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Matriptase/pdgf D/beta-Pdgfr Signaling Axis In Human Prostate Cancer: The Role Of Pten In The Regulation Of Pdgf D Expression

M. Katie Conley-Lacomb
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MATRIPTASE/PDGF D/β-PDGFR SIGNALING AXIS IN HUMAN PROSTATE CANCER: THE ROLE OF PTEN IN THE REGULATION OF PDGF D EXPRESSION

by

M. KATIE CONLEY-LACOMB

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2010

MAJOR: CANCER BIOLOGY

Approved by:

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Advisor                      Date

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DEDICATION

To my family, without whose support this process would not have been possible. My parents, Mike and Kathy Conley, always put their children’s needs ahead of their own, and did an amazing job raising five children. I would also like to thank my brothers and sisters and their spouses, Laura and Scott, Beth, Rob and Tricia, and Kevin and Teesha, as well as my nieces and nephews, Madeline, Sean, Arianna, Daija, Jacob, Hope, and Abby, who never fail to brighten my day.

Most especially, to thank my loving husband, Kevin LaComb, who has stood by me and supported me through more than I could have ever asked for.

To my friends in the Cancer Biology program and the Department of Pathology; Rose, Newton, Aaron, Jaron, Liz, and Bernadette – lunch just isn’t the same without you.

To the doctors, nurses, and support staff at the Rose Cancer Center at Beaumont Hospital, especially Dr. Samer Ballouz. I owe them more than I could ever repay, and so I can only hope to one day make their jobs a little easier through my work.

Finally, I would like to dedicate this work to all those affected by cancer, most especially the members of the Young Adult Cancer Survivors Group at Gilda’s Club Metro Detroit. I have made, and unfortunately lost, many friends through this group. These men and women are among the bravest I have ever met, and serve as a constant reminder as to why this war on cancer is so vitally important. This work is dedicated to Shannon Iezzi, who dedicated her life to supporting others with cancer. On April 16, 2010, Shannon lost her fight against breast cancer at the age of 29; the world is a better place because she was in it. I can only hope that one day her goal of a world without cancer will come true.
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I would like to thank Dr. Chen-Yong Lin, of Georgetown Medical Center, for providing us with matriptase antibodies, and Dr. Thomas Bugge at the NIH for providing access to his matriptase transgenic mouse model. I would also like to thank Dr. Yong Q. Chen, of Wake Forest University, for providing tissue sections, tumor lysate, and cell lines from the highly valuable Pten<sup>-/-</sup> mouse model.

I would like to thank Dr. Carolyn Ustach, whose work on PDGF D and matriptase laid the critical groundwork for my studies. I would also like to thank my fellow lab members, both past and present: Wei Huang, Abdo Najy, Alyssa Bottrell, Young Suk Jung, Richard Warner, Joshua Won, Rose Chirco, Newton Hurst, Yonghong Meng, Marcus Taube, Xuwen Liu, and Yaron Fridman, all of whom have made the lab a much brighter place to work, both emotionally and intellectually.
PREFACE

This work was supported by the Ruth L. Kirschstein National Research Service Award T32-CA009531 (to MK Conley-LaComb) and a National Institute of Health ROI grant (to HRC Kim). M. Katie Conley-LaComb is a member of the Graduate Program in Cancer Biology at Wayne State University. The purpose of this project is to investigate the regulation and expression of Platelet Derived Growth Factor D (PDGF D) in human prostate carcinoma.
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Chapter 1

Introduction

1.1 Prostate Cancer

Prostate carcinoma is the most frequently occurring non-cutaneous cancer and the second leading cause of cancer related deaths in men. Over 192,000 men are diagnosed with prostate cancer every year, and approximately 27,000 men die from the disease each year (68). The majority of patients that die from prostate cancer exhibit bone metastases. Prostate cancer has a high cure rate in its early stages, through the use of surgery, radiation or hormone therapy. However, not all primary tumors are able to be eradicated at an early stage, and to date, no effective therapy has been developed for metastatic prostate cancer; once the primary prostate tumor has metastasized to the bone, the disease is considered incurable. Therefore, the development of novel therapies for the treatment of both primary and metastatic prostate cancer are critical to reduce the mortality of the disease.

1.2 Platelet Derived Growth Factors

Platelet derived growth factors (PDGFs) are a family of four ligands, A, B, C, and D. First identified in the mid 1970s, PDGF was found to be an important mitogen in serum, and was first purified from platelets (75, 139, 181). Further studies revealed that the PDGF family members are also produced by epithelial and endothelial cells. Given that PDGF is a potent mitogen for mesenchymal cells, PDGF family members are reported to play a role in chemotaxis, cell survival, transformation, and apoptosis (190). The PDGFs are known to induce cell migration, proliferation, and transformation. As such, PDGF plays an important role in vivo in embryonic development, wound healing,
and inflammation. The PDGF ligands are secreted from cells as either homodimers or the heterodimer AB. Unlike the classic PDGF ligands, A and B, the recently discovered PDGF C and D are secreted from the cells in a latent form, requiring proteolytic removal of the N-terminal CUB (C1r/s, Uegf, and bone morphogenic protein-1) domain in order to activate the α- or β-PDGF receptors (PDGFRs), respectively.

The PDGF family consists of four members: the classical ligands A and B, and the newly discovered ligands C and D. Distinct chromosomes encode each PDGF ligand, with PDGF A at 7p22, PDGF B at 22q13, PDGF C at 4q31, and PDGF D at 11q22. Four dimers are known to exist, the homodimers AA, BB, CC, and DD, and the heterodimer AB. The PDGF family is characterized by eight conserved cysteine residues. These cysteine residues allow for intra- and interchain disulfide bonds to create dimers. Intrachain disulfide bonds are formed between the 1\textsuperscript{st} and 6\textsuperscript{th}, 3\textsuperscript{rd} and 7\textsuperscript{th}, and 5\textsuperscript{th} and 8\textsuperscript{th} cysteines, whereas the 2\textsuperscript{nd} and 4\textsuperscript{th} cysteines are responsible for the interchain disulfide bridges. However, PDGF D lacks the fifth conserved cysteine residue. Previous studies of PDGF B have shown that the bonds between the 1\textsuperscript{st} and 6\textsuperscript{th} as well as between the 3\textsuperscript{rd} and 7\textsuperscript{th} cysteine residues are essential for PDGF B activity, but the bonds between the 5\textsuperscript{th} and 8\textsuperscript{th} cysteine residues are not (47, 125, 143). Therefore, it is likely that the lack of the 5\textsuperscript{th} conserved cysteine in PDGF D does not critically impact the function of the protein.
1.3 PDGF Receptors

The PDGF ligands bind to and activate two membrane-bound receptors, α- and β-PDGF. PDGF-AA activates the homodimer PDGFR-αα; PDGF-BB activates homodimers PDGFR-αα and -ββ, as well as the heterodimeric receptor PDGFR-αβ; PDGF-AB is able to activate either PDGFR-αα or -αβ. PDGF-CC is able to activate PDGFR-αα and –αβ, and PDGF-DD preferentially activates PDGFR-ββ (Fig.1). Although PDGF C and D exhibit a great deal of homology to VEGF, the PDGF ligands are unable to activate the VEGF receptors (13).
The PDGF receptors are class III receptor tyrosine kinases, consisting of an extracellular region, made up of five immunoglobulin-like domains, a transmembrane region, and an intracellular region, made up of the splitted kinase domains, a kinase insert domain, and a cytoplasmic tail. α- and β-PDGFR exhibit a high degree of homology in the two halves of the kinase insert domain, sharing 85% and 75% identity between the two isoforms. The remaining regions of the receptors, however, have less similarity; the ligand binding domain, kinase insert, and C-terminus are only 31%, 27%, and 28% homologous, respectively (112, 138).

Upon activation of the PDGF receptors, dimerization and autophosphorylation of tyrosine residues occurs. Signal transduction molecules that contain SH2 domain, such as PLC-γ, RAS-GAP, PI3K, Grb 2, Shc, and Src, interact with the phosphorylated tyrosine residues on the receptors (30). This interaction leads to signal transduction through intracellular signaling molecules such as Akt, MAPK family members, and focal adhesion kinase (FAK) (30). This PDGF/PDGFR signal transduction pathway leads to induction of cell growth, proliferation, migration, cell survival, and transformation. Interestingly, α- and β-PDGFR are able to stimulate distinct downstream signaling molecules with different affinities. For example, β-PDGFR but not α-PDGFR is able to bind and activate Ras-GAP, whereas α-PDGFR but not β-PDGFR exhibits a high affinity for Crks (190). Additionally, α- and β-PDGFR differentially activate MAPK pathways; although both receptors activate ERK, α-PDGFR but not β-PDGFR activates JNK-1, (189). ERKs are mitogenic and therefore associated with cell proliferation, whereas JNK is associated with cell cycle arrest; thus, activation of α-PDGFR antagonizes the transformative effects of β-PDGFR, as previously shown by our lab (189).
1.4 PDGF C and PDGF D

For approximately thirty years, PDGF A and B were thought to be the sole ligands for \( \alpha \)- and \( \beta \)-PDGFR. However, studies of PDGF/PDGFR knockout mice revealed that loss of either \( \alpha \)- or \( \beta \)-PDGFR resulted in phenotypes that were not completely recapitulated with the loss of either or both PDGF A or B, suggesting the existence of additional ligands. A BLAST search of the expressed-sequence tag databases at the National Center for Biotechnology Information uncovered two new PDGF ligands, PDGF C and D (13, 82, 92). These newly identified ligands contain the core PDGF/VEGF domain shared by PDGF A and B; however, they also contain an additional N-terminal CUB domain. This CUB domain, consisting of approximately 110 amino acids, is similar to the CUB domains found in the complement subcomponents C1r/C1s, Uegf, and bone morphogenetic protein 1 (17). PDGF C and D consist of 345 and 370 amino acids, respectively, and are approximately 50% homologous in the PDGF/VEGF domain, while they are only \( \sim 20\text{-}23\% \) homologous to PDGF A and B (13). PDGF D is a highly conserved protein, with the human and murine versions sharing approximately 85\% similarity of their amino acids (82).

In the developing mouse (embryonic day 14.5), immunohistochemistry revealed PDGF D expression is highest in the developing heart, kidney, and lung (13). Northern blotting analysis of adult human tissue revealed that PDGF D is highly expressed in the heart, ovary, stomach, bladder, and mammary gland (13, 82). Interestingly, PDGF D is expressed at high levels in the adrenal gland, pancreas, and testis, where little to no PDGF B is detected by real time PCR, suggesting a distinct requirement for different \( \beta \)-
PDGFR ligands in specific tissues (82). It is possible that the distinct expression of PDGF D but not PDGF B in certain tissues is due to the ability of PDGF D to stimulate cells that express either β-PDGFR or both α- and β-PDGFR without stimulating nearby cells that express only α-PDGFR. Additionally, PDGF D has been shown to be expressed by the adventitial connective tissue layer of the suprarenal artery (171). Given that the arterial smooth muscle cells (SMC) have previously been shown to express β-PDGFR, this localization of PDGF D suggests a method for paracrine signaling to stimulate SMCs.

