, under both serum-starved and S1P-stimulated conditions (Fig 2A). Furthermore, in breast cancer cells, S1P treatment did not increase matriptase shedding into the culture media (Fig. 2A). Significantly higher levels of activated matriptase were also detectable in the media of breast cancer cells, compared to those in the conditioned media of immortalized mammary epithelial cells, independent of S1P (Fig. 2B). The activated matriptase, detected in the media by SDS-PAGE, migrated as a 95 kDa species and a 110 kDa species (Fig. 2 A and B), corresponding to activated matriptase, complexed to a 40 kDa fragment and to a 50 kDa fragment of HAI-1, respectively [9;12].

The differences observed in the amount of matriptase present in the media could potentially reflect, in part, a difference in cell number, possibly due to a faster proliferation rate of tumor cells, rather than an increase in the activation and the shedding of the protease. To address this possibility, we quantified, by densitometry, the western blot signal for matriptase, derived from equal amounts of conditioned media, and then we normalized these results to the amount of protein from total cell lysates for each plate (Fig. 2C). In a representative experiment, MDA MB-468 released into the media 5-fold more activated matriptase than 184A1N4 cells in the absence of S1P stimulation, and
MCF-7 and SkBr3 released 2 to 2.5-fold more activated matriptase. In addition, a higher proportion of the matriptase released by tumor cells represented the activated, two-chain form, as compared to the proportion in media from immortalized cells. Whereas only 20% of the matriptase released by the A1N4 cells was in its activated form, 46% of matriptase released by the MDA MB-468, 31% by MCF-7 and 35% by SkBr3 cells, was activated. The accumulation of activated matriptase in the conditioned media of tumor cells strongly suggests that, in contrast to the immortalized mammary epithelial cells, matriptase activation is a constitutive process in tumor cells, and that a significantly higher proportion of matriptase expressed by the cancer cells has been activated.

Localization of activated matriptase in human breast cancer cells— We have further examined the localization of activated matriptase, by immunofluorescence staining, using anti-two-chain matriptase mAb M69 (Fig. 3). Consistent with our previous studies [9], activated matriptase was detected uniformly on the surfaces of 184 A1N4 cells, only upon treatment with S1P (Fig. 3) [10]. In contrast, as our western blot results predicted, activated matriptase was detected by immunofluorescence staining at the surface of serum-starved breast cancer cells, including T-47D, MDA MB-468, and MCF-7 (Fig. 3). The distribution of activated matriptase, however, varied on individual cancer cell lines. In T-47D breast cancer cells, activated matriptase was detected on the periphery of cells. In MDA MB-468 cells, activated matriptase was also located at on the periphery of some cells; however, in other cells, a more diffuse localization was observed. In MCF-7 cells, activated matriptase localized in a diffuse pattern consistent with a cell surface localization in flatter cells or a cytosolic localization; localization at cell-cell borders was also observed in some MCF-7 cells. The levels of activated
matriptase, determined by immunofluorescence staining, on these cancer cells showed no obvious changes after S1P treatment (data not shown), consistent with the observation by immunoblot (Fig. 1).

Differential response to S1P in actin skeleton rearrangement, comparing immortalized breast epithelial cells to breast cancer cells— In mammary epithelial cells, S1P induced dramatic actin cytoskeletal rearrangement, in addition to the activation of matriptase (Fig. 4). After serum starvation, 184 A1N4 cells appeared to be more elongated, they lacked close cell-cell contacts, and they had fine actin filaments. S1P treatment resulted in a rapid and dramatic changes in cellular morphology (more square-shaped), associated with an increase in cell-cell contacts, and the accumulation of actin filaments around the cortical rings and cell-cell junctions (Fig. 4). In contrast to the immortalized mammary epithelial cells, human breast cancer cells, including T-47D and MDA MB-468 showed no responses to S1P treatment, in terms of cellular morphology, cell-cell contact, and actin cytoskeletal rearrangement (Fig. 4). The presence of lamellipodia, with a concentration of actin, was observed in T-47D cells and MDA MB-468 cells, grown both in the absence and presence of S1P. Instead, in MCF-7 cells, S1P induced the formation of actin stress fibers and spiky extensions at cell peripheries. S1P treatment did not result in the formation of cortical actin structures, in any of breast cancer cells, similar to those observed in 184A1N4. These observations suggest that the organization of actin in mammary epithelial cells is directly regulated by S1P, as is matriptase activation. In contrast, the effect of S1P in breast cancer cells was different from 184A1N4 immortalized mammary epithelial cells; the actin structure was not
affected by S1P in T-47D nor MDA MB 468; in the case of MCF 7, S1P treatment resulted in a different actin organization than the one observed in A1N4.

