Spring 5-21-2018

Regulation of the PI3-Kinase/PTEN Signaling Pathway by TGF-β in Prostate Cancer Cells

Mawiyah Kimbrough-Allah
mawiyah.allah@students.cau.edu

Follow this and additional works at: http://digitalcommons.auctr.edu/cauetds

Part of the Biochemistry Commons, Biology Commons, Cancer Biology Commons, Cell Biology Commons, Male Urogenital Diseases Commons, and the Molecular Biology Commons

Recommended Citation
Kimbrough-Allah, Mawiyah, "Regulation of the PI3-Kinase/PTEN Signaling Pathway by TGF-β in Prostate Cancer Cells" (2018). Electronic Theses & Dissertations Collection for Atlanta University & Clark Atlanta University. 123.
http://digitalcommons.auctr.edu/cauetds/123

This Dissertation is brought to you for free and open access by the Clark Atlanta University at DigitalCommons@Robert W. Woodruff Library, Atlanta University Center. It has been accepted for inclusion in Electronic Theses & Dissertations Collection for Atlanta University & Clark Atlanta University by an authorized administrator of DigitalCommons@Robert W. Woodruff Library, Atlanta University Center. For more information, please contact cwiseman@auctr.edu.
ABSTRACT

DEPARTMENT OF BIOLOGICAL SCIENCES

KIMBROUGH-ALLAH, MAWIYAH N. B.S. WINSTON-SALEM STATE UNIVERSITY, 2000

M.S. NORTH CAROLINA AGRICULTURAL & TECHNICAL STATE UNIVERSITY, 2003

REGULATION OF THE PI3-KINASE/PTEN SIGNALING PATHWAY BY TGF-β IN PROSTATE CANCER CELLS

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

Dissertation dated May 2018

Transforming growth factor -β (TGF-β) plays an important role in the progression of prostate cancer. It acts as a tumor suppressor in normal epithelial cells but as a tumor promoter in advanced prostate cancer cells. The PI3-kinase pathway has been shown to play integral roles in many cellular processes including cell proliferation, survival, and cell migration in many cell types. PI3-kinase pathway mediates TGF-β effects on prostate cancer cell migration and invasion. Phosphatase and tensin homolog (PTEN), a tumor suppressor gene, inhibits PI3-kinase pathway and is frequently mutated in prostate cancers. In this present study, we investigated possible roles of PTEN in TGF-β effects
on proliferation, migration, and the activation of PI3-kinase/AKT pathway in prostate cancer cells. PTEN was expressed in DU145 cells; however PC3 cells lack PTEN expression. TGF-β1 and TGF-β3 had no effect on PTEN mRNA levels but both isoforms increased PTEN protein levels in DU145 and RWPE1 cells, suggesting that TGF-β may mediate regulation of PTEN protein stability. TGF-β1 and TGF-β3 increased PTEN protein levels even in the presence of cycloheximide, a protein synthesis inhibitor, in DU145 cells. In addition, TGF-β upregulated phosphorylation of PTEN, stabilizing PTEN protein. Increase of PTEN protein levels in these cells may also indicate that PTEN may mediate TGF-β effects on cell proliferation. Knockdown of PTEN in DU145 cells resulted in significant increase in cell proliferation which was no longer affected by TGF-β isoforms. PTEN overexpression in PC3 cells inhibited cell proliferation. Knockdown of endogenous PTEN enhanced cell migration in DU145 cells, whereas PTEN overexpression reduced migration in PC3 cells and reduced phosphorylation of AKT in response to TGF-β. Based on these results, we conclude that PTEN plays a role in inhibitory effects of TGF-β on cell proliferation whereas its absence may enhance TGF-β effects on activation of PI3-kinase pathway and cell migration.
REGULATION OF THE PI3-KINASE/PTEN SIGNALING PATHWAY BY TGF-β 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
MAWIYAH NATHIFAH KIMBROUGH-ALLAH

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

MAY 2018
ACKNOWLEDGEMENTS

First, I want to give all praises and gratitude to Allah (God), the Almighty, the Beneficent, the Merciful, for his love, mercy, and blessings in my life. I would like to thank Dr. Shafiq A. Khan for serving as a great research mentor throughout my academic journey at Clark Atlanta University in the Center for Cancer Research and Therapeutic Development, and for giving me the opportunity to conduct research in his laboratory. Furthermore, I would like to extend my gratitude to my research committee members (Dr. Jaideep Chaudhary, Dr. Nathan Bowen, Dr. Joann Powell, and Dr. Myron Williams) for their advice and expertise. Special thanks to my lab members with whom I worked closely these past couple of years as a graduate student. I thank them for their knowledge, advice, support, friendship, and good times in the lab. My deepest gratitude goes to my parents for their love, support, and encouragement. I thank God for my entire family.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

TABLE OF CONTENTS .......................................................................................................... iv

LIST OF FIGURES ............................................................................................................... vii

LIST OF TABLES ................................................................................................................ ix

LIST OF ABBREVIATIONS .................................................................................................. x

CHAPTER

1. INTRODUCTION ............................................................................................................. 1

2. REVIEW OF THE LITERATURE .................................................................................. 7
   2.1 The Prostate ............................................................................................................. 7
   2.2 Prostate Cancer ........................................................................................................ 8
   2.3 Metastasis ................................................................................................................ 11
   2.4 Transforming Growth Factor-β (TGF-β) ............................................................. 13
   2.5 TGF-β Signaling Pathway ...................................................................................... 13
   2.6 TGF-β Isoforms ..................................................................................................... 15
   2.7 TGF-β in Normal Development ............................................................................ 17
   2.8 TGF-β Isoforms and Cancer .................................................................................. 18
   2.9 The Phosphatidylinositol 3-Kinase (PI3-Kinase) Signaling
       Pathway ...................................................................................................................... 19
CHAPTER

2.10 Phosphatase and Tensin Homolog Deleted on Chromosome 10

\textit{(PTEN)} ................................................................. 22

2.11 TGF-\(\beta\) and the PI3-Kinase/AKT/PTEN Signaling Pathway ............ 24

3. MATERIALS AND METHODS .......................................................... 25

3.1 Chemicals and Reagents ............................................................... 25

3.2 Cell Lines and Cell Culture ............................................................ 25

3.3 RNA Isolation, cDNA Synthesis, and RT-PCR ................................... 26

3.4 Quantitative RT-PCR (qRT-PCR) ..................................................... 28

3.5 Cell Treatments ......................................................................... 28

3.6 Western Blot Analysis ................................................................. 29

3.7 Transwell Cell Migration Assay ...................................................... 29

3.8 Invasion Assay ......................................................................... 30

3.9 Cell Proliferation Assay ............................................................... 31

3.10 Transfection with Specific Plasmids and Small Interfering (si) RNAs

............................................................................................................. 31

3.11 Statistical Analysis ................................................................. 31

4. RESULTS ......................................................................................... 33

4.1 Expression of PTEN in Prostate Cell Lines ........................................ 33

4.2 Effects of TGF-\(\beta\) on PTEN Expression in DU145 and RWPE1

Cells ................................................................. 35
CHAPTER

4.3 TGF-β Regulates PTEN Protein Stability ............................................. 38

4.4 The Effects of TGF-β Isoforms on the Phosphorylation of Smad2

and Smad3 ................................................................................................. 40

4.5 TGF-β Inhibits Proliferation in DU145 Prostate Cancer Cells .......... 40

4.6 The Role of PTEN on TGF-β Effects on Proliferation in Prostate

Cancer Cells .................................................................................................. 41

4.7 TGF-β Effects on Migration in Prostate Cancer Cell Lines ............. 43

4.8 TGF-β Effects on Invasive Properties in DU145 Prostate Cancer

Cells .............................................................................................................. 45

4.9 The Role of PTEN on TGF-β Effects on Migratory Behavior in

Prostate Cancer Cells .................................................................................. 46

4.10 The Role of PTEN in TGF-β Effects on the Phosphorylation of

AKT in Prostate Cancer Cells ..................................................................... 47

5. DISCUSSION ......................................................................................... 49

6. CONCLUSION ....................................................................................... 55

REFERENCES ........................................................................................... 56
LIST OF FIGURES

Figure

1. 2017 Estimates for leading cases and deaths in the United States .......................... 2
2. The adult prostate and surrounding structures ......................................................... 8
3. Remote metastasis formation ..................................................................................... 12
4. The canonical transforming growth factor β (TGF-β) signaling pathway .......... 15
5. Amino acid sequence alignments of TGF-β1, TGF-β2, and TGF-β3 ...................... 16
6. Schematic representation of the PI3-Kinase/AKT signaling pathway ..................... 21
7. mRNA expression of PTEN in prostate cell lines ..................................................... 33
8. Protein levels of PTEN and phosphorylation of AKT in prostate cell lines ........... 34
9. PTEN knockdown increased protein levels of phosphorylation of AKT in DU145 prostate cancer cells ...................................................... 35
10. TGF-β isoforms have no effect on PTEN mRNA levels in a dose and time-dependent manner in DU145 cells .................................................. 36
11. TGF-β isoforms increase PTEN protein levels in prostate cell lines ...................... 37
12. PTEN protein stability in response to TGF-β isoforms ......................................... 39
13. TGF-β induced Smad signaling in DU145 prostate cancer cells ......................... 40
14. Effects of TGF-β isoforms on proliferation in DU145 cells ................................. 41
15. PTEN is involved in inhibitory effects of TGF-β on cell proliferation in DU145 and PC3 cells .............................................................................................................. 42
16. Effects of TGF-β isoforms on cell migration in prostate cancer cells .................. 44

17. TGF-β isoforms have no effect on invasion in DU145 cells .......................... 45

18. Lack of PTEN induces cell migration in the presence of TGF-β in prostate cancer cells ................................................................. 47

19. PTEN overexpression reduces TGF-β effects on phosphorylation of AKT in PC3 cells ................................................................. 48
LIST OF TABLES

Table

1. The Origins of the Cell Lines Used......................................................... 27
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTRII/ACTRIIB</td>
<td>Activin Receptor II/ Activin Receptor IIB</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic Lymphoma Kinase</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>BMPRII</td>
<td>Bone Morphogenic Protein Receptor II</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein Kinase 2</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating Tumor Cells</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-Diamidine-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transformation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-Like Growth Factor I</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-Like Growth Factor I Receptor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IRS-I</td>
<td>Insulin Receptor Substrate 1</td>
</tr>
<tr>
<td>KSFM</td>
<td>Keratinocyte Serum Free Medium</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-Epithelial Transition</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>pAKT</td>
<td>Phosphorylated AKT</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-Dependent Kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol -4,5 bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol -3,4,5 bisphosphate</td>
</tr>
<tr>
<td>pPTEN</td>
<td>Phosphorylated PTEN</td>
</tr>
<tr>
<td>pSMAD</td>
<td>Phosphorylated Smad</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</td>
</tr>
<tr>
<td>R-Smads</td>
<td>Receptor-Regulated Smads</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SER</td>
<td>Serine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming Growth Factor-α</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TGF-βRI</td>
<td>TGF-β Receptor I</td>
</tr>
<tr>
<td>TGF-βRII</td>
<td>TGF-β Receptor II</td>
</tr>
<tr>
<td>TGF-βRIII</td>
<td>TGF-β Receptor III</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Prostate cancer is the most common malignancy among men in the United States. According to cancer statistics, there will be an estimated 161,360 new cases diagnosed and 26,730 deaths in 2017, which represent 19% of all cancer cases and 8% of cancer-related deaths among American men, respectively (Figure 1).\(^1\) Well established risk factors for prostate cancer include age, race/ethnicity, family history of the disease, and certain inherited genetic conditions.\(^2\) In black men, the risk of prostate cancer is 74% higher than whites for reasons that remain unclear, but may include inherited susceptibility.\(^3\) Overall, prostate cancer death rate has been decreasing in men of all races and ethnicities by about 3% per year since 1999, although death rates remain more than twice as high in black men compared to any other group of men.\(^3\) Men diagnosed with local or regional prostate cancer have almost a 100% 5-year survival rate, however, men diagnosed with prostate cancer that has spread to other parts of the body have a 5-year survival rate of only 29%.\(^4\) This decrease in survival due to metastasis is cause for ongoing research in the area of invasion and metastasis.

