MMP-7 is Required for TGF-β and EGF Induced Migration and Invasion in Prostate Cancer Cells

Clement Bolton II
clement.bolton@students.cau.edu

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ABSTRACT

BIOLOGICAL SCIENCES

BOLTON II, CLEMENT J. B.A. HAMPTON UNIVERSITY, 2005
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MMP-7 IS REQUIRED FOR TGF-β AND EGF INDUCED MIGRATION AND
INVASION IN PROSTATE CANCER CELLS

Committee Chair: Shafiq A. Khan, Ph.D.

Dissertation dated August 2018

Prostate cancer micrometastasis allows cancer cells to vacate their original tumor sites and migrate to distant parts of the body via the bloodstream, lymphatic system, or by direct extension. Cells synthesize and secrete matrix metalloproteinases (MMPs) that degrade proteins of the surrounding extracellular matrix (ECM); thus allowing them to escape into the lymphatic or circulatory systems to invade other tissues. Transforming growth factor β (TGF-β) induces the migration and invasion of cancer cells and the expression of matrix metalloproteinases (MMPs), specifically MMP-2, and -9 in several malignancies. In this study, we examined the role of MMP-7, a known activator of MMP-2 and MMP-9, in TGF-β signaling in cell proliferation, migration, and invasion in prostate cancer cells. Basal expression levels of MMP7 mRNA, protein, and secreted protein were determined using RT-PCR, western blot analysis, and ELISA, respectively. Our data show that MMP7 mRNA and proteins were differentially expressed in several
cell line models representing different stages of prostate cancer. TGF-β1 induces MMP-7 gene expression and protein levels 24 and 48 hours after treatment in PC3 cells. Our data also show that TGF-β induces cell migration and invasion in PC3 and E006AA cells; however, the selective knockdown of MMP7 expression using siRNA resulted in a significant decrease in control and TGFβ-induced cell migration and invasion in both PC3 and E006AA cells. MMP-7 knockdown also caused significant reduction in cell proliferation in PC3 cells. Our data suggest that MMP7 is essential for cell migration and invasion in prostate cancer cells indicating that it may be required for TGFβ-induced cancer metastases.
MMP-7 IS REQUIRED FOR TGF-β AND EGF INDUCED MIGRATION AND
INVASION IN PROSTATE CANCER CELLS

A DISSERTATION
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
CLEMENT J. BOLTON II

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

AUGUST 2018
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BM</td>
<td>Basement Membrane</td>
</tr>
<tr>
<td>BMPs</td>
<td>Bone Morphogenetic Proteins</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<tr>
<td>CSCs</td>
<td>Cancer Stem Cells</td>
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<tr>
<td>DAPI</td>
<td>4’-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>dNTP</td>
<td>Dinucleotide Triphosphate</td>
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<tr>
<td>EBRT</td>
<td>External-beam Radiation Therapy</td>
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<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>ERK1/2</td>
<td>Extra-cellular Signal Regulated Kinases</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>KSFM</td>
<td>Keratinocyte Serum Free Medium</td>
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<tr>
<td>LAP</td>
<td>Latency-associated Peptide</td>
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<td>Abbreviation</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated Protein Kinases</td>
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<td>MEM</td>
<td>Minimum Essential Medium</td>
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<td>MMP-2</td>
<td>Matrix Metalloproteinase-2</td>
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<tr>
<td>MMP-7</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>NCBI</td>
<td>National Center for Bioinformatics</td>
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<td>NIH</td>
<td>National Institutions of Health</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>Prostate Cancer</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>Prostate Specific Antigen</td>
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<td>Polyvinylidene Difluoride</td>
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<tr>
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<td>RT</td>
<td>Reverse Transcriptase</td>
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<td>Rh</td>
<td>recombinant human</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>SARA</td>
<td>Smad Anchor for Receptor Activation</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>Smad</td>
<td>Similar to Mothers Against Decapentaplegic</td>
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<tr>
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<td>Transforming Growth Factor-β Type I Receptor</td>
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<td>Transforming Growth Factor-β Type II Receptor</td>
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x
1.1 Background and Significance

1.1.1 Normal Prostate

The prostate gland is a part of the male reproductive system responsible for the production and storage of seminal fluid. In adult men, a normal prostate is nearly 3 centimeters long and weighs approximately 20 grams.\(^1\) The prostate is positioned below the urinary bladder and directly in front of the rectum. The prostate surrounds part of the urethra, the tube that carries urine from the bladder during urination and semen during ejaculation (Figure 1).\(^2\)

![Figure 1. The prostate gland and prostate cancer.](source)
The adult prostate is organized into three zones, a central zone, a transition zone, and a peripheral zone. The paired central zone is posterior to the stromal region in which approximately 20-25% of prostate cancers cases arise in this area of the gland. Positioned interiorly to the central zone is the transition zone, which is located on either side of the urethra, and represents the smallest zone in the normal prostate. Prostate cancers arising from the transitional zone are larger at the time of diagnosis due to their central location, and the decreased likelihood of detection on digital rectal exam (DRE) or extended prostate needle biopsy schemes. Despite their greater total cancer volume at diagnosis, transition zone cancers are associated with lower Gleason scores at the time of prostate biopsy and prostatectomy.

The peripheral zone is the largest region of the normal adult prostate, and contains the majority of prostatic glandular tissue. This zone is located on the posterior side of the prostate at the back of the gland closest to the rectal wall; and it is in this zone, that a doctor performs a DRE. This peripheral zone is extremely important because approximately 70% - 80% of prostate cancers originate in this peripheral zone (Figure 2). The significance of this architectural anatomy is based upon the relationship of these zones to prostatic disease. Benign prostatic hyperplasia (BPH), a nonmalignant overgrowth that is fairly common among aging men, occurs mainly in the transition zone, whereas prostate carcinoma arises primarily in the peripheral zone. Because of the prostate’s location, those affected may experience changes in urinary or sexual function, which may indicate the presence of an enlarged prostate or prostate cancer.
In Figure 2, the central zone is the area that surrounds the ejaculatory ducts. The transition zone surrounds the urethra as it enters the prostate gland, and is responsible for benign prostatic hyperplasia. The peripheral zone contains the majority of prostatic glandular tissue. The largest area of the peripheral zone is at the back of the gland, closest to the rectal wall.

1.1.2 Prostate Cancer

Prostate cancer (PCa) is the third most common cause of male cancer-related deaths and the most common male non-cutaneous malignancy in Western population.\textsuperscript{12,13} Globally, PCa is the second most common cause of cancer and the fifth leading cause of cancer-related death in men.\textsuperscript{14} More than 80\% of men will develop prostate cancer by the age of 80.\textsuperscript{15-17} However, the outcomes of this disease \textit{ultimately} depend on a person’s
age and other health problems as well as how aggressive and extensive his cancer is. Most men diagnosed with prostate cancer do not succumb to the disease.  

The development of prostatic tumor in men is generally slow, taking up to 4 to 10 years to develop a 0.4 inch-size tumor. The initiation of PCa begins when the semen-secreting prostate gland cells mutate into tumor cells, proliferating at higher mitotic levels. The prostate cells begin to proliferate leading to the formation of a tumor in the peripheral zone of the prostate gland. Over time these cancer cells continue to proliferate and begin to invade nearby organs, such as the seminal vesicles, rectum, bladder and urethra. In the early stages, malignant cells from the primary tumor detach from their original site(s) and migrate through blood and lymphatic vessels. Moreover, the lymph nodes adjacent to the primary tumor are often the first site of metastases. As this cancer progresses, cancer cells eventually spread to more distal organs, including bones, liver, and lung and the patient finally succumbs to this disease. Unfortunately, most deaths from cancer occur as a result of metastasis.

1.1.3 Risk Factors

Comprehensive understandings of the exact cause(s) of prostate cancer have not yet been clearly established. However, researchers have found several factors that may increase the risk of a man developing prostate cancer. Some of these factors include: age, race/ethnicity, family history, and obesity. Prostate cancer diagnoses are fairly uncommon in men under the age of 45, and become more prevalent as a man advances in age. The median age for prostate cancer diagnosis in men is 70 years of age.
Several research studies have demonstrated that prostate cancer disproportionately affects African Americans and Caribbean men, which suggest that aspects associated with African ancestry, may be a factor in prostate cancer etiology.\textsuperscript{28,29} Additionally, African American men have the world's highest incidence of prostate cancer and a more than two fold higher mortality rate as compared to their white counterparts.\textsuperscript{27,29} In contrast, the incidence and mortality rates for Hispanic men are one third lower than for non-Hispanic white.\textsuperscript{27}

Approximately one in six American men diagnosed with prostate cancer will die from the disease.\textsuperscript{27} The world's highest mortality rates (30.3 to 47.9 per 100,000 person-years) were seen in men of African descent in the Caribbean nations of Barbados, the Bahamas, and Trinidad and Tobago.\textsuperscript{27}

A recent study, found that African Americans and Hispanics were generally younger, less educated, poorer, and less likely to have had a previous PSA (prostate specific antigen) test than non-Hispanic whites.\textsuperscript{27} Furthermore, non-Hispanic whites were less likely than African Americans or Hispanics to have urinary symptoms, to be unemployed, on public insurance (Medicare or Medicaid), or uninsured.\textsuperscript{27} Among all study subjects, African Americans had the most comorbidity and the highest PSA levels.\textsuperscript{28} Overall, African Americans (16.8\%) and Hispanics (12.9\%) had a higher proportion than non-Hispanic whites (10.5\%) of poorly differentiated cancers which coincide with Gleason scores of 8–10.\textsuperscript{28,29}

Genetic background has been implicated in the development of prostate cancer via associations with race, family, and specific gene variants.\textsuperscript{29} For instance, men who
have a first-degree relative (father or brother) with prostate cancer have twice the risk of
developing prostate cancer compared to those who do not have a first degree relative.\textsuperscript{12}
Additionally, those with two first-degree relatives affected have a fivefold greater risk
compared to those with no family history.\textsuperscript{12} Moreover, African-American men have
twice the risk of non-Hispanic whites for presenting with advanced-stage prostate
cancer.\textsuperscript{28,29}

Several genes have been implicated in the development of prostate cancer.\textsuperscript{30}
Mutations within BRCA1 and BRCA2, (Breast Cancer Genes 1 & 2), genes have been
shown to be important risk factors in the development of ovarian cancer and breast cancer
in women.\textsuperscript{31,32} These genes have also been implicated in the development of prostate
cancer.\textsuperscript{33} Other genes implicated in the development of prostate cancer are hereditary
prostate cancer gene 1 (HPC1), the androgen receptor, and the vitamin D receptor.\textsuperscript{27}

Two genome-wide association studies linking single nucleotide polymorphisms
(SNPs) to prostate cancer were published in 2008.\textsuperscript{14,15} These studies identified several
SNPs which have substantial effects on the risk of developing prostate cancer. For
instance, individuals with the TT allelic pair at SNP rs10993994 were reported to have an
approximately 1.6 times greater risk of developing prostate cancer than those with the CC
allele pair.\textsuperscript{34,35} This SNP explains part of the increased prostate cancer risk of African-
American men as compared to American men of European descent, since the T allele is
much more prevalent in African-American men.\textsuperscript{35,36} This SNP is located in the promoter
region of the microseminoprotein-Beta (MSMB) gene, thus affecting the amount of
MSMB protein synthesized and secreted by epithelial cells of the prostate.\textsuperscript{14} Although it
is not known whether the risk factors explaining the observed patterns are related to the environment, lifestyle, or genetics, it is likely that a complex interplay of these factors is associated with prostate cancer etiology.\textsuperscript{28,29}

1.1.4 Prostate Cancer Diagnosis

Prostate cancer is a relatively asymptomatic disease at the early onset and does not show symptoms until the later stages of the disease. Consequently, men must be screened to detect evidence of cancer. As a result, many medical organizations and health care professionals have strongly advocated that men begin screening in their 50s, or sooner for those who have been determined to be at greater risk for developing PCa.

