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Interplay of Perlecan and MMP-7/Matrilysin Regulates Metastatic Prostate Cancer Cell Behavior: Basic and Clinical Implications

by

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ABSTRACT

Interplay of Perlecan and MMP-7/Matrilysin Regulates Metastatic Prostate Cancer Cell Behavior: Basic and Clinical Implications

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Perlecan/HSPG2 is a large extracellular heparan sulfate proteoglycan concentrated at tissue borders and separating epithelium and stroma. Along with its proteolytic consumers, the matrix metalloproteinases (MMPs), perlecan helps orchestrate development and homeostasis in nearly all studied multicellular organisms. However, both molecule classes can be coopted by prostate cancer (PCa) to advance the disease to its most deadly metastatic form. This work aimed to understand that relationship both at the basic and clinical level. Perlecan with its HS chains and tight domain structure is generally resistant to proteolysis, but a PCa cell must produce an associated enzyme to cleave the border proteoglycan in order to metastasize. This work was the first to identify an active protease produced by PCa cells that can completely digest intact perlecan. Following in silico proteolytic analysis, matrilysin/MMP-7, was identified as a likely candidate for in vitro assays. MMP-7, unlike other enzymes tested, cleaved perlecan when presented in multiple contexts. Perlecan and a subdomain, domain IV-3 (Dm IV-3), but not other subdomains (Dm I, IV-1 and IV-2), induced a striking clustering phenotype. MMP-7 incubation completely reversed this effect to favor cell dispersion and adhesion. Proteomic signaling arrays point towards global Src kinase activation as a major influence of perlecan DmIV-3 effects. To determine if this perlecan/MMP-7 relationship exists in PCa subjects, I performed a tissue microarray, along with β2-microglobulin (β2M), a GF that binds perlecan and induces MMP secretion. Besides increased levels of the two proteins within the patients (cancer/normal), MMP-7
and perlecan levels statistically correlated in multiple grades and localized at tissue interfaces. Additionally, I developed a new assay probing the perlecan fragment signature in the same PCa subjects’ serum. Perlecan fragments were largely increased in PCa and some of the fragments were associated with MMP-7 expression in the subjects. Overall, this work demonstrates a unique interplay between perlecan and its efficient proteolyzer, MMP-7, a relationship that is relevant from the cell and tissue to the clinic and which is likely to contribute to PCa progression to metastatic lethal disease.
I dedicate this work to my lovely wife, Deborah. She always supported me with her love and kindness through this long process.
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<tbody>
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<td>ANCOVA</td>
<td>analysis of co-variance</td>
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<td>AR</td>
<td>androgen receptor</td>
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<td>β2M</td>
<td>β2-microglobulin</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BM</td>
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<td>ELISA</td>
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<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>kDa</td>
<td>kiloDalton</td>
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<td>LC-MS</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LG3</td>
<td>laminin globular domain 3</td>
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<td>lymph node</td>
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<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
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<td>MCSF</td>
<td>macrophage/monocyte colony stimulating factor</td>
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<tr>
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<td>MMP</td>
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<td>membrane type 1 matrix metalloproteinase</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NE</td>
<td>neuroendocrine</td>
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<td>neuroendocrine differentiation</td>
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<td>NFκB</td>
<td>nuclear factor κB</td>
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<td>NG</td>
<td>normal gland</td>
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<tr>
<td>NSE</td>
<td>neuron specific enolase</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PBST</td>
<td>phosphate buffered saline, tween-20</td>
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<td>PDGF</td>
<td>platelet derived growth factor</td>
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<td>PIN</td>
<td>prostatic intraepithelial neoplasia</td>
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<td>PSA</td>
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<td>receptor activator of nuclear factor κB ligand</td>
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<td>reactive oxygen species</td>
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<td>room temperature</td>
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<tr>
<td>SEA</td>
<td>sperm enterokinase agrin</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SFK</td>
<td>src family kinase</td>
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<tr>
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<td>sonic hedgehog</td>
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<td>SJS</td>
<td>Schwartz-Jampel Syndrome</td>
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<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
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<td>TACE</td>
<td>tumor necrosis factor α converting enzyme</td>
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<td>TBST</td>
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<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
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<td>transcription factor</td>
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<td>TGF-β</td>
<td>transforming growth factor β</td>
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<td>trifluoroacetic acid</td>
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<td>TMA</td>
<td>tissue microarray</td>
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<tr>
<td>TMPRSS</td>
<td>transmembrane protease, serine</td>
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<td>TNF-α</td>
<td>tumor necrosis factor α</td>
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<td>tumor node metastasis</td>
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<td>ToF</td>
<td>time of flight</td>
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<td>TZ</td>
<td>transitional zone</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VP</td>
<td>ventral prostate</td>
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<tr>
<td>Wnt</td>
<td>wingless</td>
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Chapter 1: General Introduction

The prostate

This dissertation focuses on the mechanisms of prostate cancer (PCa) and metastasis. Therefore, understanding normal physiology and development of the prostate is necessary. Much of the prostate development patterns and normal functions are co-opted in the cancerous state.

Anatomy and physiological function

The prostate is a small exocrine gland (20 gram (g) walnut sized in a human adult) at the bladder neck and wrapped around the urethra. Among the vertebrates only male mammals have a prostate gland (Marker et al. 2003). The prostate functions as a male sex accessory organ, aiding in the fitness of spermatozoa, ultimately increasing fertility (Thomson & Marker 2006). The seminal vesicles and the prostate secretes a variety of enzymes, ions and other proteins that make up a bulk of the seminal fluid or ejaculate to help in gelation, liquefaction, and coagulation of the semen (Aumüller & Seitz 1990; Hayward & Cunha 2000). In humans, the seminal fluid is largely composed of fructose, zinc, salts, spermine (a polyamine), prostaglandins, and proteases such as prostate specific antigen (PSA), but components vary among mammals. For mammals in general, the prostatic seminal vesicle secretions are thought to regulate pH, provide energy for the sperm, and act as an antibacterial agent (Thomson & Marker 2006). However, species specific functions have been observed. For example, transglutaminase activity promotes coagulation to produce a copulation plug to disrupt superfecundity in rats and other rodents (Williams-Ashman 1984).
Preliminary descriptions of the prostate date back to the late 1600s by Gerard Blasius as a glandulae, but more detailed descriptions began in the early 20th century using wax models of the developing prostate (Timms 2008). Divisions of the human prostate into zones was controversial, going through several iterations before being established in the 1980s by McNeal (McNeal 1984; Selman 2011; Timms 2008). The adult human prostate is composed of three primary zones (figure 1.1A): the central zone (CZ), the peripheral zone (PZ), and the transitional zone (TZ). The CZ surrounds the ejaculatory ducts, the proximal urethra goes through the TZ, and the PZ surrounds most of the CZ and the distal urethra. They each contain acini that ultimately secrete their fluids through the prostatic duct openings into the urethra and ejaculatory ducts. The most anterior portion of the prostate is largely fibromuscular stroma (FMS). The prostate is highly innervated on the periphery by the prostatic nerves derived from the left pelvic plexus. Despite being highly referenced the “McNeal” prostate is still debated, especially regarding the ambiguous TZ (Selman 2011). The ambiguity is owed to the compact structure of the human prostate gland, which is similar to other mammals such as dogs. However, rodents and insectivores have highly branched, multilobed prostates (Thomson & Marker 2006). Rodents such as mice and rats have three distinct lobes (figure 1.1B): the anterior prostate (AP), the dorsolateral prostate (DLP), and the ventral prostate (VP) (Sugimura 1986). They all surround the central urethra and function analogous to the human prostate. Histology and specific staining of the prostate gland identifies key cell types of the prostate (figure 1.1E). The luminal epithelial cells facing into the ductal lumen are responsible for the secretions, basal supporting cells surround the luminal epithelial cells. Immediately outside the acini are
neuroendocrine cells and rare stem cells. The stroma is composed of smooth muscle cells and other fibroblasts, which is much denser in humans (figure 1.1C, D) (Marker et al. 2003).

Prostate Development

Embryonic and fetal development of the prostate is similar between rats and humans, but variations exist that result in the differing structure described above. Most of the experiments delineating the signals involved in prostate development are from mice and rat models (Timms 2008). The prostate is derived from the urogenital sinus (UGS), an endodermally derived structure. The Wolffian duct is responsible for generating the seminal vesicles, and in females this structure ultimately degrades (Dyche 1979). The UGS forms around 7 weeks of gestation in humans and 13 days post fertilization in mice in both males and females (Marker et al. 2003). The structure only differentiates into prostatic structures at day 17.5 post fertilization in mice and 12 weeks in humans due to the fetal testes production of androgens (Marker et al. 2003; He et al. 1991; Brown et al. 1988). Lack of androgens results in part of the vagina. If androgens and growth factors (GFs) are present, epithelial cells of the UGS begin to bud and invade into the UGS mesenchyme (UGM) which utilizes epithelial to mesenchymal transition (EMT) at the leading edges (Sugimura 1986). Following budding, the epithelia begins to branch off as solid chords (without a lumen), further invading the UGM. Invasion completely occurs within the UGM in humans, but in rodents, it extends into loose connective tissue. The UGM differentiates into FMS, completely encasing the newly developed glands in humans. While the process of developing a prostate ends before childbirth in humans, rodents continue to develop the
prostate after birth. Although most of the rapid enlargement of the prostate occurs during puberty, it continues to grow slowly throughout adulthood.

Initial budding is highly dependent on the androgen receptor (AR) and the ligand testosterone and its more potent derivative, dihydrotestosterone (DHT) (made by conversion with the enzyme 5-α reductase type 2) (Wilson & Gloyna 1970; Prins & Putz 2008). Early tissue recombination studies demonstrated that the AR in the mesenchyme, but not epithelium, is actually required for the budding (Cunha & Lung 1979). Therefore, paracrine signals from the mesenchymal stroma directed by AR activity are responsible for prostatic budding and branching. In fact, after initial budding, removing androgens does not halt budding and outgrowth, but merely slows it (Donjacour & Cunha 1988; Marker et al. 2003). Meanwhile, high doses of estrogens negatively affect prostate growth and branching in the rat model. However, low doses of estrogens and estrogenic compounds actually lead to an increased number of ducts and overall prostate size which may sensitive the individual to developing a neoplasia in the future (Judy et al. 1999). This is known as developmental estrogenization, or hormonal imprinting. Many transcription factors (TFs), cytokines, and GFs have been implicated in the branching and continued growth of the prostate, with others likely to be found. Positive and negative regulators that are spatially and temporally expressed determine the branching pattern and growth of the prostate (Prins & Putz 2008). Each one negatively or positively affects the other as well to produce fine-tuned branching patterns and areas of growth and growth inhibition once the process is complete. The major signaling pathways and TFs implicated are: fibroblast GFs (FGF-7, 10), sonic hedgehog (SHH) and Wnts, transforming GF β (TGF-β 1, bone morphogenetic
protein (BMP-7), activins), Notch1 axis, and TFs including homeobox (Hoxa,d,b13), NK homeobox (Nkx3.1), and forkhead box (FoxA1, A2). These pathways and their complex interplay to produce the final rat prostate are reviewed in detail elsewhere (Prins & Putz 2008; Thomson & Marker 2006; Marker et al. 2003).

**Prostate cancer overview**

**Cancer incidence, morbidity, and detection**

PCa originating in the epithelial glands is an adenocarcinoma and is the most common form of the disease. Given the male exclusive origin of the prostate gland, PCa only affects men. In 2014, the American Cancer Society estimates 233,000 new cases and over 29,000 deaths in the United States. In the U.S., the chance of being diagnosed with PCa is 1 in 7, and is the second leading cause of cancer related deaths in men (American Cancer Society 2014). In the African American population, however, men suffer almost double the incidence rate of whites and die from PCa at over twice the rate of whites as well (American Cancer Society 2014). However, many men die with primary PCa and not from it, as revealed after autopsy (Bubendorf et al. 2000; Sakr et al. 1994). It becomes deadly once the cancer escapes and metastasizes. Metastasis to a distant region reduces the 5 year survival rate of prostate cancer from nearly 100% to 28% (American Cancer Society 2014).

In the clinic, PCa largely is detected through two procedures, the digital rectal exam (DRE) and measuring PSA levels in serum (Schroder et al. 1998; Thompson et al. 2004). In men over 40, both the DRE are performed and PSA levels measured as a first level diagnostics to establish prostate cancer risk. In the DRE, doctors massage the prostate via the rectum to feel for unusual size or hardness of the prostate. DRE is less sensitive than the PSA test. PSA is generally thought to be in normal range with less than 4.0 nanograms (ng)/milliliter
(mL) and greater than this increases risk of having prostate cancer. However, some studies have shown PCa exists with PSA levels lower than this (Thompson et al. 2004). DRE also often fails to detect prostate cancers with <4.0 ng/mL PSA (Schroder et al. 1998). Despite these failures, these tests remain to be the first screen for PCa. Approximately only 10% of patients who are first diagnosed with PCa have observable metastasis (Coleman 2006). Issues related to oversensitivity, whether to “watch and wait”, lack of risk stratification and the resulting overtreatment are still present with PSA and DRE, however (Vickers et al. 2012). Future tests could complement the PSA measurement for better clinical outcomes.

Noncancerous lesions, Gleason grade, stages and metastatic sites
PCa is a slow growing disease and several noncancerous conditions may be present before and during PCa development. Benign prostatic hyperplasia (BPH) is a noncancerous condition of prostate cell overgrowth. It occurs primarily in the TZ (in contrast to the PZ in PCa) and results in growth in both the stromal and glandular cells. Evidence links chronic inflammation (such as from infection) to BPH, but it is not a precursor or associated directly with prostate cancer (De Nunzio et al. 2011). Unlike BPH there are several conditions that are considered precursors of PCa. Prostatic intraepithelial neoplasia (PIN) (or intraductal dysplasia) is commonly seen with PCa and is described as atypical proliferations of the glandular epithelium, but remain non-invasive. Prostatic inflammatory atrophy (PIA), a focal glandular atrophy (reduction in size, partially in cytoplasm volume) in combination with inflammatory cell influx, also is considered a precancerous lesion. Many times, a continuum of PIA and PIN next to adenocarcinoma are found in patients, which point to PIA and PIN as potential precursors of PCa (Merrimen et al. 2013).
Prostatic adenocarcinoma is divided into 5 grades based on the Gleason grade index (figure 1.2) (Gleason & Mellinger 1974; Gleason 1992). The Gleason grade is essentially a description of how well differentiated the PCa glands are and how much tumor has infiltrated into the prostatic stroma. The less differentiated and more invasive the particular cancer, the higher the grade is. The two most common grades observed by the pathologist are combined to give the sum (e.g. grade 3+4 =7 and 4+4=8). Gleason sum correlates positively with lower survival and even patients presenting a higher primary Gleason score with the same Gleason sum (i.e. 4+3 versus 3+4) will have a lower survival index (Lau et al., 2001).

Prostate cancer is staged clinically through the guidelines outlined in the American Joint Committee on Cancer and can be found in the updated manuals, currently in the 7th edition. Clinicians follow the Tumor, Node, and Metastasis (TNM) staging for prostate cancer (Egner 2010). Tumor type is divided into four groups (1-4) with some having subgroups. Generally, as the cancer becomes larger, bilobal, or appears in other structures (e.g. T4 expands beyond the prostatic capsule to the bladder neck), the higher the T number. N refers to presence of cancer in the regional pelvic lymph nodes. M refers to metastasis and is subdivided into M0 (no distant metastasis), M1a (non-regional lymph nodes), M1b (bone), M1c (other sites with or without bone disease). The TNM classifications are further grouped together with Gleason grades to produce overall stage grouping. The larger and more invasive the tumor, the higher the overall stage grouping. Stage IV indicates the presence of metastasis and is the most deadly form of the disease (Dunsmuir et al. 2001).
In a study analyzing men over 40 years old who had died from PCa revealed the most common site to be bone (90% of patients), followed by lung (46%), liver (25%), lung and pleura (21%), and adrenals (13%) (Bubendorf et al. 2000). The spine was heavily favored by PCa metastasis and did not depend upon previous lung metastasis. This pattern suggested a backward venous spread (via the Batson’s plexus) to the lower spine rather than through cava dissemination. This hypothesis was first established over 60 years ago (Batson 1940). However the common presence of bone metastases at more distal sites (skull and long bones) shows that PCa can travel via the venous and lymphatic system as well (Bubendorf et al. 2000; Ye et al. 2007)

**Mechanisms of prostate cancer**

*Early carcinogenesis mechanisms*

Similar to other cancers, PCa is initiated by a series of somatic genome alterations that activate oncogenes and deactivate tumor suppressor genes. Initial mutations may induce a feed forward mechanism resulting in additional mutations and epigenetic dysregulation to eventually produce a cancerous lesion (Shand & Gelmann 2006). A major factor in oncogenesis is inflammation. Prostate inflammation can be instigated by infectious agents, physical trauma from concretions, heterocyclic amines from diet, estrogens, and urine reflux (Platz & De Marzo 2004). One mechanism by which inflammation may ultimately lead to prostate cancer is when immune cells release highly reactive compounds (superoxide, hydrogen peroxide, singlet oxygen and nitric oxide). These compounds lead to epithelial cell death resulting in renewal where errors in DNA replication may occur (De Marzo et al. 2007). Many dysregulated genes have been implicated in carcinogenesis and can be divided into groups: tumor-suppressor genes, oncogenes (tumor promoting genes),
housekeeping or caretaker genes, and epigenetic control genes. Tumor suppressor genes such as PTEN and TP53 and CDKN1B encode proteins designed to negatively regulate cell division to ensure cell and DNA integrity. Lower expression or inactivating mutations lead to carcinogenesis (Palapattu et al. 2005). Oncogenes such as MYC (a transcription factor) are erroneously activated, causing several pro-growth changes including telomerase activation to maintain cells beyond their normal lifespan (Gil et al. 2005). A glutathione transferase (GSTP1 caretaker gene) promoter is commonly hypermethylated (leads to downregulation of the gene product) in PCa leading to increased reactive oxygen species (ROS) from a failure to detoxify carcinogens (Schnekenburger et al. 2014). Finally, in PCa certain oncogenic transcription factors of ETS family are commonly translocated in front of promoters of AR driven genes. For example, the AR driven promoter of transmembrane protease, serine 2 (TMPRSS2) is translocated in front of the ETS family member ERG in approximately 50% of PCa cases (Gasi Tandefelt et al. 2014).

*Epithelial-stromal interactions: reactive stroma*

Most research has focused solely on the epithelial cancer itself, largely ignoring the stromal or extracellular matrix (ECM) contribution to disease progression. As cancers expand, the cells bidirectionally interact with the stroma. This interaction confers a drastic response described as the reactive stroma or desmoplasia (Cher et al. 2006). The reactive stroma actually is a normal response during wound healing that is co-opted by the invading PCa cells. Hence, the reason adenocarcinoma is referred to as the “wound that never heals” (Park et al. 2000). Prostate fibroblasts in the stroma are induced to differentiate to a myofibroblast phenotype and the prostate smooth muscle is displaced. The cancer differentiated prostate fibroblasts increase production of ECM proteins (tenascin-C and
pro-collagen I) proteases (fibroblast activation protein α (FAP)) and certain markers (α smooth muscle actin (α-SMA), vimentin) while downregulating calponin (Tuxhorn, Ayala, et al. 2002). Transforming GF β 1 (TGF-β1) is a primary mediator of this response, acting as a paracrine factor on stromal cells derived from the epithelial cancer cells (Tuxhorn, Ayala, et al. 2002). Clinically, higher volume of reactive stromal staining (Masson’s trichrome stain) by itself is associated with recurrence in patients (Ayala et al. 2003). The cancer promoting effects of prostate cancer associated fibroblasts (CAFs) has been shown. Non-tumorigenic BPH cells lines when combined with prostate CAFs resulted in tumorigenesis in a rat model (Hayward et al. 2001). Other studies show that combining non-tumorigenic LNCaP cells with an ECM (Matrigel®), and prostate stroma cells increased tumorigenicity and vessel density even more than just ECM or stromal cells alone (Tuxhorn, McAlhany, et al. 2002). These studies emphasize that stromal signals from both the cells and the matrix itself are important regulators of prostate tumor progression. One stromal component they did not examine for PCa was perlecan, which will be described later and is the focus of this dissertation.

*Epithelial to mesenchymal transition*

As the primary tumor progresses a subset of cells may undergo a transition from an epithelial to a mesenchymal-like phenotype referred to EMT in order to establish secondary sites of metastasis. This, similar to a reactive stromal response, is a cancer co-opted developmental mechanism to become highly migratory and invasive (Nauseef & Henry 2011). As described above, normal cells during prostate development must invade the stromal matrix during the glandular budding. This co-opting of developmental EMT is described by increased motility, loss of apical/basal polarity, the expression of
mesenchymal markers (vimentin, N-cadherin, Twist, and ZEB1) and the downregulation of epithelial markers (E-cadherin) (Polyak & Weinberg 2009). Several inductors of EMT are well studied including the hypoxia inducible factor-1α (HIF-1α), insulin like GF (IGF, FGF, Wnt/β-catenin, and the TGF-β signaling axes (Cho et al. 2013; Acevedo et al. 2007; Kypta & Waxman 2012; Nauseef & Henry 2011). EMT is considered a transitory state for establishing metastasis, but reverses through mesenchymal to epithelial transition (MET) once the cancer has found a suitable site. Many PCa bone metastases have glandular differentiation, high membranous E-cadherin and β catenin, and lower than expected EMT markers indicative of an epithelial state (Saha et al. 2008; Roudier et al. 2003; Nauseef & Henry 2011).

*Androgen independence and neuroendocrine differentiation*

In development, prostate growth is initiated by the activity of the AR. PCa co-opts the normal growth axis to grow in the primary prostate site by utilizing enhanced AR activity. AR is a nuclear receptor family transcription factor that is activated by its ligand testosterone and DHT. The androgen/AR signaling axis is readily exploited to treat PCa patients, as a common treatment is androgen deprivation therapy (ADT). ADT drugs target the ligand binding domain as antagonists (e.g. bicalutamide, flutamide) and the steroidogenic machinery (e.g. Abiraterone, CPY17A1 enzyme inhibitor) (Chan & Dehm 2014). Despite clinically extending the lives of patients, ADT drugs ultimately fail due to “androgen independence” mechanisms (D’Angelillo et al. 2015). These produce the next phase of the disease known as castration resistant prostate cancer (CRPC). PCa cells circumvent the drugs’ effects through several means including AR overexpression/gene duplication, point mutations, splice variants removing the ligand binding domain but
retaining the activity, synthesizing autocrine acting steroids, and exploiting cross activation with other signaling networks (e.g. src-dependent activation) (Chan & Dehm 2014; Gelman 2014).

A common occurrence of androgen refractory disease is the development of neuroendocrine differentiation (NED) (Santoni et al. 2014). NE cells exist in the prostate under normal circumstances to help regulate the mature gland and NED associated PCa cancer is separate from NE cell derived small cell carcinoma (Shariff & Ather 2006). Through a process of PCa transdifferentiation and/or a strong selection for the NE type cells under ADT, a larger than usual population of NE-like cells occur (Vlachostergios & Papandreou 2015). These NE cells make the following: serotonin, chromogranin (CgA/B), secretogranin, neuron specific enolase (NSE), thyroid stimulating hormone like peptide, and in smaller quantities: calcitonin, katablecin, PHRP, bombesin-like peptide, and β human chorionic gonadotropin-like peptide (Parimi et al. 2014; Santoni et al. 2014). Some of these proteins/peptides are thought to support the assailed PCa cells until the population can be recovered after treatment stops. NE differentiated cells are also Ki67 negative and AR negative, which may explain why this cell population survives under ADT and chemotherapy. Clinically, utilizing NED markers to stratify risk in early PCa is controversial, but NED among androgen refractory advanced PCa or metastasis predicts poor outcomes (Shariff & Ather 2006; Krijnen et al. 1997).

Bone microenvironment overview, osteomimicry and the vicious cycle

As stated before, the most common site of metastasis for PCa is the bone. This phenomenon was explained in part by Paget’s seed and soil theory of directed metastasis which was based upon breast cancer and extended to other cancers including PCa (Langley
PCa cells, the “seed”, prefer the bone microenvironment, the “soil”, and the molecular and cellular mechanisms are being continually explored. Predilection for the bone is partially explained by the chemokine and selectin signaling.

Bone stromal cells expressing the chemokine CXCL12 and PCa cells expressing their cognate receptor CXCR4 is one such homing mechanism (Lee et al. 2014; Langley & Fidler 2011). Additionally, bone marrow endothelial cells highly express E-selectin and PCa cells present the tetrasaccharide ligand, sialyl Lewis X (Barthel et al. 2009). Once in the bone/bone marrow microenvironment PCa cells interact with reticular bone marrow stroma, osteoblasts, osteoclasts and a variety of marrow-derived immune cells. Osteoblasts are bone morphogenic and GF activated mesenchymal stem cells and express receptor activator nuclear factor κB ligand (RANKL). Osteoclasts are derived from RANK positive monocytes that have been activated by RANKL and macrophage colony stimulating factor (MCSF). Osteoclasts and osteoblasts are the “yin and yang” of the bone microenvironment by removing bone (osteoclasts) and relaying bone matrix (osteoblasts). This balance is lost during PCa bone metastasis and is referred to as the “vicious cycle” (Josson et al. 2010).