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is a secreted cytokine and a major regulator of many cellular processes implicated as factors in cancer formation and progression such as proliferation, survival, migration/invasion and metastasis.\(^5\) TGF-\(\beta\) acts as a tumor suppressor inhibiting proliferation in normal epithelial cells and early
stages of many cancers and while in later stages of many cancers, it exerts prooncogenic roles promoting epithelial to mesenchymal transformation (EMT) which converts static epithelial cells into highly invasive mesenchymal cells, a necessary pre-requisite for tumor cell metastasis. The three TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3 have been identified in mammalian cells and share a 70%-80% sequence homology in most organisms and play redundant roles in cancer cells. Although TGF-β1 is the most commonly studied TGF-β isoform concerning TGF-β signaling and function, and is ubiquitously expressed among cell lines and in cancer, all three isoforms bind to the same receptors and exert similar biological effects on target cells. These isoforms signal by binding to TGF-β Receptor II (TGF-βRII) which activates TGF-β Receptor I
(TGF-βRI) which recruit and phosphorylates receptor associated-Smads (R-Smads), Smad 2 and Smad 3. These phosphorylated R-Smads (Smad 2 and -3) then form heterodimeric complexes with Smad 4 that are then translocated into the nucleus where it functions to regulate responses.\textsuperscript{10,15} While it has been shown that all three TGF-β isoforms signal by binding TGF-βRII and TGF-βRI, it has been suggested that TGF-β1 and TGF-β3 share a similar receptor binding affinity to TGF-βRII and can exert similar biological effects on target cells, however TGF-β2 differs in that it binds to TGF-βRII with an affinity that is 100-1,000 fold lower and requires TGF-β Receptor III (TGF-βRIII) (betaglycan) to promote receptor assembly with TGF-β2.\textsuperscript{9,16,17}

It has been shown in earlier studies that TGF-β isoforms can differentially regulate cancer during different stages of the disease. For instance, evidence indicates that TGF-β1 is frequently overexpressed in carcinoma cells, including prostate cancer cells, and leads to paracrine stimulation and modification of cellular and extracellular matrix components of the tumor microenvironment.\textsuperscript{18,19} TGF-β2 has been shown to confer resistance to TNFα-induced apoptosis in prostate cancer cells.\textsuperscript{15,20} It has also been demonstrated that TGF-β3 increased the invasiveness of endometrial carcinoma cells via a Phosphatidylinositol-3 Kinase (PI3-kinase) –dependent pathway, which were distinct from TGF-β1.\textsuperscript{21,22} TGF-β3 expression has been previously studied in a variety of human cancers, including prostate cancer\textsuperscript{9,23,24}. Our laboratory has shown TGF-β effects on migration and invasion of prostate cancer cells, and these effects are dependent on both TGF-βRI and Smad3, and are mediated via the PI3-kinase pathway.\textsuperscript{9} Moreover, TGF-β1 and TGF-β3 isoforms have differential effects on proliferation of different prostate cancer
cell lines. Both isoforms inhibit proliferation in DU145 cells; however, PC3 cells do not respond to the inhibitory effects of either of the two isoforms,\textsuperscript{9,25–27} which indicates TGF-β differential effects on cell proliferation depending on the context of the cell.

Previous evidence has clearly suggested the involvement of the PI3-kinase signaling pathway mediating TGF-β effects on cancer cell invasion and metastasis.\textsuperscript{9,28,29} PI3-kinase has been shown to regulate diverse cellular processes such as metabolism, survival, proliferation, apoptosis, growth, and cell migration in both normal and diseased tissues.\textsuperscript{30,31} It has been demonstrated that TGF-β can rapidly induce PI3-kinase activation as determined by increased phosphorylation of AKT, a downstream target of PI3-kinase, to promote EMT, cell migration and survival.\textsuperscript{32–35} Furthermore, one study indicated that mechanistically, the PI3-kinase regulatory subunit, p85, appears to be constitutively bound to the TGF-βRII and, upon TGF-β stimulation, TGF-βRI is recruited to the complex activating PI3-kinase to initiate signaling downstream.\textsuperscript{36}

The PI3-kinase/AKT signaling pathway is negatively regulated by phosphatase and tensin homolog (PTEN), a tumor suppressor, through its lipid phosphatase activity by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP\textsubscript{3}) to phosphatidylinositol-4, 5-bisphosphate (PIP\textsubscript{2}), and therefore counteracts cell survival mechanism elicited by this signaling.\textsuperscript{30,37} PTEN is deleted or mutated in a variety of human cancers, including prostate cancer,\textsuperscript{38,39} allowing for the increase in PIP\textsubscript{3} level fueled by PI3-kinase that in turn allosterically activate AKT protein kinase.\textsuperscript{40–42} Activating mutations of the PI3-kinase pathway and loss of PTEN are extremely common in advanced cancer tumor progression.\textsuperscript{43} It has been previously shown that PTEN mutations in many cancers have
led to higher basal levels of phosphorylated AKT (pAKT\textsuperscript{ser473}) and increased survival of cells.\textsuperscript{43–45} A recent report has confirmed that an isoform-specific role of TGF-β in migration and invasion of metastatic PC3 prostate cancer cells, which are dependent on the activation of the PI3-kinase pathway, but did not see similar effects of TGF-β isoforms on the activation of PI3-kinase and pAKT in DU145 prostate cancer cells.\textsuperscript{9} This suggests that one possible contributor of differential effects of TGF-β on the activation of PI3-kinase may be PTEN. PTEN is expressed in the DU145 prostate cancer cell line, but the gene is deleted in PC3 cells which lack PTEN expression.\textsuperscript{46–49} Another study indicated that the loss of PTEN expression in human cancers may contribute to a role for TGF-β as a tumor enhancer with specific effects on cellular motility and invasion.\textsuperscript{50}

Although previous studies confirmed an isoform-specific role of TGF-β on migration and invasion of metastatic prostate cancer cells which are dependent on the activation of the PI3-kinase pathway, the role of PTEN in TGF-β effects on the activation of PI3-kinase in prostate cancer cells has yet to be elucidated. Therefore we hypothesize that PTEN may play a role in TGF-β induced effects on cell proliferation, migration, invasion, and the activation of PI3-kinase/AKT pathway in prostate cancer cells. Furthermore, reports have indicated that PTEN activity is regulated by multiple post-translational mechanisms,\textsuperscript{51,52} however, the role of TGF-β in the regulation of PTEN activity in prostate cancer have not been investigated. To test this hypothesis, the following aims were investigated:

1. To determine the expression of PTEN and pAKT in prostate cancer cells, and determine the effects of TGF-β on PTEN expression.
2. To determine the role of PTEN in TGF-β on the activation of PI3-kinase/AKT pathway, proliferation, migration, and invasion.

3. To determine the regulation of PTEN activity by TGF-β in prostate cancer cells.
CHAPTER 2
REVIEW OF THE LITERATURE

2.1 The Prostate

The prostate gland is an exocrine organ of the male reproductive system located in the pelvic cavity. Its secretes the prostatic fluid that is slightly acidic (pH of ~6.4) that forms 20% (by volume) of the semen. The location of the prostate gland is posterior to the lower portion of the symphysis pubis, anterior to the rectum, and inferior to the urinary bladder in the subperitoneal compartment between the pelvic diaphragm and the peritoneal cavity (Figure 2A). The prostate is enveloped by a thin fibromuscular layer divided into three zones which are the transition zone, central zone, and the peripheral zone (Figure 2B). These zones produce and drain prostatic secretions into the urethra, account for approximately 70% of the total prostate bulk with the fibromuscular stroma, comprising of connective and smooth muscle, making up the remaining 30%. Testosterone, the primary androgen is produced by the testes; in peripheral tissues such as the prostate, testosterone is converted locally to dihydrotestosterone (DHT) by the action of the enzyme 5α-reductase. DHT is more potent than testosterone and has a higher affinity for the nuclear androgen receptor. Various mechanisms can activate the androgen receptor which results in cell proliferation and cell growth. Growth factors such as TGF-β and EGF are expressed by prostate cells during prostate development. In non-diseased prostate, TGF-β is believed to play a role in regulating cell growth through
its anti-proliferatory effects due to the ability to inhibit the mitogenic effects of EGF/TGF-α on epithelial cells\textsuperscript{60} and of basic FGF (bFGF) on stromal cells.\textsuperscript{61}

Furthermore, prostate fluids, from non-diseased human prostates, contain large amounts of EGF\textsuperscript{62} which appears to be an important regulator for normal growth in both rat and human prostate.\textsuperscript{63}

\textbf{Figure 2.} The adult prostate and surrounding structures. (A) The prostate gland is located below the bladder. The urethra runs through the center of the prostate, from the bladder to the penis. Image is taken from http://www.healthhype.com/tips-for-living-with-a-large-prostate.html.\textsuperscript{64} (B) The prostate divided into three zones which are transition zone, central zone, and the peripheral zone. Image is taken from Toivanen and Shen (2017).\textsuperscript{55}

\textbf{2.2 Prostate Cancer}

Prostate cancer is the second leading cause of cancer deaths among men especially in the United States and in all racial groups. Although older age and family history are risk factors for being diagnosed with prostate cancer, race/ethnicity is a strong
risk factor especially among African American men. In 2017, according to the American Cancer Society, 161,360 men will be diagnosed, and 26,730 will die of prostate cancer. Early stages of prostate cancer are located in the prostate gland, where they are more easily cured by surgery and radiation therapy. However, in later stages, prostate cancer metastasizes to secondary sites, usually in the bone, lung, or liver. The standard of care for treating metastatic prostate cancer is hormonal therapy and chemotherapy. Hormonal therapies are aimed at the inhibition of the production or activity of androgens. Prostate cancer cells in advanced stages become resistant to hormonal therapy treatments, which results in castration-resistant prostate cancer. Currently, there is no effective treatment or preventative option for advanced stage androgen-independent prostate cancer. Thus, the development of therapeutic strategies targeting the development or progression of metastatic prostate cancer will lead to increased survival of prostate cancer patients. More recently, prostate cancer has been shown to exhibit a racial disparity among African American men, and the incidence of mortality rates for African American men are 1.5 and 2.3 times higher than for Caucasian men and even higher compared with all non-African-American men, respectively. Many explanations for health disparities have attributed them to modifiable factors such as low socioeconomic status and lack of access to health care; however, when such factors are controlled for, factors may include biological and/or genetic differences. Previous studies demonstrated that TGF-β1 is hyperexpressed frequently in African Americans men compared to Caucasians. A recent study reported that peripheral blood TGF-β1 protein levels are positively associated with plasma renin activity, systolic blood pressure, diastolic blood pressure,
body mass index, metabolic syndrome, and microalbuminuria are more predictive in African Americans but not in whites. Furthermore, the overexpression of TGF-β1 was significantly more frequent in African American patients with hypertension, end-stage renal disease, diabetes, and glaucoma.

