Expression of TGFB isoforms and their effects on migratory and invasive behavior of prostate cancer cells: involvement of PI3-kinase/AKT Signaling pathway

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ABSTRACT

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EXPRESSION OF TGFβ ISOFORMS AND THEIR EFFECTS ON MIGRATORY AND INVASIVE BEHAVIOR OF PROSTATE CANCER CELLS: INVOLVEMENT OF PI3-KINASE/AKT SIGNALING PATHWAY

Committee Chair: Shafiq Khan, Ph.D.

Dissertation dated December 2012

Transforming growth factor-β (TGFβ) is a secreted protein that is involved in the regulation of many cellular processes and has been implicated as a factor in cancer cell invasion and metastasis. Studies have indicated that different TGFβ isoforms may exert differential effects on cancer cells during different stages of the disease, however very little is known about the expression patterns of the 3 isoforms in prostate cancer. Non-traditional signaling pathways including PI3-kinase have been associated with TGFβ-mediated effects on cancer cell invasion and metastasis. Whether or not TGFβ isoforms play a differential role in migration and invasion of prostate cancer, and act through PI3-Kinase, has not been investigated. In the present study, we have carried out expression analysis of TGFβ isoforms and signaling components in cell line models representing different stages of prostate cancer and studied the differential effects of specific isoforms on migratory, invasive behavior and induction of the PI3-Kinase and MAP-Kinase/ERK
pathways. TGFβ1 and TGFβ3 were expressed in all prostate cell lines, with TGFβ3 increasing in metastatic DU145, PC3 and PC3M cell lines. TGFβ1 and TGFβ3 induced motility and invasive behavior in PC3 cells, with TGFβ3 being more potent in inducing invasive behavior. TGFβ3 caused a significant increase in the phosphorylation of AKT (pAKT\(^{473}\)), a downstream target of PI3-Kinase, in PC3 cells. LY294002, a PI3-kinase inhibitor, blocked this induced migration and phosphorylation of AKT. Inhibitors of TGFβRI (SB431524) and Smad3 (SIS3) blocked TGFβ isoform induced motility and TGFβ isoform induced pAKT\(^{473}\). There was no differential isoform effect on the phosphorylation of ERK (pERK). PD98059, a MEK inhibitor of MAP-Kinase/ERK, did inhibit TGFβ isoform induced migration and pERK, but did not affect isoform induced pAKT\(^{473}\). Furthermore, TGFβ isoforms phosphorylate both Smad2 and Smad3 in a similar manner in PC3 cells. Based on these results, we conclude that TGFβ3 is expressed in metastatic prostate cancer cell lines and is involved in induction of invasive behavior in these cells. Furthermore, these effects of TGFβ3 are mediated via the PI3-Kinase pathway and are TGFβRI and Smad3 dependent.
EXPRESSION OF TGFβ ISOFORMS AND ITS EFFECTS ON MIGRATORY AND INVASIVE BEHAVIOR OF PROSTATE CANCER CELLS: INVOLVEMENT OF PI3-KINASE/AKT SIGNALING PATHWAY

A DISSERTATION
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
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THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
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DEPARTMENT OF BIOLOGICAL SCIENCES
ATLANTA, GEORGIA

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.................................................................ii
TABLE OF CONTENTS.....................................................................iii
LIST OF FIGURES........................................................................vi
LIST OF TABLES...........................................................................x
LIST OF ABBREVIATIONS............................................................xi