In PCa metastases the vicious cycle generally results in osteoblastic lesions, meaning unwoven/unorganized bone matrix is deposited more than is resorbed by osteocytes (the reverse is true for breast cancer) (Ye et al. 2007). In the cycle, PCa cells express a variety of factors (e.g. endothelin-1, BMP, platelet derived GF (PDGF) that activate osteoblasts. The activated cells then deposit new unorganized matrix PCa cells favor. Activated osteoblasts and tumor cells express RANKL, which activates the osteoclasts to resorb the matrix, releasing non-collagenous protein (e.g. fibronectin) and embedded GFs. These released components further activate the PCa cells, which continue the cycle. This model
continues to expand with new players including a variety of stromal cells, macrophages, T-cells and even nerve cells (Cook et al. 2014). PCa cells do not usually express RANKL and other bone related proteins (e.g. osteocalcin, osteopontin, bone sialoprotein, osteonectin); this acquisition of a bone-like phenotype is called osteomimicry (Josson et al. 2010). A key player in instigating osteomimicry is β2-microglobulin (β2M), which operates through the cyclic AMP responsive element binding protein (Huang et al. 2006). Without PCa cells mimicking their bone stromal environment, the cells would be less likely to survive and never initiate the vicious cycle that leads to the death of the patient.

**Perlecan**

The basement membrane (BM) heparan sulfate proteoglycan (HSPG) perlecan/HSPG2 has been largely absent from the above descriptions concerning the mechanisms of prostate development, cancer progression and metastasis. However, this dissertation will make clear the sizeable impact of perlecan on the physiology of the prostate and PCa. Perlecans acts as a nexus to impact PCa cells in multiple niches important to PCa progression and metastasis. The relationship between perlecan and cancer will be better explored in the next chapter. This section aims to briefly describe the perlecan gene and protein along with its known functions impacting development, differentiation, growth.

**Gene and promoter description**

The first biochemical isolation of what would be called perlecan was in 1984 from colon carcinoma cell (HT-29, formerly misclassified as WiDr) conditioned media and was simply referred to a “basement membrane heparan sulfate proteoglycan” (Iozzo 1984). The *HSPG2* gene is on the first chromosome at position 1p36.12 (starts at 22,148,725 and ends at 22/263/790 bp from pter) (Grindel & Farach-Carson 2009). Originally described in
1993, the *HSPG2* gene was thought to be composed of 94 exons spanning over 120 kilo base pairs (kbp), but currently the annotated exon count is as high as 104 on the NCBI Gene database (Gene ID: 3339) (Cohen et al. 1993). The *HSPG2* promoter has been described and has several GC boxes, but lacks TATA or CAAT boxes, and has multiple transcriptional start sites, resulting in a mRNA of 14,294 bp (Cohen et al. 1993). The promoter is activated by TGF-β through its nuclear factor-1 element and repressed by interferon gamma (IFN gamma) via signal transducers and activators of transcription 1 (STAT-1) (Iozzo et al. 1997; Sharma 1998). Oddly, *HSPG2* promoter studies halted after 1998 until 2014 when the Farach-Carson lab re-cloned the promoter and showed enhanced activity with tumor necrosis factor α (TNF-α) mediated through the nuclear factor kappa B (NF kappa B) transcription factor (Warren et al. 2014).

**Protein function, domains and binding partners**

The *HSPG2* transcript encodes for a secreted protein of 4,391 amino acids (cDNA of 13,173 bp). The molecular weight (MW) of the perlecan core is 468 kiloDaltons (kDa). However, perlecan has upwards of 4 HS and/or chondroitin sulfate (CS) O-linked chains potentially resulting in a MW of approximately 850 kDa. Perlecan largely functions as an extracellular scaffolding protein of the BM and stromal matrix, GF reservoir, and receptor co-activator. Perlecan is best known as a component of the BM where it binds and tethers collagen IV, nidogen, and laminin to increase basal lamina integrity (Whitelock et al. 2008). Perlecan is composed of 5 multimodular domains as seen in figure 1.3 with many of its binding partners. Following a 21 amino acid signal peptide is the N-terminal domain I (Dm I), spanning amino acids 22-193 (Murdoch et al. 1992). Dm I contains 3 SGD sequences for glycosaminoglycan (GAG) chain addition on serine residues, and a 120
amino acid SEA (Sperm protein, Enterokinase, Agrin) module. The SEA module has no definitive function, but deletion studies in domain I suggest it increases HS/CS chain attachment (Dolan et al. 1997). Depending on the cell type and degree of differentiation, the HS/CS chemical composition (e.g. extent of sulfation) can be modified (Molist et al. 1998). Much of the activity regarding heparin binding GF (e.g. basic fibroblast growth factor(b-FGF/FGF-2)) delivery and storage (high capacity, low affinity) is due to the HS chains (Casper et al. 2007). The HS/CS chains also act as co-activators of the GF receptor, stabilizing the complex in varying stoichiometry (Schlessinger et al. 2000). The 210 amino acid domain II (Dm II) (amino acids 194-403) contains 4 cysteine-rich low-density lipoprotein (LDL) receptor-like modules and one immunoglobulin (Ig)-like repeat (residues 404-504) (Costell et al. 1996). This domain is implicated in extracellular lipoprotein retention as chicken perlecan domain II binds to LDL (Hummel et al. 2004).

Domain III (Dm III) (1,172 amino acids; residues 505-1676) consists of modules homologous to the short arm laminin α-chains including 3 laminin domain IV-like modules and eight laminin epidermal growth factor (EGF)-like repeats. This domain contains an RGDS integrin binding sequence in mice but not humans (Chakravarti et al. 1995). Domain IV (Dm IV) is the largest domain (2010 amino acids; residues 1677-3686), containing 21 IgG-like repeats (murine perlecan has only 14 IgG-like repeats, missing IgG repeats 5-12) similar to neural cell adhesion molecules (N-CAM). This domain has only been studied mice (until this work) and has been shown to bind various GFs (e.g. PDGF) and ECM molecules (e.g. nidogen, laminin, fibronectin) (Hopf et al. 1999; Gohring et al. 1998). Domain V (Dm V) (705 amino acids; residues 3687-4391) has 3 modules with sequence homology to the globular domain of laminin α-chains (LG1-3). In addition, this
domain contains 4 interspersed EGF-like repeats, and another GAG chain attachment site (Friedrich et al. 1999).

**Bioactive fragments: domain V**

Dm V of perlecan is most notable, not as a part of the whole molecule, but as a separate entity known as ‘endorepellin’ or simply Dm V (Bix et al. 2004). Full length perlecan with intact HS chains is pro-angiogenic owing largely to its sequestration and presentation of vascular endothelial growth factor (VEGF) and other pro-angiogenic molecules to their receptor (Jiang & Couchman 2003; Jiang et al. 2004; Tapanadechopone et al. 2001). However, perlecan is processed by certain proteases to release the C-terminal portion of perlecan resulting in a bioactive fragment having antagonistic functions of the whole molecule. The Dm V perlecan fragment was originally referred to as endorepellin because it inhibits the VEGF-mediated migration of endothelial cells (HUVEC) and chorioallantoic membrane vessel formation was inhibited strongly as well (Mongiat et al. 2003).

The effects of endorepellin are cell specific as it is anti-adhesive for endothelial cells and fibrosarcomas, but not breast carcinoma cells (Mongiat et al. 2003). The adhesion and anti-angiogenic effect is mediated by the interaction with the α2-β1 integrin receptor; an interaction that is not available when the whole perlecan molecule is presented (Bix et al. 2004). The Dm V fragment also is being studied in stroke induced ischemia where Dm V of perlecan is released from the vascular BM (Fukuda et al. 2004). Dm V, via its interaction with α-dystroglycan and the integrin receptors α2β1 and α5β1, modulate astrocytes undergoing late astrogliosis by promoting secretion of nerve growth factor (NGF) (Al-Ahmad et al. 2011). For this reason Dm V is being investigated as a neuroprotective treatment in focal ischemia induced by stroke (Bix et al. 2013). While perlecan is secreted
as a whole molecule and has homeostatic roles, the functions of Dm V clearly show perlecan can act as a biologic sensor when proteolyzed. The next chapter will delve further into the proteolysis of perlecan and the work described in this dissertation is geared towards finding new enzymes capable of cleaving the protease resistant molecule.

Diseases associated with reduced perlecan expression

Two hereditary diseases demonstrate the importance of perlecan to human life. Schwartz-Jampel Syndrome (SJS) and Dyssegmental dysplasia, Silverman-Handmaker type (DDSH) result in a deficiency of secreted perlecan (Arikawa-Hirasawa et al. 2001; Nicole et al. 2000). SJS is a rare autosomal recessive disease characterized by skeletal dysplasias and myotonia, a neuromuscular disorder resulting in prolonged muscle contraction. With respect to myotonia, perlecan was shown to localize acetylcholinesterase (AChE) to the neuromuscular junction along with α-dystroglycan. With less functional perlecan, AChE is largely absent at the synapse, resulting in a higher concentration of acetylcholine (ACh). This aberrantly stimulates the ACh receptor causing muscle contractions associated with myotonia (Arikawa-Hirasawa, Rossi, et al. 2002; Peng et al. 1999). Despite its severity, patients with the disease survive after birth likely because some partially functional perlecan can be secreted albeit in smaller amounts than wild type (Arikawa-Hirasawa, Le, et al. 2002). DDSH, meanwhile, is perinatally lethal resulting from a functional null mutation of perlecan. As a rare autosomal recessive lethal disease, DDSH is characterized by severe skeletal dysplasias, anisospondyly (irregular vertebral bodies) and micromelia (small extremities). DDSH patients also have a flat face, cleft palate, low joint mobility, micrognathia (undersized jaw), and encephalocoele (brain sac grows outside of the skull). The endochondral growth plate has shortening defects, the resting cartilage shows mucoid
degeneration, and hypertrophic chondrocytes produce calcospherites that fail to fuse (Arikawa-Hirasawa, Wilcox, et al. 2002). As evidenced by severe bone deficiencies, perlecan is required for normal bone and cartilage development (Farach-Carson et al. 2005). In the murine embryo, perlecan is expressed as early as day 10.5 in vascular tissue and by day 11-13 it is highly expressed in the developing cartilage at the chondro-osseus junction during endochondral ossification (long bone elongation/maturation). DDSH associated lethality is not surprising given the widespread expression during embryogenesis and its continued expression into adulthood. During embryonic day 13-17.5 perlecan is expressed in many other tissues such as lungs, liver, and kidneys, potentially aiding in differentiation and development of these tissues (Handler et al. 1997). Intense perlecan staining also is found in the glomerular and tubular BMs, cardiac tissue, muscle fiber borders and broad epithelial BM including the salivary glands. (Murdoch et al. 1992; Pradhan et al. 2009; Sasaki et al. 1998). Perlecan null mice are also lethal with similar defects to DDSH patients demonstrating the cross species importance of the molecule (Arikawa-Hirasawa, Rossi, et al. 2002). In general, perlecan is found at tissue borders, maintaining integrity and maturation of many tissue types (Farach-Carson et al. 2014).

**Extracellular proteases relevant to cancer**

Extracellular proteolytic enzymes are critical to life, but misregulation of the finely tuned control mechanisms can aid in the progression of cancer. This work focused on the contribution of proteases, especially matrix metalloproteinases (MMPs), in the destruction of the ECM and production of new signaling cues important to cancer.

**Matrix metalloproteinases overview**
**Classes of MMPs and description**

MMPs are a class of 26 known extracellular zinc dependent endopeptidases together capable of cleaving every known component of the structural ECM (Amălinei et al. 2007). MMPs are divided into several classes based on structural homology and enzymatic specificity (figure 1.4). MMPs minimally have to have a signal peptide, pro-peptide domain, and a catalytic domain. The catalytic domain is where actual protein proteolysis takes place with the divalent zinc ion ($\text{Zn}^{2+}$). The zinc binding motif coordinates the zinc ion with three histidines. The divalent zinc ion along with the glutamate stabilizes the water for attack on the carbonyl group of the substrate to establish the tetrahedral intermediate. The propeptide domain contains the cysteine switch that interacts with $\text{Zn}^{2+}$ to block catalytic activity, keeping the enzyme latent (zymogen form) (Dormán et al. 2010).

Once the propeptide domain is lost through proteolytic cleavage (sometimes by other MMPs) the enzyme is free to be active. Much of the substrate specificity is determined by the C-terminal four bladed β propeller hemopexin domain that interacts with other regulatory proteins. The unstructured flexible hinge domain connects the catalytic domain to the hemopexin domain. Additional regulatory domains include the furin-like domain and Ig like domain. Some MMPs have a transmembrane domain and a glycophosphoinositol (GPI) domain which allows the membrane anchorage with the catalytic domain facing the ECM. Several classes of MMPs exist including collagenases (e.g. MMP-1 and 13), gelatinases (e.g. MMP-2 and 9), stromelysins (e.g. MMP-3), matrilysins (e.g. MMP-7), membrane type MMPs (e.g. MT1-MMP) and other specialized proteases like enamelysin (MMP-20) and epilysin (MMP-28) (Rowe & Weiss 2008).

Lastly, MMPs are regulated at several levels. Besides, epigenetic, transcriptional and
translational control, MMPs are regulated in the extracellular environment by tissue inhibitor of MMPs (TIMPs). Currently there are four members of the family, TIMP1-4. TIMPs act as a substitute pro-peptide coordinating its conserved cysteines with the catalytic domain zinc ion (Gomis-Rüth et al. 1997)

**Matrilysin/MMP-7 overview**

MMP-7 (matrilysin-1) is the founding member of the matrilysin group. The protease was originally called rat uterine protease, as it is the primary MMP responsible for ECM catabolism in postpartum uterine involution (i.e. when uterus returns to normal size after birth) (Woessner & Taplin 1988). MMP-7 is the second smallest MMP at 28 kDa with a 19 kDa active form. While most literature is dedicated to MMP-7’s role in cancer and disease, the protease is expressed in a variety of normal polarized glandular tissue (e.g. salivary parotid gland) (Harrell et al. 2005). Additionally, MMP-7 is important in maintaining innate immunity by activating pro-defensins in the lung and intestinal tissue (Burke 2004). The substrates for MMP-7 are expansive including ECM proteins (elastin, type IV collagen, fibronectin), shed proteins (E-cadherin, Fas ligand, TNF-α, β 4 integrin), and other MMPs (proMMP-2, proMMP-9) (Ii et al. 2006). The substrate specificity may be flexible because MMP-7 lacks the hemopexin domain. In fact it only has the pro-peptide domain and the catalytic domain. The S1` site in MMPs is largely responsible for the substrate specificity. However, the MMP-7 S1` site is very shallow and rigid with no induced fit. This structure allows greater substrate promiscuity which may explain its wide range of substrates (Dormán et al. 2010). The wide acting protease profoundly affects the organism, being involved in angiogenesis (both anti and pro), inflammation, apoptosis,
invasion and proliferation. Given its roles, dysregulation of this enzyme at multiple levels contributes to cancer progression.

**MMP function in stages of cancer**

This section outlines how MMPs are involved in the progression of cancer at multiple levels with selected examples. In terms of early growth, both MMP-3 and MMP-7 can cleave pro-HB-EGF in a complex with cluster of differentiation 44 (CD44), allowing the mature GF to bind its receptor ErbB4 (Yu et al. 2002). MMPs can influence angiogenesis which is crucial to the continued survival of the tumor through a variety of mechanisms. For example, in cooperation with CD44, MMP-2 and 9 activate latent TGF-β leading to increased angiogenesis in mouse tumor models and endothelial tube formation assays (Yu & Stamenkovic 2000). Early inflammation can be mediated by TNF-α activity and is common to many cancers (Keerthy et al. 2014). MMP-7 activates latent TNF-α, albeit with a much lower specificity constant than TNF-α converting enzyme (TACE) (Mohan et al. 2002). The gelatinases have also been implicated in processing chemokines such as CXCL5 to attract infiltrating immune cells which occur commonly in cancer induced inflammation (Di Lorenzo et al. 2011; Song et al. 2012). Of course, several MMPs are suggested to degrade the BM in order for cancer cells to metastasize. Specifically, the transmembrane proteases (MT1-3) confer cancer cells the ability to breach the BM (Hotary et al. 2006). However, it is still debatable which key protease (or even if there is a single or specific group) is responsible for invading the BM (Rowe & Weiss 2008). Given MMPs activate one another, one enzyme could be the initiator and the other, the executioner, but discriminating which is which in cell culture, let alone *in vivo*, would be difficult.

**Extracellular serine proteases in prostate cancer**
While this dissertation focuses on the MMP class of proteases, attempts were made to cleave perlecan with other extracellular proteolytic enzymes. I focused on extracellular proteases relevant to PCa, notably PSA (kallikrein-3 peptidase), hepsin, FAP, and TMPRSS2. All of these enzymes are serine proteases. They are named so because in the catalytic site (composed of the catalytic triad: histidine, aspartic acid, and serine) the serine hydroxyl group forms a tetrahedral intermediate with the substrate to break the peptide bond. PSA is best known as a biomarker for PCa diagnostics; however, it has been shown to contribute to PCa metastasis by altering osteoblastic gene expression (Chirgwin & Guise 2006). Even though PSA is a chymotrypsin-like serine protease, it has not been tested thoroughly on extracellular substrates, but it does cleave fibronectin (Deperthes et al. 1996). Hepsin (TMPRSS1) is a type 2 transmembrane serine protease that is implicated in PCa metastasis. Specifically, inhibition of hepsin by a small molecule in a mouse model reduced metastasis to the bone (Tang et al. 2014). Unexplored is if hepsin can cleave extracellular structural components like perlecan, since most of the research focuses on its ability to activate pro-hepatocyte growth factor (HGF) (Wu & Parry 2007). FAP is a member of the transmembrane serine dipeptidyl peptidase family (DPP4) but is also an endopeptidase (other members only remove two peptides from the N-terminus). Despite extensive research into FAP on the clinical side (especially with respect to reactive stroma and wound healing reviewed above) only two known substrates exist for FAP: α 2 antiplasmin and denatured collagen type 1 (Hamson et al. 2014). The substrate list needs to be expanded for its function to be truly understood. TMPRSS2 is also a transmembrane serine protease. It is best known for its promoter being in front of the ERG transcription factor (TMPRSS2-ERG fusion reviewed above). TMPRSS2 is most studied as a protease
with respect hemagglutinin activation in the influenza virus (Meyer et al. 2013). However, it was shown to activate a G-protein coupled receptor, PAR-2, on PCa cells leading to intracellular calcium signaling (Wilson et al. 2005). TMPRSS2 is androgen regulated, yet very little is known about its potential extracellular substrates with respect to PCa.
Figure 1.1 Prostate gland structure and cell types. The human prostate gland structure (A) is more compact than the multilobed mouse prostate (B). The human prostate is composed of the peripheral zone (PZ), the transitional zone (TZ) surrounding the proximal urethra (UP), central zone (CZ) surrounding the ejaculatory duct (E), and anterior fibromuscular stroma (AFS). At the base is the exiting distal urethra (UD). The mouse prostate is composed of the dorsolateral prostate (DLP), anterior prostate (AP), and ventral prostate (VP) surrounding the center urethra (Ur). Histologically, the human prostate is more compact with dense fibromuscular stroma (C) than the mouse prostate (D) with loose connective tissue. E) Prostate glands are made of several cell types and structures designed to ultimately secrete prostatic fluid into the apical ductal opening. Figure adapted from Marker, Donjacour, Dahiya, & Cunha, 2003; Thomson & Marker, 2006; Timms, 2008.
Figure 1.2 Gleason grade scoring. Prostate cancer is assessed by pathologists using the Gleason grade scoring system. Shown are hematoxylin and eosin stained prostate sections from grade 1 to 5. Loss of glandular differentiation and increased stromal invasiveness result in higher Gleason grades. The two most common scores are combined to give the Gleason sum. Figure adapted from images at http://www.phoenix5.org/Infolink/GleasonGrading.html
Figure 1.3 Human perlecan domain structure and interacting partners. Perlecan module structure based on UniProt, PDB and PHYRE predicted structures. Not to scale. See text for abbreviations of binding partners.
Figure 1.4 Matrix metalloproteinase domain structure. Matrix metalloproteinases (MMPs) are divided into several classes based on domain homology and substrate specificity. Matrilysin-1 (MMP-7) is of the simplest domain structure containing only the signal peptide (SP), pro-peptide domain (Pro), and catalytic domain. Other MMP variations include a hemopexin domain next to a hinge region (Hg), fibronectin like (FN), and furin motifs. Some MMPs are cell surface associated by a transmembrane (TM) or glycosylphosphatidylinositol (GPI) linked sequence.
Chapter 2: Perlecan actions on prostate cancer cells and susceptibility to prostate cancer associated proteases

As modified and expanded from published manuscript in Matrix Biology, 2014¹

¹ Grindel, B.J. et al., 2014. Matrilysin/matrix metalloproteinase-7(MMP7) cleavage of perlecan/HSPG2 creates a molecular switch to alter prostate cancer cell behavior. Matrix Biology, 36, pp.64–76.
**Introduction**

For metastasis and dispersion of cancers originating in epithelial tissues to occur, epithelial cancer cells adopt an invasive mesenchymal-like phenotype that allows them to first remove and then move through the underlying protein-rich BM and blood vessel rich connective tissue matrix (Polyak & Weinberg 2009; Rowe & Weiss 2008). The large HSPG, perlecan, is a core component of the BM along with laminin, collagen IV, and nidogen/entactin (Whitelock et al. 2008). For prostate cancer (PCa) cells to metastasize to bone, as happens in over 95% of lethal cases, cells must migrate through at least five perlecan-rich ECM layers including the sub-epithelial BM, the endothelial BM at both origin and exit and the territorial matrices in glandular connective tissue and upon arrival in bone marrow. The over 4000 amino acid long perlecan protein core, which constitutes an innate tissue border (Farach-Carson et al. 2014), also binds GFs such as PDGF whose release by proteolysis can occur during wound healing (Gohring et al. 1998), or sonic hedgehog whose association with perlecan core and GAG chain enhances PCa growth and metastasis (Datta et al. 2006). Endogenous perlecan was necessary for prostate cancer cell (C4-2B, castration resistant bone metastatic cell line) survival in soft agar colony formation assays and responsiveness to VEGF-A and FGF-2. Mice inoculated with tumor cells lacking perlecan also were much smaller and less vascularized (Savore et al. 2005). An intriguing idea is that perlecan, when intact, serves as a tissue barrier and GF reservoir that is readily available during wound responses to limit cell mixing and to mount an immediate GF-mediated tissue healing response (Farach-Carson et al. 2014). To maintain their
unregulated growth, cancer cells including PCa cells can hijack this wound healing response to feed themselves constantly from the HS-bound GF reservoir bound to perlecan in the ECM, an action that involves the GF-liberating action of heparanase (Reiland et al. 2004). Additionally, as the tumor mass expands and invades the surrounding tissue in early stages of metastasis, perlecan must be cleaved and degraded in the underlying epithelial BM, in reactive stroma ECM (Warren et al. 2014) and in the vascular BM to allow intravasation to occur. While this perlecan turnover clearly involves the enzymatic activity of PCa proteases and GAGases such as heparanase and sulfatases (Brown et al. 2008; Lamanna et al. 2008), the proteases that cleave perlecan have not been identified in the context of PCa invasion.

Previous studies have found perlecan to be relatively resistant to proteolysis, although several extracellular proteases including MMP-3 (stromelysin-1), MT1-MMP, MT2-MMP, and collagenase-1 can cleave perlecan to varying extents (d’Ortho et al. 1997; Whitelock et al. 1996). It should be noted that MT1 and 2-MMP was not tested against full length perlecan but only recombinant fragments (d’Ortho et al. 1997). Not all extracellular proteases cleave perlecan, which resists cleavage by the gelatinases MMP-2 and 9 (Whitelock et al. 1996). MMP-2 and 9 are strongly associated with invasive cancer potential; so their inability to cleave perlecan suggests another protease is primarily responsible for cleaving perlecan during cancer progression. An important extracellular protease in PCa invasion is MMP-7, although its ability to cleave perlecan has not been examined. Expression of MMP-7 mRNA was found to be upregulated in primary and invasive PCa (Pajouh et al. 1991). Protein expression of MMP-7 and its inhibitor, TIMP-
1, reveals a higher MMP-7 to TIMP-1 ratio in more advanced PCa versus localized PCa or BPH, indicating that the enzyme is active in invasive PCa tissue (Hashimoto et al. 1998).

Interestingly, immunohistochemistry (IHC) studies showed increased MMP-7 stromal deposition in high grade PCa and bone marrow metastatic lesions (Cardillo et al. 2006; Hart et al. 2002). When transfected with MMP-7, DU-145 PCa cells showed enhanced invasiveness after intraperitoneal injection into immunodeficient mice (Powell et al. 1993).