**Colocalization of matriptase and HAI-1 on the plasma membrane**

HAI-1, a transmembrane, Kunitz-type serine protease inhibitor, was initially identified in the conditioned media of gastric cancer cells, as one of the two inhibitors of a blood-borne protease, HGF activator (HGFA) [14,15]. Independently, HAI-1 was isolated from human milk in a complex with matriptase [12]. Both matriptase and HAI-1 contain putative transmembrane domains, and both are coexpressed in breast cancer cells, indicating a close functional link between these two proteins. The presence of HAI-1 may provide a mechanism, whereby unwanted and prolonged proteolysis on the cell surfaces can be prevented. In mammary epithelial cells, both binding of HAI-1 to activated matriptase and shedding of the matriptase/HAI-1 complexes closely follow the activation of matriptase induced by S1P, present in serum [9]. In Figure 2, we observed that activated matriptase, in the conditioned media of breast cancer cells, was mainly detected in complexed forms, indicating a similar role of HAI-1 in preventing unwanted proteolysis in these cells. Using T-47D breast cancer cells as model system, we have further examined the localization of HAI-1 and matriptase on the surface of cells. In Figure 5, colocalization of matriptase and HAI-1 in T-47D breast cancer cells was carried out by dual labeling, using fluorescent dyes, directly coupled to anti-matriptase and anti-HAI-1 mAbs. These experiments revealed that matriptase and HAI-1 are colocalized on the cell peripheries, at cell-cell contacts, and at membrane ruffles. The colocalization on cell surfaces (Fig. 5, A, B, and C), and the formation of stable complexes, in conditioned
media, (Fig. 2) of matriptase and HAI-1 suggest that HAI-1 plays similar roles in breast cancer cells and in mammary epithelial cells, preventing the unwanted proteolysis by matriptase. In fact, the colocalization of HAI-1 with matriptase and the potent inhibitory activity of HAI-1 against matriptase could be the main reasons for the observation that the functional form of matriptase (the noncomplexed, activated matriptase) only represents a very small proportion of the activated matriptase under steady state conditions. Dynamic regulation of matriptase activity, via its constitutive activation of matriptase, its rapid inhibition by HAI-1, and the removal of matriptase-HAI-1 complexes from cell surface (by ectodomain shedding) reveals that this proteolytic activity is under a close surveillance and control even in cancer cells.

The localization of matriptase at the membrane ruffles was further confirmed by dual labeling, using a fluorescent dye coupled anti-matriptase mAb, and Texas Red-labeled phalloidin (Fig. 5 D, E, and F). Both matriptase and actin were observed to be at the membrane ruffles and some cell-cell contacts (Fig. 5 D, E, and F).

*EGF treatment induces an accumulation of activated matriptase at cell ruffles*-- Localized proteolysis at the migrating edges of invading cells has been thought to be an important element for cancer invasion and metastasis. Actin recruitment and polymerization occurs at migrating edges of cells. This dynamic of actin and the formation of membrane ruffles can be regulated by various growth factors, including EGF [16]. The colocalization of matriptase with F-actin at the site of membrane ruffles, in T-47D breast cancer cells (Fig. 5), suggests that EGF may influence the dynamic movement of the protease on the cell surfaces. Therefore, we set out to investigate whether EGF affects the distribution of matriptase, in particular, activated matriptase, on
the surfaces of cells. In serum-starved T-47D breast cancer cells, uniform and diffuse localization of activated matriptase and actin filaments was observed on the periphery of T-47D breast cancer cells. EGF stimulation of the cells, within 10 min, induced the formation of membrane ruffles and the accumulation of activated matriptase at the site of lamelopodia actin polymerization. This result suggests that EGF affects the distribution of matriptase, in addition to the distribution of lamelopodia formation in T-47D breast cancer cells.
Discussion

