# PROTEOLYTIC PROCESSING OF LAMININ-332 BY HEPSIN AND MATRIPTASE AND ITS ROLE IN PROSTATE CANCER PROGRESSION

By

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Dedicated to my Parents, my Alma Mater and Bhagavan Shri Sai Baba.

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## PREFACE

Prostate cancer is the second most common form of cancer in men. It is the second leading cause of death in American men. Despite these alarming figures and decades of research, a cure still eludes prostate cancer patients. The morbidity associated with advanced prostate cancer is due to the metastasis of the primary tumor primarily to the bone. Studies have shown that 80% of prostate cancer patients have clinical evidence of bone metastases. These metastases severely reduce the quality of life of the patient due to the associated morbidity due to bone fractures and eventually cause mortality. Factors that affect prostate cancer progression are poorly understood. Some of these factors are genes that are upregulated or downregulated during the progression of the disease. Identifying these factors could be key to the treatment of prostate cancer. Hepsin, a cell surface protease, is widely reported to be overexpressed in more than 90% of human prostate tumors and also hepsin expression correlates with tumor progression, therefore assumes tremendous relevance in prostate cancer as a therapeutic target in the future. Recently, targeted expression of hepsin in the mouse prostate promoted tumor progression and metastasis when crossed with the 12-T7f mouse model of prostate cancer. Intriguingly, the hepsin transgenic mice displayed reduced Ln-332 expression in the prostate. Despite this evidence that hepsin promotes prostate tumor progression, its physiological function remains largely uncharacterized. Matriptase is another gene that is over-expressed in prostate cancer patients. The expression of both, hepsin and matriptase positively correlates with the aggressiveness of human prostate tumors. Though both genes are known to be over-expressed in human prostate cancer, their mechanism of

action is still unknown. Both hepsin and matriptase fall within a category of genes known as proteases that have the potential ability to cleave or digest the extra cellular matrix (ECM) that immediately surrounds the tumor cells (called basement membrane) in a primary tumor and inhibits cells from escaping and eventually metastasizing. Tumor cells escape the primary tumor by interacting and digesting the basement membrane and travel through the blood stream to reach the bone environment.

This study reveals the interaction between the ECM component Ln-332, which is lost in prostate cancer and two type II transmembrane serine proteases, hepsin and matriptase that are consistently over-expressed in human prostate cancer cases. We have found in our study that hepsin and matriptase individually cleave or digest Laminin-332 and this cleavage facilitates movement of human prostate cells in tissue culture. Further, we have found that there is degradation of Ln-332 in the prostate tumor tissue of the hepsin transgenic mice. Furthermore, hepsin or matriptase overexpressing LNCaP prostate cancer cells also exhibited increased migration on Ln-332. Additionally, matriptase over-expression causes increased persistence of LNCaP cells on Laminin-332. This cleavage of Ln-332 by hepsin and/or matriptase may play the critical mechanistic role for the metastatic spread of human prostate cancer from primary tumors. This study therefore assumes relevance towards finding future therapeutic targets that inhibit tumor growth and metastasis in human prostate cancer.

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| | |
|---|---|
| µg | Micro gram |
| µl | Micro liter |
| BM | Basement membrane |
| ECM | Extracellular matrix |
| EV | Empty vector |
| H&E stain | Haematoxylin and eosin stain |
| IHC | Immunohistochemical |
| KD | Kunitz domain |
| kDa | Kilo Dalton |
| Ln-332 | Laminin-332 (Laminin-5) |
| MMP | Matrix metallo-proteinase |
| mRNA | Messenger ribonucleic acid |
| PB | Probasin |
| PBS | Phosphate Buffered Saline |
| PCa | Prostate Cancer |
| PIN | Prostatic Intraepithelial Neoplasia |
| SDS | Sodium dodecyl sulphate |
| siRNA | Small interfering RNA |
| SV40 | Simian Virus 40 |
| Tag | Simian Virus 40 Large T antigen |
| TTSP | Type II transmembrane serine proteases |
| α3 | Alpha 3 |
| β3 | Beta 3 |
| γ2 | Gamma2 |

# CHAPTER I

# INTRODUCTION

## Cancer

According to the World Health Organization (WHO), one in eight deaths is due to cancer, and death from cancer will continue to rise worldwide with a projected 12 million deaths by 2030 (Thun, DeLancey et al. 2010). Cancer is a group of different diseases characterized by unrestrained proliferation of cells. The progression of cancer is marked by genetic alterations in oncogenes and tumor suppressor genes that facilitate development of the tumor. Most cancers develop in various steps and stages over a period of many years. Cancer cells have tendency to spread to distant sites (metastasis), one of the main causes of death in cancer patients. Cancer can be found in most types of tissue or organs, and is named for the organ or cell type of origin. Carcinomas, for example, are the most common type of cancers and are derived from epithelial cells. Cancer treatment includes radiation, chemotherapy, surgery, hormones and immunotherapy. As a general rule, cancers diagnosed before they metastasize have a better prognosis. Therefore, deciphering the biomolecular events leading to metastasis is critical to define new targets for cancer therapies.

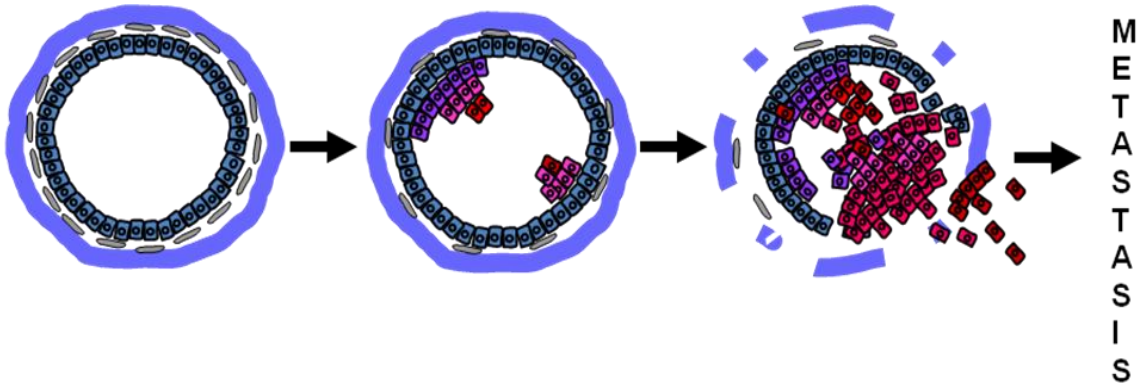


Figure 1: Prostate cancer progression model. Prostate cancer progresses from normal epithelium to prostatic intraepithelial neoplasia (PIN) to invasive carcinoma and finally to metastasis. In each step there is increased proliferation of cancer cells. Invasive carcinoma is characterized by cells breaking off of the primary tumor. Proteases help in the processes of invasion by digesting basement membrane components. Prostate cancer metastasizes primarily to bone.

## Prostate Cancer

The worldwide incidence of prostate cancer is 25.3 per 100,000 men, making it the second most common cancer in men (Nelen 2007). Prostate cancer is also the second leading cause of cancer death in men in the USA with an estimated 217,730 new cases in the USA in 2010, 70% of which are in men above the age of 80 (Jemal, Siegel et al. 2010).

Prostate cancer progression is pathologically defined as a four-step process: dysplasia, high grade prostatic intraepithelial neoplasia (HGPIN), locally invasive carcinoma, systemic disease or metastases. Prostate cancer primarily metastasizes to the

lung, liver and bone (Pinthus, Pacik et al. 2007; Witte 2009). Metastasis dramatically increases this disease's morbidity and mortality (Chambers, Groom et al. 2002; Mundy 2002; Kalluri 2003; Sherwood 2006) (Figure 1).

The two main methods for prostate cancer screening are digital rectal examination (DRE) and prostate-specific antigen (PSA) levels (Pinthus, Pacik et al. 2007). In the 1980s, the discovery and use of PSA as a marker of prostate cancer revolutionized the diagnosis of the disease (Kuriyama, Wang et al. 1980; Papsidero, Wang et al. 1980; Brawer, Chetner et al. 1992). Although prostate cancer has a poor prognosis if diagnosed at late stages, the use of PSA as a diagnostic marker has allowed for detecting the disease in its early stage, and thus has contributed to continuously decreasing death rate in patients. However, the specificity of PSA and PSA levels as markers of prostate cancer is being questioned (Stamey, Johnstone et al. 2002), initiating a search for new prostate cancer markers.

## Extracellular matrix and Cancer

About a decade ago, Hanahan and Weinberg defined cancer cells with six hallmarks: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg 2000). During the progression of human prostate cancer, these hallmarks correlate with various genetic and epigenetic changes, including the loss or downregulation of various tumor suppressor genes like Nkx3.1, PTEN, EPHB2, pRb and KLF6 (Shaw, Rabinovitz et al. 1997; Wang, Parsons et al. 1998; Narla, Heath et al. 2001; Vasioukhin 2004) and

upregulation or overexpression of genes like, MYC, BCL2 and AR (Jenkins, Qian et al. 1997; MacGrogan and Bookstein 1997; Vasioukhin 2004; Turner and Watson 2008).

As in most of cancer research, the focus in prostate cancer has been on the tumor itself but, during the last few years, the nonmalignant tumor microenvironment is being increasingly recognized for its active contribution to the disease progression (Albini and Sporn 2007). The tumor microenvironment broadly consists of the cellular component (endothelial cells, myoepithelial cells, pericytes and inflammatory cells) and the noncellular components (growth factors, proteolytic enzymes and extracellular matrix [ECM] macromolecules) (Tlsty and Coussens 2006), which create a favorable environment for the cancer cells. In the tumor microenvironment, various ECM components get processed and remodeled by proteases, which in turn affects cancer cell behavior in terms of adhesion, migration, proliferation, survival and differentiation (Cudic and Fields 2009).

*Basement Membrane*

In the 1980s, scientists realized that ECM was not just an inert scaffold, but initiated key signaling events crucial for normal cell functions (Kenny, Lee et al. 2007). ECM has since been involved in cell differentiation, proliferation, survival, polarity and migration (Liu, Wu et al. 1995; Kenny, Lee et al. 2007). Epithelial cells secrete a specialized ECM: the basement membrane (BM). Although the basement membrane is comprised of more than 50 distinct macromolecules, the major constituents are: type IV collagen, laminins, nidogen/entactin and proteoglycans, such as perlecan, agrin and bamacan (Schittny and Yurchenco 1990; Yurchenco and Schittny 1990). Most of these

proteins have a large molecular weight and have multiple functional domains. Laminins and type IV collagen are the only BM constituents that are able to self-assemble into polymers (Miner and Yurchenco 2004; Yurchenco, Amenta et al. 2004). Until recently, the consensus about BM organization was that type IV collagen was secreted first, and was acting as a scaffold on to which laminins get anchored. However, recent studies indicate that laminins form the initial template for BM assembly (Li, Liquari et al. 2005; McKee, Harrison et al. 2007). In addition to ECM macromolecules, many growth factors such as fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs) bind to ECM proteoglycans like heparin and heparin sulfate, directly linking ECM with cell growth (Hynes 2009). Further, many integrins—ECM receptors—are well known signal transduction receptors that modulate growth factor receptor downstream activity (Hynes 2009). Mutations or deletions of ECM and BM component genes can lead to serious tissue disorganization and potentially be lethal (Liu, Wu et al. 1995; Jobsis, Keizers et al. 1996; Liu, Wu et al. 1997; Bonaldo, Braghetta et al. 1998; Mayer, Kohfeldt et al. 1998; Smyth, Vatansever et al. 1998; Colognato, Winkelmann et al. 1999; Colognato and Yurchenco 1999; Buzza, Wang et al. 2001).

*Basement membrane and cancer*

In the field of cancer research, BM has conventionally been viewed as a protective barrier against cancer spread, since tumor cells have to remodel or degrade it in order to metastasize to secondary sites via blood or lymphatic circulation (Birkedal-Hansen 1995; Chambers, Groom et al. 2002; Sherwood 2006). However, it is now known that tumor cells use BM functions to their advantage to boost their own ability to

migrate, proliferate and invade (Hanahan and Weinberg 2000). For example, the remodeling of certain ECM molecules by proteolytic activity, may lead to increased tumor cell motility (Noel, Gilles et al. 1997; Giannelli, Quaranta et al. 2003; Schenk and Quaranta 2003; Woodward, Holen et al. 2007). It is now well established that tumor associated proteolytic enzymes play an important role in altering the BM to make it permissive to tumor cells. Such BM remodeling, mainly accomplished by matrix metalloproteinases (MMPs) and TTSPs (Type II transmembrane serine proteases) (Kalluri 2003; Lopez-Otin and Matrisian 2007), has been repeatedly linked to cell migration, a critical step of invasion and metastasis (Noel, Gilles et al. 1997; Hintermann and Quaranta 2004; Woodward, Holen et al. 2007).

## Proteases and cancer

Proteases are enzymes that cleave proteins by hydrolysis of peptide bonds. They are classified into five protease families; metalloproteinases, serine proteases, threonine proteases, cysteine proteases and aspartic acid proteases, and they account for more than 2% of the total genes in the human genome (Puente, Sanchez et al. 2003; Lopez-Otin and Matrisian 2007; Lopez-Otin and Bond 2008; Lopez-Otin and Hunter 2010). Under normal physiological conditions, the activity of proteases is tightly controlled: they are produced as inactive zymogens that need to be activated. Proteases are associated with a wide variety of pathological conditions, such as cancer progression. Proteases facilitate tumor development by suppressing cell death, activating cell survival, promoting inflammation, initiating angiogenesis and stimulating tumor cell migration and invasion. Their proteolytic activity regulates the activities of growth factors, signaling receptors

and the composition of the ECM. Therefore, proteases have been considered as potential targets for cancer therapies (Lopez-Otin and Matrisian 2007).

Intracellular proteases are associated with degrading endocytosed proteins. Their tumor suppressor roles occur via activation of autophagy (Teitz, Wei et al. 2000; Marino, Salvador-Montoliu et al. 2007), induction of apoptosis (Maisonnave 1965; Mandruzzato, Brasseur et al. 1997; Teitz, Wei et al. 2000; Harada, Toyooka et al. 2002; Soung, Lee et al. 2003; Soung, Lee et al. 2004; Stupack, Teitz et al. 2006), negative regulation of NFκB pathway (Bignell, Warren et al. 2000; Massoumi, Chmielarska et al. 2006; Masuya, Huang et al. 2006; Hellerbrand, Bumes et al. 2007), stabilization of p53 (Masuya, Huang et al. 2006) and inhibition of proliferation (Reinheckel, Hagemann et al. 2005; Wahlstrom, Cutts et al. 2007). In contrast, extracellular proteases are commonly associated with tumorigenesis, as they are often overexpressed in cancer (Egeblad and Werb 2002). But the failures of clinical trials using small-molecule MMP inhibitors to treat cancer implied a more complex role for these proteases in cancer progression, with fewer MMPs having possible anti-tumor roles (Coussens, Fingleton et al. 2002; Overall and Kleifeld 2006).

*Serine Proteases*

With a total of 176 identified members in humans, serine proteases are the largest family of proteases (Lopez-Otin and Matrisian 2007). All serine proteases have a serine residue at their active site, which is part of a catalytic triad serine-histidine-aspartic acid. They play important roles in various physiological processes, such as digestion, blood

coagulation, regulation of blood pressure, pathogen host interaction and wound healing (Netzel-Arnett, Hooper et al. 2003).

*Type II Transmembrane Serine Protease*

Type II Transmembrane Serine Proteases (TTSPs) are S1-class serine proteases with N-terminal transmembrane domain, a C-terminal extracellular serine protease domain of the chymotrypsin (S1) fold and highly variable stem region (Hooper, Bui et al. 2001; Netzel-Arnett, Hooper et al. 2003; Szabo, Wu et al. 2003). The extracellular C-terminal has the catalytic activity of the enzyme, while the cytoplasmic tail at its N-terminal interacts with cytoskeletal and signaling molecules. The enzymatic activity of these TTSPs is dependent on the presence of three catalytic residues—histidine, aspartate and serine (HDS)—in its proteolytic domain. These TTSPs are synthesized as single chain zymogens and are activated by cleavage after a Arg or Lys residue at the activation motif (Vu, Liu et al. 1997; Afar, Vivanco et al. 2001; Szabo, Wu et al. 2003). Unlike secreted serine proteases, TTSPs have a transmembrane domain therefore they are localized at the cell surface, making them ideal candidates for mediating signal transduction between the cell and its environment. TTSPs play an important role in normal development and they are overexpressed in cancer. Currently, 17 TTSPs are known in human, divided into four subfamilies based on the phylogenetic analysis of their serine protease domains and the domain structure of the stem region: HAT/DESC subfamily, hepsin/TMPRSS/enteropeptidase subfamily, matriptase subfamily and corin subfamily (Szabo, Wu et al. 2003) (Figure 2).

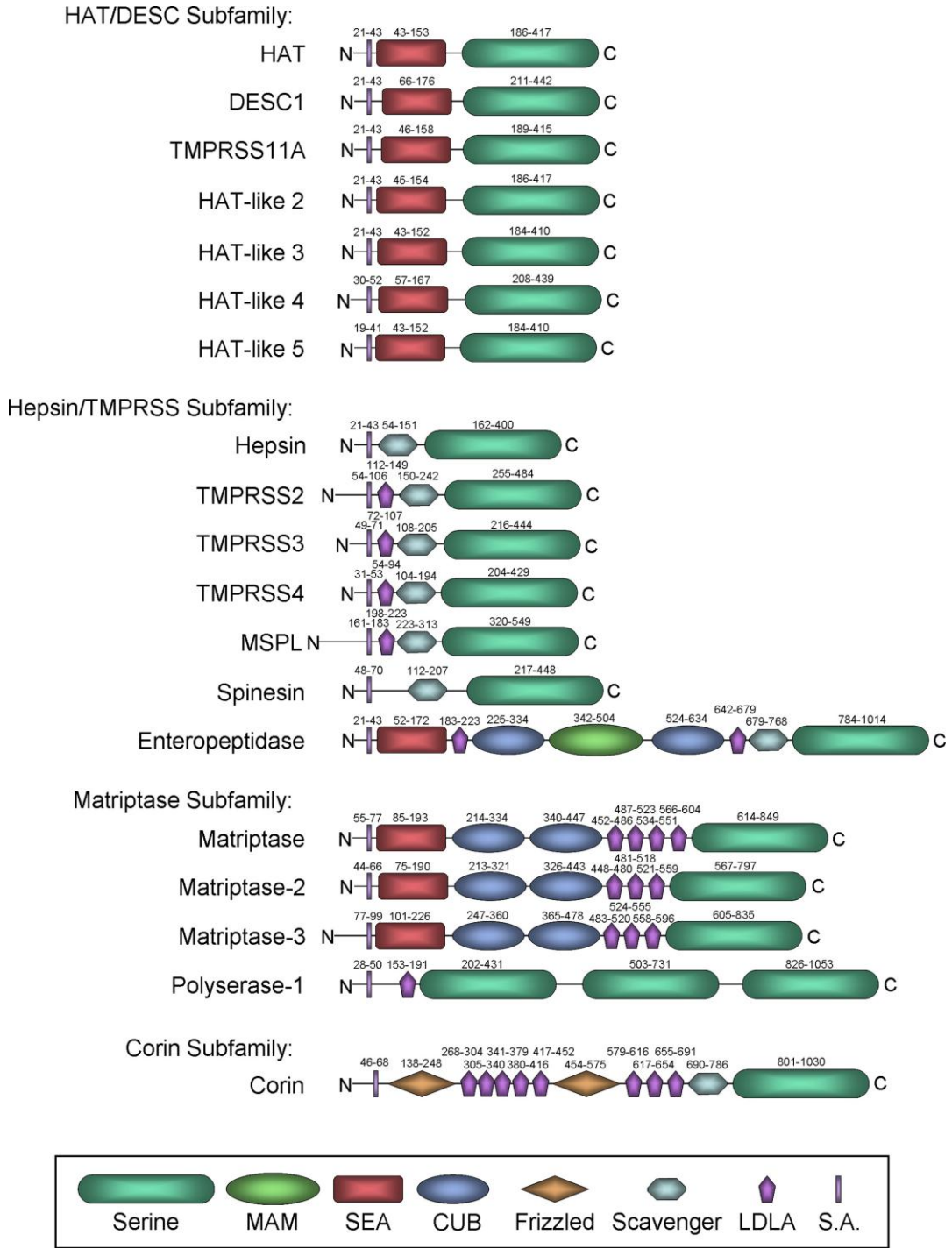


Figure 2: Summary of the human type II transmembrane serine protease family. The subdivision is based on the predicted domain structures. (Bugge, Antalis et al. 2009).

*TTSPs in Cancer*

In recent years, numerous studies have investigated the roles of TTSPs in various cancer types: TTSPs have been linked to tumor progression through the regulation of cell proliferation, migration, invasion and metastasis (Szabo and Bugge 2008; Choi, Bertram et al. 2009). Several TTSPs, such as TMPRSS2, corin, hepsin, enteropeptidase, matriptase, YMPRSS3, TMPRSS4, matriptase-2, and differentially expressed in squamous cell carcinoma gene 1(DESC1), are deregulated in cancer (Netzel-Arnett, Hooper et al. 2003; Szabo and Bugge 2008). For example, TMPRSS2 is overexpressed in androgen-dependent prostate cancer (Lin, Ferguson et al. 1999; Vaarala, Porvari et al. 2001). It is also altered via chromosomal rearrangement where TMPRSS2 fuses with transcription factors of ETS family, ERG, ETV1, or ETV4 (Tomlins, Rhodes et al. 2005; Soller, Isaksson et al. 2006; Tomlins, Mehra et al. 2006). Enteropeptidase expression is elevated in pancreatic cancer (Rinderknecht, Renner et al. 1978). TMPRSS3 is overexpressed in ovarian, pancreatic and breast cancers (Iacobuzio-Donahue, Ashfaq et al. 2003; Iacobuzio-Donahue and Hruban 2003; Iacobuzio-Donahue, Maitra et al. 2003; Sawasaki, Shigemasa et al. 2004; Carlsson, Petersson et al. 2005), whereas TMPRSS4 is overexpressed in pancreatic, gastric and colorectal tumors (Wallrapp, Hahnel et al. 2000). Interestingly, in contrast to most of the TTSPs, DESC1 is either reduced or lost in squamous cell carcinoma of head and neck (Lang and Schuller 2001; Aimes, Zijlstra et al. 2003). We discuss in detail two of these TTSPs, hepsin and matriptase, since they are subject to this work.

## Hepsin

Hepsin is a member of the TTSP family. It is highly expressed in hepatocytes, and also expressed in thyroid, thymus, lung, pancreas, pituitary gland, prostate and kidney (Tsuji, Torres-Rosado et al. 1991; Tsuji, Torres-Rosado et al. 1991; Aimes, Zijlstra et al. 2003). The physiological function of hepsin remains unclear (Wu and Parry 2007), but *in vitro* studies have identified blood coagulation factors as substrates of hepsin (Kazama, Hamamoto et al. 1995). Pro-hepatocyte growth factor and pro-urokinase-type plasminogen activator are also substrates for hepsin (Kirchhofer, Peek et al. 2005; Moran, Li et al. 2006). Intracellular downstream targets of hepsin remain unknown. Hepsin is not essential for development: hepsin-deficient mice are viable, fertile and have no reported developmental defects (Wu, Yu et al. 1998; Yu, Chen et al. 2000; Wu and Parry 2007). However, hepsin is required for normal auditory function (Guipponi, Tan et al. 2007).

Level of hepsin expression varies with the tumor type (Table 1). Hepsin is reported to be over expressed in ovarian cancer (Tanimoto, Yan et al. 1997; Bignotti, Tassi et al. 2007). In the case of breast cancer hepsin expression seems to be dependent on tumor type; estrogen-receptor alpha (ERα)-positive breast tumors had upregulation of hepsin as compared with ERα-negative breast tumors (Tozlu, Girault et al. 2006). Elucidating the role of hepsin in prostate cancer is of great interest because it is overexpressed in more than 90% of human prostate cancer. Its expression even correlates with the progression of the disease (Dhanasekaran, Barrette et al. 2001; Luo, Duggan et al. 2001; Magee, Araki et al. 2001; Stamey, Warrington et al. 2001; Stephan, Yousef et al. 2004; Riddick, Shukla et al. 2005; Xuan, Schneider et al. 2006). Moreover, a study searching for genetic factors involved in prostate cancer susceptibility in men of

European origin identified many single nucleotide polymorphisms (SNPs) in the *hepsin* gene that were associated with increased risk for the disease, (Pal, Xi et al. 2006).