MMP-7 is a secreted MMP consisting of a catalytic and activation blocking pro domain. The specificity matrix of 180 reported substrate cleavages in MEROPS (http://merops.sanger.ac.uk) reveals that the only defining feature of MMP-7 is its general preference for a leucine in the P(1)' site (Turk et al. 2001; Welch et al. 1996), which has been shown previously using tropoelastin as substrate (Heinz et al. 2010). While the MMP-7 ECM substrate specificity has been investigated (Amălinei et al. 2007; Welch et al. 1996), it has been impossible to predict if perlecan possesses sites subject to MMP-7 cleavage. However, MMP-7 is a strong candidate for perlecan proteolysis because the protease generally cleaves cartilaginous proteoglycans (Woessner & Taplin 1988), is activated by certain heparin moieties that may be similar in perlecan (Ra et al. 2009; Yu & Woessner 2000), and cleaves aggrecan, a CS proteoglycan (Fosang et al. 1992).

This study used biochemical and in silico approaches to determine if MMP-7 was a likely candidate enzyme to cleave perlecan during cancer cell tissue invasion. Susceptibility to cleavage was tested with purified perlecan, various recombinantly expressed subdomains of perlecan and with perlecan bound to other proteins in the context of the BM. The
identification of discrete fragments from Ig repeat Dm IV, thought to be an essential
cOMPONENT of the perlecan tissue barrier (Farach-Carson et al. 2014) was sought. Finally,
I performed experiments to determine if MMP-7 cleavage of perlecan and the BM not only
destroyed the barrier, but also created perlecan fragments with properties that could support
PCa cell invasion.

**Materials & Methods**

**Reagents and Antibodies**

The rabbit anti-human perlecan genomic antibody 3135 was generated using a proprietary
technology developed at Strategic Diagnostics, Inc. (Newark, DE). Antibody 3135 binds
to Dm IV-3 between amino acids 3295 and 3394. Mouse anti-perlecan domain I antibody
A71 was purchased from Pierce (Rockford, IL) and rat anti-Dm IV antibody A7L6 was
purchased from Life Technologies (Grand Island, NY). Heparitinase I, II, III,
chondroitinase ABC, and MMP-7 (cat no. M4565) were purchased from Sigma-Aldrich
(St. Louis, MO). FAP was purchased from R&D Systems (Minneapolis, MN), and PSA
was purchased from EMD Millipore (Darmstadt, Germany). Cultrex® placental human
BM extract (cat no. 3415-001-02) was purchased from Trevigen (Gaithersburg, MD). For
mass spectrometry (MS) analysis sinnapic acid matrix and trifluoroacetic acid were
purchased from Sigma-Aldrich, water and acetonitrile were from J.T. Baker of Avantor
(Center Valley, PA), formic acid was from Thermo-Fisher Scientific, and the Ascentis
Express C-18 peptide ES column was purchased from Supelco (Bellefonte, PA). All other
materials used were reagent grade or better.

**Purification of Perlecan and Dm I**
HT-29 cells (formerly known as WiDr colon carcinoma cell line) purchased from ATCC (cat no. CCL-218) were cultured in Eagles minimal essential media (EMEM) (ATCC) with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1X penicillin/streptomycin (Life Technologies) and 1X L-glutamine (Life Technologies). Cells were incubated at 37°C in a 5% (v/v) CO₂ atmosphere and passaged at 80-90% confluency. HT-29 cells were described previously to produce perlecan as their primary HSPG (Fuki et al. 2000; Iozzo 1984; Iozzo 1987), thus were used as a cell factory protein source. When cells were nearly 100% confluent in Cell Culture Vessel Hyperflasks, (Corning, Tewskbury, MA) they were switched to 2% FBS media. Recovered conditioned media was processed through a 0.45 μm Stericup filter units (Millipore, Billerica, MA). Phenylmethyl sulfonylfluoride (PMSF), benzamidine, and (ethylenediaminetetraacetic acid) EDTA at 0.5 mM each and 0.02 % (w/v) sodium azide were included in the conditioned media. The solutions were processed through a S34100 Amicon spiral wound 100 kDa molecular weight cutoff media concentrator cartridge (Millipore). The resulting high molecular weight concentrated solution was subjected to diethyl aminoethyl (DEAE) anion exchange chromatography (GE Healthcare). Equilibration buffer contained 2 M urea, 50 mM PIPES pH 7.0, 250 mM NaCl, 2.5 mM EDTA, 0.5 mM benzamidine, 0.5 mM PMSF, and 0.02% sodium azide. Elution buffer was identical to equilibration buffer except the NaCl was 750 mM. Eluted 1.5 mL fractions were collected and the absorbance read at 280 nm. Fractions were analyzed individually by dot blot immunoassay with anti-perlecan Dm IV antibody A7L6 (described below). Pooled fractions containing perlecan were dialyzed in MilliQ water and centrifuged in a speed vac until the desired concentration was obtained. The resulting perlecan enriched
solution was separated by Sepharose CL-4B (Sigma) gel filtration chromatography in the presence of 0.8 M NaCl phosphate buffered saline (PBS). A perlecan peak containing immunopositive fractions was pooled, dialyzed in MilliQ water and centrifuged in a speed vac. Perlecan-rich protein pools were subjected to gradient heparin Sepharose 6 Fast Flow (GE Healthcare) chromatography in a PBS buffer containing 0.2, 0.3, 0.5, 1.0, or 1.5 M NaCl. Perlecan, as determined by A7L6 dot blot, eluted at 0.3 M NaCl. Perlecan was concentrated in a speed vac and dialyzed into a final PBS buffered working solution. Samples were aliquotted and stored at -80°C until used. Purity was assessed by silver stain as described below.

Additionally in some preparations after gel filtration chromatography perlecan was further purified by A74 antibody affinity chromatography. A74 is mouse hybridoma antibody that recognizes the C-terminal region of perlecan. This antibody (after buffer exchange into 0.1 M NaCO3, 0.5 M NaCl, pH 8.3) was coupled to a CNBr activated sepharose CL-4b resin (Sigma, no. C9142) according to manufactures instructions. Perlecan containing fractions (from DEAE and gel filtration chromatography) were diluted in wash buffer (1 M NaCl PBS, pH 7.4) and allowed to bind to the column overnight at 4 degrees C. After cold buffer washing, perlecan was eluted from the column with elution buffer (6 M urea, PBS, pH 7.4). Peak fractions as determined by dot blot were pooled and dialyzed (10 kDa cutoff) into PBS, aliquotted and stored at -80 degrees C. Therefore, perlecan is full length from the HS chains of Dm I to A74 purified at Dm V.

Dm I of perlecan was purified and characterized as described previously (Casper et al. 2007; Yang et al. 2005). The Dm I construct (amino acids 22-194) was designed for the protein to be secreted into mammalian cell culture media for purification. Briefly, stably
transfected human embryonic kidney 293 (HEK293) EBNA cells (Life Technologies, discontinued product) were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies cat no. 11965) supplemented with 10% (v/v) heat inactivated FBS, 2 mM L-glutamine, 0.2 Units/mL penicillin, 0.2 µg/uL streptomycin (Life Tech), 10 ng/mL puromycin, and 250 µg/mL geneticin (G418) (Life Tech) and kept at 37°C in a 5% CO₂ incubator. For conditioned media collection, cells were cultured in Hyperflasks (Corning) with DMEM reduced to 2% FBS. Conditioned media was processed through a 10 kDa molecular weight cutoff spiral wound media concentrator. The concentrated conditioned media was passed twice through a DEAE column at 4°C, washed extensively in a HEPES buffered solution, pH 8.0, containing 250 mM NaCl with protease inhibitors and sodium azide. Dm-1 was eluted from the column in the same buffer but containing 750 mM NaCl. Fractions were assessed through a domain I specific A76 and N-20 (Santa Cruz Biotechnology) antibody dot blot. Dm I was concentrated and exchanged into a 25 mM HEPES, pH 7.4, 50 mM NaCl, and 1% (v/v) glycerol solution using a 10 kDa molecular weight cutoff centrifugal filter (Millipore), aliquotted, and stored at -80°C. Purity was assessed through reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4-12% acrylamide gradient gels (Life Technologies) in MOPS buffer) and subsequent Coomassie staining and N-20 antibody western blots. Approximately 20-30 mgs of Dm I was obtained from 4 L of conditioned media.

**Cloning and purification of domain IV subdomains**

Dm IV of perlecan (21 Ig repeats encompassing amino acids 1677-3662 and cDNA base pairs 5029-10986 [accession no. NM_005529.5]) was recreated and expressed using recombinant technology as three separate pieces, each containing 7 Ig repeats. Linking
regions between Dm IV-1, Dm IV-2, and Dm IV-3 were determined by Protein Homology/analogY Recognition Engine version 2.0 (PHYRE V2.0) (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) (Kelley & Sternberg 2009). Dm IV was divided at these points to avoid disrupting Ig module folding. HT-29 mRNA was collected with Trizol Reagent (Life Technologies) according to manufacturer’s directions. Monsterscript 1st-Strand cDNA Synthesis Kit (Epicentre, Madison, WI) (Cat no. MS040910) with random nonamer primers was used to generate the cDNA. Dm IV-1 (Ig repeats 1-7, amino acids 1677-2338, and bp 5029-7014), was cloned into a vector similar to pCEP-pu (Life Technologies) containing a CMV promoter, a BM40 signal sequence for secretion, and a C-terminal V5 tag and poly histidine tag. The vector also contains an internal ribosomal entry site for a green fluorescent protein (GFP) sequence and a separate puromycin resistance for selection in mammalian cells. Dm IV-1 PCR products were amplified with flanking Nhe1 and Not1 restriction sites, separated and excised from an agarose gel, and ligated into the vector after digestion with Nhe1 and Not1 restriction enzymes (New England Biolabs, Ipswich, MA). An enterokinase cleavage site sequence was introduced through two rounds of site directed mutagenesis with a QuikChange II XL kit (Agilent Technologies, Santa Clara, CA). Dm IV-2 (Ig repeats 8-14, amino acids 2338-3010, and bp 7012-9030) was synthesized by GENEWIZ (South Plainfield, NJ) and cloned into the same vector as Dm IV-1. Dm IV-3 (Ig repeats 15-21, amino acids 3011-3662, base pairs 9031-10986) PCR products were amplified with a forward BM40 signal sequence and a reverse primer flanked with an enterokinase site, inserted into a pENTR/D-TOPO entry vector (Life Technologies) and transferred to a pcDNA-Dest40 mammalian expression vector (Life Technologies). Dm IV-3a, a subpart
of Dm IV-3, is the peptide between amino acids 3126-3408 (base pairs 9376-10224). This was cloned similarly to Dm IV-1, except an enterokinase site was not included. Sequence verified vectors containing Dm IV were transfected into HEK293-Adherent (HEK293-A) cells (Life Technologies) with Lipofectamine 2000 transfection reagent (Life Technologies). After antibiotic selection (Dm IV-1, Dm IV-2, Dm IV-3a: 400 ng/mL puromycin; Dm IV-3: 1mg/mL Geneticin, Life Technologies) in DMEM, 10% FBS, 1X penicillin/streptomycin (Life Technologies), 1X L-glutamine (Life Technologies), serum free conditioned media was collected. Conditioned media was processed through a Stirred Cell Millipore media concentrator with a 10,000 Dalton cutoff concentrator filter. Nickel chromatography with Ni-NTA agarose resin (Qiagen, Valencia, CA) was performed on the concentrated conditioned media and eluted with 300 mM imidazole 50 mM sodium dihydrogen phosphate, 300 mM sodium chloride, and 0.05% (v/v) Tween-20, pH 8.0. Equilibration and wash buffers were identical, but instead contained 10 mM and 20 mM imidazole, respectively. Appropriate fractions were concentrated and buffer exchanged to remove imidazole using Millipore Amicon Ultra Centrifugal Filters (Ultra Cel-50 kDa MWCO membrane, cat no. UFC505024).

**In silico analysis of MMP-7 proteolysis of perlecan**

Using the free Site Prediction website ([http://www.dmbr.ugent.be/prx/bioit2-public/SitePrediction/index.php](http://www.dmbr.ugent.be/prx/bioit2-public/SitePrediction/index.php)) (Verspurten et al. 2009) the amino acid sequence of perlecan (uniprot ID: P98160) was subjected to *in silico* digestion by MMP-7 under default settings. Sequence cleavage specificity was extracted from the MEROPS database for MMP-7 from all organism sequences available (*Bos taurus*, 3; *Homo sapiens*, 137; *Mus musculus*, 22; *Rattus norvegicus*, 7). All sequence specificities reported are greater than
99 percent. The specificity percent as described by Site Prediction is the chance that a proteolytic site above a given threshold will actually be cleaved. Site Prediction also was performed on Dm IV of perlecan, amino acids 1677-3662 [accession no. NM_005529.5], and Dm IV-3 recombinant protein sequence.

Protease assays, silver stains and western blots

Perlecan and perlecan Dm-IV were subjected to enzymatic digestion by MMP-7, as well as PSA, FAP and hepsin, three other proteases common to the prostate (Kelly et al. 2012; Tripathi et al. 2008; Webber et al. 1995). Perlecan was incubated at varying enzyme to substrate ratios as noted in the results for individual experiments. For basic MMP-7 digestions, the buffer contained 10 mM HEPES buffer pH 7.0, 3 mM calcium acetate, and 1 mM EDTA. For placental BM extract proteolysis (6 μg per 0.2 μg MMP-7 in 40 μl total), the MMP-7 digestion buffer was identical, but lacked Triton X-100. Additionally, time points for BM extract digestion were stopped with 2.5 mM final EDTA and allowed to depolymerize at 4°C before subsequent processing. During MMP-7 co-incubation with FAP, all buffers were identical and contained 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 3 mM calcium chloride, and 0.1% (v/v) Triton X-100. For a FAP positive control, 10 mg of rat tail collagen type 1 (Sigma, cat no. C7761) was dissolved in 10 mL 0.1 M acetic acid for 1 hr at room temperature (RT) and heat denatured at 68°C for 24 hrs before digestion with MMP-7 and FAP. Hepsin digestion (0.5 μg per 6 μg perlecan construct in a 30 μL reaction volume) was attempted using two conditions; 30 mM Tris HCl, pH 8.4, 200 mM NaCl, 0.1% (v/v) DMSO and alternately 50 mM Tris HCl, pH 7.5, 250 mM NaCl. PSA (2 μg per 6 μg perlecan peptide in a 30 μL reaction volume) digestion buffer contained 50 mM Tris-HCl, pH 7.9, 10 mM NaCl, 0.01% Tween 20. All were incubated at 37°C for
varying times noted in results. Heparitinase I, II, III and chondroitinase ABC digestions of full length perlecan were performed in PBS, pH 7.4 with 2 mM calcium chloride at 0.1 units (as defined by Sigma) per enzyme and 20 µg of perlecan in a reaction volume of 40 µL at 37°C for at least 6 hrs.

For purified perlecan, digestion reactions and controls were denatured at 99°C with reducing sample buffer (final 60 mM Tris-HCl, 1% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, pH 6.8 with tracking dye) for 5-10 min. Samples were separated with SDS-PAGE in 1 mm Novex NuPAGE 4-12% polyacrylamide gradient bis-tris buffered gels (Life Technologies) in a Novex NuPAGE system (Life Technologies) with 1x MOPS SDS buffer (Life Technologies) at 150 constant volts for approximately 80 min. See Blue +2 molecular weight marker (Life Technologies) and Broad Range Marker (BioRad) were used to determine apparent molecular weights. Silver staining was performed with the Silverquest Silver Stain kit (Life Technologies) according to manufacturer’s directions. For western blots, separated protein was transferred to 0.45 µm pore nitrocellulose (Bio-Rad, Hercules, CA) in a tris-glycine buffer at 40 volts for 5 hr. Membranes were blocked in 3% (w/v) bovine serum albumin (BSA) tris buffered saline with 0.05% Tween-20 (TBST) for 2 hrs at RT. A71 antibody (200 ng/ml, 1:1000) or antibody 3135 (1:10,000) was added to the block solution overnight on a 4°C shaker. Following three by 5 min TBST washes, the membranes were incubated with 1:200,000 sheep anti-mouse HRP conjugated antibody (Jackson) in 3% BSA TBST for 2 hr at RT. Blots were washed again and incubated with chemiluminescence substrate (West Dura extended substrate, Pierce) for 5 min and exposed to film. For perlecan dot blots, 20 µL of sample was added to a BioRad slot blot apparatus and allowed to bind a pre-PBS rinsed
0.45 μm nitrocellulose membrane for 2 hr at RT. After vacuum suction, the dot blot was placed into BSA blocking solution and processed identically as the A71 western blot, except a 1:5,000 A7L6 antibody was used.

**N-terminal sequencing and mass spectrometry**

Dm IV-3 recombinant protein was incubated with MMP-7 overnight as described above and the digestion products separated by SDS-PAGE as described above. The protein was transferred to a methanol pre-soaked 0.22 μm pore polyvinylidene difluoride (PVDF) membrane in 25 mM Tris, 192 mM glycine buffer with 20% (v/v) methanol at 50 V for 5 hrs at 4°C. The PVDF membrane was washed with distilled water several times to remove glycine background, and dipped in methanol again. The membrane was stained with 0.1% (w/v) Coomassie Blue R-250 in 40% methanol, 1% (v/v) acetic acid for 1 min, and destained with 50% methanol in water solution. After extensive MilliQ water washes, the membrane was left to air dry. The membrane was sent to Midwest Analytical Inc. Protein Sequencing (St. Louis, MO) for N-terminal Edman degradation protein sequencing.

MALDI MS analysis was carried out using a Bruker Autoflex II Time of Flight (ToF)-ToF (Fremont, CA) instrument. Perlecan Dm IV-3 MMP-7 digests performed without Triton X-100 were analyzed using a saturated sinnapic acid matrix that was prepared in a solvent system of 60/40 acetonitrile/water containing 0.25% (v/v) TFA. 10 μL of perlecan Dm IV-3 digest (10 μg of perlecan Dm IV) were combined with 10 μL of matrix solution, mixed and then spotted for analysis. Positive ion MALDI analysis was carried out in both the linear and reflectron modes.

LC-MS analysis of perlecan Dm IV-3 MMP-7 digests were carried out using a Thermo-Fisher LTQ-Orbitrap Mass Spectrometer interfaced with a Shimadzu Prominence ADXR
UPLC system (Columbia, MD) through an Ion Max Electrospray Ionization (ESI) source. The LTQ-Orbitrap was operated in positive ESI mode. MS spectra were acquired at a mass resolving power (m/∆m) of 30,000. MS/MS spectra were acquired using data dependent scanning. LC separations were accomplished using a 150 mm x 1.0 mm ID, 2.7 micron Ascentis express C-18 peptide-ES column operated at a flow rate of 0.15 mL/min. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The LC gradient for the analytical separation was as follows: initial conditions 5% (v/v) B to 35% B at 32 minutes, to 65% B at 40 minutes, and to 90% B at 42 minutes. For small peptides (m/z ≤ 2500, z ≤ 5) data analysis was carried out using Matrix Science Mascot (Perkins et al. 1999) and mMass (Strohalm et al. 2010; Strohalm et al. 2008). For larger peptides (m/z > 2500, z > 5), molecular weights were computed using MagTran deconvolution software (Zhang & Marshall 1998).

Prostate cancer cell culture

C4-2 bone metastatic PCa cells were cultured as described previously (Thalmann et al. 2000; Wu et al. 1994) and cultured in T-media (Gibco) with 5% heat inactivated FBS (Atlanta Biologicals) and 1X penicillin/streptomycin (Gibco). PC-3 cells were cultured in F-12K media (ATCC) with 10% FBS, 1X penicillin/streptomycin and 1x L-glutamine (Gibco). Cells were passaged at 90% confluence and incubated at 37°C in 5% CO₂ atmosphere.

Perlecan coating and dispersion assays

In triplicate, Dm IV-3 (12.5 µg per well), MMP-7 pre-digested Dm IV-3, BSA (12.5 µg per well), or BSA incubated with MMP-7 were adsorbed to a flat bottom 12-well plate (Corning) in PBS, pH 7.4 (Life Technologies). Additionally, full length perlecan, Dm IV-
1, Dm IV-2, and Dm I was treated and coated the same in other experiments similar to that of Dm IV-3. Samples were allowed to adsorb overnight at 37°C. The remaining solution was removed, washed with PBS and replaced with full culture media (1% FBS media was also used and will be noted in results). C4-2 cells were passaged with 0.25% (w/v) trypsin-0.38% (w/v) EDTA (Life Technologies) and each well seeded with 100,000 cells. Cells were placed in the incubator and viewed and imaged by phase bright field microscopy at certain time points. Three 4X objective images were taken per well providing nine different images of each substrate type. Addition of soluble digest fragments and peptide to a Dm IV-3 coated wells was performed in 96-well plates. Plates were coated as before, but with less protein (1.75 µg Dm IV-3 per well). Digests were performed overnight as described before to a final concentration of 0.5 µg/mL of substrate. C4-2 cells (7,500 per well) were seeded into the wells along with digestion mixtures or peptides to a final volume of 100 µL. Each well received 2.75 µg in 100 µL media of either BSA, BSA and MMP-7, IV-3, or IV-3 and MMP-7. The domain IV peptide (TWSKVGGHLRPQIVQSG) used in previous studies was added at 20 µg/mL (Farach-Carson et al., 2008). After 48 hrs the wells were imaged at 4X objective. Using ImageJ software, batch images were quantified for particle count, particle size, and area fraction. Cell dispersion values are equal to (particle count)/(particle size)*(area fraction). A higher value is more dispersed and a lower value is more clustered. To compare dispersion values, the cells have to be of the same type, seeded at the same density, and subconfluent when imaged. An unpaired student's T-test was utilized to determine statistical significance.

**Basement membrane Transwell ® assay**
Corning Transwell® inserts (8 µm pore size, polycarbonate membrane) were coated in triplicate with 6 µg BM extract (Cultrex®) in the presence of serum free T-media in three experimental conditions. The BM extract was either coated and left undigested, pre-digested with 0.2 µg MMP-7 for 8 hours and coated, or coated and subsequently incubated with 0.2 µg MMP-7 for 8 hrs. After a brief media wash, 30,000 cells were seeded into each Transwell® in serum free T-media in the upper chamber. In the lower chamber, 10% FBS acted as the chemoattractant. After 80 hours, the cells were fixed in cold methanol and stained with 0.1 mg/mL crystal violet in 10% (v/v) ethanol and 1% (v/v) methanol and water for several minutes. The non-invaded top cells were removed with a cotton swab. With a dissecting microscope, a 2X objective image of migrating cells was taken and quantified by Image-J. An unpaired student’s T-test was used to determine statistical significance.

**Reverse phase protein array**

A reverse phase protein array (RPPA) was performed per the instructions of M.D. Anderson RPPA proteomics core at the following site: http://www.mdanderson.org/education-and-research/resources-for-professionals/scientific-resources/core-facilities-and-services/functional-proteomics-rppa-core/antibody-lists-protocols/functional-proteomics-reverse-phase-protein-array-core-facility-antibody-lists-and-protocols.html. Dm IV-3 was pre-treated overnight with MMP-7 or mock as described above (1 µg of Dm IV-3 or BSA was incubated with/without 0.1 µg MMP-7) at RT. This mixture is coated on plates at 4 µg per well of a 12 well plate overnight in a cell incubator. Additionally DmIV-3 was incubated at 60°C in PBS for 10 minutes and this was plated as well in PBS (see appendix E for details). After removal and
washing, 150,000 C4-2 cells per well were seeded onto the different substrates, incubated for 48 hrs, washed with PBS, and lysates collected per M.D. Anderson directions (1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl$_2$, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na$_3$VO$_4$, 10% (v/v) glycerol, containing freshly added protease and phosphatase inhibitors from Roche Applied Science Cat. # 05056489001 and 04906837001, respectively). After BCA assay (Pierce) to determine concentration (must be between 1-1.5 mg/mL), samples were boiled for 10 min in 4x SDS sample buffer without any dye. Samples were stored at -80°C until submission to core site. All samples were done in triplicate.