1.5 Activation of PDGF D

Unlike the classical PDGF ligands A and B, PDGF-C and –D are produced with an N-terminal CUB domain in addition to the C-terminal PDGF/VEGF domain. When secreted by the cell, the dimeric forms of PDGF C and D (PDGF CC and PDGF DD, respectively) are inactive. The CUB domain must be proteolytically removed from the latent full-length form to release the growth domain in order for the protein to activate PDGFR. It has been suggested that this requirement for removal of the CUB domain serves as a method of regulation of PDGF C or D activity by cells expressing proteases capable of cleaving the CUB domain (82). Previous research has indicated that PDGF-CC is processed by tissue plasminogen activator (tPA), whereas PDGF-DD can be activated by urokinase plasminogen activator (uPA) (41, 169). However, as discussed in more detail later in this paper, it is likely that PDGF-DD can be processed into its growth domain by another protease aside from uPA; PDGF-DD is also a substrate for the protease matriptase (manuscript submitted). Matriptase processes PDGF D in a multi-step manner; first, the CUB domain is removed through cleavage at R247GR249S, creating a PDGF DD dimer consisting of one full length PDGF D monomer and one growth factor
domain alone PDGF D monomer, referred to as a hemidimer. Subsequently, matriptase cleaves the second CUB domain from the dimer, releasing the active PDGF DD dimer. Additionally, matriptase can further deactivate PDGF DD by cleaving within the growth factor domain at R340R341GR343, resulting in an inactive PDGF DD dimer (Fig. 2).

1.5A Matriptase, an Epithelial Cell Specific Serine Protease. Matriptase (also known as MT-SP1 or TAGD-15), encoded by the St14 gene, is a type II transmembrane serine protease, consisting of a short intracellular N-terminal domain and a transmembrane domain, followed by one SEA (sperm protein, enterokinase, and agrin), two CUB, and four LDLRA (low density lipoprotein receptor class A) domains, in addition to the C-terminal serine-protease domain. First
identified in human breast cancer cells, matriptase has subsequently been found to be produced by many epithelial tissues, including the epidermis, intestines, prostate, breast, and ovaries (101, 122). This epithelial cell specific protease has previously been shown to activate uPA, protease-activated receptor 2 (PAR2), hepatocyte growth factor (HGF), and profilaggrin (87, 103, 160). Additionally, matriptase is regulated by its cognate inhibitor, HGF activator inhibitor (HAI)-1 (HAI-1) (95). This regulation of matriptase function is brought about through regulation of expression, activation, and trafficking (76, 86, 123). Although, paradoxically, HAI-1 has been traditionally thought to be required for matriptase activation, recent studies have suggested that matriptase can be activated in the absence of HAI-1 (32, 73, 114).

Matriptase has been shown to be critical in the regulation of the epithelial barrier and permeability of the intestines, as loss of the protease resulted in decreased transepithelial electrical resistance and increased permeability (19). Similarly, loss of matriptase is perinatally lethal due to skin permeability defects that lead to dehydration (100). Mice that exhibit a 100-fold reduction in epidermal matriptase expression display symptoms similar to the human disease autosomal recessive ichthyosis with hypotrichosis (ARIH) (99). The skin of these mice display ichthyosis, or scaly skin, along with thin whiskers, patchy and uneven fur, tooth defects, and a hyperproliferative epidermis with an impaired barrier function. Accordingly, patients with ARIH exhibit a missense mutation of G827R in the catalytic region of matriptase (10). Increased expression of matriptase in the skin has also been studied with a transgenic mouse model (102). In these mice, matriptase expression was driven by the keratin-5 promoter. Thus, matriptase was overexpressed in keratin-5-expressing tissues such as the epidermis. Matriptase
transgenic mice exhibited interfollicular hyperplasia, epidermal dysplasia, follicular
transdifferentiation, dermal fibrosis, and inflammation. Increased expression of
matriptase in the skin lead to the spontaneous development of epidermal neoplasias in
100% of mice, and the formation of squamous cell carcinoma in 70% of transgenic mice.
A single treatment of DMBA was sufficient to induce tumor formation in 95% of
matriptase transgenic mice, as compared to just 1.7% of wild type mice. The
matriptase/DMBA-induced tumors in these mice were capable of invading through the
dermis, adipose, and muscle, and lead to metastatic disease in 25% of cases. Thus,
matriptase has been shown to have a potent oncogenic potential.

Matriptase has been shown to be overexpressed in mesothelioma as well as
cervical, ovarian, breast, esophageal, liver, and prostate cancers (25, 61, 70, 85, 121, 136,
141, 142, 162, 164, 165). Although matriptase expression is associated with cancer
development and progression, little is known as to the mechanism of the association
between matriptase and cancer. Thus, further investigation of the activation of PDGF D
by matriptase may help to elucidate the role of the matriptase/PDGF D/β-PDGFR
signaling axis in oncogenesis.

1.6 Roles of PDGFs

PDGFs are known to stimulate cell proliferation, chemotaxis, survival, and
transformation. PDGF has also been shown to be involved in inflammation and wound
healing. Additionally, PDGFs are able to control the expression of collagen, collagenase,
proteoglycan, hyaluronic acid, and fibronectin, suggesting that PDGFs play a role in
tissue remodeling (16, 20, 28, 54, 117, 145). PDGFs have been shown to play a role in
fibroproliferative diseases in organs such as the kidneys and lungs, as well as in plaque
formation during atherosclerosis. PDGFs also have pro-angiogenic effects, as activation of β-PDGFR on endothelial cells results in recruitment of pericytes to immature blood vessels [reviewed in (6, 55)].

Many studies have been performed looking at the loss of PDGF ligands and their receptors through use of knockout or transgenic mice. These studies have helped to elucidate the functions during embryogenesis, as loss of PDGF A, PDGF B, PDGF C, α-PDGFR, or β-PDGFR results in either embryonic or perinatal death. α-PDGFR knockout mice die between E8 and E16 (154). Lethality was shown to be due to skeletal defects, spina bifida, and cleft face. Additionally, mice engineered to express α-PDGFR with the activating mutation D842V die at day E12.5 due to hemorrhage in both the abdomen and pericardial cavity. The phenotype was determined to be caused by an enlarged dorsal aorta with disorganized vasculature and a decrease in vascular smooth muscle cells. In addition, these mice also exhibited improper folding of the neural tube due to a lack of mesenchymal cells (78). Loss of β-PDGFR is also embryonically lethal, due to malformations in both the kidneys as well as blood vessels (96, 153). Additionally, increased expression of an autoactivated β-PDGFR is also embryonically lethal, due to defects in the formation of the placenta and fetal blood vessels (107).

Mice lacking PDGF A exhibit defective pulmonary alveoli development, in addition to skin and brain defects, leading to death between embryonic day 10 (E10) and shortly after birth (18, 42). Loss of PDGF B causes kidney defects and cardiovascular defects caused by a lack of pericycle migration to new blood vessels. These PDGF B deficient mice die during late gestation (90, 97). PDGF C knockout mice die shortly after birth due to difficulty breathing and feeding brought about by secondary palate defects
Given that this phenotype closely resembles that of the α-PDGFR knockout mice, it is likely that PDGF C plays a larger role than PDGF A in α-PDGFR signaling during embryogenesis.

Unfortunately, no PDGF D knockout mouse has been reported to date, although one model is in development (4). Thus, the role of PDGF D in embryogenesis has yet to be determined. However, given that PDGF B and β-PDGFR knockout mice exhibit similar phenotypes, it is likely that PDGF D does not play a large role during embryogenesis. It remains to be seen, though, what role PDGF D / β-PDGFR signaling plays subsequent to embryonic development. PDGF D has, however, been conditionally overexpressed in the heart (131). In these mice, PDGF D expression was driven by the heart-specific α-myosin heavy chain promoter. Heart-specific PDGF D transgenic mice developed many abnormalities in heart physiology, including enlarged hearts and cardiac fibrosis, which contributed to heart failure and death of the mice. Additionally, PDGF D expression lead to dilation of the blood vessels, vascular remodeling, thickening of blood vessel walls, proliferation of arterial smooth muscle cells. Interestingly, while PDGF B is expressed only in endothelial cells of capillaries (56), PDGF D was found to be colocalized with β-PDGFR on arterial vascular smooth muscle cells, suggesting that the two ligands may play different roles in the vasculature.

1.7 PDGFs in Cancer

PDGF family members have been shown to play a potential role in cancer development and progression. The correlation between PDGF and cancer was first indicated by the fact that PDGF-B shares a 92% homology with the oncogenic v-sis. Subsequently, PDGFs and their receptors have been found to be upregulated in numerous
cancers, as listed in Table 1. Given that PDGFs play a key role in angiogenesis, the PDGF ligands and receptors can also promote tumor growth through the promotion of the development of tumor vasculature. PDGFs have also been shown to regulate the differentiation of stromal mesenchymal cells into osteoprogenitor cells, thus potentially having a role in bone metastases. PDGF D specifically has been shown to be associated with human glioblastoma, medulloblastoma, lung cancer, and ovarian cancer (81, 105). PDGF D has also been found to be expressed in human breast adenocarcinoma, alveolar cell carcinoma, prostate adenocarcinoma, renal cell carcinoma, Wilms tumor, and erythroleukemia cell lines (171).

The mechanism by which the ligands and receptors are associated with cancer varies according to the type of cancer. For example, α-PDGFR has been found to be upregulated in glioblastoma due to gene amplification; autoactivating mutations of α-PDGFR are also associated with gastrointestinal stromal tumors (GIST) (53). Chronic myelomonocytic leukemia is commonly associated with a constitutively active form of β-PDGFR due to fusion of the kinase domain of the receptor with the promoter of the TEL gene, a member of the ets family (48).