The primary diagnostic tools in the diagnoses of PCa are the detection of serum level concentration of PSA and the DRE. The majority of prostate cancers diagnosed in the United States are diagnosed by PSA testing, although many experts have concluded that the efficacy of PSA screening is insufficient.\textsuperscript{19,37,38} Nevertheless, it has been shown that PSA screening reduces PCa-related mortality by 21\% - 44\%.\textsuperscript{39,40} On the other hand, nearly 18\% of all patients’ PCa is detected by a suspect DRE alone regardless of the detected PSA levels.\textsuperscript{41} DRE remains an important staging component for more advanced disease.\textsuperscript{42} However, if one or more of these screening examinations are abnormal, further investigation should ensue via transrectal ultrasound (TRUS) guided biopsy.

Upon a positive diagnosis of cancer by a trained pathologist, the sample obtained from the biopsy is then staged by the Gleason grading system. The Gleason system was developed by Dr. Donald F Gleason and members of the Veterans Administration Cooperative Urological Research Group (Minneapolis, Minnesota), and is the most
widely utilized grading scheme in the United States and worldwide.\textsuperscript{43-46} The Gleason grading system is based exclusively on the histological microscopic arrangement of cancer cells in Haemotoxylin and Eosin (H&E) stained prostatic tissue sections\textsuperscript{43,46,47} obtained from patient biopsies. The system initially analyzed nine different growth patterns and consolidated them into five basic grade patterns which are used to generate a histologic score, ranging from 2 to 10 (Figure 3).\textsuperscript{45-48}

\textbf{Figure 3.} The Gleason grading system.

\textit{Source:} Figure adopted from Gleason, D. F. \textit{The Veteran’s Administration Cooperative Urologic Research Group: Histologic Grading and Clinical Staging of Prostatic Carcinoma.} Tannenbaum: Philadelphia, 1977; pp 185-189.
The Gleason score is calculated by adding the primary grade pattern and the secondary grade pattern. The primary pattern is the one that is predominant in area, by simple visual inspection, and the secondary pattern is the second most common pattern. However, if only one grade is in the tissue sample, that grade is multiplied by two to give the score.\textsuperscript{45-48} The Gleason score assigned by the pathologist at the time of diagnosis significantly influences the treatment options offered to the patient, because carcinomas designated with higher Gleason scores indicate more aggressive cancers and higher risks of mortality \textsuperscript{44} (refer to Figure 4).

![Gleason Scores in Categorical Order](image)

**Figure 4.** Gleason scores for prostate cancer diagnosis.

*Source: Figure obtained from www.cancer.gov*

Once prostate cancer has been confirmed by a biopsy, it is imperative to learn the stage (location) and grade (aggressiveness) of the tumor, since a patient’s treatment options are generally tailored to the patient’s age and tumor stage. Although there has been some controversy surrounding the issue of tumor staging, the Gleason system scoring method is emphasized and remains the grading system of choice \textsuperscript{42-44} (refer to Figure 5)
1.1.5 Treatment Options

Following subsequent diagnosis of PCa, a patient’s treatment options vary and are based upon the risk factors of their prognosis as being either: low risk, intermediate risk, or high risk.\textsuperscript{49-51} A low risk diagnosis comprises a PSA concentration less than or equal to 10 ng/mL, a Gleason score which less than or equal to 6, and cancer localized within the prostate gland being staged 1-2a. Patients in this category are often candidates for an active surveillance program, previously referred to as “watchful waiting/active surveillance therapy.”\textsuperscript{44} These patients also have the option(s) to undergo radical prostatectomy, external beam radiotherapy, brachytherapy with permanent implants or high dose rate brachytherapy with temporary implants.\textsuperscript{44,52}

An intermediate risk diagnosis entails a patient’s PSA levels that are between 10-20 ng/mL, have a Gleason score of 7, and possess a clinical stage of 2b. Patients in this category are likely to benefit from radical prostatectomy, radical surgery, external beam radiotherapy, brachytherapy with permanent implants or high dose rate brachytherapy

**Figure 5.** The stages of prostate cancer.

*Source: Figure obtained from www.cancer.gov.*
with temporary implants, or active surveillance) and do well with these treatment.\textsuperscript{51,52} Patients deemed as high risk patients and classified by PSA levels greater than 20 ng/mL, Gleason scores of 8-10, and have a cancer clinically staged as 2c-3 normally undergo radical prostatectomy or external beam radiotherapy plus (neo)adjuvant treatments.\textsuperscript{51}

The relative 5-year survival rate for PCa patients with localized disease is nearly 100\%.\textsuperscript{53,54} Moreover, the 10-year and 15-year relative survival rates are 98\% and 95\%, respectively.\textsuperscript{54} However, when discussing treatment options for patients diagnosed with PCa, one must also consider patients diagnosed with advanced and aggressive forms of the disease, whose 5-yr survival rates swiftly decline to 28\%.\textsuperscript{53,54} For these men, treatment options include androgen deprivation therapy (ADT), chemotherapy, bone-directed therapy, radiation, or a combination of these treatments.\textsuperscript{55} Nevertheless, there is always the fear of recurrence of the disease, the increased probability of death due to cancer, and the complications of treatment.

\textbf{1.1.6 Growth Factors that Promote Prostate Cancer}

Growth factors (GFs) are naturally occurring regulatory molecules, which bind to receptors on the cell surface. They stimulate cell and tissue function through influencing cell differentiation and by changing their biochemical activity and cellular growth, and regulating their rate of proliferation.\textsuperscript{56} Structurally GFs are peptide-like hormones; however they are not limited to defined tissues.\textsuperscript{56} They act on target tissues in both diffusible (endocrine, autocrine and paracrine) and nondiffusible (juxtacrine or metacrine) manners and regulate a variety of cellular events which include cell migration, survival, adhesion, proliferation and differentiation.\textsuperscript{57} GFs possess diverse modes of
action, and many utilize the tyrosine kinase receptor pathway amongst other common signal transduction pathways such as MAP kinase signaling, JAK/STAT signaling, and PI3K/AKT1/MTOR signaling pathways. Therefore, any mutation(s) affecting signal transduction may affect several growth factor pathways simultaneously.58

GFs are present in a variety of tissues, both adult and embryonic, and are thought to be released by many, if not all, cells in culture.59 Membrane receptors for growth factors are also highly ubiquitous with most cells having receptors for more than one growth factor.60 Numerous studies have demonstrated that multiple growth factors are required for maximum stimulation of specific cell types; and exposure of a cell to one growth factor can lower the threshold for mitogenicity of a second growth factor.61-63

Research has also shown that GFs operate at different points of the cell cycle, and these GF are essential in several phases of tumor progression.58,63 Moreover, there is evidence to support a major role in normal and cancerous growth of the prostate from a variety of GF families which include the fibroblast growth factor (FGF) family, the insulin-like growth factor (IGF) family, epidermal growth factor family (EGF), and transforming growth factor α (TGF-α), all of which are predominantly stimulators of proliferation; retinoic acid, which causes differentiation and invasiveness; the TGF-β family, which are predominantly inhibitors of prostatic growth in normal cells and a tumor promoter in malignant cells; and the vascular endothelial growth factor (VEGF) family, which predominantly stimulate angiogenesis.64 According to numerous studies, these families of Gfs are, most involved in the normal and malignant growth of the prostate.65,66
The human FGF gene family of growth factors consists of at least 23 different genes encoding related polypeptides. FGFs are expressed in almost all tissues and play important roles in a variety of normal and pathological processes, including development, wound healing and neoplastic transformation. The FGFs are mitogenic for many cell types, both epithelial and mesenchymal. Moreover, FGFs have been shown to increase the motility and invasiveness of a variety of cell types and can even inhibit cell death in the appropriate context. Thus, FGFs have a broad range of biological activities that can play an important role in prostate tumorigenesis.

FGFs interact with a family of four distinct, high-affinity tyrosine kinase receptors, designated FGFR-1 to -4. Binding of FGFs to the extracellular domains of FGF receptors results in receptor dimerization and transphosphorylation of tyrosine residues in the intracellular domain that is required for FGF receptor kinase activation. Ultimately, activation of FGF receptors leads to signal transduction through multiple pathways including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinases (MAPK), and signal transducers and activators of transcription (STATs). These effectors in turn disseminate the receptor tyrosine kinase signals by activating many target proteins, including transcription factors in the nucleus.

The human IGF gene family consists of: two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR), six high-affinity binding proteins (IGFBPs 1–6), a large group of IGFBP proteases and a new group of proteins, low-affinity IGFBP-related proteins (IGFBP-rPs). Members of this family form a network of interactions both
among themselves and with other GF families and their signaling pathways, including MAPK, ERK, and PI3K.\textsuperscript{71}

The EGFR family is comprised of four members; c-erbB-1 (EGFR), c-erbB-2 (HER2/neu), c-erbB-3 (HER3) and c-erbB-4 (HER4).\textsuperscript{72,73} This family of GFs signals primarily via Receptor tyrosine kinases (RTKs) and is a key factor in epithelial malignancies. The activity of this GF enhance tumor growth, invasion, and metastasis not only tumor cells, but also tumor-associated endothelial cells. To date, six mammalian ligands that bind to EGFR have been characterized, including: epidermal growth factor (EGF), transforming growth factor-\(\alpha\) (TGF\(\alpha\)), amphiregulin, heparin-binding EGF-like growth factor, betacellulin, and epiregulin.\textsuperscript{74,75} Once activated by ligand binding, the receptor subsequently stimulates multiple signal pathways which include: Ras/MAPK, PI3K/AKT, NF\(\kappa\)B, and others.\textsuperscript{76-78}

EGF and TGF-\(\alpha\) are structurally similar and hence are similar in their ability to bind and regulate the EGFR.\textsuperscript{65} Their biological activities over-lap and include roles in embryogenesis, cell differentiation, and angiogenesis.\textsuperscript{79} The normal prostate contains large amounts of EGF produced by the epithelial cells, and Immunohistochemical studies have located TGF-\(\alpha\) production principally in the stromal cells in normal prostatic tissue.\textsuperscript{80} Moreover, increased expression of EGF/TGF-\(\alpha\) has been linked to prostate cancer development.\textsuperscript{81}

Three proteins of the TGF-\(\beta\) superfamily, TGF-\(\beta\)1, TGF-\(\beta\)2, and TGF-\(\beta\)3, are expressed during prostate development and in the adult prostate in both normal and malignant tissue.\textsuperscript{82,83} Of the three isoforms, TGF-\(\beta\)1 predominates in all tissues, whereas
the expression of TGF-β2 and TGF-β3 are more tissue-restricted. However, all three isoforms share a multiplicity of biological effects. Although TGFβRI and TGFβRII are transmembrane serine-threonine kinases, they can trigger decreases in the expression of members of the Src family of tyrosine kinases affecting protein tyrosine kinase signaling and hence growth regulation. Additionally, TGF-β can oppose the mitogenic effects of the stimulatory growth factors of IGF-I, EGF, TGF-α, bFGF, and KGF in normal tissues.