**Results**

**MMP-7 in silico analysis and proteolysis of perlecan and BM**

MMP-7, an enzyme that is active in PCa progression and a candidate to cleave perlecan under physiologically relevant conditions, was subjected to *in silico* digestion using free online Site Prediction software (Verspurten et al. 2009). Figure 2.1A shows the predicted cut sites in numbered rank of Average Score, a score related to the similarity of a known cut site (all predicted sites shown have >99% specificity) and the amino acid cleavage site. A majority of the predicted cut sites occur in Dm III and Dm V, with only three sites predicted to be cleaved within Dm IV. A Site Prediction MMP-7 digest including the sequence within perlecan Dm IV alone produced only 5 of the 20 predicted sites with specificity greater than 99% (not shown). Therefore, other parts of the perlecan core protein not in Dm IV are predicted to have preferable MMP-7 cleavage sites *in silico*, which, if accurate, would release Dm IV fragments.
Given the many MMP-7 cleavage sites in the perlecan core protein predicted by *in silico* analysis, I investigated the enzyme’s true *in vitro* ability to digest intact full length, HS-decorated perlecan. To do this, perlecan was purified from media conditioned by HT-29 cells and either directly incubated with MMP-7, or pre-digested with heparitinases and chondroitinase (H/C) to remove the HS and/or CS chains and then incubated with MMP-7 for 2.5 hours. The western blot for detection of perlecan (antibody A71) shown in figure 2.1B demonstrates that perlecan is susceptible to MMP-7 cleavage even when fully decorated with HS/CS. A time-course digestion of perlecan, as shown in figure 2.1C, produced certain fragments originating in Dm IV (black arrows) detected using a Dm IV specific antibody, 3135. Because cancer cells degrade perlecan in the context of the other proteins in the BM that might protect against digestion by MMP-7, I conducted experiments to use MMP7 to degrade perlecan entrapped in whole BM preparations. I used human BM extract rather than murine sourced Matrigel® to avoid issues with the mouse A71 antibody and better correlate with the human perlecan and recombinant fragments tested in this study. Human BM extract was allowed to polymerize at RT and then incubated with MMP-7 over an 8 hr period. Figure 2.2 displays a silver stain (figure2.2A, left), a western blot with Dm I-specific A71 (figure 2.2B, center) or Dm IV-specific 3135 antibody (figure 2.2C, right) that were performed to detect perlecan after either control or MMP-7 digestion. Of note, the rat Dm IV antibody A7L6 works well with dot blot during purification, but does not work consistently with western blots. Moreover, A7L6 binds the first 7 Ig repeats of Dm IV (IV-1) (data not shown, via dot blot), while 3135 binds the last 7 Ig repeats of Dm IV (Dm IV-3). The silver stain demonstrates that many proteins are present in the BM extract and that various bands are created/destroyed
over time (for example those in white boxes) by MMP-7 digestion, indicating that MMP-7 can cleave BM proteins even when they are in association with one another. Especially noted is the removal of a smeary high MW protein(s) (black arrowhead), which migrates in the same region as fully HS/CS-decorated perlecan in this system. The western blot of the same samples shown in figure 2.2A indicated new bands of perlecan (black arrows) were produced during the time course of MMP-7 digestion, possibly derived from the ~190 kDa band (white arrow). The smeary protein above the 200 kDa marker also disappeared with increasing MMP-7 incubation time (black arrowhead). Various fragments are detected when blotting for Dm IV as shown in figure 2.2C. It is of interest that the Dm IV antibody better detects the large diffuse band that was lost during incubation with MMP7 than the A71 antibody, which poorly detects glycosylated forms of perlecan. Similar sized fragments were detected during digestion of BM as was found in full length perlecan digestion (black arrows). Taken together, these experiments showed that in vitro, full length perlecan is a ready substrate for MMP-7 both in its purified state and in a BM matrix in the context of its BM binding partners.

Dm IV of perlecan consists of 21 Ig repeats that are non-uniform and represent distinct Ig module subtypes (Farach-Carson et al. 2014). The in silico digest prediction suggested that Dm IV of perlecan is largely devoid of consensus MMP-7 proteolytic sites, yet this domain encompasses nearly half of the molecule’s length. To determine the susceptibility of Dm IV to MMP-7 digestion, human perlecan Dm IV was cloned, expressed as individual recombinant proteins, and purified as three separate modules each consisting of 7 Ig repeats (Dm IV-1, 2, 3) and as a sub-part of IV-3, Dm IV-3a that was previously showed to contain an adhesion promoting motif (Farach-Carson et al. 2008). The schematic of the
subdomains created is outlined in figure 2.3A. Each recombinant protein was incubated individually with MMP-7. Figure 2.3B displays a silver stained gel of each subdomain alone or after incubation with MMP-7. Every subdomain of perlecan Dm IV was found to be susceptible to MMP-7 cleavage, producing a downshift and several new bands not present in the control digest. Dm IV-3a was purified as a smaller part of Dm IV-3, and even this produced a single downshifted band near the 38 kDa marker. This band mirrors the same fragment found in digested intact perlecan and in the BM when blotted for perlecan Dm IV with 3135. This antibody binds in the same region for Dm IV-3 or IV-3a. Interestingly, expressed Dm IV-3 and IV-3a have additional glycosylation, producing a smeary purification product. Digestion of Dm IV-3a with an endoglycosidase, PNGase F, caused a slight downshift, but the smeary band remained (Figure 2.4), indicating other forms of glycosylation or post-translational modifications exist. MMP-7 produces similar size fragments. These experiments provide further evidence that MMP-7 is remarkable in its ability to digest perlecan and that it can specifically produce Dm IV peptides whose size is consistent with those from either full length perlecan or perlecan-rich BM digestion.

**Serine protease susceptibility of perlecan**

Other proteolytic enzymes related to PCa also were tested for their ability to cleave intact perlecan or perlecan domains. PSA, hepsin and fibroblast activation protein (FAP) were tested on different portions of perlecan core protein and the results are shown in figure 2.5. The enzymes tested failed to cleave full length perlecan or Dm IV-1, 2, or 3 when perlecan and protease were incubated alone. However in the findings shown in figure 2.5A, the His Tag antibody western blot of Dm IV-3a alone, after incubation with MMP-7, hepsin, or PSA showed that MMP-7 completely digested Dm IV-3a, but PSA and hepsin each created
slightly downshifted bands. This indicates that MMP-7 cleaves at the C-terminus (removing the polyhistidine tag) and PSA and hepsin digest Dm IV-3a at the N-terminus, conserving the polyhistidine tag. While FAP alone displayed no ability to cleave perlecan Dm IV-3 (figure 2.5B, lane 4), FAP digestion combined with MMP-7 produced new bands (boxed portion in lane 5) not observed with MMP-7 alone (lane 3). The experiment was repeated to verify the two additional boxed faint bands. Also note that FAP migrates near the same molecular weight as Dm IV-3 in these gels, and is not due to presence of undigested Dm IV-3 or improper loading. Therefore, other PCa proteases may further cleave perlecan once it has been fragmented by more robust proteases, such as MMP-7, to unveil cryptic proteolytic sites.

Identification and characterization of perlecan Dm IV-3 sites of cleavage by MMP-7

To determine the representative MMP-7 site specificity for perlecan, a mixture of MMP-7 digested perlecan Dm IV-3 was analyzed by N-terminal sequencing, Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS), and liquid chromatography MS (LC-MS). Figure 2.6 presents a summary of the identified limit fragments produced by MMP-7 digestion of intact perlecan. In figure 2.6A, perlecan Dm IV-3 was digested with MMP-7 and the resultant PVDF membrane was stained with Coomassie to reveal five major bands that were subjected to N-terminal sequencing. This fragmentation banding pattern was the most common seen in multiple experiments, hence was used in analysis. Patterns seen were altered by differences in digestion time, enzyme to substrate ratios, and method of detection, as expected for an enzyme digestion. These variations account for the slight variances in patterns seen between figure 2.3 and figure 2.5. A schematic in figure 2.6B shows the cleavage products that were created for each band by taking into account
the approximate MALDI MS discriminated mass and smaller peptides found through LC-MS. MALDI generated peaks are in figure 2.7, and confirmed LC-MS peptides are mapped out in figure 2.8 and table 2.1. Note that APLD (written in italics) is the beginning of the recombinant protein as a part of the BM-40 signal sequence; the actual perlecan Dm IV-3 sequence begins at PSEG which was sequenced in bands 4 and 5. Additionally, full length Dm IV-3 only has a predicted MW of 75.46 kDa, yet the MALDI data indicates an intact MW of approximately 79.2 kDa. Therefore, matching predicted MW and protein fragment was not exact because the post translational modification makes sizes vague by approximately 4 kDa. However, the fragments laid out in figure 2.6 likely represent the largest fragments generated by MMP-7. Fragments range from approximately 17 kDa to 53 kDa, and several of the bands represent Dm IV-3 internal cleavage sites, meaning both the C and N-termini can be deciphered reasonably. Data from the LC-MS peptides and the N-terminal sequencing indicate MMP-7 has a strong preference for aliphatic amino acids at the P1’ site, generating mostly leucine (25 sites), valine (6 sites), isoleucine (5 sites), or alanine (3 sites) at the N-terminal sites in Dm IV-3. The C-termini do not seem to require the presence of any particular amino acid, but the most common amino acid at the P1 site is glutamine. Additionally, PHYRE modeling suggests that cut sites (shown as red in figure 2.6B) appear in the unstructured transitions between Ig modules (Ig20 and 22) and between connections to the interfacing β sheets (Ig17). Further validating the N-terminal sequencing data, the heptapeptide LEQRTYG (highlighted as red in the Ig17 model in figure 2.6B) appeared in LC-MS data, which would be generated when MMP-7 cleaved perlecan at those N-terminal sequence identified regions. Performing a Site Prediction analysis on the sequence of recombinant Dm IV-3 produces 20 predicted sites, albeit with
much lower Specificity percentage than full length perlecan (not shown). Interestingly, only six of the predicted sites match the peptide cleavage sites found through MS and N-terminus sequencing data. Band 3 is a common fragment produced by MMP-7 regardless of how perlecan is presented. In figure 2.9, BM extract, purified perlecan, Dm IV-3, and HT-29 concentrated conditioned media (the source of full length perlecan in this study) were digested with MMP-7 overnight. Each produced a similar fragment below 41 kDa and is detected by antibody 3135. Also note that bands 4 and 5 from figure 4A are not detected with antibody 3135 (lane 6), which coincides with the epitope range of 3135. Taken together, the combined data clearly shows that MMP-7 cleaves Dm IV of perlecan with non-predicted site specificity, and by doing so creates multiple small peptides and larger internally cleaved fragments some of which are post-translationally modified.

**C4-2 cell BM invasion with MMP-7**

To test the potential pro-metastasis effects of MMP-7, a Transwell® invasion assay through perlecan-rich human BM was utilized. BM extract was incubated in serum free media with MMP-7 to ensure that similar results were matched to the time course digestion shown in figure 2.2C, which included cell culture incompatible Triton X-100. Figure 2.10A demonstrates that even in cell culture media without any detergents, perlecan was cleaved efficiently and similarly to previous experimental conditions. Additionally, the same sized perlecan Dm IV fragments were generated in cell culture conditions. Figure 2.10B-D show representative images from an 80 hr C4-2 cell invasion assay through a BM extract coated 8 µm pore polycarbonate membrane. Panel B shows cells that migrated through membrane that contained BM extract only (condition 1), panel C is BM predigested with MMP-7 and coated (condition 2 in figure), and panel D is coated BM subsequently digested with MMP-
7 (condition 3). As quantified in panel E, invasiveness through BM is enhanced if perlecan-rich BM is digested with MMP-7. In this experiment, invasion through the polycarbonate membrane was increased regardless of whether the coated BM extract was predigested with MMP-7 or was digested after the BM was coated onto the membrane, but was greatest when BM was digested after assembly. Overall, this experiment demonstrates that MMP-7 activity creates holes in the BM that allow C4-2 cells to break through the barrier normally associated with the perlecan-rich BM border.

**Perlecan and MMP-7 proteolysis effects on prostate cancer cells**

To determine if the MMP-7 digestion products of full length have biological activity different from the parent proteoglycan, I used bone metastasis derived C4-2B cells. For this undertaking, a basal C4-2B cell response to HS intact full length perlecan first was established. Perlecan was adsorbed to plastic wells in a 96 well plate, plates were washed, and C4-2B cells then plated onto pre-coated dishes. Figure 2.11 shows the results after plating cells on perlecan coated plates (A-C), BSA controls (D-F), or the tissue culture plastic only (G-I) over a 96 hr period. While C4-2B cells on BSA coated plates ultimately adhered and spread out, cells plated onto perlecan failed to adhere and formed irregular shaped clusters. By 15 hrs after plating, the clusters have enlarged (boxed inset image, figure 2.11B), and they continued to grow larger into day 4. Some clusters maintained attachment during a media change at 48 hrs and continued to grow (boxed inset image, figure 2.11C), but many were too poorly adherent to remain on the plates. Clearly, perlecan with its HS chains intact induces cell-cell interactions that favor C4-2B clustering, a behavior that was not seen in tissue culture plate or BSA controls.
C4-2B cells next were plated onto dishes that had been pre-coated either with perlecan pre-digested with the H/C mixture of glycosidases, pre-digested with MMP-7 or with various treatment controls. Cells were imaged and in some cases stained for F-actin and nuclei. Figure 2.12 shows that the C4-2B cells plated onto intact perlecan with HS chains (A), MMP-7 only (B), or H/C only (C) failed to adhere to and spread on the plate after 1 hr. Staining the control cells at this point for F-actin was impossible because they had not yet adhered to the plates. In contrast, cells plated onto H/C pre-digested perlecan (D-E) readily adhered to the coated dishes, and remained attached throughout the staining process (E and F). C4-2B cells plated onto perlecan pre-digested with MMP-7 (G-I) also adhered to the plate surface within one hr. While the cells could be stained after one hr (H and I), they were not as firmly attached as those plated onto the H/C pre-digested perlecan coated wells. Interestingly, the cell morphologies at the early time points for H/C and MMP-7 pre-digested perlecan displayed a morphology possessing fanning ruffled edges and outstretched podia (figure 2.12, boxed enlarged images E,F,H,I). Organized F-actin was apparent, and a number of the cells appeared to be highly motile. Thus, PCa cells on intact perlecan with HS chains form spheroids dominated by cell-cell interactions, whereas those on digested perlecan form substratum adherent migrating cells consistent with an invasive phenotype.

Given the anti-adhesive effects of full length HS intact perlecan on C4-2B cells it was hypothesized that Dm I of perlecan may mediate the effects via the HS/CS chains. However, as seen in figure 2.13, after 24 hrs Dm I fails to show enhanced clustering effects displayed by full length perlecan (5 µg coated in a 96 well plate each). Therefore, an
additional motif must exist in perlecan to produce the striking cell-cell adhesion effect seen in perlecan.

Dm IV-3 represents at least one of the modules of perlecan that creates discrete large fragments after MMP-7 digestion. I investigated the effects of Dm IV-3 on the behavior of two invasive PCa cell lines, PC-3 and C4-2. While coating dishes with other recombinant generated subdomains IV-1 and IV-2 did not produce marked effects on cells, coating with Dm IV-3 produced a striking clustering effect as seen in figure 2.14 using C4-2 cells. A similar effect was seen with the PC-3 cells in the presence of Dm IV-3 (figure 2.14C), but to a somewhat lesser extent than seen with C4-2 cells. However, clustering was cell-type specific. For example, HS5 bone marrow stromal cells did not cluster at all on Dm IV-3. I controlled for general non-adherence by coating heat inactivated BSA at the same concentration as Dm IV-3. However, BSA at this low concentration did not induce clustering of any type. Plates coated with high concentrations of BSA resulted ultimately in dead floating single cells (see figure 2.15). For both cell lines, predigestion of Dm IV-3 with MMP-7 abrogated the clustering effect, favoring cell dispersion and spreading as seen with either BSA controls or other perlecan fragments. Quantification of the cell dispersion/spreading versus clustering behavior shown in figures 2.14E and F clearly demonstrates the strong clustering effect of Dm IV-3, and further that MMP-7 cleavage of Dm IV-3 destroys its clustering activity.

I wanted to know if Dm IV-3 was purely a non-adhesive phenomenon, and the clustering effect is secondary to cells being unable to attach. I performed a small experiment using a hanging drop method (10,000 C4-2 cells per 20 uL of 1% FBS T-media) and either added
1 µg of BSA or equivalent Dm IV-3. Figure 2.16 shows that despite being completely non-adhered, cells given Dm IV-3 actually cluster even more than BSA alone. Therefore, Dm IV-3 itself triggers cell-cell favored adhesion over substratum adhesion.

**Cell cluster dispersion activity by MMP-7**

To understand the context specific activities of soluble and substratum-fixed Dm IV-3, I carried out the experiments shown in figure 2.17. Plates pre-coated with Dm IV-3 were simultaneously seeded with C4-2 cells and a digestion mixture of either control BSA, MMP-7 and BSA, IV-3, IV-3 and MMP-7, or the Dm IV peptide used in previous studies (Farach-Carson et al. 2008). Dm IV-3 with control BSA produced clustered cells, as before. Soluble Dm IV-3 enhanced clustering, but only slightly. Soluble Dm IV-3 or BSA pre-incubated with MMP-7 strongly reversed the Dm IV-3 induced clustering in comparison to control BSA and IV-3. The PCa pro-adhesive peptide (Dm IV peptide that lies within Dm IV-3) did not counteract the effects of Dm IV-3 as a cluster inducing substrate. Together, these experiments provide evidence that Dm IV-3 induces positive clustering effects on PCa cells and that MMP-7 activity reverses this to favor cell dispersion and spreading normally associated with cancer.

**Cell signaling changes induced by perlecan domain IV-3**

Attempts were made by quantitative polymerase chain reaction (qPCR) and western blot to discriminate the changes induced by Dm IV-3 (and by proxy perlecan). However, results obtained in Appendix F could not be replicated in this lab. This was attributable to slightly different cells lines being used (LNCaP transfected with RANKL and neomycin cassette versus untransfected cells). Regardless, I sought to understand the
clustering effects of Dm IV-3 on C4-2 cells using an RPPA, an antibody array capable of probing over 200 different type of proteins and phosphorylation states. Cells were plated on either BSA or Dm IV-3 +/- MMP-7 for 48 hrs and analyzed by the RPPA. Additionally, cells were plated on Dm IV-3 that had been heated to 60°C for 10 min in 1:5 volume PBS. As seen in appendix D IV-3 heated to this temperature increased the phenotypic clustering effect. Effectively, this additional triplicate resulted in the same signaling pathway activation as Dm IV-3 without MMP-7. From the array, the largest change (approximately 4 fold) was in the Src family kinase activation (SFK) at tyrosine 416 between Dm IV-3 alone and Dm IV-3 preincubated with MMP-7. Dm IV-3 (and heated Dm IV-3) had strong activation of SFKs while cleavage of Dm IV-3 with MMP-7 completely abolished the effect. Initial western blots confirm this array discriminated activation of SFKs are seen in figure 2.18A. Additionally, this same band was not induced by the hanging drop method (figure 2.18B). However, this band may not be the actual Src band but another band of unknown origin. Dasatinib (a SFK inhibitor) specifically inhibits another band that is below 60 kDa and is much stronger in signal as seen in figure 2.18C. Changing the western blot procedure also eliminated the higher band seen before (figure 2.18C) and accentuated the smaller band that is of the SFK origin.

**Discussion**

The overall goals of this study were to identify proteolytic enzymes present in the tumor microenvironment that could be responsible for cleavage of perlecan in matrices and at tissue borders, and to determine what, if any, cellular behaviors are influenced by perlecan
processing by proteases. A likely candidate proteolytic enzyme with the potential to cleave perlecan was MMP-7 or matrilysin. MMP-7 expression and activity have been well linked to cancer and PCa, specifically (Cardillo et al. 2006; Hart et al. 2002; Hashimoto et al. 1998; Lynch et al. 2005; Powell et al. 1993). MMP-7 has many demonstrated ECM substrates including collagen I, III, IV, V, fibronectin (Woessner & Taplin 1988), vitronectin (Imai et al. 1995), and aggrecan (Fosang et al. 1992). MMP-7 may dissolve the BM well in vivo, but its ability to digest perlecan, a critical BM component, to this point had been unexplored. MMP-7 earlier has been reported to digest unspecified cartilage derived “proteoglycans” (Woessner & Taplin 1988), and it was hypothesized that this might include perlecan. The in silico prediction’s top 18 sites ranked above 99% in Specificity, indicating perlecan was likely to be a strong substrate. The top ranked MMP-7 in silico digest of full length perlecan predicted cleavage to favor production of large Dm IV fragments, which was subsequently verified using either BM or intact perlecan as substrates for digestions.

Current in vitro experiments verified the substrate recognition of full length perlecan by MMP-7. Unlike other MMPs such as MMP-3/stromelysin (Whitelock et al. 1996), MMP-7 can extensively cleave perlecan even with the HS/CS chains intact. The lack of GAG chain inhibition also was observed with aggrecan, which is cleaved by MMP-7 in the keratan sulfate rich interglobular domain (Fosang et al. 1992). The ability to digest perlecan with intact GAG chains might have been predicted because MMP-7 binds to and is activated by highly sulfated heparin-like regions potentially found on perlecan (Ra et al. 2009; Yu & Woessner 2000). Therefore, perlecan GAG chains potentially could serve as an available docking site for efficient proteolysis during cancer invasion, releasing cryptic
fragments and GFs associated with perlecan’s protein core and HS/CS chains. Interestingly, MMP-3 does not bind heparin sepharose (and by extension HS) so the protease would not have the advantage of MMP-7 (Whitelock et al. 1996). In the context of the BM, our new data suggests perlecan is cleaved from the C-terminus inward given the continued recognition by the domain I antibody, which was not observed when perlecan was incubated alone with MMP-7. Earlier reports that the C-terminus of perlecan is exposed more in the BM lattice at the stromal interface add credence to the results (Heremans et al. 1989). Additionally, MMP-7 releases Dm IV positive fragments either when incubated with intact perlecan or perlecan in the BM complex. Interestingly, the fragments are nearly identical in apparent molecular weight despite the different presentation. Figure 2.9 suggests that the most commonly shared fragment from each digestion type (BM, full length perlecan, Dm IV-3, and conditioned media) is below 41 kDa, and that it correlates strongly to band 3 from figure 2.6. While placental BM extract has detectable Dm I and Dm IV positive smeary bands that disappear upon MMP-7 incubation, the primary tight band is slightly below 200 kDa. Unknown is if this perlecan band represents a pre-digested product or a potentially novel variant perlecan form specific to placental tissue. Regardless, the experiment demonstrates that perlecan fragments are generated by MMP-7 activity even when perlecan is bound into a complex BM network. Invasive cancers, including PCa, degrade ECM proteins in the BM and in tissues and in the process may release perlecan fragments. Especially interesting will be determining if perlecan fragments increase in patients with early metastatic bone lesions, given the considerable presence of perlecan in bone marrow (Klein 1995). These studies were performed to some extent as described in the next chapter.
The proteolytic release of perlecan derived fragments also is relevant to several biological processes beyond cancer cell invasion through perlecan-rich matrices. As a proteomic screen for premature rupture of amniotic membranes, a 19 kDa fragment of perlecan was found only in the amniotic fluid but not the maternal blood of pregnant females (Vuadens et al. 2003). Additionally, a C-terminal fragment of perlecan was found in the follicular fluid, but not serum, of women undergoing successful in vitro fertilization (Jarkovska et al. 2010). A 25 kDa C-terminal fragment of perlecan also was found in urine of hemodialysis patients with end stage renal failure (Oda et al. 1996). Even a 90 kDa fragment derived from perlecan Dm IV is high in the vitreous humour and serum of a chick embryo, but rapidly disappears upon hatching (Hummel et al. 2004). By far the most studied bioactive perlecan fragment is Dm V/LG3/endorepellin (Mongiat et al. 2003) that is released by the proteases BMP-1 and cathepsin-1 (Gonzalez et al. 2005; Cailhier et al. 2008). The bioactive fragment affects angiogenesis by modifying the behavior of endothelial cells and vascular smooth muscle cells (Bix et al. 2004). Also, following ischemic stroke in a rodent brain model, perlecan Dm V is proteolytically released to mediate angiogenesis ultimately being neuroprotective (Lee et al. 2011). These studies demonstrate perlecan as an intact molecule can induce distinctly different behaviors than do its protease produced fragments, consistent with the findings of the current study with MMP-7. Perlecan degradation and fragment release may be common to basic development and pathologies involved with ECM turnover and it will be interesting to explore further the contribution of Dm IV fragments.
Every subdomain of Dm IV was found to be susceptible to MMP-7 digestion despite the lack of predicted high confidence cleavage sites. This finding re-emphasizes that MMP-7 has promiscuous substrate specificity, potentially more dependent upon interaction with the 3D protein structure than the specific primary substrate sequences. Other extracellular proteolytic enzymes were incubated with perlecan, but MMP-7 was the only enzyme with high proteolysis ability directed toward perlecan. In fact, none of the other enzymes tested (PSA, hepsin, and FAP) cleaved intact perlecan with or without its HS chains. FAP is a protease that is upregulated in reactive stroma surrounding PINs and PCa tumors (Tuxhorn, McAlhany, et al. 2002), but only a short list of physiological substrates exists (Kelly et al. 2012). FAP only was able to produce fragments on a specific domain of perlecan in combination with MMP-7. Similarly, FAP only produces collagen I and III degradation peptides after pre-digestion with MMP-1 (Christiansen et al. 2007). PSA degrades some ECM components including laminin and fibronectin (Webber et al. 1995), but this is the first report of perlecan as a weak substrate. However, the recognition is only of a small portion of perlecan, indicating that prior digestion by another proteolytic enzyme is likely to be necessary. The same may be true of hepsin, which downshifted Dm IV-3a migration only very slightly. Hepsin has a confirmed ECM molecular cleavage site within laminin-322’s β-3 chain (Tripathi et al. 2008). The resulting hepsin-generated laminin fragment induced migration in LNCaP cells (Tripathi et al. 2008). In this regard, it may be possible for hepsin to release cryptic fragments of perlecan if the right substrate is presented. Overall, this demonstrates that MMP-7 can efficiently cleave perlecan, and its action may generate new substrate peptides for other extracellular proteases to digest. Based on this evidence, we proposed that MMP-7 remains the strongest candidate responsible for the in
in vivo perlecan BM and stromal matrix destabilization necessary for cancer invasion and migration to occur during metastasis.