Importantly, our lab has shown that in a mouse model, LNCaP cells transfected with PDGF D exhibit accelerated onset of prostate tumor growth as well as drastically enhanced prostate carcinoma cell invasion into surrounding stromal cells, demonstrating a potential oncogenic activity of PDGF D in the development and/or progression of prostate cancer (170).
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<td>IF, IHC</td>
<td>PDGF B, α-PDGFR</td>
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<td>PDGF B</td>
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<td>Esophageal</td>
<td>Northern blot</td>
<td>PDGF A, B, PDGFR</td>
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<tr>
<td>Glioma</td>
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<td>(59, 105, 110, 118, 119, 130, 180)</td>
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<td>(5, 71, 81)</td>
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<td>Melanoma</td>
<td>Northern blot</td>
<td>PDGF A, B, α-, β-PDGFR</td>
<td>(77, 137)</td>
</tr>
<tr>
<td>Myelomonocytic leukemia</td>
<td>FISH, Northern blot, Southern blot</td>
<td>β-PDGFR</td>
<td>(48)</td>
</tr>
<tr>
<td>Neurofibroma</td>
<td>IHC, RT-PCR</td>
<td>β-PDGFR</td>
<td>(69)</td>
</tr>
<tr>
<td>Osteoblastoma</td>
<td>IHC</td>
<td>PDGF A, α-PDGFR</td>
<td>(157)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>IHC</td>
<td>PDGF A, α-PDGFR</td>
<td>(93, 157)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>IHC, ELISA</td>
<td>PDGF A, B, D, α-, β-PDGFR</td>
<td>(58, 81)</td>
</tr>
<tr>
<td>Prostate</td>
<td>IHC, Microarray</td>
<td>PDGF A, α-, β-PDGFR</td>
<td>(44, 62, 74, 129, 135, 147, 150, 152)</td>
</tr>
<tr>
<td>Stomach</td>
<td>ISH, IHC</td>
<td>PDGF B</td>
<td>(29)</td>
</tr>
<tr>
<td>Soft tissue tumors</td>
<td>ISH, IHC</td>
<td>PDGF B, β-PDGFR</td>
<td>(126, 178)</td>
</tr>
</tbody>
</table>

**Table 1. Literature review of PDGF/R association with cancer.** ISH: *In situ* hybridization; IF: Immunofluorescence; FISH: Fluorescence *in situ* hybridization; IHC: Immunohistochemistry; RT-PCR: Reverse transcription polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay.
1.8 PDGFs in Prostate Cancer

Prostate cancer is the most diagnosed non-cutaneous cancer of men in the United States, and the second leading cause of cancer-related mortality, accounting for 10% of cancer-related mortality among men (67). Previous studies have found that β-PDGFR is upregulated in prostate cancer, with 80% of both primary and metastatic prostate cancer expressing β-PDGFR (74). Importantly, expression of β-PDGFR has been identified by microarray as part of a five-gene model, along with chromogranin A, HOXC6, ITPR3, and sialyltransferase-1, that is able to predict prostate cancer recurrence (150). PDGF B, originally thought to be the sole ligand for β-PDGFR, is not detected in prostate cancer (PCa) (43, 44, 62, 74). As discussed below, PDGF D, which is also able to activate β-PDGFR, is upregulated in PCa, as shown by immunohistochemistry (manuscript in preparation). Confirming these results, a search of microarrays performed on metastatic prostate cancer samples compiled in the Oncomine database (Compendium Biosciences) revealed an upregulation of PDGF D and β-PDGFR as well as matriptase in the majority of cases, with increased expression in 75%, 60%, and 100%, respectively, of metastatic samples relative to localized prostate cancer (Table 2) (33, 79, 80, 108, 172, 173, 191). However, no previous studies have elucidated the mechanism that resulted in the switch between PDGF B and PDGF D/β-PDGFR expression in prostate cancer.

In a mouse model in which LNCaP-PDGF D cells were injected subcutaneously, PDGF D expression accelerates early onset of prostate tumor growth, and drastically

<table>
<thead>
<tr>
<th>Marker</th>
<th>Metastatic Studies No. Upregulated/Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF D</td>
<td>3/4 (75%)</td>
</tr>
<tr>
<td>β-PDGFR</td>
<td>3/5 (60%)</td>
</tr>
<tr>
<td>Matriptase</td>
<td>5/5 (100%)</td>
</tr>
</tbody>
</table>

Table 2. Prostate cancer cDNA microarray expression profile.
enhances prostate carcinoma cell invasion into surrounding stromal cells, demonstrating a potential oncogenic activity of PDGF D in the development and/or progression of prostate cancer (170). Additionally, in mice that were injected intratibially with the prostate cancer cell line PC-3MM2, the receptor tyrosine kinase inhibitor Gleevec (STI571, imatinib mesylate), which inhibits Bcr-Abl, PDGFR, and c-Kit, was shown to reduce tumor incidence and growth, and an increase in apoptosis in the tumor cells as well as tumor associated endothelial cells (167). Thus, a growing body of evidence suggests that the PDGF/PDGFR signaling pathway plays a role in prostate carcinoma.

1.9 PTEN/PI3K/Akt Pathway

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN, also known as MMAC1/TEP1) is a lipid and protein phosphatase. Its key role is to serve as a negative regulator of the phosphatidylinositol-3 kinase (PI3K) pathway [reviewed in (23)]. PI3K family members are categorized as class IA, IB, II, or II. Class IA PI3Ks consist of heterodimers composed of a catalytic subunit (p110α, p110β, or p110γ) and a regulatory subunit (p85α, p85β, or p55γ). When cells are stimulated with growth factors, class IA PI3Ks generate phosphatidylinositol 3,4,5-triphosphate (PIP3) through phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2). PIP3 is then able recruit proteins that contain pleckstrin homology domains, such as the serine/threonine kinase Akt/PKB, to the plasma membrane. Subsequent to phosphorylation, Akt is able to phosphorylate its downstream targets such as BAD, GSK3, FOXO, MDM2, NFκB, and TSC2. PTEN has also been shown to inhibit MAPK and FAK pathways (51, 161). Through its attenuation of the PI3K pathway, PTEN is able to regulate key cellular
processes such as cell proliferation, motility, protein synthesis, glucose metabolism, genomic stability, and survival.

1.10 PTEN in Cancer

Also known as MMAC (mutated in multiple advanced cancers), PTEN is located on chromosome 10q23 (91, 155). Mutations of PTEN are associated with several diseases, including Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome (94, 111). In addition, loss of PTEN has been shown to be associated with many types of cancer, such as glioblastoma, endometrial carcinoma, and breast cancer. As shown in Table 3, previous studies have found that PTEN is lost or mutated in 70-80% of primary prostate cancer, and 50% of prostate cancer bone metastases. In mouse models, it has been shown that loss of PTEN is critical for tumor initiation, and the level of PTEN expression is inversely associated with prostate tumorigenesis (34, 163).
<table>
<thead>
<tr>
<th>Ref.</th>
<th>Method of PTEN Detection</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>(49)</td>
<td>PCR, allele typing, SSCP, sequencing</td>
<td>Tumor suppressor gene near the 10q23-24 boundary was deleted in 96% of tumors</td>
</tr>
<tr>
<td>(39)</td>
<td>LOH analysis w/ PCR, SSCP, sequencing</td>
<td>LOH in 49% of primary PCa</td>
</tr>
<tr>
<td>(127)</td>
<td>PCR for microsatellite markers</td>
<td>LOH in 55% and mutation in 17% of primary PCa</td>
</tr>
<tr>
<td>(50)</td>
<td>Mutation analysis, Northern analysis</td>
<td>5 PTEN mutations identified; 70% of tumors had loss or alteration of at least 1 allele</td>
</tr>
<tr>
<td>(124)</td>
<td>LOH, FISH, Sequencing</td>
<td>LOH in 11% of Japanese patients with PCa</td>
</tr>
<tr>
<td>(140)</td>
<td>LOH</td>
<td>LOH in 14% node negative PCa, 43% node positive PCa; 10q23.3 is a marker for progression</td>
</tr>
<tr>
<td>(36)</td>
<td>SSCP, direct DNA sequencing</td>
<td>PTEN mutated in 16% of Chinese patients with primary PCa, 33% of American patients with metastatic PCa</td>
</tr>
<tr>
<td>(83, 138)</td>
<td>LOH, microsatellite instability, SSCP, PCR</td>
<td>Frequent alterations linked to late stage PCa; microsatellite instability was increased in adenocarcinomas</td>
</tr>
<tr>
<td>(89)</td>
<td>LOH, CGH, SSCP</td>
<td>LOH at the PTEN gene is frequent but mutations in the remaining allele were not detected by SSCP-screening</td>
</tr>
<tr>
<td>(174)</td>
<td>Array-based CGH, FISH, SSCP, IHC</td>
<td>Bi-allelic deletion is a major mechanism of PTEN inactivation in locally progressive PCa</td>
</tr>
<tr>
<td>(188)</td>
<td>FISH, IHC</td>
<td>Deletions were found in 3/13 (23%) of high-grade PIN and 24/35 (68%) of PCa</td>
</tr>
<tr>
<td>(144)</td>
<td>Multiplex PCR-based microsatellite analysis</td>
<td>LOH observed in 85% of prostate cancer cases; PTEN associated with the occurrence of circulating tumor cells as well as a sign of early biochemical recurrence</td>
</tr>
<tr>
<td>(187)</td>
<td>FISH</td>
<td>Hemizygous PTEN loss present in 39% of prostatic adenocarcinomas, with a homozygous PTEN deletion observed in 5% tumors</td>
</tr>
<tr>
<td>(133)</td>
<td>SSCP</td>
<td>Patients w/ PCa who had PTEN mutation had also a significantly greater Gleason score, poorer prognosis, and higher rate of metastasis</td>
</tr>
<tr>
<td>(79)</td>
<td>Array CGH, FISH</td>
<td>PCa develop via a limited number of alternative preferred genetic pathways, including loss at 10q23 (PTEN)</td>
</tr>
<tr>
<td>(111, 113)</td>
<td>FISH, IHC</td>
<td>Heterogeneous PTEN gene deletion was observed in 23% of hormone sensitive tumors, 52% of hormone-refractory tumors</td>
</tr>
<tr>
<td>(151)</td>
<td>IHC, FISH, SNP Array Analysis</td>
<td>PTEN deleted in 77% of PCa cases</td>
</tr>
<tr>
<td>(65)</td>
<td>Array CGH, FISH</td>
<td>25% of intermediate-risk group exhibits deletion of PTEN</td>
</tr>
</tbody>
</table>

**Table 3. Literature review of PTEN expression in prostate cancer.** PCR: polymerase chain reaction; SSCP: Single strand conformation polymorphism; LOH: Loss of heterozygosity; FISH: Fluorescence *in situ* hybridization; CGH: Comparative genomic hybridization; IHC: Immunohistochemistry; SNP: Single nucleotide polymorphism.
1.11 PTEN Mouse Models

Loss of PTEN throughout the mouse is embryonically lethal, as embryonic stem cells lacking Pten form aberrant embryoid bodies and are unable to properly differentiate into ectoderm, mesoderm, and endoderm (34). Thus, heterozygous as well as tissue specific knockout models were developed to study the effects of PTEN expression. Pten\(^{+/−}\) mice develop hyperplasia of the prostate as well as spontaneous colon and thyroid carcinomas (34). In order to study the role of PTEN in prostate carcinoma, a prostate specific Pten knockout mouse model was developed using the Cre/Lox system. In this model, Cre recombinase expression was driven by the androgen responsive, prostate specific probasin promoter, while exon 5 of Pten was flanked by LoxP sequences (88, 182). Upon expression of androgens, Cre recombinase is expressed specifically in the prostate, and recognizes the LoxP sequences in the PTEN gene and eliminates the sequence flanked by LoxP. Exon 5 is critical for the phosphatase activity of PTEN. Thus, exon 5 of Pten is deleted, rendering the resulting protein inactive. In this system, Pten\(^{+/−}\) mice develop mPIN lesions (mouse prostate intraepithelial neoplasia) at 12-16 months; however, no progression to carcinoma was observed. Pten\(^{−/−}\) mice, however, developed mPIN at the age of six weeks, followed by adenocarcinoma at 9 weeks (14, 15). Thus, this prostate specific loss of PTEN recapitulates human prostate carcinoma.