CHAPTER

1. INTRODUCTION........................................................................1
2. REVIEW OF LITERATURE...........................................................5
   2.1 The Prostate .......................................................................5
   2.2 Prostate Cancer.................................................................6
   2.3 Metastasis.........................................................................7
   2.4 Transforming Growth Factor- β.............................................8
      2.4.1 TGFβ Isoforms..............................................................9
      2.4.2 Signaling Pathway.........................................................10
      2.4.3 TGFβ isoforms in normal development.........................11
      2.4.4 TGFβ isoforms in cancer .............................................12
   2.5 The Phosphatidylinositol 3-kinase (PI3-Kinase) pathway...........13
   2.6 TGFβ and the PI3-Kinase Pathway: A link?.............................15
   2.7 The Mitogen-Activated Protein Kinase (MAPK) Pathways........16
3. MATERIALS AND METHODS.....................................................18
4. RESULTS..................................................................................24
4.1 Gene Expression of TGFβ isoforms, Receptors and Smad proteins in prostate cell lines .................................................. 24
4.2 Protein Expression of TGFβ isoforms and TGFβRII in prostate cell lines ................................................................. 25
4.3 Effects of TGFβ isoforms on proliferation of Mink Lung Cells ...................................................................................... 25
4.4 Effects of TGFβ isoforms on proliferation of prostate cell lines .................................................................................. 26
4.5 Differential effects of TGFβ isoforms on migration of prostate cancer cells ................................................................. 26
4.6 Differential effects of TGFβ isoforms on invasion of prostate cancer cells ................................................................. 27
4.7 The role of TGFβRI and Smad3 on TGFβ isoform induced migration and invasion ..................................................... 27
4.8 Differential effects of TGFβ isoforms on migration are mediated by PI3-Kinase pathway .............................................. 28
4.9 Differential effects of TGFβ isoforms on invasion are mediated by PI3-Kinase pathway .................................................. 29
4.10 Differential effects of TGFβ isoforms on activation of PI3-Kinase pathway ................................................................. 29
4.11 The role of TGFβRI, Smad3 and PI3-Kinase on TGFβ isoform-induced phosphorylation of AKT473 .......................... 31
4.12 The role of TGFβRI, Smad3 and PI3-Kinase on TGFβ isoform-induced phosphorylation of Smad2 and Smad3 ..........................................................31

4.13 The effects of TGFβ isoforms on the activation of the Mitogen-Activated Protein Kinase extracellular signal-regulated kinases 1/2 (ERK1/2) ........32

4.14 The role of the Mitogen-Activated Protein Kinase /ERK pathway in TGFβ isoform-induced migration .........................................................32

5. DISCUSSION ..................................................................................34

6. CONCLUSION ..................................................................................42

7. APPENDIX .....................................................................................43

8. REFERENCES ..................................................................................88
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2012 Estimates for Leading Cancer Cases and Deaths in the United States</td>
<td>46</td>
</tr>
<tr>
<td>2. The Metastatic Cascade</td>
<td>47</td>
</tr>
<tr>
<td>3. Amino acid sequence alignments of human TGFβ isoforms</td>
<td>48</td>
</tr>
<tr>
<td>4. The Transforming Growth Factor Signaling Pathway</td>
<td>49</td>
</tr>
<tr>
<td>5. The PI3-Kinase Pathway</td>
<td>50</td>
</tr>
<tr>
<td>6. The Mitogen-Activated Protein Kinase (MAPK) Cascades</td>
<td>51</td>
</tr>
<tr>
<td>7. mRNA expression of TGFβ isoforms and signaling components in prostate cell lines</td>
<td>54</td>
</tr>
<tr>
<td>8. Protein expression of TGFβ isoforms in prostate cell lines</td>
<td>55</td>
</tr>
<tr>
<td>9. Protein Expression of the TGFβRII in Prostate Cancer Cell lines</td>
<td>56</td>
</tr>
<tr>
<td>10. Mink Lung Cell (MV1Lu) TGFβ Bioactivity Assay</td>
<td>57</td>
</tr>
<tr>
<td>11. Dose dependent effects of TGFβ1 and TGFβ3 on proliferation of PZ-HPV7 prostate epithelial cells</td>
<td>58</td>
</tr>
<tr>
<td>12. Dose dependent effects of TGFβ1 and TGFβ3 on proliferation of DU145 prostate cancer cells</td>
<td>59</td>
</tr>
<tr>
<td>13. Dose dependent effects of TGFβ1 and TGFβ3 on proliferation of PC3 prostate cancer cells</td>
<td>60</td>
</tr>
</tbody>
</table>
14. Effects of TGFβ1 and TGFβ3 on migration of PC3 prostate cancer cells

15. Effects of TGFβ1 and TGFβ3 on migration of DU145 prostate cancer cells

16. Effects of TGFβ1 and TGFβ3 on migration of LNCaP prostate cancer cells

17. The invasive properties of PC3 cells

18. Number of invading PC3 cells through Matrigel after treatment with TGFβ1 or TGFβ3 for 48h

19. The invasive properties of DU145 cells

20. The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on migration of PC3 cells in the presence of inhibitor of TGFβRI (SB431542)

21. The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on migration of PC3 cells in the presence of inhibitor of Smad3 (SIS3)

22. The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on invasion of PC3 cells in the presence of inhibitor of TGF PI3-kinase (LY294002)

23. Number of migrating PC3 cells after various treatments

24. The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on invasion of PC3 cells in the presence of inhibitor of TGFβRI (SB431542)

25. The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on invasion of PC3 cells in the presence of inhibitor of Smad3 (SIS3)
26. The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on invasion of PC3 cells in the presence of inhibitor of TGF β1-kinase (LY294002). .................. 73