Proteases have been implicated in cancer progression and metastasis. The increase in proteolytic activity, present in cancer, can arise from the deregulation of protease expression, protease activation, or an imbalance in the expression of proteases relative to their endogenous inhibitors. In cultured breast cancer cells, elevated expression of proteases, such as uPA and members of the MMP family, correlates with invasiveness [17-20]. Furthermore, elevated levels of uPA and MMPs, in lysates of human breast cancer, are correlated with poor prognosis [21;22]. Tissue inhibitors of MMPs (TIMPs) exhibit anti-invasion and metastasis activity in various in vivo and in vitro model systems [23-26], and this anti-invasion and anti-metastasis activity of TIMPs could partially result from an imbalance in the expression of MMPs, relative to the TIMPs. In this study, despite comparable levels of matriptase between mammary epithelial cells and breast cancer cells in vitro and in vivo [6], we have shown that, in breast cancer cells, deregulation of matriptase lies post-translationally, at the level of its activation. Our results suggest that a critical regulatory mechanism for the activation of matriptase may be lost during breast cancer progression. Cultured human breast cancer cells constitutively activate matriptase, even when deprived of the known activators of matriptase, such as S1P, or serum. In contrast, the expression level of “total” matriptase (activated plus latent) appears to be similar, when cancer cells are compared to surrounding mammary epithelium in immunohistochemical studies [6]. Therefore, these results suggest that mammary carcinoma cells alter the pathway(s) that normally regulates the activity of matriptase, and instead constitutively activate this serine protease.
The role of S1P in the formation of cortical actin structures and in cell adherent junctions has been demonstrated in HUVEC endothelial cells [27]. A similar activity of S1P on the formation of cortical actin structures was also observed in mammary epithelial cells (Fig. 4). In HUVEC cells, the bioactivity of S1P is mainly mediated by the G-protein coupled receptors, Edg-1 and Edg-3, and by the small GTPases, Rho and Rac; both also regulate cell-cell adhesion in epithelial cells [28;29;30]. S1P simultaneously induces the formation of cortical actin structures and the activation of matriptase in mammary epithelial cells [9;10]. While the relationship between the formation of cortical actin and matriptase activation remains to be further investigated, the formation of cortical actin profoundly changes the cellular morphology and cell-cell contacts for mammary epithelial cells. This may lead to a redistribution of matriptase, including activate matriptase, to cell surfaces and cell-cell contacts. This relocalization of activated matriptase may be crucial for its function. The cortical actin structure may contribute to multiple, but currently uncertain, aspects of the biology of these cells. Activated matriptase on the surfaces of epithelial cells may serve as an activator of growth factors, such as HGF and other proteases, such as uPA, which could further initiate the activation of many other proteases and growth factors [7;8]. Therefore, it is of interest, in future studies, to determine if S1P regulates the differentiation, proliferation and survival of mammary epithelial cells via both matriptase activation and rearrangement of actin filaments.