Recently, a prostate cancer mouse model demonstrated that hepsin overexpression causes disorganization of the BM and promotes prostate cancer progression and metastasis (Klezovitch, Chevillet et al. 2004). The mechanism involved in this disorganization of the BM was not revealed, however the authors did report that prostate tissues from hepsin-overexpressing mice exhibited weaker immunohistochemical staining of basement membrane components like Laminin-332 (Ln-332) compared to wild-type mice. The transgenic mice used in that study had an overexpression of hepsin in the prostatic epithelial cells, by using the prostate specific probasin promoter (PB). These mice display a disorganized BM caused by a weakened epithelial-stromal adhesion, despite normal differentiation, proliferation and apoptosis. Further, it was shown that the areas of disorganized BM correlated with the area of higher hepsin expression (Klezovitch, Chevillet et al. 2004). This corresponds to a weakening or sometimes absent Ln-332 staining in the PB-hepsin mice. Similarly, type IV collagen staining showed disorganized and diffused localization of the protein in the PB-hepsin mice as compared to the wild type control. Integrin α6β4, an important Ln-332 receptor that is associated with formation of hemidesmosomes (specialized cell adhesion structure linked to intermediate filaments that connect epithelial cells to the ECM), is also decreased in human prostate cancer (Murant, Handley et al. 1997; Bonkhoff 1998; Davis, Cress et al. 2001). Immunofluorescent staining of α6β4 integrin demonstrated perturbations of hemidesmosomes in the PB-hepsin mice (Klezovitch, Chevillet et al. 2004). Taken together, the staining for markers of the BM and adhesion structures of cell-substratum

suggests that the BM structure is compromised in the hepsin overexpressing transgenic mice.

Interestingly, the hepsin overexpressing mice did not show any other major abnormalities of the prostate, therefore a bigenic mouse was created by crossing the PB-hepsin mice with another mouse model of prostate cancer, the LPB-Tag 12T-7f (Lady transgenic model). The LPB-Tag 12T-7f mouse was made by expressing SV40 large T antigen (Tag) under the control of PB promoter (Kasper, Sheppard et al. 1998; Masumori, Thomas et al. 2001). While the LPB-Tag 12T-7f mice develop HGPIN and adenocarcinoma, but no metastasis, the PB-hepsin/LPB-Tag 12T-7f bigenic mice show metastases in the liver, lung and bones, along with a lower prostate expression of type IV collagen and β4 integrin (Klezovitch, Chevillet et al. 2004). The decreased staining of Ln-332 following hepsin overexpression led us to hypothesize that Ln-332 is processed by hepsin, which may be a step in the progression of prostate cancer.

In a very recent study, another group has shown that hepsin cooperates with myc in the progression of adenocarcinoma in a prostate cancer mouse model (Nandana, Ellwood-Yen et al. 2010). These investigators crossed the PB-hepsin mice with the myc mice (Ellwood-Yen, Graeber et al. 2003), another model of prostate cancer. Myc mouse (Hi-Myc mouse) was generated using a probasin promoter (ARR2PB) to target high levels of the human c-myc gene to the mouse prostate. These mice develop androgen dependent invasive adenocarcinoma in the prostate by 6 months of age. This breeding program to increase the low endogenous levels of hepsin found in the myc transgenic mice. Interestingly, the hepsin/myc bigenic mice display accelerated tumor progression and develop adenocarcinoma of higher grade as compared with the c-myc transgenic mice.

Taken together, these observations indicate that hepsin deserves to be investigated in more depth as to its effect on prostate cancer progression.

**Table 1**: Expression of TTSPs, Hepsin and Matriptase in human tumors (modified from (Bugge, Antalis et al. 2009).

| **Protease** | **Cancer type** | **Changes in gene expression** |
|---|---|---|
| Hepsin | Prostate | Significant overexpression, high levels correlate with Gleason score |
| | Ovarian | Significant overexpression in tumors compared to normal tissue |
| | Breast | Elevated expression in ERα-positive compared to ERα-negative breast tumors |
| | Hepatocellular | Decreased expression, low expression correlates with shorter patient survival |
| Matriptase | Ovarian | 5 to 18-fold overexpression compared to normal tissue |
| | Breast | Elevated levels correlate with tumor stage |
| | Prostate | Increased expression correlates with Gleason score |
| | Cervical | Increased expression correlates with histopathological grade |
| | Pleural mesothelioma | More than 800-fold overexpression in malignant tumors compared to normal tissue |
| | Lung | Expression in malignant but not normal adjacent tissue |
| | Liver | Elevated expression in tumors compared to normal tissue |
| | Kidney | Elevated expression in tumors compared to normal tissue |
| | Pancreatic | Overexpression in hypoxic areas of the tumor |
| | Gastric | Downregulation of expression |
| | Colorectal | Downregulation of expression but increased matriptase/HAI-1 ratio |

## Matriptase

Matriptase, another member of TTSP, is expressed in the epithelial compartment of many embryonic and adult tissues: gastrointestinal tract, hair follicles, kidney, epidermis, trachea, thymus, urinary bladder, prostate, seminal vesicle, epididymus, uterus and oviduct (Takeuchi, Shuman et al. 1999; Indyk, Chen et al. 2003; Johnson, Oberst et al. 2003; Oberst, Singh et al. 2003; Oberst, Williams et al. 2003; List, Bugge et al. 2006; Netzel-Arnett, Currie et al. 2006; Bugge, List et al. 2007; List, Currie et al. 2007; Szabo, Molinolo et al. 2007). Recently, matriptase was reported to be expressed in monocytes and macrophages (Kilpatrick, Harris et al. 2006; Bhatt, Welm et al. 2007). Matriptase-deficient mice die shortly after birth due to a severely impaired water barrier function in the epidermis of the skin and oral epithelium (List, Haudenschild et al. 2002). The strong oncogenic potential of matriptase has been firmly established most recently by a report of transgenic mice with overexpression of matriptase in the skin that leads to malignant transformation and potentiates chemical carcinogenesis (Oberst, Williams et al. 2003).

Matriptase regulates physiological and pathological functions by proteolytically processing or cleaving its substrates (Uhland 2006; Bugge, List et al. 2007; Darragh, Bhatt et al. 2008; List, Kosa et al. 2009). A few studies have demonstrated that matriptase can cleave the pro forms of Hepatocyte growth factor (HGF), macrophage-stimulating protein1 (MSP-1), urokinase-type plasminogen activator, prostasin zymogen, Trask (transmembrane and associated with src kinases) and protease-activated receptor-2 (Lee, Dickson et al. 2000; Takeuchi, Harris et al. 2000; Bhatt, Erdjument-Bromage et al. 2005; Netzel-Arnett, Currie et al. 2006; Bhatt, Welm et al. 2007; Seitz, Hess et al. 2007; Darragh, Bhatt et al. 2008).

Matriptase is overexpressed in several carcinomas (Table 1), and has recently been recognized as a potential marker for prostate cancer progression (Saleem, Adhami et al. 2006) by many studies showing that its expression is significantly increased in prostate tumor samples compared to normal tissue and correlates with disease progression (Riddick, Shukla et al. 2005; Saleem, Adhami et al. 2006; Warren, Twohig et al. 2009). In addition, Forbs *et al.* reported that inhibition of matriptase in prostate cancer cells by siRNA reduces invasive growth potential *in vitro* (Forbs, Thiel et al. 2005). This group also reported a similar effect using a synthetic matriptase inhibitor. Another recent study found that a small molecule inhibitor of matriptase reduced growth of tumors in prostate cancer xenograft models (Galkin, Mullen et al. 2004). These authors also showed that the inhibition of tumor growth was through the attenuation of cancer cell invasion, rather than cell proliferation. A similar study showed reduction of cell migration and invasion using both *in vitro* and *in vivo* xenograft models by inhibiting matriptase by siRNA (Sanders, Parr et al. 2006). Matriptase is inhibited by the Kunitz domain (KD) of hepatocyte growth factor (HGF) activator inhibitor-1 (HAI-1), which is a Kunitz-type transmembrane serine protease inhibitor (Lin, Anders et al. 1999; Lin, Anders et al. 1999; Kirchhofer, Peek et al. 2003; Cao and Ye 2005; List, Szabo et al. 2005; Shia, Stamos et al. 2005; Zeng, Cao et al. 2005; Fan, Brennan et al. 2007; Szabo, Molinolo et al. 2007). Using this inhibitor, it has been shown that inhibition of HAI-1 expression in prostate cancer cells results in increased cell invasion and migration *in vitro* (Sanders, Parr et al. 2007).

In summary, identification of critical substrates for hepsin and matriptase is an important approach for targeting these proteases therapeutically for the treatment of prostate cancer. This was a key motivation for our study.

## Laminins

Laminins are high-molecular weight, heterotrimeric glycoproteins and are an essential component of all BM (Marinkovich 2007). The trimers are composed of one α, one β and one γ chain held together by disulphide bonds, forming a cruciform structure when viewed by rotary shadowing electron microscopy (Martin and Timpl 1987). To date five α, four β and three γ chains have been identified, forming at least 15 members of a laminin family. (Aumailley, Bruckner-Tuderman et al. 2005). The molecular weight of Laminins ranges from 0.5 million to one million Daltons. Epithelial cells use laminins as ligands to adhere, spread and migrate (McGowan and Marinkovich 2000). Laminin functions also depend on the tissue distribution. For example, laminin-111 is an embryonic isoform that is essential at the blastocyst stage (Li, Harrison et al. 2002). Laminins-511 and -521 are located in the epithelial BM, and are the most abundant laminins (Zhang 1996; Ekblom 1998; Kikkawa 1998). Mutation of laminin-211 causes congenital muscular dystrophy (Zhang 1996; Allamand 1997). Deficiency in laminin-411 causes developmental defects in the peripheral nervous system (Iivanainen 1995). Therefore, Laminins are essential players for many important biological processes including tissue development, wound healing and tumorigenesis (Figure 3) (Miner and Yurchenco 2004; Marinkovich 2007).

*Receptors for Laminins*

Laminins have two types of cell-surface receptors: integrins and non-integrins. Integrins are a large family of heterodimeric glycoproteins formed by non-covalent association of two transmembrane subunits: α and β. Out of 18 α and 8 β subunits discovered, 24 distinct integrin heterodimers are known. The extracellular domain of integrins binds to the ECM, and the cytoplasmic domain binds to the actin cytoskeleton and affiliated proteins, such as vinculin, talin, and α-actin (Chrzanowska-Wodnicka and Burridge 1996; Hannigan, Leung-Hagesteijn et al. 1996). Integrins integrate the intracellular and extracellular environment via bidirectional signaling. "Outside-in" signaling occurs when ligand binds to integrins and activates intracellular pathways (the signal received by the integrin comes from outside of the cell) and "inside-out" signaling occurs when intracellular signaling pathways activate the extracellular domain of integrins by acting on their cytoplasmic domain (in this case, the signal received by the integrin comes from inside of the cell) (Hynes 2002; Hynes 2002; Hynes, Lively et al. 2002). Integrin activation involves two processes: 1) conformational change of the integrin to a high affinity state, and 2) clustering of integrins at the cell-matrix interaction site (Lee, Bankston et al. 1995; Schwartz, Schaller et al. 1995; Parsons 1996; Takagi, Erickson et al. 2001). Integrins regulate cell adhesion, spreading and migration via their interaction with ECM ligands (Carman and Springer 2003). An important motif for integrin-ligand interactions is the RGD (Arg-Gly-Asp) motif: a cell attachment site that is recognized by nearly half of the members of integrin family. Short peptides containing RGD motif have been shown to bind integrins and have been used as integrin activity blockers. Interestingly, such peptides are able to influence tumor growth, invasion and

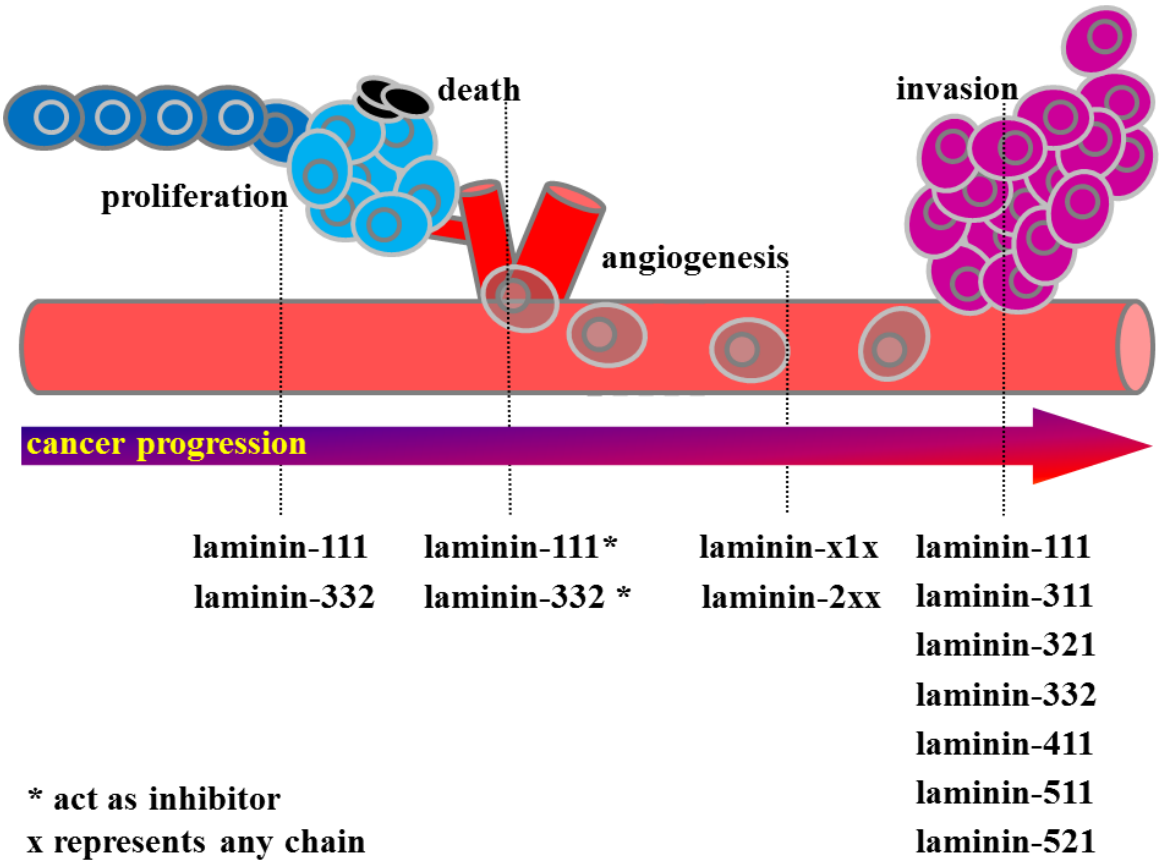


Figure 3: Laminins and Cancer Progression. Laminins involved in four main cell processes of cancer progression; proliferation, death, angiogenesis and invasion. Jourquin et al (chapter from book Laminins and cancer progression" in "*Cell- Extracellular Matrix Interactions and Cancer*." Zent R and Pozzi A (editors), Springer-Verlag New York (Publisher) 2009.

metastasis, and are used in the design of drugs for the treatment of diseases like thrombosis, osteoporosis and cancer (Pierschbacher, Hayman et al. 1981; Brooks 1994; Brooks, Clark et al. 1994; Pierschbacher 1994; Ruoslahti 1996; Ruoslahti 1996).

Integrins α3β1 and α6β4 are the main receptors for laminin-332 (Carter, Ryan et al. 1991; Delwel, de Melker et al. 1994; Niessen, Hogervorst et al. 1994). Integrin α3β1 links laminin-332 to the intracellular actin cytoskeleton and integrin α6β4 links laminin-332 to the intracellular intermediate filaments (Carter, Ryan et al. 1991; Georges-Labouesse, Messaddeq et al. 1996). In resting epithelia, like skin, gut, breast and prostate, integrin α6β4 is the main integrin engaged with unprocessed laminin-332, leading to the formation of hemidesmosomes (Baker, Hopkinson et al. 1996). Hemidesmosomes mediate the adhesion of epithelial cells to the underlying basement membrane and link the cytoskeleton to the ECM (Borradori and Sonnenberg 1996). Interestingly, in the resting epithelia, α3β1 is mostly present at the lateral cell-cell adhesion sites (Carter, Ryan et al. 1991; Bartolazzi, Kaczmarek et al. 1993; Baker, DiPasquale et al. 1996; Baker, Hopkinson et al. 1996), but is redistributed to the basal cell surface during wound healing, when it promotes adhesion, spreading and migration of keratinocytes (Borradori and Sonnenberg 1996; Goldfinger, Hopkinson et al. 1999). As a confluent layer gets reconstituted, integrin α3β1 disappears from the basal surface and gets replaced by integrin α6β4 which forms hemidesmosomes, leading to a stronger cell-BM adhesion (Jones, Kurpakus et al. 1991; Ryan, Christiano et al. 1996).

Laminins are also known to bind to proteoglycans. For example, dystroglycan, highly expressed in muscle tissue, is a receptor for laminin-211(Ervasti, Ohlendieck et al. 1990; Campbell 1995; Henry and Campbell 1998; Henry, Williamson et al. 1998).

Dystroglycan interacts with LG45 module (LG modules are globular domains present at the C-terminus of alpha chains of Laminins) (Campbell 1995). This interaction between laminin-211 and α-dystroglycan is critical for the muscle sarcolemma, which is the cell membrane of muscle cells and alterations in it can cause muscular dystropy (Ervasti and Campbell 1991; Ervasti, Kahl et al. 1991; Ohlendieck, Ervasti et al. 1991; Ohlendieck, Ervasti et al. 1991). Syndecan-4, another proteoglycan, binds to LG4 module of laminin-332 (Utani, Nomizu et al. 2001).

## Laminin-332

One essential component of BM is laminin-332 (ln-332: previously known as laminin-5, kalinin, nicein, ladsin and epiligrin (Aumailley, Bruckner-Tuderman et al. 2005), which plays an important role in epithelial homeostasis, remodeling, wound healing and tumorigenesis (Ryan, Christiano et al. 1996). Ln-332, just like other laminins, is a large, multi-domain glycoprotein consisting of α3, β3 and γ2 disulfide-bonded subunits (Figure 4). It has a cross-shaped structure, with its long arm consisting of domains I and II of the three subunits wound in a coiled-coil and holding the molecule together (Marinkovich 2007). At its C-terminus, the α3 chain has 5 large globular (LG) domains that interact with various cell surface receptors, including α3β1, α6β4 and heparin proteoglycan syndecans, which leads to the establishment of cell adhesion and migration phenotype (Hintermann and Quaranta 2004; Marinkovich 2007). Through its N-terminus, the β3 chain of Ln-332 interacts with other ECM molecules like laminin-321, laminin-411 and type VII collagen. These interactions affect BM assembly and cell-survival signaling (Hintermann and Quaranta 2004; Marinkovich 2007). The γ2 chain

contains epidermal growth factor (EGF)-like domains III-V (Marinkovich 2007) and interacts with the epidermal growth factor receptor (EGFR), which also stimulates cell migration (Schenk, Hintermann et al. 2003; Hintermann and Quaranta 2004). Of note, several studies have shown that all three Ln-332 chains can be processed by different protease families (Hintermann and Quaranta 2004). Previous reports have suggested that these proteolytic events regulate cell motility of both normal and transformed cells which may have implications in cancer progression (Marinkovich 2007).

The importance of Ln-332 in the BM assembly was established by the discovery of the occurrence of a lethal skin blistering disorder, junctional *epidermolysis bullosa*, which is due to mutation in any of the three chains of Ln-332 (Meneguzzi, Marinkovich et al. 1992). Ln-332 is a ligand for important epithelial integrin receptors, α3β1 and α6β4 (Carter, Kaur et al. 1990; Carter, Ryan et al. 1991; Delwel, de Melker et al. 1994; DiPersio, Hodivala-Dilke et al. 1997; Fuchs, Dowling et al. 1997; De Arcangelis 1999; Belkin and Stepp 2000; DiPersio, van der Neut et al. 2000). Based on the integrin it is interacting with, Ln-332 promotes the formation of two different types of cell attachment structure: interaction with α3β1 leads to focal adhesion formation (promotes cell adhesion and migration of normal and malignant cells), and interaction with α6β4 leads to formation of hemidesmosomes *in vivo* (Gil, Brown et al. 1994) (Carter, Kaur et al. 1990; DiPersio, Hodivala-Dilke et al. 1997; De Arcangelis, Mark et al. 1999). Hemidesmosomes are formed with the interaction of Ln-332 with α6β4 and of β4 integrin with type XVII collagen and plectin (Nievers, Schaapveld et al. 1999; Litjens, de Pereda et al. 2006). Interestingly, focal adhesions are short time adhesion structures that

are critical to epithelial cell migration, whereas hemidesmosomes are stronger adhesion structures that are much more resistant to mechanical stress.

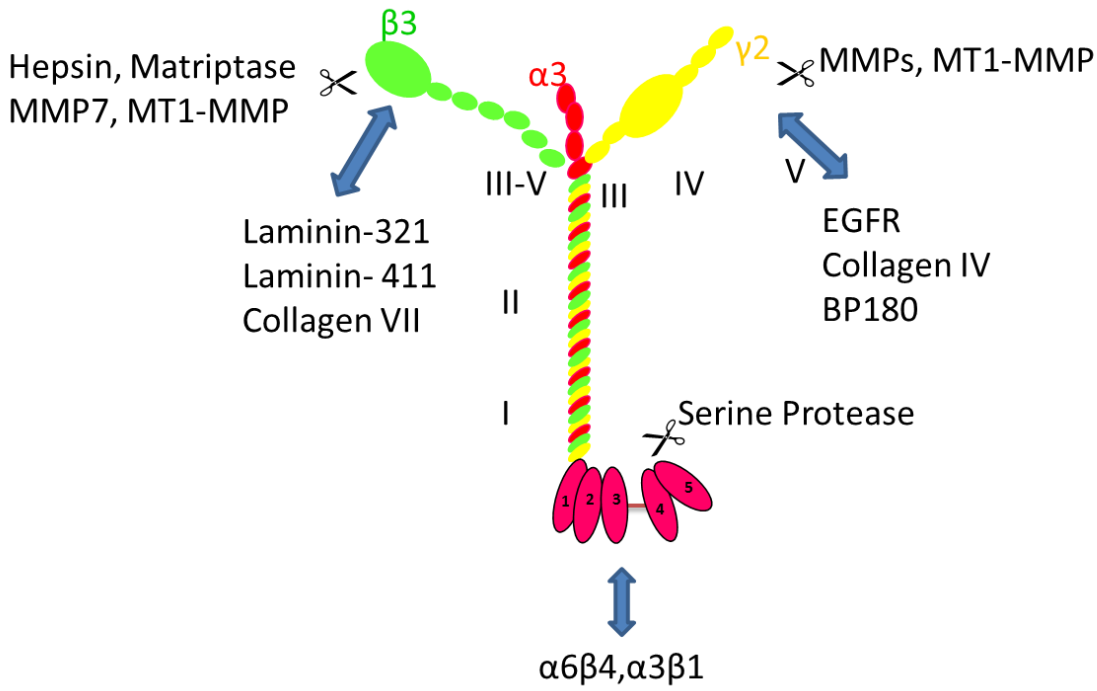


Figure 4: Ln-332 with its interaction partners. Ln-332 consists of three chains, α3, β3 and γ2, which associate through the carboxyl-terminal coiled coil long arm. The α3 chain comprises of globular structure composed of five homologous laminin G-like or LG repeats, that interact with integrins α3β1 and α6β4. The amino-terminal region interacts with CollagenVII, Ln-6 and Ln-7. The γ2 chain is processed by MMPs including MT1-MMP, liberating domains III–V, which contain EGF-Iike repeats. The collagen VII interacting domain VI of β3 chain gets cleaved by MMP7, MT1-MMP, hepsin and matriptase.

*α3 chain of Ln-332*

The α3 chain is directly involved in the interaction of Ln-332 with integrins. Our laboratory reported that LG3 and LG4 domains of α3 chain contain binding sites for integrin α3β1 (Shang, Koshikawa et al. 2001; Yamashita, Shang et al. 2010). α3 chain is also part of laminin-331 and laminin-321 heterotrimers. Of note, Ln-332 interacts with these two laminins. Recently, it has been reported that LG4-5 domains of α3 chain are highly expressed in carcinoma (Tran, Rousselle et al. 2008), correlating with an increase tumor growth and invasion via activation of phosphoinositide 3-kinase (PI3K) and increase in MMP activity.

*β3 chain of Laminin-332*

The non-collagenous domain of type VII collagen (NC1) binds to the β3 chain of Ln-332 (Rousselle, Keene et al. 1997). This interaction which occurs at the domain VI of β3 chain is necessary for the adhesion activity of Ln-332 (Nakashima, Kariya et al. 2005). Very recently, this interaction has been reported to promote tumorigenesis in squamous cell carcinoma (Waterman, Sakai et al. 2007), through the activation of anti-apoptotic and tumor invasion factors promoting PI3K pathways.

*γ2 chain of Laminin-332*

The γ2 chain is secreted and incorporated in the BM either as a part of the Ln-332 heterotrimer or in a monomeric form (Gagnoux-Palacios, Allegra et al. 2001). The γ2 chain of Ln-332 has been extensively studied when it comes to its role in cancer: its expression correlates with invasion, particularly at the leading edge of the invading

tumor. In hepatocellular cancer, γ2 chain expression is associated with the occurrence of metastasis (Giannelli, Quaranta et al. 2003). Patients with pancreatic ductal adenocarcinoma and liver metastases have elevated levels of γ2 fragment in their serum (Katayama, Funakoshi et al. 2005), making γ2 chain expression a potential marker for cancer prognosis. Our laboratory, recently published a report suggesting that decreased ratio of Ln-332 β3 to γ2 subunit mRNA is associated with poor prognosis in colon cancer (Guess, LaFleur et al. 2009). This finding raises the possibility that Ln-332 γ2 might be a therapeutic target against metastatic colon cancer.