1.12 The Serine/Threonine Kinase Akt

The Akt family of serine/threonine kinases consists of three isoforms: Akt1/PKB\(α\), Akt2/PKB\(β\), and Akt3/PKB\(γ\). Once recruited to the plasma membrane, Akt is activated through phosphorylation of two regulatory sites. Thr308/Thr309/Thr305 (in Akt1/Akt2/Akt3, respectively), in the P-loop of the protein kinase domain, is
phosphorylated by PDK1; Ser473/Ser474/Ser472 (in Akt1/Akt2/Akt3, respectively), in the carboxy-tail region, is phosphorylated by mTORC2 (1). Phosphorylation of the threonine site is required for activation, although phosphorylation of the serine site is required for maximal activity (37, 57).

Many studies have shown that the Akt isoforms have compensatory functions, although the individual isoforms also play non-redundant roles. Although little is known regarding the distinct mechanism as to how each isoform regulates different cellular processes, knockout mouse models have established unique roles of each Akt isoforms, in which the phenotypes seen in mice lacking one Akt isoform are not compensated for by the remaining two isoforms [reviewed in (184)]. Loss of Akt1 leads to decreased vascularization and size of the placenta, as well of a lack of differentiation of trophoblasts. The placental defects caused by the loss of Akt1 results in decreased birth weight and increased infant mortality (27). Mice lacking Akt2 develop severe diabetes due to impaired glucose metabolism (26, 46). It was later discovered that stimulation of the PI3K pathway by insulin results in activation of Akt2, which in turn is critical for the translocation of GLUT4 to the plasma membrane (60). Therefore, mice lacking Akt2 are unable to transport glucose into the cell upon insulin stimulation. Akt3 knockout mice exhibit a 20-25% decrease in brain size, brought about by both decreased cell size as well as cell number (38, 166).

Akt has also been linked to prostate cancer. As shown through immunostaining Akt that is phosphorylated at Ser473, prostate tumors exhibit increased activation of Akt (158). Additionally, mice that overexpress constitutively active Akt1 specifically in the prostate develop mPIN, with hyperplasia, dysplasia, and intraepithelial lumen formation,
leading to bladder obstruction, similar to the phenotype seen in Pten$^{+/-}$ mice (109). Interestingly, studies of the prostate cancer cell lines DU145, PC3, and LNCaP have shown that, while Akt1 and Akt2 are uniformly expressed, Akt3 expression is upregulated in the androgen-insensitive DU145 and PC3 as compared to the androgen responsive LNCaP cell line. Akt3 enzymatic activity was also 20-40 fold higher in DU145 and PC3 as compared to LNCaP (116). Given the role of Akt in promoting cell survival and proliferation, it is possible that this increased expression and activity of Akt3 could contribute to the aggressive nature of androgen-insensitive prostate carcinoma.
Chapter 2
Materials and Methods

2.1 Cell Culture. All cell lines were cultured at 37°C in a humidified incubator with 5% CO₂, and all media was supplemented with 2mM glutamine, 100units/ml penicillin and 100mg/ml streptomycin (Life Technologies Inc., Carlsbad, CA). Human prostate carcinoma cell lines and resultant transfectant cell lines (170), (169) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum. Prostate specific Pten\textsuperscript{lox/+} (referred to hereafter as Pten\textsuperscript{+/+}), Pten\textsuperscript{+/}, and Pten\textsuperscript{−/−} mice were established as previously described (14). Pten\textsuperscript{−/−}, Pten\textsuperscript{+/−}, and Pten\textsuperscript{+/+} mouse prostate epithelial cells were isolated from anterior prostates of 8-10 week-old corresponding mice (15) using the method previously described (9). Pten\textsuperscript{lox/lox} cells were isolated from anterior prostates of 8 week-old mice. In vitro deletion of Pten was achieved by infecting Pten\textsuperscript{lox/lox} cells with a self-deleting Cre-recombinase lentivirus (128). Cells were clonally selected using serial dilution method (8) and Pten status was confirmed by genotyping and Western blotting. Cells were maintained in Advanced DMEM supplemented with 5% FBS, 2mM glutamine, 100units/ml penicillin, and 100mg/ml streptomycin.

2.2 Reagents. Generation of antibody that recognizes the growth domain of PDGF D was described previously (169, 170). Anti-matriptase antibodies M32 and S5 were characterized and described previously (95). Antibodies recognizing PTEN, phosphorylated β-PDGFR and phosphorylated Akt were purchased from Cell Signaling Technology. Akt1, Akt2, and Akt3 antibodies were purchased from Upstate.

2.3 Microarray. Stably transfected cell lines LNCaP-neo (vector control) and LNCaP-PDGF D were cultured in 150mm plates, and total RNA was extracted using 3mls of
Trizol Reagent per 150mm plate. Total RNA from 6 plates were pooled together to make a master stock for each cell line. Total RNA was purified further using the RNeasy Kit (Qiagen, Valencia, CA). A quality check of the total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The Bioanalyzer was used to determine if the 18S and 28S ribosomal bands are defined and to ensure no RNA degradation was present. The RNA samples were also analyzed with a spectrophotometer and 260/280 ratio in the range of 1.9-2.2 were defined as acceptable. After determination of quality, 10 µg of RNA for each sample was reverse transcribed and labeled with Cyanine 5. Stratagene’s Universal Human reference RNA (Stratagene, La Jolla, CA) was used as a reference sample and was labeled with Cyanine 3. Agilent’s fluorescent direct labeling kit (Agilent Technologies) was used to synthesize the labeled cDNA. The labeled cDNA was purified using QIAquick PCR Purification kit from Qiagen (Qiagen, Valencia, CA). The Cyanine 3 and Cyanine 5 samples were mixed together and allowed to co-hybridize on the array for 17 hours at 65°C. Each sample was run in triplicate against the reference sample. The arrays used are Agilent’s Human 1 cDNA Microarrays (Agilent Technologies) with 12,814 unique human clones on each array. After hybridization the microarrays were washed and dried according to Agilent's cDNA microarray hybridization protocol. Slides were immediately scanned with the Agilent dual laser scanner. The photo multiplier tube (PMT) settings were set at 100% for both channels. Tiff images were analyzed using Agilent’s feature extraction software to obtain fluorescent intensities for each spot on the arrays. LOWESS normalization was performed on the intensity values. ANOVA analysis and clustering was performed using Rosetta Resolver software (Rosetta Biosoftware, Kirkland, WA). Differential gene
expression between the LNCaP-neo and LNCaP-PDGFD:HIS samples of each cell line was identified by selecting genes with an ANOVA p-value < 0.01.

2.4 Immunohistochemical Analysis of PDGF D, Matriptase, and Phosphorylated β-PDGFR in Human and Murine Tissue. Slides of sixty-six formalin fixed, paraffin-embedded human prostate specimens, including sections of benign, benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), and prostate carcinoma, were obtained from the Wayne State University Pathology Research Core Facility. Murine skin sections from wild type, matriptase transgenic, and matriptase/HAI-1 transgenic mice were obtained courtesy of Dr. Thomas Bugge of the NIH. Pten\textsuperscript{+/+} and Pten\textsuperscript{−/−} prostate tissue sections were obtained courtesy of Dr. Yong Q. Chen of Wake Forest University. Tissue slides were deparaffinized with xylene, then rehydrated sequentially with decreasing concentrations of EtOH from 100% to 70%, followed by water. Antigen retrieval was performed by steaming for twenty minutes in a sodium citrate buffer. Slides were then washed twice with PBS and blocked with Cas-Block solution (Zymed). Endogenous peroxidase activity was blocked with 3% H\textsubscript{2}O\textsubscript{2}. Slides were incubated overnight at 4\textdegree C in a humidified chamber with either anti-PDGF-D polyclonal Ab (custom antibody 8D2, 1:300 dilution), anti-matriptase monoclonal Ab (custom antibody S-5, 1:3000 dilution), or anti-phosphorylated β-PDGFR polyclonal Ab (Cell Signaling Technology, 1:50). Sections were then washed twice with PBS and incubated with ABC Vectastain Kit (Vector Labs) according to manufacturer’s protocol, followed by incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector Labs). Mayer’s hematoxylin was used to counterstain the nuclei (Sigma-Aldrich). Sections were then
dehydrated with increasing concentrations of EtOH and washed with xylene twice, and mounted with Permount (Fisher Scientific).

2.5 Statistical Analysis. Immunohistochemistry of tissue sections were analyzed by uropathologist Dr. Mingxin Che, in which a blind assessment of the percentage of tumor cells positive for PDGF D or matriptase was analyzed for semi-quantitation. Statistical analysis was performed by biostatistician Dr. Judith Abrams using Kendall’s tau analysis to assess the association between the American Joint Committee on Cancer (AJCC) tumor stage and Gleason score with PDGF-D and matriptase staining. In this method, a coefficient value of 1 indicates a perfect direct correlation and a coefficient value of -1 indicates a perfect inverse correlation, while a coefficient value of 0 indicates no correlation.

2.6 In Situ Hybridization. Digoxigenin-labeled antisense probes were synthesized from pCR2.1-PDGF D using the DIG RNA Labeling Kit (Roche Applied Science) according to the manufacturer’s instructions. PDGF D sense probes, created with pcDNA3.1-PDGF D, were used as a negative control. Paraffin embedded sections of human prostate carcinoma were deparaffinized with xylene followed by serial dilutions of ethanol. Slides were treated with prehybridization solution for 1hr at 65°C, then incubated with either PDGF D antisense or sense probes overnight at 65°C. After blocking with CAS-Block solution (Zymed), the probe was detected using anti-digoxigenin antibodies and visualized using 3,3’-diaminobenzadine tetrahydrochloride (DAB, Vector Labs). The sections were then dehydrated with serial dilutions of ethanol, washed with xylene, and mounted with Permount.
2.7 Immunofluorescent Double Staining of Matriptase and PDGF D in Human Prostate Tissue. For immunofluorescent staining, slides were deparaffinized with xylene, and double probed with anti-PDGF D rabbit polyclonal antibody and ant-matriptase mouse-monoclonal antibody M32. Texas Red-conjugated-anti-mouse and FITC-conjugated-anti-rabbit secondary antibodies were used to detect PDGF D and matriptase, respectively (Jackson Immunoresearch Laboratories, West Grove, PA). Confocal immunofluorescence microscopic analysis was performed using a Zeiss LSM 510 confocal microscopy system equipped with a C-Apochromat (NA = 1.2) 63x korr objective lens (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Images for figures were colored and resized with associated microscope software available through Wayne State University Imaging Core Facility.