The site specificity of MMP-7 for perlecan falls in line with the established specificity matrix found in the MEROPS database (http://merops.sanger.ac.uk/cgi-bin/pepsum?id=M10.008). Notably, MMP-7 has the same preference for leucine in the P1’ site, and a slight preference for glutamine at the P1 site and for proline at the P2 site in perlecan as reported in MEROPS. Multiple bands with the same N-terminus as in bands 1, 2 and 3 suggest perlecan is sequentially degraded by MMP-7, perhaps preferentially cutting at the N-terminus sequenced site and degrading from the C-terminus inward to create sub fragments. In the process, multiple peptides in the 5-20 amino acid range are released. Testing the biological effects (e.g. adhesion, motility, and chemotaxis) of these specific peptides and protein limit fragments on cells related to PCa, including the LNCaP derived cell line, bone stromal cells, and immune surveillance cells could be done in follow up studies. PHYRE modeling reveals that MMP-7 initially may cleave at transition areas (between Ig repeats and between β sheets) to reveal additional sites for cleavage. This type of preference suggests that perlecan Dm IV is tightly structured and needs to be loosened before additional cleavage can occur, which coincides with mouse Dm IV structure observed through rotary force shadowing (Hopf et al. 1999). Additionally, we found discrepancies between the expected and observed MW of Dm IV-3 by approximately 4-5 kDa. Likely, Dm IV-3 is O- or N-glycosylated but not decorated with GAG chains as was determined previously in mouse derived Dm IV (Hopf et al. 1999).
The ability of MMP-7 to destabilize the BM and increase the invasive/migratory capacity of C4-2 cells must involve perlecan degradation. Perlecan acts as an organizer of the BM matrix, linking together collagen, laminin, and nidogen into a network (Behrens et al. 2012; Farach-Carson et al. 2014). With perlecan degraded by MMP-7, the matrix is expected to be compromised, providing an escape route for invasive cancer cells. Besides a mechanical border, perlecan degradation creates cryptic bioactive fragments. It is likely that MMP-7 acts upon perlecan in a fashion similar to that by which MMP-2/gelatinase acts upon laminin-5. MMP-2, which does not effectively cleave perlecan, cleaves laminin-5 exposing a pro-migratory cryptic site that induces breast cancer cells (MCF-7) to become motile (Giannelli et al. 1997). MMP-7 processes other ECM proteins to generate bioactive protein fragments, such as neostatin-7 derived from collagen XVIII (Chang et al. 2005). The same phenomenon is likely to occur during MMP-7 perlecan fragmentation.

Full length perlecan and a subpart Dm IV-3 induced a strong clustering effect when presented as a fixed substratum. The effects were observed with three PCa cell lines. The poorly adherent C4-2 clusters are highly reminiscent of C4-2B cell morphology that was observed previously in 3D hyaluronic acid based hydrogels (Gurski et al. 2009). The effect is unlikely to be entirely from non-adherence, given that heat inactivated BSA, a non-adherent protein, adsorbed to plates did not induce this clustering when present at the same concentration. When plates are completely blocked with BSA, the cells eventually die. Moreover, other subdomains, IV-1 and IV-2, did not produce this strong clustering effect at the same coating amount. Also, IV-3 induced further cell-cell adhesion in hanging drop C4-2 cell culture over BSA/media alone, showing that it is not just a non-adherence phenotype but perhaps a repellent cue.
For full length perlecan, the intact GAG chains were thought to be the mediator of non-adherence/clustering, but Dm I which contains HS/CS chains did not strongly induce the effect even at higher molar concentrations. Removal of the GAG chains to expose further the perlecan core protein, as may happen when cancer cells produce heparanase (Reiland et al. 2004), may reveal a cryptic binding site. Therefore, the actual clustering effect for full length perlecan may be exposed at Dm IV to affect the cells, but removing the chains or through limited cleavage by MMP-7 reveals a stronger acting adhesive site on perlecan.

Our findings that MMP-7 digestion reverses the clustering effect of perlecan and Dm IV-3 hints that a receptor/Dm IV-3 complex that induces the clustering may exist. Currently being investigated is the functions of the various fragments and peptides produced by Dm IV-3 degradation by MMP-7. Previously, an 18 amino acid peptide from Dm IV was found to have pro-adhesive and focal adhesion kinase (FAK) activating properties on certain cell types (Farach-Carson et al. 2008). Dm IV-3 contains this peptide, specifically after the N-terminal sequenced amino acid cleavage site 3481 in figure 2.8. Adding this peptide in solution when seeding cells, however, did not overcome the pro cell-cell contact induction by intact Dm IV-3. This suggests that there is a strong pro-clustering peptide sequence or tertiary structure that is destroyed by MMP-7 proteolysis. This is further supported by the observation that addition of soluble MMP-7 (either pre-incubated with BSA or IV-3) prevented the clustering of adsorbed Dm IV-3 on the plate. It is interesting to speculate that the switch between cell-cell clustering and cell dispersion induced by MMP-7
digestion of perlecan-rich BM may be mimicking an epithelial to mesenchymal transition occurring in these cells as they acquire invasive potential.

The degree to which MMP-7 and perlecan interact in PCa progression and metastasis remains unknown; however, in other cancers an association has been demonstrated. While oral carcinoma in situ has an epithelial MMP-7 expression, squamous cell carcinoma has a more stromal appearance, matching perlecan stromal expression (Tilakaratne et al. 2009). MMP-7 also shows enhanced stromal expression in higher grade PCa cancer (Cardillo et al. 2006). In chicken cornea, active wound healing stromal sites, which are analogous to reactive stroma in cancer, upregulate perlecan expression as determined by staining with a Dm IV antibody (Ritchey et al. 2011). Therefore, perlecan deposition in reactive stroma may be a widespread phenomenon. If perlecan and MMP-7 co-localize at reactive stromal sites, MMP-7 is likely to be active. PCa and reactive stromal compartments, in particular, have a high oxidative character (Josson et al. 2010; Platz & De Marzo 2004). Oxidative species in vitro activate MMP-7 by removing the pro-domain inhibiting cysteine from the active site (Fu et al. 2001). Additionally, oxidative species themselves can destabilize the BM and reactive stromal proteins, including perlecan, making them more susceptible to proteases (Kennett et al. 2011). Therefore, studies have been performed with clinical specimens to determine if perlecan and MMP-7 co-localize in PCa tissue as seen in the next chapter.
Figure 2.1 MMP-7 cleavage of full length perlecan \textit{in silico} and \textit{in vitro}. A) Perlecan sequence (UniProt: P98160) was queried against MMP-7 proteolytic sites with online Site Prediction software under default conditions (http://www.dmbr.ugent.be/prx/bioit2-public/SitePrediction/index.php). Shown are the top ranked 18 positions by Average Score not including the signal sequence. All have specificities greater than 99% with a majority of the predicted sites occurring in domain III and domain V. The asterisks indicate the approximate epitope site of the A71 monoclonal antibody (in domain I) and 3135 polyclonal antibody (in domain IV). B) Western blot (A71) of HT-29 (formerly WiDr) purified perlecan either pre-treated or not with combination of heparitinases I, II, III and chondroitinase ABC (H/C) and then incubated with or without MMP-7 (2.5 hr at 37°C 4 μg perlecan, 0.4 μg MMP-7). Lane 1: Control perlecan incubation with no enzyme; Lane 2: perlecan with MMP-7; Lane 3: perlecan pre-treated with H/C; Lane 4: perlecan pretreated with H/C and incubated with MMP-7; Lane 5: H/C combination control. C) Western blot (3135) of an 8 hr time course digestion of perlecan with or without MMP-7. The arrows indicate discrete fragments of approximately 50 kDa and 35 kDa after incubation with MMP-7, but absent without.
Figure 2.2 MMP-7 time course digestion of placental human basement membrane (BM) extract. Polymerized BM extract was incubated with or without MMP-7 for various time points up to 8 hrs and subjected to A) silver stain, B) antibody A71 western blot, or C) antibody 3135 western blot. The first lane in A and B is the MW standard marker and the second lane contains control BM never polymerized. The black arrowhead indicates high molecular weight glycosylated protein. The white boxes in panel A highlight bands created or destroyed with MMP-7 digestion. The black boxes indicate the primary MMP-7 band, not digestion products. The white arrow is the primary A71 and 3135 positive perlecan band and the black arrows highlight the bands generated during MMP-7 incubation.
Figure 2.3 Recombinant perlecan Dm IV cleavage by MMP-7. A) Diagram of recombinant subdomains Dm IV-1, 2, 3, and Dm 3a compared to full length perlecan. B) Digestion of recombinant domain IV protein by MMP-7. Various subdomains of Dm IV: IV-1 (lanes 1, 2), Dm IV-2 (lanes 3, 4), Dm IV-3 (lanes 5, 6), Dm IV-3a (lanes 8, 9) were incubated alone or with MMP-7 (1 µg of Dm IV with 0.1 µg MMP-7) overnight at 37°C. Shown is a silver stain after SDS-PAGE separation on a 4-12% (w/v) acrylamide gradient gel. MMP-7 can cleave every subdomain. The black boxed portion in lane 7 (MMP-7 alone) indicates the MMP-7 band.
Figure 2.4. PNGase F digestion of Dm IV-3a. Silver stain of Dm IV-3a that was incubated with or without PNGase F (from NEB, catalogue no. P0704S) according to manufacturer’s instructions. With PNGase F, but not control, Dm IV-3a has a slight downshift in molecular weight, indicating Dm IV-3a has N-linked glycosylation.
Figure 2.5 PSA, hepsin, and FAP Dm IV susceptibility. A.) Perlecan Dm IV-3a was incubated with MMP-7, hepsin or prostate specific antigen (PSA) for 37°C for 2.5 hr and western blotted for the C-terminal polyhistidine tag. Neither intact perlecan nor any other subdomain besides Dm IV-3a demonstrated susceptibility to hepsin or PSA. B.) A silver stain after perlecan Dm IV-3 was incubated alone (lane 2), with MMP7 (lane 3), fibroblast activation protein (FAP) (lane 4), or in combination (lane 5). Boxed portion indicates new bands generated during MMP-7/FAP combination digestions of Dm IV-3. Lane 1 is the molecular weight standard and lane 6 was left blank. FAP alone had no effect on perlecan or its subdomains, and cleaved perlecan sequences only in combination with MMP-7.
Figure 2.6 Perlecan Dm IV-3 cleavage patterns by MMP-7. A) The Coomassie stained PVDF membrane of Dm IV-3 either alone (intact) or incubated with MMP-7. The five primary protein bands were analyzed for N-terminal sequencing by Edman degradation. B) Likely cleavage patterns for each band of MMP-7 digested Dm IV-3 given the sequenced N-termini, MALDI mass measurements and LC-MS/MS identified peptides. The left side of each indicated fragment identifies the band N-terminus sequencing found, and the right indicates the likely C-terminus amino acids (amino acid, aa) MMP-7 cleavage site and the resultant apparent molecular weight (kDa) that corresponds approximately to the masses found by MALDI and in the Coomassie stained PVDF. Above intact Dm IV-3 are the PHYRE modeled Ig modules with the indicated MMP-7 cleavage sites shown in red. Cleavage mostly occurs at amino acids in the linking region between Ig modules or in the middle of the Ig module linking the interfacing beta strands. Additionally, each MMP-7 cleavage site produces an aliphatic N-terminus such as leucine, isoleucine, or valine. The small black rectangle at the end of the Dm IV-3 schematic indicates the V5 epitope and polyhistidine tag. The asterisks indicate the 3135 polyclonal antibody binding site on Dm IV-3.
Figure 2.7 MALDI generated peaks of Dm IV-3 cleavage by MMP-7. Panels show matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) generated peaks from intact and MMP-7 digested PLN Dm IV-3. A.) MALDI MS peak of intact PLN measures an approximate mass to charge ratio (m/z) of 79.2 kDa. B-E) MALDI MS measurements of PLN Dm IV-3 digested with MMP-7. Each panel represents a different range of m/z for resolution of peaks.
Figure 2.8 MMP-7 cleavage generated Dm IV-3 sequences discriminated by mass spectrometry. Sequence of PLN Dm IV-3 with MMP-7 cleavage sites identified through both N-terminal sequencing and LC-MS. The underlined portions are not part of the natural PLN Dm IV-3 sequence. The paragraph break points indicate the N-termini sequenced through Edman degradation. The gray and pink highlighted sequences (separate colors to distinguish adjacent cut sites) are peptides found through LC-MS (found in supplemental Table 2.1). If the gray or pink highlighted sequence becomes darker in shade and/or italicized, a smaller peptide was found within the larger highlighted sequence. The red dipeptides indicate the P1 and P1′ site that matched the cut sites predicted by Site Prediction when this Dm IV-3 sequence was queried. The orange-colored amino acids represent the approximate transition points between adjacent immunoglobulin (Ig) repeats, where to the left and right of the amino acid are different Ig repeats.
**Figure 2.9 MMP-7 mediated cleavage of perlecan, conditioned media, basement membrane, and Dm IV-3.** Different preparations of perlecan were incubated alone (-) or with (+) MMP-7 and western blotted for perlecan Dm IV (antibody 3135). Lanes 1/2: Basement membrane (BM) extract; lanes 3/4: A74 purified perlecan; lanes 5/6: perlecan domain (Dm) IV-3; lanes 7/8: 20X concentrated HT-29 (formerly WiDr) conditioned media (CM) processed with a 100 kDa molecular weight cutoff membrane. Each digested lane produces a band below the 41 kDa marker (white arrowhead). In lanes 5 and 6, the IV-3 signal was so intense that it was covered partially for most of the development.
Figure 2.10 Transwell® invasion assay of C4-2 PCa cells through a basement membrane (BM) extract. A) BM extract was incubated in serum free medium either with or without MMP-7 over 18 hrs to replicate the conditions in the Transwell® invasion assay. A western blot using 3135 antibody reveals the same fragmentation pattern as does digestion in the presence of detergents. Images of crystal violet stained C4-2 cells that have entered the Transwell® membrane through (B) BM extract alone, (C) BM extract pre-digested with MMP-7, or (D) BM extract coated and then digested on the plate with MMP-7. E) Quantification of invasion assay on various conditions: 1) BM extract alone; 2) BM extract pre-digested with MMP-7; 3) BM extract coated and then digested with MMP-7. Unpaired student’s t-test comparing to condition 1; *= p value <0.05.
Figure 2.11 Full length perlecan’s effect on C4-2B cells over time. C4-2B cells were seeded into wells coated with PLN (A-C), BSA (D-E), or tissue culture plate only (F-I) and imaged over a 96 hr period. Cells on PLN begin clustering at one hr and is more pronounced later (15 hrs) (white boxed inset enlarges clusters). The cells survive for at least 4 days. Cells seeded on BSA coated plates lack any observable difference between the plates only, becoming adherent and spreading out in both cases.
Figure 2.12 Effect of perlecan enzymatic processing on C4-2B cells. C4-2B cells were seeded onto various types of perlecan (PLN) and control samples pre-adsorbed to the plate and imaged by phase bright field and then stained for F-actin (green) and nuclei (blue) after 1 hr. PLN digested with a mixture of heparitinases and chondroitinase ABC (H/C) (panels D-F) or MMP-7 (panels G-I) adhered to the plates within one hr and remained attached through the staining procedure (E, F and H, I). Neither intact PLN (A), MMP-7 alone (B), nor H/C mixture alone (C) supported this rapid adherence to substratum, and cells remained round or formed loose clusters. Boxed insets provide an enlarged image to show cell morphology.
Figure 2.13 C4-2B response to perlecan and Dm I of perlecan. Full length perlecan (A) and Dm I (B) are coated onto dishes and seeded with C4-2B cells. Shown is 24 hrs later at 10x objective. Perlecan causes an increased clustering phenotype absent in Dm I coated dishes at 24 hrs post seeding.
Figure 2.14 Cell clustering induced by perlecan Dm IV-3 and reversed by MMP-7. Cell clustering induced by perlecan Dm IV-3 and reversed by MMP-7. C4-2 (A, B) and PC-3 PCa cells (C, D) seeded on adsorbed Dm IV-3 (A, C) or Dm IV-3 pre-digested with MMP-7 (B, D). Cell clustering occurred for both cell types on Dm IV-3 but was reversed with MMP-7 digestion. Quantification of cell dispersion is shown for C4-2 (E) and PC-3 (F) cells on various matrix types. A lower value is indicative of cell clustering. A *** indicates a p value <0.005 and ** is a p value <0.01 in comparison to Dm IV-3 + MMP-7 using an unpaired student’s t-test.
Figure 2.15 Total BSA plate blocking effects on C4-2 cells. C42B cells plated on purified perlecian and BSA control. 96 well plates were coated with either 5 µg of BSA (A, B) or 5 µg of perlecian (C, D) overnight at 4°C. After blocking with additional BSA for 1 hour at room temperature, 5000 cells/well were plated and observed 1 day (A,C) and 5 days (B,D) later. Scale bar represents 100 µm.
Figure 2.16 Hanging drop C4-2 cell culture with soluble IV-3. C4-2 cells were suspended at 10,000 cells per 20 µL drop either with 1 µg of BSA (A), 0.5 µg of soluble Dm IV-3 (B), or 1 µg of Dm IV-3 (C). As the concentration of Dm IV-3 increases, so does the cell-cell adhesion. Taken at 4X objective.
Figure 2.17 Soluble digest mixtures and peptide effect on dispersion/spreading of C4-2 cells introduced to a Dm IV-3 substrate. Dm IV-3 was adsorbed to wells and seeded simultaneously with C4-2 cells with the following overnight digests in soluble form: IV-3 alone, IV-3 incubated with MMP-7, BSA alone, BSA incubated with MMP-7, and 20 µg/mL Dm IV peptide. The lower the value the more clustered (left representative image) and the higher the value the more dispersed/spread (right representative image) and are presented in % relation to control BSA addition. Significance values are in relation to control BSA soluble addition; * p <0.05, *** p <0.001.
Figure 2.18 Src family kinase western blots of C4-2 cells. A.) Western blot and densitometry on nitrocellulose probing for the Src family kinase antibody phosphorylated at Tyr416 (phosphoSFK, Y416, Cell Signaling Technology, 2101) (below 72 kDa) and β-actin (at 46 kDa). A band increases with Dm IV-3. B.) Hanging drop (HD) does not induce the band as Dm IV-3 (on 2D substrate). C) PVDF membrane western blot of phosphoSFK, Y416 all on Dm IV-3 with or without MMP-7 and with increasing concentrations of dasatinib which inhibits SFK phosphorylation. This reactive band is lower than the bands found in A and B.
Table 2.1 The characteristics of MMP-7 generated perlecan Dm IV-3 peptides detected by LC-MS/MS and discriminated by Mascot. [Charge is z, mass is m, parts per million is ppm, Dalton is Da]
Chapter 3

As modified from Grindel et al, in preparation for submission

Perlecan/HSPG2, Matrilysin/MMP-7, and β2-Microglobulin as Indices of Tissue Invasion: Tissue Localization and Circulating Perlecan Fragments in a Cohort of 288 Radical Prostatectomy Patients
**Introduction**

PCa in the United States presents an estimated 233,000 new cases and over 29,000 deaths a year. While organ confined PCa has a five-year survival rate of nearly 100% in the US, metastasis reduces this figure to near 28% (American Cancer Society 2014). Further understanding the mechanisms of PCa metastasis are vital to identify the subset of PCa patients who will progress rapidly or relapse after hormone deprivation therapy, and which of these might benefit from more aggressive treatment earlier in disease course. Locally invasive PCa cells depend on proteases, such as MMPs, to move through the ECM to reach the final site of metastasis (Lynch & Matrisian 2002). PCa has a predilection for metastasis to bone marrow, where osteoblastic/sclerotic lesions are hallmarks (Ye et al. 2007). To arrive at the sites of distant metastasis, PCa cells must degrade and cross five separate borders containing perlecan/HPSG2. Perlecan is a multimodal five domain ECM proteoglycan with three N-terminal HS and variably CS chains attached to the core protein in Dm I (Murdoch et al. 1992). By interacting with a wide variety of GFs and ECM components, perlecan modulates many fundamental biological processes including angiogenesis, differentiation, growth, and early development (Whitelock et al. 2008). Given perlecan’s actions, it is not surprising that it is involved in cancer biology (Iozzo & Sanderson 2011). Perlecan is a major constituent of the glandular BM, the reactive stroma that surrounds the tumor in response to cancer produced signals, the vascular BM at sites of both intravasation and extravasation, and as a reactive reticular network in the bone marrow metastatic niche which is illustrated in figure 3.1 (Heremans et al. 1989; Farach-Carson et al. 2014; Klein 1995). With respect to PCa, it has been shown previously that
perlecan expression is upregulated as a part of the TNF-α induced desmoplastic response to expanding and invading PCa tumors (Warren et al. 2014).

The work in this chapter investigated the tissue and serum levels of perlecan and MMP-7 as well as those of the tissue modulator and activator of metalloproteinases, β2M. MMP-7 previously was found to cleave perlecan efficiently regardless of how it is presented: within intact BM, fully decorated with HS/CS chains, or as a recombinantly produced subdomain of domain IV. Notably, the MMP-7 induced degradation of perlecan completely reversed its ability to trigger PCa cell clustering, leading to tumor dyscohesion and favoring a migratory single cell phenotype (Grindel et al. 2014). MMP-7 is the single best catabolizer of perlecan core protein among over a dozen proteases that have been tested in our lab and others (Grindel et al. 2014; Whitelock et al. 1996; d’Ortho et al. 1997). MMP-7 is a small secreted zinc dependent endopeptidase composed of only an inhibitory pro domain and catalytic site with a wide range of substrates in the ECM (Dormán et al. 2010). The MMP-7 transcript ratio to its inhibitor: TIMP-1 is higher in more aggressive cancers and MMP-7 stromal deposition is increased with higher Gleason grade (Hashimoto et al. 1998; Cardillo et al. 2006). Forced induction of MMP-7 expression in DU-145 PCa cells increased invasion in a murine model of metastasis (Powell et al. 1993). Likewise, perlecan is increased in PCa tumor specimens over normal prostate (Datta et al. 2006). Therefore, this study sought to investigate the presence and levels of perlecan, MMP-7 and β2M in a cohort of 288 PCa patients undergoing radical prostatectomy from which both tissue and serum were collected using a standardized procedure.
β2M is best known as the ubiquitous protein necessary for major histocompatibility (MHC) class 1 presentation of antigens for immune recognition and development (Koller et al. 1990). However, β2M also is a GF implicated in inducing EMT and osteomimicry in PCa (Cher et al. 2006). It also plays a role in tissue homeostasis and can modulate ECM turnover by degradative enzymes, including MMPs (Moe et al. 2000). In fact, β2M as a GF increases MMP-1 secretion and activation but does not concomitantly increase its inhibitor TIMP-1 in synovial fibroblasts (Moe et al. 2000) Overexpression of β2M in PCa cells enhances bone metastasis in a mice model (Huang et al. 2006).

This study examined the levels of β2M in serum or tissue of PCa patients at time of prostatectomy, and determining whether it might be a useful marker for identifying tissues at high risk of metastasis. Additionally, I sought to learn if the expression of perlecan, MMP-7 or β2M in well characterized tissue specimens was correlated to indices of grade or progression, and to relate this to the presence of perlecan fragments and β2M in sera that might result from local or regional cancer invasion.

**Materials & Methods**

**Tissue and serum procurement and processing**

All tissue and serum specimens were collected with consent under institutional IRB approved protocols at the Winship Cancer Center, Emory University, Atlanta, GA. Samples were collected at the time of radical prostatectomy after a PCa diagnosis. Serum samples were centrifuged at 2,400xg for 10 min at 18 °C. The serum supernatant was removed, and stored in aliquots at -80 °C until used. Tissue samples were
formalin fixed and paraffin embedded (FFPE). From each tissue block a cancerous tissue and normal adjacent tissue core of 0.6 mm were removed and inserted into a recipient block. Tissue cores were obtained using a thin-walled, sharpened stainless steel tube instrument (Beecher Instruments, Silver Spring, MD USA). Recipient array paraffin blocks were sectioned at 5 μm onto glass slides for subsequent IHC.

**Subject Population**

Table 3.1 describes the population characteristics of PCa subjects participating in this study. The test population consisted entirely of men undergoing prostatectomy in whom cancers usually are more indolent. None of the subjects had observable or known bone or other soft tissue metastases at the time of surgery. Nevertheless, a subset of subjects experienced biochemical relapse/recurrence during the follow up period. Sera from 288 patients were analyzed and approximately half of these subjects (157) were used for the tissue microarray (TMA). It thus was possible to match the serum data to the IHC data for each subject in this study. Normal serum was pooled from 12 healthy male adult subjects and provided as a custom product by SeraCare (Milford, MA).