The VEGF family is a family of glycosylated protein that exists in at least five isoforms. Members of the VEGF family include VEGF/VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and Placental Growth Factor (PIGF). VEGF is one of the most important promoters of angiogenesis and vascular permeability, especially during embryogenesis, skeleton growth and reproductive functions. This family of GFs signal primarily through tyrosine kinases VEGFR1 and VEGFR2 and stimulate cell survival, proliferation, migration, and/or adhesion. The expression of VEGF is regulated by hypoxia, and hypoxic induction leads to up-regulation of VEGF and a then a subsequent increase in blood vessels formation. VEGF appears to play a role in the growth of prostate cancer, contributing to angiogenesis thus permitting tumor progression. The human prostate relies on a range of growth factors for its normal growth and development. Many of these pathways have been shown to be upregulated in prostate cancer and there is strong evidence linking each of these pathways to prostate cancer initiation and progression.
1.1.7 Rationale for Focusing on MMP-7

Matrilysin (MMP-7) is a member of the MMP gene family that has an essential role in tumor invasion. Following activation, MMP-7 displays broad proteolytic activity against a variety of ECM substrates which include: proteoglycans, elastin, laminin, fibronectin, and casein.\textsuperscript{92-94} MMP-7 has been shown to be overexpressed in malignant tumor cells including, prostate, gastric, head, and neck, lung, hepatocellular, and colorectal carcinomas.\textsuperscript{93} MMP-7 mRNA has also been shown to increase as colorectal cancer progresses.\textsuperscript{92}

Compared to other MMPs, MMP-7 is unique due to the lack of a C-terminal domain, which gives it a low molecular mass of 28 kDa.\textsuperscript{95,96} This unique structure and pattern of localization (being secreted by malignant cancer cells, versus being secreted from stromal cells such as MMP-2,-9 and -13) suggest that this enzyme may function in a manner distinct from other MMPs, and may contribute directly to the invasive potential of colorectal carcinomas and other epithelial cancers.\textsuperscript{92} Furthermore, the expression of MMP-7 has been significantly correlated with the presence of nodal or distant metastases.\textsuperscript{97} In addition to invasion, migration, and metastasis, active MMP-7 has also been shown to cleave (activate) the propeptides of proMMP2 and proMMP9 (two of the most studied MMPs) to facilitate tumor invasion.\textsuperscript{97,98} However, relatively little is known about the function of MMP-7, in prostate cancer.

Therefore, the aim of this study was to examine the relationship between TGF-\(\beta\) and matrix metalloproteinase-7 (MMP-7) expression in prostate cancer cells and to determine the role of MMP-7 in the proliferation, migration, and invasion of prostate
cancer cells. This study is the first to investigate the role of TGF-β1 in the induction of matrix metalloprotease-7 (MMP-7) and the effects on cell proliferation, migration, and invasion in PC3 and E006AA cell lines.

1.2 Research Questions

In normal epithelial cells, TGF-β has been shown to induce growth inhibition of most cell types by causing cell cycle arrest in the G1 phase. This process is initiated when TGF-β induces the expression of the cyclin-dependent kinase (CDk) 4/6 inhibitor p15Ink4B (p15) and represses the expression of c-Myc. In certain cell types, TGF-β also upregulates p21, a CDK2 inhibitor, and down-regulates cdc25A, a phosphatase that activates CDK2. Induction of CDK inhibitors appears to represent key events in TGF-β induced growth arrest. However, as normal cells transform into cancer cells, they develop a resistance to the growth inhibitory effects of TGF-β, and TGF-β acts as a tumor promoter within these cells and exerts positive effects on migration, invasion, angiogenesis, and metastasis.

Our laboratory has demonstrated the increased migratory and invasive behaviors induced by TGF-β. Moreover, previous studies have shown that MMPs are essential to the migratory and invasive behaviors of advanced carcinoma cells. Yet no study has determined the correlation of the effects TGF-β on the regulation of MMP-7 in prostate cancer cells. Therefore, the aim of this study was to answer the following research questions:

RQ1: What are the effects of TGF-β on the regulation of matrix metalloproteinases-7 in prostate cancer cells?
RQ2: What are the function(s) of MMP-7 in TGF-β mediated cell migration, invasion, and proliferation?

1.3 Hypotheses

The hypotheses of this research are as follows:

HO1: TGF-β can regulate both the expression and protein levels of MMP-7 in prostate cancer cells.

HO2: MMP-7 plays a pivotal role in the processes of TGF-β mediated cell proliferation, migration, and invasion in prostate cancer cells.

1.4 Specific Aims

To test the hypotheses and answer pertinent research questions associated with this current study, the following aims were adopted:

1.4.1 Specific Aim 1

1A. To determine the basal expression of MMP-7 mRNA, relative protein levels, and secreted protein levels of normal prostate epithelial cells and prostate cancer cell lines.

1B. To determine the effects of TGF-β1 on MMP-7 expression and protein levels in prostate cancer cells.

Rationale: In the initial stages of invasion and metastasis of neighboring tissues metastatic cancer, cell migration and invasion have been shown to be contingent upon the degradation of the extracellular matrix (ECM). This process leads to the subsequent escape of cancer cells from their confined positions within the solid tumor mass. This phenomenon is attained via the expression, secretion, and
activation of various MMPs. Moreover, previous studies have shown TGF-β to regulate the expression of various MMPs. Moreover, MMPs produced by either cancer cells or residents’ stromal cells activate latent TGF-β in the extracellular matrix, together facilitating the enhancement of both tumor development and progression [35]. Thus, MMPs have been chosen as promising targets for cancer therapy on the basis of their aberrant up-regulation in malignant tumors and their ability to promote cancer metastasis in response to TGF-β signaling [38]. Therefore, the researcher investigated the potential involvement of the TGF-β pathway and its effects on the regulation of MMP-7 in TGF-β mediated cell migration, invasion, and proliferation in PC3 and E006AA cell lines.

**Experimental Design:** The basal expression levels of MMP-7 mRNA and protein was determined using prostate and prostate cancer epithelial cells seeded in 10 cm dishes and allowed to grow for 48 hours. Immediately after this culturing period, the cell culture media was collected to analyze the amount of protein contained in the conditioned culture media. After removing the conditioned culture media from the cells, the cells were lysed and both RNA and protein was extracted and analyzed for MMP-7 mRNA and protein expression using reverse transcription polymer chain reaction (RT-PCR) and Western Blot respectively. In order to determine the effects of TGF-β1 on MMP-7 mRNA expression and relative protein levels, PC3 prostate cancer cells lines were seeded to ~80% confluency in 6-well plates and treated with 5ng/ml of exogenous TGF-β1 at varying time points (4, 8, 24, and 48 hours).
1.4.2 Specific Aim 2

To determine the role of MMP-7 in TGF-β mediated cell migration, invasion, and proliferation of prostate cancer cells.

**Rationale:** The process of metastasis includes the multifaceted process of proliferation, migration, and invasion. Moreover, numerous studies have shown that MMPs are required for the processes of cell migration and invasion due to their role in degrading the extracellular matrix and basement membrane, thus facilitating metastasis and angiogenesis. Therefore, in efforts to develop more effective therapeutic strategies we will determine the function of MMP-7 in the multifaceted processes of TGF-β mediated cancer cell migration, invasion, and proliferation in prostate cancer cell lines.

**Experimental Design:** PC3 and E006AA cells lines were be seeded in 6-well plates and grown to ~80% confluency; the cells were transiently transfected to knockdown the MMP-7 protein using siRNA specific for MMP-7. The transfected cells will be used to perform, migration, invasion, and proliferation assays.
2.1 Transforming Growth Factor Beta (TGF-β)

The transforming growth factor beta (TGF-β) superfamily consists of more than 30 related members in mammals, including 3 TGF-βs, 4 activins, inhibins, and over 20 bone morphogenetic proteins (BMPs). TGF-β and its receptors are widely expressed in all tissues of the body and its signal transduction pathways have been implicated in the pathogenesis of many diseases, ranging from autoimmune disorders and infectious diseases to fibrosis and cancer. TGF-β modulates cell proliferation, differentiation, apoptosis, adhesion, and migration, in various cell types and favors the production of extracellular matrix proteins, and is the dynamic inducer of ECM generation known thus far. TGF-β also plays an important role in the regulation of extracellular matrix synthesis, degradation and remodeling by stimulating the synthesis of collagens, fibronectin, proteoglycans, tenascins, thrombopondin, plasminogen activator inhibitor-1 (PAI-1), and tissue inhibitor of metalloprotease-1 (TIMP-1). Most cell types, including immature hematopoietic cells, activated T and B cells, macrophages, neutrophils, and dendritic cells produce TGF-β, and all human cell types are responsive to TGFβ.
There are three known isoforms of TGF-β, and each isoform is encoded by a unique gene on different chromosomes. TGF-β1 is located on chromosome 19 (19q13.1), TGF-β2 on chromosome 1 (1q41), and TGF-β3 on chromosome 14 (14q24). These growth factors are secreted by most cell types and are expressed in mammalian tissues. However, they display distinctive and at times overlapping, spatial and temporal expression patterns. These isoforms contain highly conserved regions, but diverge in several amino acid regions. Yet, all three isoforms function via the same receptor signaling pathways. TGF-β1 and TGF-β2 share 71% sequence homology, whereas TGF-β3 shares an 80% sequence homology with both TGF-β1 and TGF-β2 (Figure 6).

**Figure 6:** Multiple sequence alignments of TGF-β1, β2, and β3.
TGF-β1 is the most abundant and ubiquitously expressed isoform, and its mRNA and/or protein have been localized in cartilage, endochondral, and bone and skin, which suggest their role in the growth and differentiation of these tissues. TGF-β2 was first described in human glioblastoma cells. Physiologically, TGF-β2 is expressed by neurons and astroglial cells in the embryonic nervous system. It is also important in tumor growth enhancing cell proliferation in an autocrine way and/or reducing immune-surveillance of tumor development. The levels of TGF-β3 have been significantly detected in cartilage, bone, brain and lung, and mesenchymal cells.

