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Matriptase Mediated C-Met Signaling In Breast Cancer

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MATRIPTASE MEDIATED c-MET SIGNALING IN BREAST CANCER

by

GINA ZORATTI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

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MAJOR: CANCER BIOLOGY

Approved by:

Advisor

Date

DEDICATION

I would like to dedicate my dissertation to my parents, **Edward and Kandis Zoratti**. You two have inspired me throughout my life to go after what I want and have shown me the importance of following everything through to the end and giving it 110%.

And, also to my husband, **Jeremy Savickas**. Your constant love, support and understanding is what made this body of work possible.

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TABLE OF CONTENTS

Dedication	ii
Acknowledgments	iii
List of Tables	x
List of Figures	xi
List of Abbreviations	xiv
Chapter 1: Introduction	1
1.1 Breast cancer- a 2013 review.....	1
1.1.1 The normal breast.....	1
1.1.2 Breast cancer types.....	3
1.1.3 Breast cancer diagnosis, staging and treatment.....	8
1.2 Proteases involved in breast cancer.....	16
1.2.1 Classes of proteases-a brief overview.....	18
1.2.2 Intracellular proteases.....	19
1.2.3 Extracellular proteases.....	21
1.2.4 Aspartate proteases and threonine proteases.....	24
1.2.5 Secreted serine proteases.....	25
1.2.6 Membrane anchored serine proteases.....	26
1.2.6.1 Type I transmembrane serine proteases.....	27
1.2.6.2 GPI anchored serine proteases.....	28
1.2.6.3 Type II transmembrane serine proteases.....	29
1.2.7 Elucidating the role of extracellular proteases in breast cancer <i>in vivo</i>	35

1.3 Matriptase.....	38
1.3.1 Expression, structure and substrates.....	38
1.3.2 Physiological functions.....	41
1.3.3 Pathological implications.....	43
1.3.4 Defining the role of matriptase in cancer using genetic mouse models.....	45
1.3.5 Matriptase in breast cancer.....	47
1.4 HGF/c-Met in breast cancer.....	48
1.4.1 Localization and activation of pro-HGF and c-Met in breast cancer.....	48
1.4.2 c-Met signaling.....	50
1.5 Models used to study invasive ductal breast carcinoma and inflammatory breast carcinoma.....	51
1.5.1 Genetic mouse models of IDC.....	52
1.5.2 Xenografting models.....	57
1.5.3 Human breast cancer cell lines.....	58
Chapter 2: Matriptase mediated c-Met signaling in breast cancer.....	60
2.1 Hypothesis and specific aims.....	60
Chapter 3: Materials and Methods.....	63
3.1 Animals.....	63
3.2 Mammary gland whole mount analysis.....	64
3.3 Immunohistochemistry.....	64
3.4 Breast cancer tissue array.....	65

3.5 Isolation of primary mammary epithelial cells and carcinoma cells.....	66
3.6 Human breast cancer cell lines.....	66
3.7 Pro-HGF/SF activation assays.....	67
3.8 3D cell culture.....	68
3.9 RNAi silencing.....	69
3.10 Western blot analysis.....	69
3.11 Proliferation assays in human breast cancer cell lines.....	70
3.12 Transient transfection of SUM159 cells.....	70
3.13 Matriptase gelatin zymography.....	70
3.14 Statistical analysis.....	71
Chapter 4: Explanation of genetic mouse models.....	72
4.1 Matriptase hypomorphic mice.....	72
4.2 Matriptase conditional knockout mice.....	73
Chapter 5: Matriptase mediated c-Met signaling- Results	
in invasive ductal breast cancer.....	75
5.1 Introduction.....	75
5.2 Matriptase is expressed in the epithelium of the mammary gland and is upregulated in mammary carcinomas.....	77
5.3 Matriptase hypomorphic mice have greatly reduced levels of matriptase in their mammary glands.....	78
5.4 Reduced matriptase leads to impairment of mammary carcinogenesis.....	80
5.5 Reduced matriptase impairs carcinoma cell proliferation in mammary	

tumors.....	81
5.6 Genetic ablation of matriptase abolishes pro-HGF induced c-Met signaling in primary mammary carcinoma cells.....	85
5.7 Matriptase silencing causes abrogated c-Met signaling and proliferation in human invasive ductal breast cancer cell lines.....	87
5.8 Matriptase re-expression restores c-Met signaling in human breast cancer cells in response to HGF.....	90
5.9 Matriptase mediates pro-HGF induced invasion of human invasive ductal breast cancer cell lines.....	91
5.10 Matriptase and c-Met expression are strongly correlated in human invasive ductal carcinomas.....	94
5.11 Inhibition of matriptase proteolytic activity abrogates pro-HGF induced c-Met signaling in mouse mammary carcinoma cells and human invasive ductal carcinoma cell lines.....	95
Chapter 6: Matriptase mediated c-Met signaling in breast cancer- Results	
in inflammatory breast cancer.....	99
6.1 Introduction- Targeting matriptase/c-Met in IBC.....	99
6.2 Matriptase silencing causes abrogated c-Met signaling and proliferation in human inflammatory breast cancer cell lines.....	99
6.3 Matriptase mediates pro-HGF induced invasion in human inflammatory breast cancer cell lines.....	101
6.4 Matriptase and c-Met are both expressed in human inflammatory breast carcinomas.....	102

Chapter 7: Discussion and future directions.....	106
Appendix A: Supplementary Figures.....	112
References.....	116
Abstract.....	141
Autobiographical Statement.....	143

LIST OF TABLES

Table 1: Phenotypic characteristics of matriptase deficient mice.....	43
Table 2: Relevant human breast cancer cell lines.....	59

LIST OF FIGURES

Figure 1: Histology and tissue types of the normal breast.....	2
Figure 2: Depiction of the progression to breast cancer.....	4
Figure 3: Hallmarks of inflammatory breast cancer.....	7
Figure 4: Characteristics of invasive lobular carcinoma.....	8
Figure 5: The pyramid of the five classes of proteases.....	19
Figure 6: Depiction of the membrane anchored serine proteases.....	30
Figure 7: Schematic of matriptase.....	38
Figure 8: Tissue specific expression of matriptase.....	40
Figure 9: Schematic of pro-HGF and the c-Met signaling pathway.....	49
Figure 10: Histology of mammary intraepithelial dysplasia in MMTV-Neu mice.....	53
Figure 11: Mammary whole mounts of cancer in MMTV-Wnt-1 mice.....	55
Figure 12: Depiction of tumor progression in MMTV-PymT mice.....	56
Figure 13: Schematic of the hypothesis.....	62
Figure 14: Matriptase in the mammary gland upon acute ablation.....	74
Figure 15: Expression of matriptase in the MMTV-PymT mammary carcinoma model and in the matriptase hypomorphic model.....	79
Figure 16: Reduced matriptase decreases mammary tumor burden, growth rate, latency and multiplicity.....	82
Figure 17: Decreased carcinoma cell proliferation in mammary tumor with reduced matriptase.....	84
Figure 18: Abrogation of activated c-Met and downstream signaling proteins in matriptase deficient primary mammary carcinoma cells.....	88
Figure 19: Matriptase is required for activation of the pro-HGF-c-Met signaling and promotes cell proliferation in invasive ductal breast cancer cells....	89
Figure 20: Re-expression of matriptase in human invasive ductal breast cancer	

cells induces robust c-Met signaling and proliferation.....	92
Figure 21: Matriptase mediates pro-HGF induced invasion in human invasive cells induces robust c-Met signaling and proliferation.....	93
Figure 22: Matriptase and c-Met are correlated in human invasive ductal carcinomas.....	97
Figure 23: Inhibition of matriptase activity impairs c-Met pathway activation in primary mammary carcinoma cells and in human invasive ductal breast cancer cell lines.....	98
Figure 24: Matriptase is required for activation of the pro-HGF/c-Met signaling pathway and promotes cell proliferation in inflammatory breast cancer cells.....	103
Figure 25: Matriptase mediates pro-HGF induced invasion in human inflammatory breast cancer cells.....	104
Figure 26: Matriptase and c-Met are expressed in human inflammatory carcinomas.....	105
Supplementary Figure 1: Activation of c-Met and downstream signaling proteins in matriptase sufficient primary mammary carcinoma cells.....	112
Supplementary Figure 2: Matriptase is required for activation of the pro-HGF/c-Met signaling pathway and promotes cell proliferation in BT20 and SUM229 breast cancer cell lines.....	113
Supplementary Figure 3: Matriptase mediates pro-HGF induced invasion in human breast cancer cells.....	114
Supplementary Figure 4: Matriptase and c-Met are expressed in mouse human breast cancer cells.....	115

LIST OF ABBREVIATIONS

2D	Two dimension
3D	Three dimension
ADAMs	A disintegrin and metalloproteinase
ADAMTSs	A disintegrin and metalloproteinase with thrombospondin motifs
ANP	Atrial natriuretic peptide
APC	Anaphase promoting complex
ARIH	Autosomal recessive ichthyosis with hypotrichosis
Atg4c	Autophagin 3
BCL3	B cell lymphoma 3
CAP-1	Channel activating protease
CCL2	Chemokine ligand 2
CNS	Central nervous system
COX-2	Cytochrome oxidase subunit 2
CtsB	Cathepsin B
CtsZ	Cathepsin Z
CUB	Complement C1r/C1s, Uegf, Bmp1
CYLD	Cylindromatosis gene
DCIS	Ductal carcinoma in situ
DESC	Differentially expressed in squamous cell carcinoma
DMBA	7,12-dimethylbezanthracene
DNA	Deoxyribonucleic acid
DUBs	Deubiquitinases

EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERG	ETS related gene
Gab1	Grb-2 associated binding protein
GPI	Glycophosphatidylinositol
Grb-2	Growth factor receptor bound protein-2
HAI-1/2	Hepatocyte growth factor activator inhibitor-1/2
HAT	Human airway trypsin-like protein
HER2	Hormone growth factor receptor 2
HGF/SF	Hepatocyte growth factor/scatter factor
HGFA	Hepatocyte growth factor activator
IBC	Inflammatory breast cancer
IDC	Invasive ductal carcinoma
IGFBP-rP1	Insulin-like growth factor binding protein related protein-1
IL-13	Interleukin-13
ILC	Invasive lobular carcinoma
K5	Keratin 5
LDLA	Low density lipoprotein A
LEKTI	Lympho-epithelial Kazal-type related inhibitor
MIN	Mammary intraepithelial dysplasia
MMPs	Matrix metalloproteinases
MMTV	Mouse mammary tumor virus
MRI	Magnetic resonance imaging

mRNA	messenger ribonucleic acid
MSP-1	Macrophage stimulating protein-1
NFκB	Nuclear factor κB pathway
PAR-2	Protease activated receptor-2
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol kinase 3
PR	Progesterone receptor
PSA	Prostate specific antigen
PymT	Polyoma middle T antigen
RNA	Ribonucleic acid
SEA	sea urchin sperm protein
ST14	Matriptase
TgMMTV	Transgenic mouse mammary tumor virus
TMPRSS	Transmembrane serine protease
TNF	Tumor necrosis factor
TRASK	Transmembrane protein that is activated by src
TTSP	Type II transmembrane serine protease
uPA	Urokinase type plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

CHAPTER ONE- INTRODUCTION

1.1 Breast cancer- a 2013 review

This section is meant to serve as a broad review of the breast and breast cancer was information was compiled from Cancer.net, American Cancer Society Breast Cancer Figures and Statistics, Arpino et al., Vargo-Gogola et. al., Kleer et al., Simpson et. al., Kittaneh et. al., Wong et. al., and Chabner and Longo.

It is estimated that one in every eight women will develop breast cancer throughout the course of their life, with 232,340 new cases of invasive breast cancer diagnosed in 2013 in the United States (American Cancer Society). While researchers have identified a considerable number of risk factors, their ability to accurately predict the development of invasive carcinoma is inadequate. Currently, breast cancer is still the second leading cause of cancer death in women, responsible for 1 in every 36 deaths. Therefore, extensive efforts have been put forward to identify novel targets in this disease and develop drugs to help treat patients once a diagnosis of invasive breast cancer has been determined.

1.1.1 The normal breast

Breast cancer has many different forms and can arise from multiple cell types within the breast. The breast is primarily composed of adipose tissue, and throughout this adipose tissue is a vast network of specialized epithelial structures that comprise the milk ducts and lobules. In addition to these ducts,

there are also blood and lymph vessels that bring blood to nourish the cells and drain waste products produced in the breast, respectively.

The main function of the human breast is to produce milk to nourish newborns. Normal breast development occurs in three distinct stages throughout a woman's life. The breasts begin to form *in-utero* where the mammary ridge develops. The mammary ridge is a thickening of the chest wall. By infancy both the nipple and milk-duct system have been formed. Near adolescence the breast begins to change again. The milk ducts (pictured below) grow in a segmentally branching pattern stemming from the nipple. Each of these branches forms separate lobes within the breast. The lobes are separated from each other by adipose tissue and are composed of stroma and lactiferous ducts. The stroma is a network of supporting structures for the cells usually composed of connective tissue. Each of these lactiferous ducts drains one lobe of the breast and leads to the nipple. The development of these lobes, 15-24 in total, depends on hormones, which are present first during puberty. Between the lobes, connecting to the skin, are ligaments called Cooper's Ligaments. These provide the support to the breast. The final stage of breast development is involution, where the breast tissue regresses back to a non-secreting form after lactation.

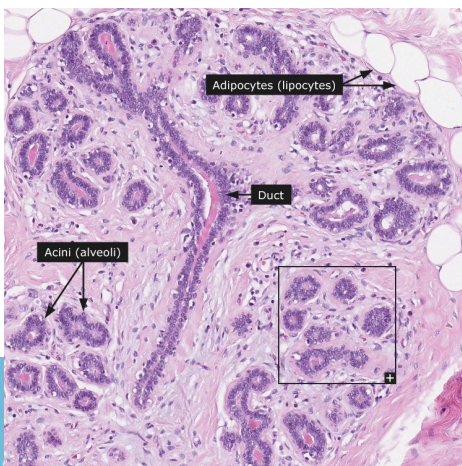
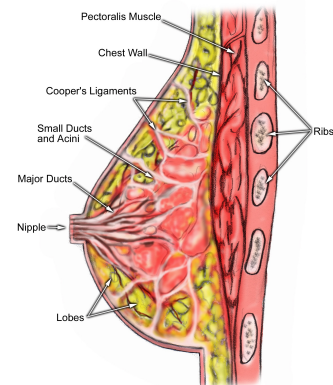


Figure 1: (Left) Shows histology of human breast tissue including adipocytes, alveoli, and ducts. (Image reprinted with permission from proteintlas.com) (Right) Cartoon depicts the make-up of a normal human breast. (Image reprinted with permission from Patrick J. Lynch (medical illustrator, Yale University))



Normal breast remodeling

Changes in hormone levels during the menstrual cycle lead to continuous remodeling of the breast. Estrogen, which is produced during the first half of the menstrual cycle, causes growth of the milk ducts. Once ovulation takes place progesterone replaces the estrogen for the second half of the cycle, which causes the formation of the milk glands. If the woman does not become pregnant during the cycle, progesterone levels drop and the breasts return to normal size. For this reason the breast tissue is constantly remodeling and turning over even in normal breast tissue.

An even greater extent of remodeling occurs during pregnancy. As mentioned above, progesterone is critical for the formation of milk glands. Once a fertilized egg is implanted in the uterus, cyclical hormone production halts and the body begins to continuously produce incrementally higher amounts of progesterone instead of the levels tapering off to begin a new cycle. As the levels of progesterone increase the milk glands continue to grow and as a result, both epithelial and endothelial cell growth increases. By the fifth or sixth month of the pregnancy, the breast structure has remodeled with a greatly increased number of lobes and ducts, and the breast becomes capable of producing milk. Upon termination of breast feeding the process of involution, or the shrinking of the milk ducts and breasts, takes place and the breast tissue returns to normal size.

1.1.2 Breast cancer types

Non-invasive and Invasive ductal carcinoma

The vast majority of breast cancers diagnosed originate in the ductal epithelium and are either non-invasive or invasive at the time of diagnosis. Ductal carcinoma in situ (DCIS) is the most common type of non-invasive breast cancer. DCIS is considered non-invasive because it has not penetrated the basement membrane and invaded beyond the milk duct into the surrounding tissue. Physicians will commonly recommend frequent check-ups for patients with DCIS since it increases the risk of developing an invasive breast cancer in the future. Occasionally physicians will perform a lumpectomy to remove the DCIS (discussed later), but in general a diagnosis of DCIS does not usually require treatment. Once the cancer cells break through the basal membrane of the ducts and into the surrounding tissues and/or lymphatics it is considered an invasive ductal carcinoma (IDC). IDC can be diagnosed at many distinct stages (discussed later in breast cancer staging) but if left untreated can metastasize to distant parts of the body. Once metastases have occurred, the five year survival rate drops from 88% down to 15%.

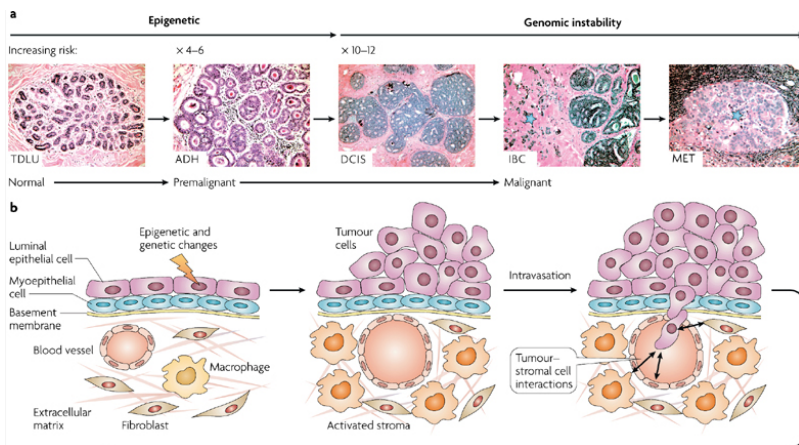


Figure 2: Depiction of the progression to breast cancer. DCIS occurs when cancer cells block the milk ducts but do not invade through them. IDC occurs when the cancer cells invade out of the milk ducts into the surrounding tissue. If left untreated, IDC will metastasize to distant part of the body. Image reprinted with permission from Vargo-Gaola et al., 2007.

Subtypes of invasive ductal carcinoma

Within the diagnosis of IDC there are various subtypes of tumors with specific properties that can determine prognosis and treatment for the individual. Hormone receptor status is arguably the most important of these properties. The presence of the progesterone receptor (PR), estrogen receptor (ER) and the human epidermal growth factor receptor 2 (HER2), two hormone receptors and a commonly amplified growth factor receptor respectively, are determined after diagnosis to accurately screen for phenotypic variation and to estimate prognosis. Several drugs that selectively target the hormone receptors and therefore curb the growth of the tumor with limited systemic cytotoxic effects are available. These targeted therapies (discussed in section 1.3) have led to better prognoses for patients whose tumors express one or more of these receptors. Tumors that do not express any of these three receptors are commonly referred to as triple negative tumors. These patients have a poor prognosis and suffer from a very aggressive and invasive phenotype. Along with analyzing hormone status, physicians also look at the morphology of the tumor to properly diagnose and determine a course of treatment.

Medullary, papillary and tubular breast cancer

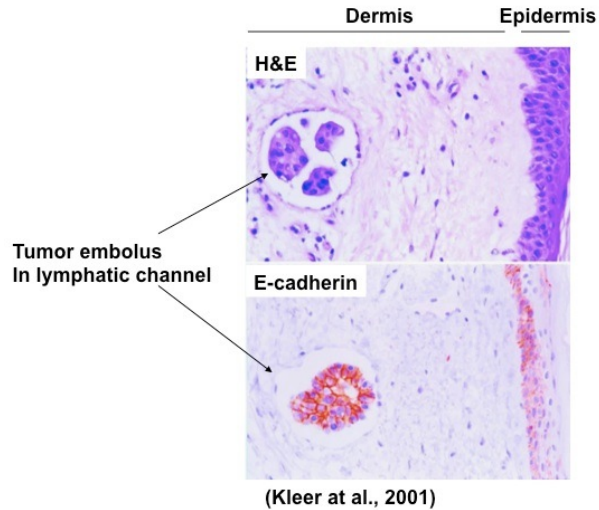
One form of breast cancer with a unique morphology is medullary carcinoma. This type of breast cancer starts in the ducts but has a very different morphology from non-medullary IDC. Medullary IDC is more profuse with a large fleshy mass that comprises the tumor and is characterized by an extensive lymphocytic infiltration. Another rare form of IDC is mucinous breast carcinoma,

characterized by pools of mucin which encapsulate the cancer. Papillary and tubular breast carcinomas are two more types of IDC that are very similar to each other. Papillary carcinoma is usually a well-defined tumor with finger-like projections whereas tubular carcinoma presents with cancer cells forming tube-like structures in the breast. Medullary, mucinous, papillary, and tubular IDC are very rare types of breast cancer and together account for about 10% of all invasive ductal carcinomas. In general, these four types of IDC are slower growing and less aggressive than triple negative IDC or the other more common forms of IDC.

Inflammatory breast cancer

Another rare type of breast cancer is inflammatory breast cancer (IBC). While IBC is only responsible for 1-3% of all diagnosed breast cancers, it has the most aggressive phenotype. Due to its rarity and presentation that is distinct from IDC, the awareness about this subtype of breast cancer is generally low. While IDC usually presents as a lump in the breast, IBC usually presents as a red, inflamed breast, which is often warm to the touch. Patients with this disease can also have unusual skin appearance, called "peau d'orange" stemming from the skin's resemblance to an orange peel. IBC is commonly misdiagnosed as mastitis, a bacterial infection of the breast. For this reason, as well as a lack of information among the general population about this type of breast cancer, patients are usually diagnosed at a later stage and have only a 34% five-year survival rate.

Figure 3: Image shows lymphatic ducts blocked by epithelial carcinoma cells in the dermis of the breast. This is a hallmark of the aggressive type of breast cancer called IBC. Most IBC tumors are positive for the epithelial marker E-cadherin as shown by immunohistochemistry in the lower panel. Image reprinted with permission from Kleer et al., 2001)



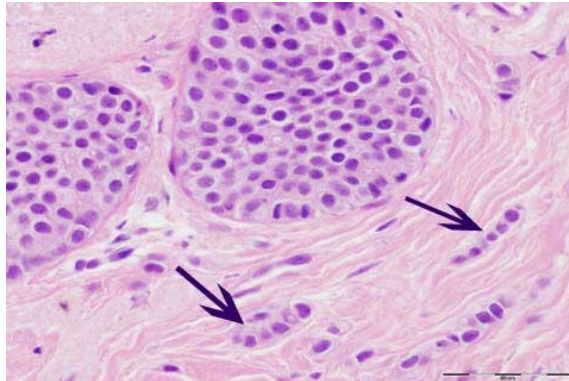
One of the defining characteristics of this type of breast cancer is tumor emboli (Figure 3). A distinguishing feature of IBC compared to IDC is the fact that the cancer cells are blocking the lymphatic vessels in the breast. These lymphatic vessels are responsible for transporting waste products out of the breast. When these are blocked as in IBC, inflammation and swelling occur. Adding to the difficulty of early diagnosis is the fact that IBC does not generate the typical lump in the breast and can typically not be detected by palpation, mammography or ultrasound examination.

Infiltrating lobular carcinoma

Another type of breast cancer is infiltrating lobular carcinoma (ILC). Many times “infiltrating” is used interchangeably with “invasive” for many types of cancer. ILC is more common than IBC but less common than IDC, and is responsible for approximately 10% of breast cancers. This type of breast cancer features small cancerous cells that originate in the lobules and infiltrate the tumor

stroma. This type of breast cancer does not typically form a mass nor does it

Figure 4: Image shows the characteristic mark of ILC: The single file carcinoma cells invading the tumor stroma (shown with arrows). Image reprinted with permission from breastpathology.net



destroy the features of the surrounding breast tissue.

Due to the distinct growth patterns and the absence of a well-defined mass this type of carcinoma is difficult to detect early and is hard to treat with surgery. ILC invades the surrounding stroma in uniform single lines. This type of invasion makes the detection on mammography or other screening modalities extremely difficult; usually a biopsy is used to make a diagnosis of ILC. Typically a diagnosis of ILC carries a good prognosis.

1.1.3. Breast cancer diagnosis, staging and treatment

Breast cancer screenings

Many advances have been made in the past decade in detecting breast cancers at earlier stages. In addition to self-breast exams, the most effective tool physicians have is mammography screening. Mammography uses a low energy X-ray to image the breast. A radiologist then examines the images to look for any early signs of breast cancer. Since mammography became readily available in the 1990s, the number of women who die of breast cancer has decreased by almost 30%. Detecting breast cancer at an early stage vastly increases the odds of survival. The NIH recommends that women receive a mammography every

two years between the ages of 50 and 74. However, mammography has a substantial false-positive rate of about 7%. There are various reasons for false-positives; some women naturally have denser breasts, which makes it difficult for the radiologist to determine if the tissue is normal or cancerous. Other times the lump detected by mammography can be a benign case of breast disease, which requires no course of action.

If screening mammography results are positive, an ultra-sound, magnetic resonance imaging (MRI) or a diagnostic biopsy will be performed. An ultrasound uses sound waves to produce an image. Ultrasounds are often used to help the physician determine if a biopsy is needed or if the breast appearance on mammography was likely secondary to a benign high density compartment of the breast. Finally, MRI uses a strong magnetic field to produce an image. Similar to an ultrasound in diagnostic use, an MRI can either confirm the need for biopsy or suggest a lesion that is likely to be benign. For diagnostic biopsies a small amount of the tissue in question is removed and sent to a pathologist to determine if cancer cells are present. This is usually a minimally invasive procedure and can confirm or refute a diagnosis of cancer.

Breast cancer staging and treatment determination

The treatment of breast cancer is typically dependent on five major factors: 1) grade and stage upon diagnosis, 2) hormone receptor status, 3) Age and general health of the patient, 4) pre/post-menopausal status and 5) presence of known mutations within the tumor.

The three grades of a tumor are characterized by the morphology, especially the level of differentiation and the size and shape of the nucleus in the tumor cells, of the individual cancer cells when observed under the microscope. Grade one, or low grade means, that their morphology is very similar to the normal breast cells (well differentiated with small nuclei). If the cancer cells are growing at an accelerated rate compared to the normal breast cells it is considered a grade two tumor (moderately differentiated with moderate nuclear pleomorphism. If morphologically the cancer cells appear very different from the normal breast cells and are growing quickly, it is considered a grade three or a high-grade tumor (poorly differentiated with pronounced nuclear pleomorphism). While grading is based on microscopic observation, staging combines two nomenclature systems. The first is a number-staging system. Briefly, stage one tumors are less than or equal to 2 cm and are localized to the site of primary origin (in this case the breast), stage two tumors are either larger than stage one or cancer cells can be detected in the nearby lymph nodes, stage three refers to a tumor is larger than stage 2 and/or has spread to nearby tissues and lymph nodes, and finally a stage four tumor indicates that the cancer has metastasized or spread to distant parts of the body. Combining this number system with the TNM staging system gives a complete picture of the tumor status in a patient. T describes the size of the tumor, so for example a T2 tumor would mean that the tumor was greater than 2 cm but less than 5 cm. The N in the TMN staging refers to the lymph node status. There are only two choices in the N stage, either N0 or N1. N0 means there are no cancer cells present in the lymph nodes where their

presence is detected in N1. Finally, the M refers to the presence of metastases again with only the choices of M0 or M1.

As mentioned, tumors are also analyzed for their hormone receptor status. The PR, ER and HER2 receptors mediate signaling that control breast cancer cells ability to grow and divide. Since most types of breast cancers (with the exception of triple negative breast cancers) express one or more of these hormone receptors, these tumors may allow treatment with a low-toxicity targeted therapy that selectively blocks the signaling through these individual receptors.

Physicians also take into consideration the overall health and age of the patient. Even targeted therapy can have substantial adverse effects when combined with more toxic chemotherapy drugs to completely eliminate the cancer resulting in debilitating-treatment related side-effects. The overall health of the patient may help determine whether they will be able to tolerate the most aggressive treatments or if a less aggressive treatment regimen would be advantageous.

The fourth factor is whether or not the patient has gone through menopause. Post-menopausal women generally have a higher risk for developing hormone independent types of breast cancers than pre-menopausal women. If the patient is of childbearing age a physician will be less likely to prescribe hormone therapies or other therapies that may permanently impact pregnancy or pregnancy-related outcomes. Drugs that affect estrogen production are not tolerated as well by pre-menopausal women compared to women who

have already gone through menopause so physicians generally use anti-estrogen therapies sparingly in pre-menopausal patients.