2.8 Inhibition of Signaling Pathways. Cells were cultured with complete growth media, and treated with increasing concentrations of inhibitors or vehicle control. Cells were treated with JNK Inhibitor II (Fisher Scientific), PD98059 (Sigma Aldrich), LY294002 (Sigma Aldrich), wortmannin (Calbiochem), or Akt Inhibitor IV (Fisher Scientific) for eighteen hours. Cells were treated with rapamycin (Sigma Aldrich) for 48 hours. mRNA was collected from cells at the designated time point and subjected to RT-PCR and real time RT-PCR.
2.9 RT-PCR. mRNA was purified from cells using the RNeasy kit (Qiagen). cDNA synthesis was performed with Superscript III First-Strand Synthesis System (Invitrogen), followed by PCR using GoTaq Flexi DNA Polymerase (Promega). Murine and human specific forward and reverse primers used are shown in Tables 4 and 5, respectively.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF A forward</td>
<td>5’-TGTGCCCATTCGAGGAAGAG-3’</td>
</tr>
<tr>
<td>PDGF A reverse</td>
<td>5’-TTGCGCACCTTGACACTGCG-3’</td>
</tr>
<tr>
<td>PDGF B F</td>
<td>5’-GCCTGAGACTAGAAGTCTTG-3’</td>
</tr>
<tr>
<td>PDGF B R</td>
<td>5’-GTCATGGGTGCTTAAACT-3’</td>
</tr>
<tr>
<td>PDGF C F</td>
<td>5’-TACCTGGTAATGGGAGCATC-3’</td>
</tr>
<tr>
<td>PDGF C R</td>
<td>5’-CGTCTAAAACACTCCACT-3’</td>
</tr>
<tr>
<td>PDGF D F</td>
<td>5’-CAGGGAAGACAGTGAGAAG-3’</td>
</tr>
<tr>
<td>PDGF D R</td>
<td>5’-GAGCTGCAGATACAGTCACA-3’</td>
</tr>
<tr>
<td>α-PDGFR F</td>
<td>5’-GTACTTTTCCTGCTTGAG-3’</td>
</tr>
<tr>
<td>α-PDGFR R</td>
<td>5’-ACTATCTCGGTAGGTGTA-3’</td>
</tr>
<tr>
<td>β-PDGFR F</td>
<td>5’-CATCATGAGGACTCAAACCT-3’</td>
</tr>
<tr>
<td>β-PDGFR R</td>
<td>5’-GATGAGCATTGAAACTGTTG-3’</td>
</tr>
<tr>
<td>Matriptase F</td>
<td>5’-AGGTCCAGTTTGTGGTGA-3’</td>
</tr>
<tr>
<td>Matriptase R</td>
<td>5’-CTCTGAGGAGACACTTT-3’</td>
</tr>
<tr>
<td>uPA F</td>
<td>5’-GTGCCGGACACTGCTTCATT-3’</td>
</tr>
<tr>
<td>uPA R</td>
<td>5’-CTGCTGCTTACGTATCTTTCA-3’</td>
</tr>
<tr>
<td>PTEN F</td>
<td>5’-ACACCGCAATTTAACTGC-3’</td>
</tr>
<tr>
<td>PTEN R</td>
<td>5’-TGAGGGTTTCTCCTGGTCTG-3’</td>
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<tr>
<td>Akt1 F</td>
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<tr>
<td>Akt1 R</td>
<td>5’-GACACAATCTCAGACCATAGAAG-3’</td>
</tr>
<tr>
<td>Akt2 F</td>
<td>5’-GAGGAGCCAGGATATTACAAG-3’</td>
</tr>
<tr>
<td>Akt2 R</td>
<td>5’-GACAGGCTACCTCATCATCTCGAG-3’</td>
</tr>
<tr>
<td>Akt3 F</td>
<td>5’-GAGTACCTGGACACGAGGT-3’</td>
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<tr>
<td>Akt3 R</td>
<td>5’-AGAAAGGCAACCTCCACAC-3’</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>5’-ATCACCATCTTCCGAGGAG-3’</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>5’-GCCAGTGAGGCTTCCGTTCA-3’</td>
</tr>
</tbody>
</table>

Table 4. Murine specific primer sequences used for PCR.

2.10 Real time RT-PCR. mRNA was purified from cells using the RNeasy kit (Qiagen). cDNA synthesis was performed with Superscript III First-Strand Synthesis System (Invitrogen). Real time RT-PCR was performed using SYBR Green QPCR Master Mix
(Stratagene) and the Stratagene MX4000 qPCR System according to the manufacturer’s protocol. Relative values of gene expression were calculated using the $2^{\Delta \Delta Ct}$ method, where $\Delta \Delta Ct = (\Delta Ct_{\text{target gene}} - \Delta Ct_{\text{GAPDH}})_\text{sample} - (\Delta Ct_{\text{target gene}} - \Delta Ct_{\text{GAPDH}})_\text{control}$. The fold change in relative expression was then determined by calculating $2^{\Delta \Delta Ct}$.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF A forward (F)</td>
<td>5’-TGAGGATTCTTTTGACACCA-3’</td>
</tr>
<tr>
<td>PDGF A reverse (R)</td>
<td>5’-GGGCCAGATCAGGAAGTTG-3’</td>
</tr>
<tr>
<td>PDGF B F</td>
<td>5’-CATTCCCCGAGGAGCTTTATG-3’</td>
</tr>
<tr>
<td>PDGF B R</td>
<td>5’-CTCAGCAAATGGTCAGGAAC-3’</td>
</tr>
<tr>
<td>PDGF C F</td>
<td>5’-TCCAGCAACAAAGGACAGAA-3’</td>
</tr>
<tr>
<td>PDGF C R</td>
<td>5’-GGGTCTTCAAGCACAATCT-3’</td>
</tr>
<tr>
<td>PDGF D F</td>
<td>5’-GAACAGCTACCACAGGACAC-3’</td>
</tr>
<tr>
<td>PDGF D R</td>
<td>5’-CTTGTGTCACACCACATCGTC-3’</td>
</tr>
<tr>
<td>$\alpha$-PDGFR F</td>
<td>5’-GAACCTACGGTGCTGCTG-3’</td>
</tr>
<tr>
<td>$\alpha$-PDGFR R</td>
<td>5’-ACCTTCATGACAGCTTTGG-3’</td>
</tr>
<tr>
<td>$\beta$-PDGFR F</td>
<td>5’-TTTTGCACCCACATGACTC-3’</td>
</tr>
<tr>
<td>$\beta$-PDGFR R</td>
<td>5’-CCAATGGTGTTTTGAGAT-3’</td>
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</tr>
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| Table 5. Human specific primer sequences used for PCR. |

2.11 Immunohistochemical analysis of PDGF D in murine prostate tissue. Slides of formalin fixed, paraffin-embedded prostate sections from Pten$^{+/+}$ and Pten$^{-/-}$ mice were
obtained from Dr. YQ Chen at Wake Forest University. Tissue slides were deparaffinized with xylene, then rehydrated sequentially with decreasing concentrations of EtOH from 100% to 70%, followed by water. Endogenous peroxidase activity was blocked with 3% H$_2$O$_2$. Antigen retrieval was performed by steaming for twenty minutes in Antigen Retrieval Citra Plus Solution (BioGenex). Slides were then washed twice with PBS and blocked with Cas-Block solution (Invitrogen). Slides were incubated overnight at 4°C in a humidified chamber with either anti-PDGF-D polyclonal Ab (8D2, 1:500 dilution) or anti-phosphorylated-β-PDGFR polyclonal Ab (1:100 dilution). Sections were then washed twice with PBS and incubated with ABC Vectastain Kit (Vector Labs) according to manufacturer’s protocol, followed by incubation with 3,3’-diaminobenzidine tetrahydrochloride (DAB, Vector Labs). Mayer’s hematoxylin (Sigma-Aldrich) was used to counterstain the nuclei. Sections were then dehydrated with increasing concentrations of EtOH, washed with xylene twice, and mounted with Permount (Sigma-Aldrich).
Chapter 3

Results

3.1 Expression of PDGF D and Matriptase in Human Prostate Carcinoma

A. Expression of PDGF D results in increased matriptase expression in the human prostate carcinoma cell line LNCaP

While it has been shown that PDGF D can be processed by uPA into its active form, LNCaP cells express little to no uPA but are able to process PDGF D into the active growth domain (GD), suggesting an additional protease is able to process PDGF D (169). Given that recombinant PDGF D is more readily processed into the active GD in LNCaP-PDGF D cells as compared to LNCaP-neo cells, it is likely that a PDGF D-inducible protease is responsible for processing PDGF D in LNCaP cells (170). Matriptase, a type II transmembrane serine protease, was identified by microarray to be increased in LNCaP-PDGF D cells (Table 6). This increase in matriptase expression was confirmed by Western blot and immunofluorescence (Fig. 3A and B). Previous results from our lab have shown that incubation of PDGF D with increasing nanomolar concentrations of the serine protease domain of matriptase results in processing of PDGF

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<tr>
<td>ST14</td>
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<td>Chemokine (C-C motif) receptor-like 2</td>
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Table 6. Identification of PDGF D responsive genes in LNCaP cells by microarray analysis. Data courtesy of Dr. Carolyn Ustach.
Fig. 3. Increased matriptase expression in LNCaP-PDGF D cells and its role in PDGF D processing. A. Immunoblot analysis of matriptase in LNCaP-neo and LNCaP-PDGF D cell lysates using M32 antibody. B. Immunofluorescent staining of matriptase in LNCaP-neo and LNCaP-PDGF D cells using the anti-matriptase antibody M32 and nuclear staining with DAPI. C. LNCaP-PDGF D cells were transfected with vector control or HAI-1 expression plasmid. Immunoblot analysis of HAI-1 and β-actin (as a loading control) in control and HAI-1 transfected cell lysates (top and middle panels, respectively) as well as immunoblot analysis of PDGF D using conditioned medium (bottom panel) are shown. D and E. LNCaP-PDGF D cells were transfected with non-targeting siRNA control (Cont. siRNA) or matriptase-targeting siRNA. Equal concentrations of cells lysates (D) or equal volume of conditioned medium (E) were analyzed by immunoblot analyses for matriptase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, as a loading control) in panel D and PDGF D in panel E. F. Serum-starved NIH3T3 cells were stimulated for 10 minutes with CM collected from non-target siRNA control or matriptase-knockdown cells. Phosphorylated-β-PDGFR was analyzed using anti-phosphorylated-β-PDGFR (pY751) Ab (top panel). The blot was reprobed with anti-β-PDGFR Ab (bottom panel). G. Recombinant PDGF D (rPDGF D) was incubated with serum-starved control or matriptase-knockdown cells for 4 hr, after which the medium was collected from the cells. PDGF D dimer species were analyzed by immunoblot analysis under non-reducing condition (right panel). The input rPDGF D is shown in the left panel. FL-M, full-length PDGF D monomer; GD- M, growth factor domain monomer; FL-D, full-length PDGF D dimer; GD-D, growth factor domain dimer. Data courtesy of Carolyn V. Ustach.
D into its active 18kDa growth factor domain, followed by further processing into an inactive 15kDa form (manuscript in preparation). As determined by fellow lab member Dr. Wei Huang, transfection of LNCaP-PDGF D cells with HAI-I, an inhibitor of matriptase, resulted in decreased processing of PDGF D into its active growth factor domain (Fig. 3C). Additionally, knockdown of matriptase expression with siRNA similarly resulted in decreased processing of PDGF D (Fig. 3D and E). Consistently, the decreased processing of PDGF D in matriptase-knockdown cells resulted in diminished activation of β-PDGFR, as determined by Dr. Huang (Fig. 3F). These results suggest that matriptase is able to process full length (FL) PDGF D into its growth factor domain in vitro.