2.2 TGF-β Signal Transduction

Members of the TGF-β family are always synthesized as an inactive complex that must be activated to enable binding to its receptor and subsequent function. Following activation, most TGF-β superfamily members convey signals via heteromeric complexes of transmembrane serine/threonine kinases, TGF-β type I (TβRI) and TGF-β type II (TβRII) receptors. The TGF-β signaling pathway is activated by ligand binding to the extracellular domain of the type II receptors. This binding induces a conformational change resulting in subsequent phosphorylation and activation of type I receptors. The activated type I receptor then phosphorylates the appropriate Smad protein and initiates the intracellular signaling cascade (Figure 7).
Figure 7. TGF-β and SMAD activation pathway with co-activators, co-repressors, and transcription factors.


The Types I and II receptors have an N-glycosylated extracellular domain which is rich in cysteine residues, one transmembrane domain, and an intracellular serine/threonine kinase domain. The type II receptor kinase is a constitutively active kinase, whereas the type I receptor kinase needs to be activated by the type II receptor kinase. Upon ligand-induced formation of the heteromeric complex, the type II
receptor phosphorylates the type I receptor in a region rich in glycine and serine/threonine residues (termed the GS domain). This phosphorylation changes the conformation of the type I receptor, thereby activating its kinase. The activated type I receptor then propagates the signal by phosphorylating specific intracellular proteins. Thus, the type I receptor acts downstream of the type II receptor and consistent with this notion, has been shown to determine signaling specificity.

The Smad family can be divided into three distinct subfamilies: receptor-regulated Smads (R-Smads: 1,2,3,5,8 and 9), common-partner Smads (Co-Smads: Smad4) and inhibitory Smads (I-Smads: Smad 6 and 7). Activation of the various R-Smads: is predicated upon which member of the TGF-β superfamily is binding to the receptor initiating signal transduction. BMPs activate R-Smads Smad1, Smad5, and Smad8, whereas TGF-β or activin activate R-Smads: Smad2 and Smad3. Following phosphorylation, the activated R-Smads recruit Co-Smads (Smad4) which forms a heteromeric complex and translocates into the nucleus.

Nuclear Smad complexes then bind to DNA directly or indirectly through other DNA-binding proteins and additional cofactors in order to achieve high binding affinity and selectivity to regulate the transcription of specific target genes. Smad4 lacks the C-terminal motif found in all R-Smads and is not phosphorylated following activation of TGF-β receptors. It functions as a convergent node in the Smad pathways downstream of TGF-β superfamily receptors, complexing not only with TGF-β/activin-activated Smad2 and Smad3 but also with BMP-activated Smad1, Smad5, and Smad8. Smad4 can translocate to the nucleus only when complexed with R-Smads.
Conversely, ligand-activated Smad2 and Smad3 translocate to the nucleus in a Smad4-independent fashion. This implies, by default, that the principal function of Smad4 is to regulate transcription rather than to transmit the signal from the cytoplasm to the nucleus.

### 2.3 TGF-β in Cancer

As previously stated, TGF-β has a biphasic role in tumorigenesis and cancer cells use TGF-β to enhance their characteristic properties and features. In the later stages of cancer, malignant cells can evade the suppressive effects of TGF-β either through inactivation of core components of the pathway, such as TGF-β receptors, or by downstream alterations that disable just the tumor-suppressive arm of this pathway. By doing so, cancer cells can hijack the remaining TGF-β regulatory functions to their advantage, thus acquiring invasive capabilities, producing autocrine mitogens, and/or releasing prometastatic cytokines.

It is worth noting, that as cancer advances, so too does the overexpression of TGF-β in many late-staged and even metastatic human tumors. During this process, epithelial polarity is gradually lost, organ structure is disrupted and epithelial cells dedifferentiate. Progression to metastatic carcinomas requires additional changes, such as proteolytic degradation of the basement membrane, conversion from a sessile to a migratory phenotype, survival in the blood stream and formation of metastases at distant sites. Moreover, TGF-β has been shown to play a role in these caveats of cancer progression.
Recent experimental studies demonstrate the ability of TGF-β to fully induce polarized, non-invasive epithelial cells so that they acquire a mesenchymal, spindle cell phenotype (which is EMT).\textsuperscript{153} The mesenchymal cells obtained after EMT \textit{in vitro} or cultivated from tumors were invasive in several \textit{in vitro} assays. They also displayed autocrine production of TGFβ, which is required to maintain the mesenchymal, spindle-like, invasive cell phenotype. Additionally, neutralizing antibodies to TGFβ caused reversion to a polarized, epithelial phenotype.\textsuperscript{153} Interestingly, TGF-β production by the tumor cells and the acceleration of EMT by exogenous TGFβ was demonstrated \textit{in vivo}.\textsuperscript{153}

Experimental studies show that blocking TGF-β1 signaling in the primary tumor reduces metastasis. In patients with hereditary non-polyposis colorectal cancer (HNCC), TβRII is inactivated and the cells no longer respond to TGF-β signaling and display reduced metastasis and longer survival rates than patients with sporadic colon cancers.\textsuperscript{154} Equally expression of a dominant negative truncated TβRII in highly metastatic colon carcinoma\textsuperscript{155} and breast carcinoma cell line\textsuperscript{156} blocked metastasis. These findings are evidence of the essential role that TGF-β plays in the multifaceted process of cancer metastasis.

It has been established that TGF-β signals via the canonical Smad signaling cascade. However, evidence suggests that the non-canonical signaling pathways are primarily involved in the induction of EMT by TGFβ. Signaling through integrin β1,\textsuperscript{157} p38MAPK,\textsuperscript{158} phosphoinositide 3-kinase (PI3K),\textsuperscript{108,159,160} Ras homologous (Rho)
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Jagged/Notch, nuclear factor κβ (NF-κβ) have all been shown to be required for TGFβ-induced EMT (Figure 8).

**Figure 8:** TGF-β canonical and non-canonical signaling pathways.

Source: Figure adopted from Costanza, B.; Umelo, I. A.; Bellier, J.; Castronovo, V., et al. Stromal Modulators of TGF-β in Cancer. *J. Clin. Med.* 2017, 6 (1), 7. Canonical and non-canonical TGF-β signaling pathways. (A) The canonical signaling pathway, biologically active TGF-β ligand binds to TGFβRII, which activates TGFβRI. TGFβRI-regulated SMAD2/3 proteins are phosphorylated at C-terminal serine residues and form complexes with SMAD4 (co-SMAD), initiating transcriptional regulation of target genes. (B) The non-canonical signaling pathways, the TGF-β receptor complex transmits its signal through other factors, such as the mitogen-activated protein kinases (MAPKs), phosphatidylinositide 3-kinase (PI3K), TNF receptor-associated factor 4/6 (TRAF4/6) and Rho family of small GTPases.

The initiation of multiple signaling pathways downstream of the activated receptor complex reveal the true pleiotropic effects of TGF-β. Emerging evidence revealed that both TGF-β canonical and non-canonical signaling cascades can simultaneously occur through crosstalk of core pathway components and combined utilization of SMAD/non-SMAD transcription factors. Although it is widely believed that the non-Smad pathways are predominantly involved in TGFβ-mediated EMT, the
Smad-dependent pathway has also been implicated in select cases. TGFβ signaling through TβRI and TβRII has also been implicated for TGFβ-mediated EMT and Smad overexpression has been shown to cause synergistic induction of EMT when combined with activated TGFβ receptors.155

2.4 Matrix Metalloproteinases (MMPs)

MMPs are a family of over 25 genetically distinct but structurally related zinc-dependent matrix-degrading enzymes110 who share similar structures, and possess the ability to degrade virtually all components of the ECM.165-168 MMPs are initially synthesized in an enzymatically inactive state due to the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site.169 Based on their domain structure and substrate preferences, MMPs are traditionally grouped into the following categories (1) collagenases, including MMP-1, -8, -13; (2) stromolysins, MMP-3 and MMP-10; (3) gelatinases, MMP-2 and MMP-9; (4) matrilysins, MMP-7 and MMP-26; (5) membrane-type MMPs (MT-MMPs); and others (Figure 9).167
Figure 9. Classifications, structures, and domain of the Matrix Metalloproteinases.

Sources: Figure adopted from Radisky, E. S.; Radisky, D.C. Matrix Metalloproteinase-Induced Epithelial-Mesenchymal Transition in Breast Cancer. J Mammary Gland Biol Neoplasia. 2010;15(2), 201-212.
Ting-Yen, C-M; Tsaoab, Chao-BinYehac; Shun-FaYang, Matrix Metalloproteinases in Pneumonia. Clinica Chimica Acta. 2014;43(10), 272-277. The various domain organizations of human MMPs are illustrated; S, signal peptide; Pro, propeptide; CAT, catalytic domain; F, fibronectin repeats; PEX, hemopexin domain; TM, transmembrane domain; GPI, glycoposphatidylinositol membrane anchor; C, cytoplasmic domain; CA, cysteine array; Ig, immunoglobulin-like domain. The flexible, variable length linker or hinge region is depicted as the curved black line connecting domains.

Under normal physiological conditions, the proteolytic activities of MMPs influence plethora of cellular process like cell proliferation, migration, and adhesion, as well as many fundamental physiological events involving tissue remodeling such as angiogenesis, bone development, wound healing, and uterine and mammary
involution.\textsuperscript{170,171} They also play an integral role in opposing the effects on angiogenesis; regulation of cell growth via cleavage of cell surface-bound growth factors and receptors; release of growth factors sequestered in the ECM (including Transforming Growth Factor-\(\alpha\) (TGF-\(\alpha\));\textsuperscript{172} regulation of apoptosis via release of death or survival factors; alteration of cell motility by revealing cryptic matrix signals or cleavage of adhesion molecules; and effects on the immune system and host defense.\textsuperscript{173} Moreover, MMPs have also been shown to affect the bioavailability of TGF-\(\beta\), by releasing it from an inactive complex.\textsuperscript{174}

The expression of many MMPs is precisely regulated at the level of transcription by a variety of growth factors, cytokines, and chemokines, though post-transcriptional pathways may contribute to this regulation in specific cases.\textsuperscript{175,176} This usually results in relatively low basal levels for these enzymes under normal physiological conditions.\textsuperscript{177} The proteolytic activity of MMPs is mainly regulated by tissue inhibitors of MMPs (TIMPs). Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been described and they can inhibit all active MMPs, however, not with the same efficacy.\textsuperscript{178-180} A loss of activity control may result in diseases such as arthritis, cancer, atherosclerosis, aneurysms, nephritis, tissue ulcers, and fibrosis.