The final factor to take into consideration when determining a course of treatment for a patient is whether or not they have any known genetic mutations within the tumor. Breast cancer cells are able to utilize many pathways to grow and drugs have been developed to specifically block some of these pathways. A common mutation in tumors is overexpression or the activation of a growth factor receptor without the presence of a ligand (i.e. BRCA1 and BRCA2 mutations). While normal cells typically require the presence of a receptor ligand to control growth factor signaling, cancer cells may exploit these growth factor receptor transduction pathways to signal for aberrant growth and proliferation. Many antibodies are used in the clinic to neutralize the receptor and decrease tumor burden. Identification and testing for common markers of breast cancer allow physicians to tailor a specific treatment to each individual patient to provide the best outcome.

Treatment types

Surgery

A common course of action for tumors that are contained within the breast and have not invaded surrounding tissues (early stage) is surgical resection of the tumor. Two surgical procedures are commonly used to remove tumors in the breast based on the size of the tumor. A lumpectomy removes just the tumor and a small amount of normal surrounding breast tissue to ensure clean margins. A mastectomy is more aggressive and involves the removal of the whole breast

and some underlying muscle tissue. Usually physicians will recommend adjuvant chemotherapy after surgery to ensure any cancer cells left behind are killed. This adjuvant therapy can include radiation, chemotherapy, targeted therapy and/or hormonal therapy.

Radiation

Radiation is the adjuvant therapy typically given to patients who have had a lumpectomy. This type of therapy is normally given for a set number of weeks to lower the number of residual cancer cells after the surgery that may cause cancer recurrence. Although there are side effects of radiation therapy, modern advances in medical physics have made radiation therapy much safer. In certain circumstances physicians may recommend radiation therapy as the primary course of treatment instead of surgery. This normally occurs if the tumor is slow growing and/or there are other circumstances, which make surgery a less attractive option.

Chemotherapy

Chemotherapy is often used alone but can also be prescribed after surgery. Multiple drug regimens are usually mixed and given systemically to reach cancer cells throughout the body. Although there are many different chemotherapy drugs, most can be categorized in the following main classes: 1) Antifolates 2) Alkylating Agents 3) Antimitotic Drugs 4) Platinum Analogs 5) Cytidine Analogs 6) Purine Antimetabolites and 7) 5-Fluoropyrimidines. These seven different classes of chemotherapeutic agents all have different mechanisms of action but most all target deoxyribonucleic acid (DNA) replication

and repair and subsequently cause apoptotic cell death. Unfortunately, chemotherapeutic drugs are unable to selectively target cancer cells and are toxic to actively replicating cells in the body. This leads to the common side effects of chemotherapy including hair loss, low blood cell counts and GI toxicity as the hair follicles, bone marrow and GI lining represent some of the body's most rapidly dividing normal cells. However, as cancer cells are more rapidly replicating their DNA the drugs preferentially affect the cancer cells over the normal cells.

The antifolate class of drugs was discovered in 1947 by Dr. Sidney Farber who developed the common anti-leukemia drug, aminopterin. Drugs within this class are effective against cancer cells because they interfere with purine and thymidylate synthesis. By depleting the store of DNA precursors within the cells, the cells cannot replicate their DNA accurately and undergo cell death.

The alkylating agents class covalently add alkyl groups to DNA through the formation of reactive oxygen species causing DNA strand breaks. These alkyl groups prevent the DNA repair mechanisms from being carried out within the cell in response to misreplicated DNA. Therefore, the integrity of the DNA is compromised triggering apoptosis.

The antimetabolic drugs have a slightly different way of affecting DNA replication. Unlike the previous classes of drugs, which affect DNA replication during the G2/S phase of the cell cycle, antimetabolic drugs disrupt the microtubules during mitosis leading to apoptotic cell death. The microtubules are responsible for pulling apart sister chromatids in the mitotic cells, one into each of the

daughter cells. If the microtubules are inhibited the genomic stability of the cells is compromised and apoptosis is triggered.

The next set of drugs is the platinum analogs. These drugs covalently bind purine bases together forming DNA adducts. When these DNA adducts accumulate faster than repair mechanisms can repair them the cells typically become apoptotic. Similar to these analogs are the cytidine analogs that mimic the DNA base cytosine and get incorporated into elongating DNA strands. These analogs inhibit DNA polymerases from extending the chain any further which compromises genomic integrity, killing the cell. The purine antimetabolites also incorporate into the elongating DNA strand. These drugs cause DNA mutations because the purine analogs pair with an otherwise non-complementary DNA base causing the mutation to be propagated. The accumulating mutations result in stress on the cell, which eventually leads to apoptosis. The final class of chemotherapy drugs is the 5-fluoropyrimidines. These drugs mimic RNA and DNA components and trigger DNA strand breaks. Once these breaks accumulate apoptosis is triggered.

Targeted/hormonal therapies

Since the drugs described above affect the replication of DNA in normal non-cancerous cells, it is not surprising that there are many side effects from using chemotherapeutic agents. Most chemotherapeutic agents are very neurotoxic and myelosuppressive causing neuropathy and muscle weakness in patients. Other common limiting toxicities for standard chemotherapy are the damage they do to cells in the kidneys and liver. Since even otherwise healthy

patients may become ill while receiving chemotherapy, research is focused on developing drugs to more selectively target cancer cells, thereby reducing the toxic side effects seen with standard chemotherapy. As mentioned previously, there are three common signaling receptors, ER, PR, and HER2, that are common in breast tumors. Many targeted therapies such as trastuzumab (monoclonal anti-HER2 antibody) inhibit a single receptor or ligand to reduce the growth signaling in the cancer cells. Another common type of a “targeted therapy” is hormonal therapy. This is often given to women past childbearing age but is also used when tumors are positive for ER and/or PR as a second line of treatment if they do not respond to initial chemotherapeutics. An example is tamoxifen, a selective estrogen receptor modulator, that blocks estrogen from binding to ER and PR. Anti-angiogenic therapies are also often used to treat breast cancer. Drugs in this class, like bevacizumab, target the growth of new blood vessels. These vessels are required to supply blood and nutrients to the tumor while allowing the cancer cells to metastasize to different areas of the body. Such cell-selective targeted therapies typically have a relatively favorable adverse effect profile but are not without side effects. However, in general they are frequently less detrimental to the health and are often a more attractive choice when compared to standard chemotherapeutics, especially when health or age concerns do not allow for the most aggressive treatments.

While much progress has been achieved in treating and diagnosing breast cancer, especially regarding early detection, there were still roughly 40,000 women that died of this disease in 2013 in the United States and there remains a

substantial need to identify new therapeutic targets and to develop more effective agents to help women once this devastating diagnosis has been made.

1.2 Proteases involved in breast cancer.

As described above, the breast (mammary gland in mice) is a highly dynamic organ, which undergoes growth and remodeling during pubertal development and subsequently as the gland adapts to the physiological requirements of lactation and postlactational involution. These normal developmental and morphogenic events are believed to influence the susceptibility of the mammary gland to carcinogenesis in both mice and humans.

Several classes of proteolytic enzymes have been implicated in mammary gland development and in the progression of preneoplastic and neoplastic breast lesions. It is widely believed that carcinogenesis often involves developmental pathways, including proteolytic pathways, that have gone awry in breast cancer. The vast majority of studied proteases are expressed in the mesenchymal compartment (e.g. fibroblasts and inflammatory cells) of the healthy mammary gland and in the stromal compartment of breast tumors, whereas others are expressed by epithelial cells. Below is a brief description of the protease classes most studied in connection with breast development and carcinogenesis. This section will focus on serine proteases, and specifically type II membrane anchored serine proteases, and provide a review of the literature that describes the roles of these enzymes in health and disease, including cancer.

1.2.1 Classes of proteases – a brief overview

Proteases were first described in 1905 as enzymes that cleaved other proteins (Levene *et al.* 1905). Today it is known that the human genome encodes at least 569 proteases. Based on the mechanism of catalysis, proteases are classified into five distinct classes: 1) aspartate proteases 2) threonine proteases 3) metalloproteinases 4) cysteine proteases and 5) serine proteases. The names of the classes refer to the amino acid residue used in their active site, except the metalloproteinase class, where the name refers to the zinc in the active site that interacts with a conserved cysteine residue. Proteases in general share a mechanism of performing a nucleophilic attack on the carbonyl-carbon of an amide bond (Chapman *et al.* 1997). Different types of proteases use different mechanisms to achieve this, but an irreversible cleavage of an amide bond is the end result for all these protease families. The ability of a tumor to metastasize into a distant site is directly related to its ability to invade into the surrounding tumor stroma and eventually into the blood stream and lymphatics (Mason *et al.* 2011). Historically, proteases have only been considered to play roles in the degradation of the extracellular matrix during cancer cell invasion, however in recent years they have gained attention for playing critical roles in many different processes in carcinogenesis including proliferation, apoptosis, signaling, migration and protein quality control. There have been a multitude of proteases studied in relation to breast cancer and this section introduces proteases and reviews previous protease studies.

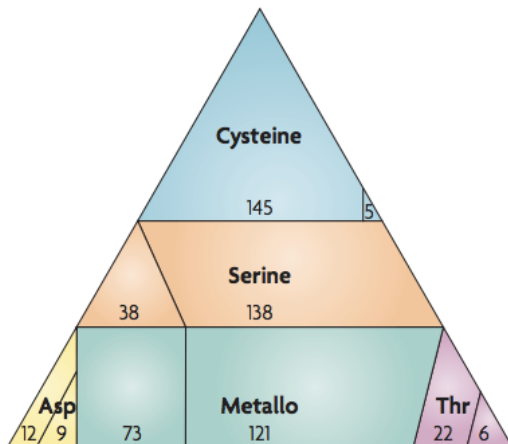


Figure 5: Graphical representation of the five classes of proteases in humans. Metalloproteases is the largest class with 194 members, followed by serine proteases with 176 members, cysteine protease class with 150 members, threonine protease class with 28 members, and the aspartate proteases with 21 members. Numbers on the left correspond to the number of intracellular proteases and the numbers on the right correspond to extra or pericellular proteases within the classes. Image reprinted with permission from Lopez-Otin *et al.* 2007.

1.2.2 Intracellular proteases

Each of the five main classes of proteases have members that are classified as intracellular proteases, members that are extracellular proteases, and some that may function either intracellularly or extracellularly depending on the cell types and circumstances. Typically, researchers associate intracellular proteases with the removal of damaged or harmful products from the cell (Lopez-Otin *et al.* 2007). These include lysosomal cysteine and aspartate cathepsins which aid in the degradation of damaged proteins in the cell. There are three main families of intracellular proteases: 1) caspases 2) deubiquitinases and 3) autophagins. The caspases are a family of cysteine proteases that play a crucial role in programmed cell death or apoptosis. One of Weinberg's "Hallmarks of Cancer" is the dysregulation of apoptosis and many studies have focused on alterations of caspases in malignancies (Hanahan *et al.* 2011, Teitz *et al.* 2000). Caspase 8 was at the forefront of caspase research when it was found to be epigenetically silenced in neuroblastomas, which overexpressed the *MYC* oncogene (Teitz *et al.* 2009). Caspase 8 was subsequently shown to be silenced

in many other tumor types and this silencing confers an increased risk of metastasis (Stupack *et al.* 2006). Caspases 3, 5, 6, 7, and 10 have also been found to be dysregulated or mutated in human tumors but it is unclear whether the mutations are the driving mutation or whether the mutations occur due to genomic instability once the cell is transformed (Park *et al.* 2002, Soung *et al.* 2004, Offman *et al.* 2005, Lee *et al.* 2006, Soung *et al.* 2003).

Protein quality control is imperative to cells in order to perform normal biological functions. The second major family of intracellular proteases, deubiquitinases (DUBs), is responsible for a large part of protein quality control in the cell. DUBs can be either cysteine proteases or metalloproteinases and are responsible for detaching ubiquitin from proteins that have been tagged for degradation and have been identified as tumor suppressors (Lopez-Otin *et al.* 2007). The cylindromatosis gene (CYLD), is a DUB that is mutated in patients with familial cylindromatosis, which causes tumors of skin appendages (Bignell *et al.* 2000, Lopez-Otin *et al.* 2007). CYLD's normal function; that is linked to limiting tumor progression, is to deubiquitinate components of the nuclear factor- κ B (NF κ B) pathway e.g. tumor necrosis factor (TNF) and B-cell lymphoma 3 (BCL3) (reviewed in Simonson *et al.* 2007).

The final major family of intracellular proteases is the autophagins. These are cysteine proteases responsible for the cell death process called autophagy. Typically thought to be a response to overwhelming cellular stress, autophagy has now been shown to be a tumor suppressive mechanism that cells undergo when genomic stability is compromised (Marino *et al.* 2007). More research

needs to be conducted to further elucidate the role of the autophagins. However, autophagin 3 (Atg4c) is knock-out mice display a higher incidence of fibrosarcomas, indicating that this autophagin is a tumor suppressor protease (Marino *et al.* 2007).

1.2.3 Extracellular proteases

The second class of proteases are the extracellular proteases, meaning their catalytic function occurs outside of the cellular membrane. The extracellular proteases can be divided into five different families 1) matrix metalloproteinases (MMPs), 2) a disintegrin and metalloproteinase or a disintegrin and metalloproteinase with thrombospondin motifs (ADAM/ADAMTSs), 3) cathepsins, 4) aspartate and threonine proteases and 5) serine proteases. These extracellular proteases play a wide variety of roles in development and homeostasis, as well as disease. The first four families will be briefly described below along with a more comprehensive focus on the serine proteases.

The family of MMPs can be further divided into three distinct but not all encompassing groups: 1) The collagenases consisting of MMP-1,8,13, and 14, 2)The stromelysins containing MMP-3,7, and 10, and 3) The metalloelastases, containing MMP12. There are 24 members in this entire family and many of the members have redundant functions (Brinckerhoff 2002). Thirteen of the MMPs are secreted proteases, 7 of them are membrane anchored and 4 can be either secreted or membrane anchored. A shared characteristic of all of the MMPs is the ability to degrade all components in the extracellular matrix, which lends to their role in tumor progression. Due to this function, the MMPs were initially

thought to predominantly be involved in tumor invasion and metastasis by direct cleavage of extracellular matrix components. Further implicating MMPs in cancer, many different MMPs are documented as being overexpressed in a variety of cancers (Liotta *et al.* 1980). It is now clear that MMPs play diverse roles in tumorigenesis. A challenge that comes with classifying so many different proteases in the MMP family is the fact that different MMPs are expressed in different cellular compartments and cell types. All MMP members have been detected in tumor tissue and about half have been confirmed to have an association with cancer using genetic mouse models. The substrates of MMPs include growth factors, cell surface receptors, adhesion molecules, protease zymogens and cytokines which are all intimately linked to cancer progression. As a result of MMP-mediated proteolytic cleavage, activation or inactivation of these substrates influence key processes such as proliferation, apoptosis, inflammation, invasion and angiogenesis (reviewed in Fingleton *et al.* 2006). MMPs in breast cancer will be reviewed later in the section in “Elucidating the Role of Extracellular Proteases in Breast Cancer *in-vivo*”.

The ADAM and ADAMTSs families contain 22 and 19 members, respectively. These proteases are closely related to the MMPs, however, genetic deficiency models have shown that ADAM/ADAMT proteases, in contrast to MMPs, are crucial for development and survival. Thus, several knockout models have resulted in embryonic lethality (Edwards *et al.* 2008, Apte *et al.* 2009). These severe phenotypes have implicated the ADAMs and ADAMTSs in fundamental biological functions including organogenesis. Although some

ADAMs are expressed in tumors, confirmation of a causal relationship has not established (Lopez-Otin *et al.* 2007, Apte *et al.* 2009, Joyce *et al.* 2011). Many ADAMTSs have been identified as potential targets for anti-angiogenic therapies. The most studied ADAMTS in this respect is ADAMTS1. ADAMTS1 inhibits angiogenesis by binding to the vascular endothelial growth factor (VEGF) which dampens the ability of VEGF to bind its receptor, vascular endothelial growth factor receptor (VEGFR)-2 which stimulates the growth of new blood vessels (Luque *et al.* 2003). Lack of ADAMTS1 allows VEGF to bind to its receptor to promote vascular growth in both humans and mice (Iruela-Arispe *et al.* 2003). ADAMTS1 has been shown to be downregulated in breast, colorectal and lung carcinomas, implicating it in the metastasis of these cancers (Lopez-Otin *et al.* 2007).

The next family of extracellular proteases is the cathepsins. There are 15 members in total, 2 serine cathepsins, 2 aspartate cathepsins and 11 cysteine cathepsins. The cathepsins that have been most studied are the cysteine cathepsins. Cysteine cathepsins are usually located in the lysosomes and are typically considered intracellular. However, in malignancies several cysteine cathepsins been shown to be secreted from cells (reviewed in Mason *et al.* 2011, Sloane *et al.* 2013). The causal relationship between cysteine cathepsins and cancer progression has been proven through inhibition studies or mutant mouse models (Mohamed *et al.* 2006). Cathepsin K is a cysteine cathepsin expressed in osteoclasts and inflammatory cells such as macrophages. This cathepsin has received attention for its role in tumor metastasis to bone. Herroon *et al.* showed

that cathepsin K activity is integral for prostate cancer cells ability to metastasize to bone possibly by contributing to the activation of chemokine ligand 2 (CCL2) and cytochrome oxidase subunit 2 (COX-2) related pathways (Herroon *et al.* 2012).

1.2.4 Aspartate proteases and threonine proteases

The aspartate protease family is a relatively small group of proteases that have not been studied as extensively as the other classes. It encompasses intracellular and extracellular proteases, however, the vast majority are intracellular (reviewed in Mason *et al.* 2011 and Ghosh *et al.* 2010). The aspartate proteases use an aspartate residue for catalysis of their peptide substrates and are optimally active at acidic pH. While research is underway, only limited information is available about this class of proteases as a whole. An example of an aspartate protease is cathepsin D. This protease is localized in the lysosomes of hepatocytes and is involved in insulin degradation (Ghosh *et al.* 2010). Another example of an aspartate protease is pepsin. Pepsin is one of the principal proteases that aid in the digestion of food in the gut (Ghosh *et al.* 2010). Threonine proteases use a threonine residue in their active site and the prototypic members of this class of enzymes are the catalytic subunits of the proteasome. These components of the proteasome are located in both the cytoplasm and nucleus of eukaryotic cells and function to degrade damaged or misfolded proteins (Reviewed in Kruger *et al.* 2001).

1.2.5 Secreted serine proteases

Classically, secreted serine proteases have been associated with biological processes like digestion, blood clotting and the complement system. As this class of proteases has expanded, researchers are finding a wide variety of novel functions, both in biological and pathological processes. A class of protease inhibitors called serpins serves as endogenous regulators of proteolytic activity for many members of the serine protease family.

One of the most studied secreted serine protease systems, not only in breast cancer research but in a variety of biological processes including thrombolysis/fibrinolysis and wound healing, is the urokinase type plasminogen activator (uPA) and plasminogen/plasmin (plasminogen activation system). Although it has the ability to cleave multiple targets, uPA's primary physiological substrate is the zymogen plasminogen, which is cleaved into the active serine protease plasmin. Plasmin efficiently degrades thrombus-bound fibrin in the vasculature as well as extravascular tissue fibrin. In order for the proform of uPA (pro-uPA) to become activated it must be bound to its receptor, urokinase plasminogen activator receptor (uPAR). Upon binding to uPAR, pro-uPA is cleaved and activated by plasmin, thus creating a uPA/plasmin activation feedback loop (Dano *et al.* 2005, Mason *et al.* 2011). The plasminogen activation system demonstrates a complex interplay between the tumor cells and the tumor stroma as uPA is expressed by stromal cells in the breast microenvironment (including myofibroblasts and macrophages) and uPAR is expressed on stromal cells and on epithelial- derived breast carcinoma cells. uPA and uPAR are over-

expressed in the invasive areas of tumors and their microenvironments of the breast, lung, esophagus, stomach and colorectum (Dass *et al.* 2008, Duffy *et al.* 2004, Gandolfo *et al.* 1996, Dano *et al.* 2005, reviewed in Degen *et al.* 2007). This uPA/uPAR/plasminogen activation system has also been extensively studied for its role in the degradation of the extracellular matrix (mainly fibrin) allowing breast cancer cells to metastasize (Edwards *et al.* 1998, Mason *et al.* 2011, Qiu *et al.* 2007, Degen *et al.* 2007, Dano *et al.* 2005).

Another commonly studied family of secreted serine proteases is the tissue kallikreins. This family contains 15 members that are expressed in a wide variety of tissues. The most well-known member of this family is kallikrein 3, also known as prostate-specific antigen (PSA). PSA is used as a biomarker in men for early detection of prostate cancer or as an indicator of prostate cancer progression (Diamandis *et al.* 1998). Although the biology of the kallikrein proteases is incompletely understood, numerous studies show they are dysregulated in cancers. In addition to kallikrein 3, kallikreins 2 and 11 are also emerging as potential biomarkers for prostate cancer and kallikreins 5, 6, 7, 10, 11 and 14 have shown promise as predictive factors in ovarian cancer. In addition, studies have revealed kallikrein 6 as being activated in the early events of squamous cell carcinogenesis (reviewed in Paliouras *et al.* 2007).

1.2.6 Membrane anchored serine proteases

All members of the membrane anchored serine protease family have a S1 peptidase catalytic domain and many have been shown to play integral roles in tissue homeostasis and disease including cancer progression (Rawlings *et al.*

1993, Bugge *et al.* 2009). These proteases are synthesized as inactive zymogens and must undergo a cleavage event to become functionally active enzymes. The membrane anchored serine proteases can be divided into three groups based on the way they are tethered to the plasma membrane: 1) a carboxy terminal glycosylphosphatidylinositol (GPI) anchor, 2) a carboxy-terminal transmembrane domain (Type I), or 3) an amino terminal proximal transmembrane domain (Type II). This dissertation project focuses on a specific Type II transmembrane serine protease (TTSP), matriptase, and this review details the TTSP family of proteases but briefly describes other membrane serine proteases.

1.2.6.1 Type I transmembrane serine proteases

The only known type I transmembrane serine protease is tryptase- γ 1. This protease is expressed exclusively in cells of the hematopoietic lineage and has been most studied in mast cells (Reviewed in Antalis *et al.* 2011). The carboxy-terminal signal anchor domain causes it to be retained on the outside of the cell when mast cells are stimulated to release their secretory granules. Since mast cells play a large role in immunosurveillance, they have been implicated in asthma as well as other pathological conditions (Antalis *et al.* 2011). Recombinant tryptase- γ 1 in the trachea of mice induces an increased expression of interleukin-13 (IL-13) in fluids of the lungs and airway hyper-responsiveness (Caughey *et al.* 2007). Beyond this, the physiological functions of tryptase- γ 1 remain unknown.

1.2.6.2 GPI anchored serine proteases

There are two proteases in this group, testisin and prostasin. Both of these proteases are anchored to the membrane via a GPI anchor that is added posttranslationally in the endoplasmic reticulum. Testisin (PRSS21) was initially discovered by Hooper *et al.*, in 1999 as a tumor suppressor expressed in the germ cells in the testis and in eosinophils. One biological function of testisin is to induce maturation of spermatozoa and their fertilizing ability. In testisin deficient mice, spermatozoa have decreased mobility and abnormal morphology (Netzel-Arnett *et al.* 2009). In cancer, it has been shown that epigenetic silencing of the testisin gene promotes testicular carcinogenesis (Manton *et al.* 2005). Conversely, in ovarian cancer, increased testisin levels have been correlated with increased tumor stage (Shigemasa *et al.* 2000). When ovarian cancer cells lines overexpressing testisin are injected into severe combined immunodeficient mice the tumors grow larger and at a faster rate (Tang *et al.* 2005). More work is needed to determine the molecular mechanisms by which testisin influences testicular and ovarian carcinogenesis.

Prostasin is also known as channel-activating protease 1 (CAP-1) since it was initially found to activate epithelial sodium channels (Vallet *et al.* 1997). Prostasin is more widely expressed than testisin and has been found in the epithelial cells of the prostate, colon, lung, kidney, pancreas, salivary gland, liver and bronchi (Yu 1994). Interestingly, similarly to testisin, prostasin levels have been found to be lost in prostate cancer but upregulated in ovarian cancer. Studies assessing prostasin levels in serum of ovarian cancer patients that have undergone oophorectomy suggest that prostasin is shed from the surface of

ovarian cancer cells and may be a candidate for an ovarian cancer biomarker (Costa *et al.* 2009, Mok *et al.* 2001, Chen *et al.* 2004). In addition to its implications in ovarian and prostate cancer, levels of prostasin have also been shown to be down-regulated in bladder and colorectal cancers (Chen *et al.* 2009, Selzer-Plon *et al.* 2009)

Particularly relevant to this work, the type II transmembrane serine protease matriptase is the physiological activator of the pro-form of prostasin in the epidermis (Netzel-Arnett *et al.* 2006). Recent studies have suggested, that in certain cells/tissues there is a complex reciprocal activation mechanism between the two proteases (Szabo *et al.* 2012, Friis *et al.* 2013). Another study has placed prostasin upstream of matriptase in a critical pathway involving activation of protease activated receptor-2 (PAR-2) in neural tube closure (Camerer *et al.* 2010). Cell culture studies have revealed that prostasin has the ability to cleave the epidermal growth factor receptor(EGFR), however, the physiological relevance of this cleavage has yet to be determined (Chen *et al.* 2008, Chen *et al.* 2009). Finally, prostasin has also been implicated in breast cancer as part of a matriptase-prostasin proteolytic cascade and prostasin is co-expressed with matriptase in the epithelial compartments of human invasive ductal carcinomas (Bergum *et al.* 2011).

1.2.6.3 Type II transmembrane serine proteases

A new family of serine proteases has emerged from the Whole Genome Sequencing project that was completed at the turn of the century. The Type II Transmembrane Serine Protease (TTSP) family contains 17 known members in

humans and each have an intracellular N-terminal signal anchor domain and an extracellular C terminus, which contains a serine protease domain. Each member of this family contains varying other domains, which make up the stem region, whereas the N-terminal signal anchor and the C-terminal serine protease domains are characterizing features of this family (Bugge *et al.* 2009, Antalis *et al.* 2013). [Figure 6] In contrast to many other types of serine proteases (i.e. the plasminogen activation system and several cathepsins) TTSP's have been shown to be mainly involved in maintaining homeostasis rather than returning the tissue to homeostasis after tissue damage (Bugge *et al.* 2009). In addition to maintaining homeostasis, matriptase has been shown to play a crucial role in development. Knockout mouse models have shown that matriptase deficiency is lethal due to malformed tight junctions in epidermal and intestinal epithelia (see 1.3.2 Physiological Functions) (List *et al.* 2002). The 17 members of the TTSP family have been divided into four subfamilies: 1) Human airway trypsin-like protease/differentially expressed in squamous cell carcinoma (HAT/DESC)

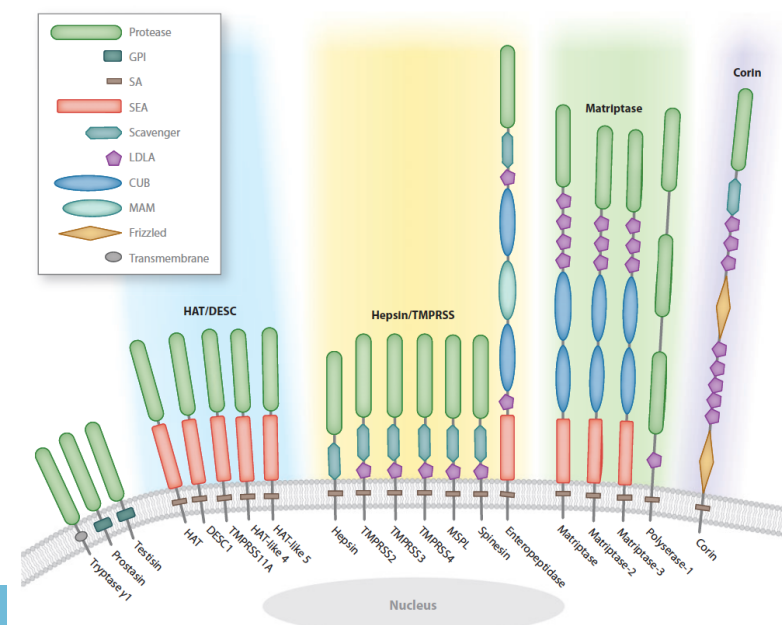


Figure 6: Depiction of all the membrane anchored serine protease. Shows the type I transmembrane serine protease, trypsin- γ 1, the GPI anchored proteases prostatic and testin and the entire family of type II transmembrane serine protease. Figure reprinted with permission from Szabo *et al.* 2011.

subfamily, 2) Hepsin/Transmembrane Serine Protease (TMPRSS) subfamily, 3) Matriptase subfamily and 4) Corin subfamily.