Major processes and molecular events are believed to represent important contributing factors in prostate carcinogenesis and have been associated with possible roles in cancer initiation and progression. These events include inflammation, oxidative stress, DNA damage, genetic factors, tumor suppressor/promotor genes, growth factor production, and receptor signaling. Of these molecular mechanisms, the tumor suppressor gene, PTEN, is frequently mutated or deleted in many cancers, including prostate. PTEN negatively regulates the PI3-kinase pathway and considerable evidence indicates that PTEN loss of function results in up-regulation of the PI3-kinase/AKT/mTOR signaling pathway in prostate cancer, primarily through the activation of AKT. In addition to the PI3-kinase signaling pathway and its involvement in prostate cancer, members of the TGF-β superfamily also play important roles in the progression of cancer. TGF-β exhibits tumor suppressor activities in the early stages of epithelial cancers. However, in the later stages of disease, TGF-β acts as a tumor promoter and is associated with aggressive form of cancers due to its effects on angiogenesis, immune suppression, and metastasis. Identifying differences in the effects of TGF-β superfamily on cellular behavior may facilitate the development of successful therapies and treatment of prostate cancer.
2.3 Metastasis

The complex process of metastasis remains the main cause for cancer related deaths. It involves the progression from the primary tumor development to the expansion of tumor cells at the secondary site of metastasis. Since the metastatic cascade involves many complex steps, generally it is considered to be an inefficient process.\textsuperscript{80} Although, common sites of secondary metastasis for prostate cancer are lung, liver, and pleura, in prostate cancer metastasis, it invariably goes to bone, forming characteristic osteoblastic lesions.\textsuperscript{81–83}

Many different stages of metastatic progression have been hypothesized which include tumorigenesis, dissemination and expansion of tumor cells at the secondary site. Mechanisms such as microRNAs, germline polymorphisms and the tumor microenvironment combined explain the metastatic cascade.

A critical process during tumor invasion and metastasis, as well as embryonic development, is the epithelial-mesenchymal transition (EMT). EMT occurs when epithelial cells lose or modify their apical-basal polarity and are converted to a mesenchymal phenotype.\textsuperscript{84,85} During metastasis, EMT disrupts the intercellular tight junctions and promotes migration, and self-renewing and stem-like properties to facilitate metastatic colonization.\textsuperscript{86,87} In this process, single tumor cells dissociate from the primary tumor, adopt mesenchymal properties to invade into the blood stream, and after extravasation circulating tumor cells (CTCs) regain epithelial characteristics, thus they can seed in secondary tissues, forming a metastatic bud (mesenchymal to epithelial (MET)) (Figure 3).\textsuperscript{88} EMT is influenced by the microenvironment and has been observed
primarily at the tumor-stromal interface.\textsuperscript{84,89} EMT is commonly characterized by the suppression of the cell-cell adhesion receptor E-cadherin and endows cells with more motile, invasive properties.\textsuperscript{87,90} Extracellular matrix metalloproteinases (MMPs) can degrade the cell basal lamina and extracellular matrix (ECM), and these enzymes maintain a balance with tissue inhibitors of metalloproteinases, which play vital roles in the invasion and metastasis of malignant tumors.\textsuperscript{91–93} Furthermore, the distinct features of EMT in tumor progression are also initiated and maintained by members of the TGF-\(\beta\) family of growth factors, activating major signaling pathways and transcriptional regulators integrated in the extensive signaling networks.\textsuperscript{90}

\textbf{Figure 3.} Remote metastasis formation. Circulating tumor cells (CTCs) undergoing epithelial to mesenchymal transition-mesenchymal-to-epithelial transition (EMT-MET). Image taken from Kölbl et al. (2016).\textsuperscript{88}
2.4 Transforming Growth Factor-β (TGF-β)

Transforming growth factor-β (TGF-β), a multifunctional polypeptide, is a member of a large family of cytokines that regulate many aspects of cellular function, including mammalian development, cellular proliferation, differentiation, homeostasis, migration, apoptosis, adhesion, angiogenesis, immune surveillance, and survival. These signaling consequences of TGF-β are dependent on factors including cell type, growth conditions and the presence of other growth factors.

The TGF-β superfamily is composed of more than 30 proteins in humans including TGF-β isoforms, activins, nodal, and bone morphogenetic proteins (BMPs). Activins, nodal, and BMPs play important roles in early embryogenesis; activins induce dorsal mesoderm in (Xenopus) embryos, whereas BMPs induce ventral mesoderm and also play critical roles in morphogenesis of various tissues. Nodal inhibits differentiation, maintains the pluripotency of human embryonic stem cell (hESCs), and promotes the self-renewing capacity of mouse ES cells. The highly similar TGF-β isoforms TGF-β1, TGF-β2, and TGF-β3 potently inhibit cellular proliferation of many cell types, including those from epithelial origin, and most mesenchymal cells are stimulated in their growth by TGF-β. Many growth factors belonging to the TGF-β superfamily play important roles in embryonic development. These growth factors may also play more crucial roles at relatively late stages of development and in adult tissues.

2.5 TGF-β Signaling Pathway

The canonical TGF-β signaling pathway involves three types of receptors which are type I, II, and III. There are seven members of the type I receptor (TGF-βRI) family
which include activin receptor-like kinase (ALK) (1–7), and five known members of the type II receptor (TGF-βRII) family have been characterized in mammals including TGF-βRII, activin receptor II and IIB (ActRII, ActRIIB), bone morphogenetic protein receptor II (BMPRII), and anti-Müllerian hormone receptor 2 (AMHRII).\textsuperscript{12,97,99} The TGF-β ligand signals through the TGF-βRII and the ALK5 complex in most cells.\textsuperscript{12,25} The type III receptor (TGF-βRIII), also known as betaglycan, and the related molecule endoglin, act as enhancers of growth factor access to corresponding signaling receptors.\textsuperscript{100} The initiation of TGF-β signaling involves the binding of TGF-β ligands to its (serine and threonine) kinase receptors which are TGF-βRII and TGF-βRI located on the cell membrane. Ligand binding leads to formation of the receptor heterocomplex, in which TGF-βRII phosphorylates TGF-βRI and thus activates TGF-βRI (Figure 4).\textsuperscript{101–103} The Smad family consists of eight Smad proteins which are classified into three subgroups: receptor-activated Smads (R-Smads) (Smads1, 2, 3, 5, and 8), the common Smad (Smad4), and the inhibitory Smads (I-Smads) Smad6 and Smad7.\textsuperscript{103} Activated TGF-βRI recruits and phosphorylates R-Smad proteins, which include Smad 2 and Smad 3 for TGF-β isoforms and activin, while Smad 1, Smad 5, and Smad 8 are for BMP signaling, which leads to the formation of a heterocomplex with their constitutive co-Smad, Smad4.\textsuperscript{101,104,105} This Smad complex is then translocated in the nucleus where it functions to regulate transcriptional target genes.\textsuperscript{101,104,106,107}
Figure 4. The canonical transforming growth factor β (TGF-β) signaling pathway. Figure taken from Hui and Friedman (2003). Expert Reviews in Molecular Medicine

2.6 TGF-β Isoforms

The TGF-β multifunctional cytokines are a family that exerts important roles in a variety of effects on normal and transformed cells. TGF-β exist as three mammalian isoforms which are TGF-β1, TGF-β2, and TGF-β3. The three isoforms, TGF-β1-3 have more than 97% sequences identity in mammalian tissue and signal through activation of TGF-β receptors.\textsuperscript{108,109} It has been revealed that the amino acid sequences of the three mammalian TGF-β isoforms share a high level of similarity between the active domains; TGF-β3 is 86% similar to TGF-β1, while it shares 91% similarity with that of TGF-β2 (Figure 5).\textsuperscript{14} TGF-β2 and TGF-β3 share the highest level of sequence similarity out of the three isoforms; however, TGF-β2 binds to the TGF-β receptor II (TGF-βRII) differently.
from TGF-β1 and TGF-β3 through different residues. Both TGF-β1 and TGF-β3 bind to type II receptor. Previous studies showed that the soluble extracellular domain of receptor II binds TGF-β1 and TGF-β3 with apparent dissociation constants of 200pM and 500pM, respectively, but the recognition of TGF-β2 was supported by the presence of receptor III, which is a membrane-anchored proteoglycan without any apparent signaling motif.

Figure 5. Amino acid sequence alignments of TGF-β1, TGF-β2, and TGF-β3. Figure taken from Laverty et al. (2009).