2.5 Matrix Metalloproteinases in Cancer

Tumor cells fulfill their metastatic potential after acquiring advantageous characteristics, which allow them to escape from the primary tumor site, migrate and invade surrounding tissues, enter the vasculature, circulate and reach secondary sites, extravasate and establish metastatic foci.\textsuperscript{181-183} However to complete this feat, tumor
cells require the assistance of a variety of proteolytic enzymes. The MMPs, as their name implies, are associated with degradation of the extracellular membrane (ECM), including the basement membrane, which is a specialized matrix composed of type IV collagen, laminin, entactin, proteoglycans, and glycosaminoglycans.\textsuperscript{184}

In cancer, MMPs are believed to promote tumor progression by enhancing growth via angiogenesis, disrupting local tissue architecture to allow tumor growth, and degrading basement membrane barriers to facilitate metastatic spread.\textsuperscript{166} As a result, the expression and activity of MMPs in a variety of human cancers have been studied intensively, and their expression and activity have been found to be increased in virtually every type of human cancer.\textsuperscript{185}

These findings also correlate with advanced tumor stage, increased invasion, and metastasis, and shortened survival.\textsuperscript{186} Mounting evidence demonstrates that MMPs are involved in the earlier stages tumorigenesis (e.g., in malignant transformation, angiogenesis, and tumor growth both at the primary and metastatic sites).\textsuperscript{175,187,188} Thus, MMPs have been considered as potential diagnostic and prognostic biomarkers in many types and stages of cancer.\textsuperscript{189}

Cancer progression involves different stages including tumor growth, invasion, metastasis, and angiogenesis all of which can be modulated by MMPs.\textsuperscript{190,191} The expression of MMPs in the tumor microenvironment depends not only on the cancer cells, but also on the neighboring stromal cells. MMPs exert their proteolytic activity to degrade the physical barriers, thus facilitating angiogenesis, tumor cells invasion and metastasis. Tumor growth and angiogenesis also depend on the increased availability of
signaling molecules, such as growth factors and cytokines, by MMPs making these factors more accessible to the cancer cells and the tumor microenvironment. This occurs by liberating them from the ECM (IGF, bFGF and VEGF) or by shedding them from the cell surface (EGF, TGF-α, HB-EGF). Angiogenesis is also tightly modulated by the release of negative regulators of angiogenesis, such as angiostatin, tumstatin, endostatin, and endorepellin. MMPs also modulate the cell–cell and cell–ECM interactions by processing E-cadherin and integrins, respectively, affecting both cell phenotype (EMT) and increasing cell migration (Figure 10). 192-194

**Figure 10:** Pivotal roles of MMPs in the four hallmarks of cancer: migration, invasion, metastasis, and angiogenesis.

MMPs have been chosen as promising targets for cancer therapy on the basis of their aberrant up-regulation in malignant tumors and their ability to promote cancer metastasis.\textsuperscript{180,195} Moreover, MMPs have recently been proposed as pivotal regulators within the tumor stroma and regulate responses of both the cellular and noncellular microenvironment).\textsuperscript{196} The tumor stroma consists of several types of resident cells and infiltrating cells derived from bone marrow, which together play crucial roles in the promotion of tumor growth and metastasis.\textsuperscript{165,196} In cancer cells, TGF-\(\beta\) regulates MMPs expression. Conversely, MMPs produced by either cancer cells or residents’ stroma cells, activate latent TGF-\(\beta\) in the extracellular matrix, and in combination facilitate the enhancement of tumor progression.\textsuperscript{165,192} Moreover, MMPs can promote EMT by proteolytic activation of TGF-\(\beta\), and the same activation can be involved in the suppression of T-lymphocyte reaction against cancer cell proliferation.\textsuperscript{165} For a tumor cell to metastasize from the primary tumor requires disruption of cell-cell interactions from the surrounding cells.\textsuperscript{197} In addition to this, cells must also undergo detachment from the ECM and develop a resistance to anoikis, (apoptosis upon cell detachment from ECM).\textsuperscript{197} Subsequent attachment, movement and invasion of cancer cells are functionally facilitated by the actin cytoskeleton and tubulin as the structural component of microtubules.\textsuperscript{197}

TGF-\(\beta\) has tumor-inhibitory activity in the early stages of tumorgenesis, but it promotes tumor invasive characteristics in metastatic disease. Recent evidence implicates active (dephosphorylated) cofilin, an F-actin severing protein required for cytoskeleton reorganization, as an important contributor to altering TGF-\(\beta\)’s characteristics from a
growth suppressor to that of a promoter of prostate cancer invasion and metastasis. Consequently, cancer cells eventually lose their ability to adhere to adjacent neighboring cells, as well as ECM proteins. This process leads to subsequent EMT, and cells acquire invasive and metastatic characteristics.

2.6 MMPs in Prostate Cancer

In prostate cancer tissue, there is an imbalanced expression of MMPs and TIMPs. This imbalance is demonstrated as a general loss of TIMPs and an upregulation of MMPs. Elevated MMP activity promotes PCa progression not only by facilitating metastasis, but also by overwhelmingly influencing various steps of cell proliferation, apoptosis, angiogenesis, and EMT. According to numerous studies, MMPs are more active in the advanced stages of carcinoma. To confirm these findings, analysis of MMP mRNA and protein levels in the serum and tissue samples from PCa patients has shown that increased expression of MMP-2, -3, -7, -9, -13, -14, -15, -and 26 are correlated with advanced or metastatic disease. The expression of these MMPs promote PCa progression, however with subtle differences in their patterns of expression, biological function, regulation, and prognostic value (Figure 11).
Figure 11: The roles of various MMPs in the hallmarks of prostate cancer progression.

Source: Figure adopted from Yixuan Gong, UDC-V; William, K. Oh. Roles of Matrix Metalloproteinases and their Natural Inhibitors in Prostate Cancer Progression. Cancers. 2014;6, 1298-1327.

MMP-2 belongs to the gelatinase subfamily, which is a group of proteolytic enzymes distinguished by their fibronectin-like gelatin-binding domain, which allows it ability to degrade gelatin into its sub-compounds (polypeptides, peptides, and amino acids). Moreover, increased expression of MMP-2 has been extensively reported in PCa, and, higher MMP-2 expression has been correlated with increased tumor burden, higher Gleason score, and more advanced pathological Tumor, Lymph Node, Metastasis (TNM) stage. To substantiate these findings, it was found that MMP-2 expression was not present in micro metastasis and surrounding stromal cells, but rather present in metastatic disease, which strongly suggest that elevated MMP-2 expression was associated with PCa progression and metastasis. Although IHC staining and in situ hybridization have demonstrated the increased expression of MMP-2 in PCa tissue...
specimens, in vitro studies of MMP-2 expression in cultured PCA cells have given somewhat inconsistent result.\textsuperscript{212} For instance, a study by Lokeshwar et al. conditioned media from freshly cultured malignant prostate explants contained a higher proportion of the active form of MMP-2 than normal tissues.\textsuperscript{212}

However, in another study to examine MMP-2 secretion from cultured normal and neoplastic prostate cells derived from different zones of the prostate, only prostate stromal cells secreted the pro-enzyme form of MMP-2, whereas conditioned media from epithelial cells of various origins demonstrated little to no pro-MMP-2 as examined by zymography.\textsuperscript{213} The absence of MMP-2 expression in tumor epithelial cells in the study can be potentially explained by the low-grade tumor samples used in the study or lack of stromal support in cell culture. It was demonstrated that addition of fibronectin to cell culture induced high expression of pro and active forms of MMP-2 in prostate cancer cell lines,\textsuperscript{214} suggesting that a cell culture model that more closely mimics the in vivo tumor microenvironment is critical when studying MMP expression and function in vitro.

MMP-7 is the smallest known member of the MMP family, and is secreted as a 28 kDa proenzyme which can be activated in vitro by APMA (4-Aminophenylmercuric Acetate), trypsin and high temperatures and in vivo by MMP-3 to a 19 kDa active MMP-7 enzyme.\textsuperscript{215} Activated MMP-7 mediates the cleavage of ECM and basement membrane proteins such as fibronectin, collagen type IV, laminin, and others, as well as mediates the ectodomain shedding of pro- and anti-tumor molecules such as tumor necrosis factor-\alpha, Fas ligand, heparin-binding epidermal growth factor, E-cadherin and \(\beta4\)-integrin.\textsuperscript{216-218} MMP-7 has been also been shown to display biphasic characteristics in regulating
angiogenesis not only by mobilizing endogenous pro-angiogenic factors, but also by generating angiogenic inhibitors such as endostatin.\textsuperscript{219}

Elevated MMP-7 expression has been detected in a variety of epithelial and mesenchymal tumors.\textsuperscript{220,221} Likewise, in prostate cancer, 77\% and 50\% of prostate tumors were found to focally express MMP-7 by \textit{in situ} hybridization analyses and western blotting, respectively.\textsuperscript{222} To validate these findings, a study aimed at investigating serum levels of various MMPs in the prostate cancer, found that circulating MMP-7 was significantly elevated in individuals with distant metastases, suggesting that MMP-7 may play a role in facilitating distant metastases.\textsuperscript{223}

A recent publication has linked MMP-7 to bone metastasis from prostatic adenocarcinoma. Prostate and breast cancers are unique among solid tumors in their strong propensity to metastasize to bone.\textsuperscript{224,225} It has been discovered that approximately 84\% prostate cancer patients have bone metastases upon autopsy\textsuperscript{225} which further substantiates the need for effective MMP inhibitors. Of the MMPs that are highly expressed within the tumor-bone microenvironment (MMP-2, -3, -7, -9, and -13), only osteoclast-derived MMP-7 significantly contributed to human breast-to-bone metastatic tumor growth and tumor-induced osteolysis in experimental mice,\textsuperscript{226} suggesting that MMP-7 can be an effective target for advanced PCa therapies. An additional point to consider in the case of MMP-7 is that studies have shown that active MMP-7 can activate both MMPs -2, and -9 to exacerbate cancer progression.\textsuperscript{97}

Similar to MMP-2, MMP-9 also belongs to the gelatinase subfamily. As previously stated, MMPs are synthesized as inactive zymogen and require cleavage of the
prodomain to achieve activation. MMP-9 has been shown to be activated by other members of the MMP family such as MMP-2, -3,227 and -7.97 There have been discrepancies in reports detailing the expression of MMP-9 in prostate cancer tissue. Protein expression has been reported to be either absent228,229 or present208,230 and the localization of MMP-9 expression reported in the literature also varies as well. In some studies, MMP-9 mRNA expression was detected only in macrophages in areas of prostatic inflammation222 or along the invasive edge of higher Gleason score tumors.208 However, according to one published study, 94.1% of prostate cancer cells actually expressed MMP-9 in the cytosol and intracellular MMP-9 expression was directly correlated with Gleason score, but not with prognosis.230 This was an interesting finding because it is challenging the established paradigm of MMP expression and localization.