B. Expression of matriptase in murine skin results in an increase in PDGF D expression and activation of β-PDGFR

To examine the processing of PDGF D by matriptase in vivo, we obtained skin sections from matriptase transgenic mice through collaboration with Dr. Thomas Bugge at NIH. In these mice, matriptase expression was driven by the keratin-5 promoter. Thus, matriptase was overexpressed in keratin-5-expressing tissues such as the epidermis (102). Matriptase transgenic mice exhibited interfollicular hyperplasia, epidermal dysplasia, follicular transdifferentiation, dermal fibrosis, and inflammation. In the control mice, weak staining of both PDGF D and phosphorylated β-PDGFR was detected in the epidermis and sebaceous glands. However, matriptase transgenic mice exhibited markedly increased expression of PDGF D and activated β-PDGFR in the epidermis and
Fig. 4. PDGF D/β-PDGFR signaling axis in matriptase transgenic mice. Sections of skin were taken from newborn mice (A), 10-12 month old mice (B), 10-12 month old skin treated with DMBA (C), or from a spontaneous tumor (D). Sections from wild type, matriptase transgenic, HAI-1 transgenic, and matriptase/HAI-1 double transgenic mice were immunostained with antibodies against PDGF D or phosphorylated β-PDGFR. Images were taken at 20X magnification.
hair follicles. Matriptase/HAI-1 transgenic mice exhibited weak staining of both PDGF D and phosphorylated β-PDGFR (Fig.4). Consistently, these matriptase/HAI-I double transgenic mice do not exhibit the premalignant changes found in the matriptase transgenic mice (102).

List et al reported that that 100% of the matriptase mice developed epidermal neoplasias by the age of 23 months, with 70% of these lesions being squamous cell carcinoma. Additionally, a single treatment with DMBA induced tumors in 38/40 matriptase transgenic mice within 40 weeks after exposure, as compared to just 1/59 wild type littermates (102). Immunohistochemistry revealed high levels of PDGF D and activated β-PDGFR in these tumors (Fig. 4D). These results suggest a strong correlation between matriptase expression and the PDGF D/β-PDGFR signaling axis.

C. PDGF D and matriptase are upregulated in human prostate cancer.

To examine the association of PDGF D and matriptase expression with prostate carcinoma (PCa) stage, immunohistochemical staining for PDGF D and matriptase was performed on both normal prostate and PCa specimens. A blind assessment of the percentage of cells stained for PDGF D and matriptase was performed for quantitation (Tables 7 and 8). Kendall’s tau analysis was used to assess the association between tumor stage and Gleason score with PDGF-D and matriptase staining. As shown in Table 9, PDGF-D staining is associated with both higher tumor stage (p=0.006) and higher Gleason score (p=0.01). Matriptase has a weaker association with both tumor (p=0.08) and Gleason score (p=0.11). When the staining patterns of PDGF D and matriptase were analyzed, expression of both markers in normal prostatic glands was predominantly in the
Table 7. Analysis of PDGF D staining in human PCa. Cases were grouped according to tumor stage and Gleason score and the percent of tumor cells stained in each tissue section, as determined blindly by a uro-pathologist.

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<th>Tumor Stage</th>
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Table 8. Analysis of matriptase staining in human PCa. Cases were grouped according to tumor stage and Gleason score and the percent of tumor cells stained in each tissue section, as determined blindly by an uro-pathologist.

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Fig. 5. Expression of PDGF D and matriptase in human prostate carcinoma. Sections of benign glands, high-grade prostatic intraepithelial neoplasia (HGPIN), and tumor glands from Gleason scores 6 and 9 were immunostained with antibodies against PDGF D (A) and matriptase (B). Images were taken at 200X magnification. Insert shows the enlargement of boxed area. Basal cells and secretory cells in the prostate glands are indicated by the black arrows and red arrow heads, respectively. In negative control experiments, immunohistochemistry was performed on human prostate carcinoma without the corresponding primary antibody.
peripheral basal cells but not in luminal cells. Increased expression of both markers was also observed in some of the glands with high-grade prostatic intraepithelial neoplasia (HGPIN). The neoplastic glands in well to moderately differentiated PCa (Gleason score 6) largely showed focal and variable staining. Significantly increased staining of both markers was observed in poorly differentiated PCa (Gleason score 8 or higher) (Fig. 5). In situ hybridization of PDGF D

### Table 9

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<td>Matriptase</td>
<td>62</td>
<td>0.18 (0.08)</td>
<td>0.17 (0.11)</td>
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**Table 9.** Kendall’s tau analysis was used to assess the association between AJCC tumor stage and Gleason score with PDGF D and matriptase staining. p-value is shown within parentheses.

**Fig. 5.** Expression of PDGF D mRNA in human prostate carcinoma. In situ hybridization was performed on sections of benign glands (A) and tumor glands from Gleason scores 6 (B) and 8 (C) using PDGF D antisense probes. Images were taken at 200X magnification. Insert shows the enlargement of boxed area. PDGF D sense probe was used as a negative control on human prostate carcinoma (D).

**Fig. 6.** Expression of activated β-PDGFR in benign and malignant human prostate glands. Sections of benign, high-grade prostatic intraepithelial neoplasia (HGPIN), low grade (Gleason score 7) and high grade (Gleason score 9) prostate carcinoma were immunostained with an antibody against phospho-β-PDGFR. Images were taken at 200X magnification.
Fig. 8. Expression of PDGF-D, matriptase, and phosphorylated-\(\beta\)-PDGFR in PNI and mucinous prostate carcinoma and bone metastases. A, B) Serial sections of neoplastic glands with perineural invasion (PNI, at 400X) (A) and sections of the mucinous variant of poorly differentiated prostate cancer (mucinous PCa, at 200X) (B) were immunostained with anti-PDGF D growth factor domain, anti-matriptase, and anti-phospho-\(\beta\)-PDGFR antibodies. Carcinoma and nerve cells are indicated by the red arrow heads and black arrows, respectively. C) Prostate cancer bone metastases were immunostained with anti-PDGF D growth factor domain and anti-matriptase antibodies. Right panels are enlargements of boxed areas in left panels.
mRNA was also performed, in which PDGF D mRNA was found to be expressed in epithelial cells. Consistent with the IHC, PDGF D mRNA was located in the basal cells, but not secretory cells, of benign prostatic glands, whereas expression was increased in poorly differentiated prostate carcinoma cells (Fig. 6). Staining for the phosphorylated form of β-PDGFR revealed increased activation of the receptor in high grades of prostate cancer (Fig. 7). Additionally, both PDGF D and matriptase were observed in aggressive prostate cancer cases, such as areas of perineural invasion (PNI), a mucinous variant of prostate cancer, and bone metastases. β-PDGFR was also found to be activated in these cases (Fig. 8).

Immunofluorescence was also used to examine colocalization of PDGF D and matriptase in PCa. Colocalization of PDGF D and matriptase were detected in prostate cancer with Gleason score 8 (A) and in a mucinous variant of prostate carcinoma (B) was performed at 630X magnification. Cell nuclei were stained with DAPI (blue). Yellow in the merged panel indicates co-localization of the proteins. C) A negative control of co-localization study was performed with indicated secondary antibody without primary antibody.

**Fig. 9. Co-localization of PDGF D and matriptase.** Immunofluorescence analysis of matriptase (Texas Red) and PDGF D (FITC-Green) in human prostate carcinoma with Gleason score 8 (A) and in a mucinous variant of prostate carcinoma (B) was performed at 630X magnification. Cell nuclei were stained with DAPI (blue). Yellow in the merged panel indicates co-localization of the proteins. C) A negative control of co-localization study was performed with indicated secondary antibody without primary antibody.
carcinoma specimens as well as in the mucinous variant of PCa (Fig. 9).

Taken together, these results suggest that PDGF D is able to be processed by matriptase in prostate cancer, allowing for activation of β-PDGFR signaling and cancer progression.

3.2 Regulation of PDGF Expression by PTEN

A. PDGF Expression Changes with PTEN Expression

Given these findings of an increase in PDGF D expression in PCa, the mechanism for this change in expression was next explored. The phosphatase PTEN is an important regulator of growth factor signaling. Additionally, PTEN is frequently lost or mutated in human prostate cancer. Therefore, Pten⁻/⁻ mice provide a clinically relevant setting PDGF D in PCa. To this end prostate epithelial cells were obtained from Pten⁺/+, Pten⁺/-, and Pten⁻/-

![Fig. 10. Loss of PTEN modulates expression of PDGFs/PDGFR. A) mRNA expression levels of PTEN, PDGF ligands and receptors, matriptase, and uPA in PTEN +/+, +/−, and −/− cells were analyzed by RT-PCR. B) CM was collected from serum-starved PTEN +/+, +/−, and −/− cells, resolved on reducing SDS-PAGE, and immunoblotted with anti-PDGF D antibody. rPDGF D was used as positive control. FL-M: Full length monomer; GD-M: Growth domain monomer.](image-url)
mice. In these mice, Cre expression was driven by the prostate specific, androgen responsive probasin promoter. Therefore, expression of androgens after five weeks of age results in deletion of exon 5 of Pten in the Pten$^{+/+}$ and Pten$^{-/-}$ mice. Exon 5 contains the phosphatase domain of PTEN, thus deletion of this exon results in loss of function of PTEN. By 8 weeks of age, all Pten$^{-/-}$ mice developed prostate carcinoma (14). PDGF expression in all three cell lines was determined through RT-PCR. PDGF B was expressed in Pten$^{+/+}$ and Pten$^{+/-}$ cells but not Pten$^{-/-}$ cells. Conversely, PDGF D and β-PDGFR were expressed in Pten$^{-/-}$ but not Pten$^{+/+}$ or Pten$^{+/-}$ cells (Fig. 10A). To verify protein expression, western blotting was performed. Consistent with mRNA levels, PDGF D expression increased in Pten$^{-/-}$ cells as compared to Pten$^{+/+}$ or Pten$^{+/-}$ cells (Fig. 10B). These results suggest that PTEN plays an important role in regulating the expression of PDGF family members.

B. PDGF D and Activation of β-PDGFR Are Increased in Pten$^{-/-}$ Mice

To confirm that PDGF status is altered in vivo, tissue sections and lysate were obtained from the prostate of eight week old Pten$^{+/+}$ and Pten$^{-/-}$ mice. Immunohistochemistry was performed on the tissue sections using antibodies directed against either PDGF D or activated, phosphorylated β-PDGFR. As shown in Figure 11A, PDGF D expression was increased in Pten$^{-/-}$ mice as compared to Pten$^{+/+}$ mice, consistent with what was found in the cell lines. Additionally, phosphorylated β-PDGFR levels also increased in Pten$^{-/-}$ mice, suggesting that the increased levels of PDGF D were able to activate the receptor. Western analysis of tissue lysate from Pten$^{+/+}$ and Pten$^{-/-}$ prostate
Fig. 11. Activation of PDGF D/β-PDGFR in prostate tumors in PTEN−/− mice. A, B) Prostate tissue sections from PTEN+/+ and PTEN−/− mice were immunostained with antibodies against PDGF D (A) or active, phosphorylated β-PDGFR (B). Images were taken at 200X. VP: ventral prostate; DL: dorsolateral prostate; AP: anterior prostate. C, D) Prostate tissue lysate from PTEN+/+ and PTEN−/− mice were resolved on non-reducing SDS-PAGE, followed by immunoblot analysis with anti-PDGF D (C) or anti-matriptase (D) antibody. Two mice per group were analyzed, as designated by numbers 1-4. FL-D: Full length dimer; GD-D: Growth domain dimer. *: 70kD Matriptase with 55kD HAI-I; **: 70kD Matriptase with serine protease domain of HAI-I
also revealed increased levels of PDGF D. Importantly, PDGF D was found in both the inactive, full-length form as well as the activated, growth domain form (Fig. 11B). Additionally, Pten\(^{\text{--}}\) mice exhibited increased levels of matriptase (data courtesy of Dr. Wei Huang) (Fig. 11C). These results suggest that loss of PTEN is also associated with PDGF expression in vivo.