27. Number of invading PC3 cells after various treatments ........................................... 74

28. Comparative expression of phosphorylation of AKT \(^{\text{ser473}}\) in prostate cell lines .............................................................................................................. 75

29. Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \(^{\text{ser473}}\) in PC3 .................................................................................................................. 76

30. Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \(^{\text{ser473}}\) in PC3 cells after treatment with the two isoforms for 15 and 60 min................... 77

31. Band density analysis of pAKT \(^{\text{ser473}}\) in PC3 cells after treatment with TGFβ isoforms for 15 or 60 min................................................................. 78

32. Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \(^{\text{ser473}}\) in DU145 cells after treatment with the two isoforms for 15 and 60 min ............... 79

33. Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \(^{\text{ser473}}\) in LNCaP cells after treatment with the two isoforms for 15 and 60 min......... 80

34. Effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \(^{\text{ser473}}\) in PC3 cells in the presence of inhibitor of TGFβRI (SB431542) ....................................................... 81

35. Effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \(^{\text{ser473}}\) in PC3 cells in the presence of inhibitor of Smad3 (SIS3) ................................................................. 82

36. Effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \(^{\text{ser473}}\) in PC3 cells in the presence of inhibitor PI3-Kinase inhibitor (LY294002) ......................... 83

viii
37. Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells

38. Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells in the presence of inhibitor of TGFβRI (SB431542)

39. Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells in the presence of inhibitor of Smad3 (SIS3)

40. Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells in the presence of inhibitor of PI3-Kinase inhibitor (LY294002)

41. The effects of TGFβ1 or TGFβ3 on the phosphorylation of ERK, a downstream target of the Mitogen-Activated Protein Kinase Pathway

42. The effects of MAPK inhibitor PD98059 on the TGFβ1 or TGFβ3 induced phosphorylation of AKT$^{ser473}$ in PC3 cells

43. The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on migration of PC3 cells in the presence of inhibitor of MAPK (PD98059)
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The Origins of the Cell Lines Used</td>
<td>52</td>
</tr>
<tr>
<td>2.</td>
<td>Gene-Specific Primers Used for RT-PCR Amplification</td>
<td>53</td>
</tr>
</tbody>
</table>
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTRII/IIB</td>
<td>Activin Receptor-2/2B</td>
</tr>
<tr>
<td>AKT (PAN)</td>
<td>Total AKT</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>AMHRII</td>
<td>Anti-müllerian hormone receptor 2</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>BMPRII</td>
<td>Bone Morphogenic Protein Receptor 2</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostate Hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor I</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N terminal Kinase</td>
</tr>
<tr>
<td>KSFM</td>
<td>Keratinocyte Serum Free Medium</td>
</tr>
<tr>
<td>L-TGFβ</td>
<td>Latent Transforming Growth Factor- β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPKinase/ERK Kinase 1</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear Factor-KappaB</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>pAKT</td>
<td>Phosphorylated AKT</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pERK</td>
<td>Phosphorylated ERK</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>pSmad</td>
<td>Phosphorylated Smad</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDK1/2</td>
<td>Phosphoinositol-dependant kinase 1/2</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>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</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>TGFβ</td>
<td>Transforming Growth Factor- β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>THR</td>
<td>Threonine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked Inhibitor of Apoptosis</td>
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</tbody>
</table>
Prostate cancer is the most commonly diagnosed cancer in men, with the chances of diagnosis rapidly increasing after the age of 50.\textsuperscript{1} It is estimated that in 2012, roughly 242,000 new cases will be diagnosed, and nearly 28,000 deaths will occur due to prostate cancer (Figure 1).\textsuperscript{2} Though prostate cancer is known to grow at a slow rate, risk factors such as diet and late detection can lead to increased fatality rates.\textsuperscript{1} Men diagnosed with low-grade, localized and regional prostate cancers have a 100% 5-year survival rate, however, men with late-stage, metastatic prostate cancers that have spread to distant areas such as lymph nodes, bones and other organs only have a 31% 5-year survival rate.\textsuperscript{3} This dramatic decrease in survival due to metastasis is cause for increased research in the area of invasion and metastasis.