*Ln-332 and cancer*

The expression of Ln-332 is altered in various types of cancers (Katayama and Sekiguchi 2004; Guess and Quaranta 2009). Ln-332 is overexpressed in many tumor types, including: oesophageal, cutaneous, oral, laryngeal, colon, tracheal, and cervical cancers (Skyldberg, Salo et al. 1999; Lenander, Habermann et al. 2001; Marinkovich 2007). In these tumors, Ln-332 is found to accumulate at the interface of the tumor and the surrounding stroma. Interestingly, Ln-332 expression is instead decreased or lost in prostate cancer (Davis, Cress et al. 2001; Hao, Jackson et al. 2001; Nagle 2004; Calaluce, Beck et al. 2006), basal cell carcinoma (Guess, LaFleur et al. 2009), lung(Guess, LaFleur et al. 2009), breast (Martin, Kwan et al. 1998) bladder cancers (Sathyanarayana, Maruyama et al. 2004) and prostate (Hao, Jackson et al. 2001; Nagle 2004). Studies of Ln-332 in mouse models of cancer have been carried out to determine the role of the Ln-332 heterotrimer in tumor progression and in the underlying mechanism(s) (Figure 5). Ortiz-Urda et al. showed that Ln-332 acts as a tumor promoter in squamous cell

**Table 2**: Laminin-332 Proteolytic Processing.

| Ln-332 Chain | Processing Protease | Cellular Phenotype | References |
|---|---|---|---|
| α3 chain | Plasmin | Migration and Hemidesmosome assembly | Lawrence E. Goldfinger |
| β3 Chain | MT1-MMP | Migration | (Udayakumar, Chen et al. 2003) |
| | MMP7 | Migration | Remy, Trespeuch et al. 2006) Tripathi 2008 |
| | Hepsin | Migration | Tripathi 2010 |
| | Matriptase | Migration | |
| γ 2 Chain | MMP2 and MT1-MMP | Migration | (Giannelli, Falk-Marzillier et al. 1997; Koshikawa, Schenk et al. 2004) |
| | MMPs 3, 8, 12, 13, 14, and 20 | Migration | (Pirila, Sharabi et al. 2003), (Wang, Sun et al. 2006) |
| | cathepsin S | Angiogenesis | (Veitch, Nokelainen et al. 2003) |
| | mTLD, | | Amano, Scott et al. 2000) |
| | BMP-1 | | Mydel, Shipley et al. 2008) |
| | neutrophil elastase | Migration | |

carcinoma of the epidermis, through the interaction of its β3 chain and type VII collagen (Ortiz-Urda, Garcia et al. 2005; Waterman, Sakai et al. 2007). This finding is in sharp contrast with the study by Yuen et al. that shows that Ln-332 is a tumor suppressor in tumors generated with the human head and neck cancer cell line, JUH-022 (Yuen, Ziober et al. 2005). The authors suggested that the increased cell motility induced by Ln-332 suppression was responsible for increased tumorigenicity. Taken together, the role played

by the Ln-332 in tumor progression is still being debated, and seems to be dependent on the type of cancer.

*Laminin-332 and migration*

Ln-332 has been known as a motility inducing agent for normal and cancerous cells (Pyke, Romer et al. 1994; Giannelli, Falk-Marzillier et al. 1997; Plopper, Domanico et al. 1998; Koshikawa, Giannelli et al. 2000; Schenk, Hintermann et al. 2003; Hintermann and Quaranta 2004). It has been shown that in the absence of Ln-332 during wound healing, keratinocytes migrate to the wound edge and attach to the wound bed through focal adhesions and β1 integrin, with activation of the GTPase Ras homolog gene family, member A or RhoA (Nguyen, Gil et al. 2000; Nguyen, Ren et al. 2001). After producing Ln-332, these keratinocytes switch their attachment to Ln-332 and the GTPase activation changes to Ras-related C3 botulinum toxin substrate 1 or Rac1 due to the interaction of Ln-332 with α6β4 integrin. Another study has shown that cells migrating on Ln-332 have downregulated RhoA and Rho-associated, coiled-coil containing protein kinase 1 or Rock1 (Zhou and Kramer 2005). Further, it has been shown that α3β1 integrin binds to Ln-332 if binding with α6β4 integrin is blocked, resulting in enhanced cell migration via RhoA activation. Therefore it appears that Ln-332 promotes cell migration via two different mechanisms depending on its binding to alternate integrins and downstream signaling. It is interesting that RhoA and Rac1 mediated keratinocyte migration have notable differences: RhoA-mediated migration is accompanied with decreased cell-cell contact, whereas Ras-related (C3 botulinum toxin substrate 2 (Rac2)-mediated) migration occurs as multi-cellular epidermal sheets

(Russell, Fincher et al. 2003). The Ln-332/α6β4 integrin-induced migration with Rac1 activation is directional whereas RhoA-mediated migration is non- directional (Pullar, Baier et al. 2006; Sehgal, DeBiase et al. 2006).

## Proteolytic processing of Laminin-332

Secretion of Ln-332 into the ECM is followed by its proteolytic processing (Figure 6 and Table 2): the α3 chain gets processed from 190-200 to 160kDa, the β3 chain gets processed from 145 to 80kDa and the γ2 chain from 155 to 105kDa and further to 80kDa (Meneguzzi, Marinkovich et al. 1992; Giannelli, Falk-Marzillier et al. 1997; Udayakumar, Chen et al. 2003). Previously, our group has shown that Ln-332 γ2 chain is cleaved by MMP2 and MT1-MMP (Giannelli, Falk-Marzillier et al. 1997; Koshikawa, Schenk et al. 2004); others have shown that this chain is also cleaved by MMPs 3, 8, 12, 13, 14, and 20 (Pirila, Ramamurthy et al. 2003; Pirila, Sharabi et al. 2003; Wang, Sun et al. 2006), cathepsin (Wang, Sun et al. 2006), mTLD (Veitch, Nokelainen et al. 2003), BMP-1 (Amano, Scott et al. 2000), and neutrophil elastase (Mydel, Shipley et al. 2008) (Table 2). Furthermore, processing of Ln-332 γ2 chain by these proteases regulates cell migration (Giannelli, Falk-Marzillier et al. 1997; Pirila, Sharabi et al. 2003; Mydel, Shipley et al. 2008). The β3 chain of Ln-332 was believed to be relatively resistant to proteolytic processing, until recently when studies have shown that Ln-332 β3 chain is processed by MT1-MMP, which enhances prostate cancer cell migration compared to uncleaved Ln-332 (Udayakumar, Chen et al. 2003). Another study reported that Ln-332 β3 chain is a ligand for MMP7, which enhances cell motility of a colon carcinoma cell line (Remy, Trespeuch et al. 2006). It has also been reported that Ln-332 β3 chain is

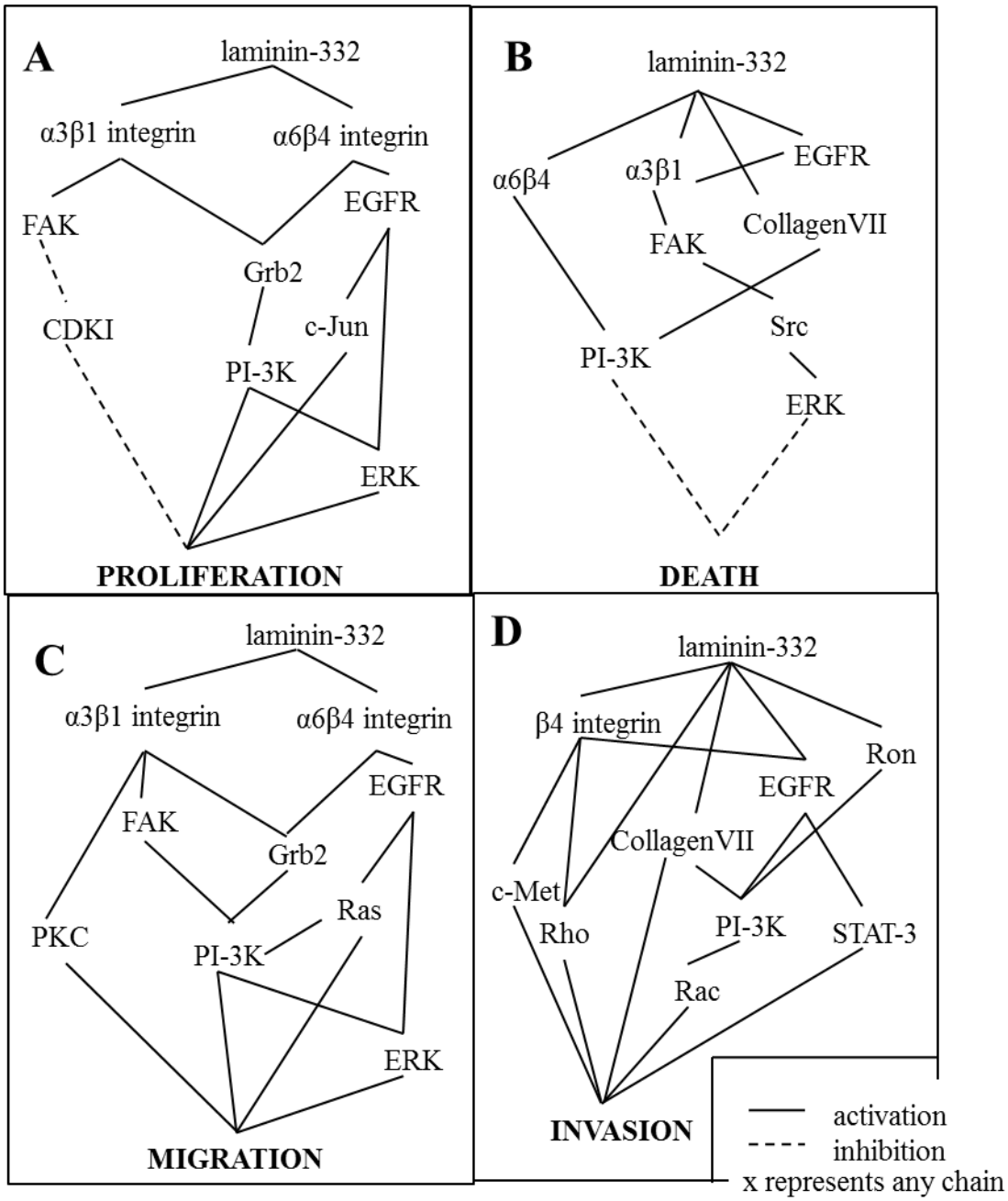


Figure 5: Laminin-332 and the signaling pathways involved in cancer progression. These four diagrams represent the relationships between laminin-332 and cell proliferation (a), cell death (b), migration (c), and cancer invasion (d). Solid lines denote activation, whereas dot lines represent inhibition. Modified from Jourquin et al (chapter from book Laminins and cancer progression" in "*Cell- Extracellular Matrix Interactions and Cancer*." Zent R and Pozzi A (editors), Springer-Verlag New York (Publisher) 2009.

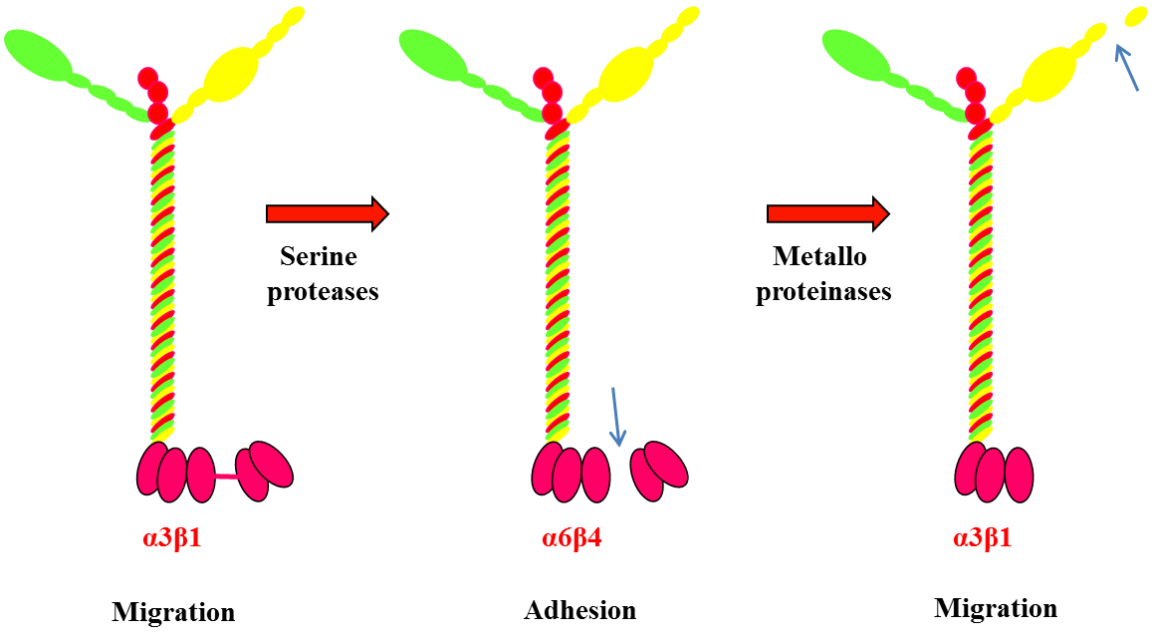


Figure 6: Proteolytic processing of Ln-332 chains and its effect on cellular motility. Full length Ln-332 interacts with integrin α3β1 and supports haptotaxis. Serine protease cleavage releases LG4/5 from the α3 chain that might expose a cryptic site on LG1-3, which then binds with integrin α6β4. Binding to α6β4 leads to static adhesion structure formation, hemidesmosome. Further processing of γ2 releases domains that contain EGF-like repeats, DIII, and DIV/V. Domain DIII can bind and activate erbB1 leading to chemotaxis.

cleaved at its N-terminus by endogenous proteases in human keratinocytes and other cell lines (Nakashima, Kariya et al. 2005); however, the specific proteases involved in this cleavage have not been identified. Taken together, these studies establish that the proteolytic processing of Ln-332 occurs physiologically and can alter cellular functions such as adhesion and migration (Carter, Wayner et al. 1990; Belkin and Stepp 2000), both important in cancer progression.

## Hypothesis Aims and Rationale for the Study

### *Hypothesis*

**Type II Transmembrane Serine Proteases proteolytically process Ln-332, facilitating basement membrane degradation and contributing to prostate cancer progression.**

*Aim 1*

Determine the role of cleavage of Ln-332 by hepsin in prostate cancer progression.

*Aim 2*

Determine the role of cleavage of Ln-332 by matriptase in prostate cancer progression.

### *Rationale*

**Ln-332 is lost in prostate cancer progression. Ln-332 facilitates migration of cancer cells when cleaved by various cell surface proteases, including MMPs. We have found that Ln-332 is individually cleaved by two serine proteases, hepsin and matriptase, and that this cleavage enhances migration of human prostate cancer cells *in vitro*. Hepsin is overexpressed in more than 90% prostate cancer cases. Similarly, matriptase is overexpressed in human prostate cancer cases and expression of both proteases correlates with tumor progression. However, the mechanisms by which these two serine proteases regulate prostate cancer progression are unknown.**

# CHAPTER II

# MATERIALS AND METHODS

## Cell culture

Prostate cancer cell line DU145 (American Type Culture Collection, Manassas, VA) and 804G bladder squamous cell carcinoma cells (previously described in Falk-Marzillier *et al.* (Falk-Marzillier, Domanico et al. 1998)) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Gemini, Irvine, CA) and 1% glutamine/penicillin/streptomycin antibiotics (g/p/s) (Life Technologies) in an incubator with 5% $CO_2$ at 37°C.

## Generation of Hepsin or matriptase overexpressing LNCaP cells

LNCaP-17 (low hepsin-expressing) and LNCaP-34 (hepsin-overexpressing) prostate cancer cells were created at Genentech, previously described by Moran et al. (Moran, Li et al. 2006). Briefly, a LNCaP clone expressing luciferase gene (LNCaP-luc) was used for hepsin transfection experiments. Full-length hepsin cDNA was inserted into a mammalian expression vector containing the puromycin resistance gene for antibiotic selection (Genentech). The LNCaP-luc clone was transfected with full-length hepsin construct with a C-terminal FLAG tag, and the cells were selected with 0.5 μg/ml puromycin (Sigma-Aldrich). Two clones, LNCaP-34, the high hepsin expressor and LNCaP-17, the low hepsin expressor were used in this study.

LNCaP (low matriptase expressing, LNCaP-wt) and matriptase-overexpressing LNCaP (LNCaP-mt) prostate cancer cells were also created at Genentech in the laboratory of Dr. Kirchhofer. The cDNA of full-length matriptase was inserted into a mammalian expression vector containing the puromycin resistance gene for antibiotic selection (Genentech). The LNCaP-luc clone (Moran, Li et al. 2006) was transfected with the construct encoding full-length matriptase with a C-terminal FLAG tag, and the cells were selected with 0.5 μg/ml puromycin (Sigma-Aldrich). The clones were analyzed by FACS for matriptase surface expression using an anti-FLAG monoclonal antibody (Sigma-Adrich). Two clones, the high matriptase expressor LNCaP-mt and the one low matriptase expressor LNCaP-wt, were selected for further experiments.

LNCaP-17, LNCaP-34, LNCaP-wt and LNCaP-mt cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 500 μg/ml Geneticin (Invitrogen, Carlsbad, CA), 0.5 μg/ml puromycin (Sigma, St. Louis, MO), and 1% g/p/s antibiotics and incubated with 5% $CO_2$ at 37°C.

**Purification of rat Laminin-332**

Rat Ln-332 was purified from spent medium of 804G, bladder squamous cell carcinoma cells. Briefly, 804G cells were cultured in 10% FBS containing DMEM in 150-mm dish. Cells were then washed with PBS twice and cultured in serum-free conditioned medium for 2 days in roller bottles. The serum- free conditioned medium was collected and concentrated by ammonium sulfate at 80% saturation and dialyzed against 20 mM Tris-HCl (pH 7.5)/0.5 M NaCl / 0.005% Brij-35 (TNB buffer). The concentrated serum- free conditioned medium was then used for immunoaffinity

chromatography. The Protein A-Sepharose column (0.8 X 4.0 cm; Bio-Rad Laboratories, Hercules, CA) chemically conjugated with non-functional Ln-332 mouse antibody, TR-1 (Plopper, Falk-Marzillier et al. 1996) was equilibrated with TNB buffer at a flow rate of 15 ml/h. The concentrated and dialyzed sample was applied to the TR1 column. The column was then washed with TNB and absorbed Ln-332 was eluted with 10 ml of 0.05% trifluoroacetic acid (TFA), pH 2.5. The eluted fractions were neutralized by 300 µl of 1 M Tris-HCl, pH 8.0, and then 1% CHAPS was added to each fraction.

## Recombinant Proteins

The proteases, hepsin and matriptase and the inhibitor KD1 used in this work was provided by Dr. Daniel Kirchhofer at Genentech. The construction, expression, and purification of recombinant hepsin is described in detail in the original publication by Moran et al (Moran, Li et al. 2006). Briefly, a secreted His-tagged hepsin cDNA was made by fusion of cDNA coding for the signal sequence of honeybee melittin with cDNA coding for the extracellular domain of human hepsin. This cDNA construct was inserted in a baculovirus expression vector under the control of a polyhedrin promoter and was expressed in T.in.Pro cells. Hepsin was purified by nickel-nitrilotriacetic acid affinity chromatography.

The cloning, expression, and purification of matriptase protease domain is also described in Moran et al (Moran, Li et al. 2006). Briefly, full-length clone of matriptase was obtained by PCR from human cDNA libraries. The nucleotide sequence encoding the mature protease domain was cloned by PCR from the full-length clone into plasmid pSTII.TIR3 variant 4 (Simmons and Yansura 1996). The plasmid contained a phoA

promoter, the stII signal sequence, and the λto transcriptional terminator. The matriptase protease domain was produced in E. coli and purified using Ni-NTA metal chelate column. The matriptase protease domain was analyzed by N-terminal sequencing and mass spectrometry.

Production and purification of KD1 is described in Shia et al (Shia, Stamos et al. 2005). Briefly, the first Kunitz domain of human HAI-1B was expressed in the Escherichia coli using the plasmid pKD1. This pBR322-based construct has phoA promoter for transcription and trp ribosome-binding site for translation initiation. The poly-histidine leader sequence was used before the coding sequence for the Kunitz domain for purification. The KD1 protein was purified on a Ni-NTA metal chelate column.

**Cleavage of Laminin-332**

The cleavage of rat Ln-332 was studied with human recombinant hepsin consisting of the entire extracellular domain and the human recombinant protease domain of matriptase (Moran, Li et al. 2006). To study the cleavage of Ln-332 by hepsin and matriptase, purified rat Ln-332 (0.2 μM) was incubated with either the recombinant protease domain of hepsin (at both 0.13 and 1.3 μM), or the human recombinant protease domain of matriptase (0.6, 2 and 6 μM) and reaction buffer containing 250 mM NaCl and 50 mM Tris (pH 7.5) for 1.5 hours (for hepsin) or 2 hours (for matriptase) at 37°C. For the time course experiment, Ln-332 (0.8 μM) was incubated with either hepsin (5.2 μM) or matriptase (24μM) and reaction buffer containing 250 mM NaCl and 50 mM Tris pH 7.5 for 0, 2, and 6 h (for hepsin) or 0, 3, 6 and 12 hours (for matriptase) at 37°C. After

incubation, hepsin and Ln-332 or matriptase and Ln-332 reaction mixtures were electrophoresed on 4%-12% precasted sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel under reducing/non-reducing (as indicated) conditions and then stained with SimplyBlue™ Safe Coomassie Blue stain (Invitrogen). A standard marker (identified as M in figures; Precision Plus™ protein dual color standard; BioRad, Hercules, CA) was also run for comparison.

Western blot analysis was performed after transferring the untreated and treated protein on a PVDF membrane (Perkin Elmer, Waltham, MA), from reducing gel. Polyclonal antibody (pAb; 1:500) against the C- terminus of Ln-332 β3 chain (sc-20775; H-300; Santa Cruz Biotechnology, Inc.), and secondary anti-rabbit IgG HRP mAb (1:5000; GE Healthcare, UK), were used for western blot. Protein bands were visualized with the ECL plus system (Perkin Elmer). *ImageJ* was used for quantification of bands in the scanned western blot film. Briefly, in the "*Analyze*" function of *ImageJ* we *"Set Measurements"* for area, mean gray value, and integrated density. The band of interest was selected and the parameters were measured. The raw intensity measurement for each band was normalized to its corresponding β-actin control.

**Mass spectrometry**

The cleaved product of Ln-332 by hepsin or matriptase was further identified using mass spectrometry analysis performed by the Mass Spectrometry Research Center at Vanderbilt University (Nashville, TN). After digestion, the proteins in the reaction mixture were separated by SDS-PAGE under non-reducing conditions and visualized using Coomassie Blue stain. The protein bands of interest were excised from the SDS-

PAGE gel, and then equilibrated in 100 mM $NH_4HCO_3$, reduced with 3 mM DTT in 100 mM $NH_4HCO_3$ at 37$^oC$ for 15 min. Alkylation was carried out with iodoacetamide (6 mM in 100 mM $NH_4HCO_3$ for 15 min). After destaining, the gel slices were dehydrated with 50% acetonitrile in 50 mM $NH_4HCO_3$, followed by 100% acetonitrile. Gel slices were rehydrated with 15 µl of 25 mM $NH_4HCO_3$ containing 0.01 µg/µl modified trypsin. Trypsin digestion was performed for 2 h at 37$^oC$. Peptides were extracted with 60% acetonitrile with 0.1% trifluoroacetic acid, dried by vacuum centrifugation, and reconstituted in 10 µl 0.1% trifluoroacetic acid. After desalting, peptides were concentrated into 2 µl 60% acetonitrile with 0.1% trifluoroacetic acid using ZipTipC18 pipette tips. For the preparation of sample for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), 0.4 µl of the sample was applied to a target plate and overlaid with 0.4 µl alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 60% acetonitrile, 0.1% trifluoroacetic acid). MALDI-TOF MS and tandem TOF/TOFMS/MS were performed using a Voyager 4700 mass spectrometer (Applied Biosystems, Foster City, CA). TOF/TOF fragmentation spectra were acquired in a data-dependent fashion based on the MALDI-TOF peptide mass map for the protein. Both types of mass spectral data were collectively used to examine the protein databases to generate statistically significant candidate identification using GPS Explorer software (Applied Biosystems) running the MASCOT database search algorithm (Matrix Science). Searches were performed against the SWISS PROT and the NCBI databases.

## Edman degradation sequencing

For N-terminal sequencing, 20 pmole (10 ug) of Ln-332 was digested and transferred to PVDF membrane. The membrane was then stained with Commassie blue (R250, acetic acid/methanol/water destain), destained, washed with water and air dried. N-terminal sequencing of the PVDF membrane containing the cleaved protein was carried out on an Applied Biosystems (AB) Procise® 494 cLC at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). The detected amino acids were matched to the theoretical sequence for all the possible matches. The amino acids with the strongest signal (NH2-LQGSCFC) was further analyzed on the basis of predicted published sequence for hepsin (Herter, Piper et al. 2005). Further the mass spectrometry data of the same band has this sequence (LQGSCFCHGHADR) as first peptide sequence confirmed to be present in the band by MS/MS.