C. PDGF Expression Is Regulated through the PI3K/Akt Pathway

PTEN is known to regulate many signaling pathways, including PI3K and MAPK pathways. Therefore, inhibitors of PI3K, MEK, JNK, and mTOR were used to identify the signaling pathway that regulates PDGF expression downstream of PTEN. LY294002 and wortmannin inhibit PI3K activity through two distinct mechanisms. Inhibition of PI3K by LY294002 arises from its competitive inhibition of the ATP binding site on the p85\(\alpha\) subunit, whereas wortmannin exhibits an irreversible, covalent interaction with the p110 subunit (134, 176, 183). PD98059 inhibits the MAPK pathway by binding to MEK (MAPK kinase), preventing its activation by Raf-1; this inhibition of MEK results in an inhibition of ERK1/2 activation (2). JNK Inhibitor II binds to the ATP binding site of JNK (12, 148). mTOR is inhibited by rapamycin through its binding of mTOR in complex with FK506-binding protein (FKBP12), thus blocking binding of the lipid second messenger phosphatidic acid (PA) to mTOR (24, 40).

Treatment of Pten\(^{\text{--}}\) cells with MEK, JNK, and mTOR inhibitors did not alter PDGF mRNA expression (Fig. 12A-C). However, inhibition of PI3K with either LY294002 or wortmannin resulted in decreased expression of both PDGF D and \(\beta\)-PDGFR (Fig. 12D and E). These results suggest that PDGF/PDGFR mRNA expression is regulated through the PI3K pathway.
D. Akt3 Expression Increases with Loss of Pten

Given that Akt is a downstream effector of PI3K, the expression of the three Akt isoforms were next analyzed. RT-PCR revealed that Akt1 mRNA levels are consistent throughout the three cell lines, whereas Akt3 is increased in Pten\(^{-/-}\) cells as compared to Pten\(^{+/+}\) and Pten\(^{+/-}\) cells (Fig. 13A). Similar results were found with protein levels of the three isoforms (Fig. 13B). Furthermore, inhibition of Akt, downstream of PI3K, also reduced expression of PDGF D and β-PDGFR in Pten\(^{-/-}\) cells (Fig. 13C). These results suggest that Akt3 is responsible for changes in PDGF expression in the Pten\(^{-/-}\) cell line.

Fig. 12. The PTEN/PI3K pathway regulates PDGF expression. PTEN \(-/-\) cells were treated for 2 days with Rapamycin (mTOR inhibitor) (A), or overnight with increasing concentrations of JNK Inhibitor II (B), PD98049 (C), LY294002 (D), and wortmannin (E), inhibitors of JNK, MAPK, and PI3K, respectively. mRNA expression levels were analyzed by RT-PCR.
E. **In vitro Loss of Pten Results in Changes in PDGF Expression**

To further confirm the role of PTEN in PDGF expression, the wild type Pten^loxP/loxP^ cell line was infected with Cre through the use of a lentivirus, thus allowing for loss of PTEN *in vitro*. Similar to what is observed upon PTEN loss *in vivo*, cells in which PTEN expression was lost *in vitro* exhibit decreased expression of PDGF B and increased expression of PDGF D, relative to wild type Pten^loxP/loxP^ cells (Fig. 14A). Expression of the Akt isoforms was also examined by western blot; Akt1 protein expression was similar amongst all cell lines, whereas Akt2 and Akt3 expression was increased in the pooled population of the *in vitro* PTEN knockout cells (Fig. 14B).

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**Fig. 13. Loss of PTEN leads to increased Akt3 expression.** Expression of Akt isoforms in PTEN +/-, +/-, and -/- cells were determined by RT-PCR (A) and western blotting (B). C). PTEN -/- cells were treated overnight with increasing concentrations of Akt Inhibitor IV. mRNA expression levels were analyzed by real time RT-PCR.
Fig. 14. In vitro loss of PTEN regulates PDGF expression through PI3K/Akt pathway. A) mRNA expression levels of PTEN, β-PDGFR and its ligands, PDGF B and PDGF D, in PTEN wild type (LoxP/LoxP) and knockout cells were analyzed by RT-PCR. B) Expression of Akt isoforms in PTEN wild type and knockout cells were determined by western blotting. C) PTEN knockout cells (clone 2) were treated overnight with increasing concentrations of LY294002 (upper panel) or Akt Inhibitor IV (lower panel). mRNA expression levels were analyzed by real time RT-PCR. PP: PTEN knockout pooled population; #2: PTEN knockout clone 2; #3: PTEN knockout clone 3; #4: PTEN knockout clone 4
**Fig. 15. The PTEN/PI3K pathway modulates PDGF/PDGFR expression in human PCa.**

**A)** mRNA expression levels of PTEN, PDGF ligands, and Akts were analyzed by RT-PCR in human PCa cell lines DU145 and PC3. **B)** DU145 and PC3 lysate was collected and resolved on non-reducing SDS-PAGE, and immunoblotted with antibodies directed against Akt isoforms and phosphorylated Akt. **C,D)** PC3 cells were treated overnight with increasing concentrations of LY294002 (C) or Akt Inhibitor IV (D). mRNA expression levels were analyzed by real time RT-PCR.
Additionally, when PTEN$^{+/+}$ CreIIa cells were treated with inhibitors of PI3K or Akt, expression of PDGF D decreased (Fig. 14C).

**F. Inverse Association between PTEN Status and PDGF Expression in Human Prostate Cancer**

To investigate the role of PTEN in PDGF expression in human prostate cancer, the cell lines DU145 and PC3 were analyzed for PDGF expression. As shown previously,

![Diagram](image)

**Fig. 16. Working model of Matriptase/PDGF D/β-PDGFR signaling axis in human prostate carcinoma.** Loss of PTEN, a common occurrence in PCa, results in increased expression of PDGF D and β-PDGFR. PDGF D is then able to be processed by matriptase/uPA, allowing for autocrine and paracrine signaling through activation of β-PDGFR on both tumor and stromal cells.
DU145 expresses wild type PTEN, whereas PC3 does not express PTEN (177). Additionally, DU145 and PC3 express similar levels of both Akt1 and Ak2 mRNA and protein, whereas expression of Akt3 mRNA is increased in PC3 cells (Fig. 15A-B). Similar to the PTEN-/- murine cells, PC3 exhibits decreased levels of PDGF B mRNA and increased levels of PDGF D mRNA, relative to DU145. Furthermore, inhibition of either PI3K or Akt resulted in decreased levels of PDGF D mRNA in PC3 cells (Fig. 15C). Taken together, these results suggest that the PTEN/PI3K/Akt pathway regulate the PDGF D/β-PDGFR signaling axis in human prostate carcinoma (Fig. 16).
Chapter 4  
Discussion

Previous studies have shown that β-PDGFR expression is upregulated in human prostate carcinoma, while its classical ligand PDGF B is not detected. β-PDGFR has also been indicated to be one of five key genes, along with chromogranin A, HOXC6, IPTR3, and sialyltransferase-1, that are able to predict prostate cancer recurrence (150). PDGF B was originally thought to be the sole ligand for β-PDGFR; thus, the lack of detection of PDGF B in prostate cancer lead to the question of a clinically relevant ligand for β-PDGFR in human prostate cancer. Through the use of immunohistochemistry and in situ hybridization, the results shown in this dissertation reveal for the first time that PDGF D expression is increased in human prostate carcinoma (Figs. 5-6). Thus, the novel finding of an association of PDGF D with increasing prostate cancer tumor stage and Gleason score indicates for the first time the PDGF ligand that is able to activate β-PDGFR in prostate cancer.

Previous work in our lab has established that the serine protease matriptase is capable of processing PDGF D into its active growth factor domain form in vitro. In an effort to establish the ability of matriptase to process PDGF D in vivo, immunohistochemistry and immunofluorescence studies were performed with human prostate cancer samples, as well as in a murine model. In the matriptase transgenic mouse model, in which matriptase is upregulated in keratinocytes, increased expression of matriptase resulted in increased activation of β-PDGFR in the skin of these mice (Fig. 4). Matriptase expression is also associated with human prostate cancer tumor stage and Gleason score, in a manner similar to PDGF D (Fig. 5). Importantly, PDGF D and
matriptase colocalize in human prostate cancer (Fig. 9). These results support the hypothesis that matriptase can cleave the CUB domain from PDGF D, resulting in an active growth factor.

Taken together with previous findings, the results of this dissertation show that PDGF B and PDGF D exhibit different expression patterns in prostate cancer. Importantly, our findings that PDGF D and PDGF B have distinct expression signatures is not unique to the prostate, as determined by previous studies. PDGF D is expressed at high levels in the adrenal gland, pancreas, and testis, where little to no PDGF B is detected by real time PCR (82). Additionally, Bergsten et al has reported that PDGF D and PDGF B are expressed in distinct cellular components of the embryonic kidney (13). Immunohistochemistry revealed that PDGF D is expressed in the fibrous capsule of the kidney, the metanephric mesenchyme of the cortex, and the basal aspect of the branching ureter. β-PDGFR is also expressed in the metanephric mesenchyme as well as vascular smooth muscle cells (7, 96). PDGF B, however, is expressed only in the endothelial cells (96). These expression patterns suggest that PDGF B and PDGF D play separate roles in the developing kidney, with PDGF D playing a role in the proliferation of the metanephric mesenchyme that express β-PDGFR and PDGF B suggested to be important in the vascularization of the kidney. Given the frequent expression of one, but not both, ligands for β-PDGFR, it is likely that PDGF B and PDGF D may serve distinct functions in specific tissues. For example, it has been suggested that it is possible that the distinct expression of PDGF D but not PDGF B in certain tissues is due to the ability of PDGF D to stimulate cells that express either β-PDGFR or both α- and β-PDGFR without stimulating nearby cells that express only α-PDGFR.
Interestingly, PDGF D expression in benign prostatic glands was seen exclusively in the basal cells but not secretory cells, whereas PDGF D expression in neoplastic glands was seen in luminal cells (Fig. 5). Current research indicates the possibility that prostate cancer arises from the transformation of basal cells to neoplastic secretory cells (3, 106). Thus, it is possible that PDGF D plays a role in the neoplastic transformation of these cells. Additionally, PDGF D and matriptase expression was detected in areas of neoplastic glands surrounding nerves, known as perineural invasion (PNI) (Fig. 8). Although controversial, PNI has been shown to be associated with increased tumor size, risk of recurrence, and metastasis (11, 84, 175).