**Development of custom antibodies**

The rabbit anti-human perlecan antibodies were generated using a proprietary technology developed at Strategic Diagnostics, Inc. (Newark, DE). The eight genomic antibodies chosen for this study were designated 3100, 3105, 3107, 3132, 3134, 3135, 3136, and 3139 (see Figure 3.8) for locations on perlecan core protein). All are rabbit polyclonal antibodies that recognize unique and regularly spaced 100 amino acid perlecan sequences spanning the length of the molecule. Additionally, the rabbit polyclonal β2M antibody used in the
enzyme linked immunosorbant assay (ELISA) was generated using the same proprietary technology. All antibodies are the property of Strategic Diagnostics, Newark DE.

**Other antibodies used for immunohistochemistry**

The antibodies used for the tissue microarray (TMA) were the rabbit anti-HSPG2 prestige antibody (Sigma-Aldrich, St. Louis, MO), the mouse anti-MMP-7 ID2 antibody (EMD Millipore, Billerica, MA) which recognizes both the pro and active form of MMP-7, and the rabbit anti-β2M antibody (Dako, Glostrup, Denmark).

**Tissue microarray staining and quantification**

Staining for perlecan was performed as described previously (Warren et al. 2014). MMP-7 and β2M also were stained with variation in antigen retrieval method as noted below. Briefly, tissues were deparaffinized with xylene and graded ethanol, incubated with hydrogen peroxide in methanol, washed, and subjected to antigen retrieval. Perlecan was retrieved with bovine testicular hyaluronidase (Sigma-Aldrich) for 30 min at RT, MMP-7 was retrieved with Diva Decloaker (Biocare Medical, Concord, CA) for 30 min in a steamer, and β2M was left unretrieved per manufacturer instructions. Following blocking in Background Punisher (Biocare), tissue was incubated with primary antibody (anti-HSPG2, 1:100; anti-MMP-7, 1:200; anti-β2M, 1:800) overnight at 4 °C. The staining was visualized with Diagnostic Biosystems’ (Pleasanton, CA) Polymer Penetration Enhancer, anti-mouse/rabbit PolyVue horseradish peroxidase, and (3, 3’-diaminobenzidine) DAB chromogen. Nuclei were counterstained with CAT hematoxylin (Diagnostic Biosystems) and a sodium bicarbonate blueing agent. Tissue was dehydrated with graded ethanol and xylene and mounted in Cytoseal 60 (Fisher Scientific, Hampton, NH).
Each stained slide was imaged and quantified with a Leica Biosystems (Buffalo Grove, IL) instrument and software. For each core, a certified pathologist identified and circled several annotation types if present: normal gland (NG), Gleason grade 3, 4, 5 (G3/4/5), normal stroma (NS) and tumor-related stroma (TS). These regions of interest were quantified for “intensity values” (the lower the intensity the higher/darker the actual stain amount) by the Leica Biosystem software and converted to “concentration values” with a formula including absorbance and pixel area. Instead of using both values in this study we determined that intensity values and concentration values were correlated statistically to be inverse with a Pearson coefficient of -0.89 and a p value of 2.2E-16 (figure 3.2). Therefore, we could reliably use one value for this study. Concentration values were used in the detailed statistical study (see below) because concentration values actually increases with the amount of stain (darker) which makes understanding the associations with other factors such as serum protein quantities more straightforward.

**Sandwich ELISA setup and procedure**

All eight custom anti-perlecan antibodies, β2M antibody, and non-immune rabbit IgG negative control antibodies were printed in triplicate onto Whatman 16 Pad nitrocellulose Fast Slides (GE Healthcare, Pittsburgh, PA) with an Arrayit NanoPrint® (Arrayit, Sunnyvale, CA) instrument according to manufacturer’s specifications. Slides were placed in Whatman Fast Slide Frames (GE Healthcare) capable of isolating each nitrocellulose pad for individual processing. All steps were performed at RT on a 120 rotations per min (RPM) shaker unless otherwise stated. Pads with printed antibodies were blocked with Blockit® Blocking Buffer (Arrayit cat no. BKT) for 1 hr and washed 6 times with PBST.
PCa subject serum and normal serum was diluted by a third in PBST (phosphate buffered saline, 0.05% (v/v) Tween-20) (Sigma-Aldrich). PCa cell line PC-3 (American Type Culture Collection (ATCC), Manassas, VA, cat no. CRL-1435) in serum free conditioned media (F-12K (ATCC) medium with 1X penicillin/streptomycin (P/S) (Life Technologies, Carlsbad, CA) was diluted by half in PBST for a positive control consisting of full length perlecan. The samples were placed into eight separate pads, incubated for 1 hr and then washed 6 times with PBST. Detector antibodies conjugated to biotin were incubated individually on separate pads at 2 μg/mL in PBST for 1 hr followed by 6 PBST washes. Pads were incubated with 1 μg/mL streptavidin Cy-5 (GE Healthcare) in PBST for 1 hr. Following 6 PBST washes, pads were removed from cassettes, spin dried and scanned at 633 nm with a LS Reloaded™ Series Microarray Laser Scanner (Tecan, Mannendorf, Switzerland) and analyzed with Array Pro® Analyzer software. Intensity values were normalized to account for general background by subtraction. Background subtracted values were divided by buffer control (same pad, same spot) to control for pad to pad variability. This value then had the negative control rabbit IgG values subtracted from it. Therefore, the assay may produce negative values for analysis.

**Western blot and Coomassie stain of subject serum**

Serum samples were obtained from Bioreclamation, Inc. (Baltimore, MD). Four age and sex-matched normal sera (HMSRM BRH506649-52) and four stage IV prostate cancer sera (HMSRM-PROSTATE BRH506645-48) were analyzed. Aliquots of each subject were thawed, combined (320 μL total), Protease Inhibitor Cocktail added (Pierce, Rockford, IL) and diluted with PBS. Protein AG magnetic beads (25 μL) (Chromatrap®, Ashland, VA)
were added to the mixture and allowed to rotate end over end for 1 hr at 4 °C and 30 min at RT. Sera was removed and the beads washed 4x with PBS. After washing, 20 uL of PBS were added along with 5x SDS-reducing sample buffer (Pierce) and heated to 99 °C for 10 min. The supernatant was removed and separated with 4-12% (w/v) polyacrylamide SDS-PAGE in MOPS buffer (Life Technologies) and transferred to nitrocellulose (BioRad, Hercules, CA) in Tris-glycine buffer at 4 °C at 40 volts for 5 hrs. Membranes were blocked in 3% (w/v) BSA Tris buffered saline with 0.05% Tween-20 (TBST) for 2 hrs at RT. Antibody 3135 (1:10,000) was added to the block solution overnight on a 4 °C shaker. Following three by 5 min TBST washes, the membranes were incubated with 1:200,000 goat anti-rabbit HRP conjugated antibody in 3% BSA TBST for 2 hr at RT. Blots were washed again and incubated with chemiluminescence substrate (West Dura extended substrate, Pierce) for 5 min and exposed to film. For the Coomassie stain and N-terminal sequencing the protein was transferred to a methanol pre-soaked 0.22 μm pore PVDF membrane in 25 mM Tris, 192 mM glycine buffer with 20% (v/v) methanol at 50 V for 5 hrs at 4°C. The PVDF membrane was washed with distilled water several times to remove glycine background, and dipped in methanol again. The membrane was stained with 0.1% (w/v) Coomassie Blue R-250 in 40% methanol, 1% (v/v) acetic acid for 1 min, and destained with 50% methanol in water solution. After extensive MilliQ water washes, the membrane was left to air dry. The membrane was sent to Midwest Analytical Inc. Protein Sequencing (St. Louis, MO) for N-terminal Edman degradation protein sequencing.

**Statistical analysis**
Each statistical model utilized is described specifically along with the results of each study. In general a linear mixed-effect model was used to compare differences between and among groups. Staining concentration values were normalized by taking the square root. For strength of correlation, the Pearson correlation coefficient was determined. Significance was set a priori at \( p < 0.05 \).

**Results**

**Perlecan, MMP-7 and β2-microglobulin stain with formalin fixed paraffin embedded tissue**

Staining for perlecan in paraffin fixed tissue previously has been difficult, leaving many studies to resort to frozen tissue where tissue architecture is often difficult to preserve. Hence, initial effort in this study was devoted to developing a reliable stain for perlecan on paraffin fixed tissue such that it could be used with both archived and new tissue blocks. The perlecan antibody used in this tissue microarray was verified by the Human Protein Atlas project to work on FFPE tissue ([http://www.proteinatlas.org/ENSG00000142798/antibody](http://www.proteinatlas.org/ENSG00000142798/antibody)). However, the antigen retrieval protocol was modified to allow visualization of stromally deposited perlecan by pre-digestion with hyaluronidase. The stain obtained was generally lighter than from acidic heat induced epitope retrieval. However, there was less extracellular loss of perlecan and staining was typically not intracellular as often the case with earlier protocols, resulting in a superior stain overall.

Figure 3.3 displays the various staining patterns with the anti-perlecan antibody and controls to verify the specificity of the primary and secondary antibody detection systems,
and lack of background interference with analysis. Figure 3.3A shows a wide view of a representative prostate cancer tissue where perlecan stain is largely diffuse and extracellular, as it should be for a component of the territorial matrix. Additionally and as expected, the perlecan present in the vascular basement membrane is darkly stained (arrow). Negative and low staining cores also were found (figure 3.3B). Extensive digestion with proteinase K led to epitope destruction, and demonstrated the low background associated with the perlecan primary antibody (figure 3.3C), and the anti-GFP antibody shows the low background associated with the secondary visualizing system (figure 3.3D). The procedure commonly stained the perlecan-dense vasculature basement membrane heavily, but in general the thin glandular basement membrane remained difficult to visualize in many sections, attributable either to under retrieval or epitope blockade. The staining intensity of deposited perlecan in the stroma varied in intensity and was different in various sections, which was quantitated for the total specimen pool in Figure 3.6. Overall, the FFPE perlecan staining protocol was judged to be suitable to carry out statistical analysis.

The antibody used for detection of MMP-7 was specific for both the latent and active forms of the enzyme, meaning the protease is not necessarily active when stain is present. The antibodies for MMP-7 and β2M are commonly used in FFPE tissue IHC, and yielded staining patterns similar to that found in literature (see figure 3.4) (Cardillo et al. 2006; Mink et al. 2010).

Expression of perlecan, MMP-7, β2M
Protein expression patterns of perlecan, MMP-7, and β2M next were assessed by TMA in serial sections of the entire specimen pool (see Table 3.1). Each subject of the 157 used in this study provided two associated cores; a cancerous core and normal adjacent core. A new value was produced comparing the cancerous tissue with the normal adjacent stain concentrations ‘[cancer]/[normal]’ for G3/4 over NG and TS over NS. This created three new ratiometric values: G3/NG, G4/NG, and TS/NS. If a value was reported to be greater than 1.0, the tissue had more stain concentration in cancerous tissue than normal tissue, and the opposite was the case if the value was lower than 1.0. Figure 3.5 shows a standard box plot for the expression profiles of perlecan, MMP-7, and β2M based upon analysis of tissues from almost all subjects in this study. G5/NG values were not included because the low sample size (3-4 values) was insufficient for statistical relevance to be achieved. Below the graph is the number (N total) of mined annotations, the number of values above 1 (N>1), the percentage of values above 1 (% >1), and the mean value for each ratiometric comparison group. This value normalizes any slide-to-slide stain variability and accounts for inherent subject variability. If no variation in marker expression existed between normal and cancerous tissue, the mean would be 1.0 and the % > 1 would be 50%. As shown, perlecan mean [cancer]/[normal] value for G3, G4 and TS show an approximately 40-45% increase in cancer tissue stain over normal. Additionally, a majority of the values are above 1.0 (55-71%). MMP-7 showed the same trend as did perlecan, but the specimen to specimen variation was higher than that seen for perlecan (~8X). MMP-7 values varied from near 32 to below 0.031 (MMP-7 G3/NG), meaning some glands had as much as 32 times more or alternatively 32 times less stain in G3 versus its normal adjacent tissue counterpart. MMP-7 expression also demonstrated the highest [cancer]/[normal] mean
value deviation from 1.0 with 2.54 and 2.38 for G4/NG and TS/NS, respectively. The mean value is shifted up greatly for MMP-7 in TS/NS by a few “superexpressers”, because only 44% of total values are above 1.0, yet the mean is high at 2.38. The mean value shifts for β2M are modest in comparison to the other biomarker stains, save for G4/NG (1.40). In fact, the mean for TS/NS was almost exactly 1.00, indicating lack of a stromal response affecting β2M expression. Overall, these findings demonstrate the expression variability and heterogeneity in staining patterns between cancer and normal tissue within the patient specimen set studied. In general, the staining concentration for perlecan and MMP-7, but only one grade (G4) for β2M, for the entire tissue pool examined was higher in PCa versus normal for tissues of both glandular and stromal origin.

**Perlecan deposition is increased in Gleason grade 4**

Perlecan stain in tissues was quantified for concentration as described in Materials and Methods, and normalized for each assigned Gleason grade in addition to the normal gland. Figure 3.6 shows the results comparing overall perlecan stain for all tissues assigned a grade of G4 versus the NG. In contrast to figure 3.5, these results were analyzed over the absolute intensity values over the entire pool of G4 tumors, and not normalized to cancer vs. normal tissue for each subject specimen. The quantification of perlecan stain in normal vs. G4 tissues is displayed in figure 3.6A. Each dot represents a single specimen with the black bar as the mean and the gray bars as the standard deviation (SD) of the mean. The means differ with high significance (p value 0.0006) where G4 staining is higher for perlecan (20.15) than the normal gland (16.98). Figure 3.6B and 3.6C show representative images of perlecan stain (brown) with nuclear counterstain (blue) of normal (B) and G4
(C) prostate tissue. While the staining of the individual cells was largely low or absent, as expected for a secreted ECM protein, the immediate stroma around the cancerous lesions and the territorial matrix in between cells often had increased perlecan staining (figure 3.6C) over normal glands (figure 3.6B). Staining showed a pericellular pattern of perlecan within the gland (arrow in figure 3.6C) that was largely absent in the normal glands (arrowhead in figure 3.6B). This result corroborates the data shown in figure 3.5 where 71% of the G4 subjects had higher perlecan stain in cancer over their normal adjacent gland and a 44% increase in the mean ratiometric value.

**Perlecan and MMP-7 stain concentration are highly correlated in prostate tissue**

Next determined was if perlecan stain is correlated with MMP-7 stain, given that MMP-7 efficiently proteolyzes perlecan core protein (Grindel et al. 2014). Each cancer and normal tissue type was quantified for perlecan and MMP-7 stain concentration, normalized, and the correlation coefficients analyzed. Therefore, this correlation coefficient is not a direct measure of co-localization in series, but rather an index of whether within a given area of analysis, the levels of the two are likely to be correlated. Table 3.2 provides Pearson’s correlation coefficient values and associated significance (p values) for perlecan and MMP-7 staining in tissue sections with the annotations G3, G4, NG, and TS, and NS. In every annotation (save for G5, which was not analyzed due to low sample size) there was a positive correlation between perlecan and MMP-7 staining levels in the specimens. These correlation coefficients ranged from 0.45 (G3) to 0.65 (NS) with all significant p values less than 0.05. Therefore, perlecan and MMP-7 are statistically likely to be found together high or together low in the same encircled area of tissue, be it normal or cancerous, but do
not typically directly overlap. Figure 3.7 shows a representative stain showing the directly appositional localization of perlecan (A) and MMP-7 (B) in one illustrative serially sectioned tissue specimen from the TMA in G3 tissue. The immunostained areas are shown in brown and the nuclei are stained blue. The arrows placed in approximately the same location in serial sections shown in figure 3.7 indicate the junction where perlecan and MMP-7 intersect in the G3 tissue. While the regions of intense staining for the proteins do not overlap, they are found in the same area of the tissue, which can explain why the annotated stains are positively correlated with one another (positive correlation coefficients in Table 3.2).

**Perlecan fragment and β2-microglobulin variation in PCa subject serum**

Given the intersecting co-localization and statistical correlation of perlecan and its protease MMP-7 in tissues, I next sought to determine if perlecan fragments potentially created by MMP-7 lysis of perlecan substrate in tissues could be detected in the serum of the same prostate cancer patients and in serum of additional patients for whom we had serum collected at time of prostatectomy. Initial experiments demonstrated that perlecan fragments can be measured in serum, do not bind to the fibrin clot, and do not need to be analyzed in plasma, which would be problematic for this analysis. To test for serum fragments of perlecan, we developed a capture-detector ELISA utilizing a series of paired peptide sequence-specific rabbit polyclonal antibodies that we developed across human perlecan’s core protein spanning over 4,000 amino acids from Dm II to Dm V (Figure 3.8A). Intact perlecan and its fragments could be visualized by this method, spanning from peptide sequences recognized by antibodies 3100 to 3139 (“whole molecule”), in
conditioned media of cells secreting full length unproteolyzed perlecan (figure 3.8A, white bar). The far N-terminal region was omitted from this assay, as it is the site of attachment of the large GAG chains that sterically impede antibody access to the protein core. Every capture-detector pair that we employed produced signal when used to detect perlecan in conditioned media from cell lines producing large amounts of perlecan (positive control). When analyzing subject sera from prostatectomy patients, several antibody capture-detector pairs showed positive signal after normalizing for background buffer control only and non-immune rabbit IgG (figure 3.8A, B). The fragments in serum that we detected are shown below the molecule in black and gray shaded lines spanning from the various perlecan-specific antibody capture-detector pairs. Assaying mouse serum did not produce a detectable signal (data not shown). Despite assaying over 288 patients (12 subjects were omitted from the final analysis for erroneous signal), positive signal from the N-terminal recognizing capture antibody 3100 was never found. Therefore, the N-terminal portion of perlecan could not be recognized, which suggests epitope destruction likely via proteolytic fragmentation or retention of this portion of the molecule in tissue. The most robust and common signal was derived from antibodies recognizing sequences in domain IV in perlecan. Only the most C-terminal Dm V-IV antibody capture-detector pair, 3139-3135, in this assay produced a positive signal, pointing to enhanced cleavage at the C-terminus as well. Antibody pair 3136-3139 (the most C-terminal pair possible) was not included in the 288 subject assay, but it gave signal in preliminary experiments using a full 8x8 capture-detector array. We were unable to perform an 8x8 capture-detector array for all samples because of the subject serum volume constraints. In comparison to a pooled normal serum control, some peptides detected with the shown capture-detector pairs were significantly
elevated (p value <0.05 and <0.001) in cancerous sera over normal sera (gray diamond) using a student’s T-test (figure 3.8B). The Dm V fragment (3139-3135), however, was slightly higher in pooled normal when compared to cancer samples. In figure 3.8A the gray bars represent a statistical difference was found between the prostate cancer subjects and the normal pooled serum. The black bars were present, but not significantly different from the pooled serum.

In addition to perlecan, β2M, a potential perlecan regulator studied by our group, was analyzed in the same serum samples (Figure 3.8C) where β2M (1-4) was processed in conjunction with each of the four different perlecan detector antibodies (Biotinylated 3105, 3107, 3132, 3135). Thus, β2M levels were analyzed in quadruplicate per serum sample. Serum levels of β2M were found to be statistically higher than in a pooled normal sample (gray diamond) in each of the repeated assays (p value <0.001).

**Levels of serum perlecan are associated with MMP-7 stain concentration**

We next explored if any association existed between the MMP-7 expression in the tissue and its matched serum sample in terms of soluble perlecan fragments. The hypothesis being tested was that higher MMP-7 staining concentration would translate into greater proteolysis of any nearby perlecan at the “intersections” seen in the tissue arrays, and therefore would increase the levels of circulating perlecan fragments. Overall MMP-7 staining concentration per patient was compared through analysis of co-variance (ANCOVA) with the perlecan capture-detector pairs. Table 3.3 displays the statistics derived from the ANCOVA analysis. Shown are the capture-detector pairs and related F-values and p-values. Four of the peptides identified using indicated capture-detector pairs
shown in bold were found to be significantly associated with MMP-7 stain concentration. All of those perlecan peptides detected were derived from domain IV-IV antibody pairs, 3105-3102, 3107-3107, 3132-31312, and 3134-3132 with p-values less than 0.006. Therefore, overall MMP-7 prostate expression in cancerous subjects has an associative relationship with some portion of proteolyzed perlecan and associated fragment(s) present in serum.

**Evidence of perlecan domain IV fragments in serum of Stage IV PCa subjects**

This study largely focused on the tissue to serum relationship with regards to MMP-7 and its substrate, perlecan, at the time of radical prostatectomy and androgen withdrawal. None of the patients had known metastatic diseases at the time of resection. To examine if the fragments we identified were present in patients with metastatic disease, we conducted a small scale (4 subjects), study in which perlecan levels were assayed by western blot using the 3135 antibody in a pooled stage IV prostate cancer serum samples and a normal age/sex matched pooled control as seen in figure 3.9. Lane 1 shows the results of analysis of normal sera and lane 2 shows the stage IV prostate cancer sera. Attempts were made to immunoprecipitate with the perlecan specific antibody 3135, but the same results were obtained regardless of whether the protein AG magnetic beads only, non-immune rabbit IgG or the 3135 antibody were used as the precipitation agent. While not useful in immunoprecipitation, the 3135 antibody worked very well in detecting differences in perlecan fragments between the normal and stage IV sera in western blots. Three primary bands (100 to 200 kDa range) were found in the stage IV sera that are not present in the normal sera. The goat anti-rabbit secondary antibody by itself failed to detect these bands.
The secondary antibody only detected the IgG heavy chain protein at 50 kDa. Transfer to a membrane and Coomassie staining highlighted the differences in the bands between normal and cancer sera that were positive for the 3135 antibody reactivity (right arrows). Attempts to identify the bands by N-terminal sequencing were unsuccessful because of the high molecular weight resulting in low pM amounts of protein that were present. The dashed arrow above 54 kDa that was not recognized by the perlecan antibody was identified as complement factor C4. Nevertheless, these data demonstrate that as the disease progresses toward metastasis, new perlecan fragments in serum appear.

**Discussion**

Dynamic changes in the cancer and the stroma surrounding PCa foci play a major role in determining the course of the disease, including its ability to invade local connective tissue. Perlecan is a key component of the reactive stroma (Warren et al. 2014) that must be removed in order for cancer cells to invade and colonize nearby tissue. This study sought to reveal the relationship of levels of perlecan, perlecan fragments, the perlecan degrading metalloproteinase MMP-7, and a multifunctional tissue factor and activator of metalloproteinase, β2M, in PCa tissue and serum collected at the time of prostatectomy from a large 288 patient cohort. Retrieval conditions were improved to allow visualization of extracellular deposited perlecan in paraffin-embedded blocks, unlike a previous study that only revealed largely intracellular signal in prostate cancer samples (Datta et al. 2006). This allowed us for the first time to systematically analyze cancer-associated, stromal and extracellular staining of perlecan in organ confined prostate cancer as a potential index of grade.
A clear observation from this study is that the levels of perlecan were higher in many Gleason grade 3 and especially grade 4 prostatectomy tissues and surrounding stroma, compared to normal gland. In grade 4 specimens, 71% of cancer specimens had elevated expression of perlecan over normal gland. Likewise, more than half (55%) of tumor stroma had elevated expression compared to normal stroma. Perlecan is a highly conserved GF binding component of ECM that also plays a key role in separating cell layers in normal tissues (Farach-Carson et al. 2014). Earlier knockdown studies showed that perlecan augments VEGF-A and FGF2 mediated growth in cultured PCa cells, and in PCa xenografts where loss of perlecan decreased the overall tumor growth and tumor vascularization (Savore et al. 2005). The increased levels of perlecan seen in Gleason grade 4 tissues and stroma thus may indicate that increases in perlecan expression in the tumor microenvironment are part a tissue reaction to the presence of the cancer, and that increases in expression in the cancer cells may be part of an adaptive survival and growth response.

Cancer cells produce MMPs and other degradative enzymes that permit tissue invasion. In the chapter previous, I showed that MMP-7 is the most efficient protease examined in degrading perlecan in its native context (Grindel et al. 2014). In this study of perlecan and MMP-7 in 157 PCa tissues, perlecan stain was positively correlated with MMP-7 stain and both typically were found in apposition to one another. This relationship has been reported in one other cancer. In dysplasia of oral mucosa and oral carcinoma in situ, perlecan and MMP-7 were commonly juxtaposed to one another (Tilakaratne et al. 2009). Therefore, perlecan upregulation and subsequent MMP-7 mediated destruction may be a common feature among several cancers as they invade local tissues. This idea is consistent with our findings in this study that MMP-7 and perlecan immunostaining levels, together high or
together low, were highly correlated with each other in individual specimen blocks. Noted is the presence of MMP-7 does not mean it is active, as the antibody used recognizes both latent and active forms of the protease. The correlative co-expression of perlecan and MMP-7 observed in normal tissue (0.67 Pearson coefficient) can be explained if endogenous inhibitors (TIMP-1) are expressed along with MMP-7 as has been reported (Hashimoto et al. 1998). In cancerous tissue, MMP-7 expressing cells can come into contact with and degrade perlecan in the matrix, and, interestingly, it has been shown that certain HS moieties that could be present on perlecan can actually bind and activate MMP-7 (Yu & Woessner 2000), creating a positive “feed forward” response that could speed local tissue invasion. It is worth considering, in this context, that some patients who are “superexpressers” of MMP-7 in regions of high perlecan expression could be considered of higher grade than those without this condition, and that such patients might be at higher risk of rapid disease progression. Unfortunately, the number of patients having a biochemical relapse during this prostatectomy study was insufficient to determine if this is the case.