## Enzyme inhibition assay

HGF activator inhibitor-1 derived KD1 was used to inhibit enzymatic matriptase and hepsin activity. KD1 is the N-terminal Kunitz domain of HAI-1 and was produced in *Escherichia coli* as described by Shia *et al*. (Shia, Stamos et al. 2005). As described in the *cleavage of Ln-332* section above, purified rat Ln-332 (0.2 μM) was incubated alone or with either recombinant matriptase (6 μM) or recombinant hepsin (1.3μM) and reaction buffer with or without KD1 inhibitor (15 μM for matriptase inhibition) or (5.6 μM or 11.2 μM for hepsin inhibition) for 2 h at 37°C. SDS-PAGE was then performed and the gel was stained with Coomassie Blue.

## Inactivation of hepsin enzymatic activity by Glu-Gly-Arg chloromethyl ketone (EGRcmk)

Inactivation of hepsin was performed at Genentech in the laboratory of Dr. Kirchhofer. Briefly, purified recombinant hepsin as described (40) was incubated either with 10-fold molar excess of an irreversible covalent inhibitor, EGR-cmk (Hematological Technologies, Essex Junction, VT), or with buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl (for Ctrl-hepsin) for 3-hours at room temperature. EGR-hepsin complex was separated from un-reacted EGR-cmk by size exclusion chromatography using a Superdex S-200 column (GE Healthcare Inc.) with a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl. Ctrl-hepsin was also subjected to size exclusion chromatographic purification as described above. Relative to Ctrl-hepsin, EGR-hepsin had lost >99% of its catalytic activity as assessed by the rate of substrate hydrolysis with a small synthetic substrate, S2366 (Diapharma, West Chester, OH).

## EGR-hepsin assay

EGR-hepsin was used to further examine the specificity of Ln-332 cleavage by hepsin. As with earlier assays, 0.2 µM purified rat Ln-332 was incubated with reaction buffer alone or with recombinant EGRcmk- inactivated hepsin (EGR-hepsin; 1.3 µM), control hepsin for EGR-hepsin (Ctrl-hepsin; 1.3 µM), or the recombinant hepsin (1.3 µM) used for the initial cleavage reactions at 37°C. The reaction mixtures were electrophoresed on 4%-12% pre-casted gradient gel under reducing conditions and then stained with Coomassie blue.

## Transwell migration assays

Cell migration assays were performed using 8.0 μm pore size Transwell™ permeable supports (Corning Costar, Lowell, MA). For Ln-332 and hepsin experiments, the underside of the filters were coated with either untreated or hepsin- treated rat Ln-332 (1 μg/ml), Ln-332 coincubated with hepsin and KD1, phosphate buffered saline solution (PBS), hepsin (1.3 μM), or KD1 (5.6 μM) overnight at 4°C. For Ln-332 and matriptase experiments, the lower side of the filters were coated with either untreated or matriptase-treated rat Ln-332 with and without KD1, phosphate buffered saline solution (PBS), or Ln-332 coincubated with matriptase and KD1 overnight at 4°C. Transwells were then blocked with 5% milk in PBS with 0.2% Tween 20 for 1 h. DU145 or LNCaP cells were trypsinized, resuspended in serum-free medium, washed twice with serum-free medium, and cells (20,000 or 50,000, respectively) were seeded in the upper chamber of inserts. After 5 h (DU145) or 24 h (LNCaP) incubation in 5% $CO_2$ at 37°C, cells remaining on the upper filter were scraped off gently using a cotton swab and the inserts were gently washed with PBS. Those cells that migrated to the lower chamber were fixed with 400 μl of fixation solution (Hema-3® stain kit, cat. # 122-911, Fisher Scientific Company LLC, Kalamazoo, MI) for 10 min, stained with 400 μl of staining solution for 20 min, and imaged with a Zeiss LSM-510 inverted microscope (Zeiss, Germany). Five representative images (10x magnification) were randomly captured for each insert and used to manually count the number of cells present. Results are presented as mean number of cells per field ± standard deviation. Student's t-tests ($\alpha = 0.05$) were performed on final data to test significance of effects.

## Cell Adhesion Assay

Cell adhesion assays were performed in 96-well plates. Plates were first coated with cleaved or uncleaved Ln-332 overnight at 4 °C. Unbound protein was removed with phosphate-buffered saline (PBS) washing. Cells were detached with 0.025% trypsin and 0.53 mM EDTA (Life Technologies) with 10% FBS was used to neutralize trypsin. Cells were washed twice with serum-free media. Cells were then resuspended in serum-free media and counted. $8.0 \times 10^4$ cells were plated per well in 100 μl of serum-free media, and allowed to attach for 1 h at 37 °C. After one hour non-adherent cells were removed by washing; adherent cells were fixed with glutaraldehyde (3% (v/v) in phosphate-buffered saline) and stained with crystal violet (3% (w/v) in methanol); and absorbance was measured at 560 nm.

## Hepsin overexpressing cell mediated cleavage of Laminin-332

LNCaP-17 (low hepsin-expressing) or LNCaP-34 (hepsin-overexpressing) cells ($9.0 \times 10^4$) in 100 μl of RPMI 1640 were incubated with either 100 μg/ml of Ln-332 or of PBS for 12 h at 37°C. After 12 h, Ln-332 solution with media and cells was collected and centrifuged for 5 min at 15,000 rpm and supernatant was collected. SDS-PAGE analysis was performed under reducing conditions and protein was transferred to a nitrocellulose membrane. A pAb against the C- terminus of Ln-332 β3 chain (1:200; sc-20775; H-300; Santa Cruz Biotechnology, Inc.) and the secondary anti-rabbit IgG HRP antibody (1:5000) was used for visualization in western blot. Protein bands were visualized with the ECL plus system (Perkin Elmer).

## LNCaP cell protein isolation and Western blot analysis

LNCaP-wt and LNCaP-mt cells were lysed with RIPA buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Roche, Indianapolis, IN). Total protein levels of samples were measured using a bicinchoninic acid assay (Pierce, Thermo Fisher Scientific, Rockford, IL) and normalized. Denatured proteins were separated by SDS-PAGE and transferred for western blotting. Nonspecific binding to membranes was blocked for 1 h with blocking buffer (5% milk in PBS). Blots were incubated overnight in primary antibody (1:1000 pAb matriptase; Bethyl Laboratories, Inc., Montgomery, TX) or 1:1000 monoclonal anti β-actin antibody (AC-74, Sigma) in blocking solution at 4°C, and subsequently in HRP conjugated anti-rabbit IgG and anti-mouse IgG secondary antibody (NA493v: GE Healthcare) for 1 h at room temperature. Protein bands were visualized with an ECL Plus system (Amersham Pharmacia Biotech, Piscataway, NJ).

## Single cell motility assay

LNCaP-wt or LNCaP-mt cells were plated (20,000 cells) overnight on 60-mm dishes coated with Ln-332 (10 μg/ml). Cell density was kept low to avoid interacting cell populations. Cells were monitored using the phase-contrast optics in a Zeiss Axiovert 200M inverted microscope with a monochrome, cooled CCD camera (CoolSNAP HQ, Roper Scientific, Trenton, NJ) equipped with temperature-controlled, humidified chamber. Cellular images were captured using *Metamorph* (Molecular Devices Corporation, Sunnyvale, CA) for data acquisition and analysis. Time-lapse images were collected at a magnification of 10x (1 pixel=0.98 μm) using a sampling time interval of 1

min. All cells were equilibrated in the humidified, temperature-controlled (37 $^{\circ}$C) microscope chamber for 30 min and media was replaced with fresh growth media before data collection. The cells were tracked for at least 12 h in all experiments.

## Cell tracking

Each cell was tracked by following the cell nucleus using the "track objects" function in *Metamorph*. Only single cells were considered for the analysis; cells that remained stationary, moved outside the viewing area, underwent cell division during the course of the experiment, did not migrate over a distance of at least 2 cell bodies (<20 μm), or that adhered to other cells were excluded from the tracking procedure. Applying this criterion, ~60% cells were retained. Results are presented in combined box-and-whisker and scatter plots, which show the mean speed per population (dark horizontal line), quartiles (box), 95% confidence intervals (whiskers), and raw data points (scatter). Mann-Whitney U tests were performed on final data to test significance of effects, with values less than 0.05 accepted as significant.

## Immunohistochemistry

Immunohistochemistry was performed on 5 μm thick paraffin sections. Sections were deparaffinizied and rehydrated though a series of ethanols. Following washing with PBS, the tissue sections were blocked using 10% serum of appropriate species for 20 mins at room temperature. Tissue sections were then incubated with primary antibody diluted in blocking buffer for overnight at 4°C. Primary antibody were polyclonal antibody (pAb) against the C-terminus of Ln-332 β3 chain (1:1000; sc-20775; H-300;

Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Hepsin Cayman Chemical cat# 100022 (1 µg/ml). Vectastain ABC kit (Vector Laboratories Inc, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Dako) were used to visualize the staining. Sections were counterstained with hematoxylin, dehydrated, and permanently mounted.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from LNCaP-17 and LNCaP-34 cells using RNeasy Mini kit (Qiagen, Valencia, CA). The mixture of 2 µg of RNA, 50 ng random hexamer, and 1 mM NTP was incubated at 65 ºC for 5 min, briefly chilled on ice, and added to a 20 µl reaction mixture containing 2 µl of 10x RT buffer (Invitrogen), 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNase OUT™, and 1 µl of SuperScript™. Reaction mixture was incubated at 50 ºC for 1 hour. Single-stranded cDNAs were amplified by 40 cycles of PCR using Taq DNA polymerase (Promega, Madison, WI). Denaturation was carried out at 95 ºC for 2 min, annealing at 71 ºC (hepsin) and 52 ºC (GAPDH) for 30 sec, and extension at 72 ºC for 2 min. The amplified PCR products were electrophoresed on 1% (w/v) agarose gels. RT-PCR was performed using the following primer sets: hepsin forward primer, 5' – TGGTCTTTGACAAGACGGAAGGGA - 3'; hepsin reverse primer, 5'-GCAATCACAACGGAGATGACCT - 3'; GAPDH forward primer, 5'-ATGACATCAAGAAGGTGGTG -3'; and GAPDH reverse primer, 5' - CTGTAGCCAAATTCGTTGTC - 3'.

**Quantitative real-time reverse transcriptase polymerase chain reaction (q RT-PCR)**

Total RNA was extracted from LNCap-WT and LNCaP-MT cells using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration of RNA was measured using a spectrophotometer. Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and quantitative real-time q RT-PCR was done using iQ SYBR green supermix (Biorad, Hercules, CA). Relative quantitation of matriptase expression was performed by ΔΔct method normalized to GAPDH. The matriptase primers were generated based on the Human sequence of matriptase. The forward matriptase primer (5' TGTGGATGCCTACGAGAACTCCAA 3') and reverse matriptase primer (3' TGTCCTGGGTCCTCTGTACTGTTT5') generated a 170 bp amplimer. GAPDH forward primer, 5'-ATGACATCAAGAAGGTGGTG -3'; and GAPDH reverse primer, 5' - CTGTAGCCAAATTCGTTGTC - 3'.

**Isolation of protein from tumor tissue and Western blot analysis**

Tumor tissues for 12 month old wild type, myc and hepsin/myc mice were frozen in liquid nitrogen and then crushed using a mortar and pestle with RIPA buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate , and 0.1% SDS) containing protease inhibitor cocktail (Roche, Indianapolis, IN). Further homogenization of the tissue was done by sonicator. The lysate was centrifuged for 15 min at 15000 rpm to collect the supernatant. Total protein levels of samples were measured by BCA assay (Pierce, Thermo Fisher Scientific, Rockford, IL) and normalized. Denatured proteins were separated by SDS-PAGE and then transferred for

Western blotting. Nonspecific binding to membranes was blocked for overnight with blocking buffer for near infra-red fluorescent Western blotting (Rockland, Gilbertsville, PA). Blots were incubated overnight in primary polyclonal antibody to C-terminus of Ln-332 β3 chain (1:500 sc-20775; H-300; Santa Cruz Biotechnology, Inc.) or mAb Anti-β-actin (1:5000, Sigma,St. Louis, MO). Secondary antibodies used were Alexa Fluor 680 anti-rabbit IgG (Invitrogen) and IRDye 800 anti-mouse IgG (Li-COR Biosciences, Lincoln, NE) and subsequently signals were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

# CHAPTER III

# LAMININ-332 IS A SUBSTRATE FOR HEPSIN, A PROTEASE ASSOCIATED WITH PROSTATE CANCER PROGRESSION

## Introduction

Hepsin, a cell surface protease, is widely reported to be overexpressed in more than 90% of human prostate tumors (Dhanasekaran, Barrette et al. 2001; Stephan, Yousef et al. 2004). Interestingly, hepsin expression correlates with tumor progression, making it a significant marker and target for prostate cancer (Xuan, Schneider et al. 2006). Despite overwhelming evidence that hepsin expression promotes prostate tumor progression, the physiological function of hepsin remains largely unknown (Wu and Parry 2007). Recently, it was reported that a hepsin transgenic mouse model, displayed disorganized basement membrane (Klezovitch, Chevillet et al. 2004). Further, crossing these mice with LPB-Tag 12-T7f mice, another prostate cancer model, resulted in significant tumor progression and induced metastasis. The underlying mechanisms, however, remain largely uncharacterized. Hepsin transgenic mice displayed reduced Ln-332 expression in prostate tumors. This is an intriguing cue, since proteolytic processing of extracellular matrix macromolecules such as Ln-332 is believed to be involved in cancer progression, and Ln-332 expression is lost during human prostate cancer progression. In this study, we provide the first direct evidence that hepsin cleaves Ln-332. Direct cleavage of Ln-332 may be one of the mechanisms by which hepsin promotes prostate tumor progression and metastasis, possibly by upregulating prostate cancer cell motility.

## Results

*Processing of Laminin-332 by hepsin*

To determine whether hepsin cleaves Ln-332, purified rat Ln-332 was incubated with recombinant hepsin at different concentrations in reaction buffer for 1.5 h with the enzyme : substrate (i.e., hepsin : Ln-332) molar ratio of 1.0 : 1.5 and 1.0 : 0.15 (latter ratio used in all follow-up experiments), as described in Materials and Methods. After incubation, protein samples were separated under reducing conditions by SDS-PAGE and stained with Coomassie blue (Figure 7A). We observed a unique band at ~100 kDa in the lanes containing Ln-332 incubated with hepsin at both concentrations tested (arrow; lanes 2 and 3), but not in untreated samples (lane 1). The intensity of the cleaved band correlated with increasing hepsin concentration (lane 3). Additionally, lanes 2 and 3 produced a unique band at ~30 kDa, which corresponds to the protease domain of hepsin. Lane 4, which includes hepsin alone, also revealed this ~30 kDa band. Ln-332, both in the absence and presence of hepsin, was also electrophoresed under non-reducing conditions (Figure 7C). Under both reducing and non-reducing conditions, Ln-332 appeared proteolytically degraded by hepsin on SDS-PAGE; however under non-reducing conditions the bands appeared less defined, which was due to the absence of the reducing agent (DTT). This electrophoretic behavior under non-reducing conditions is consistent with the presence of disulfide bonds between the Ln-332 chains, as mentioned in the *Introduction*.

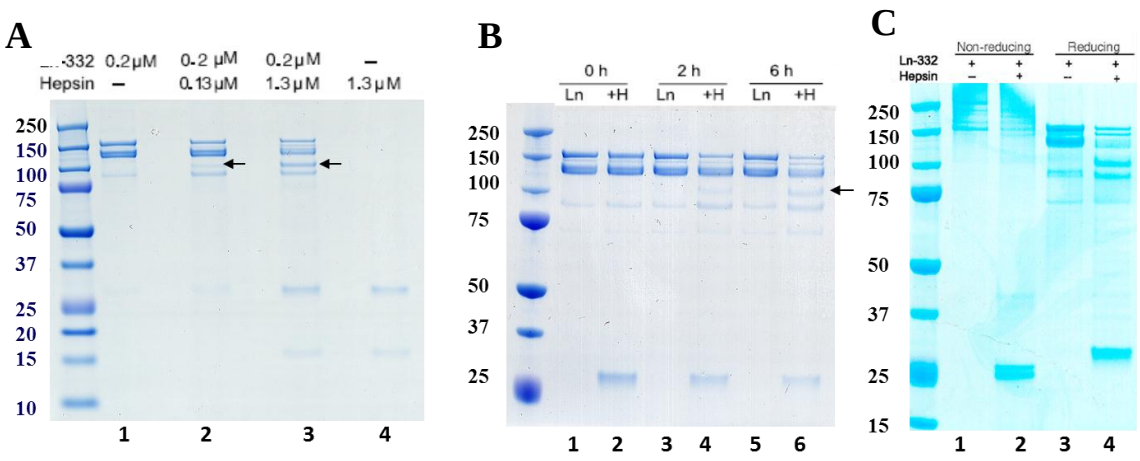


Figure 7: SDS-PAGE analysis of hepsin cleavage of purified rat laminin-332. (A) Purified rat Ln-332 (0.2 µM) was incubated alone or with the recombinant extracellular domain of hepsin for 1.5 h at 37°C, electrophoresed on 4%-12% gradient gel under reducing conditions, and stained with Coomassie Blue. After incubation of Ln-332 alone, the gel included bands identified as the α3 (190 kDa), β3 (145 kDa), and γ2 (155 kDa and 80 kDa) chains (lane 1). However, upon incubation of Ln-332 with 0.13 or 1.3 µM hepsin (lanes 2 and 3, respectively), an additional ~100 kDa band was seen (indicated by solid arrow), indicating a cleavage event. Those lanes including hepsin treatment (lanes 2-4) also produced an ~30 kDa band, which represents the protease domain of hepsin (indicated by open arrow). (B) After incubation of Ln-332 alone at various time points (0, 2, and 6 h), the same uncleaved Ln-332 chains are visible. However, co-incubation of Ln-332 (0.2 µM) with hepsin (1.3 µM) resulted in the generation of a new band (~ 100 kDa, indicated by closed arrow), again indicating cleavage. Again, the ~30 kDa band corresponds to the hepsin protease domain (indicated by open arrow; lanes 2,4, and 6). (C) Non-reducing SDS-PAGE analysis of hepsin cleavage of laminin-332. Ln-332 (0.2 µM) was incubated alone or with hepsin for 1.5 h at 37°C. Upon incubation of Ln-332 hepsin, an additional band was seen indicating a cleavage event. However, Ln-332 incubated with hepsin electrophoresed under non-reducing conditions demonstrated degraded Ln-332, compared to Ln-332 control (lane 2), but less obvious changes than those seen in reducing conditions (lane 4).

In a time-course experiment, we incubated Ln-332 and hepsin in reaction buffer for 0, 2, or 6 h (Figure 7B). As expected, no cleavage product of Ln-332 was observed at 0 h (lane 2), whereas the intensity of the ~100 kDa band increased from 2 h (lane 4) to 6 h (lane 6) ( arrow). As shown in Figure 7B, those treatments including hepsin (lanes 2, 4, and 6) also showed the ~30 kDa hepsin band. These results confirmed that untreated Ln-332 resolved as bands corresponding to α3 (190 kDa), β3 (145 kDa), and γ2 chains (155 kDa and 80 kDa), and that no cleavage band was detectable in the absence of hepsin (lanes 1, 3, and 5). These results suggest that the ~100 kDa band is a unique product of hepsin cleavage.

*Laminin-332 is specifically cleaved by hepsin*

In order to confirm that Ln-332 was cleaved by hepsin specifically and not by another contamination protease, we performed an enzyme inhibition assay. We added an inhibitor of hepsin, KD1, in the cleavage reaction with Ln-332 and hepsin during incubation, and analyzed by SDS-PAGE (Figure 8A). Hepsin-treated Ln-332, as expected, contained a ~100 kDa band, not present in untreated Ln-332. However, addition of the inhibitor KD1 nearly abolished this band. Those lanes with hepsin treatment again revealed ~30 kDa protease domain bands. Additionally, those treatments with KD1 inhibitor produced a band at ~10 kDa. This experiment indicates that the ~100 kDa band is a product of cleavage of Ln-332 by hepsin.

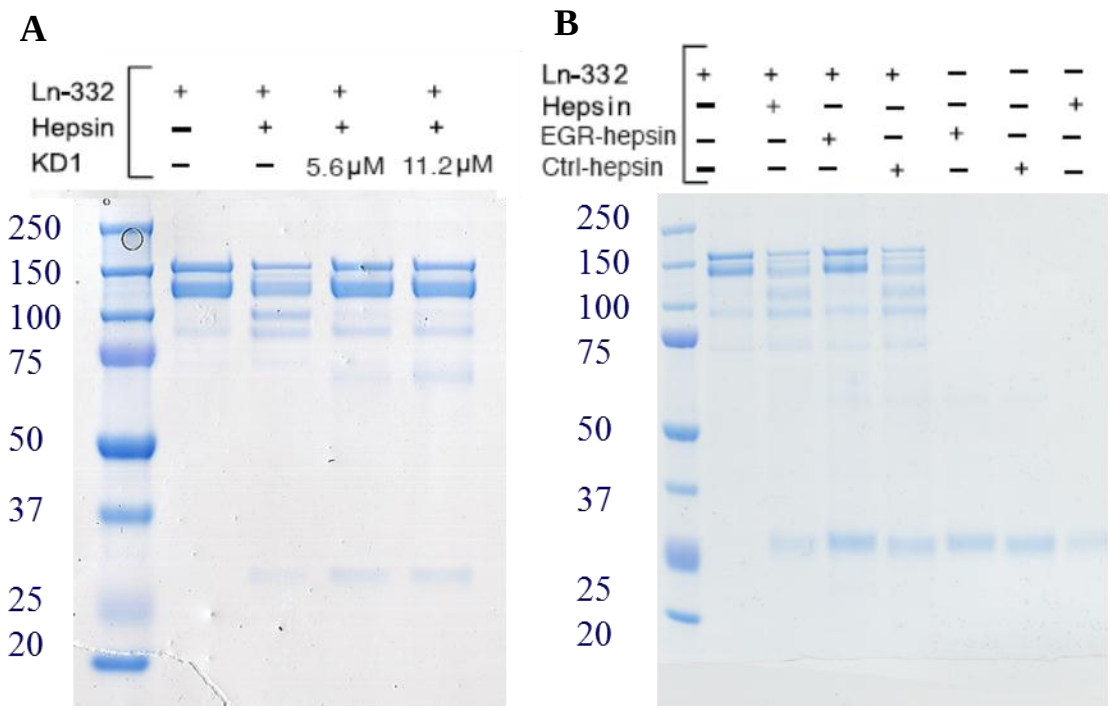


Figure 8: SDS-PAGE analysis to show specificity of hepsin-cleavage of laminin-332. (A) Ln-332 (0.2 μM) was incubated alone, with recombinant hepsin (1.3 μM), or with hepsin and KD1 inhibitor for 1.5 h at 37°C. Ln-332 coincubated with hepsin displayed an additional band of cleavage product. However, Ln-332 incubated with both hepsin and KD1 inhibitor displayed greatly diminished bands of cleavage product compared to the band in the absence of KD1 . Those treatments with hepsin produced an ~30 kDa band. Lanes with KD1 also showed ~11 kDa band, representing the presence of this inhibitor. (B) Ln-332 was incubated either alone, or in the presence of hepsin, inactive EGR-hepsin, or Ctrl-hepsin. Ln-332 incubated with catalytically active hepsin or Ctrl-hepsin resulted in an ~100 kDa band. In contrast, Ln-332 alone, or Ln-332 incubated with inactive EGR-hepsin did not produce this band. Those lanes with only hepsin (7), Ctrl-hepsin (6), or inactive EGR-hepsin (5) only produced a single band at ~30 kDa.

*Inactive hepsin does not cleave Laminin-332*

To further examine the specificity of Ln-332 cleavage, we performed experiments with catalytically inactive EGR-hepsin. In enzymatic assays with synthetic S2366 substrate, EGR-hepsin displayed less than 1% activity of uninhibited Ctrl-hepsin. In the experiments (Figure 8B), we incubated Ln-332 either alone, or in the presence of hepsin, inactive EGR-hepsin, or Ctrl-hepsin. As shown in Figure 8B, Ln-332 incubated with active hepsin or Ctrl-hepsin resulted in an ~100 kDa band. In contrast, Ln-332 alone, or Ln-332 incubated with inactive EGR-hepsin, did not produce this band. Those lanes with only hepsin, Ctrl-hepsin, or inactive EGR-hepsin only produced a single band at ~30 kDa. These results further support the conclusion that Ln-332 is cleaved specifically by catalytically active hepsin molecule, and not by any other contaminating protease.