Transforming growth factor-β (TGFβ) is a secreted cytokine involved in the regulation of many cellular processes and has been implicated as a factor in cancer formation and progression leading to increased migration and invasion.\textsuperscript{4,5} TGFβ exerts pleiotropic effects on numerous cellular functions that include differentiation, proliferation, motility and extracellular matrix (ECM) production in many normal cell types.\textsuperscript{6,7} In normal epithelial cells and early stage cancers, TGFβ inhibits cell proliferation and acts as a tumor-suppressor.\textsuperscript{4,8,9} However, in later stages of many cancers, the tumor cells become resistant to growth inhibitory effects of TGFβ. In these cells, TGFβ can act as a tumor promoter due to its role in stimulating angiogenesis, epithelial to mesenchymal
transformation (EMT), or promoting the degradation of ECM; all of which aid in invasion and metastasis.\textsuperscript{5,10,11}

The three TGFβ isoforms, TGFβ1, TGFβ2 and TGFβ3, share a 70-80\% sequence homology in most organisms and are expressed throughout most mammalian tissues.\textsuperscript{6,7} Of the three isoforms, TGFβ1 is the most commonly studied and tends to be ubiquitously expressed among cell lines and in diseased tissues such as cancer.\textsuperscript{13,14} In numerous in vitro studies, all three isoforms bind to the same receptors and exert similar biological effects on target cells.\textsuperscript{15} However, TGFβ isoforms can have non-redundant specific effects during development as indicated by gene knock-out studies.\textsuperscript{16-19} Binding affinity studies show that the three isoforms signal by binding to TGFβRI and TGFβRII.\textsuperscript{20} However, TGFβ2 differs from TGFβ1 and TGFβ3 in that it binds to TGFβRII with an affinity that is 100-1000 fold lower and requires TGFβRIII (β-glycan) to promote receptor assembly with TGFβ2.\textsuperscript{12,21-25} Additionally, crystal structure of TGFβRII-TGFβ3 complex showed that interfacial residues of TGFβ3 are identical to TGFβ1 but are different from TGFβ2 at three positions.\textsuperscript{26} These studies suggest that although both TGFβ1 and TGFβ3 share a significant homology, receptor binding affinity and can exert similar biological effects on target cells, they may retain the ability to have non-redundant differential effects.

Earlier studies have indicated that individual TGFβ isoforms may exert differential effects on cancer cells during different stages of the disease. In one such study, TGFβ3 increased the invasiveness of endometrial carcinoma cells via a PI3-Kinase-dependent pathway, and these effects were distinct from those of TGFβ1.\textsuperscript{27} In prostate cancer, there is very little known about the expression levels of the three
isoforms, specifically TGFβ3, in prostate cancer cell lines and clinical tissue samples. Evidence indicates that TGFβ3 expression increases 2 fold in prostate tumors and that the TGFβ3 gene is over-expressed in an androgen-independent derivative of prostate cancer cell line compared to the androgen-dependent parental cell line. \(^{28}\)

Recent studies have suggested the involvement of the phosphatidylinositol 3-kinase (PI3-Kinase) pathway in TGFβ-mediated effects on cancer cell invasion and metastasis. PI3-Kinase has been shown to play an integral role in many cellular processes including cell proliferation, growth and survival in both normal and diseased tissues. \(^{29,30}\) Activating mutations of the PI3-Kinase pathway and loss of PTEN are common in many types of cancers and are associated with tumor progression. \(^{31}\) It has been shown that TGFβ can activate PI3-Kinase, as determined by increased phosphorylation of AKT, a downstream target of PI3-Kinase. \(^{32-36}\) Prior studies have also revealed that TGFβ isoform-specific effects on the PI3-Kinase pathway can be either dependent or independent of Smad signaling depending on the experimental conditions and cellular context. \(^{27,35,36}\) The PI3-Kinase pathway has also been implicated as a contributing pathway to TGFβ induced EMT as well as fibroblast proliferation and morphological transformation, all precursors to invasion and metastasis. \(^{36}\) Whether or not TGFβ isoforms play a differential role in invasion and metastasis of prostate cancer, and act through non-Smad pathways such as PI3-Kinase, is still unclear.

Therefore, it is important to determine what differential effect, if any, TGFβ isoforms have on the induction of the PI3-Kinase pathway and their role in invasion and metastasis in prostate cancer.

Preliminary results from our laboratory led us to the hypothesis that the TGFβ3
isoform may play a more significant role in the invasive behavior of late stage prostate cancer epithelial cells. Furthermore, the action of TGFβ3 may also have a significantly higher effect on the phosphorylation of AKT and be mediated via the PI3-Kinase Pathway. To test this hypothesis, we asked at the following questions:

1. Do the TGFβ isoforms have a differential effect on the migratory and invasive properties of different prostate cancer cells?