The discrepancies related to MMP-9 expression could be partly explained by differences in the degree of invasiveness of the tumor samples used in the studies or by the sensitivity of detection methods. Nonetheless, it is interesting to note that in a study of fresh prostate tissue obtained from 22 radical prostatectomies, the overall collagenolytic and gelatinolytic activities was relatively low in comparison to other malignancies such as basal cell carcinomas, which may help explain why the majority of localized primary prostate tumors remain confined to the prostate for relative long periods of time compared to other more invasive cancers.231

Similarly to MMP-2, MMP-9 is derived from both tumor cells and tumor microenvironments and plays as imperative role in the process of cancer metastasis. Additionally, MMP-9 expression detected on both the cell surface and in its secreted
forms is thought to contribute to enhancement of prostate cancer growth, metastasis and angiogenesis. LNCaP, DU-145, and PC-3 cells are commonly used prostate cancer cell lines that have demonstrated low, moderate, and high metastatic potential in Matrigel invasion assays, respectively.\textsuperscript{231,232} PC-3 cells show increased expression of MMP-9 compared with LNCaP and DU-145, which correlate with the highest invasive activity among the cell lines,\textsuperscript{232} substantiating evidence that MMP-9 is important to invasion and metastasis of PCa cells. Stable expression of human MMP-9 in poorly metastatic LNCaP cells produced a 2 to 3-fold increase in MMP-9 activity with an associated increase in invasiveness, further validating the role of MMP-9 in the metastatic process.\textsuperscript{233}

MMP-9 has also been shown to be involved in the regulation of angiogenesis; antisense ablation of MMP-9 expression in DU-145 and PC-3 cells produced concomitant inhibition of the gene expression of the proangiogenic factors such as vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1).\textsuperscript{234} MMP-9 knockdown also increased the release of angiotatin, a key protein that suppresses angiogenesis and decreases secretion of VEGF, the most common and potent angiogenic factor, in PC-3 cells.\textsuperscript{234} Furthermore, MMP-9 can also activate urokinase plasminogen activator (uPA), serpin protease nexin-1 (PN-1) and other matrix proteins involved in the process of invasion and angiogenesis.\textsuperscript{235,236}

MMP-9 expression is regulated by tumor-stromal interactions in prostate cancer.\textsuperscript{237} Experimental studies which co-cultured prostate cancer and stromal cells \textit{in vitro} demonstrated an enhanced expression of pro-MMP-9 in prostate cancer cells, and down-regulating TIMPs in stromal cells.\textsuperscript{237} MMP-9 expression was also induced in
metastatic PC-3 cells grown in human fetal bone implants in severe combined immunodeficient (SCID) mice. An additional co-culture experiment culturing endothelial cells with prostate cancer cells also showed significant enhancement of MMP-9 expression and subsequent invasiveness of cancer cells through increased IL-6 secretion from endothelial cells, suggesting that growth factors or cytokines secreted by tumor cells, stromal cells, and infiltrating inflammatory cells in the tumor microenvironment collectively regulate MMP-9 gene expression in an autocrine and paracrine manner, demonstrating that several cytokines and related proteins can regulate MMP-9 expression in prostate cancer. Moreover, it was shown that increased IL-6 expression, which is often seen in advanced prostate cancer, resulted in activation of MMP-9 expression through the TGF-β pathway, thus indirectly implicating TGF-β to be an inducer and enhancer or the metastatic potential of prostate cancer cells.

2.7 MMPs and TGF-β

TGF-β and MMPs have been implicated to function in a bidirectional regulatory loop associated with cancer development. TGF-β needs to be proteolytically activated by MMPs in order to exert its cellular functions, but activated TGF-β in tumors modulates the balance of ECM remodeling by regulating the expression of MMPs and their tissue inhibitors TIMPs. Moreover it has been established that TGF-β regulates EMT, a characteristic of invasive and metastatic cells, subsequently leading to increased metastases in human cancer, as well as in animal cancer models. Moreover, TGF-β is able to stimulate several MMPs in cancer cells. According to a recent study, treatment of prostate stromal cells with TGF-β moderately increased the
secretion of pro-MMP-2 protein, while the treatment of epithelial cells with TGF-β induced expression and secretion of both MMP-2 and MMP-9. These data suggest the precarious role of TGF-β and its role in the regulation of MMP-2 and MMP-9 in PCa cells.  

The regulation and effects of TGF-β on MMPs can be attributed to its capacity to activate a plethora of signal transduction pathways and different transcription factors other than Smads, thus demonstrating the complexity in the capacity of TGF-β to regulate MMP expression in cancer cells. There are at least two different regulatory domains present in various MMPs: (1) TGF-β inhibitory element, TIE and (2) the Smad binding element (SBE) [43-45]. Because MMP-1, MMP-7, MMP-9, MMP-13, and MM-P14 contain TIE binding sites in their promoters, it is postulated that the expression of these MMPs may be modulated by TGF-β (Figure 12). Conversely, TGF-β has also been shown to negatively regulate the transcription of MMP-1 and MMP-7. Molecular analyses have demonstrated that the consensus TIE found within the promoters of MMP-9, MMP-13, and MMP-14 were not required for their induction by TGF-β.
Figure 12. Regulatory elements within the promoter regions of human MMP genes. (Transcription start sites are indicated with a bent arrow and the main functionally validated cis-elements are represented within boxes. The relative positions of the different binding sites are not drawn to scale.)


Recent research has revealed that Smads can interact with members of the AP1 family, to alter the expression of MMPs.\(^{246,247}\) Additionally, TGF-β can regulate MMP-13 gene expression partially via the AP1 site and partially through interactions of Smad3 in conjunction with JunB and Runx-2.\(^{248}\) Furthermore, TGF-β directly activates other transcription factors implicated in the regulation of MMPs expression, and can induce
cell signaling which culminates in the transactivation of AP1, PEA3, NF B, SP1, and MEF-2 transcription factors to enhance MMP promoters’ trans-activity.\textsuperscript{249-251}

TGF-\(\beta\) has been shown to activate numerous intracellular signaling pathways and this may explain its extensive role in cancer, as well as its profound impact in the regulation of MMPs.\textsuperscript{165} For example, TGF-\(\beta\) has been shown to induce the expression of MMP-2 by activating the TAK1-p38 MAPK in breast epithelial cells,\textsuperscript{252,253} while simultaneously enhancing SW1990, (pancreatic cancer cells derived from a spleen metastasis), invasiveness and induction of MMP-2 expression via the activation of Rac1/ROS/NF B.\textsuperscript{254} Furthermore, MMP-9 has been shown to be upregulated by TGF-\(\beta\) through the activation of ERK1, 2, Rac1-ROS-NF\(\kappa\)B, and TAK1-NF\(\kappa\)B in transformed keratinocytes, breast, and hepatocellular carcinoma cells.\textsuperscript{252-254}

TGF-\(\beta\) also plays a major role in promoting breast cancer migration, invasion, and metastasis by acting at various levels: (1) on the stroma and neighboring cells surrounding the tumor and (2) directly on the cancer cells themselves. Moreover, these pro-metastatic responses of TGF-\(\beta\) include the ability to remodel the surrounding ECM, through stimulation of matrix metalloproteinase (MMP) expression and modulation of the plasminogen activation system, resulting in TGF\(\beta\)-mediated matrix degradation and, consequently, an increasing release of stored TGF-\(\beta\) from the ECM that acts as a TGF-\(\beta\) reservoir.\textsuperscript{255}
3.1 Chemicals and Reagents

Recombinant human TGFβ1 (#100-21C) and TGFβ3 (#100-36E) were purchased from PeproTech (Rock Hill, NJ). The antibody against MMP-7 (#sc-80205), MMP-7 siRNA (#sc-41553) control siRNA (#sc-36869) and transfection reagent (#sc-99528) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas). Antibodies against β-Actin (#A5441) and α-Tubulin (#T5168) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse IgG-HRP (#NA931) was obtained from GE Healthcare (Piscataway, NJ).

3.2 Cell Culture and Cell Treatments

Immortalized prostate luminal epithelial cell line, RWPE1, and prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from American Type Culture Collection (ATCC, Rockville, MD). E006AA cells, a prostate cancer cell line derived from an African American patient, were kindly provided by Dr. Shahriar Koochekpour (Department of Cancer Genetics, Center for Genetics and Pharmacology, Roswell Park Cancer Institute, New York, USA).²⁵⁶,²⁵⁷ RWPE1 cells were maintained in keratinocyte serum-free medium as previously described.²⁵⁸ PC3 and DU145 cells were cultured in MEM; LNCaP cells were cultured in RPMI media, and E006AA cells were cultured in
DMEM media, all supplemented with 5% FBS.\textsuperscript{258} All cells were grown and maintained at 37\textdegree{}C with 5% CO\textsubscript{2}.

To determine the effects of TGF\textbeta{}1 on MMP-7, PC3 cells were cultured in 6-well plates at the density of 1.5 x 10\textsuperscript{5} cells/well, treated with TGF\textbeta{}1 (5 ng/ml) for 4, 8, and 24 hours. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA) containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na\textsubscript{2}EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 µg/ml leupeptin and 1X protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein concentrations were determined by the Lowry HS assay using the Bio-Rad DCProtein Assay kit (Bio-Rad, Hercules, CA) according to the instruction provided by the manufacturer.

3.3 RNA Isolation, Reverse Transcription (RT), PCR and qPCR

Total RNA was isolated from prostate cell lines using TRIzol (Invitrogen, Carlsbad, CA) as previously described.\textsuperscript{259} RT-PCR reactions were performed using the Master Cycler PCR Systems, (Eppendorf) as previously described to detect MMP-7 mRNA levels. Gene specific primers were designed with NCBI Primer-Blast. The following primers were used: MMP-7 forward, 5’–GAGTGCCAGATGTTGCAGAA-3’; MMP-7 reverse, 5’–ACCCAAAGAATGGCCAAGTT-3’; L-19 forward, 5’–GAAATCGCCAATGCCAACTC-3’; L-19 reverse, 5’–TCTTAGACCTGCGACTCA-3’. L19 (a ribosomal protein) was used as an internal control. The PCR products were visualized on 2% agarose gels stained with ethidium bromide (Amresco, Solon, OH). Analyses of PCR products were carried out in at least 3 independent experiments.
The real-time PCR analysis was performed using the iCycler (BioRad, Hercules, CA) in 96 well plates. A fluorescence-based SYBR-Green Master Mix (Bio-Rad) was used in a volume of 25µl/well. The following primers were used: MMP-7, forward, 5’-TTGTATGGGGACTGACA-3’; MMP-7 reverse, 5’-GCCCATCAATGGGTAGGAGT-3’. GAPDH primer: forward, 5’–GAAGGTGAAGGTCGGAGTC-3’; reverse, 5’–GAAGATGGTGATGGGATTTC-3’. The thermal profile for the real-time PCR was as follows: (1) 95ºC for 3 min; (2) 95ºC for 10 sec; (3) 56.5ºC for 30 sec; (4) repeating steps 2 and 3 for 40 times. Melting curves were examined for the quality of PCR amplification of each sample. Relative quantification of MMP-7 mRNA expression was determined using the 2^ΔΔCT method.

3.4 Western Blot Analysis

Total cellular proteins were prepared from different prostate cell lines and were analyzed by Western Blot analysis as previously described. Cell lysates were mixed with Lammeli’s buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% glycerol). Individual samples (40–50 µg protein) were subjected to SDS-PAGE in 10% gels and transferred to PVDF membranes (Millipore) at 100V for 90 minutes. The membranes were blocked with 5% fat-free milk in TBST (50 mM Tris, pH 7.5, containing 0.15 M NaCl, and 0.1% Tween–20) and incubated with the MMP-7 primary antibody at a ratio of 1:800 overnight at 4ºC. The blots were washed, and then incubated with anti-mouse secondary antibody conjugated with Horse Radish Peroxidase (HRP) at a dilution of 1:10,000 for 1 hour at room temperature. The blots were developed in Luminata Forte Western HRP substrate mixture (Billerica, MA #WBLUF0500) and the
density of specific protein bands were acquired using the SynGene Imager (Frederick, MD) and analyzed by Image J Analysis Software (NIH). Western Blots for β-actin or α-Tubulin were probed on previously probed blots and used as loading controls.