*Characterization of cleaved Laminin-332 band*

To determine the identity of the unique band that appeared after hepsin treatment of Ln-332, we resorted to using an antibody specific for the Ln-332 chains. A polyclonal antibody raised against the C-terminal sequence of Ln-332 β3 chain (Zapatka, Zboralski et al. 2007) reacted in western blotting (Figure 9A), both with the full-length β3 chain in Ln-332 alone and the 100 kDa hepsin-cleaved fragment This result suggested that hepsin cleaves the β3 chain of Ln-332, located in the ~100 kDa C-terminal region of rat Ln-332 β3 chain (Figure 9A). This result indicates that hepsin cleaves the Ln-332 β3 chain in the vicinity of the N-terminus. To confirm this possibility, we further established the identity of the cleaved fragment by mass spectrometry (performed by Mass Spectrometry Research Center at Vanderbilt University). Briefly, trypsin digestion of the ~100 kDa

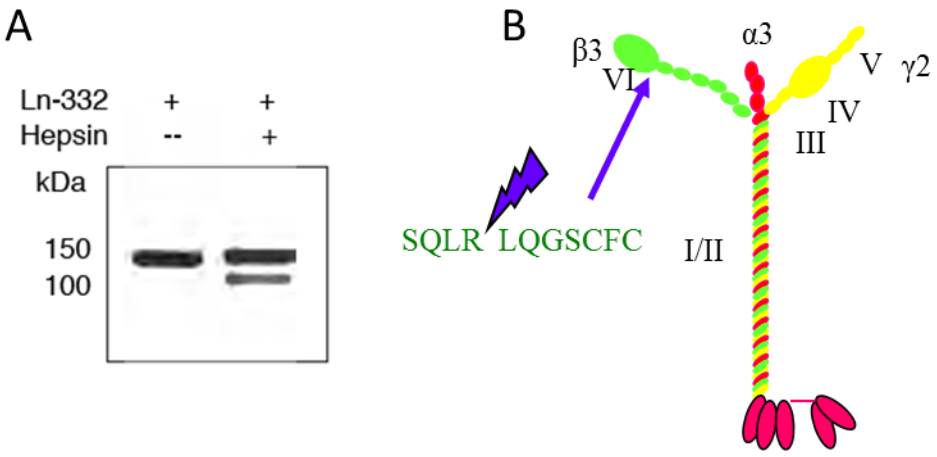


Figure 9: Identification of hepsin–cleavage site on laminin- 332 ß3 chain by Western blot analysis and N-terminal sequencing. (A) Western blot was performed on Ln-332 alone (0.1 µM) and in combination with hepsin (0.7 µM), and probed with a polyclonal antibody specific for the ß3 chain. Application of ß3 antibody identified the ~100 kDa cleaved-product of Ln-332 ß3 chain and the uncleaved ß3 chain. (B) Schematic representation of Ln-332 including the specific hepsin cleavage site determined to be between the $Arg^{245}$-$Leu^{246}$ residues of the rat Ln-332 ß3 chain. Note that cysteine residues are not detected by Edman sequencing and were deduced from cDNA. The three chains (α3, β3, and γ2) including domains I-VI and LG domains of Ln-332 are also indicated. Abbreviations for amino acid residues: S, serine; Q, glutamine; L, leucine; R, arginine; G, glycine; C, cysteine; F, phenylalanine. The sequence around the cleavage site is a perfect match to the consensus sequence found by a high-throughput combinatorial approach by Herter S.et al, 2005 *Biochem J*

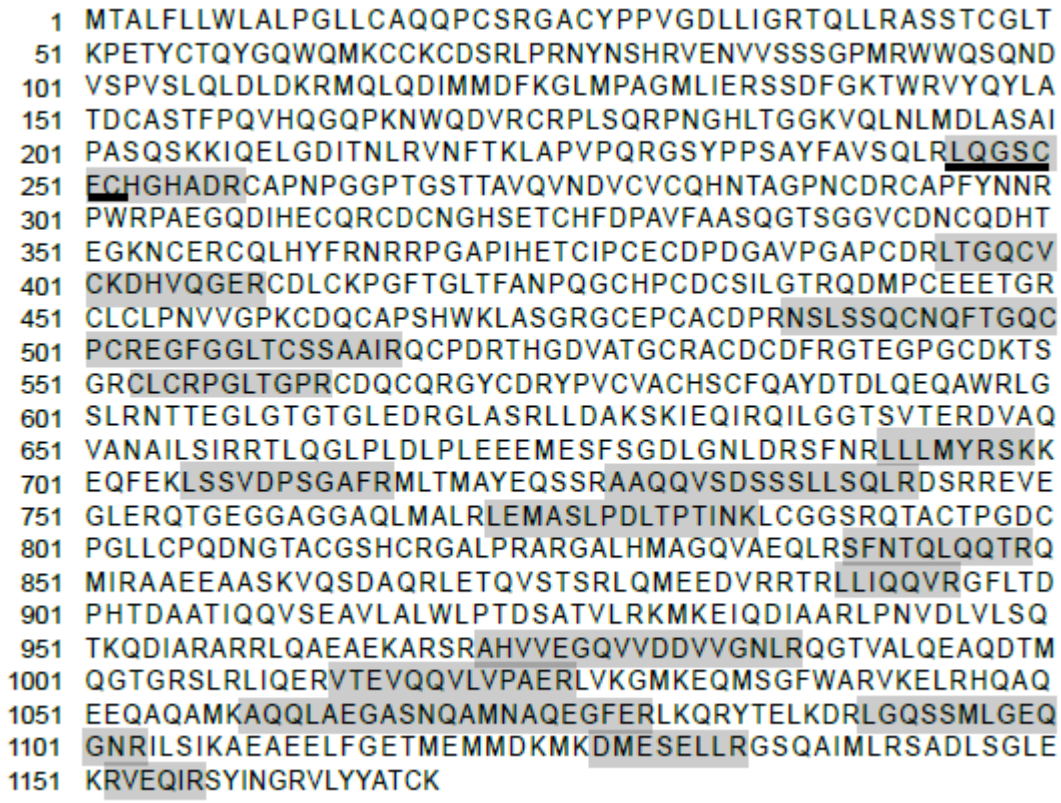


Figure 10: Identification of hepsin–cleavage on laminin- 332 ß3 chain by mass spectrometry: Trypsin digestion of the ~100 kDa band was performed for 2 hours at 37 °C before subjection to matrix- assisted laser desorption/ionization, time-of-flight mass spectrometry. The mass spectral data were used to examine protein databases to generate statistically significant candidate identifications using GPS Explorer software running the Mascot database search algorithm. Seventeen individual peptides (highlighted in gray) were identified from the digestion, which directly aligned with the sequence of rat Ln-332 ß3 chain

cleavage band produced 17 distinct peptides, all identical to peptides located in the ~100 kDa C-terminal region of rat Ln-332 β3 chain (Figure 10). This result indicates that hepsin cleaves the Ln-332 β3 chain in the vicinity of the N-terminus. To confirm this possibility, and to positively identify the cleavage site, we performed N-terminal sequencing of the cleaved band. The strongest signal was obtained for the following sequence: $NH_2$-LQGSCFC (note that cysteine residues are not detected by Edman sequencing and were deduced from cDNA), which corresponds exactly to a sequence in rat Ln-332 starting at $Leu^{246}$ (Figure 9B). Therefore, we conclude that the hepsin cleavage site on Ln-332 β3 chain is located between $Arg^{245}$ and $Leu^{246}$.

*Migration of DU145 cells on Laminin-332 is enhanced by hepsin cleavage*

To determine the potential biological significance of the cleavage of Ln-332 β3 chain, we examined the migratory behavior of DU145 prostate cancer cells on hepsin-cleaved Ln-332 versus untreated Ln-332 substrate (Figure 11A and B). Using a modified Boyden chamber assay, we applied various substrates to transwells, as described in Materials and Methods. Hepsin-cleaved Ln-332 promoted a significant increase in migration (1.7-fold) compared to untreated Ln-332 ($N = 4$, in duplicate; $P < 0.05$). To confirm that hepsin cleavage of Ln-332 caused increased migration of cells, we added inhibitor KD1, to test its ability to abolish increased activity. As expected, in the presence of KD1, cells migrated similar to cells on Ln-332 ($N = 4$, in duplicate). As a control, almost no cells were seen on PBS-, KD-1, or hepsin-treated alone inserts, (i.e., without ECM substrate). These results suggest that cleavage of Ln-332 by hepsin may physiologically enhance migration.

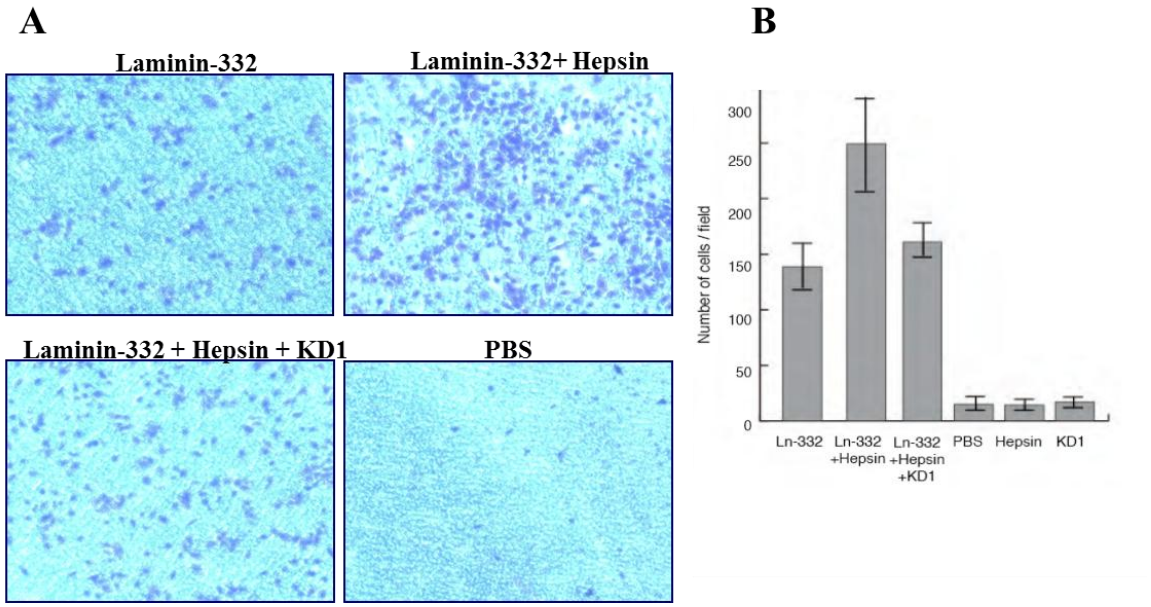


Figure 11: DU145 prostate cancer cells exhibit enhanced migration on hepsin-cleaved laminin-332. DU145 cells ($2 \times 10^4$) in serum-free DMEM were added to pretreated upper chambers of transwell inserts and allowed to migrate for 5 h at 37ºC. After incubation, non-migratory cells and media were washed from transwells, and those cells that migrated to the bottom of the filters were stained with Hema® kit, fixed, and imaged using a Zeiss LSM-510. (A) Representative images (5 fields) were taken of pretreated (Ln-332, Ln-332+Hepsin, Ln-332+Hepsin+KD1 or PBS filters with fixed cells. The scale bar is equal to 20 µm. (B) Cells plated on hepsin-cleaved Ln-332 treated inserts had a significant ($N = 4$; $P < 0.001$) increase in migration, compared to cells on either Ln-332 alone or Ln-332 with hepsin and KD1 inhibitor. Cells on PBS treated inserts migrated significantly ($N = 4$, in duplicate; $P < 0.01$ in all cases) less that all other treatments.

*Cell adhesion of DU145 cells exhibit no significant difference on hepsin-cleaved Ln-332*

To determine if cellular adhesion of DU145 cells was different on hepsin cleaved Ln-332 versus untreated Ln-332 substrate, we performed cell adhesion assay as described in the Materials and Methods Chapter. We coated Ln-332 alone (L), Ln-332 + hepsin (LH), Ln-332 + hepsin + KD1 and PBS onto 96-well plates. DU145 cells were allowed to adhere for 1 h at 37ºC in serum-free medium. Unbound cells were washed off; adherent cells were fixed, stained (crystal violet), and solubilized; and absorbance was read at 560 nm. We found that DU145 cells had no significant difference in cell adhesion on hepsin cleaved Ln-332 as compared to untreated Ln-332 or on mixture with Ln-332, hepsin and KD1 (Figure 12). Results represent mean ± S.D. (N= 2, in duplicates; $P < 0.05$).

*Migration of hepsin-overexpressing cells is enhanced on Laminin-332 substrate*

To further determine the potential biological significance of hepsin cleavage of Ln-332, we also examined the migratory behavior of hepsin-overexpressing LNCaP-34 prostate cancer cells on Ln-332 versus low hepsin-expressing LNCaP-17 cells on the same substrate. We verified LNCaP cells for hepsin expression by RT-PCR and western blot, and obtained data consistent with published findings that LNCaP-34 cells express ~5-fold higher levels of hepsin than LNCaP-17 cells (Figure 13A and B) (Moran, Li et al. 2006) . Using a modified Boyden chamber assay, we applied either Ln-332 (10 μg/ml) or PBS substrate to transwells, and allowed cells to migrate at 37°C for 24 h, as described in Materials and Methods. As shown in Figure 14A, LNCaP-34 cells exhibited a significant increase in migration on Ln-332 (~2.1-fold) compared to LNCaP-17 cells on Ln-332

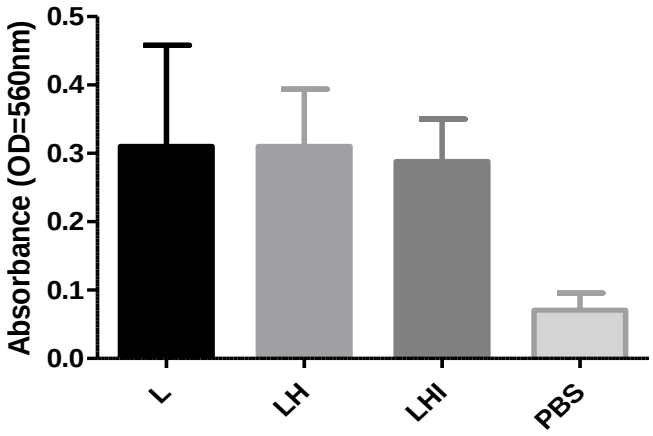


Figure 12: DU145 prostate cancer cells exhibit no change in cell adhesion on hepsin-cleaved Ln-332. Ln-332 alone (L), Ln-332 + hepsin (LH), Ln-332 + hepsin + KD1 and PBS were coated onto 96-well plates. DU145 cells were allowed to adhere for 1 h at 37ºC in serum-free medium. Unbound cells were washed off; adherent cells were fixed, stained, dried and solubilized; and absorbance was read at 560 nm. Results represent mean ± S.D. (N= 2, in duplicates; $P < 0.05$).

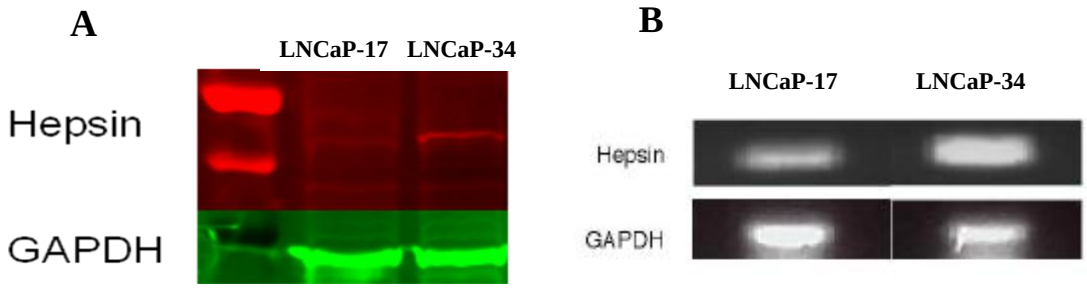


Figure 13: Hepsin expression in LNCaP-17 and LNCaP-34 by Western blot and RT PCR (A) Western blot using an antibody against hepsin confirmed that LNCaP-34 cells express more hepsin than LNCaP-17 cells. As a control, the blot was probed for GAPDH, which revealed that similar total protein was used for each cell type. (B) mRNA encoding hepsin was detected by RT-PCR on RNA extracted from LNCaP-17 and LNCaP-34 cells. Amplified PCR products were electrophoresed on 1% agarose gel. GAPDH was used as a control. Estimated size of PCR products were hepsin, 229 bp and GAPDH 196 bp.

(N = 3, in duplicate; $P < 0.01$). To confirm that degradation of Ln-332 by cells influenced migration, we also measured both cell lines on PBS-treated inserts. As expected, both cell clones migrated slowly on PBS-treated transwells (N=3, in duplicate; $P > 0.05$). Additionally, a polyclonal antibody directed against the β3 chain of Ln-332 in western blot analysis (Figure 14B) revealed an additional ~100 kDa band unique to hepsin-overexpressing LNCaP-34 cells incubated with Ln-332 (lane 2), which was not exhibited by LNCaP-17 cells (lanes 1 and 3), nor by LNCaP-34 cells in the absence of Ln-332 (lane 4). The bands in lanes 3 and 4 are background bands, possibly due to endogenous expression of β3 by these LNCaP cells. Taken together, these results suggest that cleavage of Ln-332 by hepsin may physiologically enhance migration.

*Cell adhesion of hepsin-overexpressing prostate cancer cells exhibit no significant difference on Ln-332*

To determine if cellular adhesion of hepsin-overexpressing LNCaP-34 prostate cancer cells on Ln-332 was different as compared to low hepsin-expressing LNCaP-17 cells on Ln-332, we performed cell adhesion assay as described in Materials and Methods Chapter. We coated Ln-332 or PBS onto 96-well plates. Both Cell types were allowed to adhere for 1 h at 37ºC in serum-free medium. Unbound cells were washed off; adherent cells were fixed, stained (crystal violet), and solubilized; and absorbance was read at 560 nm. We found that hepsin-overexpressing LNCaP-34 cells had no significant difference in cell adhesion on Ln-332 as compared to low hepsin-expressing LNCaP-17 cells (Figure 15). Results represent mean ± S.D. (N= 3, in triplicates; $P < 0.05$).

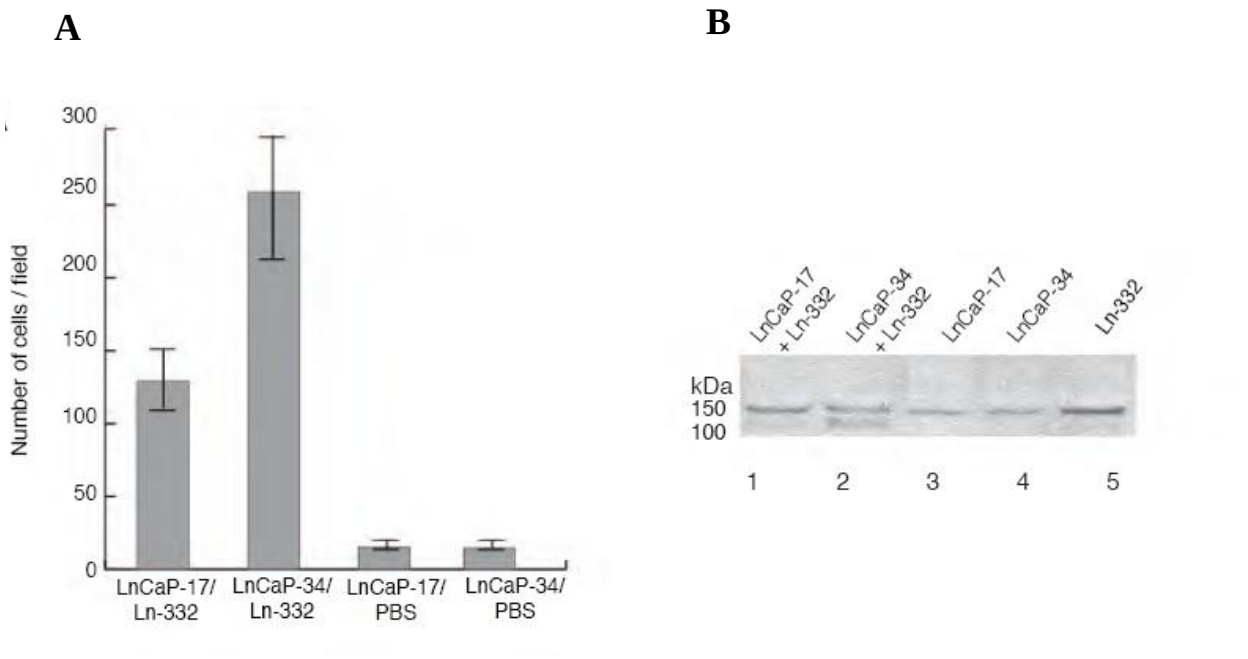


Figure: 14 Migration of LNCaP hepsin-overexpressing prostate cancer cells is enhanced on Ln-332. (A) We examined the migratory behavior of hepsin-overexpressing LNCaP-34 prostate cancer cells on Ln-332 versus low hepsin-expressing LNCaP-17 cells. Using a modified Boyden chamber assay, we applied either Ln-332 (10 μg/ml) or PBS to transwells, and allowed cells ($5 \times 10^4$) to migrate at 37°C for 24 h. LNCaP-34 cells exhibited a significant increase in migration on Ln-332 (~2.1-fold) compared to LNCaP-17 cells on Ln-332 ($N = 3$, in duplicate; $P < 0.01$). To confirm that degradation of Ln-332 by cells influenced migration, we also measured both cell lines on PBS-treated inserts. As expected, both clones weakly migrated on PBS-treated transwells ($N=3$, in duplicate; $P > 0.05$). These results suggest that cleavage of Ln-332 by hepsin may physiologically enhance migration. (B) Western blot analysis of LNCaP-17 and LNCaP-34 cells, each in the presence or absence of Ln-332, revealed that hepsin-overexpressing cells (LNCaP-34) created an addition band at ~100 kDa. The bands in lanes 3 and 4 (cells alone) are background, possibly due to endogenous expression of β3 by these LNCaP cells.

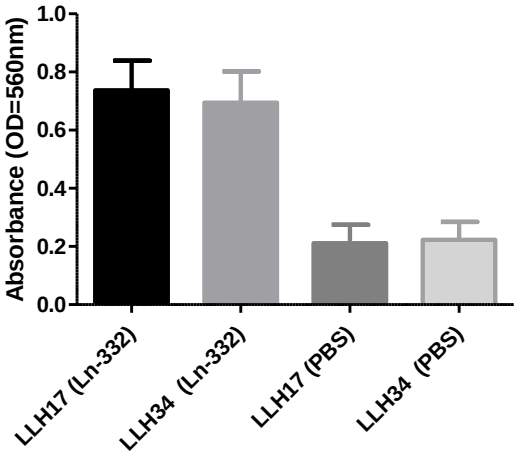


Figure 15: Hepsin overexpressing LNCaP cells exhibit no change in cell adhesion on Ln-332. Ln-332 or PBS was coated onto 96-well plates. LNCaP17 and LNCaP cells were allowed to adhere for 1 h at 37ºC in serum-free medium. Unbound cells were washed off; adherent cells were fixed, stained (crystal violet), and solubilized; and absorbance was read at 560 nm. Results represent mean ± S.D. (N= 3, in triplicates; $P < 0.05$).
Both Cell types were allowed to adhere for 1 h at 37ºC in serum-free medium. Unbound cells were washed off; adherent cells were fixed, stained (crystal violet), and solubilized; and absorbance was read at 560 nm. We found that hepsin-overexpressing LNCaP-34 cells had no significant difference in cell adhesion on Ln-332 as compared to low hepsin-expressing LNCaP-17 cells. Results represent mean ± S.D. (N= 3, in triplicates; $P < 0.05$).

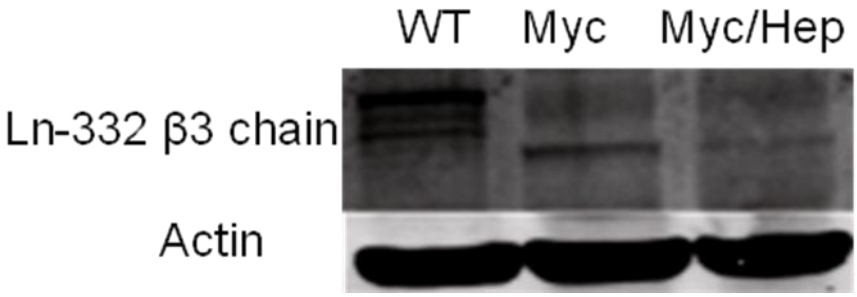


Figure 16: Hepsin/myc tumors mice display increased degradation of Laminin-332 as compared with myc alone tumors. Western blot analysis showing Laminin-332 β3 chain expression in 12 month wildtype, myc and hepsin/myc mice. Western blot is representative of the experiment performed three times, two mice for each group has been used. Lane WT shows intact Laminin-332 in 12 month wildtype mice (145 kDa). Lanes Myc and Myc/Hep show cleaved Laminin-332 (100kDa) in 12 month myc and hepsin/myc mice respectively.

*Hepsin/myc tumors mice display increased degradation of Laminin-332 as compared with myc alone tumors*

In an effort to correlate increased hepsin expression with increased hepsin activity in terms of Ln-332 cleavaged *in vivo*, we performed Western blot analysis with the tumor tissues derived from wild type, myc and hepsin/myc mice. The Western blot results showed intact Ln-332 (145 kDa), in the tissue lysate of wild type mice. The myc tumor displayed a 100 kDa band. In the hepsin/myc tumor this 100 kDa band was further diminished (Figure 16). These data correlate with the fact that hepsin cleaves laminin-332 *in vitro*. The tissue lysate from wild type mice showed intact Ln-332 beta3 chain whereas our data indicates that increased hepsin levels in hepsin/myc tumors are correlated with degradation of Ln-332, a component of the basement membrane that is lost during human prostate cancer.