TGF-β3 has also been shown to differ significantly from TGF-β1 and TGF-β2 in its detailed tertiary structure of the activated domain. According to Nuclear Magnetic Resonance (NMR) data show which that alpha helical region of TGF-β1 is structurally ordered, while the alpha 38 helical region of TGF-β3 is structurally disordered, indicating that TGF-β3 adopts a more flexible “open” state in the crystal structure and in its complex with TGF-βRII. Their difference in structural flexibility is that TGF-β1 may lock the receptor complex in a closed conformation, while TGF-β3 may have a more open conformation due to greater flexibility of the TGF-β3 dimer. These observations imply that the structures of the ligand/receptor complexes for TGF-β1 and TGF-β3 may
be significantly different and may engage the downstream signaling pathways in different ways, which leads to different biological outcomes.\textsuperscript{14}

### 2.7 TGF-β in Normal Development

TGF-β isoforms are multifunctional cytokines and members of the TGF-β superfamily of signaling proteins that are critical for growth, differentiation, control of the immunological response, tissue homeostasis, and embryonic development of many different cell types within an organism. There have been multiple reports on the critical roles for TGF-β isoforms in embryonic development using knockout null-mutant mice.\textsuperscript{121–125} Among cytokines and growth factors, numerous \textit{in vivo} and \textit{in vitro} studies in various models have shown TGF-β’s leading role in the bone fracture healing process,\textsuperscript{126–131} and TGF-β’s involvement in nerve regeneration.\textsuperscript{132–135} In addition, primary functions of TGF-β include enhancing formation of the extracellular matrix, regulation of cellular differentiation, and inhibition of proliferation of most cells.\textsuperscript{121,136,137} Tumor suppressor responses of TGF-β are essential for maintaining homeostatic control of normal cell growth and cells in early phases of tumorigenesis.\textsuperscript{138} In premalignant cells, TGF-β act as a tumor suppressor of c-Myc expression,\textsuperscript{139} and the induction of the cell cycle inhibitors p15 and p21.\textsuperscript{138} Studies have suggested that the tumor suppressor response of TGF-β is important in the early stages of tumorigenesis. One report showed that activation of TGF-β delays the appearance of primary mammary tumors, and mice deficient in TGF-β signaling are prone to earlier tumor development.\textsuperscript{140} In contrast, mice expressing an activated TGF-β receptor exhibited an increase in metastatic lung foci, which is consistent with a pro-oncogenic effect of this pathway in late-stage disease.\textsuperscript{79,138}
Advanced disease is accompanied by increased expression and the activation of TGF-β. TGF-β acts as a tumor suppressor in early stages of epithelial cancers by inhibiting proliferation and inducing apoptosis. However, in later stages of cancer, TGF-β acts as a tumor promoter facilitating tumor cell and also targets genes involved in angiogenesis and metastasis.

2.8 TGF-β Isoforms and Cancer

TGF-β has been implicated in cancer formation and progression. Generally, it has been found that there is a marked increase in TGF-β mRNA and protein in human cancers (in vivo), including those of the pancreas, colon, endometrium, prostate, breast, brain, and bone, and in many of these types of cancers high expression of TGF-β correlates with advanced stages of malignancy and decreased survival. Among multiple cancers, TGF-β plays an important role in the progression of prostate cancer. It acts as a tumor suppressor in the early stages of epithelial cancers by inhibiting proliferation and inducing apoptosis. However, in later stages of many cancers, the tumor cells become resistant to growth inhibitory effects of TGF-β, in which TGF-β acts as a tumor promoter due to its role in stimulating angiogenesis, epithelial to mesenchymal transformation, or promoting the degradation of ECM due to increased production of MMPs; all of which aid invasion and metastasis. Of the three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3), TGF-β1 is the most commonly studied and tends to be ubiquitously expressed among cell lines and in cancer. During the signaling process, TGF-β binds first to TGF-βRII and then to TGF-βRI. The ectodomain of TGF-βRII binds with higher affinity to TGF-β3 than to TGF-β1, whereas the recognition of TGF-β2 has to be
supported by the presence of TGF-βRIII (betaglycan).\textsuperscript{113,118} It is implicated that both TGF-β1 and TGF-β3 share similar sequence homology, receptor binding affinity and similar biological effects on target cells, however, they may retain the ability to have non-redundant differential effects.\textsuperscript{9} Evidence indicates that TGF-β1 is frequently overexpressed in carcinoma cells, including prostate cancer cells, and leads to paracrine stimulation and modification of cellular and extracellular matrix components of tumor microenvironment.\textsuperscript{18,19} It has also been demonstrated that TGF-β3 increased the invasiveness of endometrial carcinoma cells via a PI3-kinase-dependent pathway, which were distinct from TGF-β1.\textsuperscript{29} In several different cancers, TGF-β3 expression levels have been reported from clinical studies. For instance, an earlier study indicated in colon carcinoma biopsies that the expression of TGF-β3 was uniform across tissue stages as well as normal tissue samples, suggesting that the isoform is unlikely to play a role in cancer progression.\textsuperscript{145} However, in breast carcinomas, clinical studies reported an increase in TGF-β3 expression, which correlated with a decrease in overall survival.\textsuperscript{146,147} TGF-β3 expression has also been studied in prostate cancer. One study revealed that TGF-β3 expression increases 2 fold in prostate tumors.\textsuperscript{148} Furthermore, our laboratory has shown that TGF-β effects on migration and invasion of prostate cancer cells are primarily induced by TGF-β3 and that those effects are dependent on both TGF-β receptor I and Smad3 and mediated via the PI3-kinase pathway.\textsuperscript{9}

2.9 The Phosphatidylinositol 3-Kinase (PI3-Kinase) Signaling Pathway

The phosphatidylinositol 3-kinase (PI3-kinase) pathway regulates diverse cellular processes such as metabolism, survival, proliferation, apoptosis, growth, and cell
migration. PI3-kinase is a family of lipid kinases that phosphorylate the 3’–hydroxyl group of phosphatidylinositols and phosphoinositides. PI3-kinase is divided into three classes which show distinct substrate specificity: Class I (IA and IB), Class II, and Class III. Class I PI3-kinase isoforms are mostly studied and understood in mammalian cells and represent important druggable targets for multiple human diseases. Notably, Class IA PI3-kinase is activated by receptor tyrosine kinases (RTKs), whereas Class IB PI3-kinase are activated by G-protein coupled receptors (GPCRs), in which both RTKs and GPCRs are stimulated by growth factors and hormones. Class IA PI3-kinase is a heterodimeric signaling factor composed of a regulatory subunit (p85α/β) and a catalytic subunit (p110α/β/γ/δ), which upon activation converts phosphatidylinositol -4,5-bisphosphate (PIP2) to phosphatidylinositol – 3, 4, 5- triphosphate (PIP3) (Figure 6). PIP3 transduces activating signals by binding to the pleckstrin homology (PH) domains of protein, thereby recruiting them to the membrane. AKT (also known as protein kinase B), is a serine-threonine kinase that is an important downstream effector of PIP3, in which AKT is recruited to the membrane via PIP3 binding of its PH domain and is phosphorylated either by PDK1 (3-phosphoinositide-dependent kinase), at threonine 308, and at serine 473 by the rapamycin-insensitive mTOR complex (mTORC2) or potentially by other kinases in some contexts. The PI3-kinase/AKT signaling pathway is negatively regulated by PTEN (phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor, by dephosphorylating PIP3 to PIP2, and therefore counteracts cell survival mechanisms elicited by this signaling.
AKT is a signaling pathway that is activated downstream of PI3-kinase, and has attracted much attention because of its role in cell survival. The PI3-kinase/AKT pathway provides a mechanism in cancer progression which includes evading cell death pathways. AKT promotes cell survival and may regulate cell proliferation by means of phosphorylation of multiple targets. There are three isoforms of AKT encoded by three different genes which include AKT1, AKT2, and AKT3, and these isoforms are of the serine/threonine protein kinase B family. It has been reported that AKT1 and AKT2 have been found to be overexpressed in gastric adenocarcinomas; breast cancers, ovarian cancers, and pancreatic cancer; while studies have indicated that AKT3 may contribute to breast and prostate carcinoma. It has also been reported that levels of HIF-1α (Hypoxia-induced factor-1α) are controlled by

Figure 6. Schematic representation of the PI3-Kinase/AKT signaling pathway. Image taken from Meier et al. (2005).
PI3-kinase and represent a critical component in AKT-dependent angiogenesis and tumorigenesis. High levels of phosphorylated AKT (pAKT), which is activated AKT, is correlated with poor prognosis of prostate cancer, whereas in normal prostate tissue, pAKT is undetectable.

2.10 Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN)

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), is a well-known tumor suppressor located in the 10q23 region of chromosome 10 encoding for a 403-amino acid multifunctional protein, which possesses both lipid and protein phosphatase activities. PTEN plays a role in regulating a number of cellular processes, including cell death and proliferation, through the PI3-kinase/AKT/mTOR pathway.

Evidence suggests that the tumor suppressor status of PTEN relies on its normal cellular activity as a lipid phosphatase which reduces the cellular levels of PIP$_3$ (dephosphorylating PIP$_3$ to PIP$_2$), and negatively regulate the PI3-kinase/AKT signaling pathway, thereby antagonizing downstream activity controlled by the AKT/mTOR axis, such as cell cycle progression, induction of cell death, transcription, translation, stimulation of angiogenesis, and stem cell self-renewal. In addition to its ability to dephosphorylate lipid substrates, PTEN also exhibits protein phosphatase activity, responsible for some of its biological effects including inhibition of cell migration and cell cycle arrest.

The PTEN tumor suppressor gene is most frequently lost, either partially or fully from sporadic human tumor types, and with germline mutations causing-predisposition syndromes such as Cowden and Bannayan- Zonana syndromes. Mutations in PTEN
have been found in a variety of human tumors occurring in about 50% of glioblastoma, endometrial carcinoma, prostate carcinoma, bladder carcinoma, and melanoma cases.\textsuperscript{176,180,181} During tumor development, PTEN mutations, and deletions often occur that inactivate or decrease PTEN function, and loss of PTEN can also occur by epigenetic silencing, transcription, or disruption of competitive endogenous RNA (ceRNA) networks.\textsuperscript{176,179} More than a decade of research has demonstrated and expanded our knowledge on the role of PTEN in cancer. Previous experiments performed in transgenic mice have revealed that loss of both copies of the PTEN gene resulted in embryonic lethality, whereas PTEN heterozygous mutants develop a diverse range of dysplasias in a wide spectrum of tissues with high incidence of prostate and colon cancer.\textsuperscript{182,183} Furthermore, recent studies demonstrated a dose-dependent role of PTEN in cancer which highlighted that subtle reductions in active PTEN levels dictate cancer susceptibility.\textsuperscript{184,185} The dysfunction of PTEN plays crucial roles in the pathogenesis of hereditary and sporadic tumors. In addition to genomic inactivation, many other pathogenic mechanisms are associated with PTEN’s gene expression or subcellular compartmentalization of the protein associated with tumorigensis. PTEN’s activity can indeed, be modulated by mutations, epigenetic silencing, transcriptional repression, post-transcriptional regulation, aberrant protein localization, protein-protein interaction, and post-translational modifications.\textsuperscript{164,186} These mechanisms are known to regulate, and fine-tune PTEN’s expression and function.
2.11 TGF-β and the PI3-Kinase/AKT/PTEN Signaling Pathway