3.5 ELISA Analysis

The secreted MMP-7 protein levels in culture supernatants were analyzed using a sandwich type MMP-7 specific ELISA kit (R & D Systems, Minneapolis, MN #DY907) following the manufacturer’s instructions with minor modifications. In brief, 96-well plates were coated with MMP-7 capture antibody and incubated overnight at 4°C. Standards were prepared from 2ng/ml of recombinant human MMP-7 stock solution in concentrations ranging from 0- 2000 pg/ml using two-fold serial dilutions. Protein contents of the supernatants were determined and used at a range of 75-80 μg of protein sample; 100 μl of standards and samples were transferred into wells of ELISA plate (clear microplate #DY990, R&D) for duplicate analysis. Absorbance was read at 450 nm with a reference filter set to 540nm using a BioRad microplate reader.

3.6 Transfection with MMP-7 siRNA

To knockdown MMP-7 expression in PC3 and E006AA cells, cells were seeded in six-well plates at a density of 1.5 x 10⁵ cells per well in 1.0 ml antibiotic-free MEM or DMEM, respectively supplemented with 5% FBS. The cells were incubated overnight at 37°C. MMP-7 specific siRNAs or control siRNA were transfected into PC3 and E006AA cells using transfection reagent (Santa Cruz Biotechnology, Dallas, TX), following the manufacturer’s recommendation. Transfection complexes were mixed together in a 1:1 ratio of reagent to siRNA per 200μl of antibiotic-free normal growth
medium, and allowed to incubate at room temperature in the dark for 30 minutes. The transfection reagent and siRNA duplex were added to the cells. Seventy-two hours after transfection, cells were trypsinized (0.25% trypsin/2.21 mM EDTA) and re-plated for biological assays. Western Blot analyses were used to confirm MMP-7 protein knockdown.

3.7 Cell Proliferation Assay

Following transfections of PC3 and E006AA cells, cell growth assays were performed using manual cell counts. In brief, PC3 cells were seeded in 24 well plates at a density of $2.0 \times 10^4$ cells/well, and E006AA cells were seeded in 6 well plates at a density of $1.0 \times 10^5$ cells/well. Both cell lines were allowed to attach for 24 hours. Cells were serum starved for 3 hours and treated with TGF-β1 (5ng/ml) and TGF-β3 (5ng/ml) for 72 hours, trypsinized, and counted using a hemocytometer. Each experiment was performed in duplicates and was repeated at least three times using independent cell preparations. Statistical analyses were performed using Student’s t test with SigmaPlot Analysis Software.

3.8 Migration Assay

After transfections, cell-migration assays were performed using 24-well transwell inserts (8 µm) as previously described with minor modifications. Chemoattractant solutions were prepared by diluting TGF-β1 (5 ng/ml) or EGF (10 ng/ml) into MEM supplemented with 0.2% BSA for PC3 cells, and DMEM supplemented with 5% FBS for E006AA cells. In brief, transwell inserts were coated on both sides with 50 µg/ml Type I Collagen (#354236) purchased from Corning (Bedford, MA). PC3 and E006AA cells
were seeded at $3 \times 10^4$ cells/insert and $2 \times 10^4$ cells/insert, respectively. PC3 cells were allowed to migrate for 5 hours, whereas the E006AA cells were allowed to migrate for 24 hours. Migrating cells on the membrane were fixed in 3.7% paraformaldehyde and stained using DAPI (Fisher Scientific Hampton, NH). Pictures were taken in five different fields for average number of migrated cells to be determined. The results are expressed as migration index, defined as the average number of cells per field for test substance/the average number of cells per field for the medium control. Each experiment was performed in duplicates and was repeated at least three times using independent cell preparations. Statistical analyses were performed using Student’s t test with SigmaPlot Analysis Software.

3.9 Invasion Assay

After transfection, invasive properties of PC3 and E006AA were measured using transwell membranes pre-coated with 50μl of 1:4 matrigel, medium dilution. PC3 and E006AA cells were resuspended at a density of $5.0 \times 10^4$ cells/insert and $4.0 \times 10^4$ cells/ml, respectively in normal growth media supplemented with 0.1% FBS. 500 μl of cell suspension was added to each insert. Cells were treated with 5ng/ml of TGF-β1 or TGF-β3 or 10 ng/ml EGF, and allowed to invade at 37 °C for 48 h. Non-invading cells were removed using a cotton swab. Invading cells on the membrane were fixed in 3.7% paraformaldehyde and stained using the DAPI (Fisher Scientific Hampton, NH). Pictures were taken, and results are expressed, precisely as described in the previous section. Each experiment was performed in duplicates and was repeated at least three times using
independent cell preparation. Statistical analyses were performed using Student’s t test with SigmaPlot Analysis Software.

The results are expressed as invasion index, defined as the average number of cells per field for test substance, the average number of cells per field for the medium control. Each experiment was performed in duplicates and was repeated at least three times using independent cell preparation.

3.10 Statistical Analysis

Quantitative data are presented as mean ± S.E.M. and were analyzed with Student t test or one-way analysis of variance (ANOVA). P values < 0.05 were considered significant. All statistical data collected were calculated using Sigma Plot Software.
CHAPTER IV

RESULTS

4.1 MMP-7 Basal Gene Expression and Protein Levels in Prostate Cell Lines

An analysis of the basal expression levels of MMP-7 mRNA in several prostate cell lines representing prostate cancer progression using RT-PCR was performed. Figure 13A shows MMP-7 mRNA levels constitutively expressed in all cell lines, with significantly higher mRNA expression in E006AA, (4.016 ± 0.9 fold; p<0.05); an African American derived prostate cancer cell line. Western blot analyses were also performed to determine the relative protein levels of MMP-7 in these prostate cell lines. The MMP7 protein was present in all cell lines, and the levels were slightly higher in RWPE1 and PC3 prostate cells compared to LNCaP, DU145, and E006AA cells (Figure 13B). To further examine the basal levels of secreted MMP-7 protein in normal and prostate cancer cell lines, conditioned media were collected from all cell lines and analyzed using ELISA. The data show significantly higher secreted MMP-7 protein levels in E006AA cells (266.2 ± 54-pg/µg protein; p<0.05) compared to RWPE1, LNCaP, DU145, and PC3 cells (Figure 13C).
Figure 13: Basal levels of MMP-7 in prostatic cell lines: (A) total mRNAs, (B) Western Blot analysis, and (C) quantitative ELISA analysis.
As shown in Figure 13A, total mRNAs were extracted and semi-quantitative RT-PCR analyses were performed. L-19 was used as a loading control; mRNA expression profiles of MMP-7 in prostate cell lines show significantly higher levels of MMP-7 expression in the African-American E006AA cell line than in the RWPE1, LNCaP, DU145, and PC3 cell lines (4μg RNA). Figure 13B shows the Western Blot analysis of MMP-7 protein levels in prostate cell lines. Total cellular proteins were separated by SDS polyacrylamide gel electrophoresis and blotted using an anti-MMP-7 antibody. Anti-β-actin was used as a loading control. Quantitative analysis of relative MMP-7 protein levels present in prostatic cell lines was carried out after normalization to the signal obtained with β-actin (50μg protein) as shown in Figure
13C. Quantitative ELISA analysis demonstrates the significantly elevated levels of secreted MMP-7 protein in the conditioned cell culture media. Each bar represents Mean ± SEM (n=3). Significant differences between groups in a given category (p<0.05) are designated with different lowercase letters.

4.2 The Effects of TGF-β1 on MMP-7 Expression and Secreted MMP7 Protein in Prostate Cancer Cells

TGF-β has been shown to stimulate the expression of several MMPs in cancer cells. To determine the effects of TGF-β1 on MMP-7 mRNA and protein levels in prostate cancer cells, total RNA, protein, and secreted MMP-7 obtained from conditioned culture media were collected from PC3 cells treated with or without TGF-β1 (5ng/ml) for 24 and 48 hours. The mRNA, protein, and secreted MMP-7 protein levels were determined using RT-PCR, qPCR, Western blot analysis, and ELISA, respectively. RT-PCR data illustrated that TGF-β1 increased MMP-7 mRNA expression 24 hours after treatment (Figure 14A). Further analysis using qPCR confirmed this finding to show that TGF-β1 significantly increased MMP-7 mRNA 24 hours (24 hr. 1.403 ± 0.035-fold; p< 0.05) after treatment. The data also showed a significant increase in MMP-7 mRNA 48 hours (48hr 1.7 ± 0.05-fold; p< 0.05) after treatment when compared to the 48 hour control (Figure 14B). A significant increase in MMP-7 protein levels was observed following a 48-hour treatment with TGF-β1 (48hr 1.9 ± 0.11-fold; p< 0.05) (Figure 14C). Another finding was that TGF-β1 can moderately increase the secreted levels of MMP-7 protein following 24 hours of treatment (Figure 14D); however, these changes were not statistically significant.
Figure 14. The effects of TGF-β1 on MMP-7 gene expression and protein levels: (A) total RNAs from treatment groups, (B) quantitative real-time PCR, (C) Western Blot analysis, and (D) quantitative enzyme-linked immunosorbent assay data.
Figure 14. *Continued*

Figure 14A illustrates total RNAs from treatment groups were isolated and semi-quantitative RT-PCR was performed to determine the effects of TGF-β1 on the mRNA expression of MMP-7 in PC3 cells. L-19 was used as an internal loading control. Quantitative real-time PCR was performed to analyze the effects of TGF-β1 on the expression levels of MMP-7 in PC3 cells as depicted in Figure 14B. TGF-β1 significantly increases the expression of MMP-7 after 24 and 48 hours of treatment. The relative concentration of each PCR product was determined using the 2-ΔΔCt method. GAPDH was used as an internal control. As shown in Figure 14C, a Western Blot analysis of MMP-7 protein levels detailing the effects of TGF-β1 after 24 and 48 hour treatments and quantitative analysis of MMP-7 protein following TGF-β1 treatments in PC3 cells was conducted after normalization of signal obtained by α-
Tubulin. Figure 14D shows the Quantitative enzyme-linked immunosorbent assay data of secreted MMP-7 protein levels in conditioned cell culture media following TGF-β1 treatments. Analysis of secreted MMP-7 levels were normalized to the total cellular protein concentration of seeded cells. Data are expressed as Mean + SEM (n=3), and were analyzed by ANOVA and Duncan’s modified range test. Different letters designate statistically significant (p <0.05) among different treatments.

4.3 The Effects of MMP7 Knockdown on Cell Proliferation, Migration, and Invasion in PC3 and E006AA Cells

Next, the role of MMP-7 in TGFβ-induced cell proliferation, migration, and invasion in prostate cancer cells was determined. A transient knockdown of MMP-7 was performed using siRNA specific to MMP-7 in PC3 and E006AA cells, followed by cell proliferation, migration, and invasion assays as seen in Figures 15 and 16.