We also performed immunohistochemical analysis for Ln-322 on tumors derived from myc mice, that develops adenocarcinoma by 6 month of age (Ellwood-Yen, Graeber et al. 2003) and hepsin/myc mice, that develops invasive adenocarcinoma at 4.5 months and develops a higher grade adenocarcinoma compared with age-matched myc mice tumors (Nandana, Ellwood-Yen et al. 2010). We found that 12 month old hepsin/myc tumor displayed increased degradation of Ln-332 as compared with age-matched myc tumor. Although, the immunohistochemistry data (Figure 17) are not very clear as we see more staining of Ln-332 on hepsin/myc mice as compared to myc mice but it is possible that the staining appears to be more due to diffused Ln-332.

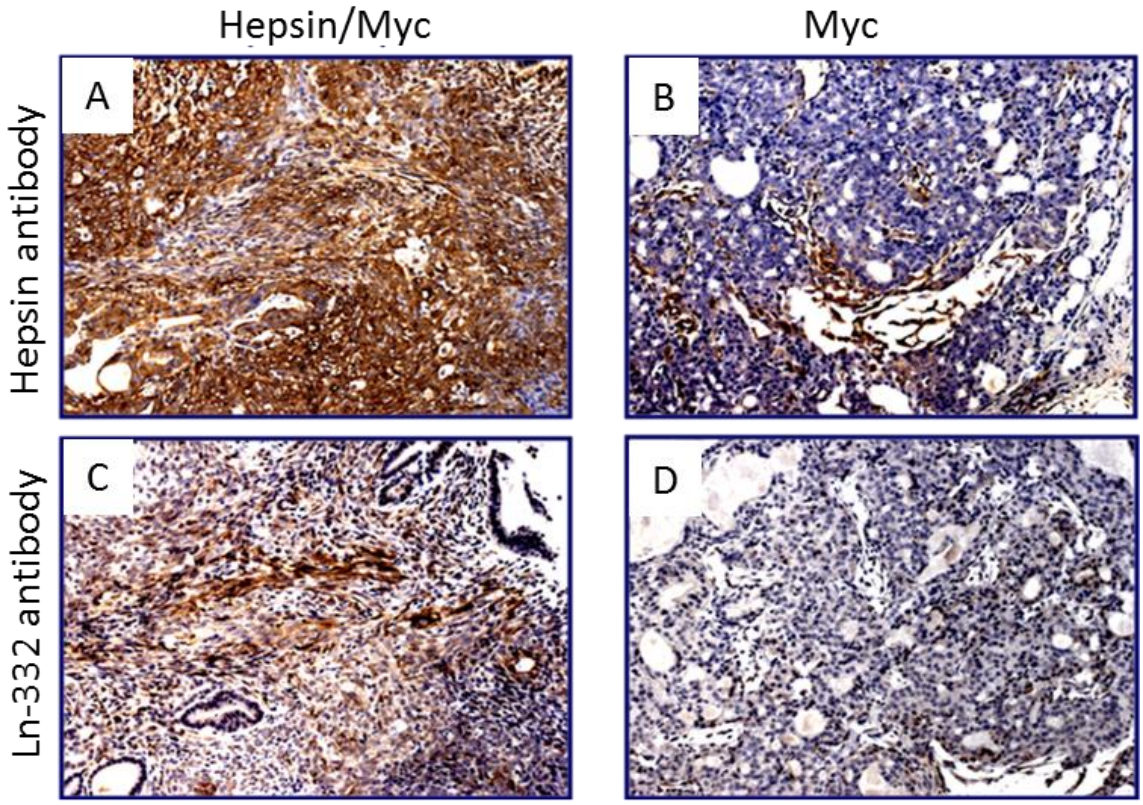


Figure 17: Immunohistochemical staining of tumor sections of lateral prostate of hepsin/myc and Myc overexpressing Mouse models of Prostate Cancer. Panel A and B showing hepsin staining in hepsin/myc (A) and myc (B) mouse and panel C and D showing Ln-332 staining in hepsin/myc (C) and myc (D) mouse.

## Conclusions

In summary, a role for either Ln-332 or hepsin in prostate cancer progression is supported by several studies. However, the roles of these two molecules have been studied separately and they have not been previously linked to date. In this chapter, we demonstrate for the first time that hepsin cleaves Ln-332. Using western blot analysis and mass spectrometry, we identified that cleavage occurs specifically in the β3 chain. Further, N-terminal sequencing identified the hepsin cleavage site between the Arg $^{245}$-Leu $^{246}$ residues in the β3 chain. Cleavage of Ln-332 was inhibited by a known hepsin inhibitor (KD1), and did not occur in the presence of catalytically inactive hepsin, confirming specificity. We also report increased migration of DU145 prostate cancer cells on hepsin-cleaved Ln-332, which was also inhibited in the presence of KD1. Similarly, we show that hepsin-overexpressing LNCaP-34 cells exhibit enhanced motility on Ln-332, compared to low hepsin-expressing LNCaP-17 cells. We show that hepsin correlated with Ln-332 degradation *in vivo* in mouse model of prostate cancer over expressing hepsin. This study suggests a physiological role of hepsin in proteolytic cleavage of Ln-332 and gives new insight into possible mechanisms for hepsin in prostate cancer progression.

# CHAPTER IV

# LAMININ-332 CLEAVAGE BY MATRIPTASE ALTERS MOTILITY PARAMETERS OF PROSTATE CANCER CELLS.

## Introduction

Matriptase, a type II transmembrane serine protease, is involved in maintaining the integrity of epithelia. Matriptase has been linked to initiation and promotion of epidermal carcinogenesis in a murine model, suggesting that deregulation of its role in epithelial integrity contributes to transformation. Overexpression of matriptase in mouse epidermis induces spontaneous skin lesions, however epidermal hyperproliferation and tumors are abolished by coexpression of its physiological inhibitor, HAI-1 (List, Szabo et al. 2005). In human prostate cancer, matriptase expression indeed correlates with progression, suggesting it could be used as a biomarker for prognosis and a target for treatment (Riddick, Shukla et al. 2005; Saleem, Adhami et al. 2006). It is therefore of interest to determine how matriptase may contribute to epithelium neoplastic progression.

One approach is to identify matriptase substrates involved in epithelial integrity and/or transformation. In this study, we provide the first direct evidence that matriptase proteolytically processes Ln-332, a key extracellular matrix macromolecule found in the basement membrane of many epithelia, including prostate. Ln-332 has a demonstrated role in maintaining epithelial integrity. Its deregulated expression has been reported in several cancers and, especially relevant to this study, it is extinguished in advanced prostate cancer. Based on these findings, we propose that proteolytic processing of Ln-

332 could be a possible mechanistic role for matriptase in prostate cancer progression via altered migration parameters and subsequent basement membrane transgression.

## Results

*Laminin-332 is cleaved by matriptase*

To determine whether matriptase cleaves Ln-332, purified Ln-332 from 804G rat bladder cells was incubated with three concentrations of the recombinant protease domain of matriptase for 2 h. After incubation, the mixtures and Ln-332 alone were electrophoresed on SDS-PAGE and then stained with Coomassie Blue. Not surprisingly, Ln-332 alone revealed 4 primary bands representing the α3 (190 kDa), β3 (145 kDa), and γ2 chains (155 and 80 kDa; Figure 18A) of the structure and matriptase alone revealed one strong band at ~27.5 kDa . Of note, the lanes containing Ln-332 and matriptase mixtures revealed a unique band at ~100 kDa, particularly at higher concentrations of the latter (arrows) (Figure 18 A). The lanes with the protease also resolved bands at ~27.5, ~18, and ~10 kDa, which were determined to be matriptase by mass spectrometry (data not shown). These results suggest that the recombinant protease domain of matriptase cleaved Ln-332. To determine if this cleavage was time-dependent, we also performed a time-course experiment whereby Ln-332 and matriptase were coincubated for 0, 3, 6 and 12 h and the mixtures were again resolved using SDS-PAGE. As expected, no cleavage product of Ln-332 was observed in the 0 h mixture, however the cleaved ~100 kDa band was present in lanes containing the mixtures from 3, 6, and 12 h (Figure 18B).

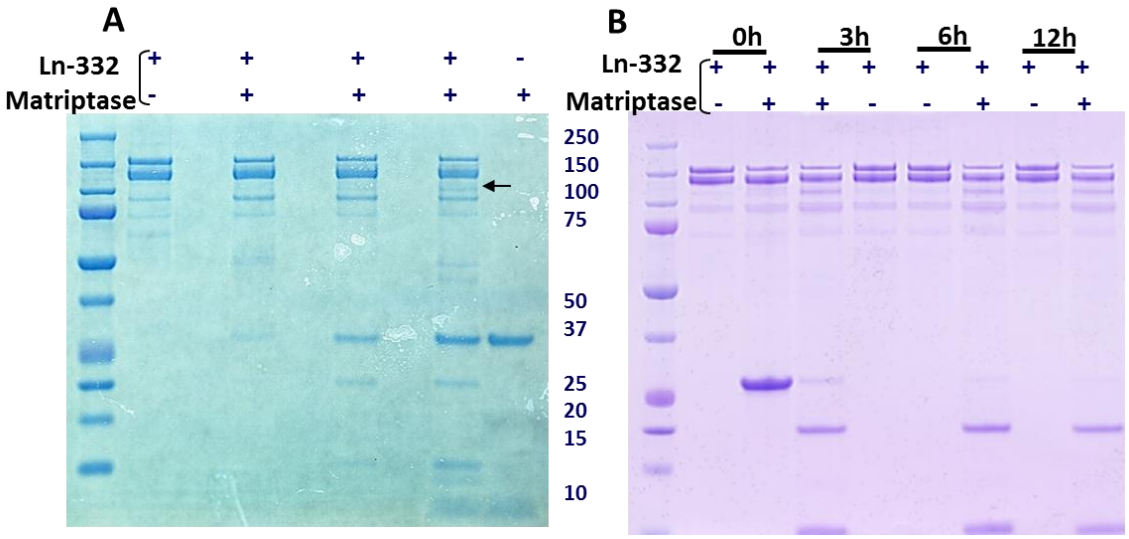


Figure 18: Ln-332 is cleaved by matriptase. A. Purified Ln-332 from 804G rat bladder cells (0.2 µM) was incubated with the recombinant protease domain of matriptase (0.6, 2, and 6 µM) for 2 h. After incubation, the mixtures and Ln-332 alone were electrophoresed on SDS-PAGE and then stained with Coomassie blue. Ln-332 alone revealed 4 primary bands representing the α3 (190 kDa), β3 (145 kDa), and γ2 chains (155 and 80 kDa). Of note, a unique band was resolved at ~100 kDa in the lanes containing Ln-332 and matriptase, particularly at higher concentrations of the latter (arrows). (B) Ln-332 (0.8 µM) and matriptase (24 µM) were coincubated for 0, 3, 6 and 12 h. No cleavage product of Ln-332 was observed from the 0 h mixture, however the cleaved ~100 kDa band was present in lanes containing the mixtures from 3, 6, and 12 h.

*Matriptase cleaves the Laminin-332 β3 chain*

To determine that the cleavage event of Ln-332 was due to addition of matriptase, we also added inhibitor, KD1, to the mixture. As in earlier experiments, purified rat Ln-332 alone revealed 4 bands, representing its respective chains (Figure 19A) and Ln-332 treated with matriptase again revealed the ~100 kDa cleavage product. Of interest, the mixtures containing KD1 lacked the ~100 kDa cleavage product, suggesting that matriptase was responsible for cleavage of Ln-332. Matriptase was again resolved as an ~27.5 kDa band, which matches up with the previous SDS-PAGE results, and KD1 was resolved as an ~11 kDa band, in line with our published report (Tripathi, Nandana et al. 2008).

To further determine the identity of the unique band that appeared after matriptase treatment of Ln-332, we used both western blotting with an antibody to Ln-332 and a proteomics approach. As shown in Figure 19B, a polyclonal antibody against the C-terminal sequence of Ln-332 β3 chain reacted with both the full-length β3 chain in Ln-332 alone and the ~100 kDa matriptase-cleaved fragment as revealed by western blotting. This result suggests that matriptase cleaves the β3 chain of Ln-332, possibly removing an N-terminal sequence. To explore this, then performed mass spectrometric analysis. The protein bands of Ln-332 and the ~100 kDa product were excised from a gel from SDS PAGE. After trypsin digestion, MALDI-TOF MS and tandem TOF/TOFMS/MS were performed, and data from both methods were collectively used to examine the protein databases. Statistically significant candidates were identified using GPS Explorer software running the MASCOT database search algorithm. Searches were performed

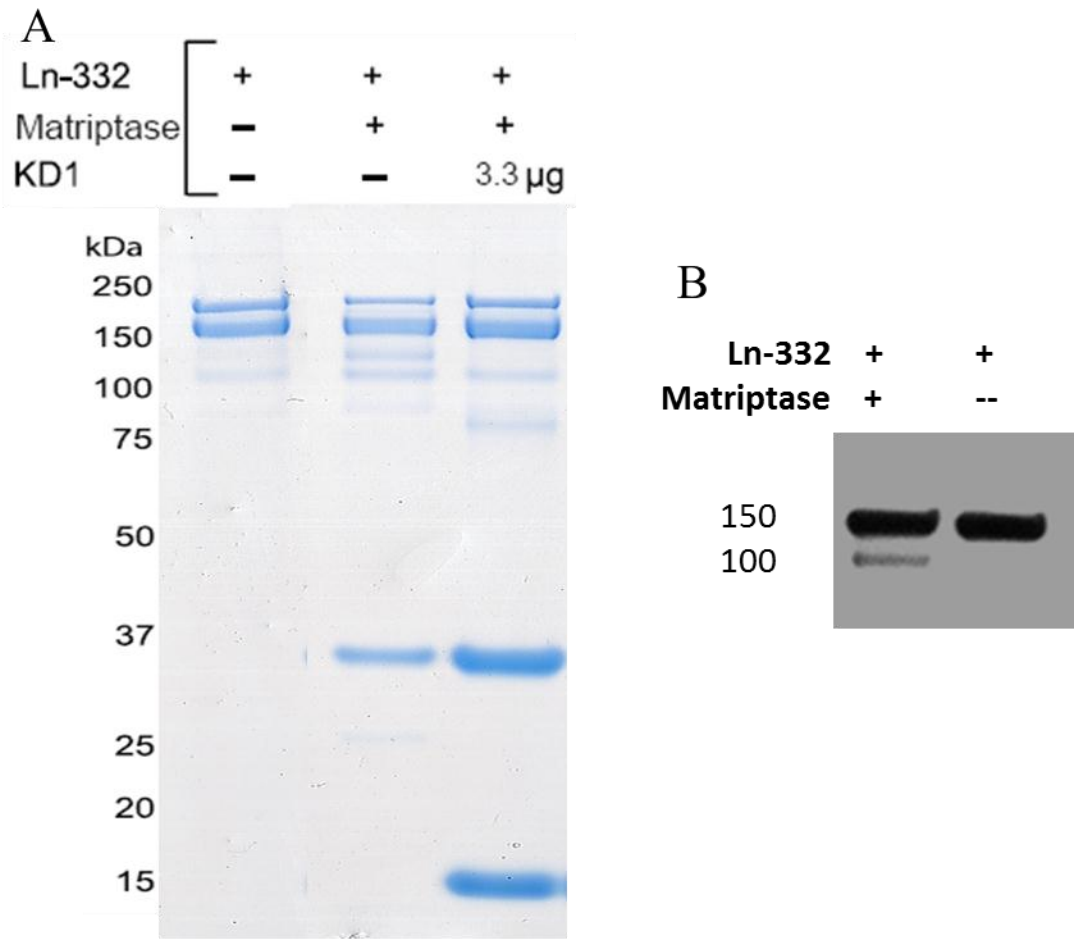


Figure 19: Matriptase cleaves the Ln-332 β3 chain. (A) A known matriptase inhibitor, KD1, was added to the Ln-332 and matriptase mixture. Purified rat Ln-332 (0.2 µM) alone revealed 4 bands, representing its respective chains. Ln-332 (0.2µM) treated with matriptase (6µM) again revealed the ~100 kDa cleavage product. The mixture also containing KD1 lacked the ~100 kDa cleavage product. (B) A western blot was performed using a polyclonal antibody against the C-terminal sequence of Ln-332 β3 chain. The antibody reacted with both the full-length β3 chain in Ln-332 alone and the ~100 kDa matriptase-cleaved fragment.

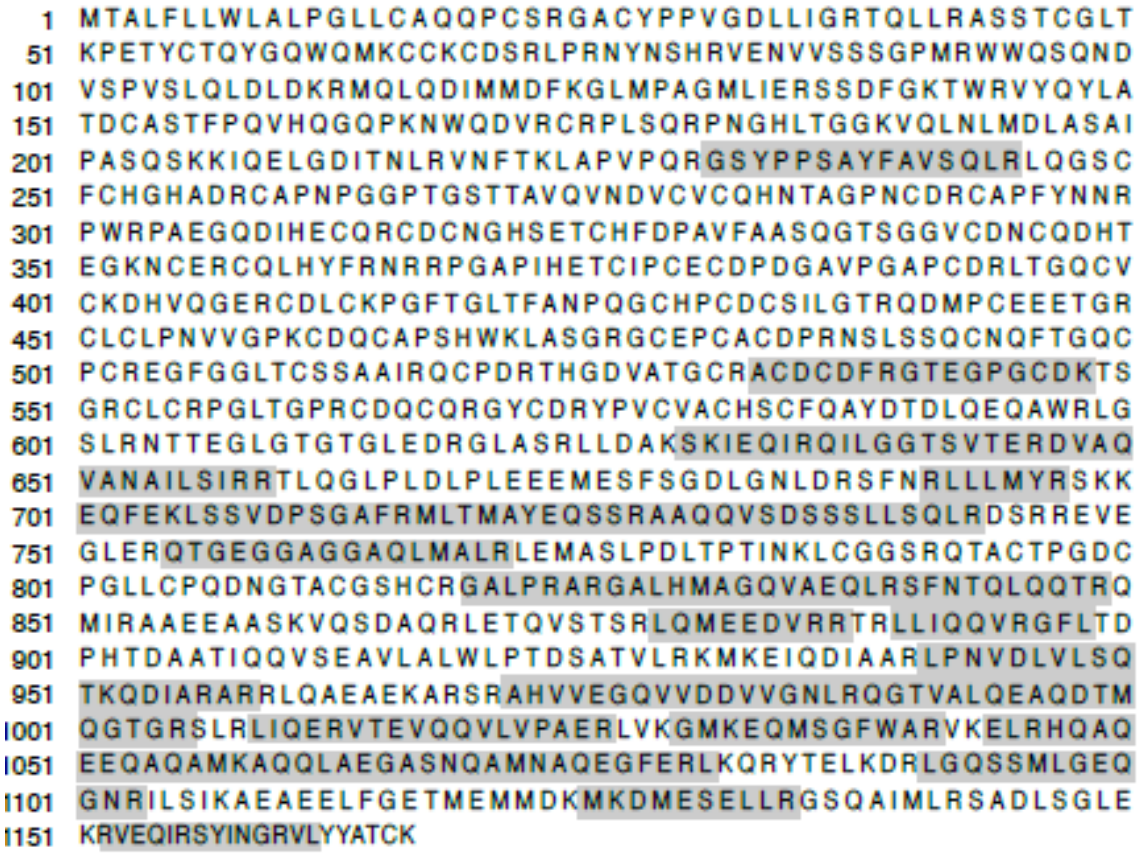


Figure 20: Mass spectrometry was performed to analyze the contents of the protein bands of Ln-332 and the ~100 kDa product from Ln-332 treated with matriptase. This analysis revealed that the Ln-332 protein band contained its 3 chains: α3 (190 kDa), β3 (145 kDa) and γ2 (155 kDa), and the digested ~100 kDa band that appeared upon treatment of Ln-332 with matriptase produced nineteen different peptides (grey) that were clearly identical to amino acid sequences of Ln-332 β3 chain.

against the SWISS PROT and NCBI databases. The Ln-332 protein band contained its three chains: α3 (190 kDa), β3 (145 kDa) and γ2 (155 kDa). In addition, the digested ~100 kDa band that appeared upon treatment of Ln-332 with matriptase produced 19 different peptides that were identical to amino acid sequences of the Ln-332 β3 chain (Figure 20; peptides in gray).

*Migration of DU145 cells is enhanced on Laminin-332 cleaved by matriptase*

Since previous studies have shown that cleavage of the Ln-332 β3 chain by other proteases leads to changes in cell migration (Hintermann and Quaranta 2004; Tripathi, Nandana et al. 2008), we also investigated the effect of the cleavage of Ln-332 β3 chain by matriptase on prostate cancer cell migration. First, we examined the motility of DU145 cells using modified Boyden chambers coated with either untreated Ln-332, matriptase- cleaved Ln-332, PBS, or a mixture containing Ln-332, matriptase, and KD1. The number of cells that passed through filters after 5 h were then manually counted under a microscope. Cells seeded in chambers coated with matriptase-cleaved Ln-332 migrated significantly more (~1.6-fold) than cells on uncleaved Ln-332 (Figure 21A and B). In addition, cells in chambers coated with the Ln-332, matriptase, and KD1 inhibitor mixture migrated significantly less than cells on matriptase-cleaved Ln-332, which was similar to migration levels on untreated Ln-332.

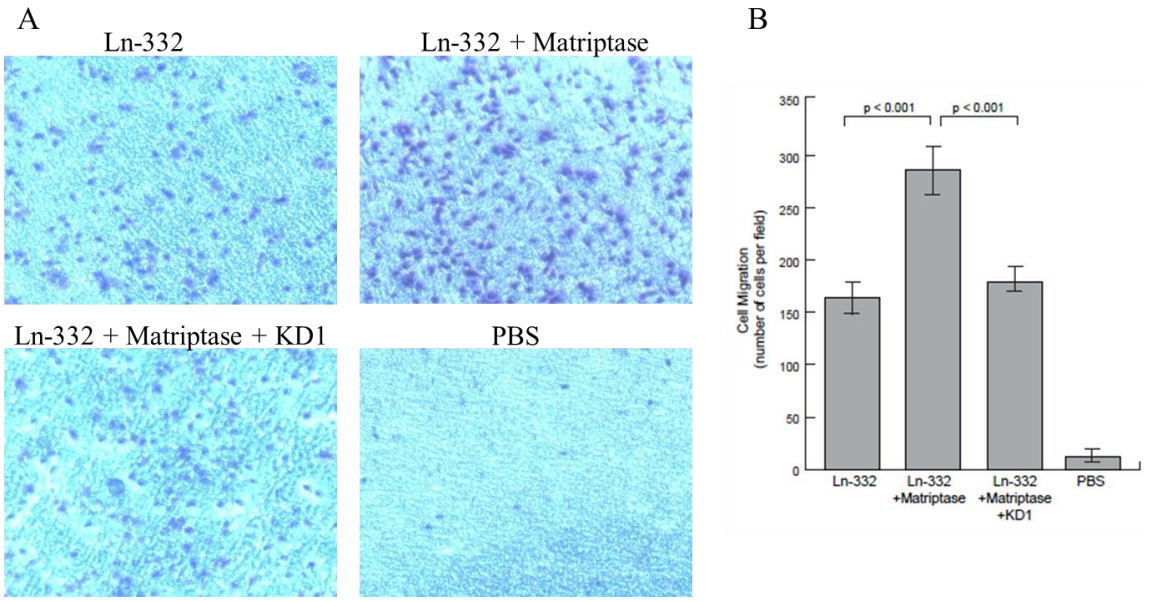


Figure 21: Migration of DU145 cells is enhanced on Ln-332 cleaved by matriptase. (A) DU145 prostate cancer cell migration was measured using Boyden chambers coated with either untreated Ln-332 or matriptase-cleaved Ln-332. Some filters were also coated with either PBS or a mixture containing Ln-332, matriptase, and KD1. The number of cells that passed through filters after 5 h were then manually counted under a microscope. (B) Cells seeded in chambers coated with matriptase-cleaved Ln-332 exhibited significantly more migration (~1.6-fold) than cells on uncleaved Ln-332 (N=2, in duplicate; $P<0.001$). Further, cells in chambers coated with the Ln-332, matriptase, and KD1 inhibitor mixture exhibited significantly less migration than those cells on matriptase-cleaved Ln-332 (N=2, in duplicate; $P<0.001$), similar to levels on untreated Ln-332.

*Cell adhesion of DU145 cells exhibit no significant difference on matriptase-cleaved Ln-332*

To determine if cellular adhesion of DU145 cells was different on matriptase cleaved Ln-332 versus untreated Ln-332 substrate we performed cell adhesion assay as described in Materials and Methods. We coated Ln-332 alone (L), Ln-332 + matriptase (LM), Ln-332 + matriptase + KD1 and PBS onto 96-well tissue culture treated plates. DU145 cells were allowed to adhere for 1 h at 37ºC in serum-free medium. Unbound cells were removed by washing and adherent cells were fixed, stained with crystal violet, solubilized; and absorbance was read at 560 nm. We found that DU145 cells had no significant difference in cell adhesion on matriptase cleaved Ln-332 as compared to untreated Ln-332 or on mixture with Ln-332, matriptase and KD1 inhibitor. Results represent mean ± S.D. (N= 2, in duplicates; $P < 0.05$) (Figure 22).