There have been numerous studies that suggest a role for TGF-β in PI3-kinase signaling. Reports have indicated that TGF-β can activate the PI3-kinase pathway as determined by increase in the phosphorylation of AKT.\textsuperscript{32–35,187} Although it has yet to be elucidated on how TGF-β activates the PI3-kinase pathway, studies have revealed that activation of AKT can be independent of Smad2/3 and that both TGF-βRI and TGF-βRII kinase activity associates with the p85 subunit of PI3-kinase protein, therefore activating the pathway.\textsuperscript{32,33,35,36,188} It has also been implicated that PI3-kinase/AKT signaling is required for TGF-β induced transcriptional responses, EMT, and cell migration.\textsuperscript{32} Previous evidence has suggested the involvement of PI3-kinase pathway in TGF-β mediated effects on cancer cell invasion and metastasis\textsuperscript{9,28} and that the effects of TGF-β isoforms on cell migration and invasion are mediated by both TGF-βRI and Smad3 dependent activation of the PI3-kinase pathway.\textsuperscript{9} Activating mutations of the PI3-kinase pathway and loss of PTEN are extremely common in advanced cancer and are associated with tumor progression.\textsuperscript{43} An earlier study indicates that the loss of PTEN expression in human cancers may contribute to a role for TGF-β as a tumor enhancer with specific effects on cellular motility and invasion.\textsuperscript{50} Another study showed that TGF-β signaling is induced in the prostate by PTEN loss or by activation of AKT, and functions to keep in check the tumorigenic effects of PI3-kinase/AKT pathway activation.\textsuperscript{189} Whether or not PTEN plays a role in TGF-β effects on the activation of PI3-kinase in prostate cancer has yet to be elucidated.
CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals and Reagents

Recombinant human TGF-β1 and TGF-β3 were purchased from PeproTech (Rocky Hill, NJ). Mammalian expression vectors pcDNA3 GFP PTEN (plasmid # 10759), and pcDNA3 GFP (plasmid #20738) were obtained from Addgene (Cambridge, MA). Human PTEN siRNA (sc-29459) and control non-silencing siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Dallas, TX). Matrigel, rat tail collagen and transwell inserts were purchased from BD Biosciences (Bedford, MA). DAPI (4’, 6-Diamidine-2-phenylindole dihydrochloride) was purchased from Roche Diagnostics, (Indiana, IN). The antibodies against PTEN, pAKT<sup>Ser473</sup>, AKT (pan), pSmad2, pSmad3, and Smad2/3 were purchased from Cell Signaling Technology (Danvers, MA). Antibody against pPTEN<sup>Ser380</sup> was purchased from Santa Cruz Biotechnology (Dallas, TX). Antibody against β-Actin (clone AC-15) was purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG HRP was obtained from Life Technologies (Grand Island, NY). Anti-mouse IgG HRP was obtained from GE Healthcare (Piscataway, NJ).

3.2 Cell Lines and Cell Culture

Prostate epithelial and prostate cancer cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD), which include RWPE1 (immortalized human prostate epithelial cell line) and prostate cancer cell lines isolated from distinct
metastatic sites from prostate cancer patients such as androgen-dependent cell line LNCaP (derived from a lymph node lesion), and androgen-independent cell lines DU145 (derived from brain) and PC3 (derived from bone) (Table 1).\textsuperscript{191} RWPE1 cells were maintained in keratinocyte serum free medium containing 50µg/ml gentamycin, 0.05mg/ml bovine pituitary extract (BPE), and 5ng/ml epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA). LNCaP cells were maintained in RPMI-1640 medium containing 4mM L-glutamine, and 50µg/ml gentamycin. DU145 and PC3 cells were cultured in Eagles minimum essential medium with Earle’s salts with 0.1 mM of the following amino acid supplements: L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and L-glycine. The medium contained 4 mM L-glutamine, 2.5 g/l NaHCO$_3$, 1.5 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 50 µg/ml gentamycin. MEM and RPMI media (Mediatech, Herndon, VA) were supplemented with 5% fetal bovine serum (HyClone, South Logan, Utah).

3.3 RNA Isolation, cDNA Synthesis, and RT-PCR

Total RNA was isolated from normal prostate and prostate cancer cells using TRIzol (Invitrogen, Carlsbad, CA). The RNA samples were quantified by optical density reading at 260nm as described previously \textsuperscript{192}; OD260/OD280 ratio for RNA samples were between 1.8 and 2.0. Total RNA (2 µg) was reverse transcribed in a 50 µl reaction
Table 1. The Origins of the Cell Lines Used

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RWPE1</td>
<td>epithelial cells from histological normal adult human prostate</td>
</tr>
<tr>
<td>DU145</td>
<td>prostate carcinoma, derived from the brain of a 69-year old Caucasian male</td>
</tr>
<tr>
<td>PC3</td>
<td>prostate adenocarcinoma derived from the bone of a 62-year old Caucasian male</td>
</tr>
<tr>
<td>LNCaP</td>
<td>prostate carcinoma, derived from the left supraclavicular lymph node of a 50-year old Caucasian male</td>
</tr>
</tbody>
</table>

mixture containing 0.5 mM dNTP (Fisher Scientific, Pittsburgh, PA), 0.5 mM dithiothreitol (Bio-Rad, Hercules, CA), 0.5 µg of oligo dT, and 400 U of M-MLV Reverse Transcriptase (Promega, Madison, WI) at 37°C for 1.5 hours. The reaction was terminated by heating the samples at 60°C for 5 min and subsequently cooled to 4°C. Polymerase chain reaction (PCR) was performed to detect mRNA levels of PTEN and L-19. The sequences of primer pairs that were used are as follows: human PTEN (forward 5’AGCTTCTGCCATCTCTCTCC-3’ and reverse 5’AATATTGTTCCTGTATACGCCTTC-3’) and L-19 (forward 5’GAAATCGCCAATGCCAACTC-3’ and reverse 5’TCTTAGACCTGCGAGCCTCA-3’). RT-PCR reactions were performed according to procedures described previously. L-19 (a ribosomal protein) was used as an internal control. The PCR products were visualized on 1.5% agarose gels (Amresco, Solon, OH) stained with ethidium bromide.
3.4 Quantitative RT-PCR (qRT-PCR)

The mRNA levels of PTEN were determined by quantitative RT-PCR. Total RNA isolation and cDNA synthesis of DU145 cells are described previously. The quantitative RT-PCR was performed with iCycler (Bio-Rad, Hercules, CA, USA) in 96-well plates. A SYBER-Green Master Mix (Bio-Rad) was used in a volume of 25µl/well. The following primers were used: human PTEN (forward 5’CGACGGGAAGACAAGTTCAT-3’ and reverse 5’AGGTTCCTCTGGTCTTGGTG-3’) and GAPDH (forward 5’GAAGGTGAAGGTCGGAGTC-3’) and reverse 5’GAAGATGGTGATGGGATTTC-3’). Relative quantification of PTEN mRNA expression was determined using the 2-ΔΔCT method with GAPDH as an internal control.

3.5 Cell Treatments

To determine the effects of TGF-β isoforms on PTEN, pAKT(Ser473), pSmad2, and pSmad3, cells were cultured in 6 well plates at a density of 2 x 10^5 cells/well in culture media with 5%FBS and allowed to attach overnight. Before each experiment, the cells were incubated in serum free or supplement free media for 2 hours, followed by treatment with TGF-β1 or TGF-β3 (5ng/ml) over various time points. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA) containing 20mM Tris-HCL (pH 7.5), 150mM NaCl, 1mM Na2 EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na3VO4, 1µg/ml leptin and 1X protease inhibitor cocktail (Calbiochem, La Jolla, CA). Protein concentrations were determined by the Lowry HS
assay using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA).

3.6 Western Blot Analysis

Cell lysates (35-50μg protein) were subjected to SDS-PAGE in 8% or 10% gels and transferred to PVDF membranes (Millipore, Billerica MA). The membranes were blocked in 5% fat-free skim milk in TBST (50mM Tris, pH 7.5, containing 0.15M NaCl, 0.05% Tween 20) for 1 hour at room temperature. The blots were then incubated overnight at 4°C in TBST containing 5% bovine serum albumin (BSA) with appropriate dilutions of specific primary antibodies (1:1000 dilution for anti-PTEN; anti-pAKT<sup>Ser473</sup>, anti-Total AKT (pan), anti-pPTEN<sup>Ser380</sup>, anti-pSmad2, anti-pSmad3, anti-Total Smad2/3; 1:5000 dilution for anti-β-Actin). After washing, blots were then incubated with anti-rabbit or anti-mouse IgG-HRP (dilution 1:20,000) in blocking buffer (TBST with 1% milk) for 1 hour and washed in TBST for 1 hour. The blots were developed in Millipore Luminata Forte (EMD Millipore, Billerica, MA) for 5 minutes, exposed by using Syngene PXI 6 imaging system (Syngene, Frederick, MD). The density of specific protein bands was determined by ImageJ Software (NIH ver1.48) for normalization of data.

3.7 Transwell Cell Migration Assay

In vitro cell migration assay was performed using a 24-well plate transwell inserts (8 μm) as previously described. Cells were washed with MEM and harvested from cell culture dishes by EDTA-trypsin into 50 ml conical tubes. The cells were centrifuged at 1000 RPM for 3 min at room temperature; the pellets were resuspended in MEM.
supplemented with 0.2% bovine serum albumin (BSA) at a cell density of 3 x 10^5 cells/ml. The outside of the transwell insert membrane was coated with 50 µl total volume. Chemoattractant solutions were made by diluting TGF-β1 and TGF-β3 (5ng/ml), and EGF (10 ng/ml) in MEM for DU145 and PC3 cells, and RPMI for LNCaP cells supplemented with 0.2% BSA. MEM containing 0.2% BSA served as a negative control. EGF was used as a positive control. The results were 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.

### 3.8 Invasion Assay

The invasive properties of DU145 cells were measured using the BD BioCoat Matrigel Invasion inserts. Inserts were coated with 50µl of a 1:4 Matrigel/medium dilution and allowed to solidify at 37°C for 48 hours. Cells were resuspended (3 x 10^4 cells/ml) in MEM with 0.1% FBS and 500µl of cell suspension were added to each insert. Cells were treated with TGF-β1 and TGF-β3 (5ng/ml), or EGF (10ng/ml) and were allowed to invade through the porous membrane coated with Matrigel for 48 hours. Matrigel and non-invading cells were removed via cotton swabs. Invading cells on the membrane were fixed in 3.7% paraformaldehyde and stained using DAPI (Roche Diagnostics, Indiana, IN). Images were taken in five different fields for average number invading cells. The results were 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.
3.9 Cell Proliferation Assay

The cell growth assay was performed by counting the total number of cells. Cells were seeded at a density of $1 \times 10^5$ cells overnight in 6 well plates and treated the next day with TGF-β1 or TGF-β3 (5ng/ml) in culture media containing 1% FBS for specific time points. Cells were then trypsinized and counted using the Cellulometer Vision System (Nexcelom Bioscience LLC, Lawrence, MA).