Figure 15. Differential effects of MMP7 knockdown on TGFβ-induced cell proliferation, migration, and invasion of PC3 cells: (A) proliferation assays, (B) migratory, and (C) invasive behavior.
Table 15. Continued
Figure 16. Differential effects of MMP-7 knockdown on the cell proliferation, migration, and invasion of E006AA cells: (A) cell proliferation assay, (B) migratory, and (C) invasive behavior.
Related to Figures 15A-15C, PC3 cells were transfected with siRNA to transiently silence the expression of MMP7 protein. Western Blots were used to confirm MMP7 knockdown (insert). For proliferation assays, PC3 cells were seeded in 24 well plates following transfection (Figure 15A). The cells were serum starved for 3 hours and then treated with TGF-β1 and TGF-β3 (5 ng/ml) for 48 hours followed by manual cell counting using hemocytometer. Following siMMP7 transfection, the migratory (Figure 15B) and invasive behavior (Figure 15C) were measured in PC3 cells treated with 5ng/ml of TGF-β1 and -β3 and 10 ng/ml EGF using transwell inserts. EGF was used as a positive control. Each bar represents Mean ± SEM (n=3). Different letters designate statistically significant (p <0.05) among different treatments. Cells were stained with DAPI and visualized under 10x objectives.
Related to Figures 16A-16C, E006AA cells were transfected with siRNA to transiently silence the expression of MMP7 protein. Western Blots were used to confirm MMP7 knockdown (insert). Following transfection, cell proliferation assay were performed on E006AA cells treated with TGF-β1 and TGF-β3 (5 ng/ml) for 72 hours followed by manual cell counting using hemocytometer (Figure 16A). Following siMMP7 transfection, the migratory (Figure 16B) and invasive behavior (Figure 16C) were measured in E006AA cells treated with 5ng/ml of TGF-β1 and -β3 and 10 ng/ml EGF using transwell inserts. EGF was used as a positive control. Each bar represents Mean ± SEM (n=3). Different letters designate statistical significance ($p <0.05$) among different treatments. Cells were stained with DAPI and visualized under 10xobjectives.

The data showed that TGF-β1 did not induce cell proliferation in PC3 or E006AA cells. However, the knockdown of MMP7 significantly decreased cell proliferation in PC3 cells ($0.34 + 0.06$ fold; $p <0.05$) (Figure 15A), but had no effect on cell proliferation in E006AA cells (Figure 16A). Moreover, TGF-β1 induced cell migration and invasion in both cells lines, and the knockdown of MMP7 resulted in a significant decrease in migration in both PC3 cells ($0.4 + 0.1$; $p <0.05$) (Figure 15B) and E006AA cells ($0.43 + 0.0034$; $p <0.05$) (Figure 16B). There was also a significant decrease in invasion in both PC3 cells ($0.5 + 0.05$; $p <0.05$) (Figure 15C) and E006AA cells ($0.5 + 0.026$; $p <0.05$) (Figure 16C); compared to the appropriate control siRNA of transfected cells. MMP-7 knockdown also attenuated TGF-β1, TGF-β3, and EGF induced cell migration and invasion.
CHAPTER V
DISCUSSION

The results presented in this study compared the relative gene expression, protein levels, and secreted protein levels of MMP-7 in a prostate epithelial cell line (RWPE1) and various established prostate cancer cell lines (LNCaP, DU145, PC3 and E006AA). The present study also determined the function of MMP-7 in PC3 and E006AA prostate cancer cells pertaining to its role in TGF-β mediated cell proliferation, migration, and invasion. Our results show that all five of the prostatic cell lines express MMP-7. However, the relative secreted basal levels of MMP-7 are significantly higher in the E006AA cell line than in the epithelial cell line, RWPE1 and the prostate cancer cell lines: LNCaP, DU145, and PC3.

Until now, there was no evidence of the levels of secreted MMP-7 protein from any of the above referenced cell lines. These results suggest that MMP-7 gene overexpression is an early event in prostate cancer tumorigenesis, as in the case of colorectal carcinomas. Moreover, a recent in vivo study has corroborated these findings by implicating that MMP7 promotes prostate adenocarcinoma through induction of epithelial-to-mesenchymal transition (EMT). Furthermore, it has been well established that EMT is considered be critical in the development of more migratory and invasive phenotypes of epithelial tumor cells. Additionally, authors found that
increased levels of MMP7 are required for tumor formation in nude mice. Therefore, if MMP-7 is required for tumor formation, elevated levels of MMP-7 would be present in the earlier stages of cancer progression versus later stages of cancer. The E006AA cell line was derived from an early stage of organ confined PCa; whereas the LNCaP cell line was derived form a supraclavicular lymph node metastasis. The DU-145 cells were derived from a brain metastasis and the PC3 cell line was derived from a bone metastasis, which can all be classified as advanced or late stage carcinomas. These data also suggest that MMP-7 gene expression patterns and secreted protein levels may participate in early events in tumor progression and that multiple members of the metalloproteinase family may work in concert to facilitate late-stage tumor invasion and metastasis. MMP-7 is a secreted protein and its substrates include other MMPs. Moreover, MMP-7 has been shown to activate MMPs -2 and -9, which further facilitate cancer progression, and would also suggest that MMP-7 is an early regulator of cancer progression.

It is well established that TGF-β can modify the expression, secretion, and activation of MMPs in various cancers. The effects of TGF-β on the expression and relative protein levels of MMP-7 had not yet been determined in prostate cancer cell lines. The results show that TGF-β1 can significantly induce the expression of MMP-7 24 hours after treatment. Moreover, TGF-β1 can moderately induce protein levels of secreted MMP-7 24 hours after treatment. However, the effects on endogenous MMP-7 protein levels cannot be seen until 48 hours following TGF-β1 treatment. These data suggest that TGF-β1 can enhance the migratory and invasive potentials of PCa cells by
upregulating MMP-7. This may signify the characteristic role of TGF-β1 acting as a tumor promoter by increasing the levels of secreted MMP-7 into the conditioned media to degrade components of the extracellular matrix (ECM) to facilitate cancer cell migration and invasion.

Although the process of cell proliferation and migration, are essential to normal development and homeostasis, these processes play a detrimental role in the progression of prostate cancer. Due to the detrimental role of MMPs in cancer cell migration and invasion, we determined the function of MMP-7 in prostate cancer cells in hopes of developing alternative therapeutic strategies for controlling metastatic cancer cell dissemination. MMP-7 protein was transiently knocked down in the PC3 and E006AA cell lines to determine its role in cell migration, invasion, and proliferation. The data showed that after silencing MMP-7, cell proliferation (PC3), migration, (PC3, E006AA), and invasion (PC3, E006AA), were significantly reduced in control cells, TGF-β1 and EGF-treated cells. These data suggest that MMP-7 may be required for TGF-β-mediated cell migration and invasion. The results from this present study also support previous findings which demonstrate that highly malignant cells become less aggressive when MMP expression is reduced.
CHAPTER VI

CONCLUSION

In conclusion, we have demonstrated for the first time the relative secreted MMP-7 protein levels from E006AA prostate cancer cells is significantly higher than the relative MMP-7 secreted levels of prostate epithelial cells and prostate cancer cells. Also determined were the moderate effects of TGF-β signaling on the expression and relative protein levels of MMP-7. Additionally, the data have shown that MMP-7 plays a fundamental role in the invasive and metastatic potential of cancer cells which is consistent with previous studies. Our findings suggest that further analysis of MMP-7 expression in prostate cancer may lead to more effective screening methods, and the development of an MMP-7 biomarker profile which can assist in the diagnosis of early stage organ-confined prostate cancer. These findings can aid in the advancement of personalized anticancer treatment plans, help discover new molecular targets for gene therapy, and benefit in the development of new therapeutic treatment options to treat metastatic PCa.
REFERENCES


100. Feng, X. H.; Lin, L. X.; Derynck, R. Smad2, Smad3 and Smad4 Cooperate with Sp1 to Induce p15(Ink4B) Transcription in Response to TGF-Beta. Embo J. 2000, 19, 5178–5193.


212. Lokeshwar, B. L.; Sezler, M. G.; Block, N. L.; Gunja-Smith, Z. Secretion of Matrix Metalloproteinases and Their Inhibitors (Tissue Inhibitors of Metalloproteinases) by Human Prostate in Explant Cultures: Reduced Tissue Inhibitor of Metalloproteinase Secretion by Malignant Tissues. Cancer Res. 1993, 53, 4493–4498.


227. Toth, M.; Chvyrkova, I.; Bernardo, M. M.; Hernandez-Barrantes, S.; Fridman, R. 
Pro-MMP-9 Activation by the MT1-MMP/MMP-2 Axis and MMP-3: Role of 
308, 386–395.

228. Bodey, B.; Bodey, B. Jr.; Siegel, S. E.; Kaiser, H. E. Immunocytochemical 
Detection of Matrix Metalloproteinase Expression in Prostate Cancer. *In Vivo.* 
**2001**, 15, 65–70.

229. Wilson, M. J.; Sellers, R. G.; Wiehr, C.; Melamud, O.; Pei, D.; Peehl, D. M. 
Expression of Matrix Metalloproteinase-2 and -9 and Their Inhibitors, Tissue 
Inhibitor of Metalloproteinase-1 and -2, in Primary Cultures of Human 

230. Trudel, D.; Fradet, Y.; Meyer, F.; Têtu, B. Matrix Metalloproteinase 9 is 
Associated with Gleason Score in Prostate Cancer But Not with Prognosis. 

231. Varani, J.; Hattori, Y.; Dame, M. K.; Schmidt, T.; Murphy, H. S.; Johnson, K. J.; 
Wojno, K. J. Matrix Metalloproteinases (MMPs) in Fresh Human Prostate 
Tumour Tissue and Organ-Cultured Prostate Tissue: Levels of Collagenolytic 
and Gelatinolytic MMPs are Low, Variable and Different in Fresh Tissue vs. 

232. Aalinkeel, R.; Nair, M. P.; Sufrin, G.; Mahajan, S. D.; Chadha, K. C.; Chawda, R. 
P.; Schwartz, S.A. Gene Expression of Angiogenic Factors Correlates with 

233. Aalinkeel, R.; Nair, B. B.; Reynolds, J. L.; Sykes, D. E.; Mahajan, S. D.; Chadha, 
K. C.; Schwartz, S. A. Overexpression of MMP-9 Contributes to Invasiveness 

234. Gupta, A.; Zhou, C.; Chellaiah, M. Osteopontin and MMP9: Associations with 
VEGF Expression/Secretion and Angiogenesis in PC3 Prostate Cancer Cells. 
*Cancers.* **2013**, 5, 617–638.

235. Xu, D.; Suenaga, N.; Edelmann, M. J.; Fridman, R.; Muschel, R. J.; Kessler, B. M. 
Novel MMP-9 Substrates in Cancer Cells Revealed by a Label-Free 


241. Long, L.; Navab, R.; Brodt, P. Regulation of the Mr 72,000 Type IV Collagenase by the Type I Insulin-Like Growth Factor Receptor. Cancer Res. 1998, 58, 3243–3247.


252. Kim, E. S.; Sohn,Y-W.; Moon, A. TGF-β-Induced Transcriptional Activation of MMP-2 is Mediated by Activating Transcription Factor (ATF) 2 in Human Breast Epithelial Cells. Cancer. 2007, 252 (1), 147–156.