HAT/DESC subfamily

The HAT/DESC subfamily is the least studied. This family has five members in humans, HAT, HAT-like 4, HAT-like 5, DESC1, and TMPRSS11A. The physiological substrates of these proteases are not well characterized, however, the proteases have been showed to be upregulated during inflammation and pathological processes such as carcinogenesis where inflammation is present (Antalis *et al.* 2013). Mucus production, airway maintenance, and cleavage of other proteases have all been proposed roles of the HAT-like proteases (Webb *et al.* 2011). DESC1 was first studied in head and neck carcinomas and found to be downregulated during carcinogenesis (Lang *et al.* 2001). DESC1 has been shown to cleave many different components of the extracellular matrix, including fibrin and collagen (Hobson *et al.* 2004, Antalis *et al.* 2013). HAT proteases have been reported to activate PAR-2 as well as uPAR. Furthermore, HAT can uncoat reovirus virions to promote infection in cell culture models (Yoshinaga *et al.* 1998, Iwakiri *et al.* 2004, Nygaard *et al.* 2011, Baron *et al.* 2012). Ongoing studies are exploring the potential of DESC1 as a therapeutic target in some types of tumors (Webb *et al.* 2011) and HATL-5 has recently been shown to be differentially expressed in epithelial tumors (Miller *et al.* 2014).

Hepsin/TMPRSS subfamily

This subfamily contains seven members, Hepsin, TMPRSS2, TMPRSS3, TMPRSS4, TMPRSS5, TMPRSS13/mosaic serine protease long form (MSPL),

and enteropeptidase. Hepsin was named due to its abundant expression in the human liver, but is also expressed in the kidney, pancreas, stomach, prostate and thyroid (reviewed in Antalis *et al.* 2013). Studies of knockout mouse models of hepsin have demonstrated that this protease plays an important role in cochlear development and that these mice also have low levels of the thyroid secreted hormone, thyroxine (Guipponi *et al.* 2007, Antalis *et al.* 2013, Klezovitch *et al.* 2004). It is still unclear if the low levels of thyroxine are linked to the cochlear defects in the hepsin null mice (Guipponi *et al.* 2007). Hepsin also appears to be crucially involved in prostate cancer metastasis. In 2004, Klezovitch *et al.* used transgenic mice to show that the overexpression of hepsin leads to a disorganized basement membrane and promotes prostate cancer metastasis to the liver, bone and lungs (Klezovitch *et al.* 2004).

The physiological role of TMPRSS2 is currently unknown, however, a protein resulting from a common fusion between the TMPRSS2 promoter and the ERG (ETS-related gene) transcription factor has been shown to be highly expressed in many prostate cancer patients. The TMPRSS2 androgen responsive promoter drives an abnormally high expression of the ERG transcription factor to promote tumor progression and invasion (Tomlins *et al.* 2005, Antalis *et al.* 2013).

TMPRSS3 was originally isolated as a protease that was overexpressed in ovarian, pancreatic and breast cancer (Underwood *et al.* 2000). However, TMPRSS3 also plays a crucial role in human autosomal recessive deafness. Scott *et al.* was the first to show that mutations within the gene causes this

condition in multiple families and it has now been demonstrated in over 20 families (Scott *et al.* 2001, Antalis *et al.* 2013). The mechanism by which TMPRSS3 contributes to hearing remains unclear. TMPRSS4 mRNA has been shown to be upregulated in several types of cancers, however, little is known about its physiological functions. Regarding pathological functions, it has been demonstrated that TMPRSS4 can activate influenza virus hemagglutinin and promote infection (Chaipan *et al.* 2009). TMPRSS5 is also known as spinesin and again, little is known about the physiological functions of this protease. In expression studies mRNA was detected in rat inner ear tissues possibly implicating this protease in hearing function (Antalis *et al.* 2013). TMPRSS13/MSPL cleaves and activates the pro-form of the hepatocyte growth factor (HGF) *in vitro* (Hashimoto *et al.* 2010, Szabo *et al.* 2011a). However, at this time the physiological function of TMPRSS13/MSPL remains unknown and its expression in cancer has not been described. The seventh family member was discovered in the early 1900's and has been extensively studied. Enteropeptidase is located in the intestines where it converts pancreatic trypsinogen into active trypsin, resulting in the subsequent activation of several protease zymogens that are essential for food digestion and nutrient absorption. Patients with enteropeptidase deficiency have impaired food digestion and many suffer from malnutrition and anemia (Hadorn *et al.* 1975). Replacement therapy with pancreatic extracts clinically restores that impaired proteolysis in the gut (Antalis *et al.* 2013).

Matriptase subfamily

This family only contains three family members: matriptase, matriptase-2 and matriptase-3. Despite their homology, each of these members appear to have very different biological functions, likely due to the fact that the three proteases display mostly non-overlapping expression patterns. Matriptase is widely expressed in epithelial tissues and can undergo autocatalytic activation (Takeuchi *et al.* 1999, Wu *et al.* 2003, Bugge *et al.* 2009, List *et al.* 2009, Szabo *et al.* 2011a, Antalis *et al.* 2013). Matriptase was originally discovered in 1993 as a new gelatinolytic activity in cultured breast cancer cells and subsequently cloned in 1999 by three different groups (Shi *et al.* 1993, Takeuchi *et al.* 1999, Kim *et al.* 1999, Lin *et al.* 1999). Matriptase will be further described in the next section. Studies of matriptase-2 knockout mice have identified its important role in iron metabolism. Matriptase-2 deficiency is the cause of the human autosomal recessive disorder called iron-refractory iron deficiency anemia (Finberg *et al.* 2008). Matriptase-2 is expressed in hepatocytes and suppresses the function of the hormone hepcidin, which is responsible for iron transport across the gut mucosa. When matriptase-2 is absent, the levels of hepcidin increase the rate of iron exported from hepatocytes is increased rapidly (Finberg *et al.* 2008, Antalis *et al.* 2013). Matriptase-3, the final member of this subfamily, displays a restricted expression pattern in human and mouse tissues (testis, ovary and eye) (Szabo *et al.* 2011a). Interestingly matriptase-3 is also expressed throughout the central nervous system (CNS), an organ system where no matriptase or matriptase-2 expression has been reported (reviewed in Antalis *et al.* 2013). The physiological function of matriptase-3 remains unknown (Szabo *et al.* 2011a).

Corin subfamily

This protease was named because it is abundantly expressed in the human heart. In 2009 Wu *et al.* demonstrated that corin was the physiological activator of the pro-atrial natriuretic peptide (pro-ANP). ANP is a hormone that regulates cardiac function and blood pressure (Wu *et al.* 2009). Corin deficient mice are hypertensive, have impaired cardiac function and develop cardiac hypertrophy (Wu *et al.* 2009, Antalis *et al.* 2013). Human corin variants with hypertension have been observed in the clinic and often develop accelerated hypertension and cardiovascular disease (Dries *et al.* 2005). Corin is also expressed in the pregnant uterus and plays a role in promoting trophoblast invasion (Cui *et al.* 2012). Importantly, it has recently been shown that many patients with preeclampsia have low levels of corin in the uterus, and corin gene mutations in pre-eclamptic patients have been identified (Zhou *et al.* 2013).

1.2.7 Elucidating the role of extracellular proteases in breast cancer *in vivo*

Elegant studies using knockout mouse models for different proteases demonstrate the crucial role of proteolysis during breast cancer progression. In 1997, Thomas Bugge *et al.* crossed a plasminogen (secreted serine protease) deficient mouse model with transgenic mice that carried the polyoma middle T antigen (PymT) oncogene that induces multifocal, bilateral mammary gland tumors in female mice. In this model, PymT expression is under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter. This MMTV-PymT model, which is further characterized by tumor metastasis to the lung, will be further described in section 1.5.

Plasminogen, once converted into the active serine protease plasmin, is able to degrade fibrin as well as many other extracellular matrix proteins. (Bugge *et al.* 1997). When the plasminogen deficient mice were crossed into the MMTV-PymT model the authors were unable to detect a difference in the formation of the primary mammary tumors in the plasminogen deficient mice compared to the controls. However, the incidence of lung metastasis, the total number of metastases and the total metastatic tumor burden in the plasminogen deficient mice was significantly reduced. This finding implied that plasminogen could be a viable drug target for antimetastasis therapy (Bugge *et al.* 1997).

A related study was conducted by Almholt *et al.* also used the MMTV-PymT model, but this time crossed with uPA deficient mice. uPA together with tPA are the main physiological activators of plasminogen. Again, these authors found that uPA deficient mice did not have altered primary tumor growth, whereas metastasis to the lungs was greatly reduced. This indicated that the uPA protease was also a candidate antimetastasis target and the authors suggested that inhibiting uPA may be less toxic than anti-plasminogen therapy since uPA has fewer physiological targets than plasmin (Almholt *et al.* 2005).

The MMP's have also been extensively studied in breast cancer progression and metastasis and have been associated with diverse effects in progression with some functioning as tumor suppressors whereas others have pro-tumorigenic properties. Unfortunately, likely owing to their broad functional roles, pan MMP inhibitors have largely failed in clinical trials due to off target

effects and severe side effects (Martin *et al.* 2008, Reviewed in Coussens *et al.* 2002).

Martin *et al.* examined the role MMPs in an *in vivo* mouse model of breast cancer, using the genetic MMTV-PyMT model crossed with mice that were deficient in either MMP7 or MMP9. These two MMP's have very different localization; MMP7 is located on the epithelial cancer cells whereas MMP9 is expressed in inflammatory cells in the tumor microenvironment. When the authors crossed the MMP7 deficient mice with the MMTV-PyMT mice they observed no difference in neither the primary tumor burden nor in metastatic burden compared to the control mice. When MMP9 mice were crossed into the tumor model, although there was no detectable difference in the tumor latency or growth of the primary mammary tumors, the metastatic lung tumor burden was significantly reduced. This suggests that inhibitors of MMP9 could have potential utility as therapeutics to decrease the metastatic potential of the cancer cells in breast cancer patients (Martin *et al.* 2008).

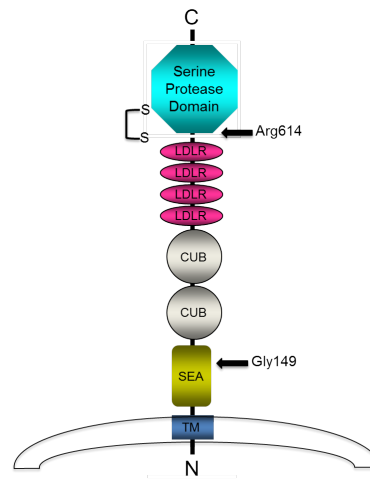
Sevenich *et al.* focused on two members of the cysteine protease class, cathepsin B (ctsB) and cathepsin Z (ctsZ). Despite the cathepsins general localization in the endosomal/lysosomal compartment, changes in distribution during neoplasia are associated with secretion of these enzymes and resultant extracellular effects. Interestingly, ctsB has been shown to relocate to the cell surface of epithelial cells in cancer and plays a role in the degradation of the extracellular matrix by activating plasminogen. Therefore they are included in this section. These investigators crossed ctsZ null mice with ctsB null mice that also

carried the PymT oncogene. Mice with double deficiency i.e. null for both *ctsB* and *ctsZ* showed a significant delay in primary tumor growth as well as reduced metastases to the lungs implying roles in primary breast tumor growth as well as in progression. Interestingly, in this model both *ctsB* and *ctsZ* needed to be simultaneously ablated to see the anti-cancer effects, potentially secondary to functional overlap between the two proteases, which allows one to compensate when the other is knocked out (Sevenich *et al.* 2010).

1.3 Matriptase

This work has focused on the TTSP matriptase. This transmembrane serine protease has recently emerged as a protease with oncogenic properties. As described below, matriptase has been implicated in many types of epithelial cancers and this study sought to specifically investigate the role of matriptase in breast cancer.

Figure 7: Schematic showing matriptase in the cell surface. Matriptase contains an N-terminal transmembrane domain, a single SEA domain, two CUB domains, four LDLA domains and a C-terminal serine protease domain. Arrows indicate cleavage points.



1.3.1 Expression, structure and substrates

Originally isolated in 1993 from breast cancer cells, matriptase, also known as ST14, contains an N-terminal signal anchor and a C-terminal serine protease domain, which is located on the extracellular side of the cell (Shi *et al.*

1993, Lin *et al.* 1997). In addition to these domains, matriptase also contains a single sea urchin sperm protein (SEA) domain, two complement C1r/C1s, Uegf, Bmp1 (CUB) domains and 4 low density lipoprotein A (LDLA) domains. Although these domains are non-catalytic they appear to play crucial roles in the cellular localization and the substrate specificity of the protease (Shi *et al.* 1993, Lin *et al.* 1997, Lin *et al.* 1999). Matriptase is synthesized as a single chain inactive zymogen in the rough endoplasmic reticulum and is transported to the plasma membrane through the Golgi apparatus (Takeuchi *et al.* 2000). The exact mechanism(s) leading to matriptase activation are not well understood, but requires two endoproteolytic cleavages (Figure 7) (Cho *et al.* 2001). The zymogen must be cleaved after Gly149 in the SEA domain, which separates the signal anchor domain from the remaining portion of the protease. However, the protease remains tethered to the cell membrane by a non-covalent bond (Cho *et al.* 2001). The second proteolytic cleavage occurs after Arg614 in the serine protease domain. Once this cleavage occurs the protease domain remains tethered to the rest of the protease by a disulfide bond (Figure 7). Once these two cleavages have occurred matriptase is an active serine protease (Takeuchi *et al.* 2000, Cho *et al.* 2001, List *et al.* 2006a). Importantly, matriptase has the ability to autoactivate, a rare property among serine proteases (Lee *et al.* 2006).

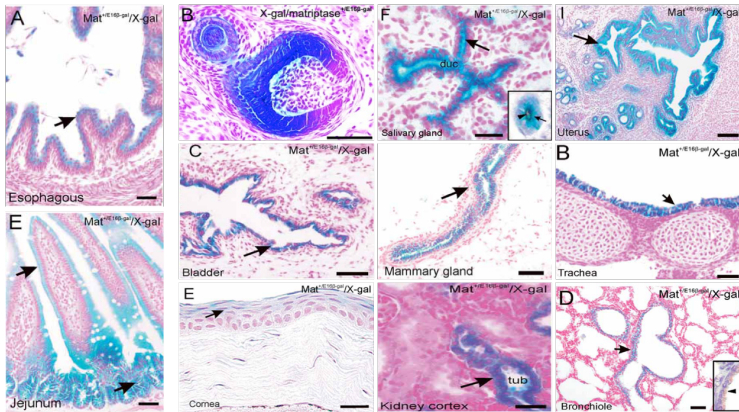


Figure 8- X-gal stained tissue pictures showing matriptase's expression (in blue). Matriptase has been shown to be expressed in the esophagus, bladder, hair follicle, mammary gland, salivary glands, uterus, trachea, jejunum, cornea, kidney and bronchioles.

Matriptase is involved in various biological and pathological processes and has a large variety of substrates of which only a few have been verified to be relevant *in vivo* (see Physiological and Pathological Functions below). Matriptase is expressed in epithelial cells throughout many tissues of the body, including skin, cornea, salivary gland, oral and nasal cavities, thyroid, thymus, esophagus, trachea, bronchioles, alveoli, stomach, pancreas, gall bladder, duodenum, small intestine, colon, rectum, kidney, adrenals, urinary bladder, ureter, seminal vesicles, epididymis, prostate, ovaries, uterus, cervix, vagina, and breast/mammary gland) (Figure 8). (Oberst *et al.* 2003, List *et al.* 2006a,b). In addition to these epithelial cells, matriptase expression can also be found in some types of immune cells, such as mast cells, monocytes and macrophages (Bhatt *et al.* 2007, Cheng *et al.* 2007). Using a cell free approach matriptase is able to cleave the inactive pro-forms of uPA, HGF, and to activate the G-protein-coupled PAR-2 (Lee *et al.* 2000, Takeuchi *et al.* 2000). In a 2007 study using transcriptional profiling, additional substrates of matriptase were identified including macrophage-stimulating protein 1 (MSP-1), a transmembrane protein that is activated by src (TRASK), VEGFR-2 and insulin-like growth factor binding

protein-related protein-1 (IGFBP-rP1) (Bhatt *et al.* 2007). The substrates that have been identified for matriptase have diverse localizations and functions, indicating that matriptase may play a key role in many molecular pathways. However, only few substrates have been verified *in vivo* including the GPI-anchored serine protease prostaticin and pro-kallikreins in the epidermis (Netzel-Arnett *et al.* 2006, List *et al.* 2006a, List *et al.* 2007, Sales *et al.* 2014).

Matriptase has two cognate inhibitors, hepatocyte growth factor activator inhibitor 1 (HAI-1) and HAI-2. Other inhibitors of matriptase have been identified including antithrombin III, α -1-antitrypsin and α -2-antiplasmin; however, HAI-1 and HAI-2 are the only inhibitors that have been shown to be physiologically relevant (List *et al.* 2006a, Nagaike *et al.* 2008, Szabo *et al.* 2008). Matriptase is usually co-expressed with HAI-1 and HAI-2 and some researchers argue that co-expression with one of these two proteins is necessary for trafficking matriptase to the cellular membrane, though this is still a controversial hypothesis (Wu *et al.* 2003, Oberst *et al.* 2005, Carney *et al.* 2007).

1.3.2 Physiological functions

Since matriptase is expressed in such a wide variety of epithelial tissues as well as immune cells, determining relevant physiological roles for matriptase involves analysis of a wide array of potential functions. Following matriptase's discovery, multiple genetic mouse models were created to learn about its function in both development and disease. Knockout mouse models have proven to be invaluable tools to determine the function of proteins, and the generation and study of matriptase null mice provided important insights into matriptase's

function (List *et al.* 2002, List *et al.* 2003, List *et al.* 2007). In 2002 List *et al.*, published a study that demonstrating that mice with a null deletion in both matriptase alleles died one to two days after birth due to severe dehydration. Matriptase was found to be critically involved in the formation of a functional epidermis and matriptase null mice are unable to prevent water loss from excessive evaporation through the skin. Further investigation led to the finding that matriptase is also crucial for several processes that take place during epidermal differentiation. These include lipid matrix formation in the cornified layer, cornified envelope morphogenesis and the shedding of corneocytes (List *et al.* 2002, List *et al.* 2006a, b). On the cellular level, matriptase deficiency affects the processing of a major epidermal structural protein, profilaggrin, which contributes to the skin impairments. Matriptase deficiency also causes the thymus to be hypoplastic and prevents hair follicles from maturing (See Table 1). Discussed more in the next section, humans with a missense mutation in their matriptase alleles have skin abnormalities further verifying matriptase's role in the formation and maintenance of a functionally intact epidermis in humans. (Basel-Vanagaite *et al.* 2007, Avrahami *et al.* 2008). Another physiological substrate of matriptase is the pro-form of the GPI-anchored serine protease prostaticin. Gene ablation studies demonstrated that matriptase and prostaticin were both required for the formation of a functional epidermal barrier and the null mice phenocopied one another (List *et al.* 2002, Leyvraz *et al.* 2005). Subsequent investigation showed that prostaticin was activated by matriptase in a proteolytic cascade which regulates terminal epidermal differentiation (Netzel-

Arnett *et al.* 2006). Since matriptase deficiency is perinatally lethal in mice, in 2009 List *et al.* generated a matriptase conditional knockout mouse using the tamoxifen-inducible Cre/Lox system. These mice can fully develop before the administration of tamoxifen ablates matriptase expression. (This model will be described in detail in section 4.) However, knocking out matriptase at a later timepoint in life was still lethal. List *et al.* demonstrated that the postnatal ablation of matriptase led to severe organ dysfunction with increased permeability, loss of tight junction function and general epithelial demise. These mice ultimately died roughly two weeks after the ablation of matriptase due to the dysfunctional epithelial structures and leaky tight junctions in the gut (List *et al.* 2009b).

Table 1:

Phenotypic characteristics of matriptase deficient mice:

Survival	<i>Postnatal lethality</i>
Body weight and body length	<i>Reduced</i>
External appearance of skin	<i>Reddish & wrinkled</i>
Hair follicles	<i>Immature</i>
Thymus	<i>Hypoplastic</i>
Inwards epidermal barrier function	<i>Impaired</i>
Outwards epidermal barrier function	<i>Impaired</i>
Corneocytes	<i>Enlarged</i>
Epidermal lipid composition	<i>Abnormal</i>
Filaggrin	<i>Absent</i>
Profilaggrin processing	<i>Impaired</i>
Epidermal differentiation markers besides profilaggrin/filaggrin	<i>Normal</i>
Pro-prostasin activation	<i>Absent</i>
Tight junctions	<i>Abnormal</i>
Saliva production	<i>Abnormal</i>
Intestinal barrier function	<i>Impaired</i>

Compiled from List *et al.*, 2002, List *et al.*, 2003, Netzel-Arnett 2006, List *et al.*, 2009

1.3.3 Pathological implications

There have been several reports describing mutations in the human matriptase alleles causing two different hereditary syndromes: autosomal recessive ichthyosis with hypotrichosis (ARIH) or ichthyosis, follicular

atrophoderma and hypotrichosis (Basel-Vanagaite *et al.* 2007, Avrahami *et al.* 2008). Patients with either of these conditions present with dry flaky skin and hair follicle dysplasia, which is associated with brittle slow growing hair. Both syndromes are indicative of an impaired skin barrier (Basel-Vanagaite *et al.* 2007, Avrahami *et al.* 2008, Antalis *et al.* 2013). Furthermore, this syndrome was phenocopied in a mouse model with low levels of matriptase (List *et al.* 2006b, described fully in section 4.1) In 2010, Sales *et al.* demonstrated that matriptase initiates a cascade that is linked to a mouse model of the human Netherton syndrome. Netherton syndrome is an autosomal recessive form of severe ichthyosis (Chao *et al.* 2005). In lympho-epithelial Kazal-type related inhibitor (LEKTI)-deficient mice matriptase was found to initiate the inflammatory pro-kallikrein-related proteases that are associated with stratum corneum detachment (Sales *et al.* 2010). LEKTI is a serine protease inhibitor and LEKTI deficiency causes excessive proteolysis and detachment of the stratum corneum in patients with Netherton syndrome (Chao *et al.* 2005, Sales *et al.* 2010). When the authors ablated matriptase expression from the LEKTI deficient mice the inflammation and symptoms of ichthyosis were diminished and the detachment of the stratum corneum was inhibited. This indicates that the kallikrein cascade (associated with Netherton syndrome) is dependent on activation by matriptase (Sales *et al.* 2010).

In addition to these skin-associated syndromes, matriptase is also being actively investigated regarding multiple links to the pathobiology of cancer. Matriptase has been implicated in cancer for over 2 decades since being detected in breast cancer cells (Shi *et al.* 1993). In many types of cancers, expression

studies have linked increasing matriptase levels to increasing tumor grade. This is true for breast cancer, prostate cancer, ovarian cancer, colorectal cancer and cervical cancer (Hoang *et al.* 2004, Saleem *et al.* 2006, Warren *et al.* 2008). Based on these findings and the fact that matriptase is very tightly regulated by HAI-1, several groups have analyzed the matriptase:HAI-1 ratio in tissues hypothesizing that a higher matriptase to HAI-1 ratio would be indicative of a higher grade tumor. Such a correlation has been verified for ovarian, prostate and colorectal cancers (Vogel *et al.* 2006, Saleem *et al.* 2006, Oberst *et al.* 2002).

1.3.4 Defining the role of matriptase in cancer using genetic mouse models

A role for matriptase as a critical factor in cancer initiation was first apparent with the generation of Keratin 5 (K5)-transgenic mice. This mouse model of matriptase and its contribution to our current understanding related to the physiological functions of matriptase and matriptase's role in cancer progression will be discussed next

List *et al.* in 2005 generated the K5 transgenic overexpression model of matriptase. This model was developed in order to gain further insight into the pro-oncogenic properties that matriptase was hypothesized to have based on prior observations showing its consistent up-regulation in epithelial tumors (Oberst *et al.* 2001, Benaud *et al.* 2002b, Kang *et al.* 2003, Oberst *et al.* 2003, Wu *et al.* 2003). The authors designed the model to express the matriptase gene under the K5 promoter which caused matriptase to be overexpressed exclusively in the basal layer keratinocytes of the epidermis. List *et. al.*, initially found that the overexpression of matriptase in the keratinocytes of these mice was sufficient to

cause spontaneous formation of squamous cell carcinomas. These tumors occurred independently of *ras* activating mutations and caused an up-regulation of the PI3K-Akt signaling pathway. This study demonstrated that matriptase is critically involved in cancer progression and that it is expressed at all stages of carcinogenesis (List *et al.* 2005). Furthermore, the spontaneous tumor formation could be exacerbated by the application of the genotoxic chemical 7,12-dimethylbenzanthracene (DMBA). When a single dose of DMBA was applied to the skin of K5-matriptase transgenic mice 95% of them formed tumors compared to just 1.7% of the control mice. The skin tumors resulting from the DMBA application were frequently accompanied by *K-ras* or *H-ras* mutations. Taken together, these studies indicate that matriptase promotes both *ras*-independent and *ras*-dependent malignant transformation (List *et al.* 2005).

Additional studies of the K-5 transgenic model demonstrated that in head and neck squamous cell carcinomas, matriptase, is co-expressed with HGF) and its receptor, c-Met. In addition, this study showed that the oncogenic potential of matriptase in these tumors is dependent on c-Met expression. Thus, when the investigators genetically ablated c-Met expression from the basal keratinocytes, they found that matriptase no longer promoted malignant transformation (Szabo *et al.* 2009a, b).

In addition to the initial matriptase null and matriptase K5 overexpression mice (List *et al.* 2002, List *et al.* 2005), two additional mouse models have been generated. The matriptase hypomorphic mice and the matriptase conditional

knockout mice (List *et al.* 2006b, List *et al.* 2009) will be explained in detail in section 4 on models used in the project.

1.3.5 Matriptase in breast cancer

Multiple studies have demonstrated the relationship between matriptase expression and breast cancer patient outcome (Benaud *et al.* 2002b, Welman *et al.* 2012, Charafe-Jauffret *et al.* 2006, Kang *et al.* 2003, Uhland *et al.* 2006). Matriptase is expressed in most breast cancer cell lines, however, its role *in vivo* and the molecular mechanisms and downstream signaling events underlying its expression in these tumors are less clear. The previously described study in squamous cells demonstrating that c-Met is essential for matriptase induced malignant transformation established matriptase as an initiator of the c-Met signaling cascade in the epidermis (Szabo *et al.* 2009a,b, Szabo *et al.* 2011b). Aberrant growth factor receptor signaling is common to many malignancies including breast cancer. In breast cancer the c-Met receptor is commonly dysregulated, although most patients do not have an activating mutation in the receptor itself (Szabo *et al.* 2011b, Uhland *et al.* 2006, Charafe-Jauffret *et al.* 2006). As mentioned, c-Met, its' ligand (HGF/SF) and matriptase are all expressed in the breast tumor microenvironment, and furthermore, all have been correlated with a poor patient outcome (Ghoussoub *et al.* 1998, Charafe-Jauffret *et al.* 2006, Camp *et al.* 1999). This expression of matriptase and c-Met and the discovery that matriptase has the ability to cleave the inactive form of HGF (pro-HGF) into active HGF prompted this study to investigate the functional relationship between matriptase and c-Met in breast cancer. (Lee *et al.* 2000).

1.4 HGF/c-Met in breast cancer

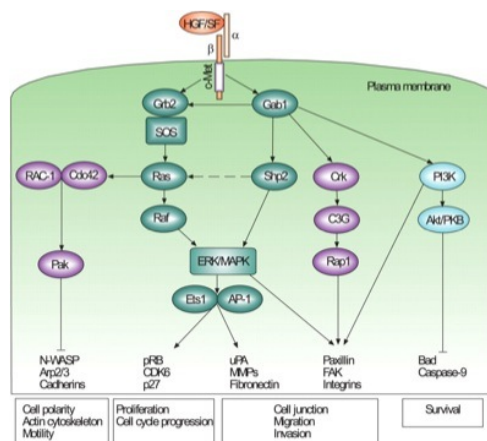
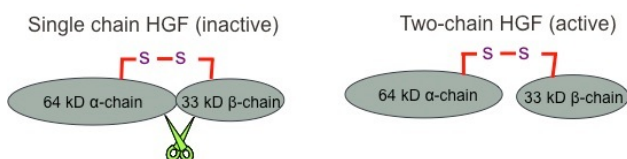
Multiple studies highlight the importance of the tumor microenvironment in breast cancer progression. However, mono-cell tumor culture models do not recapitulate potentially crucial tumor-stromal signaling exchanges. For instance, in ductal breast cancer, HGF is a mesenchymally derived growth factor and paracrine mediator that affects the proliferation, differentiation, motility, and invasiveness of the malignant ductal epithelial cells through an often dysregulated c-Met: HGF's tyrosine kinase receptor. In breast cancer patients this has been identified as a key signaling pathway that has been targeted in breast cancer therapeutics (Gherardi *et al.* 2012). In this section c-Met and HGF are reviewed focusing on their relevance to breast cancer.