3.10 Transfection with Specific Plasmids and Small Interfering (si) RNAs

Cells were seeded at a density of $1 \times 10^5$ cells in 6 well plates in 2ml of antibiotic-free normal growth medium supplemented with 5% FBS and incubated overnight at 37ºC. Plasmids (pcDNA3 GFP or pcDNA3 GFP PTEN (PTEN promoter T7 polymerase)) were transfected in PC3 cells using FuGene® HD transfection reagent (Promega, Madison, WI) following manufacturer’s instructions. Small interfering RNA (siRNA) (60nM) for the PTEN or Control siRNA were transfected into DU145 cells using transfection reagent (Santa Cruz Biotechnology, Dallas, TX) following manufacturer’s recommendations. Forty-eight to seventy-two hours after transfection, cells were either treated with TGF-β1 or TGF-β3, or subjected to different functional analyses.

3.11 Statistical Analysis

All experiments were performed at least three times using different cell preparations. Data from representative experiments are presented in the figures. One-way analysis of variance and Duncan’s all pair-wise multiple comparison tests were employed.
to assess the significance of differences among treatment groups. SigmaPlot version 11.0 for Windows (Systat Software, Inc., San Jose, CA) was used for statistical analyses.
CHAPTER 4

RESULTS

4.1 Expression of PTEN in Prostate Cell Lines

Gene expression of PTEN in prostate cells was determined using semiquantitative RT-PCR across four established prostate cell lines (RWPE1, LNCaP, DU145, and PC3). PTEN mRNA was detectable in three of the four cell lines with no detectable expression in PC3 cells (Figure 7).

![Image showing RT-PCR results](image)

**Figure 7.** mRNA expression of PTEN in prostate cell lines. RT-PCR was performed using total RNA from RWPE1, LNCaP, DU145, and PC3 cells to determine mRNA levels of PTEN. L-19 served as a loading control and was used to normalize mRNA levels in all cell line samples. No reverse transcriptase (RT) samples derived from the same RNAs were also included.

To examine the presence of PTEN protein and pAKT\(^{\text{Ser473}}\) in these cell lines, the total cell lysate proteins were analyzed using Western blotting (Figure 8). PTEN protein was detected in RWPE1 and DU145 cells, however, it was absent in LNCaP and PC3 cells (Figure 8A). LNCaP cells, which contain a deactivating frameshift mutation in the
gene encoding PTEN\textsuperscript{197}, and PC3 cells, due to the homologous deletion of both PTEN gene, do not express PTEN protein.\textsuperscript{198} The pAKT\textsuperscript{Ser473} bands were detected in all cell lines with high levels of pAKT\textsuperscript{Ser473} in PC3 and LNCaP cells compared with RWPE1 and DU145 cells (Figure 8B).

![Figure 8](image)

**Figure 8.** Protein levels of PTEN and phosphorylation of AKT in prostate cell lines. Levels of (A) PTEN and (B) pAKT\textsuperscript{Ser473} were determined by Western blot analysis in RWPE1, LNCaP, DU145, and PC3 cells. β-actin served as a loading control.

To determine whether the knockdown of PTEN in DU145 cells increased pAKT\textsuperscript{Ser473} protein levels, we used a specific siRNA to transiently knockdown PTEN expression in DU145 cells (Figure 9). Control siRNA was also transfected to serve as a negative control. Protein levels of PTEN and pAKT\textsuperscript{Ser473} were determined by Western blot analysis, which confirmed that phosphorylation of AKT at Serine 473 levels increased while total PTEN protein levels decreased in comparison with cells transfected with control siRNA (Figure 9).
Figure 9. PTEN knockdown increased protein levels of phosphorylation of AKT in DU145 prostate cancer cells. Western blot analysis determined relative protein levels of PTEN and pAKT$^{\text{Ser473}}$ in DU145 prostate cancer cells after transfection with control siRNA or PTEN siRNA. Total AKT (tAKT) and β-actin were used as loading controls.

4.2 Effects of TGF-β on PTEN Expression in DU145 and RWPE1 Cells

We investigated the effects of TGF-β isoforms on the expression of PTEN in DU145 and RWPE1 cells. DU145 and RWPE1 cells were treated with different concentrations of TGF-β1 and/or TGF-β3 for specific times. As shown in Figure 10A, TGF-β3 had no effect on the mRNA levels of PTEN in DU145 cells, as determined by RT-PCR analysis. We also confirmed the lack of significant TGF-β effects on PTEN mRNA levels by real-time PCR (qRT-PCR) (Figure 10B).
Figure 10. TGF-β isoforms have no effect on PTEN mRNA levels in a dose and time-dependent manner in DU145 cells.
PTEN gene expression in DU145 cells after treatment with TGF-β3 (5ng/ml) at specific time points and different doses of exogenous TGF-β3 for 4 hours for both (A) RT-PCR analyses; L-19 was used as a control, and (B) qRT-PCR analysis; GAPDH was used as a control.

However, as shown in Figure 11A and B, both isoforms caused a dose-dependent increase in PTEN protein levels at specific time points. Treatment with TGF-β3 (5ng/ml) significantly increased PTEN protein levels in DU145 cells at 4 hours (2.7 ± 0.34 fold; $P < 0.05$) and 8 hours (2.0 ± 0.40 fold; $P < 0.05$); and also in RWPE1 cells at 4 hours (2.0 ± 0.14 fold; $P < 0.05$) and 8 hours (1.7 ± 0.31 fold; $P < 0.05$) (Figure 11A). As shown in Figure 11B, TGF-β3 treatments significantly increased PTEN protein levels in a dose-
Figure 11. TGF-β isoforms increase PTEN protein levels in prostate cell lines. (A) Western blot analysis of PTEN protein levels in RWPE1 (upper panel) and DU145 (lower panel) cells after treatment for specific time periods with exogenous TGF-β1 and TGF-β3 (5ng/ml). Each bar represents mean ± SEM (n=3). *Significantly different (P < 0.05) compared with untreated controls. (B) DU145 cells were treated with different doses of TGF-β1 or TGF-β3 for 4 hours. β-actin was used as a loading control. Each bar represents mean ± SEM (n=3). *Significantly different (P < 0.05) compared with untreated controls.

dependent manner in DU145 cells after 4 hours at 1ng/ml (1.7 ± 0.26 fold; P < 0.05), 5ng/ml (2.2 ± 0.12 fold; P < 0.05), and 10ng/ml (2.15 ± 0.103 fold; P < 0.05). As shown in Figure 11A and B, TGF-β1 (5ng/ml) treatment also caused an increase in PTEN protein levels in both DU145 and RWPE1 cells. In DU145 cells, TGF-β1 treatment significantly increased PTEN protein levels at 4 hours (Figure 11A) (1.9 ± 0.10 fold; P < 0.05), and in a dose-dependent manner after 4 hours at 5ng/ml (1.38 ± 0.149 fold; P <
0.05) (Figure 11B), however the increase in PTEN protein levels in TGF-β1 treated RWPE1 cells was not statistically significant (Figure 11A). These results suggest that both TGF-β1 and TGF-β3 increase PTEN protein levels; however TGF-β3 effects on both cell lines are more pronounced.

4.3 TGF-β Regulates PTEN Protein Stability

We have shown in earlier experiments that TGF-β upregulates PTEN protein in DU145 cells. To determine this mechanism, we tested the effects of TGF-β on the stability of PTEN protein. In our preliminary data, we treated DU145 cells with cycloheximide (CHX) (20μg/ml), a protein synthesis inhibitor, for specific time points (Figure 12A). We observed that CHX inhibited PTEN protein levels after 24 hours in DU145 cells (Figure 12A). Next, we pretreated DU145 cells with CHX (20μg/ml) for 1 hour followed by TGF-β1 and TGF-β3 (5ng/ml) treatment for 30 hours. As shown in figure 12B, both TGF-β1 and TGF-β3 treatment increased PTEN protein levels in the presence of CHX in DU145 cells at 30 hours compared to cells treated with CHX only for 30 hours.

Previous studies have shown that PTEN post-translational modification via phosphorylation increases PTEN protein stability.\textsuperscript{199,200} It has been suggested that phosphorylation regulates PTEN activity from a wide variety of upstream signal transduction pathways.\textsuperscript{199,201–203} Previously, TGF-β has been shown to stabilize p21; a cell cycle inhibitor; through phosphorylation.\textsuperscript{204} To determine whether TGF-β regulates PTEN stability via phosphorylation, we treated DU145 cells with TGF-β1 and TGF-β3 (5ng/ml) for specific time points. Relative phosphorylated PTEN (pPTEN) protein levels
were determined by Western blot analysis (Figure 12C). Upregulation of pPTEN was observed after 4 hours of treatment with TGF-β1 and TGF-β3 (5ng/ml) (Figure 12C). These results may suggest that TGF-β mediates the regulation of PTEN protein stability via phosphorylation in DU145 prostate cancer cells.

**Figure 12.** PTEN protein stability in response to TGF-β isoforms. (A) Western blot analysis of PTEN protein levels in DU145 cells after treatment at specific time points with cycloheximide (CHX) (20µg/ml), and β-actin was used as a loading control. (B) DU145 cells were pretreated with CHX (20µg/ml) for 1 hour. Cells were then treated with TGF-β1 or TGF-β3 (5ng/ml) at specific time points to determine PTEN protein levels. β-actin was used as a loading control. Each bar represents mean ± SEM (n=3). Different letters represent significant differences among various groups (P< 0.05). (C) Western blot analysis of phosphorylated PTEN serine 380 (pPTEN<sub>Ser380</sub>) after 4 hour treatment with TGF-β1 or TGF-β3 (5ng/ml). PTEN was used as a loading control. Each bar represents mean ± SEM (n=3). *Significantly different (P < 0.05) compared with untreated controls.
4.4 The Effects of TGF-β Isoforms on the Phosphorylation of Smad2 and Smad3

TGF-β signaling is initiated by binding of the ligand to TGF-βRII that form heterodimers with TGF-βRI leading to Smad2 and Smad3 phosphorylation. Therefore, we examined whether TGF-β effects on PTEN are mediated by this canonical signaling pathway. We determined the effects of TGF-β on the phosphorylation of Smad2 and Smad3 in DU145 cells. Western blot analysis showed that TGF-β isoforms induced both Smad2 and Smad3 phosphorylation in a time-dependent manner (Figure 13).

![Western blot analysis of phosphorylated Smad2 and Smad3](image)

**Figure 13.** TGF-β induced Smad signaling in DU145 prostate cancer cells. Western blot analyses of phosphorylated Smad2 (pSmad2) and Smad3 (pSmad3) in DU145 cells after treatment with TGF-β1 or TGF-β3 (5ng/ml) for 15 and 30 minutes. Total Smad (Smad2/3) and β-actin were used as loading controls.