In an effort to determine the mechanism resulting in the increased expression of PDGF D and β-PDGFR in prostate cancer, we turned to the PTEN model due to the fact that PTEN is a critical regulator of growth factor signaling and is frequently lost or mutated in prostate cancer. To this end, the murine model in which PTEN expression is lost specifically in the prostate is a useful model for studying the regulation of PDGF expression. Given that complete loss of PTEN expression is embryonically lethal, the prostate-specific model used for these studies provides a valuable tool for studies of the role of this important gene in prostate cancer formation and progression. Upon loss of PTEN \textit{in vivo}, PDGF D expression and activation of β-PDGFR increases, as determined through immunohistochemistry as well as western blotting (Fig. 11). In cells derived from these prostate specific Pten\textsuperscript{−/−} mice, loss of PTEN also resulted in decreased PDGF B mRNA expression with a concomitant increase in PDGF D and β-PDGFR mRNA expression (Fig. 10). Through the use of inhibitors of signaling pathways regulated by PTEN, it was determined that PDGF D/β-PDGFR expression was regulated through the
PI3K/Akt pathway (Figs. 12-13). The role of the PI3K/Akt signaling pathway in the expression of PDGF D and β-PDGFR was also established in human prostate cancer cells. The human prostate cancer cell line PC3, which lacks PTEN expression, exhibits increased expression of PDGF D relative to DU145, which expresses a functional PTEN (Fig. 15). Additionally, inhibition of either PI3K or Akt in PC3 cells results in decreased expression of PDGF D, showing that the PI3K/Akt pathway is necessary to maintain PDGF D expression.

In both murine and human prostate cancer cells lacking PTEN, there is an increase in the expression of Akt3 at both the mRNA and protein level, suggesting that this Akt isoform may be responsible for controlling PDGF D and β-PDGFR expression (Figs. 13 and 15). Unfortunately, experiments in which each Akt isoform was knocked down through the use of siRNA proved to be inconclusive, as the decreased expression of one Akt isoform lead to the increase of expression of the two remaining Akt isoforms (data not shown). In these experiments, PDGF D and β-PDGFR expression was not significantly altered upon loss of any one Akt isoform, possibly due to compensatory features of the different isoforms. Thus, this study was unable to conclusively prove whether one specific Akt isoform is responsible for regulation of PDGF D or β-PDGFR expression, or if all three isoforms are capable of this regulation of expression.

Our lab has previously found that conditioned media from LNCaP cells transfected with PDGF D enhance fibroblast motility. Additionally, LNCaP-PDGF D cells exhibit accelerated onset of prostate tumor growth in a subcutaneous injection SCID mouse model; two weeks post-injection, one out of five mice injected with LNCaP-neo developed tumors, whereas five out of five mice injected with LNCaP-PDGF D
developed tumors. Additionally, in this subcutaneous injection model, LNCaP-neo cells generated well-encapsulated tumors, whereas LNCaP-PDGF D tumors displayed a drastically enhanced invasion into the surrounding stroma. These results demonstrate a potential oncogenic activity of PDGF D in the development and/or progression of prostate cancer (170). Given these previous findings, taken together with the results shown here, it is likely that increased levels of PDGF D in prostate carcinoma can stimulate the growth of the tumor.

Furthermore, unpublished data from our lab strongly supports the oncogenic effects of PDGF D. Preliminary data from our lab has shown that normal murine prostate epithelial cells transfected with PDGF D display increased migration and invasion, as compared to both control cells and cells transfected with PDGF B. These PDGF D transfected cells also expressed increased levels of phosphorylated ERK and JNK (unpublished data, courtesy of Dr. Abdo Najy). Expression of PDGF D was also able to induce osteoblastic differentiation in MC3TS cells through stimulation of β-PDGFR and Akt, as determined by staining for mineralization as well as RT-PCR for the osteoblast differentiation marker osteocalcin and transcription factor Runx2. Importantly, in an intratibial injection model, LNCaP-PDGF D cells exhibit increased tumor take and growth rate as compared to control LNCaP-neo cells. In mice with these LNCaP-PDGF D tumors, there is also an increased mixed osteolytic/osteoblastic response, as determined by TRAP staining, indicating osteolytic responses, as well as trichrome staining, indicating osteoblastic responses. Conversely, LNCaP-neo derived tumors exhibited a solely osteoblastic response (manuscript in preparation). Additionally, as discovered by Dr. Wei Huang, PDGF D is able to induce osteoclastogenesis in a RANK/RANKL
independent manner. Using RAW264.7 cells, Dr. Huang determined that treatment of pre-osteoclasts with rPDGF D induced osteoclast differentiation as determined by tartrate resistant acid phosphatase (TRAP) staining. rPDGF D treatment also resulted in increased expression of nuclear factor of activated T cells 1 (NFATc1), a key transcription factor in osteoclastogenesis. Interestingly, PDGF B was unable to induce these osteoclastic effects, suggesting that PDGF D exhibits distinct functions in osteoclastogenesis from that of PDGF B. To this end, we raise four hypotheses as to the mechanism for the unique PDGF D signaling capabilities, as depicted in Fig. 17. First, activation of a unique receptor by the CUB domain subsequent to its proteolytic removal may result in osteoclastogenic signaling in cooperation with β-PDGFR signaling that is induced by the PDGF D growth factor domain (Fig. 17, Hypothesis 1). Our second hypothesis involves the PDGF D hemidimer, which is produced by the removal of one CUB domain, creating a dimer consisting of one full length PDGF D monomer and one growth factor domain monomer, as depicted in Fig. 2. In this hypothesis, binding of the growth factor domain subunit of the hemidimer to one β-PDGFR subunit could result in the recruitment by the CUB domain of additional cell surface signaling molecules. Subsequent to further

Fig. 17. Potential methods for PDGF D induction of osteoclastogenesis.
processing of the hemidimer into the growth factor domain dimer, β-PDGFR could then activate unique signaling pathways to initiate osteoclastogenesis (Fig. 17, Hypothesis 2). The third hypothesis is that the CUB domain of the hemidimer results in heterodimerization of β-PDGFR with an unknown receptor, inducing signaling pathways leading to osteoclastogenesis (Fig. 17, Hypothesis 3). The exact mechanism of the induction of osteoclastogenesis is an exciting area of study that is under investigation in our lab.

Importantly, human prostate cancer bone metastasis is characterized by both osteolytic and osteoblastic responses (115). Thus, PDGF D expression induces a bone response typical of human prostate carcinoma. The major site for prostate cancer metastasis is the bone. Importantly, both matriptase and PDGF D expression were detected in bone metastatic samples of human prostate carcinoma (Fig. 8). Thus, targeting the matriptase/PDGF D/β-PDGFR signaling axis could provide a specific therapeutic approach for prostate cancer. While patients with primary prostate cancer have several treatment options, such as surgery, radiation, hormone therapy, or even “watchful waiting,” therapy choices for metastatic prostate cancer is more limited and, unfortunately, less effective.

β-PDGFR has been found to upregulated in 80% of both primary prostate cancer as well as bone metastases (74). Additionally, β-PDGFR is identified as part of a five-gene model that predicts prostate cancer recurrence (150). Therefore, PDGFR signaling is a potentially important target for prostate cancer therapy. In a mouse model of prostate cancer, treatment with the Bcr-Abl/PDGFR/c-Kit inhibitor Gleevec (STI571, imatinib mesylate) was shown to reduce tumor incidence and growth, and an
increase in apoptosis in the tumor cells as well as tumor associated endothelial cells (167). However, clinical trials with Gleevec were halted due to excessive side effects, such as diarrhea related to inhibition of c-kit in the intestines and cardiotoxicity associated with inhibition of c-abl in cardiac myocytes (31, 52, 72). Therefore, more specific therapies that target PDGFR signaling could be critical for developing effective targeted treatments. To this end, targeting PDGF D could result in more effective PDGFR related therapies with less toxicity, through targeting either PDGF D expression through the PTEN/PI3K/Akt pathway, or through targeting PDGF D activation by matriptase.

As shown above, the PI3K/Akt pathway regulates PDGF D expression. It has been previously shown that PDGF D expression is increased by luteinizing hormone (LH) (64). In human granulose-luteal cells, treatment with LH resulted in a 4.21-fold increase in PDGF D. Conversely, LH treatment resulted in a 41% decrease of PDGF B. Given that LH is capable of signaling through Akt, it is possible that its effects on PDGF D expression are through the PI3K/Akt pathway (21, 45). Additionally, it has previously been shown by microarray that loss of Pten in murine prostate epithelial cells resulted in a 1.42 and 1.65 increase in PDGF D and β-PDGFR, respectively (179). Thus, the results of our study confirm the role of PTEN in PDGF D and β-PDGFR expression. Previously, it has been shown that the PDGF D promoter contains sites regulated by the transcription factors Ets-1 and Sp1 (104). Interestingly, both PTEN and Sp1 have been linked to regulation of IGF-IR and VEGF (132, 192, 193). Sp1 induction of VEGF expression is brought about by Akt activation; Pore et al determined that Akt activation lead to increased phosphorylation of Sp1 as well as increased binding of Sp1 to the VEGF
promoter (132). Given the degree of similarity between PDGF and VEGF family members, regulation of PDGF D expression may be similar to that of VEGF.

In conclusion, the results discussed above, taken together with previous studies, indicate that the matriptase/PDGF D/β-PDGFR signaling pathway may be a key factor in prostate carcinoma. By elucidating the mechanism by which PDGF D expression is increased by the PTEN/PI3K/Akt pathway, future therapies for prostate cancer may be developed.
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ABSTRACT

REGULATION OF PLATELET DERIVED GROWTH FACTOR D EXPRESSION BY PTEN/PI3K/AKT IN PROSTATE CANCER

by

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Degree: Doctor of Philosophy

Platelet Derived Growth Factor (PDGF) is a family of mesenchymal growth factors that regulate cell proliferation, migration, and differentiation. Unlike the classic PDGF ligands A and B, which are secreted as active dimers, PDGF D must undergo extracellular proteolytic processing to remove its N-terminal CUB domain from the C-terminal PDGF growth domain before the ligand is able to stimulate its receptor, PDGF receptor beta (β-PDGFR). Importantly, recent clinical studies have shown that β-PDGFR is upregulated in primary prostate cancer and bone metastases. However, PDGF B, formerly thought to be the sole ligand for β-PDGFR, is not expressed in clinical prostate cancer samples. In a study of human primary prostate carcinoma and bone metastases, we found that PDGF D and matriptase are associated with prostate cancer progression. Additionally, in a clinically relevant prostate-specific PTEN (phosphatase and tensin homolog) knockout mouse model, we found an increase in PDGF D expression and β-PDGFR phosphorylation upon loss of PTEN. Upon inhibition of the PI3K pathway, PDGF D/β-PDGFR induction was abolished in PTEN-/- cells. Among Akt isoforms, downstream effectors of PI3K,
increased Akt3 expression was most prominent in PTEN-/- cells. These results suggest a molecular basis for activation of PDGF D/β-PDGFR signaling driven by the loss of PTEN, a frequent occurrence in human prostate cancer. Similarly, PTEN/Akt3 expression correlates with PDGF expression in human PCa cell lines, DU145 and PC3. Taken together, these results suggest that loss of PTEN in prostate cancer results in upregulation of PDGF D, which can then be activated by increased levels of serine proteases. The active growth domain is then able to activate β-PDGFR, thus causing subsequent downstream signaling.
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