1.4.1 Localization and activation of pro-HGF and c-Met in breast cancer

Pro-HGF is secreted by mesenchymal cells, mainly fibroblasts in the breast, as a single chain inactive enzyme that is unable to initiate the phosphorylation and signaling through its receptor c-Met. Although c-Met can bind pro-HGF, a specific cleavage event must occur converting pro-HGF into its two-chain active form for c-Met signaling to commence (Birchmeier *et al.* 2003, Owen *et al.* 2010, Gherardi *et al.* 2012). The c-Met receptor is located on the surface of epithelial cells within the breast, creating crosstalk between the mesenchymal cells, which secrete pro-HGF, to the epithelial cells which express its receptor. Upon cleavage of pro-HGF, the c-Met binding site on HGF's alpha subunit is exposed which has a much higher affinity for the receptor. Once this site binds to c-Met, cross phosphorylation of the receptor and its subsequent signaling is induced (Naldini *et*

al. 1995). This binding activates various signaling cascades through c-Met that promote survival, mitogenic, motogenic and proliferative responses (Birchmeier *et al.* 2003, Gherardi *et al.* 2012).

Figure 9: (Below) Schematic showing single chain inactive HGF and the cleavage required to convert HGF into its two chain activated form. (Right) Picture showing the c-Met signaling pathway and the possible cellular outcomes when the c-Met receptor is activated. Image reprinted with permission from <http://pharmaxchange.info/>.



Aberrant signaling of the c-Met oncogene is associated with poor patient outcomes for not only breast cancer, but also for prostate, ovarian, and head and neck cancer patients (reviewed Birchmeier *et al.* 2003, Gherardi *et al.* 2012). Logically following this observation, much attention has been focused on the identification of the protease(s) responsible for activating c-Met's cognate ligand pro-HGF in the breast microenvironment. (Lee *et al.* 2000, Owen *et al.* 2010, Oberst *et al.* 2003). The classical activator of pro-HGF is the hepatocyte growth factor activator (HGFA). This is a soluble serine proteinase, which is involved in the coagulation cascade (Shimomura *et al.* 1997, Birchmeier *et al.* 2003). HGFA is expressed in the liver and present in the blood circulation, but extensive studies of its localization throughout the the mammary gland and other areas of the body are lacking (Shimomura *et al.* 1997, Birchmeier *et al.* 2003, Owen *et al.* 2010, Gherardi *et al.* 2012). Other known proteinases that activate pro-HGF are hepsin, TMPRSS13 and matriptase (Owen *et al.* 2010, Hashimoto *et al.* 2010). Of these

known activators, the only activators known to be expressed in the breast and in breast tumors are matriptase and hepsin.

1.4.2 c-Met signaling

Once the c-Met receptor is activated, a number of effector proteins are recruited, Grb2-associated binding protein 1 (Gab1), Growth factor receptor-bound protein 2 (Grb2), phospholipase C and c-Src all bind directly to activated c-Met. Four major signaling cascades can subsequently be activated depending on other cellular processes and signals in the cell. The first pathway is the Rac/Cdc42 pathway which affects integrins and cadherins, and plays a role in cell migration and adhesion. The second pathway is the *Ras*-MAPK pathway, which is linked to proliferation, cell cycle progression, migration and invasion. The Rap/PAK pathway has been shown to activate cellular migration and invasion. The phosphatidylinositol kinase 3 (PI3K)-Akt pathway is a classical pro-survival pathway that has also been linked to proliferation and migration (Birchmeier *et al.* 2003, Gherardi *et al.* 2012).

These signaling outcomes of the activated c-Met impact many normal biological functions. Discovered for its role in liver and skin regeneration, c-Met signaling also plays major roles in cell migration during embryogenesis, wound repair and tissue regeneration (Bladt *et al.* 1995, Schmidt *et al.* 1995, Birchmeier *et al.* 2003, Gherardi *et al.* 2012).

Reciprocal interactions between epithelium and mesenchyme are believed to play essential roles in breast cancer progression. Furthermore, c-Met, like matriptase, is exclusively expressed on the surface of mammary epithelial cells.

This pathway has been commonly shown to be dysregulated in breast cancer patients, however the receptor itself is not often overexpressed and activation mutations are very rare. However, it appears that signal transduction itself is dysregulated (Ghoussaub *et al.* 1998, Owen *et al.* 2010, Raghav *et al.* 2012, Eder *et al.* 2009). To date, dysregulated c-Met has been implicated in 32 different types of cancers, including breast cancer (HGF/SF-Met in cancer-<http://www.vai.org/met>). The level of active c-Met signaling pathway factors in patient tumors is a prognostic factor, with a 5-year survival rate of 89.6% in patients lacking c-Met signaling in their tumors compared to only 52% for patients that have activated c-Met signaling (Ghoussaub *et al.* 1998, Owen *et al.* 2010, Gisterek *et al.* 2011, Raghav *et al.* 2012, Eder *et al.* 2009). There are 29 c-Met inhibitors in various stages of clinical trials. These agents have increased progression free survival and overall survival in lung cancer patients and also show promise in other cancers as well (Reviewed in Gherardi *et al.* 2012). (Clinical Trials involving HGF/SF-Met [inhibitors-http://www.vai.org/metinhibitors/](http://www.vai.org/metinhibitors/)).

1.5 Models used to study IDC and IBC

Mouse models are powerful tools that researchers can use to analyze mechanisms of disease *in vivo*. While mice can never completely mimic human disease, the knowledge gained from various animal models is invaluable. Multiple immortalized breast cancer cell lines are also important tools used to elucidate the role of various molecules *in vitro*. Often, initial studies in cell culture models are followed by studies in whole organism models, including mouse models. The

following section reviews several genetic mouse models of IDC, the benefits of xenograft models, as well as relevant human breast cancer cell line models.

1.5.1 Genetic mouse models of IDC

Numerous genetic mouse models of breast cancer have been generated (Reviewed in Herschkowitz *et al.* 2007). Although 13 different models have been developed, the three most common mouse models to specifically study IDC use the MMTV promoter to express oncogenes in the mammary gland and include: 1) Transgene MMTV promoter-Neu (TgMMTV-Neu), 2) TgMMTV-Wnt, 3) TgMMTV-PyMT (reviewed in Herschkowitz *et al.* 2007 and Vargo-Gogola *et al.* 2007). This promoter contains a glucocorticoid hormone response element which drives the oncogene expression in the mammary gland (reviewed in Vargo-Gogola *et al.* 2007).

TgMMTV-Neu

This model expresses the activated rat homolog of the oncogene c-Neu under the control of the MMTV promoter. The c-Neu receptor is also known as ErbB2 or Her2 and is a member of the epidermal growth factor family. Amplification of this gene is seen in 15-20% breast tumors in the clinic and overexpression of c-Neu in this mouse model causes the formation of mammary tumors (Guy *et al.* 1992a,b, Fantozzi *et al.* 2006). Since Neu is commonly overexpressed in breast tumors, targeted treatments have been developed (See Treatments section in breast cancer review). The MMTV-Neu model is well suited to study the efficacy of drugs designed to target this receptor. Importantly, the mice develop breast cancer in stages, similar to those stages observed in human

breast cancer (Urisini-Siegel *et al.* 2007). Precancerous lesions in mice are called mammary intraepithelial neoplasia (MIN) and in the TgMMTV-Neu model MIN closely resembles DCIS (Urisini-Siegel *et al.* 2007, See figure 10). However, advanced tumors induced with this model often coalesce to involve the entire mammary gland and have a latency of roughly seven months (Muller *et al.* 1988, Fantozzi *et al.* 2006). Overall this mouse model closely resembles human breast cancer progression resulting in the development of mammary tumors and lung metastasis (See figure 10). The drawbacks to using this model are that tumor latency is roughly seven months with only a 72% lung metastasis incidence with a latency of eight months (Reviewing in Fantozzi *et al.* 2006). Therefore, comprehensively studying the natural progression of IDC in this model is time-consuming and expensive to complete.

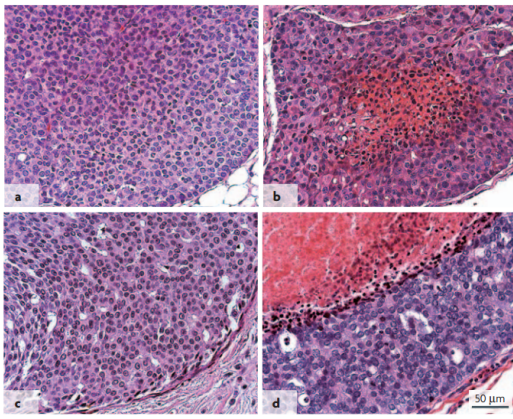


Figure 10: Histology showing MIN in MMTV-Neu mice compared to DCIS in human. (A) and (B) show MIN and MIN with a comedo pattern in MMTV-Neu mice respectively compared to DCIS and DCIS with comedo in (C) and (D). Image reprinted from Urisini-Siegel *et al.* 2007.

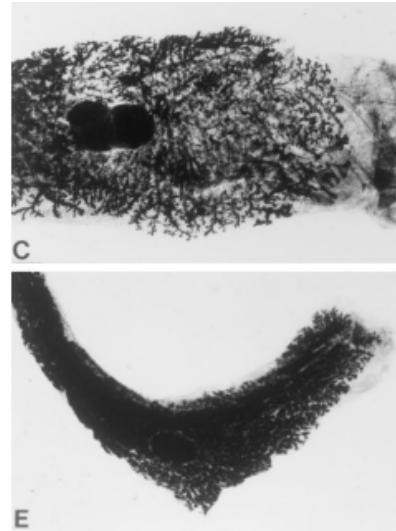
TgMMTV-Wnt-1

Wnt-1 was the first protooncogene to be discovered and inserted into mice with the MMTV controlled expression mechanism (Fantozzi *et al.* 2006). Wnt-1 is part of a large family of secreted intracellular signaling molecules. The major

event of Wnt-1 signaling is to bind to the Frizzled receptors on the cell surface to increase the level of cytosolic β -catenin. β -catenin heterodimers then translocate to the nucleus to transactivate a number of genes, including c-myc, another protooncogene (Li *et al.* 2000). Interestingly, Wnt-1 is not normally expressed in the mammary gland, so in terms of oncogene expression, this model may not be as relevant to human breast cancer as the Neu model described above (Li *et al.* 2000, Bocchinfuso *et al.* 1999). The genes that are induced by prolonged Wnt-1 expression in the MMTV-Wnt-1 model include β -catenin, c-myc, cyclin D1, E-cadherin and anaphase promoting complex (APC). Each of these genes have been found to be either dysregulated or mutated in breast cancer (Bieche *et al.* 1999) The tumors formed in this transgenic model of Wnt-1 are multifocal and also metastasize to the lymph nodes and lungs. However, the progression of mammary cancer does not replicate the multiple stages of human breast cancers, the pathology of these tumors present as normal or cancerous without stages in between (Fantozzi *et al.* 2006, Bocchinfuso *et al.* 1999). Even though Wnt1 is not normally expressed in the breast or in breast cancer, many associated components of the Wnt-1 signaling pathway are relevant. These include β -catenin, c-myc, cyclin D1, E-cadherin and APC making this an appropriate model to study the effect of experimental drugs or therapies which target these signaling molecules in breast cancer (Li *et al.* 2000). However, this model also has a long tumor latency time of about eight months with only 60% penetrance making the systems time consuming and expensive utilize. In

addition to the long tumor latency time, the incidence of metastasis is not well established (Li *et al.* 2000, Fantozzi *et al.* 2006).

Figure 11: Mammary gland whole mounts of mice expressing MMTV-Wnt-1. Top panel shows a mammary gland from a 10 week old mouse with the expression of MMTV-Wnt-1 and the bottom panel shows 6 month old mouse. Image reprinted and modified with permission from Bocchinfuso *et al.* 1999.



TgMMTV-PymT

Perhaps the most widely used mouse model of breast cancer is the MMTV-PymT model. The PymT oncogene, a component of the polyomavirus (PyV) family, is essential for transformation and tumorigenesis (Fluck *et al.* 2009). The prolonged expression of the PymT oncogene induces several signaling proteins associated with breast carcinogenesis including protein phosphatase 2A (PP2A), Src family tyrosine kinases and phosphatidylinositol kinase 3 (PI3K). Importantly, the tumors induced by this model are multifocal and tumor development more closely resembles human breast cancer progression than other models (Fluck *et al.* 2009). Another advantage to the MMTV-PymT model is the fact that tumor progression is associated with a similar evolution of hormone receptor status (ER, PR) to that seen in humans. Similar to human breast cancer, early tumors in this model are largely ER and PR positive. However,

more advanced tumors lose the expression of these receptors (Lin *et al.* 2003). Much like the MMTV-Neu model, MMTV-PymT model tumors also develop similar stages (progression model) that mimic human disease (See figure 12). This model also has the advantage of a relatively short (average of 2 to 3 months) latency time with 100% penetrance (Fluck *et al.* 2009, Fantozzi *et al.* 2006, Guy *et al.* 1992a,b). The incidence of metastasis is also favorable with a rate greater than 85% with a latency of about 3.5 months (Fantozzi *et al.* 2006, Guy *et al.* 1992a,b). The main drawback to using the MMTV-PymT model is that the PymT oncogene itself is not expressed in human cells. But much like the MMTV-Wnt-1 model, this model activates a number of relevant signaling molecules that are commonly upregulated in human breast cancers. These include but are not limited to, c-myc, ras, cyclin D1, Cdk2 and E2F (Guy *et al.* 1992a,b, Lin *et al.* 2003, Fluck *et al.* 2009). One characteristic pertaining to the MMTV-PymT model that should be taken into consideration is the fact that tumor latency is highly dependent on the mouse strain. This means that comparative studies should be performed in the same background strain, and littermate controlled studies should be used to minimize potential effects of genetic background variation (Almholt *et al.* 2013).


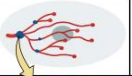
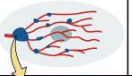

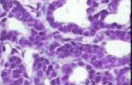
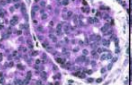
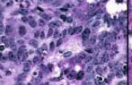
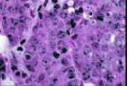
Stage	Hyperplasia	Adenoma/MIN	Early carcinoma	Late carcinoma
Gross				
H&E				
Cellular morphology	Basement membrane Lumen Epithelial cells Myoepithelium	Tumor cells Focal infiltration of leukocytes		
Biomarkers	ER++ PR+ Neu (T/D) ~ 1 Cyclin D1 + Integrin β ±	ER+++ PR++ Neu (T/D) † Cyclin D1 + Integrin β ±	ER++ PR± Neu (T/D) †† Cyclin D1 ++ Integrin β -	ER± PR- Neu (T/D) ††† Cyclin D1 +++ Integrin β -

Figure 12: Shows a summary of MMTV-PymT tumor progression. This model occurs in four stages 1) Hyperplasia 2) MIN 3) Early Carcinoma and 4) Late carcinoma. These stages are shown as a cartoon depiction of gross morphology, a hematoxylin and eosin staining of tissue, a depiction of the cellular morphology and the biomarkers of each stage are listed at the bottom. Image reprinted with permission from Fluck *et al.* 2009.

1.5.2 Xenografting Models

Xenografting is a commonly used technique where cells or tissue from one species is transplanted into another species. In the case of breast cancer, taking primary cells from human tumors or transplanting immortalized human breast cancer cell lines into mice has been a successful strategy to advance our understanding of this disease. Therefore, xenografting allows the investigation of breast cancer cell lines or tissue in an *in vivo* environment that recapitulates many tumor-stromal interactions.

However, xenografting studies must be meticulously designed and substantial preliminary data must be obtained to appropriately estimate the predictive power of this model (Reviewed in Sausville *et al.* 2006). Xenografts can either be ectopic or orthotopic. Ectopic xenografting refers to the injection of cells from one organ to a different site, (e.g. subcutaneous injections of breast cancer cells). Orthotopic xenografting refers to cells that are transplanted into the organ of origin (e.g. breast cancer cells placed into the mammary gland). Xenografts as preclinical models for breast cancer has been used successfully to evaluate drug activity, investigate signaling pathways and learn about protein expression during different stages of tumorigenesis (Reviewed in Vargo-Gogola *et al.* 2007).

Although xenografting models are useful in some situations, there are also several substantial pitfalls. Xenograft models require immunodeficient mice in which inflammatory and tumor immune responses that may be critical factors in the pathophysiology of breast cancer progression are muted or absent. In

addition, transplanting or injecting human breast cancer cell lines into a foreign host (the mouse) may not recapitulate aspects of the microenvironment present in the patient where the cancer initially developed and interacted with the surrounding stromal compartments. For instance, in recipient mice, the surrounding stromal cells are normal and have not adapted to support (or suppress) cancerous growth. Depending on the parameter being studied, these factors may affect the results and lead to inaccurate conclusions.

1.5.3 Human breast cancer cell lines

The most widely used models for breast cancer studies include human cultured breast cancer cell lines. These cell lines are developed from tumor cells collected from patients and kept alive in the laboratory. The cells are easily propagated, can be genetically manipulated and, under properly controlled conditions, can supply reproducible and quantifiable results (reviewed in Vargo-Gogola *et al.* 2007). Cell lines can be grown in two dimensions or in three dimensional (2D/3D) cultures. A drawback of using 2D culture is that the cellular microenvironment is drastically different from that in the human breast. However one can attempt to recreate this environment through the addition of extracellular matrix components, e.g. collagen. 3D culture aims to mimic the tumor microenvironment by surrounding breast cancer cells with components of the extracellular matrix and in some cases various stromal cells (Reviewed in Vargo-Gogola *et al.* 2007 and Rotherberg *et al.* 2011). Of the numerous breast cancer cell lines that have been developed, those most relevant to this project are listed in Table 2.

Table 2: Relevant Breast Cancer Cell Lines:

	ER	PR	Her2/Neu	c-Met	Matriptase	Subtype:
SUM229	-	-	-	+	+	IDC
HCC1937	-	-	-	+	+	IDC
BT20	-	-	-	+	+	IDC
SUM149	-	-	-	+	+	IBC
SUM190	-	-	+	+	+	IBC
SUM159	-	-	+	+	-	Anaplastic

CHAPTER 2- MATRIPTASE MEDIATED c-MET SIGNALING IN BREAST CANCER

2.1 Hypothesis and specific aims

The overall hypothesis of this project is that matriptase is critically involved in breast cancer progression and exerts its oncogenic functions through the proteolytic cleavage and activation of pro-HGF bound to c-Met, and that the resultant sequential signaling is critical for breast cancer progression and metastasis. To test this hypothesis three specific aims were generated:

1. Determine the significance of matriptase proteolysis in breast cancer progression.

The working hypothesis was that matriptase plays a critical role in breast cancer progression. To test this hypothesis, a novel matriptase loss-of-function mouse model that has been intercrossed with the MMTV-PymT transgenic breast cancer model has been established.

The matriptase hypomorphic model, in which the levels of matriptase protein are highly reduced in the mammary glands, was employed to study the effect of matriptase activity on mammary cancer progression.

2. Determine the underlying mechanism by which matriptase confers pro-tumorigenic properties to breast cancer cells.

The working hypothesis was that matriptase activates pro-HGF to elicit an epithelial-mesenchymal protease-mediated signaling axis where c-Met

signaling leads to the activation of mitogenic and invasive responses in breast cancer cells.

- a. *Matriptase deficient and matriptase sufficient murine primary mammary tumor cells were compared to determine the significance of the matriptase-mediated activation of the HGF/c-Met signaling pathway and the effect on cancer cell proliferation ex vivo.*
 - b. *Human breast cancer cell lines were used and matriptase expression was manipulated by RNAi silencing and by the addition of specific matriptase inhibitors. The ability of these cell lines to activate the pro-HGF/c-Met pathway and the functional consequences of matriptase inhibition on proliferation was determined.*
 - c. *Patient tissue samples of IDC were used to determine the distribution and correlation of matriptase and c-Met in human breast cancer tissue samples.*
3. Determine if matriptase proteolysis plays a role in the progression of the least characterized type of breast cancer, inflammatory breast cancer.

The working hypothesis was that matriptase plays a similar role in *inflammatory breast cancer and intraductal breast cancer.*

- a. *Human inflammatory breast cancer cell lines were used and matriptase expression was manipulated by RNAi silencing. The ability of these cell lines to activate the pro-HGF/c-*

Met pathway and the functional consequences of matriptase inhibition on proliferation was determined.

b. Human inflammatory breast cancer cell lines were used in 3D culture and matriptase expression was manipulated by RNAi silencing. The ability of these cells to grow in 3D in response to pro-HGF was determined.

c. Tissue samples from IBC patients was used to determine the distribution and correlation of matriptase and c-Met in human breast cancer tissue samples.

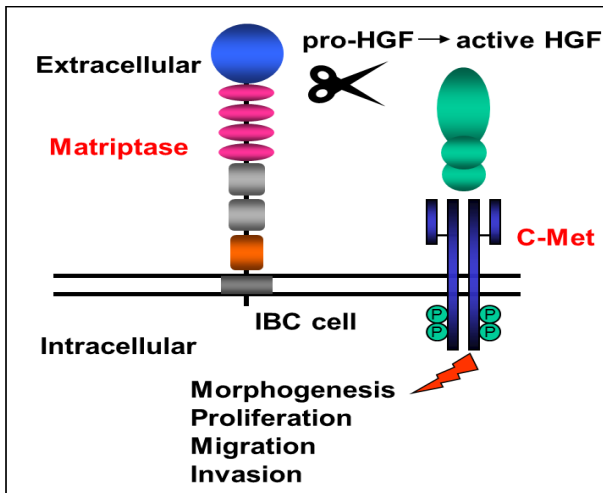


Figure 13: Matriptase is a multi-domain membrane anchored serine protease expressed on the surface of IBC cells. The HGF receptor, c-Met, is also expressed on the cell surface of IBC cells. HGF is secreted by stromal cells as an inactive precursor (pro-HGF). Matriptase plays a critical role in IBC progression by activating pro-HGF that elicits an epithelial-mesenchymal protease-mediated signaling axis whereby c-Met signaling leads to the activation of mitogenic, morphogenic, migratory and invasive responses in IBC cells.

CHAPTER 3: MATERIALS AND METHODS

3.1 Animals

All procedures involving live animals were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium following institutional guidelines and standard operating procedures. Generation of *ST14* (Matriptase) hypomorphic mammary tumor mice: Mice transgenic for the MMTV-Polyoma virus middle T oncogene (*PyVT*) in the FVB/N background (strain: *FVB/N-Tg (MMTV-PyVT)^{634Mul}*) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and crossed with females carrying an *ST14* null allele (*ST14^{+/-}*) (List *et al.* 2002) or an *ST14* knock-down/hypomorphic allele (*ST14^{+/hypo}*) (List *et al.* 2007) in the C57BL/6 background. F1 *PyVT/ST14^{+/-}* male pups were then mated to *ST14^{+/hypo}* or *ST14^{hypo/hypo}* females to produce the study cohorts of F2 hypomorphic female mice carrying the *PyVT* transgene, *PyVT/ST14^{-/hypo}* mice (referred to as: PymT-Mat^{hypo} mice) and littermate control mice, *PyVT/ST14^{+/+}*, *PyVT/ST14^{+/hypo}* or *PyVT/ST14^{+/-}* (referred to as: PymT-Mat⁺ mice. Genotyping of *ST14* wild-type, knockdown, and null alleles was performed by PCR of ear or tail biopsy DNA as described (List *et al.* 2002, List *et al.* 2007). Study animals were weighed and palpated weekly and tumor diameters in two dimensions were obtained using calipers. Study mice were euthanized by CO₂ inhalation at 90 days, 127 days and 145 days of age and tissues were removed for subsequent for analysis. Generation of *ST14* (Matriptase) conditional knock-out mammary tumor mice: The generation of the conditional matriptase knockout allele (*St14^{LoxP}*) as well as

the *ST14* null allele carrying a β -galactosidase expression cassette (*ST14*^{E16 β -gal}) have been described previously (List et al., AJP 2006, 2009). Interbreeding of mice carrying these *St14* alleles with mice carrying a 4-hydroxytamoxifen (4-OHT)-inducible Cre transgene (β -actin-Cre-ER^{tm+/0} mice, Strain: [B6.Cg-Tg(Cre/Esr1)5AMC/J] Jackson Laboratories), and FVB/N-Tg (MMTV-PyVT)^{634Mul} mice resulted in the generation of female (MMTV-PyVT)^{634Mul} / β -actin-Cre-ER^{tm+/0}; *St14*^{LoxP/E16 β -gal} study mice (referred to as PymT-Mat^{Cond. KO}) and their associated control littermates, (PyVT/ β -actin-Cre-ER^{tm+/0}; *St14*^{LoxP/+}, PyVT/ β -actin-Cre-ER^{tm+/0}; *St14*^{LoxP/E16 β -gal}, PyVT/ β -actin-Cre-ER⁰; *St14*^{LoxP/E16 β -gal}, PyVT/ β -actin-Cre-ER⁰; *St14*^{LoxP/+} (referred to as PymT-Mat⁺). The genotypes of all mice were determined by PCR of ear or tail biopsy DNA as described (List et al. 2006b, 2009).

3.2 Mammary gland whole mount analysis

Mammary glands were fixed for four hours in Carnoy's Fixative (60% Ethanol, 30% Chloroform, 10% Acetic Acid) at room temperature. The glands were then rehydrated and stained in Carmine Alum (0.2% Carmine, 0.5% Aluminum Potassium Sulfate) overnight. Upon dehydration, glands were cleared in xylene and embedded in Permount Mounting Medium (Fisher Chemicals, Fair Lawn, NJ). The area of cancerous lesions relative to the area of the entire mammary gland was determined with Image J (NIH software).

3.3 Immunohistochemistry- Mouse tissues: Mice were euthanized by CO₂ inhalation. Tissues were fixed in 10% neutral-buffered zinc formalin (Z-fix) (Anatech, Battle Creek, MI), embedded in paraffin, and cut into 5- μ m sections. The

immunohistochemical procedures were performed as previously described (List *et al.* 2009). Primary antibodies used were: sheep anti-matriptase and goat anti-c-Met (R&D Systems, Minneapolis, MN). Cell proliferation was visualized by intraperitoneal injection of 100µg/g bromodeoxyuridine (BrdU) (Sigma Chemical Co, St. Louis, MO) 2 hours before euthanasia. BrdU incorporation was detected with a rat anti-BrdU antibody (Accurate Chemical and Scientific Corporation, Westbury, NY Alternatively, a rabbit anti Ki67 (Thermo Scientific, Logan, UT) was used. As negative controls non-immune rabbit IgG (Sigma, St. Louis, MO) or non-immune rat IgG were used (Sigma, St. Louis, MO). Bound antibodies were visualized using biotin-conjugated anti-rabbit or anti-rat antibodies (Vector Laboratories, Burlingame, CA) and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The 3,3'-diaminobenzidine was used as the substrate (Sigma-Aldrich, St. Louis, MO) and arrays were counterstained with hematoxylin. All microscopic images were acquired on a Zeiss Scope A.1 using digital imaging.

3.4 Breast cancer tissue array

The "B20812 and "BRc711" breast cancer tissue arrays with pathology grading were obtained from US Biomax, Inc. (Rockville, MD). Grade II and III tumors were analyzed in this study. These two arrays contained no duplicate samples. Immunohistochemistry was performed as previously described (Bergum *et al.* 2012). Primary antibodies were mouse anti-matriptase and goat anti-c-Met (both from R&D Systems, Minneapolis, MN). As negative controls non-immune mouse IgG (Sigma, St. Louis, MO) or non-immune goat IgG were used. Bound antibodies was detected as described above (Section 3.3)

3.5 Isolation of primary mammary epithelial cells and carcinoma cells

Primary mammary carcinoma cells were isolated essentially as described (Simian, hirai et al., 2001). Briefly, tumor tissue was removed aseptically, minced and digested with hyaluronidase (300 U/ML) (EMD Chemicals, Gibbstown, NJ) and collagenase (2 mg/ml) (Worthington Biochemical Corporation, Lakewood, NJ) added to growth medium: Ham's F12:DMEM (Corning Cellgro, Manassas, VA) supplemented with 5% FCS (Atlanta Biologicals, Lawrenceville, GA), insulin (10 µg/ml), EGF (10 ng/ml), transferin (T), (10 ug/ml) (Lonza, Walkersville, Maryland) and hydrocortisone (10 µg/ml) (Sigma-Aldrich Chemicals, St. Louis, MO) and incubated for 2 hours at 37°C. Normal primary mammary cells were isolated from glands 4 and 9 (inguinal) and digested as above for 30 min at 37°C. Primary epithelial cells or carcinoma cells were then isolated by several differential centrifugation steps and plated in growth medium (Niranjan *et al.*1995, Development). Growth medium was supplemented with Penicillin/Streptomycin/Gentamicin (Thermo Scientific, Logan, UT).