Perlecan and β2M are reported to be co-localized in hemodialysis induced β-amyloidosis where perlecan acts as an early driver of fibrillogenesis (Ohashi 2001). Additionally, β2M was shown to increase the production of MMPs but not the corollary TIMP in human synovial fibroblasts (Moe et al. 2000). Perhaps a positive association could also exist for MMP-7 and β2M in prostatectomy specimens. However, co-localization was not observed in this specimen set. While perlecan and MMP-7 levels were found to have a positive correlation in tissue, β2M levels were not correlated directly with either in tissue
specimens. Thus, while β2M may play a role in cancer biology, it seems unlikely that this occurs as a direct modifier of the perlecan-MMP-7 tissue invasion pathway.

Reasoning that destruction of matrix perlecan by MMP-7 at invasion borders should be detectable by the presence of perlecan-derived fragments in serum, I examined serum from the full 288 prostatectomy patient cohort. By creating a panel of antibodies recognizing perlecan from one end to the other and using a reliable automated capture-detection epitope mapping assay, fragments in patient sera were detected that were largely derived from the 21 Ig repeat domain IV of perlecan that spans almost half of the molecule (Farach-Carson et al. 2008). This was not entirely unexpected given the domain’s length and the existence of multiple predicted and demonstrated sites for proteolysis in this region (Farach-Carson & Carson 2007). The epitope mapping assay would have identified full length perlecan if it were present in serum, as positive signal for nearly the whole molecule (Dm II to Dm V) was found in conditioned media of perlecan secreting cells and when purified full length perlecan was added to the assays. Thus, the inability to detect full length perlecan in the sera of prostatectomy patients indicates that tissue perlecan must be at least partially degraded in order to enter the serum. Also, the pattern of perlecan peptide in serum that was detected indicates that perlecan cleavage occurs predominately from the C-terminus, which is consistent with our previous finding that MMP-7 cleaves domain IV inward from the C-terminus in vitro (Grindel et al. 2014).

Interestingly, levels of some perlecan capture-detector pairs or fragments were significantly increased in prostatectomy patients over those in pooled normal sera, which is consistent with increased perlecan turnover during cancer invasion of tissue. The normal
serum pool was not, however, completely devoid of signal, as cancer is not the only condition that is expected to trigger matrix turnover. Confounders that could contribute to perlecan degradation and fragment presence in the serum of normal subjects should be considered. Perlecan destruction and release into serum is expected to occur during atherosclerotic associated intimal hyperplasia (Newby 2005), arthritis (Dodge et al. 1995), or any other inflammatory or acute wounding condition that may result in ECM destruction, including that in perlecan-rich cartilage joint disease (Srinivasan et al. 2012). Some low level of perlecan also is expected to be turned over in normal tissue homeostasis, such as during immunosurveillance or bone remodeling. For example, perlecan was shown to be turned over constitutively in the glomerular basement membrane (Beavan et al. 1989).

Nonetheless, the finding that specific perlecan fragments were elevated in serum in conjunction with MMP-7 elevation in tissue suggests that there are fragments derived from perlecan that may be useful indices of cancer tissue invasion. Current studies in the Farach-Carson laboratory seek to develop useful and specific probes for MMP-7 created fragments in tissue.

Because of its association with tissue destruction, it was suspected that patients with active bone metastases also would have elevated perlecan fragments in serum. None of the 288 patients in the original prostatectomy cohort had detectable metastasis at the time of surgery, so this question was addressed with a separate, smaller, serum pool from patients with active metastatic disease. The domain IV antibody, 3135, was used for this study because it consistently recognizes perlecan very specifically in a variety of perlecan preparations (e.g. conditioned medium, basement membrane extract, domain IV expression constructs, see figure 2.9) (Grindel et al. 2014). Western blot analysis revealed that several
large perlecan antibody reactive fragments (100-200 kDa in size) were present in patient serum with active metastasis, but not in normal serum. Full length perlecan core protein (467 kDa without GAG chains) typically would be near the top of the gel in this system. One band visible by Coomassie stain also was clearly present in sera from PCa but not normal sera, although this band did not react with antibody 3135. Mass spec analysis identified this band as complement C4, a protein reported decades ago to be upregulated in prostatic fluid of PCa patients (Grayhack et al. 1979). The measurable presence of perlecan-derived fragments in PCa patient serum indicates that it might be a useful index by which to suspect that PCa patients have relapsed or have early metastasis. Perlecan is highly expressed in the bone marrow reticular matrix and reports show that higher MMP-7 serum concentrations are associated with metastatic disease and are predictive of poor prognosis (Szarvas et al. 2011). Future studies are needed to determine if changes in perlecan fragment “velocity”, assessed in serum, is useful along with the current PSA velocity in detecting early metastatic lesions that create these fragments as a consequence of elevated MMP activity.

The GF β2M, which was examined because of its potential role as a multifunctional tissue factor and activator of MMPs, was observed by serum ELISA to be increased in patients with prostate cancer. This increase is consistent with our earlier work defining β2M as a soluble GF driving osteomimicry, prostate cancer progression and growth (Huang et al. 2006). Findings here, however, indicate that, at least within this 288 large patient cohort that was examined, while β2M is increased in serum of prostatectomy patients, it does not have a significant correlative relationship with MMP-7 or perlecan either in tissue or serum.
In summary, examination of a large cohort of 288 prostatectomy patient samples showed that the presence of MMP-7 in tissue blocks assessed by IHC correlated with the presence of specific perlecan fragments in serum. Overall, these studies revealed the enzyme-substrate relationship between perlecan and MMP-7 predicted in silico, confirmed in vitro (Grindel et al. 2014; Farach-Carson & Carson 2007), and now shown to extend to both tissue and serological patient specimens (this study), may be a key component of PCa tissue invasion. Also, on a per patient basis, the trend is increased perlecan, MMP-7, and β2M in tissue. Perlecan concentration in the entire TMA was upregulated in PCa Gleason grade 3 and, especially, 4 and MMP-7 and perlecan were highly correlated in prostatectomy tissue specimens. Additionally, an association exists between MMP-7 staining intensity and the presence of perlecan fragments even in organ confined disease. While this study focused on MMP-7 staining intensity in relation to perlecan, other MMPs and even other classes of cancer-associated proteases almost certainly contribute to the proteolysis of perlecan during cancer invasion of tissue. For example, membrane type 1 and 2 MMP (MT1/2 MMP) each have been shown to cleave perlecan and suggested to be required for cancer cell invasion (d’Ortho et al. 1997; Rowe & Weiss 2008). Future work will seek to create antibodies that can recognize a variety of cleaved forms of perlecan in tissue. Taken together, this study indicates that MMP-7-associated degradation of perlecan-rich matrices surrounding PCa cells occurs during tissue invasion, creating fragments that can enter the circulation along with other local modulators of tissue homeostasis including β2M. The utility of using these perlecan fragments and β2M as independent PCa biomarkers for invasive or recurrent disease merits further investigation.
Figure 3.1 Perlecan (PLN) is present at boundaries in metastatic prostate cancer (PCa). Perlecan (green fluorescent in primary site, red in bone marrow secondary site) is present as green at five different boundaries that must be dealt with by PCa cells: 1) The glandular basement membrane 2) The reactive stroma that is induced by the expanding/invading tumor 3) the endothelial basement membrane during intravasation, 4) the metastatic extravasation blood vessel basement membrane site, 5) and the reticular matrix of the bone marrow secondary site.
Figure 3.2 Tissue staining intensity and calculated concentration values correlate inversely. For each antibody stain (perlecan, MMP-7, and β2M), quantified staining intensity values (y-axis) were plotted against the concentration values (x-axis) that are calculated from intensity and other quantified values. “Intensity values” actually decrease with enhanced/darker staining and “concentration values” mostly increase with enhanced/darker staining. To determine if we needed to use both or just one of the values in this study we calculated their correlation. Utilizing Pearson correlation, an inverse relationship exists with a rho value of -0.89 and p-value of 0.22E-16. Since they are highly negatively correlated, only one value is needed. Concentration values, rather than intensity values, ultimately were used in the study because they increased with greater stain amount (see text).
Figure 3.3 Tissue microarray validation of staining for perlecan. Perlecan was detected in tissues as described in the text (stained brown) and shown with a hematoxylin nuclear counterstain (blue) in tissues. A) typical positive stain for perlecan with arrows highlighting prominent blood vessel staining along with strong diffuse tissue stain; B) typical low/negative tissue stain for perlecan. Other controls used in this study: C) overdigestion with proteinase K destroyed perlecan epitopes and produced a negative background control for IHC protocol; D) negative/background stain with an anti-green fluorescent protein antibody. Panels A and C are from the same tissue in series. All images at 5X objective.
Figure 3.4 Tissue microarray staining pattern for MMP-7 and β2-microglobulin. Antibody stain is brown with a blue nuclear hematoxylin counterstain. A) MMP-7 stain is largely glandular with focal areas of high stain. B) β2M stain is ubiquitous on all cells with varying concentrations. 10X objective.
Figure 3.5 Ratios of stain concentration for perlecan, MMP-7, and β2-microglobulin in TMA sections. Gleason grade 3 and 4 over normal gland (G3/NG and G4/NG), and tumor stroma over normal stroma (TS/NS) ratios were assessed in TMA sections from the same patient with N showing the number of cases examined for each (N Total). The number of cases with ratios above 1 (greater stain concentration in the cancer core) (N>1), percent of cases above 1 (% >1), and the mean ratio value for each type are shown below each graph.
Figure 3.6 Perlecan concentration is increased in Gleason grade 4 (G4) tissue over normal gland (NG) tissue in the tissue microarray. A) Graph comparing G4 tissue concentration to NG concentration with respective annotation case numbers (N), mean and standard deviation (SD). An unpaired parametric two tailed t-test was performed to test the difference between the means (see text). The p-value was 0.0006 (**). B) Representative image of normal gland core with low perlecan stain with an arrowhead indicating absence of perlecan stain between cells. C) Representative image of G4 core with perlecan stain with an arrow highlighting pericellular staining in a cancerous gland. Both B and C are 10X.
Figure 3.7 Perlecan and MMP-7 stain expression intersect in tissue microarray. Staining patterns of perlecan (A) and MMP-7 (B) are shown in the same TMA tissue specimen in Gleason grade 3 tissue. The perlecan or MMP-7 antibody-detected stain is brown and the hematoxylin nuclear counterstain is blue. Perlecan and MMP-7 positively staining regions are adjacent to one another, but not overlapping, as indicated by the arrows.
Figure 3.8 Sandwich ELISA detection of perlecan and β2-microglobulin (β2M) in serum. A) Schematic of perlecan core protein divided into domains I to V with the sites recognized by the eight antibodies used in the sandwich ELISA. The black and gray bars below show the detectable fragments (capture-detector pairs) from perlecan in patient sera. The white bar indicates full length perlecan was detectable by this assay in conditioned media of cells producing it, but not in patient sera. The fragments noted with gray bars were present in statistically significant levels from a pooled normal serum sample. B) Box plot of observable capture-detector pairs in patient serum in comparison to a pooled normal serum sample (gray diamond) with associated p-values shown above. C) Box plot with β2M serum values shown in comparison to a pooled normal serum sample (gray diamond) with associated p-values shown above. Student’s unpaired t-test p-values: * < 0.05, *** < 0.001. Serum values were normalized to buffer control and negative control (non-immune rabbit immunoglobulin; see text).
Figure 3.9 Western blot and protein stain of normal and stage IV prostate cancer sera. Lane 1 contains four pooled normal sera and lane 2 contains four pooled stage IV prostate cancer sera. Both were non-specifically precipitated with protein AG agarose beads and processed by western blot with either the 3135 perlecan domain IV specific antibody (left) or secondary antibody alone (middle). On the left, the sera were transferred to a membrane blot and stained with Coomassie. The three top solid arrows represent the bands recognized by the 3135 anti-perlecan antibody. The bottom dashed arrow was not recognized by the 3135 antibody, but was present in cancer sera but not normal sera. N-terminal sequencing identified the dashed arrow band as complement factor C4 (see text).
<table>
<thead>
<tr>
<th>CHARACTERISTICS</th>
<th>ELISA</th>
<th>TMA</th>
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<tbody>
<tr>
<td>Total Subjects</td>
<td>288</td>
<td>157</td>
</tr>
<tr>
<td>Gleason sum 6</td>
<td>113</td>
<td>38</td>
</tr>
<tr>
<td>Gleason sum 7</td>
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<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Biochemically Recurrent</td>
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<td>26</td>
</tr>
<tr>
<td>Average Time to Recurrence (months)</td>
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<td>12.9</td>
</tr>
<tr>
<td>Stage 1</td>
<td>3</td>
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<td>Stage 4</td>
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<tr>
<td>Average Age</td>
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<td>58.6</td>
</tr>
<tr>
<td>PSA &lt;10 ng/mL</td>
<td>262*</td>
<td>139</td>
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<tr>
<td>PSA 10-20 ng/mL</td>
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<tr>
<td>Annotation Normal Stroma</td>
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Table 3.1: Patient pool at prostatectomy. Table shows the patient characteristic profile for the ELISA study and for the subset of patients in the TMA analysis. PSA: prostate specific antigen levels in serum at the time of prostatectomy. The * notes that one patient data point was not available for the PSA ng/mL value in the ELISA column. Every TMA annotation is the number of annotation types (G3, G4, G5, normal gland, tumor related stroma and normal related stroma) ascribed by the pathologist to regions of interest among the three stains visualized in serial section (perlecan, MMP-7, and β2M). Multiple annotations can exist in one tissue core.
Table 3.2: Correlation between overall perlecan and MMP-7 expression in specimens among various annotation types. The rho values (Pearson correlation coefficients) are given and analysis methods described in the text. Significance p-value codes: * < 0.05, ** < 0.01, *** < 0.001.

<table>
<thead>
<tr>
<th>Annotation Type</th>
<th>MMP-7 and Perlecan Correlation Coefficient</th>
<th>p-Value</th>
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<tbody>
<tr>
<td>Gleason Grade 3</td>
<td>0.45</td>
<td>**</td>
</tr>
<tr>
<td>Gleason Grade 4</td>
<td>0.46</td>
<td>*</td>
</tr>
<tr>
<td>Normal Gland</td>
<td>0.54</td>
<td>***</td>
</tr>
<tr>
<td>Tumor Stroma</td>
<td>0.48</td>
<td>*</td>
</tr>
<tr>
<td>Normal Stroma</td>
<td>0.65</td>
<td>***</td>
</tr>
</tbody>
</table>

Significance values: 0 *** 0.001 ** 0.01 * 0.05
Table 3.3: The general model of association (analysis of covariance, ANCOVA) between MMP-7 concentration stain and perlecan fragments in the ELISA assay was determined as described in the text. Reported are the F and associated p-values. Values in bold are considered significant (p-value < 0.05).

<table>
<thead>
<tr>
<th>Perlecan Capture-Detector Pair</th>
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<th>p-Value</th>
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<td>Ab3105-3107</td>
<td>0.205</td>
<td>0.615</td>
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<tr>
<td><strong>Ab3105-3102</strong></td>
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<tr>
<td>Ab3107-3107</td>
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<td><strong>2.52E-05</strong></td>
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</tr>
<tr>
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<tr>
<td><strong>Ab3132-3132</strong></td>
<td><strong>8.135</strong></td>
<td><strong>0.0045</strong></td>
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<td><strong>7.784</strong></td>
<td><strong>0.00545</strong></td>
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<tr>
<td>Ab3134-3135</td>
<td>0.141</td>
<td>0.708</td>
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<tr>
<td>Ab3136-3135</td>
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<td>0.958</td>
</tr>
<tr>
<td>Ab3139-3135</td>
<td>1.921</td>
<td>0.166</td>
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Chapter 4

Discussion, Future Directions, and Significance

Summary and Discussion

The second chapter’s main focus was on the discovery of novel proteases capable of cleaving perlecan, a large HSPG, and what biological effect this relationship has on metastatic PCa cells. Perlecan normally is expressed in the BM underlying epithelial and endothelial cells. During PCa cell invasion, a variety of proteolytic enzymes are expressed that digest BM components including perlecan. An enzyme upregulated in invasive PCa cells, MMP-7, was examined as a candidate for perlecan proteolysis both in silico and in vitro. Purified perlecan showed high sensitivity to MMP-7 digestion even when fully decorated with HS or when presented in native context connected with other BM proteins. In both conditions, MMP-7 produced discrete perlecan fragments corresponding to an origin in Ig repeat region domain IV. While not predicted by in silico analysis, MMP-7 cleaved every subpart of recombinantly generated perlecan domain IV. Other enzymes relevant to PCa that were tested had limited ability to cleave perlecan including PSA, hepsin, and FAP. Full length perlecan and a long C-terminal portion of perlecan domain IV, Dm IV-3, induced a strong clustering phenotype in the metastatic PCa cell lines, PC-3, C4-2, and C4-2B. Dm I and other Dm IV subdomains (Dm IV-1 and 2) did not induce this clustering phenotype. MMP-7 digestion of Dm IV-3 reverses the clustering effect into one favoring cell dispersion. Moreover, removing the GAG chains of full length perlecan or digesting it with MMP-7 resulted in adhesion of C4-2B cells. In a C4-2 Transwell® invasion assay, perlecan-rich human BM extract that was pre-digested with MMP-7
showed loss of barrier function and permitted a greater level of cell penetration than untreated BM extract. Concluded is that enzymatic processing of perlecan in the BM or territorial matrix by MMP-7 as occurs in the invasive tumor microenvironment acts as a molecular switch to alter PCa cell behavior and favor cell dispersion and invasiveness.

The third chapter extends the basic findings of the second chapter to clinical specimens to investigate both the tissue and serum in PCa. During both local PCa tissue invasion and subsequent metastasis, PCa cells depend on MMPs including MMP-7 to degrade the ECM. Perlecan is degraded in this process at multiple sites including epithelial and endothelial basement membranes, nearby reactive stroma, and in the case of metastasis, perlecan-rich bone marrow. In chapter 2, I showed that MMP-7 cleaves perlecan creating bioactive fragments that modulate cell-cell adhesion and dispersion in PCa cells (Grindel et al. 2014). The follow-up study analyzed both PCa tissue and serum in 288 prostatectomy patients, with the focus of deciphering the relationship between perlecan and MMP-7 in PCa invasion and grade. Both cancerous and normal regions of the prostatic specimens were examined. The multifunctional tissue factor and activator of metalloproteinase, β2M, also was investigated as a second potential index of grade. Interestingly, cancerous tissues immunostained for perlecan were found to be statistically higher in expression as compared to their normal counterparts. In an examination of 157 of all 288 prostatectomy specimens, perlecan levels were increased in Gleason grade 4 tissues. MMP-7 and perlecan immunostaining levels also were highly correlated with each other in PCa tissue, regardless of grade. Next, serial sections were stained individually for perlecan and MMP-7 and examined for overlap. Intense immunostaining typically was found to be separate, meeting at adjacent borders, but not overlapping even in the stroma, indicating MMP-7 is likely to
degrade perlecan when co-expressed. Utilizing a panel of new N- to C-terminal spanning antibodies against perlecan created at Strategic Diagnostics Inc., I next determined if fragments of degraded perlecan were increased in PCa patient serum over a normal serum pool. Analysis of 288 PCa patient sera collected at time of prostatectomy showed that perlecan fragments were both present and elevated, with most derived from Ig-rich perlecan domain IV. The presence of perlecan fragments in PCa serum was associated positively with overall MMP-7 staining levels in PCa tissue. In serum, β2M levels were significantly increased in PCa patients at time of prostatectomy over normal controls. Overall, this study suggests that MMP7-induced degradation of perlecan-rich ECM likely occurs during tissue invasion, creating fragments that enter circulation along with other local modulators of tissue homeostasis including β2M.

Several key findings need to be emphasized and discussed along with data unmentioned from the appendices. Perlecan is largely resistant to proteolysis, but few studies actually have investigated the cleavage of perlecan, partly because perlecan is difficult to purify. While I used several methods to purify perlecan, 100% purity was never achieved despite using high salt (up to 1 M NaCl) washes to remove any interacting partners. HT-29 (formerly WiDr) cells are used as the source of perlecan because they are described as only secreting perlecan as their HSPG, but unfortunately, this proteoglycan core protein was reported to be 250 kDa (Iozzo 1984) and my results indicate it may be smaller than 470 kDa even though enough protease inhibitors were in the purifying buffers. Unknown if this represents an alternate form similar to the placental BM extract that was used in figure 2.2 and 2.9. Regardless, each form of perlecan (i.e. conditioned media, BM, and purified perlecan) produced the same Dm IV-3 antibody (3135) reactive band (figure 2.9) after
MMP-7 digestions, indicating it’s the same molecule. Moreover, as perlecan was further purified, the clustering effect was magnified (i.e. from concentrated conditioned media to DEAE purified to gel filtration to affinity chromatography), indicating that perlecan is the active molecule.

Perlecan can be cleaved by several MMPs (MMP-3, MT1 and MT2-MMP) but notably absent are MMP-2 and MMP-9, which are the dominate EMT/invasive MMPs discussed in literature (Chaudhary et al. 2013; Whitelock et al. 1996). Moreover, murine knockout models of both MMP-2 and 9 do not affect BM degrading processes (Rowe & Weiss 2008; Baluk et al. 2004; Perez et al. 2005). Therefore, finding MMP-7 as potent catabolizer of perlecan is important given its stabilizing interactions in the BM of so many tissues (Breitkreutz et al. 2013). Not included in the chapter 2 manuscript because it was discovered later, is that the transmembrane serine protease TMPRSS2 was capable of cleaving perlecan (appendix A). This serine protease is a specifically androgen regulated protease and may be another mediator of early invasion in the developing cancer (Gasi Tandefelt et al. 2014). Perhaps the two proteases, MMP-7 and TMPRSS2, work in concert to degrade the ECM components. Besides PAR-2, perlecan is now the only known human derived substrate for TMPRSS2 (Wilson et al. 2005).

The pro and anti-adhesive effects of perlecan have been reported but unknown was the actual mediator. This work indicates that Dm IV-3, the last 7 Ig repeats of perlecan that are highly conserved throughout evolution (Farach-Carson et al. 2014), is the mediator of the anti-adhesive, pro-cluster forming activity. Interestingly, a 1995 report indicated that murine derived perlecan was strongly repulsive and non-adherent to a variety of
hematopoietic cells (Klein 1995). In contrast, skin cells attached strongly to the same preparation of perlecan. The anti-adhesive effect was related to the protein core because heparitinase/chondroitinase treatment did not reverse the effect. Oddly, perlecan attenuated the pro-binding properties of fibronectin with respect to hematopoietic cells. I too observe Dm IV-3 induced clustering even in the presence of FBS which has high fibronectin. Additionally, bone marrow mononuclear cells formed non-adherent colonies on perlecan in the presence of granulocyte colony stimulating factor. Perhaps in conjugation with Dm IV binding GFs such as PDGF, perlecan Dm IV-3 could have striking effects on PCa cells as well.

Cells varied in response to Dm IV-3 and it could be from multiple factors. Some cell types may have a receptor that at least transiently binds to Dm IV to trigger the clustering phenomenon and others don’t. Attempts have been made by another graduate student, Jerahme Martinez, to find the receptor. He focused on CAMs due to the Ig repeats found in both molecule types and found some binding activity on solid phase binding assays with epithelial CAM (EpCAM), a biomarker for many epithelial cancers including PCa (Schnell et al. 2013). EpCAM after cell-cell adhesion ultimately leads to proliferation by its cleaved intracellular domain (Maetzel et al. 2009; Denzel et al. 2009). Future studies need to be assessed as to whether perlecan can assist in this function with the cancer specific biomarker. Another possible reason for a lack of response is the quick secretion and activation of perlecan degrading enzymes. As seen in appendix C, certain cells that secrete higher ng/mL amounts of MMP-7 fail to respond to Dm IV-3 as a substrate (e.g. salivary cells and LNCaP RANKL transfected cells). However, timing may discount this phenomenon. The clustering response is almost immediate (30 minutes or less) and the
experimental media is not conditioned media, so any MMP-7 would have to be quickly released or already on the cell surface (even after trypsin-EDTA treatment). While not impossible, it is less likely than a receptor complex initiating cell-cell adhesion rather than cell-substrate adhesion.