In contrast, in breast cancer cells, low nanomolar concentrations of S1P fail to induce the formation of cortical actin structures (Fig. 4), which requires both Edg-1 and Edg-3 receptors in HUVEC cells [27]. The lack of response to S1P for the formation of
cortical actin structures in breast cancer cells may partially result from the lack of expression of the Edg-1 receptor [31]. Aberrant regulation of Rho-protein activity in breast tumors may result from overexpression of Rho A, Rho C, Rac 1, and CDC 42 [32;33], and this aberrant activity of small GTPases may enable cancer cells to proliferate without appropriate physical conditions. In analogy with a failure to form cortical actin, breast cancer cells express similar levels of activated matriptase, regardless of the presence of S1P. This can be explained either by an absence of response of breast cancer cells to S1P, or by the higher basal level of activated matriptase that masks any additional small increase in matriptase activation. In both cases, constitutive activation of matriptase should occur in breast cancer cells, because the accumulation of activated matriptase was observed in culture media. In general, serine proteases require other proteases for their activation. The constitutive activation of matriptase in breast cancer cells could result from such a constitutively active activator, which may require S1P for its activity in mammary epithelial cells. Alternatively, matriptase could be activated by a trans-activation mechanism, whereby one matriptase molecule activates another one. If this is a case, both mammary epithelial cells and breast cancer cells may activate matriptase by the same mechanism. Thus, S1P may simply function to create proper microenvironments on the surfaces of mammary epithelial cells, to facilitate transactivation. The formation of cortical actin structures, resulting in profound changes on cell surfaces, may contribute to the creation of these microenvironments. In contrast, the pro-activation microenvironments probably may exist on the surfaces of breast cancer cells in the absence of S1P, resulting in constitutive activation of matriptase. While the mechanism for matriptase activation and the role of S1P in matriptase activation remain
to be further investigated, the constitutive activation of matriptase may increase the proteolytic activity on the surfaces of breast cancer cells. This increased matriptase activity may further enhance the activation of growth factors and other proteases, promoting various aspects of malignancy in breast cancer cells.

We detected activated matriptase, complexed with its inhibitor HAI-1, in human breast cancer cell lines, and HAI-1 was co-expressed with matriptase on the cell surfaces of normal, hyperplastic and cancerous human mammary epithelium [6]. In our current study we demonstrated that HAI-1 is colocalized with matriptase on the surfaces of T-47D cells (Fig. 5). Based on in vitro models, protease inhibitors have been proposed to serve as negative factors for cancer invasion and metastasis [34-37]. However, in human tumors, including primary breast cancer, expression of the plasminogen activator inhibitor-1 (PAI-1) and the MMP inhibitors TIMP-1 and -2 have been correlated with poor prognosis [38-42]. Furthermore, PAI-1 has been shown to be required for cancer cell invasion, since PAI-1 deficiency in the PAI-1 (−/−) mice prevented local invasion of implanted cancer cells, and since invasion could be restored by exogenous delivery of PAI-1 [43]. These results indicate that, in some instances, the presence of protease inhibitors is permissive, if not contributory, for malignant cell invasion and cancer progression. In our study, we have observed, in a continuous manner, the accumulation of activated matriptase in the conditioned media of breast cancer cells, suggesting that matriptase is constitutively activated. Binding of HAI-1 to matriptase requires that matriptase first be converted into its catalytically active form. Despite its binding to HAI-1, a continual process of protease activation could still result in an accumulation of proteolysis at the surface of the cancer cells. We have observed that activated matriptase
is detected in cell lysates, mainly in its free form (Fig. 1), and in the conditioned media primarily complexed to HAI-1 (Fig. 2). The binding of HAI-1 to activated matriptase may prevent prolonged excess proteolysis, that could be detrimental to cancer cell survival.

Various sizes of matriptase/HAI-1 complexes were observed in cell lysates (120-kDa) and in cell conditioned media (110- and 95-kDa); these complexes are stable at physiological pH and in 1% SDS, under non-boiled conditions. Acidic buffer (pH 2.4) can dissociate matriptase/HAI-1 complexes; reformation of the complexes can be triggered by alkalinizing buffer to pH 8 [9]. This pH-dependent binding behavior is common for serine proteases and Kunitz-type inhibitors, but different from the binding behavior of HAI-1 and another target protease, HGF activator (HGFA) [44]. The binding of HGFA to HAI-1 is regulated by the size of HAI-1. The membrane-bound HAI-1 exhibits higher binding affinity to HGFA than its fragment, which releases HGFA under physiological pH, when HAI-1/HGFA complex is shed from cell surfaces [44].