3.6 Human breast cancer cell lines

The SUM159 and SUM229 cell lines were a gift from Dr. Stephen Ethier (Medical University of South Carolina, Charleston, SC, USA). The HCC1937 and BT20 breast cancer cells were purchased from ATCC (Mannassas, VA). The growth conditions for each cell line are as follows; SUM159, SUM229 and SUM149 cells were grown in 5% IH media (Ham's F-12 media, supplemented with 5% FBS, 1µg/ml hydrocortisone, and 5µg/ml insulin), BT-20 cells were grown in Eagles + NEAA media (Eagle's MEM with 2mM L-glutamine and Earle's BSS adjusted to contain

1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids, 1mM sodium pyruvate, and 10% FBS), SUM190 cells were grown in SF1H media (Ham's F-12 media, supplemented with 1 μ g/ml hydrocortisone, and 5 μ g/ml insulin, 5mM ethanolamine, 10 mM HEPES, 5 μ g/ml transferrin, 10 nM triodo-thyronine, 50 μ M sodium selenite, and 5% BSA) and HCC 1937 cells were grown in RPMI+L-GLUT media (RPMI-1640 media with 2mM L-glutamine adjusted to contain 1.5g/L sodium bicarbonate, 4.5g/L glucose, 10mM HEPES, 1mM sodium pyruvate, and 10% FBS).

3.7 Pro-HGF/SF activation assays

Pro-HGF/SF was produced in the immortalized human fibroblasts cell line RMF-HGF as described (Kuperwasser, *et al.* 2004, Mueller *et al.* 2012). To ensure the quality and to estimate the concentration of each preparation, western blot analysis was performed using a goat anti-HGF primary antibody (R&D Systems, Minneapolis, MN) with recombinant human recombinant active HGF (R&D Systems, Minneapolis, MN) as a standard. Human breast cancer cell lines or primary mouse MMTV-PyMT mammary carcinoma cells were plated in 6- well plates and grown to confluence. Cells were then serum starved for a minimum of three hours. The media was then changed to either fresh serum free media, serum free media with 100 ng/ml recombinant active two-chain HGF/SF (R&D Systems, Minneapolis, MN) or pro-HGF/SF with or without either 2 μ M Aprotinin (MP Biomedicals, LLC, Solon, OH) 20 μ M Leupeptin (Fisher Bioreagents, Fair Lawn, New Jersey), 60nM HAI-1 or 40nM HAI-2 (R&D Systems). Inhibitors were added concomitantly with pro-HGF/SF or HGF/SF. To determine the effect of Pro-HGF/SF on activation of c-Met and downstream signaling molecules (Gab1, AKT) with time,

the cell culture medium was aspirated at various time points and the cells were lysed in ice cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.1% SDS, pH 7.4) with protease inhibitor cocktail and phosphatase inhibitor (Sodium Orthovanadate, Sigma-Aldrich, St. Louis, MO). Activation levels of cMet, Gab1 and AKT was determined by accessing the levels of the phosphorylated forms relative to the level of the total amount of the specific signaling molecules by western blotting analysis (see below).

3.8 3D cell culture

The 3D protocol described in Victor *et al.* 2011 was used. Briefly, cells were grown under conditions listed above and then plated on Matrigel Matrix Growth Factor Reduced (MMGFR)(BD Biosciences, Franklin Lakes, NJ) with serum free Mammary Epithelial Cell Growth Medium (MEGM™) media (Lonza, Walkersville, MD) containing the BulletKit™ growth supplement (BPE, hydrocortisone, GA-1000, Insulin). On overlay of MEBM growth media containing 2% MMGFR was added 1 hour after seeding. Spheroids were allowed to form overnight before addition of 100 ng recombinant HGF/SF (R&D Biosystems, Minneapolis, MN) or pro-HGF conditioned media. All conditions were serum free from the initial seeding onto MMGFR. After treatment, cells were stained with 5 uM Cell Tracker Orange (Invitrogen, Carlsbad, CA) for 45 minutes and Hoechst 33342 (Life Technologies Carlsbad, CA) for 5 minutes prior to imaging. Confocal images were acquired on the Zeiss LSM 780 scope at the Microscopy Imaging and Cytometry Resources Core at Wayne State University School of Medicine. Light microscopy

pictures were taken on the Nikon TMS microscope courtesy of Dr. Hyenong-Reh Kim (Wayne State University, Department of Pathology).

3.9 RNAi silencing

For matriptase knockdown in SUM229, BT20 AND HCC1937 breast cancer cells, three independent Stealth RNAi™ siRNA duplexes targeting matriptase (siM1-ST14HSS186125, siM2-ST14HSS186126, siM3- ST14HSS110268), as well as %GC matched negative controls were used as previously described (Invitrogen, Carlsbad, CA) (Bergum *et al.* 2012). Two days after transfection the cells were serum starved and used in the pro-HGF/HGF activation assay and in the 3D invasion assay (described above in 3.7 and 3.8) and in the proliferation assay described below (3.11)

3.10 Western blot analysis

Mouse tissues were snap frozen and homogenized in ice cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.1% SDS, pH 7.4) with protease inhibitor cocktail added (Sigma-Aldrich, St. Louis, MO). Cultured mouse and human cells were lysed in ice cold RIPA buffer with protease inhibitor cocktail and phosphatase inhibitor (Sodium Orthovanadate, Sigma-Aldrich, St. Louis, MO). The protein concentration was determined by BCA assay (Pierce, Rockford, IL) and lysates were separated by 4–12% reducing SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Invitrogen). The following primary antibodies were used for detection: sheep anti-matriptase (R&D Systems, Minneapolis, MN), rabbit anti-matriptase (CalBioChem, Philadelphia, PA), rabbit anti-phospho cMet, anti-phospho Gab1, anti-Gab1, anti-phospho Akt, anti-Pan Akt, mouse anti-human cMet (all from

Cell Signaling Technology, Beverly, MA) and mouse anti-beta-actin (Sigma St. Louis, MO). The signal was detected with secondary antibodies conjugated with either alkaline phosphatase (Sigma St. Louis, MO) or horseradish peroxidase (Chemicon Temecula, CA) using the chromogenic substrate nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate solutions (Roche, Indianapolis, IN) or Super-SignalWest Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

3.11 Proliferation assays in human breast cancer cell lines

30,000 human breast cancer cells were seeded into a six-well plate and serum starved overnight before being treated with 100 ng active HGF or pro-HGF for 24 hours. Thereafter cells were trypsinized and recounted using a hemocytometer to determine the proliferation of cells after treatment.

3.12 Transient transfection of SUM159 cells

SUM159 cells were plated using 10^6 cells/well in a 6 well plate and were transfected when they reached 85% confluency using Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. A total of 5 μ g of DNA and 10 μ L of LTX was used. The DNA construct used was the mammalian expression vector, pcDNA3.1 containing full length human matriptase DNA (a generous gift from Dr. Stine Friis, NIH) (See Friis *et al.* 2013).

3.13 Matriptase gelatin zymography

Gelatin zymography for matriptase was performed as described in List *et al.*, 2005. Briefly, serum-free media conditioned for 2 days by primary mammary cells was collected and dialyzed against distilled water overnight at 4°C. The dialyzed samples were lyophilized, and the dried protein powder was dissolved in a 1/100

volume of the initial conditioned medium in 20 mM Tris-HCl (pH 7.5). Concentrated medium was separated by SDS-PAGE under non-reducing conditions, and the gels (containing 0.1% gelatin) were subsequently incubated in renaturation buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, and 2.5% Triton X-100) for 1.5 h followed by 30 min in EDTA buffer (50 mM Tris-HCl at pH 7.5, 0.5 mM EDTA) to eliminate matrix metalloproteinase activities as described (Jin et al. 2005). The gels were incubated at 37°C for 16 h in developing buffer (50 mM Tris-HCl at pH 7.5 with 5 mM CaCl_2), and stained with Coomassie Brilliant Blue to detect zones of gelatinolysis.

3.14 Statistical analysis

All analysis was performed and graphs generated using Prism 5 software (GraphPad Software Inc., San Diego, CA). Comparisons between two groups used student's t test. Non-parametric data was compared using the Mann-Whitney test. For correlation data, two estimators of association were used. The tetrachoric correlation coefficient assumes that the variables, matriptase and c-Met, in this case, are normally distributed but that they have been dichotomized. Kendall's tau does not have the assumption of normality and is a measure of concordance. Both measures have a theoretical range from -1 to 1.

CHAPTER 4: EXPLANATION OF GENETIC MOUSE MODELS

Knockout mouse models have proved to be invaluable tools to determine the functions of oncogenes, tumor suppressor genes, and other genes associated with breast cancer progression. The perinatal lethality of matriptase null mice limits the ability to study matriptase in an *in vivo* setting. We have circumvented this problem by generating two novel genetically modified mice. This section outlines the two mouse models used in this study.

4.1 Matriptase hypomorphic mice

The matriptase hypomorphic mice were generated by inserting a retroviral targeting vector into the first intron of the matriptase gene (List *et al.* 2006b). These mice have an 80-99% reduction in matriptase mRNA in different tissues, and importantly have a >85% reduction of matriptase levels in the mammary gland. Although these mice have severely reduced matriptase messenger RNA (mRNA) levels both pre- and postnatally, these mice are viable and live a normal lifespan. It is important to note that even with reduced matriptase levels, the residual protein is sufficient to maintain normal epithelial function in the mammary gland and the female hypomorphic mice are still lactation competent. By crossing these matriptase hypomorphic mice with mice carrying the MMTV-PymT oncogene, we performed a comparative study of breast cancer progression in mammary glands with normal matriptase levels and mammary glands with greatly reduced matriptase levels. In agreement with the findings in human breast carcinomas (Lin *et al.* 1993), endogenous matriptase is expressed exclusively in the epithelium of the MMTV-PymT tumors and is overexpressed in these malignancies. Importantly, the level of

proteolytically active matriptase is also increased as demonstrated by gelatin zymography

4.2 Matriptase conditional knockout mice

While the matriptase hypomorphic model represents a chronic reduced loss of function model since matriptase levels are reduced *in utero* and postnatally, it is possible that compensatory mechanisms during mammary gland development and/or carcinogenesis, e.g. altered expression of other proteases or their inhibitors may occur. In addition, matriptase hypomorphic carcinoma cells have approximately 25% residual matriptase. To gain the most comprehensive insights into matriptase functions, we therefore used the matriptase conditional knockout mice where the matriptase gene can be acutely disrupted, rendering matriptase protein level undetectable in mammary primary mammary carcinoma cells.

We used an inducible acute matriptase conditional knockout mouse, which allows us to efficiently and temporally regulate matriptase ablation. This strain of mice carries a ubiquitously expressed form of the Cre-recombinase (β -actin-CreERTM) which can be activated by exposure to the synthetic estrogen antagonist 4-hydroxytamoxifen (4-OHT) but is not activated by endogenous estrogen (Hayashi 2002). Administering 4-OHT to the adult conditional knock-out mice (Mat^{loxP/Null}/ β -actin-CreERTM) is lethal since the global acute matriptase ablation causes rapid loss of cell-cell adhesions in the intestinal epithelium and the mice die after one week (List *et al.* 2009). However, the mammary glands can be excised from the mice and grown *ex vivo* where 4-OHT can be administered to the primary cells in culture to induce efficient matriptase ablation (See figure 14). It is important to mention that

the most commonly used Cre-strain for mammary specific loxP mediated recombination, the MMTV-Cre strain, proved to be deleterious for our purpose. This was caused by an unexpectedly high (“leaky”) expression of the MMTV-Cre-recombinase in the skin of the mice. This expression led to premature death caused by the loss of matriptase that is essential for proper epidermal function. Our strategy was therefore to use the β -actin-CreERTM as described above. We again crossed this strain with mice bearing the MMTV-PymT oncogene to determine the effect of ablated matriptase expression on signaling in the cells from these mammary tumors.

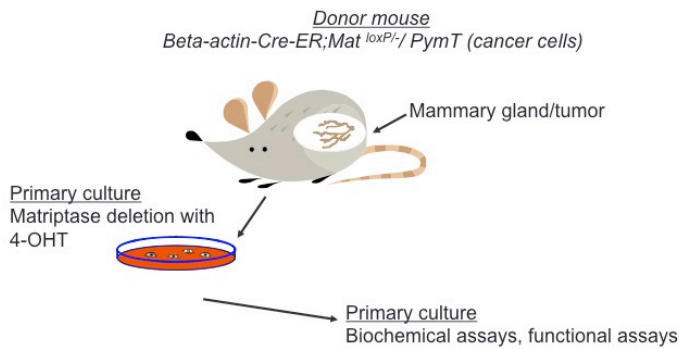


Figure 14: Matriptase in mammary gland cells upon acute ablation. A conditional matriptase knock-out mouse model was generated by flanking exon 2 of the mouse matriptase gene with LoxP sites. The mice also carry a null allele with a β -galactosidase marker gene which makes easy identification of donor cells after transplantation possible, and a β -actin-Cre ERTM transgene for quick and efficient matriptase gene inactivation upon 4-OHT administration. Matriptase-deficient and matriptase-sufficient primary mammary breast carcinomas cells (from mice with the PymT oncogene) were isolated and cultured *ex vivo* to perform western blotting to evaluate the resultant level of HGF/c-Met signaling.

CHAPTER 5: MATRIPTASE MEDIATED c-MET SIGNALING- RESULTS IN INVASIVE DUCTAL BREAST CANCER

5.1 Introduction

Pericellular proteases in cancer progression were previously primarily considered to be extracellular matrix protein degrading enzymes. While it is clear that proteases are involved in degradation events related to breaching the basement membrane and reorganization of the extracellular matrix, during invasive growth a more complex view of pericellular proteolysis has emerged in recent years. One concept in protease mechanistic research is that proteolytic modifications of targets including activation of growth factors are critically involved in carcinogenesis through the activation of oncogenic signaling pathways. Importantly, tumor progression is characterized by a complex interplay between invading tumor cells and stromal cells, which, includes paracrine pathways where growth factors secreted by stromal cells activate signaling pathways in the cancer cells.

The type II transmembrane serine protease, matriptase, has been implicated in breast cancer since it was first discovered in breast cancer cell lines, and is highly expressed in the epithelium-derived cancer cells of human breast carcinomas (Lin *et al.*, 1997, Oberst *et al.*, 2001, Jin *et al.*, 2007, Bergum *et al.*, 2012). However, it is currently not known whether matriptase plays a critical role in breast cancer progression *in vivo*. One factor that has slowed advances on this front has been the perinatal lethality of matriptase-null mice which has thus far, precluded direct studies of matriptase loss-of-function in the mammary gland (List *et al.*, 2002, 2003). This obstacle has been circumvented by employing a matriptase hypomorphic model with low levels of

matriptase in the mammary gland. When crossed into the mouse mammary tumor virus (MMTV) Polyomavirus middle T (PymT) mouse genetic mammary tumor model, we show the matriptase hypomorphic mice displayed a significant delay in tumor onset and a decreased tumor burden and tumor multiplicity. The impaired growth was caused by a profound impairment of tumor cell proliferation.

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic, paracrine growth factor and key mediator of cell migration, proliferation, survival, motility, and morphogenesis in epithelial cells (Weidner *et al.* 1991; Nakamura *et al.* 1989; Stoker *et al.* 1987). HGF/SF is biosynthesized as a single-chain zymogen-like inactive precursor (pro-HGF) and is proteolytically processed to its two-chain mature active form. The epithelial cell receptor, cMet, binds pro-HGF/SF or active HGF/SF, however, only the active form elicits the cMet signaling pathway (Cooper *et al.*, 1984, Bottaro *et al.*, 1991). Proteolytic cleavage of the Arg⁴⁹⁴-Val⁴⁹⁵ peptide bond in pro-HGF results in allosteric activation of the serine protease-like β -chain (HGF β) and the two-chain form, consisting of a disulfide-linked α/β -heterodimer, is capable of activating cMet (Lokker *et al.*, 1992, Hartman *et al.*, 1992). Pro-HGF is secreted by mesenchymal cells, mainly fibroblasts, in the breast. Importantly, c-Met is, like matriptase, expressed on the surface of mammary epithelial cells and breast carcinoma cells (Kang *et al.*, 2003, Bergum *et al.*, 2011). The HGF/c-Met signaling pathway is dysregulated in many cancer types including breast cancer and has been causally linked to breast carcinogenesis (Camp *et al.*, 1999, Ghoussoub *et al.*, 1998, Gallego *et al.*, 2003, Liang *et al.*, 1996, Kang *et al.*, 2003). Since a key post-translational regulation mechanism of HGF/SF/c-Met signaling is the proteolytic activation of pro-HGF/SF, the identification of the critical activator(s) as

potential targets for therapeutic intervention in cancer is needed (Gherardi *et al.*, 2012). This work identifies matriptase as the essential proteolytic mediator of HGF/c-Met signaling in breast cancer. Thus, genetic ablation of matriptase in mouse mammary carcinoma cells leads to the lack of c-Met activation upon stimulation with fibroblast derived pro-HGF. Silencing of matriptase in human breast cancer cells causes complete abrogation of the c-Met signaling pathway and causes severe impairment of proliferation and invasive responses. In sum, we demonstrate that matriptase, HGF and c-Met define an epithelial-mesenchymal protease-mediated signaling axis where pro-HGF is efficiently activated on the cell surface by matriptase, leading to initiation of the c-Met signaling pathway and elicitation of mitogenic and invasive responses in breast cancer.

5.2 Matriptase is expressed in the epithelium of the mammary gland and upregulated in mammary carcinoma.

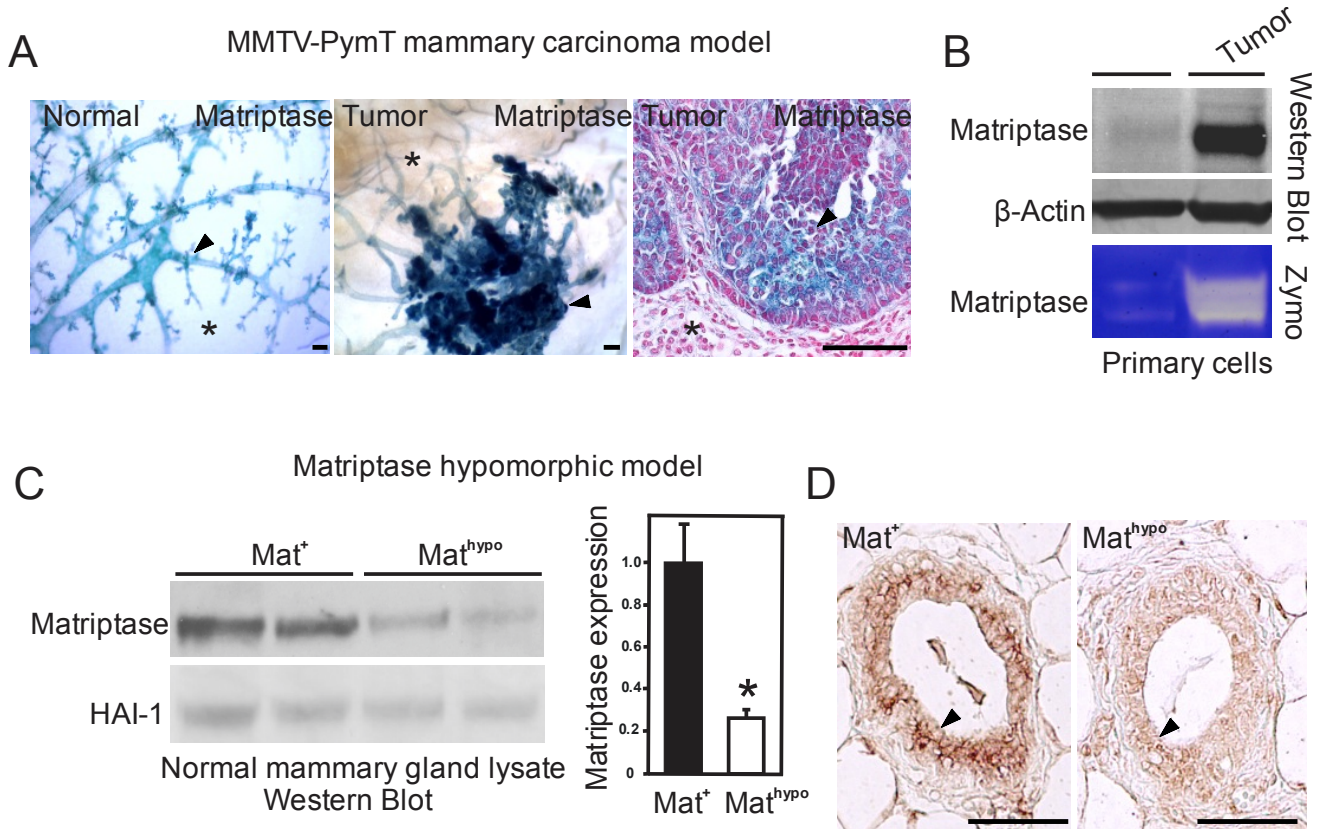
Transgenic MMTV-PymT mice are predisposed to develop multifocal mammary carcinomas with tumor progression that is very similar to that seen in human breast carcinomas (Guy *et al.*, 1992 a, b, Lin *et al.*, 2003). In order to ensure that the mouse mammary cancer model closely mimics the observations in human breast cancer, matriptase expression in normal mammary gland and mammary tumors was characterized (Fig. 15.). A knock-in mouse with a promoterless β -galactosidase gene inserted into the endogenous matriptase gene was used as a unique tool for precise assessment of endogenous matriptase expression in the mammary gland by X-gal staining [List *et al.*, 2007 a, b]. Matriptase is exclusively expressed in the epithelial cells in normal mammary glands and in the cancer cells in MMTV-PymT oncogene induced

carcinomas with no detectable expression in the stroma (Fig. 15 A). Matriptase protein is detected in normal primary cells at low levels and the expression is increased in mammary carcinoma cells by western blotting (Fig. 15 B). Furthermore, matriptase is present in its proteolytically active form in mammary epithelial cells, and the level of active matriptase is significantly increased in mammary carcinoma cells in comparison to normal mammary epithelial cells as demonstrated by gelatin zymography (Fig. 15 B). Importantly, the findings that matriptase is specifically expressed in the epithelial compartment and upregulated during carcinogenesis is in accordance with previous findings in human breast carcinomas (Bergum et al., 2011, Oberst et al., 2001), rendering this tumor model particularly well-suited to understand the role of matriptase in tumor progression in humans.

5.3 Matriptase hypomorphic mice have greatly reduced levels of matriptase in the mammary glands.

We have previously generated matriptase hypomorphic mice (Mat^{hypo}) harboring one ST14 null allele and one ST14 “knockdown” allele in which an engrailed-2 splice acceptor site had been inserted between coding exons one and two (List et al., 2007 a,b , List et al., 2006). These mice display an 80-99% reduction in matriptase mRNA levels in different tissues and have normal postnatal and long-term survival (List et al., 2007b). To assess the level of matriptase protein in the mammary gland of Mat^{hypo} mice, western blot analysis of protein lysates of whole glands was performed. The level of matriptase protein in the mammary gland in Mat^{hypo} mice is reduced >75% (Fig. 15 C) as compared to littermate control mice (Mat^+). Immunohistochemical staining of mammary glands confirmed that matriptase protein expression in the ductal epithelium

FIGURE 15: EXPRESSION OF MATRIPTASE IN THE MMTV-PyMT MAMMARY CARCINOMA MODEL AND IN THE MATRIPTASE HYPOMORPHIC MODEL



(A) A knock-in mouse with a promoterless β -galactosidase marker gene inserted into the endogenous matriptase gene was used as a unique tool for precise assessment of endogenous matriptase expression in the mammary gland by X-gal staining (blue color). Matriptase is expressed in the mammary gland epithelium (arrowhead in A) with no detectable expression in the stroma (*) in whole mounts from normal glands (left panel) and MMTV-PyMT induced carcinomas (middle panel). Histological analysis shows matriptase expression in the mammary cancer cells of MMTV-PyMT transgenic mice (arrowhead, right panel) **(B)**. Mammary epithelial cells from normal glands and tumor bearing glands were isolated and analyzed by western blotting (top panel) and gelatin zymography (lower panel) demonstrating increased levels of active matriptase in mammary carcinoma cells. **(C)** Western blot analysis of whole mammary gland extracts from matriptase hypomorphic mice (Mat^{hypo}) and control (Mat⁺) littermates with anti-matriptase (upper panel) or anti-HAI-1 antibodies. The levels is greatly reduced in the Mat^{hypo} mice (Fig. 15 D). Importantly, the residual matriptase in the of the epithelial specific, physiological matriptase inhibitor, HAI-1, is comparable in Mat^{hypo} and Mat⁺ littermate mice. **(D)** Relative matriptase expression in Mat^{hypo} (N=3) and Mat⁺ mice (N=3) as determined by densitometry. Matriptase levels were normalized to HAI-1. Error bars represent S.D. P<0.04, Student's T-test.

is greatly reduced in the Mat^{hypo} mice (Fig. 15 D). Importantly, the residual matriptase in the mammary gland suffices to sustain normal mammary gland development based on comparative morphological and histological assessment (data not shown). Furthermore, Mat^{hypo} females have functional mammary epithelium since they are lactation competent, and no reduction in litter size or litter survival has been observed. Thus, this model is well-suited to study progression of breast cancer in morphologically and functionally normal mammary glands with greatly reduced levels of matriptase.

5.4 Reduced matriptase leads to impairment of mammary carcinogenesis.

To investigate the effects of low matriptase levels in the mammary gland on mammary carcinoma progression, matriptase Mat^{hypo} and Mat⁺ mice harboring the MMTV-PymT oncogene were generated (referred to as PymT-Mat^{hypo} and PymT-Mat⁺ mice, respectively). Prospective cohorts with littermate PymT-Mat^{hypo} and PymT-Mat⁺ virgin female mice was established and carefully monitored by weekly palpation to determine the appearance of the first palpable mammary mass (tumor latency). At 145 days of age the number of glands with visible and palpable tumors was recorded (tumor multiplicity) and the area of tumors was determined by caliber measurements. Total mammary tumor burden was calculated as the total weight of all postmortem excised mammary glands from a cohort of littermates.