*Matriptase-overexpression enhances LNCaP cell migration on Laminin-332*

Since our experiments indicated that DU145 cell migration was increased on matriptase-cleaved Ln-332, we decided to also examine migration of LNCaP prostate cancer cells stably overexpressing matriptase, LNCaP-mt (described in materials and methods). Migration of these overexpressor cells was compared to that of wild-type LNCaP cells (LNCaP-wt) first using modified Boyden chamber assays. Prior to performing assays, we verified LNCaP cell expression by RT-PCR and western blot (Figure 23). These results indicated that LNCaP-mt cells expressed ~2 times more matriptase than LNCaP-wt cells. For Boyden chamber assays, inserts were coated with either Ln-332 or PBS, and cells were allowed to migrate for 24 h prior to counting the number of cells that passed

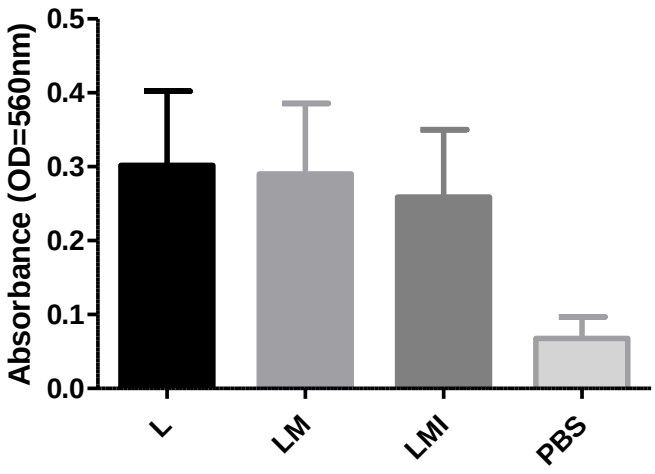


Figure 22: DU145 cells exhibit no change in cell adhesion on matriptase-cleaved Ln-332. Ln-332 alone (L), Ln-332 + matriptase (LM), Ln-332 + matriptase + KD1 (LMI) and PBS were coated onto 96-well plates. DU145 cells were allowed to adhere for 1 h at 37ºC in serum-free medium. Unbound cells were washed off; adherent cells were fixed, stained (crystal violet), and solubilized; and absorbance was read at 560 nm. Results represent mean ± S.D. (N= 2, in duplicates; $P < 0.05$)

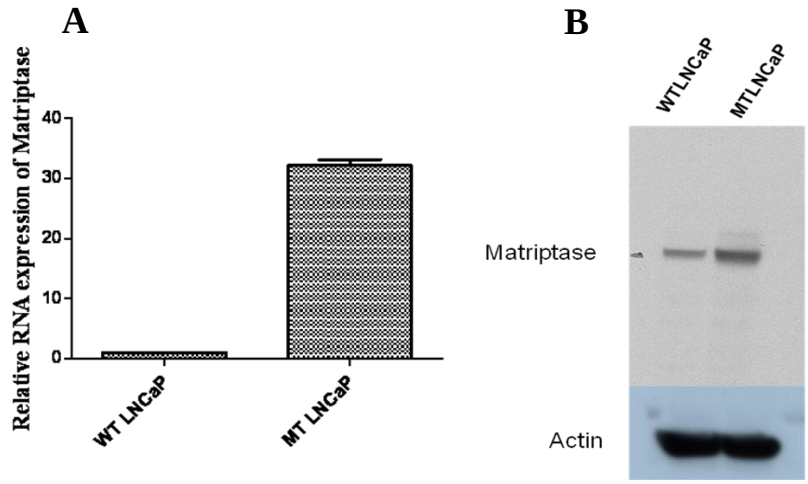


Figure 23: Matriptase expression of both types of LNCaP cells was confirmed by Western blot and RTPCR. Band intensity for each sample was normalized to its corresponding β-actin control. These results indicated that LNCaP-mt cells express ~2 times more matriptase than LNCaP-wt cells.

through filters. As expected, LNCaP-mt cells exhibited significantly more migration than LNCaP-wt cells on Ln-332 (~3-fold; Figure 24A and B). In contrast, both cell types migrated minimally on PBS-treated Transwells.

*Matriptase-overexpression enhances cell speed and directional persistence*

In order to acquire more detailed information about the motility of LNCaP cells, we also performed single-cell motility assays using high-content microscopy in collaboration with Alka Potdar (Department of Chemical Engineering). This technique involves tracking individual cell movement over time using video microscopy (Figure 25). In contrast to static Boyden chamber assays, this approach allows inspection of dynamic cell movement in real time. Further, single-cell level parameters can help to model and predict population level behavior.

In line with previous Boyden chamber results, the LNCaP-mt population moved significantly faster than LNCaP-wt cells using this technique (Figure 26). In addition, LNCaP-mt cells also moved in a more directed manner than LNCaP-wt cells, leading to significantly increased directionality ratios for this cell type (Figure 27A). This ratio represents the *linear* distance a cell travels during an assay (d) versus the *total* distance traveled by that cell (t), which essentially captures cell persistence, or the tendency of a cell to continue moving in a particular direction without turning (Pankov, Endo et al. 2005).

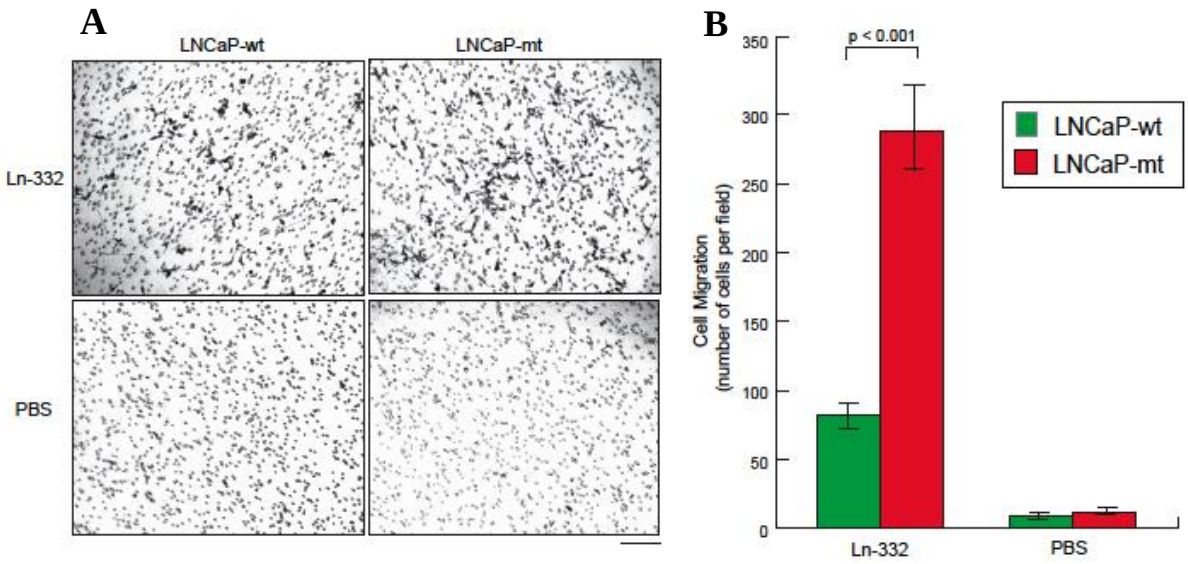


Figure 24: Matriptase-overexpression enhances LNCaP cell migration on Ln-332. (A) Boyden chamber assay was used to examine cell migration of both LNCaP-wt and LNCaP-mt cell types. Transwells were coated with either Ln-332 (10 μg/ml) or PBS, cells were allowed to migrate for 24 h, and cells that migrated across the filter were fixed, stained, and counted manually under a microscope. (B) LNCaP-mt cells exhibited significantly more migration than LNCaP-wt cells on Ln-332 (~3-fold; N=3, in duplicate; P<0.001). In contrast, both cell types migrated minimally on PBS-treated inserts.

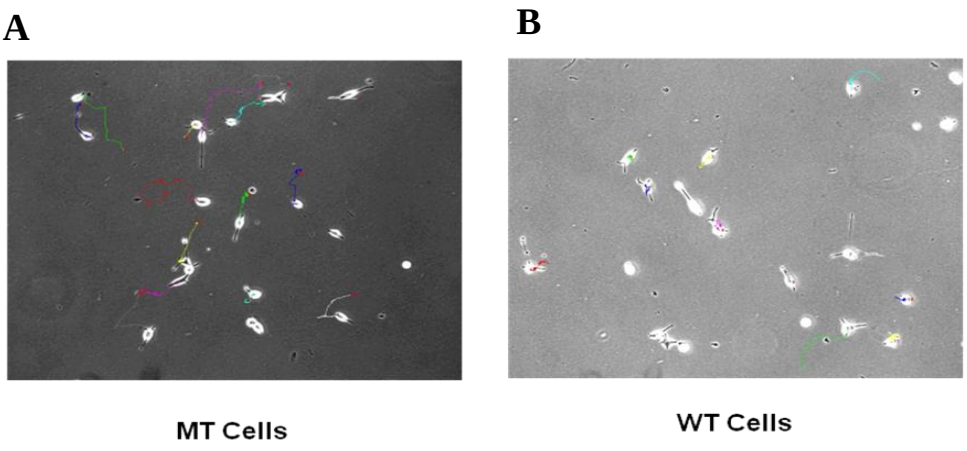


Figure 25: Migration movie tracks of LNCaP-mt and LNCaP-wt cells. Cells plated on dishes coated with Ln-332 were monitored using phase-contrast optics in a Zeiss Axiovert 200 M inverted microscope with camera. Images were both acquired and cells tracked using *Metamorph*. Cell tracks are shown here in different colors for both cell types. LNCaP-mt (A) cells have longer tracks as compared to LNCaP-wt cells (B).

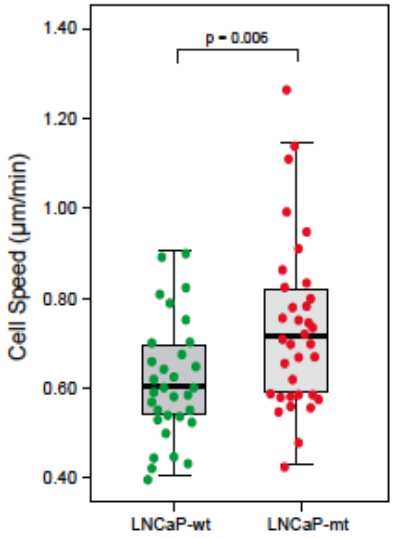


Figure 26: Matriptase-overexpression enhances cell speed. Individual cell speed was measured using Metamorph software. The LNCaP-mt cell population moved significantly faster than LNCaP-wt cells using this technique (N=32 and 35 cells, respectively; P=0.006). Box and whisker plots show the population mean (bold horizontal line), 25$^{th}$ and 75$^{th}$ quartiles (box), and 95% confidence intervals (whiskers) overlaid on raw single-cell data (scatter).

Windrose plots were also created by overlaying single cell tracks onto a single origin (0,0), in order to qualitatively assess the persistence for each cell type (Figure 27 B). Twelve-hour trajectories of LNCaP-wt (green) and LNCaP-mt (red) cells are shown, which indicate that matriptase overexpressing cells are generally more persistent and travel further than wt cells. The concentric circles superimposed on the plots indicate the root mean squared displacement for each cell population after 12 h. This value was obtained by first calculating the mean squared displacement (MSD) for each cell population, using Equation 1, where $\vec{r}(t)$ is the position vector of the cell after time *t*,

$\vec{r}(0)$ is the position at the beginning and $\langle\ \rangle$ denotes the average over the entire cell population, followed by taking the square root of the MSD.

$$MSD = \left\langle (\vec{r}(t) - \vec{r}(0))^2 \right\rangle$$
$$RMSD = \sqrt{MSD} \qquad [1]$$

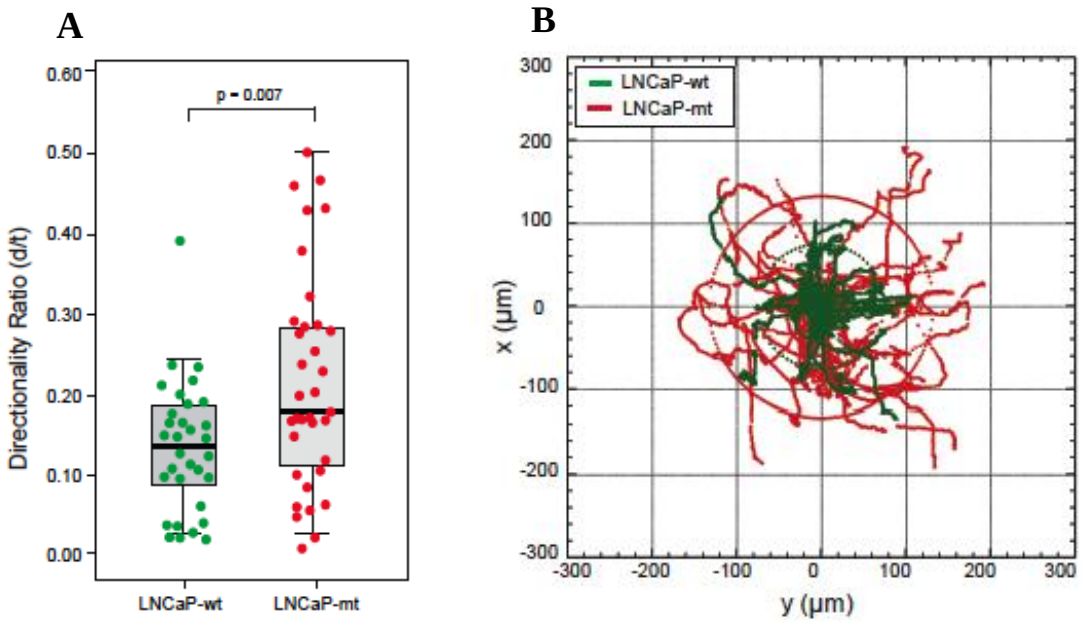


Figure 27: Matriptase-overexpression enhances directionality ratio and directional persistence. (A) LNCaP-mt cells were also found to move in a more directed manner than LNCaP-wt cells, leading to significantly increased directionality ratios for this cell type, which represents the linear distance a cell travels during an assay (d) versus the total distance traveled by that cell (t).
(B) Windrose plots were made to qualitatively examine the persistence for these cell types; these plots overlay all cell tracks (from x and y coordinates) starting with a common origin (0,0). Twelve-hour trajectories of LNCaP-wt (grey) and LNCaP-mt (black) cells are shown, which indicate that, in general, the matriptase overexpressing cells are more persistent and travel further than wt cells. The circles superimposed on the Windrose plots indicate the root mean squared dispersal for each cell type.

These results show that the LNCaP-mt cell population covered a greater area than the LNCaP-wt population. Taken together, all single-cell motility parameters indicate that the matriptase overexpressing cells exhibit a different migration phenotype than wild-type cells.

## Conclusions

In this report, we demonstrate for the first time that matriptase cleaves Ln-332. Using western blot analysis and mass spectrometry, we demonstrated that this cleavage occurs specifically in the β3 chain. We also determined that this event could be inhibited by a matriptase inhibitor, KD1, confirming specificity of the cleavage. In addition, we found that DU145 prostate cancer cell migration was significantly enhanced on matriptase-cleaved Ln-332, which was also reversible by treatment with KD1. Similarly, matriptase-overexpressing LNCaP cells exhibited increased motility on Ln-332, compared to wild-type cells. Interestingly, single-cell motility analysis revealed that these overexpressor cells exhibited both a faster and more persistent phenotype. This study suggests a physiological role for matriptase in proteolytic cleavage of Ln-332 and gives new insight into possible mechanisms for matriptase in cancer progression.

# CHAPTER V

## DISCUSSION

In this study, we found that the processing of Ln-332 by TTSPs, hepsin and matriptase enhances cell motility, and therefore might contribute to invasion processes in prostate cancer progression. The findings of this thesis are relevant to two important steps of the metastatic cascade: 1) breach of the basement membrane and 2) cell migration (Talmadge and Fidler 2010). Specifically, we focused on Ln-332, an essential basement membrane component, and hepsin & matriptase, members of the TTSP family—all of which are important players in prostate cancer progression (Szabo and Bugge 2008; Bugge, Antalis et al. 2009). The role of Ln-332 in human prostate cancer has been of special interest because it is downregulated or lost in human prostate cancer, a clear contrast with other tumor types where Ln-332 is overexpressed (Hao, Jackson et al. 2001; Nagle 2004; Calaluce, Beck et al. 2006).

The TTSPs hepsin & matriptase are overexpressed in some cancers, including human prostate cancer, and their expression has been shown to correlate with disease progression; however the mechanism of action of both TTSPs remains to be elucidated (Stephan, Yousef et al. 2004; List, Bugge et al. 2006; List, Szabo et al. 2006; Uhland 2006; Xuan, Schneider et al. 2006; Bugge, List et al. 2007; List, Kosa et al. 2009). Hepsin, for example, is upregulated in more than 90% of human prostate cancer cases (Stephan, Yousef et al. 2004; Landers, Burger et al. 2005). Matriptase is reportedly overexpressed in a wide variety of epithelial tumors, including breast, cervix, esophagus, liver, mesothelium, prostate and colorectal cancers (Oberst, Johnson et al. 2002; Kang,

Dolled-Filhart et al. 2003; Santin, Cane et al. 2003; Hoang, D'Cunha et al. 2004; Lee, Yong Song et al. 2005; Riddick, Shukla et al. 2005; Tanimoto, Shigemasa et al. 2005; Cheng, Tzao et al. 2006; Saleem, Adhami et al. 2006; Tsai, Chao et al. 2006; Tsai, Chu et al. 2008), and its overexpression correlates with tumor grade in prostate cancer (Riddick, Shukla et al. 2005; Saleem, Adhami et al. 2006; Warren, Twohig et al. 2009).

The mechanism of action of these proteases in tumor progression remains to be determined; however the possibilities include activation of growth factors, receptors, proteases, and the processing of ECM components (Bugge, Antalis et al. 2009). For example, in prostate and colon cancer cells, activation of pro-HGF to HGF by matriptase was shown to be inhibited by using siRNA to inhibit matriptase (Forbs, Thiel et al. 2005). It has been shown that overexpression of matriptase in mouse epidermis induces spontaneous skin lesions in the absence of genetic alteration and independent of carcinogen exposure, effects that can be abolished by coexpression of its inhibitor, HAI-1 (List, Szabo et al. 2005). The equilibrium between matriptase and its inhibitor HAI-1 appears to be the key to the normal functioning of this enzyme. If this equilibrium is altered then activities of matriptase lead to disease conditions like cancer (Oberst, Johnson et al. 2002). The Matriptase: HAI-1 ratio is increased in ovarian, prostate (Saleem, Adhami et al. 2006) and colorectal cancer carcinoma (Vogel, Saebo et al. 2006). These studies have established that matriptase is an important molecule for physiological and pathological pericellular proteolysis and that its activity must be tightly regulated to maintain the epithelial homeostasis. Altered matriptase expression, especially an increase, caused malignant transformation.

Intuitively, the inversely-correlated expression between Ln-332 and hepsin (or matriptase) in prostate cancer led us to hypothesize that an interaction between these proteins may occur during disease progression.

In this study, we identified and then focused on a novel substrate of hepsin and matriptase: Ln-332. The role of laminin-332 in cancer progression is yet to be completely defined; Ln-332 could have different mechanisms of action depending on the type of cancer. Expression of Ln-332 is altered in many cancers: it is increased in colon (Pyke, Romer et al. 1994; Pyke, Salo et al. 1995), cervical (Noel, Fernandez-Aguilar et al. 2005) and oral cancers (Gasparoni, Della Casa et al. 2007); on the other hand it is decreased in lung (Akashi, Ito et al. 2001) and bladder cancers (Sathyanarayana, Maruyama et al. 2004). In the case of prostate cancer expression of Ln-332 is entirely lost (Nagle, Hao et al. 1995; Davis, Cress et al. 2001; Hao, Jackson et al. 2001). We propose the loss of Ln-332 in human prostate cancer could be attributed to processing by the TTSP, hepsin and matriptase.

We have found that Ln-332 is cleaved by the serine proteases hepsin and matriptase, both of which are overexpressed in human prostate cancer (Stephan, Yousef et al. 2004; Riddick, Shukla et al. 2005; Saleem, Adhami et al. 2006; Warren, Twohig et al. 2009). Specifically, the experimental evidence that hepsin and matriptase proteolytically cleave the Ln-332 β3 chain are as follows: 1) treatment of purified Ln-332 with catalytically active hepsin or matriptase produces a ~100 kDa fragment both in a time- and dose-dependent fashion; 2) production of this fragment is abolished by KD1, an inhibitor of hepsin and matriptase; 3) catalytically-inactive hepsin (EGR-hepsin) does not promote cleavage; 4) the cleaved (~100 kDa) fragment reacts by Western blot with

antibodies against the C-terminal region of the β3 chain; and 5) the sequences of peptides from the cleaved ~100 kDa band, determined by mass spectrometry, are identical to peptides from the C-terminal region of rat Ln-332 β3 chain.

The human genome includes at least 500 proteases (Puente, Sanchez et al. 2003). Proteolysis is an important step in both physiological and pathological conditions (Lopez-Otin and Matrisian 2007; Lopez-Otin and Bond 2008; Rowe and Weiss 2008; Lopez-Otin and Hunter 2010). For example, the cleavage of Ln-332 increases cell migration (Hintermann and Quaranta 2004; Marinkovich 2007). The mechanism involves DIII, a Ln-332 domain, that is released by MMPs and binds to EGFR to stimulate mitogen-activated protein kinase (MAPK) signaling, MMP-2 gene expression, and cell migration (Schenk, Hintermann et al. 2003; Schenk and Quaranta 2003). Also, the processing of γ2 chain may influence Ln-332 turnover in basement membrane and affect epithelial morphogenesis (Koshikawa, Schenk et al. 2004). Several proteinase systems are reported to cleave Ln-332 (Schenk and Quaranta 2003; Hintermann and Quaranta 2004), but we propose that the cell surface proteases of the TTSPs family are more involved in specialized physiological and pathological events that require direct interactions with cell surface (Takeuchi, Harris et al. 2000), or direct access to ECM components.

Due to the limited availability of purified human Ln-332, most of the studies in this field (including ours) have been carried out using purified rat Ln-332. However, due to the functional interchangeability of ECM components across mammalian species (Yurchenco, Amenta et al. 2004), we are confident that similar processing of human Ln-332 by matriptase and hepsin occurs. Nonetheless, we sought additional evidence and therefore searched for the hepsin cleavage site on Ln-332, both in rat and human. N-

terminal sequencing of the hepsin-generated Ln-332 β3 fragment identified the hepsin cleavage site at $Arg^{245}$-$Leu^{246}$ of the β3 chain of rat Ln-332. The sequence around this cleavage site, SQLR↓LQGSCFC, is in agreement with the previously identified target sequences for hepsin. Using a tetrapeptide positional scanning-synthetic combinatorial library (PS-SCL) screening approach, Herter et al. identified optimal P4-P1 cleavage motifs for hepsin (P1 represents the residue immediately N-terminal to the cleavage site) on the basis of peptide profiling and amidolytic activity measurements: hepsin exhibits a strong preference for arginine (R) at the P1 position, and moderately favored threonine (T), leucine (L), or asparagine (N) at P2, glutamine (Q) or lysine (K) at P3, and proline (P) or lysine (K) at the P4 position (Herter, Piper et al. 2005). Accordingly, we found that hepsin cleaves Ln-332 after a QLR sequence (P3-P1). It is to be noted that the target sequence for pro-HGF, which turned up at the top of the list of hepsin substrates identified by Herter et al. (Herter, Piper et al. 2005), is KQLR, almost identical to the Ln-332 hepsin target sequence, SQLR. The only difference is at the P4 position (L for pro-HGF and S for Ln-332), which was found to be more promiscuous and less critical than P3-P1 (Herter, Piper et al. 2005). The same author also identified serine as a possible residue in position P4 (Figure 1 in reference (Herter, Piper et al. 2005)).

Visual inspection for homology showed that the hepsin substrate sequence SQLR↓LQGSCFC is completely conserved between rat, mouse and human Ln-332 β3 sequences (NCBI data base rat LAMB3 accession- XM_001069930, mouse LAMB3 accession- NM_008484 and human LAMB3 accession- NM_000228). Sequence conservation strongly supports a functional significance of this hepsin cleavage site.

Nonetheless, cleavage of human Ln-332 could not be verified directly, because purified human Ln-332 is not available at this time, and remains to be formally determined.

Interestingly, the $SQLR^{245}$ is repeated downstream in the rat Ln-332 β3 sequence, $SQLR^{743}$. However, this is an unlikely cleavage site of hepsin for three reasons: 1) we found no evidence of cleavage products matching a corresponding molecular weight; 2) the sequence $SQLR^{743}$ is not conserved in human Ln-332 β3 chain; and 3) residue 743 falls within the predicted coiled-coil region of Ln-332, presumably inaccessible to proteases. Therefore, it is unlikely that $SQLR^{743}$ is cleaved by hepsin. However, once the residue 245 site is cleaved, it is theoretically possible that the coiled coil opens up and exposes $SQLR^{743}$, allowing for a second hepsin-induced cut. Because this double cleavage could only occur in the rat protein ($SQLR^{743}$ is absent in human), we performed a time-course experiment to determine if cleavage occurred at this second site. We did not see additional cleavage product, even at longer incubation times (Figure 1B). In summary, the $SQLR^{245}$ cleavage sequence, directly identified by N-terminal sequencing, appears to be the main cleavage site of Ln-332 by hepsin.