4.5 TGF-β Inhibits Proliferation in DU145 Prostate Cancer Cells

In previous studies, TGF-β has been shown to exert differential biological effects in different prostate cancer-derived cell lines\(^\text{25,205}\). To confirm these studies, we first
determined the effects of TGF-β1 and TGF-β3 (5ng/ml) on cell proliferation in DU145 prostate cancer cells (Figure 14). TGF-β caused a significant inhibition of cell proliferation in DU145 cells with no differences in the potencies of the two isoforms. Treatment with TGF-β1 resulted in 54% ($P < 0.05$) inhibition and TGF-β3 resulted in 49% ($P < 0.05$) inhibition of proliferation in DU145 cells.

![DU145 Cell Proliferation](image)

**Figure 14.** Effects of TGF-β isoforms on proliferation in DU145 cells. DU145 cells were treated with TGF-β1 or TGF-β3 (5ng/ml) to determine cell proliferation. Each bar represents mean ± SEM (n=3). *Significantly different ($P < 0.05$) when compared with appropriate controls.

**4.6 The Role of PTEN on TGF-β Effects on Proliferation in Prostate Cancer Cells**

The possible role of PTEN in proliferation of prostate cancer cells and its role in the effects of TGF-β on cell proliferation were determined using siRNAs to transiently knockdown PTEN proteins in DU145 cells (Figure 15A). Expression of PTEN protein
was determined by Western blotting analysis, which confirmed reduced levels (~ 68 %) of PTEN protein in comparison with the cells transfected with control siRNA for seventy-two hours (Figure 15A). DU145 cells were treated with TGF-β1 and TGF-β3 (5ng/ml) for 48 hours after transfection with PTEN siRNA or Control siRNA (Figure 15A). As expected, after transfection with Control siRNA, we observed a significant decrease ($P < 0.001$) in proliferation of DU145 cells after treatment with both TGF-β isoforms. On the other hand, knockdown of endogenous PTEN resulted in a significant increase in cell proliferation which was further increased in TGF-β3 (80%; $P < 0.001$) treated DU145 cells (Figure 15A).

**Figure 15.** PTEN is involved in inhibitory effects of TGF-β on cell proliferation in DU145 and PC3 cells. (A) Cell proliferation in DU145 cells after transfection with control siRNA or PTEN siRNA, and (B) PC3 cells after transfection with pcDNA3 GFP empty vector or pcDNA3 GFP PTEN vector, treated with TGF-β1 or TGF-β3 (5ng/ml) for 3 days. Each bar represents mean ± SEM (n=3). Different letters denote significant differences among various groups ($P < 0.05$). Levels of PTEN proteins after transfection with control siRNA or PTEN siRNA in DU145 cells, and empty vector or PTEN vector in PC3 cells were determined by Western blotting analysis (inset).
To further confirm the possible role of PTEN in inhibitory effects of TGF-β, we overexpressed PTEN in PC3 cells which are null for PTEN. PC3 cells were transiently transfected using the plasmid pcDNA3 GFP PTEN or empty vector pcDNA3 GFP and treated with TGF-β1 or TGF-β3 (5ng/ml) to determine the effects on cell proliferation. As shown in Figure 15B, expression of PTEN proteins was determined by Western blotting which confirmed PTEN overexpression in comparison with the cells transfected with the empty vector. TGF-β1 (70%; \( P < 0.05 \)) and TGF-β3 (45%; \( P < 0.05 \)) induced a significant increase in cell proliferation in PC3 cells transfected with the empty vector (Figure 15B). However, proliferation was significantly reduced in PC3 cells overexpressing PTEN protein and TGF-β isoforms had no effects on proliferation in these cells.

4.7 TGF-β Effects on Migration in Prostate Cancer Cell Lines.

TGF-β exerts effects on migration and invasion of specific prostate cancer cells. The effects of TGF-β1 and TGF-β3 on migration in selected prostate cell lines (DU145, PC3, and LNCaP) under identical experimental conditions were determined by using transwell cell migration assay. DU145, PC3, LNCaP cells were treated with either TGF-β isoforms and allowed to migrate according to established procedures. As shown in Figure 16A and Figure 16B, treatment with both isoforms induced cell migration in PC3 cells (TGF-β1; 1.2 ± 0.03 migration index; \( P < 0.05 \)) and TGF-β3; 1.3 ± 0.13 migration index; \( P < 0.05 \), however TGF-β1 did not have any effect on migration in DU145 cells. Interestingly, TGF-β3 treatment (1.7 ± 0.20 migration index; \( P < 0.05 \)) significantly induced migration in DU145 cells (Figure 16A). In addition, we determined TGF-β
effects on migration in LNCaP cells. It has been reported that LNCaP cells lack the TGF-βRII, a necessary receptor for TGF-β signaling. To confirm this notion, LNCaP cells were treated under the same experimental conditions and allowed to migrate according to established procedures. As expected, both TGF-β isoforms had no effect on migration in LNCaP cells (Figure 16C). On the other hand, epidermal growth factor (EGF), used as a positive control, induced cell migration in DU145 (1.7 ± 0.26 migration index; $P < 0.05$), PC3 (1.6 ± 0.07 migration index; $P < 0.05$), and LNCaP (1.6 ± 0.20 migration index; $P < 0.05$) cells (Figure 16A, 16B and 16C).

**Figure 16.** Effects of TGF-β isoforms on cell migration in prostate cancer cells. (A-C) Cell migration of DU145, PC3, and LNCaP cells across transwell membranes were assayed in response to TGF-β1 (5ng/ml), TGF-β3 (5ng/ml), or EGF (10ng/ml) treatments. EGF was used as a positive control. Each bar represents mean ± SEM (n=3). *Significantly different ($P < 0.05$) compared with untreated controls. Representative images of immunofluorescent cells using DAPI to stain the nucleus of the cells. Cells were visualized under 10x objective.
4.8 TGF-β Effects on Invasive Properties in DU145 Prostate Cancer Cells.

Previous studies in our lab have shown that TGF-β isoforms induced invasive behavior in PC3 cells, but not in DU145 cells \(^9,28\). To confirm these studies, we determined the effects of TGF-β isoforms on cell invasion in DU145 cells using the BD BioCoat Matrigel Invasion inserts. As shown in Figure 17, both TGF-β1 and TGF-β3 had no effect on invasiveness in DU145 cells, however, EGF induced invasion (2.9 ± 0.66 invasion index; \( P < 0.05 \)) in DU145 cells. These results suggest that TGF-β isoforms may have differential effects on migration and invasion in prostate cancer cells.

**Figure 17.** TGF-β isoforms have no effect on invasion in DU145 cells. Invasive properties of DU145 cells treated with TGF-β1 (5ng/ml), TGF-β3 (5ng/ml), or EGF (10ng/ml) were determined by an invasion assay. Cells were allowed to invade through a Matrigel-coated porous membrane for 48 hours. Each bar represents mean ± SEM (n=3). *Significantly different (\( P < 0.05 \)) compared with untreated controls.
4.9 The Role of PTEN on TGF-β Effects on Migratory Behavior in Prostate Cancer Cells

To determine whether PTEN plays a role in TGF-β-mediated effects on migration in prostate cancer cells, we performed transient knockdown of PTEN in DU145 cells using siRNA specific for PTEN (Figure 18A). PTEN protein levels were determined by Western blot analysis, which confirmed a 54% reduction (P < 0.05) in DU145 cells transfected with PTEN siRNA compared to control siRNA transfected cells (Figure 18A). We analyzed migration of DU145 cells after knockdown of PTEN (Figure 18A). DU145 cells were treated with TGF-β1 and TGF-β3 (5ng/ml) for 48 hours after transfection with PTEN siRNA. We observed that both TGF-β isoforms induced migration with a significant increase with TGF-β3 (1.6 ± 0.23 fold; P < 0.008) and EGF (1.8 ± 0.07 fold; P < 0.008) (Figure 18A).

Next, we investigated the effects of PTEN expression on TGF-β induced migration in PC3 cells. We transiently overexpressed PTEN in PC3 cells (Figure 18B). Expression of PTEN proteins were determined by Western blot analysis which confirmed overexpression of PTEN in PC3 cells. DU145 served as a positive control for PTEN protein expression (Figure 18B). Effects of TGF-β isoforms on cell migration were significantly reduced in these cells compared with PC3 cells transfected with empty vector (Figure 18B). These results suggest that PTEN overexpression in PC3 cells inhibited TGF-β-mediated effects on migration.
Figure 18. Lack of PTEN induces cell migration in the presence of TGF-β in prostate cancer cells. (A) DU145 cells transfected with control siRNA or PTEN siRNA were treated with TGF-β1 or TGF-β3 (5ng/ml), or EGF (10ng/ml) to determine migratory properties in a transwell cell migration assay. Levels of PTEN proteins after transfection with control siRNA or PTEN siRNA in DU145 cells were determined by Western blotting analysis (inset). Each bar represents mean ± SEM (n=3). *Significantly different (P < 0.05) compared with untreated controls. (B) PC3 cells after transfected with pcDNA3 GFP empty vector or pcDNA3 GFP PTEN vector were treated with TGF-β1 or TGF-β3 (5ng/ml), or EGF (10ng/ml) to determine migration using a transwell migration assay. Each bar represents mean ± SEM (n=4). *Significantly different (P < 0.05) when compared with untreated controls. Levels of PTEN proteins and β-actin (used as a loading control) after transfection with empty vector or PTEN vector, in the presence or absence of TGF-β3 or EGF in PC3 cells were determined by Western blotting analysis (inset). DU145 was used as a positive control.

4.10 The Role of PTEN in TGF-β Effects on the Phosphorylation of AKT in Prostate Cancer Cells

We determined whether or not overexpression of PTEN will affect TGF-β effects on the activation of PI3-kinase in PC3 cells treated with TGF-β3 (5ng/ml) (Figure 19). Western blot analysis revealed that in response to TGF-β3, PTEN overexpressing PC3 cells had significantly lower levels (0.73 ± 0.06 fold; P < 0.05) of AKT phosphorylation compared to those transfected with empty vector (Figure 19). These results suggest that
overexpression of PTEN in PC3 cells attenuates TGF-β effects on the activation of PI3-kinase.

**Figure 19.** PTEN overexpression reduces TGF-β effects on phosphorylation of AKT in PC3 cells. Western blot analysis showing levels of phosphorylation of AKT (pAKT<sup>Ser473</sup>) with or without TGF-β3 (5ng/ml) treatment in PC3 cells after transfection with pcDNA3 GFP empty vector or pcDNA3 GFP PTEN vector. Total AKT (tAKT) was used as a loading control. Each bar represents mean ± SEM (n=4). *Significantly different (P < 0.05) when compared with untreated controls.
CHAPTER 5

DISCUSSION

In this study, we investigated the role of PTEN in TGF-β effects on proliferation and migration of prostate epithelial cancer cells. We report that TGF-β has differential effects on PTEN protein and RNA levels in prostate cancer cells. TGF-β increases PTEN protein levels; however, TGF-β has no effect on mRNA levels in DU145 prostate cancer cells. We also show that PTEN may mediate anti-proliferative effects of TGF-β in prostate epithelial cells. Our results also revealed that lack of PTEN may enhance TGF-β effects on cell migration in prostate cancer cells.