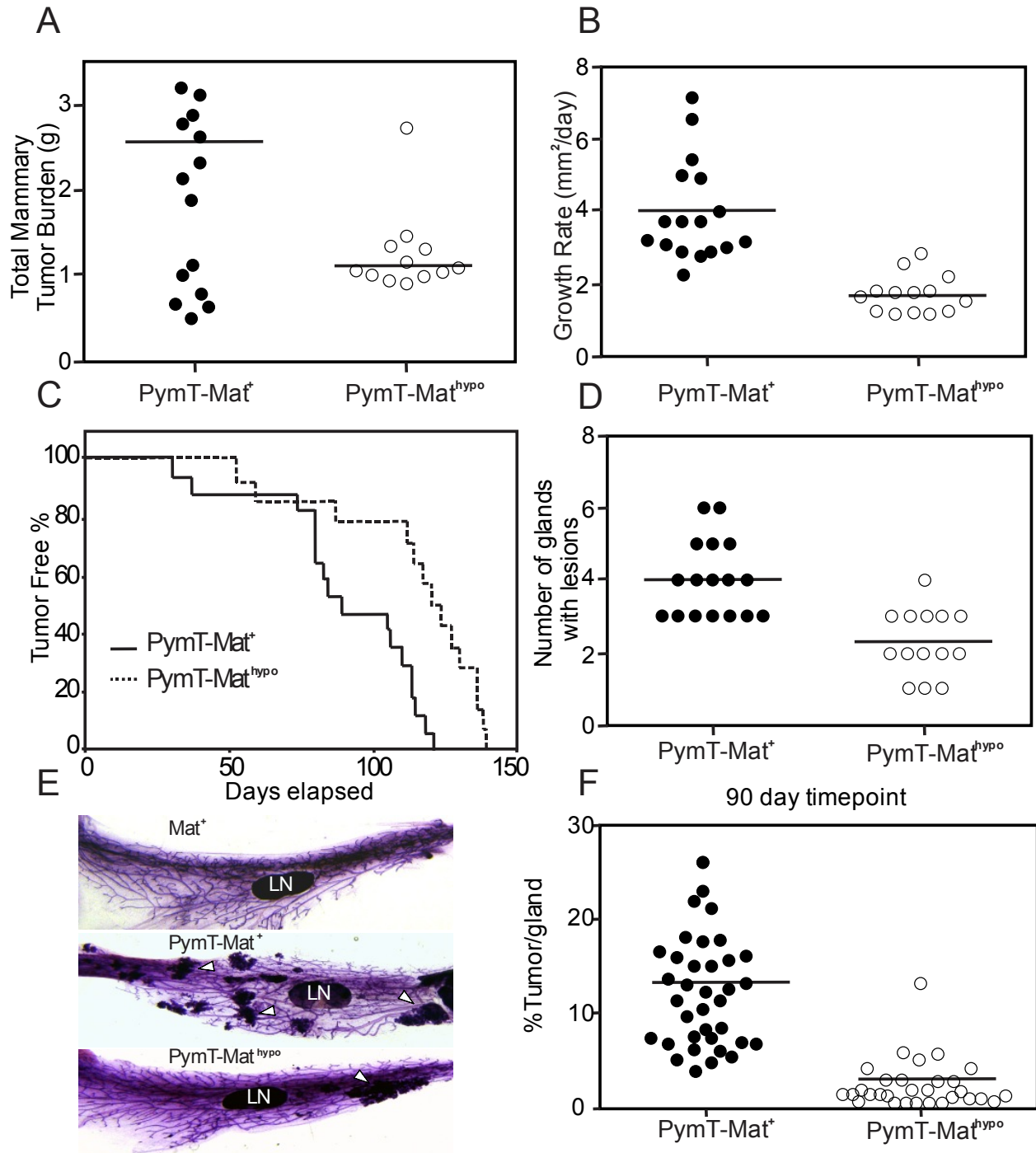
Reduced levels of matriptase in the mammary glands had significant effects on all the tumorigenesis parameters measured (Fig. 16). Thus, the total tumor burden was reduced by 58% (mean tumor burden of PymT-Mat⁺, 2.6 g; PymT-Mat^{hypo}, 1.1 g) and the total tumor area was reduced by 76% (mean tumor area in PymT-Mat⁺, 2.9 mm²/day; PymT-Mat^{hypo}, 0.7 mm²/day). Also, tumor multiplicity was reduced by 45% (mean

number of glands with tumors in PymT-Mat⁺, 4.0; PymT-Mat^{hypo}, 2.2). These results demonstrate that matriptase is an important contributor to tumor growth and progression. Importantly, there was a significant delay in initial formation of palpable mammary indicating that matriptase is critical for early carcinogenesis with a mean latency of palpable tumors of 84 days in PymT-Mat⁺ versus 120 days in PymT-Mat^{hypo} mice. At 90 days 53% of PymT-Mat⁺ mice had palpable tumors versus 21% of PymT-Mat^{hypo} mice. In order to examine the role of matriptase in early mammary carcinogenesis in further detail, an independent cohort was established and dysplastic lesions in the inguinal mammary gland were examined postmortem by whole mount analysis at 90 days of age. Importantly, total area measurements of lesions revealed a 75% reduction in the glands from PymT-Mat^{hypo} mice as compared to PymT-Mat⁺ (Fig. 16 F). Taken together, these data strongly supports a functional role for matriptase in both tumor formation and tumor growth.

5.5 Reduced matriptase impairs carcinoma cell proliferation in mammary tumors

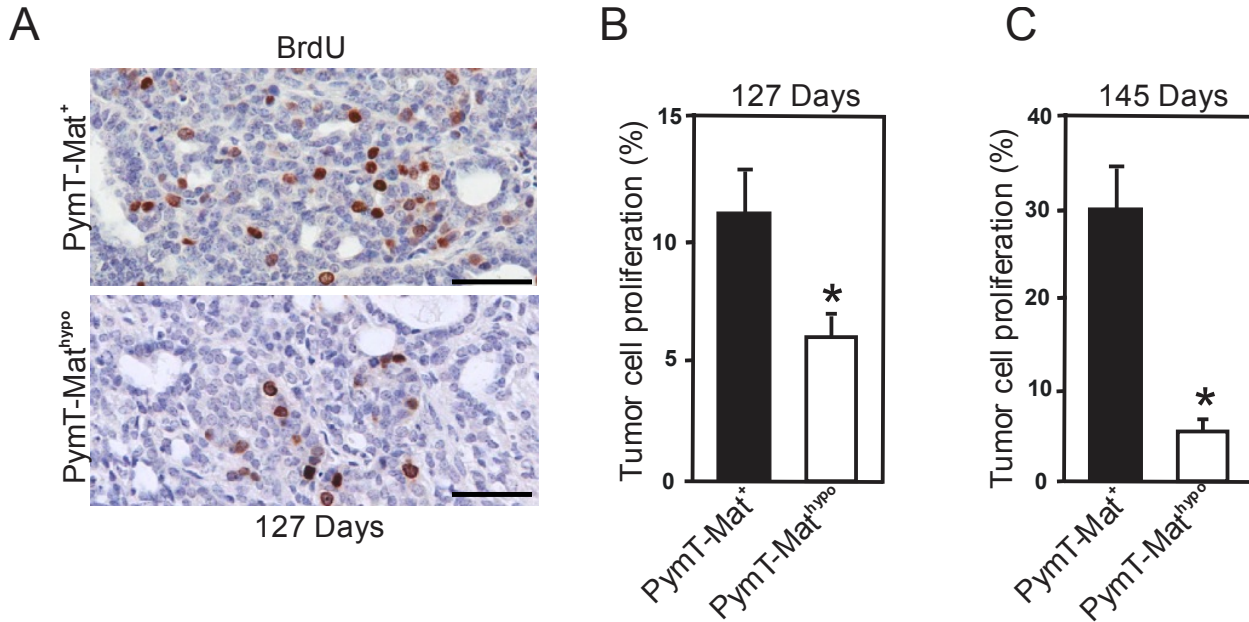
To address the basis for the diminution of mammary tumor growth in mice with low matriptase, proliferation rates of carcinoma cells were determined at two different time points by immunohistochemical detection of bromodeoxyuridine (BrdU) or endogenous Ki67 (Figure 17). At 127 days of age carcinoma cell proliferation was reduced by 47% in PymT-Mat^{hypo} mice as compared to PymT-Mat⁺ mice. A dramatic decrease in proliferation was also observed in PymT-Mat^{hypo} 145 day old mice with an 81% reduction as compared to littermate PymT-Mat⁺ mice. These data strongly suggest that matriptase-dependent carcinoma cell proliferation promotes mammary tumor growth.

FIGURE 16: REDUCED MATRIPTASE DECREASES MAMMARY TUMOR BURDEN, GROWTH RATE, LATENCY AND MULTIPLICITY



A prospective cohort of littermate PymT- Mat⁺ mice (N=13) and PymT Mat^{hypo} mice (N=14) was followed **(A)** Tumor burden at 145 days. Mice were euthanized and their mammary tumors resected and the total tumor weight was recorded for PymT Mat⁺ mice (closed circles) and PymT-Mat^{hypo} mice (open circles). Mean values were 2.6 and 1.1, respectively ($P < 0.002$) Mann-Whitney U Test. **(B)** Mammary tumor growth rate for PymT-Mat⁺ mice (closed circles) and PymT-Mat^{hypo} mice (open circles). Mean values were 2.9 and 0.7, respectively ($P < 0.0001$) Mann-Whitney U Test. **(C)** Kaplan-Meier tumor-free survival curves of PymT-Mat⁺ (black line) and PymT-Mat^{hypo} (grey line). The median tumor-free survival times were 89 days versus 122 days, respectively ($P < 0.007$) Chi-Squared Test **(D)** Total number of glands with lesions at 145 days for PymT-Mat⁺ mice (closed circles) and PymT-Mat^{hypo} mice (open circles). Mean values were 4.0 and 2.2, respectively ($P < 0.0001$) Mann-Whitney U Test. **(E)** Representative whole mounts of inguinal mammary glands from 90 day old virgin female littermates. Normal mammary epithelia in Mat⁺ gland (upper panel). Extensive growth of multiple lesions (arrowheads) in PymT-Mat⁺ gland (middle panel), small localized lesion (arrowhead) in PymT-Mat^{hypo} gland (lower panel). LN=Lymph node. **(F)** Total area of lesions in 18 inguinal mammary glands from PymT-Mat⁺ (closed circles) and 15 PymT- Mat^{hypo} (open circles) ($P < 0.0001$) Mann-Whitney U Test.

FIGURE 17: DECREASED CARCINOMA CELL PROLIFERATION IN MAMMARY TUMORS WITH REDUCED MATRIPTASE



(A) Representative pictures of mammary tumors from PymT-Mat⁺ (top) and PymT-Mat^{hypo} (bottom) stained with an anti-BrdU antibody. Mice were injected with BrdU two hours prior to euthanasia. Size bars, 50 μ m. **(B)** Quantification of carcinoma cell proliferation in 127 day old mice. Data are presented as the mean relative number of cells with positive nuclear BrdU staining in tumors from PymT-Mat⁺ (N=4, black bars), and littermate PymT-Mat^{hypo} mice (N=3, white bars) Error bars represent S.D. P<0.001, Mann-Whitney U Test **(C)** Quantification of carcinoma cell proliferation in 145 day old mice. Data are presented as the mean relative number of cells with positive nuclear Ki67 staining in tumors from PymT-Mat⁺ (N=4, black bars), and littermate PymT-Mat^{hypo} mice (N=4, white bars). P<0.001, Mann-Whitney U Test. Error bars represent S.D.

5.6 Genetic ablation of matriptase abolishes pro-HGF induced c-Met signaling in primary mammary carcinoma cells.

An important task to undertake in order to gain mechanistic insight into the pathophysiological functions of matriptase is to identify the critical proteolytic target(s). Of the candidate substrates for matriptase that have been identified thus far, pro-HGF has been shown to directly promote mammary carcinogenesis. Thus, in a transgenic mouse model with overexpression of pro-HGF in the mammary gland, hyperplastic ductal trees with highly proliferative epithelial cells and subsequent development of multifocal invasive tumors were observed (Gallego et al., 2003). Also, pro-HGF/HGF is a prognostic marker in human breast cancer where elevated expression is associated with poor prognosis (Camp et al., 1999, Ghossoub et al., 1998, Gallego et al., 2003, Liang et al., 1996, Kang et al., 2003]. Pro-HGF is expressed by stromal mammary fibroblasts in both human and mice (Niranjan et al., 1995). To date, no genetic matriptase loss-of-function studies have been published, determining whether matriptase is critical for pro-HGF activation and c-Met signaling in cancer. Therefore, we chose to employ specific gene ablation to determine whether matriptase is required for efficient pro-HGF activation and elicitation of the c-Met signaling pathway in breast cancer. For this purpose MMTV-PymT-induced primary mammary carcinoma cells from conditional matriptase knock-out mice carrying a ubiquitously expressed inducible Cre recombinase (PymT-Mat^{cond.ko}) which allows for permanent inactivation of floxed matriptase alleles upon brief exposure to 4-hydroxy-tamoxifen (4-OHT)(List et al., 2009). As shown in Figure 18 A, efficient matriptase ablation was induced *ex vivo* in PymT-Mat^{cond.ko} mammary carcinoma cells rendering matriptase protein undetectable by

western blotting. Upon exposure to serum free media conditioned with fibroblast-secreted pro-HGF (Kupperwasser *et al.* 2004), c-Met activation was assessed by western blotting using antibodies against phosphorylated c-Met (p-c-Met). In matriptase sufficient PymT-Mat^{control} cells robust c-Met activation was observed (Fig. 18 B). In addition, activation of the two down-stream cytoplasmic effector proteins: Gab1 and AKT (also known as Protein Kinase B) was observed. In contrast, activation of c-Met, Gab1 and AKT was efficiently abrogated in PymT-Mat^{cond.ko} mammary carcinoma cells. Importantly, when cells were stimulated with pre-cleaved two-chain active HGF, matriptase deficiency did not affect phosphorylation of c-Met, Gab1 and AKT (Fig. 18). This strongly suggests, that the effects of matriptase gene ablation on cell signaling is caused by the loss of matriptase-mediated proteolytic conversion of pro-HGF to active HGF and thereby the ability to initiate the c-Met signaling pathway. Also, in a parallel control experiment, where primary PymT-Mat^{control} and PymT-Mat^{cond.ko} carcinoma cells from the same primary cell isolation batches as used above were treated with vehicle instead of the gene recombination-inducing agent, 4-OHT, matriptase expression was not affected and the cells responded to pro-HGF expression by phosphorylation of c-Met, Gab1 and AKT, (Supplementary figure 1) demonstrating that the cells have the inherent ability to cleave pro-HGF and activate c-Met signaling prior to matriptase gene ablation.

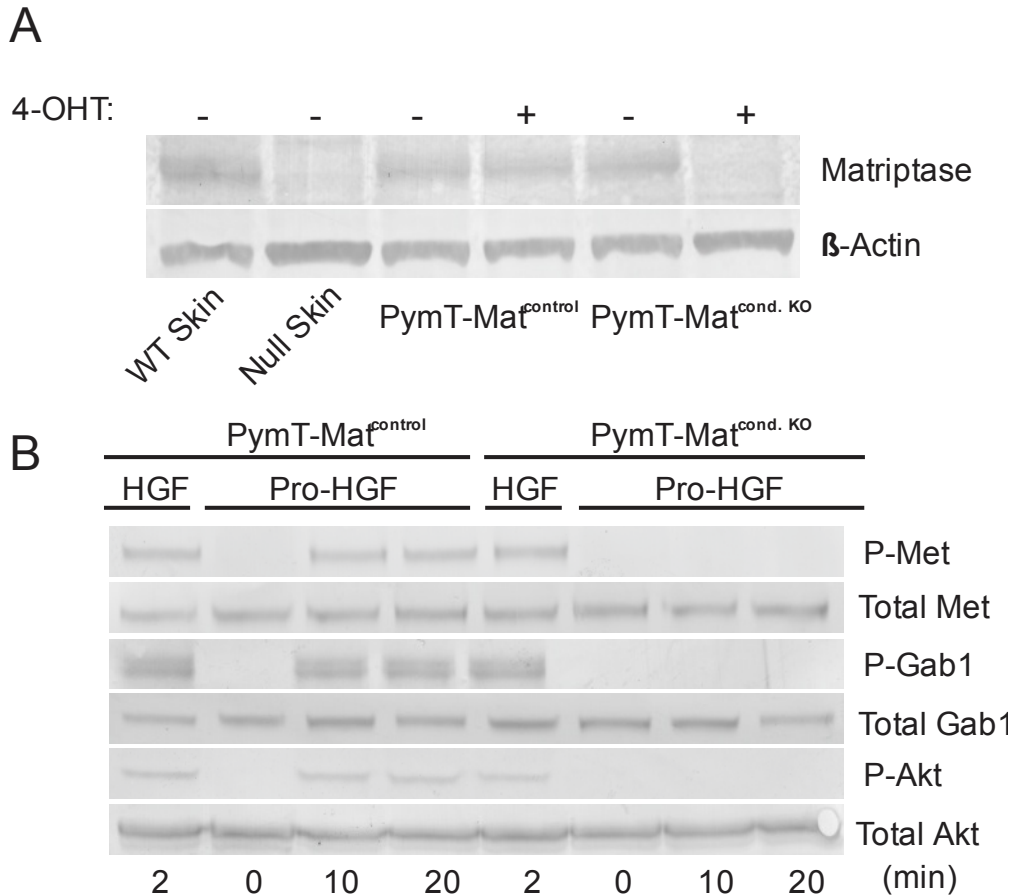
Both Gab1 and the PI3K–AKT pathway are important regulators of cell survival and proliferation downstream from pro-HGF/c-Met activation. Based on the finding that matriptase is critical for their activation, it is plausible that

matriptase promotes mammary tumor proliferation via initiation of the c-Met signaling network.

5.7 Matriptase silencing causes abrogated c-Met signaling and proliferation in human invasive ductal breast cancer cell lines.

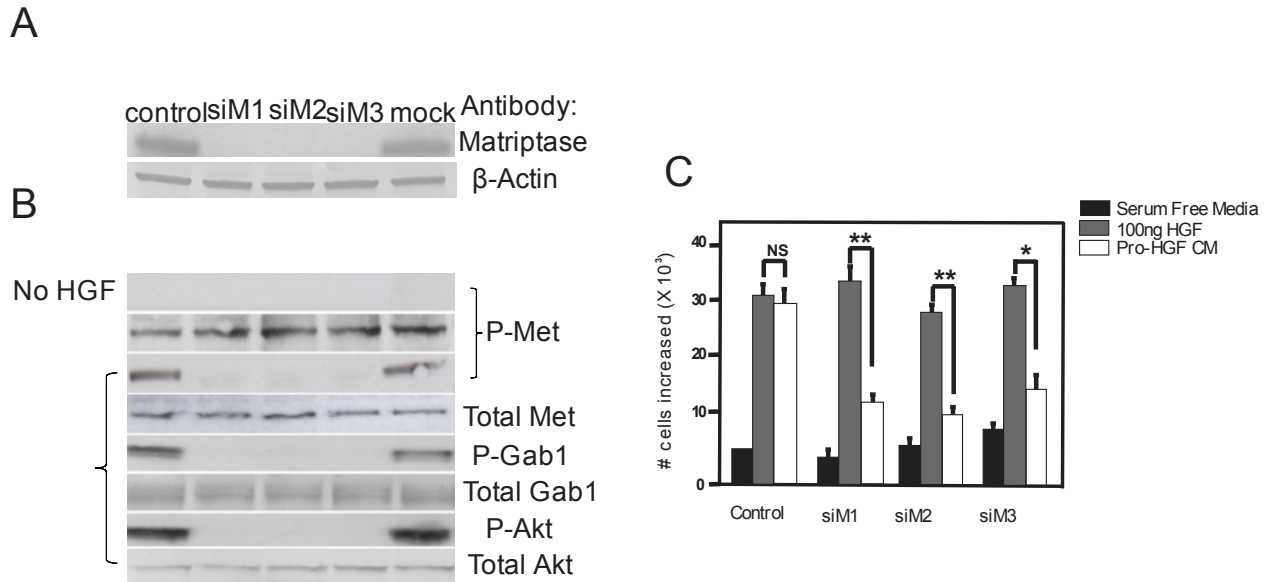
In order to verify the relevance of matriptase-dependent pro-HGF activation in human breast cancer, three different human breast cancer cell lines of diverse origin were employed: BT20 (invasive ductal carcinoma, triple negative), SUM229 (pleural effusion, triple negative) and HCC1937 (invasive ductal carcinoma, triple negative). All three cell lines express matriptase and c-Met (Fig. 19 A and Supplemental Fig 2). Matriptase was efficiently silenced using three independent non-overlapping matriptase silencing synthetic RNA duplexes in all three human breast cancer cell lines. Addition of pro-HGF to HCC1937 matriptase sufficient control cells resulted in robust activation of c-Met and the downstream targets Gab1 and Akt (Fig. 19 B). In contrast, matriptase ablation led to a greatly reduced level of c-Met pathway activation rendering the phosphorylated forms of c-Met, Gab1 and Akt undetectable by western blotting. Importantly, the matriptase silenced cells display unaltered activation of the c-Met pathway upon stimulation with pre-cleaved active HGF, demonstrating that the matriptase deficient cells have a fully functional c-Met signaling pathway and that the impaired response to pro-HGF is due to the lack of the matriptase-mediated activation cleavage (Fig. 19 B). Similar results for BT10 and SUM229 cells upon matriptase silencing were observed including efficient abrogation of the activation of c-Met, Gab1 and Akt (Supplemental Fig 2). This indicates that the mechanism observed is not limited to one cell line and may represent a general phenomenon in human breast cancer.

FIGURE 18: ABROGATION OF ACTIVATED c-MET AND DOWNSTREAM SIGNALING PROTEINS IN MATRIPTASE DEFICIENT PRIMARY MAMMARY CARCINOMA CELLS



(A) Efficient temporally controlled Cre-mediated ablation of matriptase in primary mammary carcinoma cells as demonstrated by western Blot analysis of primary cells isolated from virgin female mammary glands from PymT-Mat^{cond. KO} or PymT-Mat^{control} mice. Two days after plating 10 μ M 4-OHT (+) or vehicle (-) was added to the culture medium for 24 hours. Hereafter, the cells were kept in serum-free medium without 4-OHT for another 24 h before lysis and separation by reducing SDS-PAGE. A sheep anti-matriptase antibody was used for detection. Skin lysates from a newborn matriptase null mouse and wild-type littermate were included as controls for antibody specificity. Anti- beta-actin was used as control for equal loading **(B)** Mammary carcinoma cells were isolated from PymT-Mat^{cond. KO} and treated with 4-OHT as above to ablate matriptase expression (vehicle treated cells shown in S1) and exposed to active HGF (2 mins) or pro-HGF (0, 10 or 20 mins). Cells were lysed and analyzed for total c-met, activated c-Met, and its downstream targets by western blotting.

FIGURE 19: MATRIPTASE IS REQUIRED FOR ACTIVATION OF THE PRO-HGF/c-MET SIGNALING PATHWAY AND PROMOTES CELL PROLIFERATION IN INVASIVE DUCTAL BREAST CANCER CELLS



(A) Efficient RNAi silencing of matriptase in HCC1937 cells with three independent non-overlapping synthetic RNA duplexes (siM1, siM2 and siM3). A %GC matched negative control was used as negative control as well as a mock where no RNA duplex was added. Beta-actin staining of the membrane was performed to ensure equal protein loading **(B)** Cells were serum starved and exposed no HGF (serum free media), active HGF (2 min) or pro-HGF (15 min). Cells were lysed and analyzed for total c-met, activated c-Met, and its downstream targets by western blotting. **(C)** HCC1937 cells were grown on culture plates and matriptase was silenced using three independent non-overlapping synthetic RNA duplexes (siM1, siM2 and siM3). A %GC matched negative control was used as negative control. The cells were serum starved and left untreated (black bars) or exposed to either active HGF (gray bars) or pro-HGF (white bars) 24 hours and counted. Graphs show the proliferation at 24 hours after stimulation (* $P < 0.04$, ** $P < 0.005$, *** $P < 0.0008$, Student's T-Test). Data represent mean of three replicates. Error bars represent S.D.

To assess the functional consequences of inhibiting the matriptase-mediated c-Met signaling in human breast cancer cells, a proliferation assay was performed. As described above, matriptase was silenced with three different synthetic RNA duplexes, counted and seeded on cell culture plates and stimulated with pro-HGF or active HGF. After 24 hours the cells were recounted. As expected, there was no significant growth difference in matriptase sufficient control cells treated with active HGF or pro-HGF. In contrast, the growth in matriptase silenced cells was significantly impaired in response to pro-HGF in comparison to the response observed in HGF. These results demonstrate that silencing matriptase leads to decreased pro-HGF activation and subsequent c-Met signaling pathway activation ultimately causing growth inhibition. Taken together, with the results for mouse mammary carcinoma described above we have demonstrated that matriptase is the major, and perhaps the only, efficient activator of pro-HGF in model systems using either primary carcinoma cells or three established carcinoma cell lines. This conclusion is supported by the fact that no apparent compensation by one or more proteases upon genetic ablation or post-transcriptional silencing of matriptase was detected.

5.8 Matriptase re-expression restores c-Met signaling in human breast cancer cells in response to HGF.

The SUM159 cell line was established from a primary breast anaplastic carcinoma. Interestingly, SUM159 cells have lost the expression of endogenous matriptase and are non-responsive to pro-HGF (Fig. 20). Importantly, SUM159 cells have retained the proliferation response capability upon stimulation with

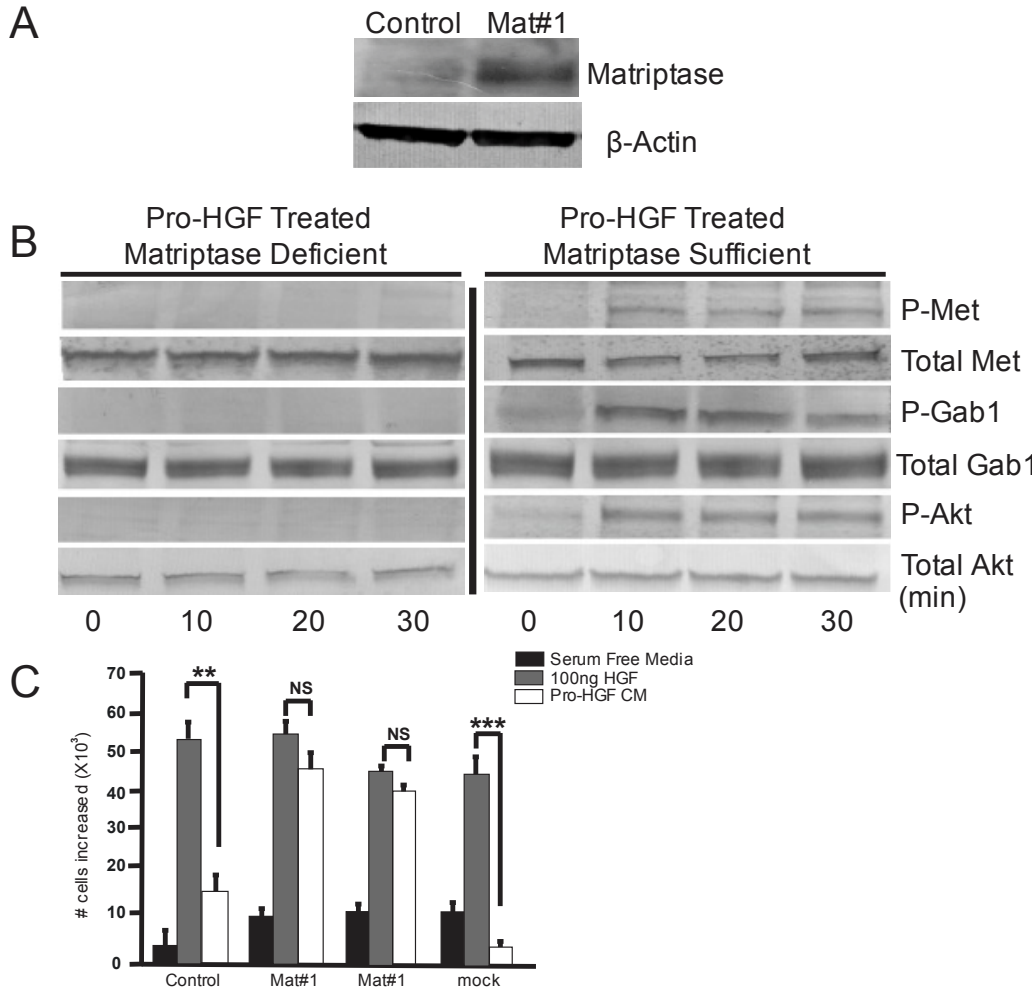
two-chain active HGF. To investigate whether re-expression of matriptase could re-store the ability to respond to pro-HGF, SUM159 cells were transiently transfected with a mammalian expression vector containing human full-length matriptase. When SUM159 cells with very low endogenous matriptase expression were exposed to pro-HGF and subsequently analyzed by western blotting, no phosphorylated c-Met, Gab1 or Akt were detected. In contrast, SUM159 expressing full-length matriptase displayed readily detectable phosphorylation of c-Met, Gab1 or Akt upon exposure to pro-HGF. The matriptase-mediated ability to activate the c-Met signaling pathway was accompanied by a strong mitogenic response to pro-HGF (Fig. 20 C). This demonstrates that gain-of-function matriptase expression confers SUM159 breast cancer cells with the capability of activating pro-HGF and thereby elicits the c-Met signaling pathway and subsequent proliferative response.

5.9 Matriptase mediates pro-HGF induced invasion in human invasive ductal breast cancer cell lines.

Many cancer cell lines form spontaneous three dimensional (3D) multicellular tumor-like spheroids when cultured in reconstituted basement membrane (rBM) overlay culture. These spheroids often resemble solid tumors, and tumorigenic processes including invasive growth, can therefore studied in this model system.