Among the Ln chains expressed in prostate, α1 (Ln-111) is expressed in the fetus and newborn, while α3 (Ln-332) and α5 (Ln-511/Ln-521) are expressed in adults (Nagle 2004). Ln-511/Ln-521 and Ln-211 are present in the normal gland and prostate cancer. In contrast, Ln-332 is present in normal gland and lost in prostate cancer (Nagle 2004). Therefore, it is of great interest for studying prostate cancer progression that Ln-332 is a substrate for hepsin: Ln-332 is the only heterotrimer featuring the β3 chain. It is unlikely that the other laminin beta chains, β1 and β2, are substrates for hepsin since, while homologous to β3, they do not contain sequences resembling the hepsin substrate

sequence (SQLR↓LQGSCFC). However, future studies will need to experimentally confirm whether other ECM macromolecules are substrates for hepsin.

We tried N-terminal sequencing to determine the cleavage site of matriptase on Ln-332 using a similar approach as to the hepsin experiments. However, the same approach failed to determine the exact cleavage site for matriptase on Ln-332, possibly due to the fact that very large proteins typically do not sequence well, sometimes not at all: the random cleavages add up and obscure the true N-terminal amino acid peaks. However, mass spectrometry and western blotting analyses indicated that catalytically active recombinant matriptase also cleaves the β3 chain of Ln-332 and produces a novel ~100 kDa fragment. It appears that matriptase might not be cleaving Ln-332 at the same site as hepsin. The mass spectrometry data for the matriptase cleaved Ln-332 shows a peptide fragment ($^{230}$GSYPPSAYFAVSQLR$^{245}$) that is located before the cleavage site of hepsin ($R^{245}$-$L^{246}$) on Ln-332 β3 chain. Although without N-terminal sequencing and further experimental proof it cannot be stated definitively, based on the mass spectrometry data matriptase might be cleaving before the hepsin cleavage site on Ln-332 β3 chain. If matriptase cleavage event of Ln-332 β3 chain occurs before hepsin cleavage, then it is possible that matriptase cleavage results in exposing the hepsin cleavage site on Ln-332 β3 chain facilitating hepsin processing of Ln-332. It is difficult to speculate which protease might be cleaving Ln-332 before the other, or if both the cleavages occur at the same time, or if only one protease cleaves Ln-332 at one time. Further, it might be possible that one cleavage event facilities the other. Extensive experiment research needs to be done to delineate these proteolytic events.

It is intriguing that the hepsin and matriptase cleavage sites are located around the predicted boundary between domains V and VI of the Ln-332 β3 chain (Aumailley, Bruckner-Tuderman et al. 2005). It is therefore possible that hepsin and matriptase cleavages release domain VI of β3 chain, a domain important for the interaction of Ln-332 with type VII collagen (Chen, Marinkovich et al. 1999; Brittingham, Uitto et al. 2006). If hepsin and matriptase were to disrupt this interaction, at least two events could be triggered: 1) hemidesmosome formation would be downregulated or prevented, because β3 chain domain VI has a key role in their assembly (Jones, Hopkinson et al. 1998; Litjens, de Pereda et al. 2006; Waterman, Sakai et al. 2007). 2) Domain FNC1, the specific region of type VII collagen that physically interacts with Ln-332 β3 chain (Chen, Marinkovich et al. 1999), would be free and could promote tumor invasion (Ortiz-Urda, Garcia et al. 2005). Taken together, hepsin and matriptase cleavage of Ln-332 would be involved in epithelial organization and in tumor development. Interestingly, it has previously been reported that hemidesmosomes are lost in prostate cancer (Nagle, Hao et al. 1995). Determining if the cleavage of Ln-332 by hepsin and matriptase induces a loss of interaction between Ln-332 and type VII collagen will be critical for a deeper understanding of the role of hepsin and matriptase in prostate cancer.

Interestingly, our laboratory and others have shown that MT1-MMP-mediated cleavage of Ln-332 impacts the expression of this ECM protein (Koshikawa, Schenk et al. 2004; Riggins, Mernaugh et al. 2010). Further, the authors even hypothesized that MT1-MMP may regulate Ln-332 turnover. Based on these studies, it is tempting to speculate that TTSPs also regulate Ln-332 expression via cleavage of the protein. To

determine this it would be necessary to first determine the expression level of Ln-332 in hepsin and matriptase knockout and overexpressing mouse models in future studies.

In this study, we have shown that hepsin-induced cleavage of Ln-332 correlates with an enhanced migration of prostate cancer cells plated on Ln-332. Similar results were obtained when matriptase was used. Furthermore, hepsin-overexpressing LNCaP-34 cells also displayed significantly increased migration on Ln-332 compared to low hepsin-expressing LNCaP-17 cells. We also found that LNCaP prostate cancer cells overexpressing matriptase migrated significantly more on Ln-332 than their wild-type counterparts. The basis for this enhancement remains to be elucidated. One possible explanation is that domain VI of Ln-332 β3 chain may support "non-specific" adhesion with the filters used in the migration assays, an artifactual cell-anchoring effect that is then released by hepsin and matriptase. Nonetheless, this enhanced, hepsin/matriptase-induced cell migration fits well with the pro-tumorigenic and pro-invasive effects linked to hepsin and matriptase in animal tumor models (Klezovitch, Chevillet et al. 2004; List, Szabo et al. 2005). These results can also explain why LNCaP-34 tumors grew larger tumors than LNCaP-17 in an orthotopic prostate cancer model, and why only LNCaP-34 tumors showed 100% contra-lateral prostate invasion (Li, Wang et al. 2009). For matriptase, its siRNA-induced inhibition in prostate cancer cells reduced their invasive growth potential *in vitro*, an effect that can be reproduced with a synthetic matriptase inhibitor (Forbs, Thiel et al. 2005). Furthermore, a small molecule matriptase inhibitor reduced tumor growth in prostate cancer xenograft models (Galkin, Mullen et al. 2004). Along the same lines, single-cell motility analysis of matriptase-overexpressing LNCaP cells showed that on Ln-332, they migrated faster and more persistently than the

corresponding control cells. Taken together in the context of our results, these reports constitute potential evidence that cleavage of Ln-332 by hepsin or matriptase may occur *in vivo*.

Virtually all cell migration studies are conducted at the population level (e.g., Boyden chamber), whereby a cell population is represented by an average measurement and some range of error. In this study, we investigated cell motility both at the population level and at the single-cell level using time-lapse video microscopy for slightly different purposes. Boyden chamber assays captured an end-point population level behavior of cells seeded at a high density; in contrast, single cell assays were performed at a lower cell density, data were collected dynamically and evaluated both at the single-cell and ensemble levels.

Recently, it was reported that two different phenotypic outcomes for cell migration may occur *in vitro*, dependent upon whether an investigator uses single-cell or cohesive migration strategies (Giampieri, Manning et al. 2009). Also, it is thought that *in vivo* single-cell migration is required for metastasis through blood, whereas cohesive migration is required for lymphatic metastasis (Ozerlat 2009). In other words, studying migration using these two different approaches investigates two different questions. In our case, change(s) in the migratory phenotype due to cleavage of Ln-332 by matriptase produced a similar result using both approaches. Intuitively, the increased number of LNCaP-mt cells that crossed through Boyden chambers can be explained by the single cell results, which show that matriptase overexpressing cells have increased speed and persistence on Ln-332. Persistence in cell motion can be defined as the property by which a cell continues to migrate in one direction without much deviation (before changing its

path) (Pankov, Endo et al. 2005). Studies have shown that cancer cell migration is directionally persistent, for instance in the case of highly invasive cancers like neuroepithelial tumors (Deisboeck, Demuth et al. 2005) or epithelial cell overexpressing HER2 (Kumar, Zaman et al. 2006). In addition, genetic modifications of biological molecules in a cell line have previously been shown to change the intrinsic pattern of cell migration (Pankov, Endo et al. 2005). To our knowledge, our report is the first study to show that overexpression of a cell surface protease leads to increased cell speed and persistence of cells plated on its substrates.

At the physiological level, hepsin overexpression has been linked to human prostate cancer in multiple studies, (Dhanasekaran, Barrette et al. 2001; Luo, Duggan et al. 2001; Magee, Araki et al. 2001; Ernst, Hergenhahn et al. 2002; Ernst, Hergenhahn et al. 2002; Lai, Wu et al. 2004; Stephan, Yousef et al. 2004; Halvorsen, Oyan et al. 2005), and reported to be as much as 10-times higher (Stephan, Yousef et al. 2004). However, the mechanism(s) by which it affects tumor progression have remained elusive (Vasioukhin 2004). When hepsin was specifically overexpressed in mouse prostate epithelium, it did not cause changes in cell proliferation or differentiation, but rather correlated with areas of disorganized basement membrane and weak or absent staining of Ln-332 (Klezovitch, Chevillet et al. 2004). Moreover, when hepsin was introduced in a mouse model of prostate cancer, the LPB-Tag 12T-7f mouse, the tumor progression was accelerated and the bigenic mice developed metastases to the bone making it the only mouse model to develop bone metastasis. This feature is of relevance to the field of prostate cancer since the predominant cause of mortality and morbidity in human prostate cancer patients is due to bone metastases. However, one caveat of this model is that it

develops a mixture of adenocarcinoma and neuroendocrine tumors at the primary site and the bone metastases are neuroendocrine in nature. In contrast, human prostate tumors are predominantly adenocarcinomas at the primary site and metastases; metastases that are neuroendocrine in nature are very rare in human prostate cancer (Grignon 2004). Another existing mouse model for prostate cancer is the c-myc model that develops adenocarcinoma by 6 months but fails to develop metastases (Ellwood-Yen, Graeber et al. 2003). In addition, the myc mice have very low endogenous levels of hepsin. In order to test if the over-expression of hepsin in the myc model would result in further progression, Dr. Matusik's laboratory at Vanderbilt University crossed the c-myc mice with the hepsin transgenic mice. Interestingly, the hepsin/myc transgenic mice showed progression of tumorigenesis at the primary site including development of higher grade tumors as compared to the myc alone mice, however the bigenic mice did not show metastasis (Nandana, Ellwood-Yen et al. 2010). These results indicate that hepsin overexpression, although incapable of initiating tumorigenesis in the prostate, is sufficient to drive an already existing tumor. These results also indicate that hepsin expression is insufficient to develop adenocarcinoma metastases. One possible explanation of why hepsin expression caused neuroendocrine metastases in the 12T-7f model is due to the extreme aggressive nature of neuroendocrine tumors. Since a mouse model of human prostate cancer would seek to incorporate as many of its features, the development of a mouse model of human prostate cancer that replicates all its features including the development of adenocarcinoma metastases to the bone is still an open issue in the field. In collaboration with Dr. Matusik's group, we studied whether the increase in tumorigenesis in the hepsin/myc transgenic mice is correlated with Ln-332

degradation. Although Klezovitch et al have shown that hepsin overexpression *in vivo* resulted in decreased staining of Ln-332, the authors did not provide direct evidence of cleavage of Ln-332 by hepsin (Klezovitch, Chevillet et al. 2004). Therefore we investigated by Western blot analysis the tumor tissues from 12-month old wildtype, myc and hepsin/myc mice. The tissue lysate of wild type mice showed intact Ln-332 β3 chain (145 kDa), the myc tumor displayed a 100 kDa band, and in the case of the hepsin/myc tumor this 100 kDa band was further diminished due to degraded Ln-332. These *in vivo* data provides biological significance to our *in vitro* results of cleavage by hepsin.

Further studies need to be performed to determine the effect of this cleavage of Ln-332 on the formation of prostate tumors *in vivo.* A follow-up experiment to our study would be to inject genetically-modified human prostate cancer cells with different hepsin or matriptase levels into mouse prostates and evaluate tumor progression and lesion formation of these cells *in vivo*. For example, it has been reported that PC3 cells, a human prostate cancer cell line, grow into primary tumors and metastasizes to lymph nodes when orthotopically injected into the mouse prostate (Bastide, Bagnis et al. 2002). Therefore, we would expect that hepsin overexpressing PC3 cells will form larger, more aggressive tumors, along with enhanced or accelerated metastases. This effect would be accompanied with reduced Ln-332 staining, more proliferation, and reduced apoptosis in these tumors. On the contrary, we would anticipate that matriptase knock-down PC3 cells will feature increased Ln-332 staining, less aggressive, smaller tumors, and reduced number or increased latency of metastases formation. Another area of interest that this study could lead to is to the elucidation of role of hepsin, and specifically the cleavage of Ln-332 by hepsin, on the formation of prostate cancer bone lesions *in vivo*. Bone is the

most common site of metastases in human prostate cancer, and is a significant cause of morbidity and mortality in patients (Mundy 2002; Higano 2008). Prostate cancer cells that metastasize from the primary tumor typically form osteoblastic lesions in the bone; however, the mechanisms determining the osteoblastic response is largely unknown (Bonfil, Dong et al. 2007). Recently, the expression of hepsin was found higher in bone metastases versus liver and lymph node metastases in human prostate cancer metastases (Morrissey, True et al. 2008), arguing that the bone microenvironment may influence hepsin expression in tumor cells. Human prostate tumors have two marked features i) hepsin is overexpressed (Stephan, Yousef et al. 2004) and ii) the majority of the bone metastases in human prostate cancer are osteoblastic in nature (Bonfil, Dong et al. 2007). Therefore it might be hypothesized that hepsin expression leads to the osteoblastic nature of the prostate cancer bone metastases. Interestingly, another correlation along this direction is that PC3 cells that do not endogenously express hepsin form osteolytic lesions when directly placed in bone environment (Armstrong, Miller et al. 2008). Therefore, to further address the role played by hepsin in the bone physiology, an elegant experiment would be to place hepsin overexpressing PC3 cells directly in contact with bone and evaluate the nature of lesions formed. One possible action of hepsin in bone metastases is to cleave Ln-332, as Ln-332 is expressed in the bone (Klees, Salasznyk et al. 2005). This could be tested by grafting PC3 cells stably overexpressing hepsin into the bone, using the mouse intra-tibial inoculation model (Yamamoto, Bonfil et al. 2007). We anticipate that hepsin overexpressing PC3 cells will form osteoblastic lesions in the bone. Another possibility is to use the intra-cardiac inoculation model (Schneider, Kalikin et al. 2005), a model in which cells travel through the blood stream, reach the bone and form

lesions. One can also use the LNCaP cells. Although intra-tibial inoculation of LNCaP cells have been reported to have failed to produce lesions in bone, it will be interesting to examine if hepsin over-expression in LNCaP cells promotes lesions. Also, in case the bone lesions do not show Ln-332 expression, one could co-inject purified Ln-332 with the cells to determine the effect of hepsin over-expression on Ln-332.

In our *in vitro* experiments, Ln-332 cleavage by hepsin and matriptase was detectable at an enzyme: substrate ratio of 1:1.5 and 1:0.3 respectively, although maximal yield of cleavage occurred at the enzyme:substrate ratio of 1:0.15 and 1:0.03, respectively. The need for this high ratio can be explained by the multi-chain nature and high molecular size of Ln-332 (490 kDa), which may hinder accessibility of cleavage sites *in vitro*. Accordingly, other studies focusing on MMP-induced cleavage of laminins used high enzyme: substrate ratios *in vitro* (Giannelli, Falk-Marzillier et al. 1997; Pirila, Sharabi et al. 2003; Veitch, Nokelainen et al. 2003; Remy, Trespeuch et al. 2006; Wang, Sun et al. 2006). In addition to substrate availability, proteolysis also depends on additional factors like temperature, pH, cations, and the relative topology of enzyme and substrate. In this respect, it is worth stressing the transmembrane location of the serine proteinase, since this topology places it in close vicinity to the BM (Somoza, Ho et al. 2003). According to the structure of hepsin, its catalytic domain is extracellular and should lie flat on the plasma membrane (Somoza, Ho et al. 2003), i.e. in an ideal position to access BM substrate such as Ln-332.

Under normal physiological conditions, proteolysis is tightly regulated. However in the case of disease, there can be increased or decreased proteolytic activity. Blocking abnormal proteolytic activity has been a major area of interest for the pharmaceutical

industry. For example, warfarin, a heparin and vitamin K analogue, has been used to treat thrombosis for the last 50 years (Gustafsson, Bylund et al. 2004). More recently, angiotensin-converting enzyme (ACE) inhibitors have been introduced to treat heart attack, heart failure and hypertension (Acharya, Sturrock et al. 2003). However, clinical trials involving small molecule inhibitors targeting MMPs failed, even if the overexpression of MMPs in cancerous tissues had led to the notion that proteases help tumor progression (Coussens, Fingleton et al. 2002). Small molecule MMP inhibitors had disappointing results probably because of their broad spectrum and the lack of detailed knowledge of their specific substrates: the best possible way to delineate the role of any proteinase in normal and disease condition is to identify the physiological substrates of the proteinase.

Overall, our findings raise the possibility that one mechanism whereby hepsin/ matriptase contribute to human prostate cancer progression is via its proteolytic activity on Ln-332 which results in increased migration. Identifying other physiological substrates of these proteases could help in unraveling their activity in diseases, such as cancer. Our study raises the possibility that other substrates of hepsin/matriptase, such as blood coagulation factors (Kazama, Hamamoto et al. 1995), pro-hepatocyte growth factor, and pro-urokinase-type plasminogen activator (Kirchhofer, Peek et al. 2005; Moran, Li et al. 2006), macrophage-stimulating protein1 (MSP-1), prostasin zymogen, Trask (transmembrane and associated with src kinases) and protease-activated receptor-2 (Lee, Dickson et al. 2000; Takeuchi, Harris et al. 2000; Bhatt, Erdjument-Bromage et al. 2005; Netzel-Arnett, Currie et al. 2006; Bhatt, Welm et al. 2007; Seitz, Hess et al. 2007; Darragh, Bhatt et al. 2008) could be involved in the hepsin/matriptase-mediated prostate

cancer progression. Therefore, further investigating the role of the BM in prostate cancer, and possibly finding other substrates of hepsin and matriptase will help us better understand the mechanism of action of these two proteases. Ultimately, this better knowledge of prostate cancer progression may stimulate designing novel therapies to prevent prostate cancer invasion and/or metastasis.

With our experimental evidence from this study and insights from the literature, we find that hepsin and matriptase cleavage of Ln-332 might be a critical step in prostate cancer progression. Therefore in accordance with the working hypothesis of our study, we believe that blocking the hepsin and/or matriptase cleavage site on Ln-322 would affect the progression of cancer cells in a tumor microenvironment. To provide experimental evidence to prove that Ln-332 cleavage is critical for prostate cancer progression, a possible experiment would be to generate a cell line expressing uncleavable Ln-332 by mutating the residues of the cleavage site. To do so, we can start with a cancer cell line that does not express Ln-332 and transfect it with Ln-332 α3, mutated β3 and γ2 chains. The mutated β3 chain construct would have its cleavage site (in case of hepsin, SQLR↓LQGSCFC) blocked using procedures like site directed mutagenesis. This cell line with uncleavable β3 chain could then be over expressed with hepsin or matriptase. These cells could then be evaluated for their tumorigenicity in a tumor microenvironment utilizing a mouse xenograft model such as the kidney capsule or the orthotopic model. Similarly, targeted prostate transgenic mice could be created with uncleavable Ln-332. These mice could be crossed with the hepsin or matriptase transgenic mice. These experiments would tease apart the biological relevance of the

proteolytic processing of Ln-332 by hepsin and matriptase in the progression in a tumor microenvironment.

In summary, from the work in this dissertation, we draw the following conclusions:

1) Hepsin and matriptase cleave Ln-332 *in vitro;*
2) The cleavage site of Ln-332 is located on the N-terminus of the domain VI of β3 chain;
3) This processing of Ln-332 by hepsin and matriptase is inhibited by an inhibitor of these proteinases;
4) Migration of DU145 and LNCap prostate cancer cells is increased on hepsin- and matriptase-cleaved Ln-332 *in vitro*;
5) Matriptase overexpression causes increased persistence of LNCap cell migration on Ln-332;
6) Cell adhesion of prostate cancer cells is not affected by hepsin or matriptase cleavage of Ln-332;
7) Hepsin overexpression induces Ln-332 degradation *in vivo***.**

To put our findings in perspective, our working model is the following: under physiological conditions, full-length Ln-332 interacts with α3β1 integrin and induces cell migration, whereas TTSP-cleaved Ln-332 interacts with α6β4 integrin and promotes static adhesion (hemidesmosomes). However, these TTSPs are overexpressed in prostate cancer, leading to an unregulated processing of Ln-332 by these TTSPs, which might

result in a) degradation of basement membrane and help the cancer cells in escaping the primary site and b) the disruption of focal adhesion complexes and increased cancer cell migration. In turn, more motile cells might become more invasive, resulting in more metastases (Figure 28).

In summary, we have identified a new substrate for matriptase and hepsin, the processing of which has been directly linked to increased cell migration *in vitro* and correlated with increased tumorigenesis *in vivo*. Our findings contribute to unraveling the roles of matriptase and hepsin in prostate cancer. By understanding the mechanisms of action of these two proteinases, we can better target their inhibition, towards developing novel therapeutic strategies in the cure of human prostate cancer. In a broader prospective, our study should promote additional studies aimed at molecular mechanisms of interaction between epithelial cells and their immediate microenvironment, the basement membrane.

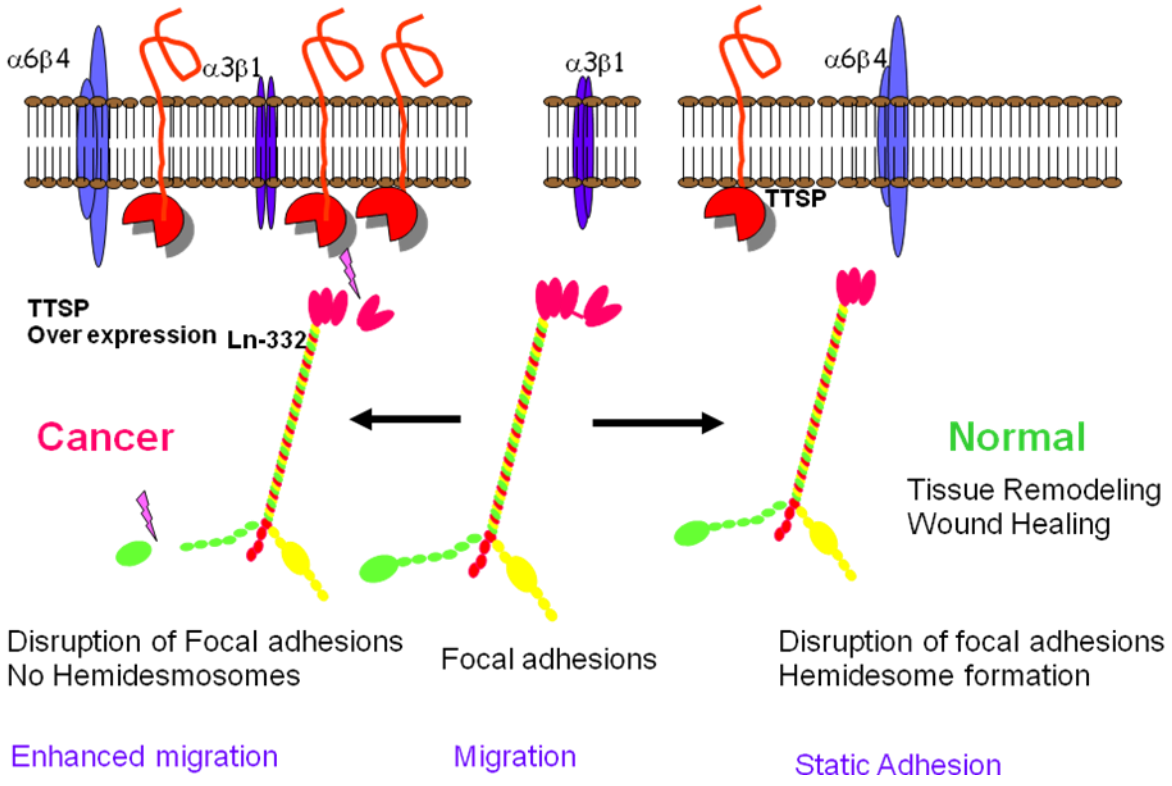


Figure 28: Hypothetical Model for Ln-332/TTSP interaction in cancer. Based on our data from this study we hypothesize that under normal conditions, full-length Ln-332 interacts with α3β1 integrin and induces cell migration, whereas TTSP-cleaved Ln-332 interacts with α6β4 integrin and promotes static adhesion (hemidesmosomes). However, these TTSPs are overexpressed in prostate cancer, leading to an unregulated processing of Ln-332 by these TTSPs, which might result in i) degradation of basement membrane and help the cancer cells in escaping the primary site and ii) the disruption of focal adhesion complexes and increased cancer cell migration. In turn, more motile cells might become more invasive, resulting in more metastases.

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