This Dm IV-3 induced non-adherence clustering effect may be related to repulsive axon guidance found in *Drosophila melanogaster*. Perlecan (Trol in *Drosophila*) genetically interacts with semaphorin 1A and plexinA repulsive signaling axis (Cho et al. 2012). Perlecan is found at axon junctions and trajectories in both the peripheral and central nervous system, helping dictate the neuronal patterning. Loss of perlecan leads to failed motor axon repulsive guidance initially thought to only be mediated by semaphorin and plexin. Notably, genetic backgrounds that remove the HS/CS chains on perlecan actually augment the repulsive guidance, meaning the perlecan core protein is responsible for these effects. I hypothesize that Dm IV is the mediator of this signaling axis. Unknown is if perlecan, and specifically Dm IV, binds and stabilizes the semaphoring/plexin complex, and experiments should be conducted determine this. Semaphorin has an Ig loop similar to Dm IV; perhaps a homophilic Ig binding complex could mediate this. In regards to repulsive guidance, cancer cells could be seeded on micro-patterned Dm IV-3 and note how the cells move to a chemoattractant coated bead. Semaphorin 4D and its receptor Plexin B1 are mutated and overexpressed in PCa (Rehman & Tamagnone 2013; Wong et al. 2007). Unknown is whether perlecan is necessary for this interaction in PCa cells like in the *Drosophila* motor axons.
The RPPA data in the second chapter aimed to identify pathways that are activated or deactivated in the presence of Dm IV-3 and alternatively its MMP-7 cleaved fragments. Of striking difference was the phosphorylation at the active tyrosine in Src family kinases (SFKs). The antibody used in the array doesn’t discriminate between different SFKs, so work must be done to IP a series of specific SFKs and western blot for the active form. Initial western blots using the same antibody and cell lysate showed a prominent band below 72 kDa that was indeed upregulated on a Dm IV-3 substrate and absent in MMP-7 cleaved Dm IV-3. Also this band was not induced by hanging drop clusters. SFKs serve as a nexus point in a variety of signaling pathways (e.g. activation of STAT-1, RhoGTPases, FAK, and PI3K) leading to growth, motility, invasion and, in PCa, androgen independence (Gelman 2014). In fact, Src is known to transactivate AR in the absence of androgens leading to growth (Asim et al. 2008). Therefore, staining or western blotting for activated AR in the nucleus of PCa cells on Dm IV-3 +/- MMP-7 in the absence of androgens (charcoal stripped media) should be done. Perhaps reticular (non BM associated) perlecan in the bone marrow may be responsible for the re-epithelialization (MET) and Src activated growth commonly seen the bone microenvironment as well (Varkaris et al. 2014). As for the clustering phenomenon, E-cadherin mediated cell-cell adhesion can activate SFKs resulting in growth (McLachlan et al. 2007). Perhaps Dm IV-3 may be initiating the cadherin dependent cell-cell adhesion leading to SFK activation seen in the RPPA. However, none of the Src activation may be truly relevant. Changing the western blotting procedure with PVDF and milk blocking while inhibiting the SFK activation with dasatinib show the real SFK band may be closer to 59 kDa. And this band has a different pattern with Dm IV-3. What the higher band may be is unknown. The
antibody recognizes a phosphorylation site similar to other proteins. Nevertheless, the RPPA identified other proteins and pathways that need to be explored with cell signaling pathway analysis software. Efforts are underway in this regard.

The TMA work in the third chapter also contributed to the study analyzing perlecan regulation in desmoplasi (Warren et al. 2014). Perlecan was found on average to be higher in tumor related reactive stroma from the arrays I stained. TNF-α, perhaps released by infiltrating immune cells such as monocytes/macrophages cells could lead to the increase of perlecan observed in patients. Correlative statistics and staining patterns indicate that MMP-7 and perlecan are juxtaposed to one another. Interestingly, in appendix C, I found that TNF-α increases the secretion of MMP-7 by C4-2 metastatic cancer cells. Reports show that TNF-α treated and hypoxic macrophages release MMP-7 that may be responsible for destabilizing carotid atherosclerotic plaques (Abbas et al. 2014). A similar phenomenon could be occurring where TNF-α is mediating the release of both perlecan and its potent catabolizer, MMP-7 at the interface of expanding cancer cells. Therefore, perlecan can help sequester the cancer associated GFs (e.g. VEGF, PDGF, and HGF) both in the GAG chains and protein core to be released when cancer cells reach the barrier of perlecan. Perhaps a co-stain of perlecan, MMP-7 and TNF-α in PCa tissue may further validate this.

GF sequestration and presentation is an important function of perlecan. The TMA aimed to see if perlecan and β2M, the pro-EMT/osteomimicry GF, were co-localized given their association in other pathologies (e.g. amyloidosis). However, no association was revealed in quantified statistics and in the staining patterns. Attempts to demonstrate binding of
β2M and perlecan failed as well in two binding assays (see appendix B). Future work should focus on finding PCa associated GFs that associate with perlecan and Dm IV given how it is readily cleaved by MMP-7. An obvious start would be verifying human PDGF AA or BB binds to human perlecan and its core (i.e. Dm IV), and then observe if MMP-7 can release these GFs from perlecan as may happen in the PCa microenvironment. Similar studies showed MMP-3 mediated bFGF release (Whitelock et al. 1996).

Several other experiments can be performed to complement the current work. At the time I stained the array, I was forced into using another perlecan antibody besides SDIX antibodies developed for the ELISA experiment because of contract disagreements. Now however, the series of rabbit polyclonal antibodies from SDIX are available from Novus Biologicals. An interesting experiment would be to stain with the series of antibodies in serial sections in PCa tissue. It could possibly demonstrate differential catabolism of perlecan in various grades of PCa, i.e. one domain or subdomain is present but another lacks staining. And in relation to perlecan proteolysis, it would be interesting to see if any of the current SDIX antibodies recognize neo-epitopes revealed after digestion with MMP-7 or other proteases. Essentially, do one of the antibodies fail to recognize full length perlecan, but after digestion allow recognition of its epitope? This antibody could be specifically used to analyze the serum and tissue to discover perlecan turnover in various stages of disease. And to expand on this, creating new antibodies in the presence of MMP-7 derived cleavage neo-epitopes could be done as well, but may be more work intensive.

The serum and tissue samples used in the ELISA and TMA were from mostly indolent cases of PCa. The serum of patients with active metastasis should be compared to age-
matched controls and men with earlier stage prostate cancer with the same ELISA system. I predict the difference in perlecan fragment signal will be substantially different from the indolent and normal subjects. Perlecan is highly enriched in the bone marrow microenvironment and cancer expansion would likely proteolyze this fraction of perlecan, releasing it into the serum. Initial experiments with a small sample size (N=4) at the end of chapter 3 (figure 3.9) suggest this may the case. However, larger populations need to be assessed for confirmation.

**Significance and Conclusions**

As described in the introduction chapter, PCa is the second most common form of cancer for men in the United States. PCa largely manifests as an indolent and slow growing tumor, with patients dying with cancer rather than from cancer. However, a subset of patients experience the full course of the disease, ultimately ending in death from metastasis. The most common site of PCa metastasis is the bone and bone marrow microenvironment resulting mostly in osteoblastic (bone forming) lesions. This unwoven matrix forms large unstable bone masses. PCa patients can die from complications of treatment (iatrogenic causes) and cachexia, wasting syndrome. Despite extensive studies on PCa the disease at metastatic stage only carries a 35% five year survival rate. Therefore, finding novel pathways and mechanisms of PCa metastasis and progression are crucially needed.

There are several steps in PCa progression that can be studied for targeted treatment mentioned in the first chapter. One may study inflammatory processes, the reactive stromal matrix induction, EMT/MET mechanisms, migration and chemokine signaling, the vicious cycle and osteomimicry in the bone microenvironment and androgen independence
mechanisms. By studying the evolutionarily important HSPG, perlecan, and its proteolysis I have touched on many of these processes crucial to PCa progression and metastasis. The work presented can be formalized into a model, incorporating both data chapters and aspects of PCa discussed in this and the introduction chapter (figure 4.1).

1) **Perlecan at borders promotes cell-cell adhesion.** Perlecan is reported to be at several borders that PCa cells would contact during progression. Full length perlecan was shown to initiate cell-cell adhesion common to an epithelial-like cell cluster. This morphology is in contrast to a mesenchymal/fibroblastic outstretched phenotype that is partially mediated by heavy cell-substratum interactions needed for motility and migration. I found that the last seven Ig repeats of Dm IV, Dm IV-3, is responsible for the clustering and not the HS/CS-rich Dm I.

2) **Perlecan deposition is increased in reactive stroma and G4 grade tissue.** Once the PCa begins to progress by instigating the reactive stroma and subsequent invasion (progressing to grade G3 and G4), perlecan is secreted at higher levels according to TMA data. Perlecan may initially promote indolent spheroids (as above) and assist in GF sequestration and presentation for tumor growth. However, for metastasis to occur, cancer cells need to invade through this perlecan-rich stroma.

3) **EMT associated MMP-7 localizes near and can cleave perlecan.** The TMA also revealed a positive correlative relationship between perlecan and the protease MMP-7. Perlecan staining is adjacent but not overlapping with MMP-7 in many grades of primary PCa. Studies conducted *in silico* and *in vitro* demonstrate that perlecan is a ready substrate for MMP-7 presented as the full length GAG protected
molecule, within a BM matrix, and as subdomains. Therefore, MMP-7 is a strong candidate for destabilizing the perlecan rich BM and stromal matrix for invasion. I also found that MMP-7 is secreted more in PCa cells after treatment with the pro-inflammatory cytokine, TNF-α. This cytokine also induces the secretion of perlecan. TNF-α may be the mediator for both MMP-7 and perlecan deposition seen in the PCa TMA. Additionally, EMT induced RANKL-LNCaP cells secrete 11 times the amount of MMP-7 as the indolent control cells (LNCaP NEO), showing that MMP-7 secretion (and its subsequent activity on perlecan) is associated with EMT and bone metastasis.

4) **Cleavage of perlecan by MMP-7 reverses cell-cell adhesion and allows for invasion.** MMP-7 cleavage of perlecan does more than simply remove the perlecan barrier, which is important by itself. Cleavage of perlecan by MMP-7 reverses this cell-cell adhesion in favor of attachment and dispersion, which is akin to an EMT morphology for PCa cells. Therefore, within perlecan a cryptic bioactive fragment or motif exists that is revealed after MMP-7 cleavage (and removal of GAG chains). Invasion of PCa cells is increased by MMP-7 as well. Exogenous MMP-7 alone could induce PCa cell invasion through a BM matrix as well. Perlecan is cleaved into large fragments during this invasion process.

5) **Perlecan fragments are found in PCa patient serum and relate to MMP-7 expression.** A novel method to assay various stretches or fragments of perlecan revealed that perlecan is not only present in PCa serum, but is dysregulated. Other methods, such as single antibody ELISAs, may have failed to observe this. For example, if I only assayed Dm I or II in serum, I would have falsely concluded that
perlecan is not present in PCa patient serum. This novel assay can be tested on other cancers and can be modified to detect other large ECM molecules to get a clearer picture. Additionally, MMP-7 expression in patients is associated with perlecan fragments present in their serum, which lends further credence to MMP-7 proteolysis of perlecan in PCa. And finally, metastatic patients (Stage IV) have detectable fragments originating from Dm IV-3 that are not present in normal age matched controls.

Some of these insights can be exploited for earlier detection and new avenues of treatment. For example, one of the perlecan fragment assays may detect bone marrow turnover before PSA biochemical recurrence arises giving valuable time for patient treatment. Additionally, novel peptidomimetics could be designed resembling MMP-7 target sites I found in perlecan to slow its proteolysis during PCa invasion. To conclude, a novel relationship between perlecan and MMP-7 was revealed through biochemical techniques. This relationship translated into PCa clinical specimens both in the serum and tissue. Further evaluation of this interplay will lead to improved insights to target and detect PCa.
Figure 4.1 Relationship of perlecan and MMP-7 in PCa. Perlecan is upregulated in reactive stroma in response to PCa that is partially induced by TNF-α. Intact perlecan promotes cell-cell adhesion with Dm IV-3 as the effector domain. MMP-7 is upregulated by TNF-α and RANKL. MMP-7 is able to cleave perlecan and is found at junctions with perlecan in PCa tissue. MMP-7 reverses the clustering phenotype to favor dispersive PCa cells. Invading tumors, possibly mediated by MMP-7 proteolyze perlecan at multiple sites including the bone marrow to release detectable Dm IV fragments into serum.
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Appendix A

TMPRSS2 activity on perlecan

TMPRSS2 was tested on perlecan and several subdomains of perlecan to observe if any cleavage could occur. Other serine proteases tested, PSA, Hepsin and FAP, could only cleave perlecan in certain contexts as seen in chapter 2. The assay was performed in 0.1 M Tris-HCl, 0.25 M NaCl, pH 8.7 buffer. The reaction was performed with 3 µg of substrate to 0.6 µg of TMPRSS2 in a total of 20 µL. Samples were incubated overnight at 37°C. Some samples were pre-treated with H/C to cleave off GAG chains in the following buffer: 10 mM HEPES, 2 mM CaCl2, 1 mM EDTA, 0.1% Triton X-100.

As seen in figure A.1 TMPRSS2 is capable of removing the bands associated with perlecan with or without HS/CS chains. TMPRSS2 was also capable of creating new bands when incubated with subdomains of perlecan, Dm IV-1, 2, 3, and 3a. Previous attempts to cleave perlecan with TMPRSS2 erroneously failed because the serine protease inhibitor PMSF were in the perlecan preparations. This further demonstrates that TMPRSS2 is causing the cleavage through serine protease actions.
Figure A.1 Perlecan and subdomains of perlecan are substrates for TMPRSS2. Perlecan preincubated with or without heparitinases and chondroitinases (left panel) and subdomains of perlecan (domain IV-1, 2, 3, 3a) (right panel) were incubated either with or without TMPRSS-2 overnight at 37°C. Shown above are silver stain gels. TMPRSS2 is able to cleave perlecan and subdomains of perlecan as seen by the creation of new bands and absence of intact bands.
Appendix B

Perlecan and β2-microglobulin interactions

Perlecan and β2M are thought to associate in amyloidosis related diseases (see chapter 3), but any binding association has not been demonstrated in vitro. Attempts were made to show binding or interaction of perlecan and perlecan domains with β2M as seen in figure B.1. Solid phase binding assays (figure B.1A) on nitrocellulose failed to demonstrate any signal on multiple substrates (perlecan, Dm I, Dm IV-3, IV-3a at 2 µg bound per well in dot blot apparatus) with β2M (Sigma Aldrich) at 0.1 µg/mL in binding assay buffer (25 mM HEPES, pH7.5, 50 mM NaCl, 2 mM CaCl2, 0.5 mM PMSF, benzamidine, 0.02% NaN3). Only the positive control (β2M antibody) gave any signal. To find weaker interactions the Hummel Dreyer method was employed (figure B.1B). Assay binding buffer as above with β2M at 0.1 µg/mL was equilibrated in a CL-4B column, perlecan was allowed to go through the column. If interactions occurred a slight bump of β2M signal should coincide with the perlecan peak. This is not the case, and therefore binding did not occur in the assay. Another indirect method to observe interactions was employed in figure B.1C. Perlecan (2 µg) with removed GAG chains was incubated with MMP-7 (1.25 µg) with or without β2M (4.24 µg) in a total of 30 uL for 2.5 hrs at 37°C. As seen in the third lane perlecan incubated with MMP-7 in presence of β2M results in a new band (white box). Therefore, β2M may in some regards interact with perlecan to limit its proteolysis by MMP-7.
Figure B.1 β2-Microglobulin (β2M) association with Perlecan (Pln). A.) Full length Pln, Dm I, Dm IV-3, 34-35 fragment, and positive (β2M rabbit antibody) and negative (Bovine Serum Albumin, BSA) controls were adsorbed to nitrocellulose in a dot blotter and incubated with β2M. β2M signal was detected with a biotinylated mouse antibody and SA-HRP. Only the positive control gives detectable signal. B.) Hummel Dreyer method detects no association between β2M and Pln. C.) Silver stain of Pln with heparitinase/chondroitinase (lane 1) to remove glycosaminoglycan chains, digested with MMP-7 (lane 2), and digested with MMP-7 in the presence of excess β2M (lane 3). Red box indicates Pln and the white box is a possible Pln limit fragment produced by β2M association.
Appendix C

MMP-7 secretion in various cell lines and response to TNF-α

Several cell lines were tested for the secretion of MMP-7 using a commercial ELISA kit recognizing pro and active MMP-7 (Human Total MMP-7 Quantikine Kit, DMP700, R&D Systems). The results are shown in table C.1 along with the respective cell line’s response to Dm IV-3 as a substrate, if tested. Of note is the higher secretion of RANKL transfected LNCaP cells over control neomycin transfected LNCaP cells (LNCaP NEO). According to qPCR there is no real difference in MMP-7 transcription after the cells in RANKL induced EMT, but this data shows an approximate 11 fold induction. Also of interest is 2.7 fold increase in MMP-7 over LNCaP parental cells in control LNCaP NEO cells. LNCaP NEO cells did not respond to Dm IV-3 like LNCaP cells. A trend exists where higher MMP-7 secretion corresponds with lower response to Dm IV-3. Another striking difference is the huge induction of MMP-7 in bone localized prostate cancers (mouse maintained prostate derived xenografts (PDXs)) of the 183 cells but not 118B cells.

I performed a small experiment in figure C.1 to see the effects of cytokines and GFs on C4-2 cells with respect to MMP-7 secretion into conditioned media. Curt Warren had previously shown an induction of perlecan in PCa cells. Given the array data showing perlecan and MMP-7 positive correlation, perhaps TNF-α also induces MMP-7 secretion. Using a dot blot procedure with an MMP-7 antibody (ID-2 clone used in the TMA in chapter 3), conditioned media was statistically higher in cells treated with TNF-α over control BSA. A slight increase is seen with FGF-2, but the difference was not significant. The assay was specific for MMP-7 given the MMP-7 dilution scheme resulted in an appropriate decrease of signal.
Figure C.1 MMP-7 secretion after cytokine and growth factor treatment. Utilizing a dot blot method with the MMP-7 antibody (clone ID2) C4-2 cells were treated with TNF-alpha and FGF-2 in serum free T-media for 72 hours at the doses indicated. Conditioned media was analyzed by dot blot and quantified with densitometry. TNF-alpha treatment statistically increased the secreted MMP-7 in C4-2 cells by approximately 4-fold. Students T-test, ** P value <0.01
### Table C.1 Secreted MMP-7 in various cell lines

<table>
<thead>
<tr>
<th>Samples</th>
<th>ng/mL MMP-7</th>
<th>Dm IV-3 Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS media</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Hepatostem</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Salivary P1 Duct</td>
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<td>No Clusters</td>
</tr>
<tr>
<td>Salivary P7</td>
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</tr>
<tr>
<td>Salivary P18</td>
<td>1.14</td>
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</tr>
<tr>
<td>Salivary P1 Mix</td>
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<td>No Clusters</td>
</tr>
<tr>
<td>183 PDX</td>
<td>21.15</td>
<td>Unknown</td>
</tr>
<tr>
<td>118 B PDX</td>
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</tr>
<tr>
<td>LNCaP</td>
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</tr>
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</tr>
<tr>
<td>LNCaP RANKL</td>
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<td>No Clusters</td>
</tr>
<tr>
<td>C4-2</td>
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<td>Clusters</td>
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<tr>
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<td>Small Clustering</td>
</tr>
<tr>
<td>HEK293</td>
<td>ND</td>
<td>Unknown</td>
</tr>
<tr>
<td>MCF7</td>
<td>ND</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

An ELISA for MMP-7 that recognizes both the pro and active form was performed on the conditioned media of several cell lines: salivary duct and acini cell lines, prostate derived xenografts (PDX), Prostate cancer cells (LNCaP, C4-2, and PC-3), LNCaP cells that have been stably transfected with RANKL (EMT inducing factor) or control neomycin cassette (LNCaP NEO), kidney cells (HEK293) and breast cancer cells (MCF7). The last column indicates if the cell lines responds to Dm IV-3. ND= not detected; NA= not applicable.
Appendix D

Dm IV-3 heating and enhanced clustering phenotype

In attempts to heat inactivate Dm IV-3, the protein was heated at various temperatures (22, 45, 62, 75, and 90°C) diluted in PBS (21 µg in 205.8 uL PBS) for 10 minutes and assessed for ability to cluster C4-2 cells as seen in figure D.1. At 75 and 90°C Dm IV-3 was not able to cluster, possibly due to precipitation. However, unexpectedly Dm IV-3 heated to 62°C exhibited strikingly higher clustering than control at RT (22°C). Refer to the dissertation of Jerahme Martinez for additional information about heated Dm IV-3. This heated Dm IV-3 was also used in the RPPA analysis and essentially served as an additional triplicate to Dm IV-3 at RT.
**Figure D.1 Heated Dm IV-3 increases clustering activity.** Perlecan Dm IV-3 was heated at several temperatures, diluted in PBS and coated onto cell culture plates. C4-2 cells were seeded on the substrate for 24 hours and imaged at 4x objective. In the left panel are C4-2 cells seeded on Dm IV-3 left at room temperature and on the right are the same cells seeded on 62 degrees C heated Dm IV-3. The clustering effect is highly pronounced on the heated IV-3.
Appendix E

Dm IV-3 effect on invadopodia of C4-2 cells in 3D hyaluronic acid culture

Perlecan Dm IV-3 was also tested for its effects in a three dimensional (3D) hyaluronic acid model. For details on the 3D hyaluronic acid system see (Gurski et al. 2009). Dm IV-3 (10 µg of soluble addition) was added (figure E.1B) or omitted (figure E.1A) in the presence of 10% FBS T-media. Normally, C4-2 cells extend projections of invadopodia. However, in the presence of Dm IV-3 invadopodia retract and the cells clusters become more spherical. This response is reminiscent of the hanging drop clusters with soluble IV-3 added. Essentially, soluble Dm IV-3 induces cell-cell adhesion even in a 3D matrix with many contact points available.
Figure E.1 Dm IV-3 retracts invadopodia in 3D cell culture. C4-2 cells embedded in hyaluronic acid (HA) hydrogels were either treated with 10 µg of Dm IV-3 in 10% FBS T-media (A) or just media (B) and imaged 3 days later. A decrease in invasive protrusions and interconnections is observed with Dm IV-3 but not the media control.
Appendix F

Dm IV-3 effects on RNA and protein expression in prostate cancer cells

Early attempts to discriminate the effects of Dm IV-3 on C4-2 PCa cells were performed at Cedars Sinai in the Chung lab. The hypothesis being that Dm IV-3 could be modifying EMT in the cells. Used was an EMT panel of primers for qPCR (Hu et al. 2011) to assess LNCaP NEO, LNCaP RANKL, and C4-2 cells on Dm IV-3 or equal amounts of BSA (figure F.1). Unfortunately, her cells did not respond to Dm IV-3, in that they did not form cell-cell adhesions and cluster. However, changes in RNA expression were observed. Notably, there was a huge induction (100 fold) of c-MET (scatter factor, HGF receptor) RNA in LNCaP NEO cells when seeded on Dm IV-3. However, attempts to repeat this data with my cell lines were unsuccessful; I failed to see any statistical change with c-MET.

Efforts also were made to do western blotting with key antibodies (figure F.2). The Chung lab was responsible for figure F.2A (only left two LNCaP blots). Strangely, LNCaP NEO and LNCaP RANKL cells responded oppositely to Dm IV-3. Dm IV-3 induced RANKL, c-MET, and phospho c-MET in LNCaP NEO cells but was suppressed in RANKL transfected cells. I also found a decrease in c-MET with PC-3 cells using less Dm IV-3. My attempts to replicate the c-MET western blot data with LNCaP RANKL cells failed (figure F.2 rightmost blot) as no change was observed.

In my attempts to do western blotting for perlecan, c-MET and MMP-7 I came across an unexpected finding that was not followed up on in this dissertation (figure F.2B). Cells treated in combination with two cytokines (TNF-α and IFN-γ) had interesting results. In PC-3 cells, the cytokine treatment increased perlecan and decreased c-MET expression.
Additionally, the prostate cancer cell line DU-145 also saw decreased c-MET in the presence of the cytokines. In some accordance with figure C.1, the cytokine treatment may have increased the amount of MMP-7. Antibodies used: Perlecan, 3135 from SDIX; c-MET from Cell Signaling Technologies, MMP-7 clone ID2 from Millipore.
Figure F.1 Assaying epithelial mesenchymal transition (EMT) in prostate cancer cells after grown on perlecan domain IV-3. Prostate cancer cells LNCaP NEO, LNCaP RANKL, and C4-2 cells were grown on plates coated with either BSA or perlecan Dm IV-3. They were assayed for control relative mRNA expression of EMT associated genes (RANKL, E-cadherin, N-cadherin, vimentin, fibronectin, c-Met, androgen receptor (AR), MMP-2, 7, and 9). Notice the large induction of c-Met in LNCaP cells grown on Dm IV-3.
Figure F.2 Effect of perlecan domain IV-3 (Dm IV-3) and inflammatory cytokines on prostate cancer cell lines. A) Noninvasive LNCaP cells (NEO), LNCaP cells stably transfected with RANKL (LNCaP RANKL), and PC-3 cells were grown on either control BSA or Dm IV-3 substrates (15 or 5 µg) for 3 days. Cells were assayed by western blot for a variety of proteins related to epithelial mesenchymal transition including RANKL, c-Met, phosphorylated c-Met, perlecan, MMP-7 and β-actin. B) Prostate cancer cells dual treated for 48 hrs in tumor necrosis factor-alpha (TNF) (25 ng/mL) and interferon-gamma (IFN) (200 U/mL) or control (BSA). Cell lysates were western blot probed for perlecan, c-Met, MMP-7 and control β-actin.