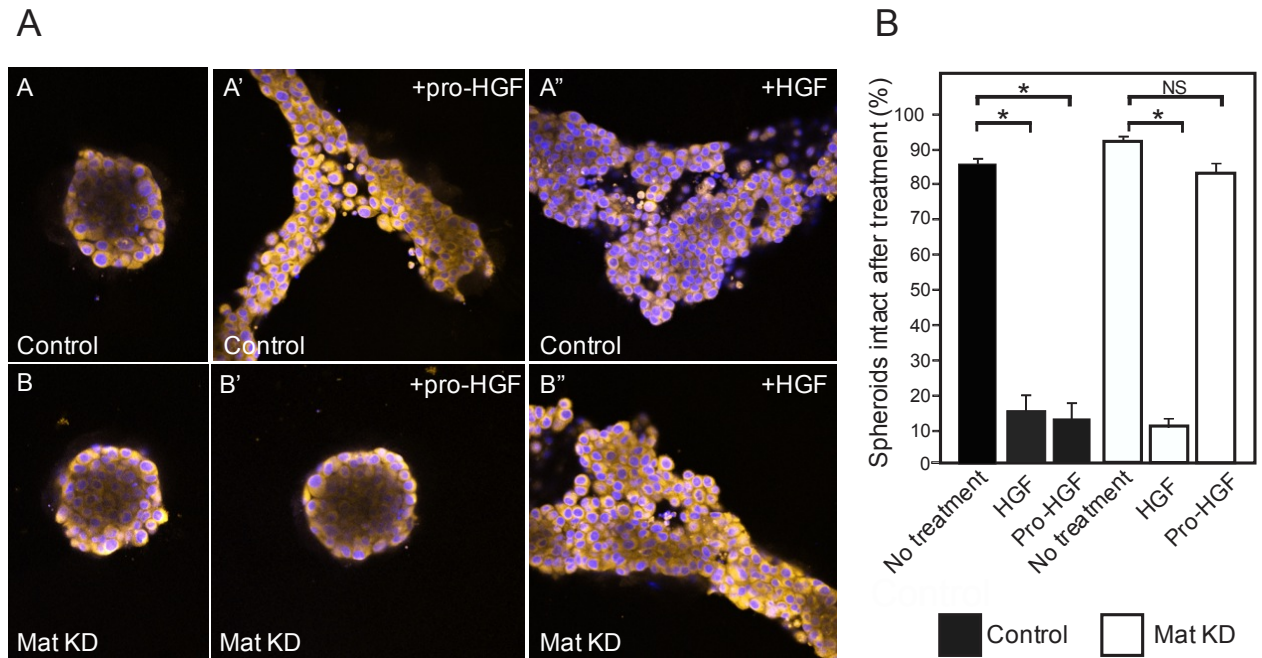
HGF is also named scatter factor (SF) because of its ability to induce scattering of MDCK cells involving the disruption of cell-cell junctions and an increase in motility in 2D culture, and formation of branching tubules when embedded in collagen (Stoker et al., 1987, Montesano et al., 1991). Since then

FIGURE 20: RE-EXPRESSION OF MATRIPTASE IN HUMAN INVASIVE DUCTAL BREAST CANCER CELLS INDUCES ROBUST c-MET SIGNALING AND PROLIFERATION



(A) The matriptase deficient human breast cancer cell line, SUM159 was transfected with an expression vector containing full-length matriptase. Beta-actin staining of the membrane was performed to ensure equal loading. **(B)** Cells were serum starved and exposed to active HGF or pro-HGF for the time periods indicated. Cells were lysed and analyzed for total and activated c-Met and downstream signaling proteins by western blotting. **(C)** SUM159 cells were grown on culture plates and matriptase transfected in using an expression vector containing full-length human matriptase. The cells were serum starved and left untreated (black bars) or exposed to either active HGF (gray bars) or pro-HGF (white bars) for 24 hours and counted. Graphs show the proliferation at 24 hours after stimulation (* $P < 0.04$, ** $P < 0.005$, *** $P < 0.0008$, Student's T-Test). Data represent mean of three replicates. Error bars represent S.D.

FIGURE 21: MATRIPTASE MEDIATES PRO-HGF INDUCED INVASION IN HUMAN INVASIVE DUCTAL BREAST CANCER CELLS



SUM229 cells were grown on culture plates and matriptase was silenced (Mat KD) using synthetic RNA duplexes (B, B', B"). A %GC matched negative control was used as negative control (A, A', A"). Cells were allowed to form spheroids in 3D rBM overlay culture for 24 hours before addition of either pro-HGF (A', B'), HGF (A'', B'') or no treatment (A, B) and imaged by confocal microscopy. When no HGF was added, control and Mat KD spheroids remained intact (black arrowheads). In contrast, addition of pro-HGF induced extensive invasive outgrowths in matriptase sufficient control cells (A') (indicated with open arrowheads) whereas the majority of Mat KD remained intact (B'). Both control and Mat KD responded to active two-chain HGF (A'', B''). Cells were labeled with Cell Tracker Orange and nuclei with Hoechst (blue). (C) 3D rBM cell cultures were scored for the number of intact spheroids when left untreated (black bars) or exposed to either active HGF (white bars) or pro-HGF (white bars). Data represent mean of the number of intact spheroids before treatment relative to the number after treatment (*P<0.04, Student's T-Test). Error bars represent S.D.

HGF/SF has been shown to induce invasiveness in a variety of cancer cells in 3D cultures, we used the 3D model system to assess the invasive response of matriptase sufficient and matriptase deficient human breast cancer cells in response to pro-HGF. When SUM229 cells were grown in rBM overlay culture they spontaneously formed spheroids (Fig. 21 A). Upon stimulation with pro-HGF for 24 hours, matriptase sufficient control cells displayed pronounced invasive growth characterized by branching cellular structures and single cells with a flattened spindle-like appearance. Matriptase silencing in SUM229 spheroids (Mat KD) rendered the cells unresponsive to pro-HGF, thus, keeping the vast majority of the cells confined in compact spheroid structures (Fig. 21 A, B and Supplemental figure 3). Similar observations were made using HCC1937 cells (data not shown). Addition of pre-cleaved active HGF restored the ability of matriptase-deficient cells to respond to pro-HGF. These data demonstrate that matriptase, in addition to promoting breast cancer cell proliferation as described above, potentiates the invasive capacity of cancer cells suggesting that matriptase-mediated c-Met signaling is critical for several pro-tumorigenic processes.

5.10 Matriptase and c-Met expression are strongly correlated in human invasive ductal breast carcinomas.

To investigate whether the functional link identified between matriptase and c-Met is likely to represent a general molecular pro-tumorigenic mechanism in human breast cancer, the expression of matriptase and c-Met in a large sample set of 153 (grades I-III) human invasive ductal carcinoma tumor tissue was analyzed by immunohistochemistry (IHC) (Fig. 22). The staining of invasive ductal carcinomas was strong and uniform for both matriptase and c-Met in the cancer cells. Furthermore, the

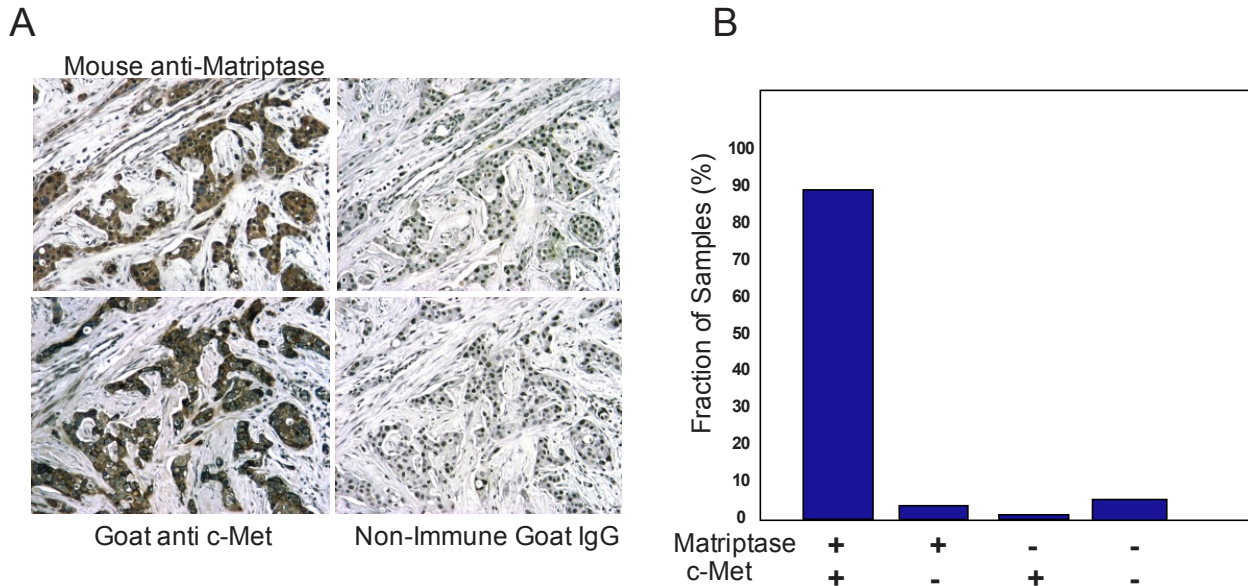
staining patterns observed for matriptase and c-Met were strikingly similar, consistent with co-localization between the two proteases in breast carcinomas (Fig. 22). No significant staining was observed in tumor stromal compartments. The vast majority of tumor samples, 88% (134/153), displayed detectable expression of both matriptase and c-Met protein. A small subset of tumors 7% (11/153), displayed no detectable expression of neither matriptase nor c-Met, whereas 5% (7/153) of the breast tumors expressed matriptase but not c-Met. In only 0.7% (1/153) of the tumors was c-Met but not matriptase protein detected. The overall comparison of groups showed a highly significant correlation between matriptase and c-Met expression in breast cancer ($p < 0.001$). Importantly, co-expression of matriptase and c-Met was also consistently observed in MMTV-PymT tumors validating this breast tumor model as being highly relevant for studying the matriptase/pro-HGF/c-Met signaling axis (Supplementary Figure. 4). The finding that matriptase and c-Met are both expressed in the vast majority of the human invasive ductal carcinomas analyzed, presents the opportunity of potential therapeutic intervention in a large number of breast cancer patients.

5.11 Inhibition of matriptase proteolytic activity abrogates pro-HGF induced c-Met signaling in mouse mammary carcinoma cells and human invasive ductal breast cancer cell lines.

Based on our findings that matriptase is critically involved in breast cancer cell proliferation and invasion, and tumor progression and growth *in vivo*, this protease represents a promising target for drug development. To determine whether inhibition of matriptase proteolytic activity leads to abrogation of the c-Met signaling activation, three matriptase inhibitors were tested in murine and human breast cancer

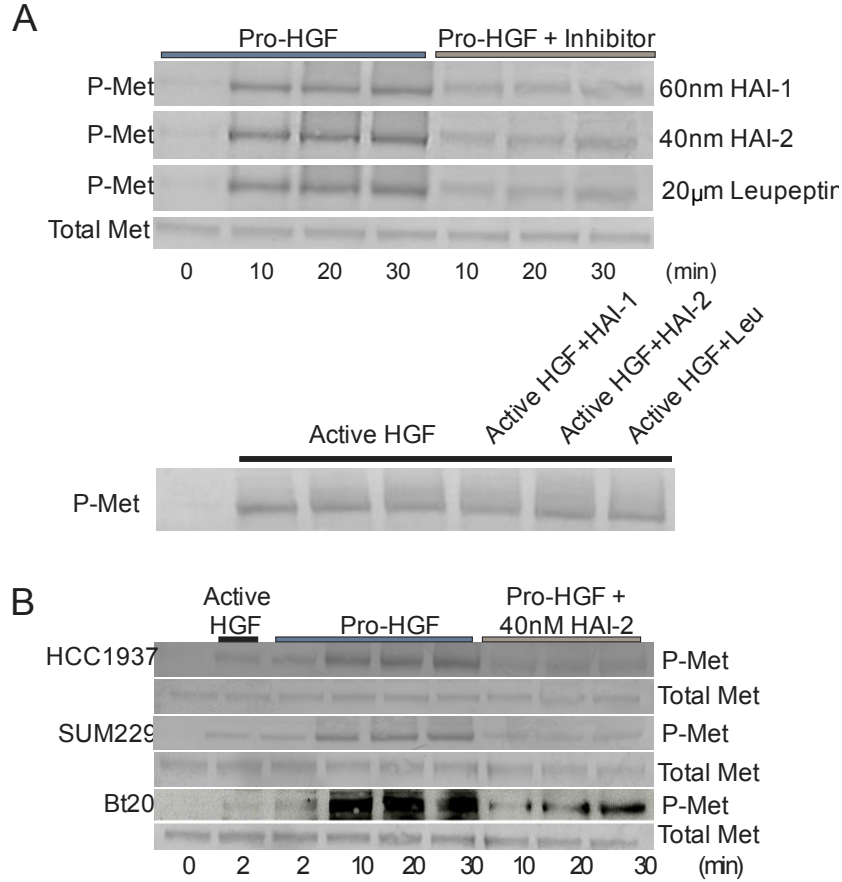
cell culture models. Leupeptin is a broad spectrum small peptide, reversible protease inhibitor. HAI-1 and 2 are reversible, competitive macromolecular Kunitz-type serine-protease inhibitors that haven been shown to be physiological inhibitors of matriptase (Benaud et al., 2001, Szabo et al., 2008, 2009a, 2009b). In addition, HAI-1 and 2 have previously been shown to inhibit several members of the TTSP family *in vitro* including matriptase, matriptase-2, HAT, hepsin and TMPRSS13, however, the physiological relevance of the inhibition of these proteases remains unknown (Maurer et al., 2013, Hashimoto et al., 2010, Tsai et al, 2013, Kato et al., 2011). When leupeptin was added to primary mouse mammary carcinoma cells concomitantly with pro-HGF, a significant inhibition of c-Met activation was observed (Fig. 23 A). A similar effect was seen in the three human breast cancer cell lines BT20, HCC1937 and SUM229 (data not shown). HAI-1 and HAI-2 also inhibited c-Met activation in both murine and human breast cancer cells (Fig. 23 A, B and data now shown). In sum, the development of selective and efficient inhibitors of matriptase proteolytic activity represents a new avenue for targeting the pro-tumorigenic c-Met signaling pathway by inhibition of the matriptase-dependent conversion the signaling-inert single-chain pro-HGF to a signaling-competent two-chain HGF.

FIGURE 22: MATRIPTASE AND c-MET ARE CORRELATED IN HUMAN INVASIVE DUCTAL CARCINOMAS



Representative staining of serial sections of invasive ductal carcinoma (**A-C**) using a mouse monoclonal anti-matriptase antibody (**A**) and a goat anti-cMET antibody (**C**). Primary antibodies were substituted with non-immune isotype control mouse IgG (**B**) or non-immune goat IgG (**D**) as negative controls. Both matriptase and c-Met (brown staining, arrows) is detected in the cancer cells (indicated with "C") with no significant staining in the mesenchymal/stromal compartments (indicated with "S"). Both proteases are primarily localized on cell surfaces and in the cytoplasm and display highly similar expression patterns. (**E**) Expression of matriptase and c-Met in 153 human invasive ductal carcinomas. Bars depicts the frequency of samples expression both matriptase and c-Met 87% (134/153) samples that express neither of the two proteases 7% (11/153), samples that express only matriptase 5% (7/153) and samples that express c-Met only as indicated 1% (1/153). The overall comparison of groups showed a highly significant correlation between c-Met and matriptase expression: Tetrachoric Rho=0.95 ($P < 0.001$).

FIGURE 23: INHIBITION OF MATRIPTASE ACTIVITY IMPAIRS THE c-MET PATHWAY ACTIVATION IN PRIMARY MAMMARY CARCINOMA CELLS AND HUMAN INVASIVE DUCTAL BREAST CANCER CELL LINES



(A) Mouse mammary carcinoma cells were isolated from Mat-PymT⁺ mice and exposed to active HGF or pro-HGF with and without matriptase inhibitors added concomitantly (60 nM HAI-1, 40 nM HAI-2 and 20 uM Leupeptin). Cells were lysed and analyzed for activated c-Met by western blotting. **(B)** Human breast cancer (HCC1937, SUM229 and BT20) cell lines were plated, serum starved and exposed to active HGF, pro-HGF or pro-HGF with 40 nM HAI-2 added concomitantly for 10, 20 and 30 minutes. Cells were lysed and analyzed for total and activated c-Met by western blotting.

CHAPTER 6: MATRIPTASE MEDIATED c-MET SIGNALING IN BREAST CANCER- RESULTS IN INFLAMMATORY BREAST CANCER

6.1 Introduction: Targeting matriptase/c-Met in inflammatory breast cancer

Inflammatory breast cancer is a rare and aggressive form of invasive breast cancer accounting for approximately 5% of breast cancer cases annually in the United States. IBC is characterized by rapid progression, local and distant metastases, younger age of onset, and lower overall survival compared with other breast cancers. IBC is a lethal disease with a 5-year survival rate of 40% when treated with surgery, or radiation therapy. IBC patients often present with a breast that looks inflamed due to extensive lymphovascular invasion of tumor emboli which block lymphatic drainage from the breast, but no palpable tumor. The rapid development of metastases with IBC results from high proliferative rates and potent ability for angiogenesis and lymphangiogenesis.

Studies of extracellular proteolysis in breast cancer progression have mainly focused on the most common form of breast cancer; non-IBC invasive ductal carcinoma. To date, no studies have assessed the expression and potential pro-cancerous role of matriptase in IBC. Based off the promising results in the IDC breast cancer and an on-going collaboration with Dr. Julie Lang, a breast cancer surgeon at the University of Southern California, we expanded the previous experiments to human IBC breast cancer cell lines and obtained IBC patient tumor samples.

6.2 Matriptase silencing causes abrogated c-Met signaling and proliferation in human inflammatory breast cancer cell lines.

It has been demonstrated that matriptase can cleave and activate the pro-form of HGF. However, no studies describing a role for matriptase as a pro-HGF activating protease in breast cancer have been published. To determine whether matriptase regulates c-Met activation via activation of pro-HGF, SUM149, and SUM190 cells were stimulated with pro-HGF or active HGF and the phosphorylation/activation state of c-Met and the downstream targets, Gab-1 and AKT, were analyzed. The source of pro-HGF was serum free conditioned media from human reduction mammoplasty fibroblasts expressing human HGF (RMF-HGF) (Kupperwasser *et al.*, 2004). In the first set of experiments, two physiological inhibitors of matriptase: HAI-1 and HAI-2 were employed. SUM149 and SUM190 cells were serum starved and stimulated with pro-HGF with either HAI-1 or HAI-2 added concomitantly. Both inhibitors efficiently abrogated activation of the c-Met activation pathway (data not shown). Since it cannot entirely be excluded that HAI-1 or HAI-2 inhibits one or more additional proteases in IBC cells, experiments using RNAi mediated silencing of matriptase were performed. Efficient RNAi silencing of matriptase with three independent non-overlapping synthetic RNA duplexes was achieved in SUM149 and SUM190 cells (Fig. 24 A and B). Addition of pro-HGF to matriptase sufficient control cells resulted in robust activation of c-Met and the downstream targets Gab1 and Akt (Fig. 24A' and 3B'). In contrast, matriptase ablation led to a greatly reduced level of c-Met pathway activation (to levels not detectable by western blotting). Importantly, the matriptase silenced cells display unaltered activation of the c-Met pathway upon stimulation with pre-cleaved active HGF, demonstrating that the impaired response to pro-HGF is due to the lack of matriptase-mediated cleavage and not caused by an unforeseen RNAi effect on c-Met functionality

per se (Fig. 24A' and B', second panel). The similar findings in two different IBC cells indicates that the mechanism observed may represent a general phenomenon in IBC.

Again, the functional consequences of abrogating the activation of the c-Met pathway was assessed (Fig. 24 A'''). The growth in matriptase-silenced cells was significantly impaired in response to pro-HGF in comparison to matriptase sufficient cells. No significant difference in growth response to HGF was observed between matriptase sufficient and matriptase deficient cells. These results demonstrate that silencing matriptase leads to decreased pro-HGF activation and subsequent c-Met signaling pathway activation ultimately causing growth inhibition in inflammatory breast cancer cells. Taken together, with the results for mouse mammary carcinoma described above we have demonstrated that matriptase is the major, and perhaps the only, efficient activator of pro-HGF in these inflammatory carcinoma cell lines.

6.3 Matriptase mediates pro-HGF induced invasion in human inflammatory breast cancer cells.

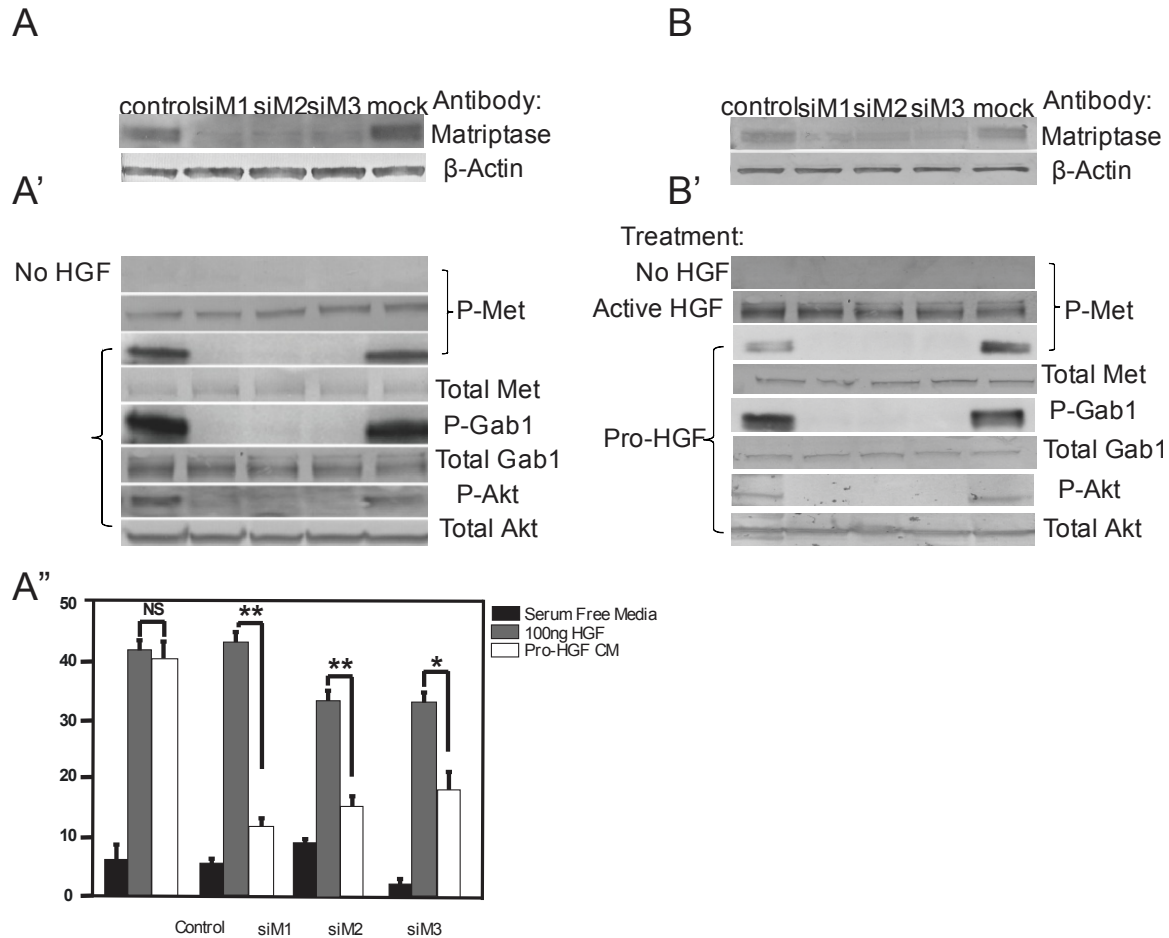
Additionally, we used the 3D model system to assess the invasive response of matriptase sufficient and matriptase deficient human inflammatory breast cancer cells in response to pro-HGF. When SUM149 cells were grown in rBM overlay culture they spontaneously formed spheroids (Fig. 25 A). Upon stimulation with pro-HGF for 24 hours, matriptase sufficient control cells displayed pronounced invasive growth characterized by formation of branching, invasive multi-cellular structures. Matriptase silencing in SUM149 spheroids (Mat KD) rendered the cells unresponsive to pro-HGF, thus, keeping the vast majority of the cells confined in compact spheroid structures (Fig. 25 A, B). Addition of pre-cleaved active HGF restored the ability of matriptase-deficient

cells to respond to pro-HGF. These data demonstrates that matriptase, in addition to promoting breast cancer cell proliferation as described above, potentiates the invasive capacity of inflammatory breast cancer cells suggesting that matriptase-mediated c-Met signaling is critical for several pro-tumorigenic processes.

6.4 Matriptase and c-Met are both expressed in human inflammatory breast carcinomas.

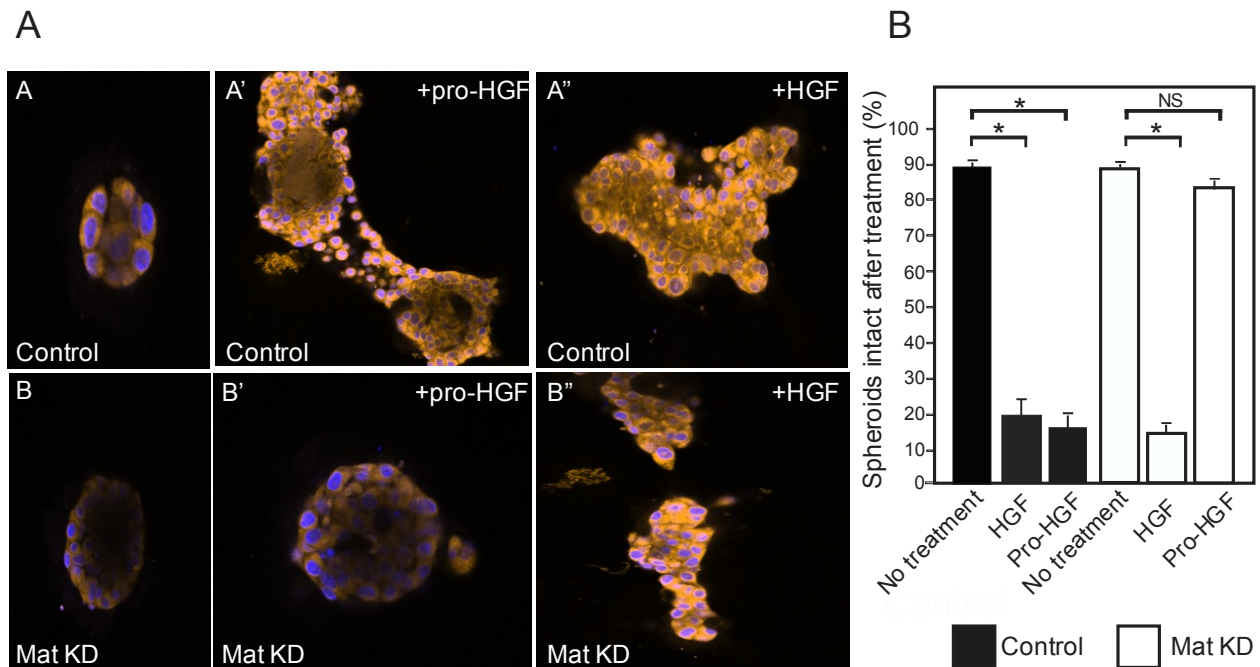
A prerequisite for matriptase and c-Met to be functionally linked *in vivo* is that they are expressed in close proximity to each other, on either the same cell or neighboring cells. Therefore, an important component to elucidate the roles of matriptase and c-Met in IBC is to determine their expression and localization in IBC patient samples. We have performed immunohistochemistry using two different anti-matriptase antibodies and observe staining in infiltrating cancer cells and in the cancer cells of lymphatic emboli from IBC patient samples (Fig. 26 and data not shown). We have also verified that c-Met protein is detected in IBC (Fig. 26) in accordance with a study by Garcia and colleagues (Garcia *et al.* 2007). As of yet, there are no data assessing the extent of co-localization in cancer cells of IBC tumors, or regarding whether co-expression is widespread or confined to certain areas. Therefore, a comprehensive expression analysis on serial sections is needed. However, preliminary data below provide a solid foundation to execute the experiments needed to determine if matriptase and c-Met are correlated in human inflammatory breast carcinomas.