In summary, both the activity of matriptase and the arrangement of actin cytoskeleton are tightly controlled by S1P in mammary epithelial cells, a likely physiological regulatory influence. This dual physiological mechanism(s) appears to be strongly altered in breast cancer cells. Active matriptase could serve as an activator on epithelial cancer cell surfaces, recruiting and activating stromal ECM-degrading proteases, such as uPA, and cell growth/motility factors, such as HGF [7]. The accumulation of activated matriptase at membrane ruffles in T-47D cells suggests a model whereby uPA and HGF might be locally and preferentially activated at the leading edge of cancer cells. Such activation and recruitment of matriptase to the invading front
of tumor cells may ultimately enhance the growth and the invasion/metastasis of breast
tumors and possible other types of cancer.
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Figure Legends

Figure 1: Human breast cancer cells constitutively expressed activated matriptase.
Immortalized, non-tumorigenic mammary epithelial cells (184 A1N4, MCF-10A) and breast cancer cell lines (MCF-7, T47D, MDA MB-468) were serum-starved for 50 hrs in 2% FBS, and then stimulated with IMEM in the presence or absence of 10ng/ml S1P for 1 hr. Total cell lysates were analyzed by western blot for total matriptase (mAb M32) and activated matriptase (mAb M69). Activated matriptase was detected as an non-complexed form (70 kDa) and as a complex with HAI-1 (120 kDa).

Figure 2. Increased ectodomain shedding of matriptase in breast cancer cells.
In panels A and B, equal numbers of non-tumorgenic human mammary epithelial cells (184 A1N4) and human breast cancer cells (MDA MB-468, MCF-7, and SkBr 3) were serum-starved, and then maintained in the presence or absence of 50 nM S1P for 24 hr. The media were collected and concentrated. Equal amounts of media were examined by immunoblot, using anti-total matriptase mAb M32 (A) and anti-two-chain matriptase mAb M69 (B). C, to compare the amounts of matriptase released into the media in the absence of S1P between immortalized non-tumorgenic mammary epithelial cells and the breast cancer cell lines, equal volumes of the conditioned media for each cell lines were analyzed by western blotting for the presence of activated (mAb M69) and total matriptase (mAb M32). Western blot results were analyzed by densitometry analysis, and the values obtained for each cell line were normalized to the total amount of cellular protein present in the tissue culture plate at the time the conditioned media were collected. (a.u., densitometry arbitrary units).
Fig 3: Immunofluorescence of activated matriptase in breast cancer cells.

Serum-deprived 184A1N4, T47D, MDA MB-468, MCF-7 cells were stimulated with 10 ng/ml S1P or vehicle control for 40 min. Cells were stained for activated matriptase with M69 monoclonal antibody followed by a FITC-conjugated anti-mouse antibody.

Figure 4: Differential response to S1P for actin skeleton rearrangement, comparing mammary epithelial cells with breast cancer cells.

Immortalized, non-tumorigenic mammary epithelial cells (184 A1N4) and breast cancer cell lines (T47D, MDA MB-468, and MCF-7) were serum-starved and then stimulated with IMEM in the presence or absence of S1P for 1 hr. Cells were stained for actin with Texas Red-labeled phalloidin (Molecular Probes).

Figure 5: Colocalization of HAI-1 and matriptase on the cell periphery, where actin filaments accumulate.

T-47D breast cancer cells were grown on cover slides in IMEM, supplemented with 5% FBS. Cells were dual stained for matriptase with Alexa Fluor 488-labeled mAb M32 (panel A, green) and for HAI-1 with Alexa Fluor 594-labeled M19 (panel B, red). The merged image (panel C, yellow) shows the colocalization of matriptase and HAI-1. The localization of matriptase and actin was carried out by dual staining for matriptase with Alexa Fluor 488 labeled mAb M32 (panel D, green) and for actin with Texas Red labeled phalloidin (Panel E, red). The merged image (Panel F) shows that both matriptase and actin localize on the membrane ruffling and some cell-cell contacts. The cell nuclei (blue) were stained with 4',6-diamidino-2-phenylindole (DAPI).
Figure 6: Activated matriptase accumulates at membrane ruffles following EGF treatment.

T47D cells were serum-starved overnight (a, b, c) and stimulated with 10ng/ml EGF (d, e, f, g) for 10 min. Cells were dual-labeled for activated matriptase (M69; FITC) (a, d) and F-actin (Phalloidin-Texas Red) (b, e) and the two images merged (c, f), or with secondary FITC-conjugated anti-mouse antibody alone (g).
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A. mAb M32 (Total)

B. mAb M69 (Activated)

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