Prostate cancer occurs and progresses as a result of accumulated genomic mutations that lead to unchecked cellular growth and survival of the mutated and dividing cells. The principle problem arising from prostate cancer and its high rate of mortality is due to metastasis of the primary tumor to secondary sites. Cellular migration and invasion play fundamental roles in cancer metastasis. Among multiple growth factors, TGF-β has been implicated in the regulation of prostate cancer cell proliferation, progression and/or metastasis. TGF-β is a multiple functional protein that acts as a tumor suppressor in normal epithelial cells and in early stages of epithelial cancers by inhibiting proliferation and inducing apoptosis. However, in later stages of cancer, cells become resistant to growth inhibitory effects of TGF-β, in which TGF-β acts as a tumor promoter due to its role in epithelial to mesenchymal transformation (EMT) or promoting the
degradation of ECM; all of which aid invasion and metastasis. In addition, previous studies also show that TGF-β effects on migration and invasion of metastatic prostate cancer cells are dependent on activation of the PI3-kinase pathway by TGF-β, as shown by increased phosphorylation of AKT. However, the intracellular mechanisms involved in TGF-β activation of PI3-kinase pathway are largely unknown. The PI3-kinase pathway mediates cellular processes such as proliferation, cell survival, and migration, and is negatively regulated by PTEN which inhibits those cellular functions. Therefore, PTEN may play a role in differential effects of TGF-β on proliferation, and migratory behavior by antagonizing PI3-kinase activity in prostate cancer cells.

PTEN mutations and deletions in prostate cancer cells lead to the loss of PTEN and constitutive activation of the PI3-kinase/AKT pathway. Several prostate cancer-derived cell lines have also been shown to lack expression of PTEN such as a homozygous deletion in PC3 cells and frameshift mutation in LNCaP cells. Other cell lines derived from normal or cancer cells such as RWPE-1 and DU145 cells maintain normal expression of PTEN. These cell lines provide convenient model systems to study the role of PTEN and PI3-kinase pathway in prostate cancer cell proliferation, migration, and invasion. Our results showed higher constitutive levels of pAKT<sup>Ser473</sup> in LNCaP and PC3 cells as a consequence of PTEN loss, but not in RWPE1 and DU145 cells. On the other hand, knockdown of endogenous PTEN by specific siRNA in DU145 and RWPE1 cells resulted in increased levels of pAKT<sup>Ser473</sup>, indicating enhanced activation of PI3-kinase pathway.
Earlier studies have addressed the interaction of the PI3-kinase/PTEN pathway and TGF-β signaling pathways in various cell types. Previous reports have shown that TGF-β1 downregulates PTEN expression in keratinocytes\textsuperscript{212} and causes a reduction of PTEN mRNA levels in pancreatic cancer cells.\textsuperscript{213} Moreover, inactivation of TGF-β signaling and loss of PTEN cooperate to induce colorectal cancer formation and progression by suppressing cell cycle inhibitors.\textsuperscript{214} Although it is known that loss of PTEN expression seems to be frequent in many human tumors, the expression of PTEN has been associated with inhibitory effects on proliferation, migration, and induction of apoptosis \textit{in vitro} and \textit{in vivo}.\textsuperscript{213,215,216} In the current study, we studied the effects of TGF-β1 and TGF–β3 on PTEN expression in DU145 and RWPE1 cells. Our results show that while TGF-β isoforms had no effect on PTEN mRNA levels; they induced an increase in PTEN protein in both DU145 and RWPE1 cells. TGF-β induced increase in PTEN protein levels may suggest that the level of PTEN may be regulated by TGF-β via other mechanisms such as protein stability and protein degradation. It has been reported previously that PTEN activity may be highly regulated on many levels by post-transcriptional and posttranslational mechanisms.\textsuperscript{186} Previous groups have demonstrated that the C-terminal end of PTEN and PTEN posttranslational modification via phosphorylation upregulates PTEN protein stability.\textsuperscript{199,200} Post-transcriptionally, PTEN can be modified as a result of phosphorylation by casein kinase 2 (CK2) and has been previously demonstrated that PTEN protein phosphorylation by CK2 was important for proteasome-mediated PTEN protein degradation.\textsuperscript{199} Other studies have shown that compared to phosphorylated PTEN, dephosphorylated PTEN has decreased stability.\textsuperscript{199–201,217} While it has been
suggested that phosphorylation regulates PTEN activity from a wide variety of upstream signal transduction pathways. Previous studies confirmed that TGF-β can regulate protein stability via posttranslational modifications. One study revealed that TGF-β increases nuclear p27 levels by preventing proteasomal degradation, specifically by downregulating ubiquitin E3 ligase subunits in endometrial carcinoma cells. Our results show that both TGF-β1 and TGF-β3 upregulated PTEN phosphorylation in DU145 cells. These results indicate that TGF-β may mediate the phosphorylation of PTEN regulating its stability by inhibiting the proteasomal degradation pathway in prostate cancer cells.

Our results suggest that increased PTEN protein levels in DU145 cells and RWPE1 cells, in response to TGF-β, may lead to a reduction in cell proliferation in these cells. Our laboratory has previously shown that TGF-β inhibits cell proliferation in RWPE1 and DU145 cells, whereas PC3 (PTEN null) prostate cancer cells are resistant to these growth inhibitory effects. Our results show that PTEN knockdown by specific siRNA resulted in a significant increase in cell proliferation in the presence of TGF-β1 and TGF-β3 isoforms. These results indicate that reduced PTEN protein levels in DU145 cells enhanced TGF-β effects and that PTEN may be required for inhibitory effects of TGF-β on cell proliferation in these cells. The possible role of PTEN in cell proliferation was also confirmed by our experiments where we transiently overexpressed PTEN in PC3 cells, which led to inhibition of cell proliferation. Our results are very similar to recently reported findings indicating that the overexpression of PTEN in PC3 cells inhibited cell
proliferation, and decreased IGF-I-induced phosphorylation of IRS-1, the downstream substrate of IGF-IR. 221

Previous studies have shown that TGF-β1 and TGF-β3 exert differential effects on migration and invasion in prostate cancer cells.9,25 It has been indicated that TGF-β3 exerts more potent effects on migratory and invasive behavior. Although both TGF-β isoforms induced migratory and invasive behavior in PC3 cells, 9,25 both isoforms had no effects on migratory behavior in DU145 cells 9. In another study, TGF-β1 induced cell migration and invasion in PC3 cells but not in DU145 cells using the transwell insert cell migration assay.25,28 TGF-β3 was not investigated in these studies. Interestingly, our results show that while TGF-β1 had no effects on migration in DU145 cells, TGF-β3 significantly induced migration in both DU145 and PC3 cells. Interestingly, TGF-β3 had no effects on invasive behavior in DU145 cells. These results may suggest that TGF-β3 is significantly more potent than TGF-β1 and exert differential effects in prostate cancer cells. These observed effects in both DU145 and PC3 cells are specific to TGF-β, since LNCaP cells (used as negative controls due to lack of TGF-βRII) were unresponsive to both isoforms of TGF-β. 9,222 Similar differential role of TGF-β isoforms is also supported in breast carcinomas, where high expression of TGF-β3 correlates with decreased overall survival rate. 146,147 It has been confirmed that the isoform-specific roles of TGF-β on cell migration and invasion are mediated by TGF-βRI and Smad3 dependent activation of PI3-kinase pathway in metastatic prostate cancer cells. 9 Previous studies have implicated a role for PTEN in repressing the pro-tumorigenic effects of TGF-β; reconstitution of PTEN expression in PTEN-null cells blocked TGF-β-induced
invasion, but did not modulate TGF-β-mediated growth regulation. \(^{50}\) Similarly, in keeping with these studies, our results show that in the presence of TGF-β, knockdown of PTEN enhanced migration in DU145 cells, whereas PTEN overexpression reduced migratory behavior in PC3 cells in response to TGF-β. These results indicate that reduced PTEN protein levels in DU145 cells enhance TGF-β effects on migration, and that PTEN may play a role in TGF-β mediated effects in regulating tumor cell metastasis. In PC3 cells, overexpression of PTEN has been shown to be associated with a decrease in the levels of pAKT\(^{\text{Ser473}}\). \(^{221}\) Previously, it has been shown that TGF-β can activate PI3-kinase pathway in prostate cancer cells as shown by increased phosphorylation of AKT, and activation of this pathway is required for its effects on increased migration and invasion of these cells. \(^{29}\) Here, we show that overexpression of PTEN in PC3 cells reduced the phosphorylation of AKT and invasive behavior in response to TGF-β3. These results suggest that PTEN inhibits PI3-kinase-dependent AKT phosphorylation, and that the ability of TGF-β to activate PI3-kinase signaling may be inhibited in the presence of PTEN.
CHAPTER 6

CONCLUSION

Our results demonstrate that TGF-β increases protein levels of PTEN in prostate cancer cells; however, TGF-β has no effect on mRNA levels of PTEN in these cells. In addition, TGF-β upregulated the phosphorylation of PTEN in prostate cancer cells. This suggests that TGF-β mediates the regulation of PTEN protein stability via phosphorylation in these cells. We also show that lack of PTEN led to increased TGF-β effects on migration in prostate cancer cells, whereas overexpression of PTEN, in PTEN null prostate cancer cells, inhibited TGF-β effects on cell migration. In addition, overexpression of PTEN in these cells decreased TGF-β-induced phosphorylation of AKT, as a result of inhibition of the PI3-kinase/AKT signaling pathway. Furthermore, increased proliferation and enhanced TGF-β effects on cell migration and invasion after knockdown of endogenous PTEN could explain the upregulation of TGF-β signaling and increased tumor growth and metastatic behavior in prostate cancer cells that have deletions or inactivating mutations of PTEN.
REFERENCES


3. Society, A. C. American Cancer Society


191. Steinmetz, N. F.; Maurer, J.; Sheng, H.; Bensussan, A.; Maricic, I.; Kumar, V.; Braciak, T. A. Two Domains of Vimentin Are Expressed on the Surface of Lymph Node, Bone and Brain Metastatic Prostate Cancer Lines along with the Putative Stem Cell Marker Proteins CD44 and CD133. *Cancers (Basel)* 2011, 3, 2870–2885.


212. Li, D. M.; Sun, H. TEP1, Encoded by a Candidate Tumor Suppressor Locus, Is a Novel Protein Tyrosine Phosphatase Regulated by Transforming Growth Factor Beta. *Cancer Res* 1997, 57, 2124–2129.