FIGURE 24: MATRIPTASE IS REQUIRED FOR ACTIVATION OF THE PRO-HGF/c-MET SIGNALING PATHWAY AND PROMOTES CELL PROLIFERATION IN INFLAMMATORY BREAST CANCER CELLS



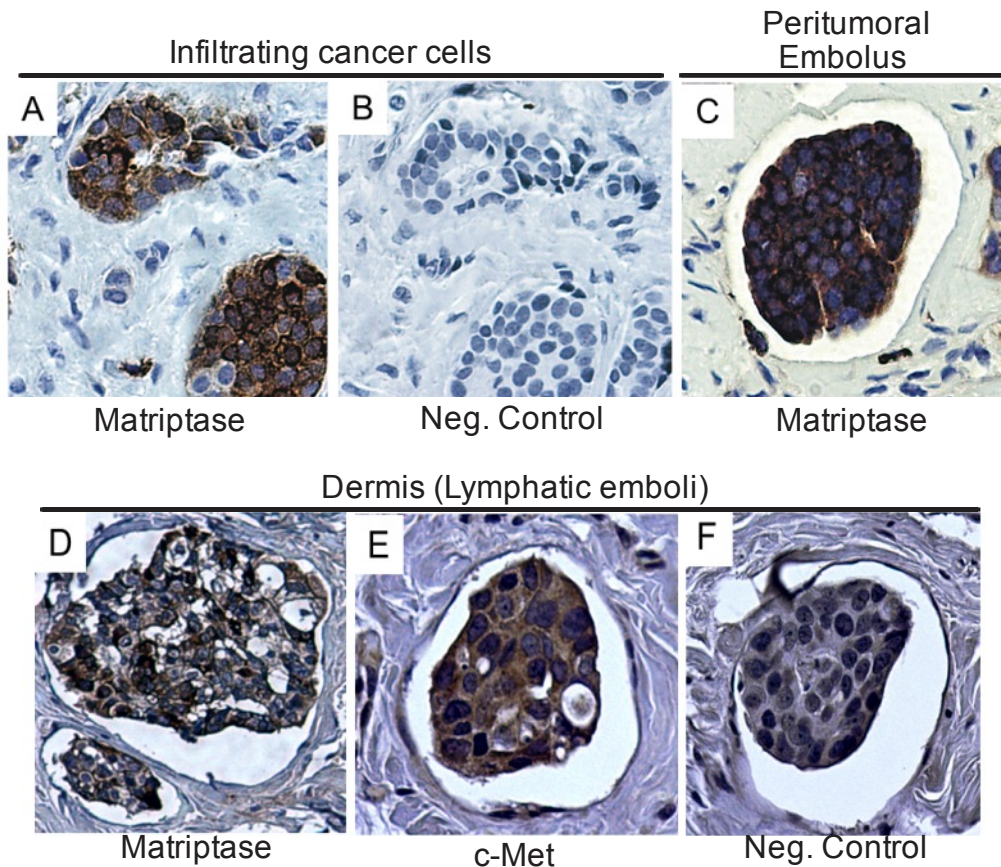
Efficient RNAi silencing of matriptase in SUM149 cells (**A**) and SUM190 cells (**B**) with three independent non-overlapping synthetic RNA duplexes (siM1, siM2 and siM). A %GC matched negative control was used as negative control as well as a mock where no RNA duplex was added. Beta-actin staining of the membranes was performed to ensure equal protein loading SUM149 cells (**A'**) and SUM190 cells (**B'**) were serum starved and exposed to no HGF (serum free media), active HGF (2 min) or pro-HGF (15 min). Cells were lysed and analyzed for total c-met, activated c-Met, and its downstream targets by western blotting. Sum149 cells (**A''**) were grown on culture plates and matriptase was silenced using three independent non-overlapping synthetic RNA duplexes (siM1, siM2 and siM). A %GC matched negative control was used as negative control. The cells were serum starved and exposed to either active HGF or pro-HGF for 24 hours and counted. Graphs show the proliferation at 24 hours after stimulation (* $P < 0.04$, ** $P < 0.005$, *** $P < 0.0008$)

FIGURE 25: MATRIPTASE MEDIATES PRO-HGF INDUCED INVASION IN HUMAN INFLAMMATORY BREAST CANCER CELLS



SUM149 cells were grown on culture plates and matriptase was silenced (Mat KD) using synthetic RNA duplexes (B, B', B''). A %GC matched negative control was used as negative control (A, A', A''). Cells were allowed to form spheroids in 3D rBM overlay culture for 24 hours before addition of either pro-HGF (A', B'), HGF (A'', B'') or no treatment (A, B) and imaged by confocal microscopy. When no HGF was added, control and Mat KD spheroids remained intact (black arrowheads). In contrast, addition of pro-HGF induced extensive invasive outgrowths in matriptase sufficient control cells (A') (indicated with open arrowheads) whereas the majority of Mat KD remained intact (B'). Both control and Mat KD responded to active two-chain HGF (A'', B''). Cells were labeled with Cell Tracker Orange and nuclei with Hoechst (blue). (C) 3D rBM cell cultures were scored for the number of intact spheroids when left untreated (black bars) or exposed to either active HGF (white bars) or pro-HGF (white bars). Data represent mean of the number of intact spheroids before treatment relative to the number after treatment (*P<0.04, Student's T-Test). Error bars represent S.D.

FIGURE 26: MATRIPTASE AND c-MET ARE EXPRESSED IN HUMAN INFLAMMATORY CARCINOMAS



Representative examples of staining of IBC paraffin sections samples with mouse anti-matriptase (**A, C, D**) and goat anti-met antibodies, respectively. Non-immune matching IgG were included as negative controls respectively (**B, F**). Matriptase staining is detected in infiltrating IBC cells (**A**), peritumoral emboli (**C**) and dermal lymphatic emboli (**D**). Expression of c-Met observed in dermal emboli (**E**).

CHAPTER 7: DISCUSSION AND FUTURE DIRECTIONS

Matriptase is an epithelial cell-surface protease that is highly expressed in human breast cancer. In this project, the specific functions of matriptase in mammary tumorigenesis were investigated by using a validated mouse model of human ductal mammary adenocarcinoma (Maglione *et al.* 2001; Lin *et al.* 2003). Mice with low matriptase expression in the mammary epithelium displayed a significant delay in tumor onset, tumor burden and multiplicity. Importantly, a significant abrogation of tumor progression was seen in early stages of carcinogenesis, where total area measurements of lesions revealed a 75% reduction in the matriptase hypomorphic mammary glands. In late stage carcinogenesis, the total tumor burden was reduced by 58%. A major contributing factor to tumor growth impairment was the dramatic decrease of carcinoma cell proliferation in matriptase hypomorphic tumors. To elucidate the molecular mechanisms underlying matriptase-mediated proliferation, an *ex vivo* model where primary mammary carcinoma cells were isolated from tumors and grown outside the body for no more than one week and without passaging was utilized. This model has the benefit of being short-term and therefore fewer cellular changes caused by adaptations to cell culture conditions are expected.

HGF/SF is an important promoter of proliferation and invasion in cancer and its receptor c-Met, like matriptase, is expressed in the epithelial compartment of the breast. The inactive pro-form binds to the c-Met tyrosine kinase receptor where it, upon proteolytic cleavage, is converted to the signaling-competent active form. HGF mediated c-Met signaling activates multiple downstream targets including the PI3/AKT pathway and the c-Met adapter protein, Gab1. Until now, the protease responsible for

pro-HGF activation in breast cancer has not been identified. Using matriptase deficient primary mammary carcinoma cells this work demonstrates that matriptase is essential for activation of fibroblast secreted pro-HGF and initiation of downstream c-Met signaling. This finding was confirmed using matriptase silencing in three different human IDC cell lines and 2 human IBC cells lines, as well as matriptase re-expression in one human IDC cell line. In addition, these data demonstrate that the functional consequence of matriptase abrogation is severe impairment of proliferation and invasion in breast cancer cell lines. This work concludes that matriptase-mediated pro-HGF activation and c-Met signaling is critical in breast cancer cells from both mice and human, and that this mechanism is conserved in two very different human breast cancer types as well as in an array of breast cancer cells lines with different origins and characteristics.

Since breast cancer cells express a variety of extracellular proteases, it is somewhat surprising that none of these appear to be capable to cleave and activate pro-HGF in mouse primary mammary carcinoma cells or human breast cancer cells when matriptase expression is ablated. Hepatocyte growth factor activator (HGFA) has been reported to be expressed in multiple breast cancer cell lines and in human breast tumors (Parr *et al.* 2001). It should be mentioned, that despite its name, no evidence has been presented that implicates HGFA as a physiological relevant pro-HGF activator. In genetic targeting studies in mice, both HGF null and c-Met null mice display an embryonic lethal phenotype, whereas HGFA-null mice develop normally to term and have no discernable phenotype in adult mice (Bladt *et al.* 1995, Uehara *et al.* 1995, Itoh *et al.* 2004). That suggests that HGFA is dispensable as a pro-HGF activator during

development and postnatal life. Studies assessing the role of HGFA in tumorigenesis *in vivo* have not been published.

Two TTSP's, hepsin and TMPRSS13, that have previously been reported as being able to cleave and activate pro-HGF *in vitro*, were readily detected by Western blot analysis in a variety of breast cancer cells lines (unpublished data). These included HCC1937, SUM229, BT20, SUM190 and SUM149 used in this study for matriptase silencing studies. Since no or minimal residual pro-HGF activity was detected upon matriptase silencing, it is unlikely that these proteases act as efficient pro-HGF activators on the cell surface of breast cancer cells. It can be argued that acute matriptase ablation may not allow breast cancer cells time to mobilize compensatory mechanisms. Countering this possibility is the fact that the SUM159 cell line that has lost endogenous matriptase and remains undetectable through several years and multiple passages, expresses high levels of both hepsin and TMPRSS13, yet requires transgenic re-expression of matriptase in order to respond to pro-HGF stimulation. Several scenarios could be imagined to explain why matriptase is the most efficient cellular pro-HGF activator. In *in vitro* experiments using recombinant soluble activate forms of hepsin and matriptase, hepsin is approximately 10 fold less efficient than matriptase in activating pro-HGF, whereas matriptase and HGFA display similar efficiency (Owen *et al.* 2010). However, the level of active matriptase in comparison to active hepsin or active HGFA in breast cancer cells is not known. Also, matriptase is able to efficiently cleave pro-HGF on the cell surface (Owen *et al.* 2010) which may give matriptase an advantage over HGFA, a soluble protease, as activated HGF can be specifically generated at its site of action. It remains to be determined whether the

membrane localization of matriptase provides the protease with a kinetic advantage and whether the stem-region domains are involved in substrate recognition or interaction with c-Met. Thus, factors including activation state, membrane-anchoring, localization in cell membrane microdomains, and interaction between protease and c-Met could favor matriptase in certain cellular microenvironments including breast cancer.

Based on these studies, the development and testing of selective matriptase inhibitors may represent a new avenue for targeted therapy in breast cancer. As demonstrated, matriptase is critically involved in the activation of c-Met dependent pro-tumorigenic processes *in vivo*, *ex vivo* and in breast cancer cell lines. Importantly, the fact that matriptase and c-Met display a strong correlated expression in human breast tumors suggest that targeting the matriptase-mediated c-Met signaling cascade has the potential for broad clinical application. One factor to consider is the potential side-effects of matriptase inhibition in patients. Based on studies in mouse genetic models, the skin and the gastrointestinal tract would like be the most sensitive tissues where matriptase targeted drugs could cause adverse effects. However, hypomorphic mice which have less than 1% residual epidermal and intestinal matriptase and display only a mild and temporary ichthyosis of the skin in very young mice, and a mild gut barrier defect with no discernible consequences for adult mouse health (List *et al.* 2005, Buzza *et al.* 2012). Furthermore, humans with a hypomorphic mutation in the *ST14* gene, resulting in a matriptase mutant protein with a 1000-fold lower activity than that of wild-type matriptase resulted in a mild temporary ichthyosis in the skin and brittle hair of affected children with significant improvement with age. No gastrointestinal symptoms were reported (List *et al.* 2007, Basel-Vanagaite *et al.* 2007). It is therefore plausible that

matriptase function in normal physiological functions in humans is predominantly critical during childhood and that targeted inhibition in adults have few and mild adverse effects.

In a recent article by a group of researchers that originally identified c-Met and since made seminal contributions regarding the biochemical properties, structure and functions of c-Met in development and disease, the rationale and progress of targeting c-Met in cancer is reviewed (Gheradi *et al.* 2012). The c-Met inhibitors developed thus far include c-Met antagonists such as antibodies that bind to c-Met and competes with pro-HGF/HGF binding, and c-Met kinase inhibitors. Some of the c-Met kinase inhibitors are highly selective for c-Met, whereas others target additional receptors such as VEGFR-2 and platelet-derived growth factor receptor- β (PDGFR β). The authors mention targeting of pro-HGF activating proteases as potential novel inhibitors of c-Met signaling and that resistance to kinase inhibitors may be circumvented by combining inhibitors with different modes of action.

The majority of c-Met inhibitors used in clinical trials are still under evaluation, however, promising results have been achieved in non-small cell lung cancer where patients with high c-Met expression showed increased progression-free survival when treated with a monoclonal anti-c-Met antagonistic antibody in combination with an EGFR-inhibitor (erlotinib) than with erlotinib alone (Spigel *et al.* 2011). In breast cancer, studies with the multi-target c-Met inhibitor XL184 (also known as cabozantinib) have shown significant activity (Gordon *et al.* 2011).

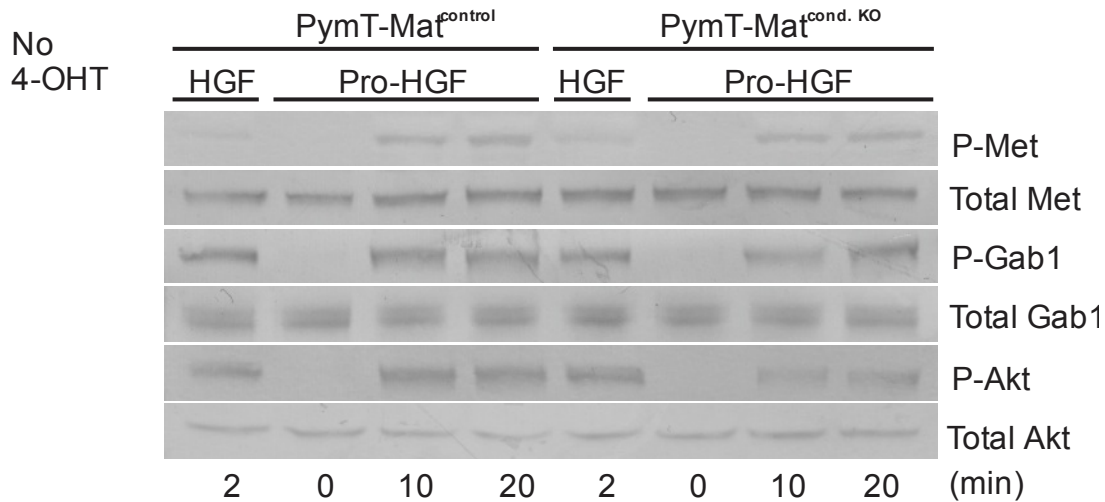
In an effort to evaluate the anti-tumorigenic properties of small-molecule matriptase inhibitors, a collaboration with Drs. Leduc, Marsault and Richter at the

Université De Sherbrooke, Québec, Canada has been initiated. This group has designed and synthesized a series of potent and selective matriptase inhibitors that have not previously been tested in cancer cell experiments (Colombo *et al.* 2012). Initially, the efficacy of the inhibitors will be tested for their ability to inhibit matriptase-mediated pro-HGF activation, c-Met signaling and proliferation in human breast cancer lines. The long-term plan is to test the anti-tumorigenic potential of selected inhibitors in mouse breast cancer models.

In conclusion, the data obtained in the dissertation work presented here demonstrates that matriptase is critical for breast carcinoma progression using multiple *in vivo*, *ex vivo* and cell culture approaches. Importantly, a mechanistic understanding of the pro-cancerous properties matriptase has been reached by identifying matriptase as an essential activator of pro-HGF and initiator of the c-Met signaling pathway. The findings described here have laid the groundwork for future studies testing small-molecule matriptase inhibitors and their potential as novel targeted therapeutic drugs in breast cancer.

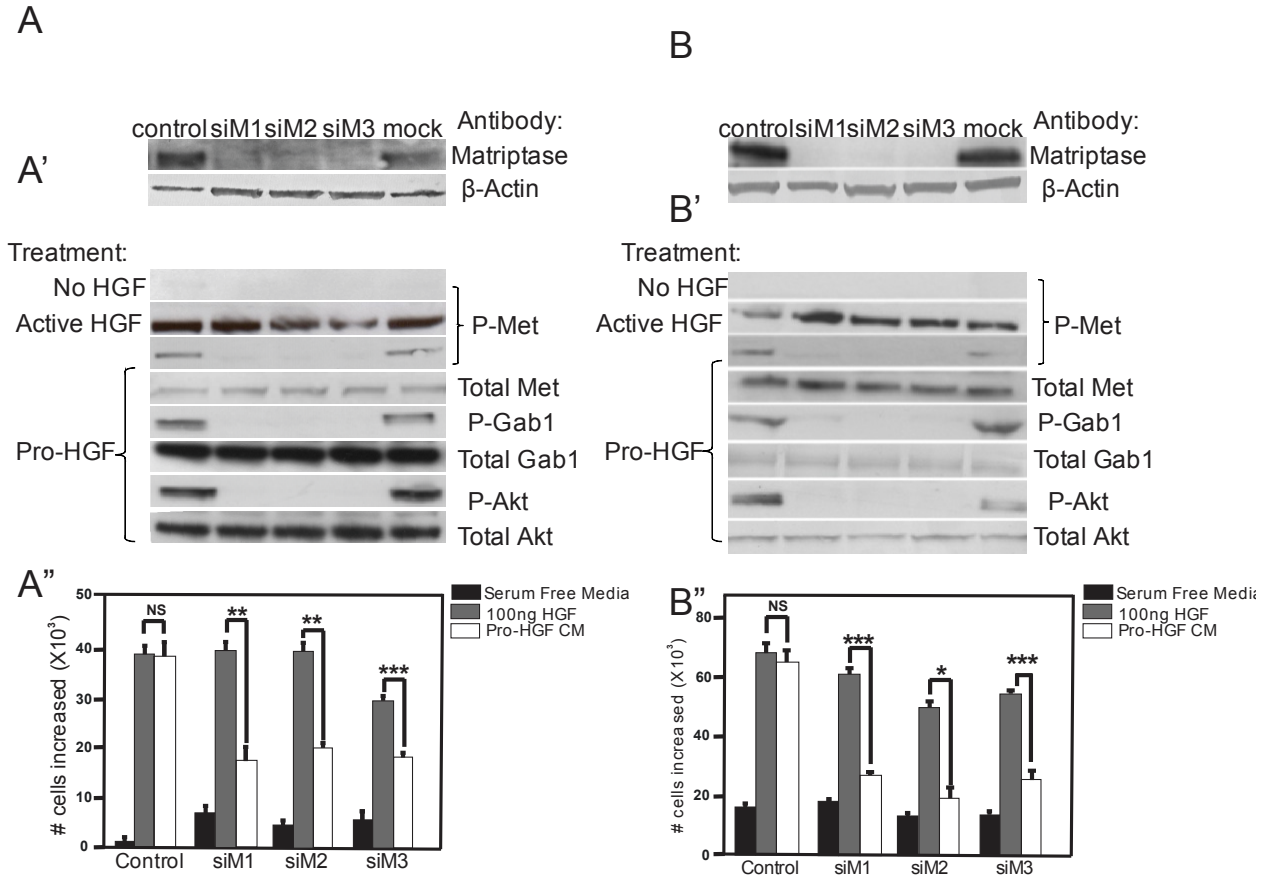
APPENDIX A: SUPPLEMENTARY FIGURES

Supplementary Figure 1: Activation of c-Met and downstream signaling proteins in matriptase sufficient primary mammary carcinoma cells.



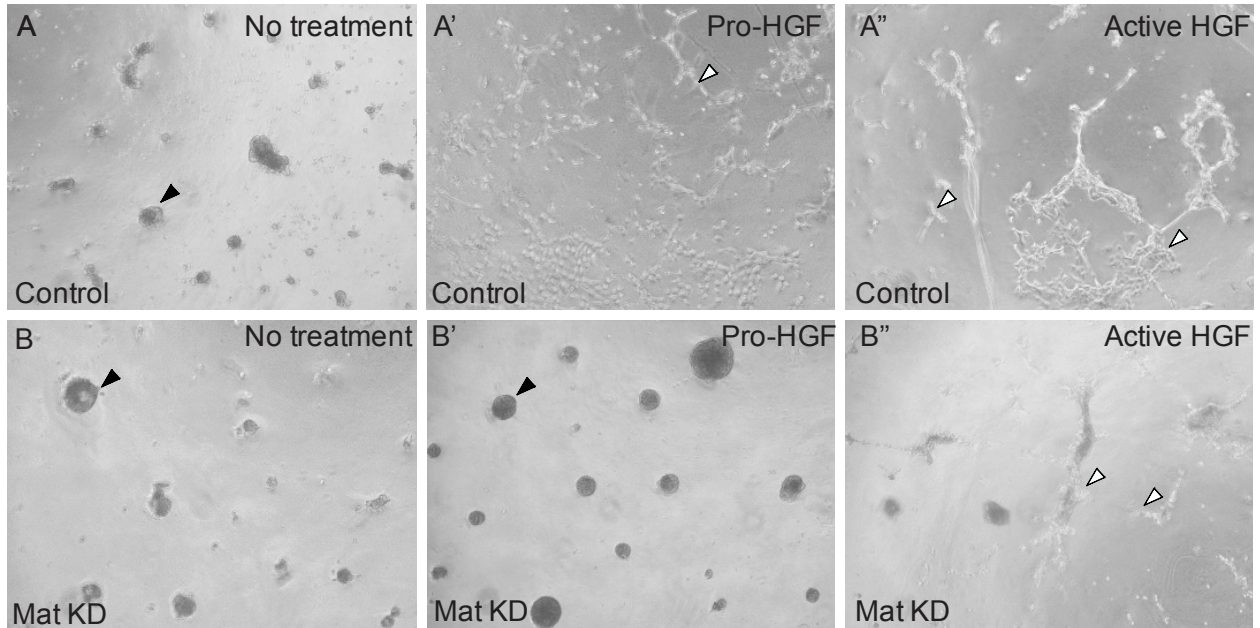
Mammary carcinoma cells were isolated from PymT-Mat^{cond KO} and treated with vehicle for 24 hours (4-OHT treatment shown in Figure 4) and exposed to active HGF (2 mins) or pro-HGF (0, 10 or 20 mins). Cells were lysed and analyzed for total c-met, activated c-Met, and its downstream targets by western blotting.

Supplementary Figure 2: Matriptase is required for activation of the pro-HGF/c-Met signaling pathway and promotes cell proliferation in BT20 and SUM229 breast cancer cell lines.



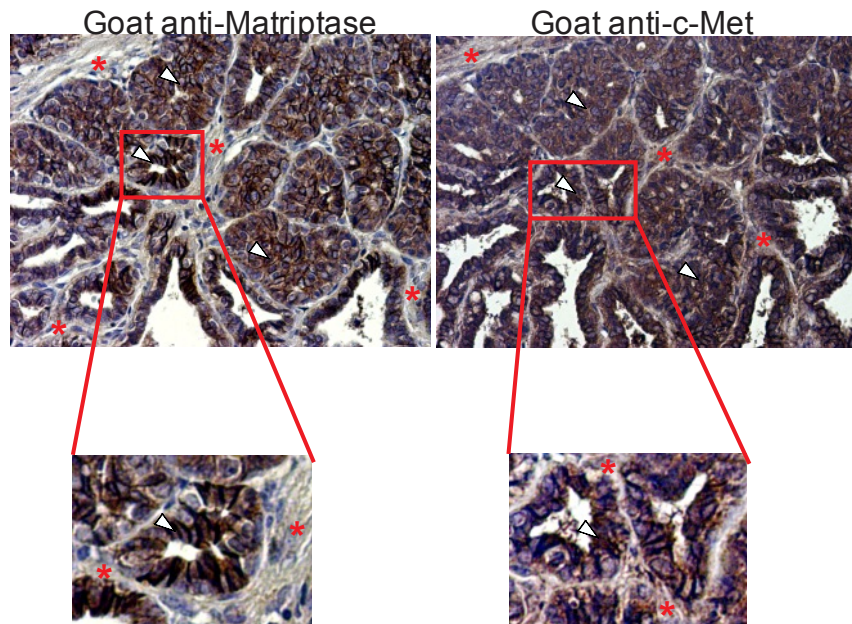
Efficient RNAi silencing of matriptase in Bt20 cells (**A**) and SUM229 cells (**B**) with three independent non-overlapping synthetic RNA duplexes (siM1, siM2 and siM3). A %GC matched negative control was used as negative control as well as a mock where no RNA duplex was added. Beta-actin staining of the membranes was performed to ensure equal protein loading Bt20 cells (**A'**) and SUM229 cells (**B'**) were serum starved and exposed to no HGF (serum free media), active HGF (2 min) or pro-HGF (15 min). Cells were lysed and analyzed for total c-met, activated c-Met, and its downstream targets by western blotting. Bt20 cells (**A''**) and SUM229 cells (**B''**) were grown on culture plates and matriptase was silenced using three independent non-overlapping synthetic RNA duplexes (siM1, siM2 and siM3). A %GC matched negative control was used as negative control. The cells were serum starved and exposed to either active HGF or pro-HGF for 24 hours and counted. Graphs show the proliferation at 24 hours after stimulation (* $P < 0.04$, ** $P < 0.005$, *** $P < 0.0008$)

Supplementary Figure 3: Matriptase mediated pro-HGF induced invasion in human breast cancer cells.



SUM229 cells grown on culture plates and matriptase was silenced (Mat KD) using synthetic RNA duplexes (B, B', B''). A %GC matched negative control was used as negative control (A, A', A''). Cells were allowed to form spheroids in rBM overlay culture for 24 hours before addition of either pro-HGF (A', B'), HGF (A'', B'') or no treatment (A, B). When no HGF was added, control and Mat KD spheroids remained intact (black arrowheads). In contrast, addition of pro-HGF induced extensive scattering and invasion in matriptase sufficient control cells (A')(indicated with open arrowheads) whereas the majority of Mat KD remained intact (B'). Both control and Mat KD responded to active two-chain HGF (A'', B'')

Supplementary Figure 4: Matriptase and c-Met are expressed in mouse mammary tumors.



Representative examples of immunohistochemical analysis of serial sections of invasive ductal carcinoma in the mammary gland of PymT-Mat⁺ mice using a sheep anti-matriptase antibody (A) and a goat anti c-Met antibody. Both matriptase and c-Met (brown staining, arrows) is detected in the cancer cells with no significant staining in the stromal compartment (*). Matriptase and c-Met are primarily localized on cell surfaces and display highly similar expression patterns. Inserts; high magnification photos or indicated tumor areas. Slides were counterstained with hematoxylin.

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ABSTRACT**MATRIPTASE MEDIATED c-MET SIGNALING IN BREAST CANCER**

by

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Historically proteases have been associated with tumor progression and metastasis through degradation of the basement membrane. However, in recent years these enzymes have also been shown to play key roles in the activation of growth factors and cytokines, thereby activating pro-oncogenic signaling pathways. Matriptase (MT-SP1, ST14) is an epithelia-specific pericellular protease which has received considerable attention in recent years spurred by the consistent dysregulation of the protease in human epithelial tumors including breast cancer. We have performed both functional and mechanistic studies of matriptase in breast cancer using parallel, complementary *in vitro* and *in vivo* genetic “loss-of-function” strategies. To determine the contribution of matriptase to breast cancer *in vivo* we used matriptase hypomorphic mice with very low matriptase expression in the mammary gland. When crossed into the mammary tumor virus (MMTV) Polyomavirus middle T (PymT) mouse genetic mammary tumor model, matriptase hypomorphic mice displayed a significant decrease in tumor burden.

The hepatocyte growth factor (HGF)/c-Met signaling pathway is dysregulated in breast cancer and has been causally linked to breast carcinogenesis. It has previously been shown that matriptase is an effective activator of the inactive pro-form of HGF. To investigate whether matriptase mediated pro-HGF activation and elicitation of the c-Met signaling pathway plays a critical role in breast cancer we isolated primary mammary carcinoma cells from conditional matriptase knock-out mice carrying the *β -actin-Cre* *ERTM* transgene. Upon brief exposure to tamoxifen, matriptase was efficiently genetically ablated allowing for comparative analysis of c-Met phosphorylation/activation in matriptase sufficient and deficient primary carcinoma cells. Addition of pro-HGF to matriptase sufficient cells resulted in robust activation of c-Met and the downstream targets Gab1 and Akt. In contrast, matriptase ablation led to a greatly reduced level of c-Met pathway activation. Parallel experiments using RNA silencing of matriptase in four different human breast cancer cell lines similarly demonstrated abrogation of c-Met signaling upon pro-HGF stimulation. This abrogation of the c-Met signaling pathway affects the breast cancer cells proliferation rate as well as their invasiveness as demonstrated in 3D culture.

Our results suggest that matriptase is critically involved in pro-HGF activation leading to downstream signaling and elicitation of mitogenic, migratory and invasive responses in breast cancer.

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