2. Does this differential effect of the TGFβ isoforms use the canonical TGFβ pathway and/or other pathways involved in invasion and metastasis of cells including the PI3-kinase/AKT pathway?
CHAPTER 2

REVIEW OF THE LITERATURE

2.1 The Prostate

The prostate is a male accessory reproductive exocrine gland whose function is the secretion of a slightly acidic seminal fluid that aids in the production of semen.\textsuperscript{37-39} This gland is described as having three distinct areas categorized as the peripheral, central and transition zones, of which the peripheral area contains the majority of epithelial cells and where most prostate carcinomas develop.\textsuperscript{37,40} The prostate consists of two cell types, stromal and epithelial. The stroma consists of mostly smooth muscle cells which are involved in the production of growth factors, whereas epithelial cells contain luminal secretory, basal, and neuroendocrine cells, all of which aid in the function of the prostate.\textsuperscript{37,41} The development of this male reproductive organ is dependent upon the expression and production of growth factors, receptors and androgens, including testosterone during fetal development. Androgens are essential for the growth and function of the prostate gland during development and have been shown to regulate growth factor gene expression in the prostate.\textsuperscript{42-45} Multiple growth factors produced in response to androgens including epidermal growth factor (EGF) and insulin-like growth factors (IGF) can help stimulate prostatic epithelial growth.\textsuperscript{46-49} Transforming growth factor-\(\beta\) (TGF\(\beta\)), on the other hand, exhibits an inhibitory role on the prostatic stromal and epithelial cell growth in multiple prostate samples.\textsuperscript{47,50-53} One study postulates that TGF\(\beta\) plays an important role in the stromal-epithelial interactions during prostate development based on the
differential expression of two TGFβ isoforms in rat ventral prostate and that testosterone does not affect this expression. The exact mechanism by which TGFβ isoforms affect prostate development and their hormonal regulation, however, remains unclear.

2.2 Prostate Cancer

Prostate cancer transpires and progresses as the result of accumulated genomic mutations that led to unchecked cellular growth and survival advantage of the mutated and dividing cells. The incidence of prostate cancer has been on a steady increase in the United States with age, ethnicity and a positive family history of the disease being the most common risk factors. Though many types of cancer grow and spread rapidly, prostate cancer is known for its slow progression and occurs at a higher incidence rate among men over the age of 50. More recently, prostate cancer has been shown to be a racial disparity in that African American men have the highest rate of incidence of any population in the world, with an incidence rate of 231.9 to 146.3 versus Caucasians per 100,000 individuals and a mortality rate of 56.3 to 23.6 versus Caucasians per 100,000 individuals. This suggests that prostate cancer is heavily determined not just by age, but by genetic, environmental and possibly socio-economic factors as well.

Most prostate cancers arise from multiple genetic shifts such as suppression or promotion within genetic polymorphisms, tumor suppressor/promoter genes, growth factor production and receptor signaling. Of these conventional tumor suppressor genes, a mutated PTEN, a negative regulator of the PI3-Kinase pathway, has been shown to be involved in the progression of prostate cancer. Multiple growth factors including IGF-1, Interleukins and TGFβ have been implicated in the regulation of prostate cancer cell proliferation, progression and/or metastasis as well.
2.3 Metastasis

Metastasis of a primary tumor is the spread of cancerous cells from one part of the body to another, leading to secondary tumor formation and growth. Frequently, secondary tumor sites can occur in vital organs or systems, with some of the most common cancer secondary sites being lung, brain, liver, and bone, eventually leading to increased medical complications and decreased survival rate. Prostate cancer, however, almost exclusively metastasizes to the bone. Metastasis, being the final stage in tumor evolution, is thought to be the cause for the bulk of cancer deaths that are associated with primary tumors.

During cancer progression, there are several general mechanisms that occur during the metastatic cascade including tumor formation and angiogenesis, cell attachment, invasion and growth of the metastases. During this process, a single cell can become genetically mutated in which cellular responses ordered to correct these mutations are no longer capable of doing so. When these mutated cells are not corrected, they can proliferate, forming a possible carcinoma. Once the carcinoma, or primary tumor, has gained the size and necessary components, it begins to degrade the basement membrane or extracellular matrix of the surrounding tissues and invades into other nearby compartments. If the carcinoma becomes malignant, cells break free from the primary tumor, enter the blood stream and disseminate throughout the body (Figure 2). This leads to extravasation to the lymph nodes, organs and/or bone, increasing the rate of poor prognosis and mortality.

Many different molecular barriers occur between a newly mutated and migratory tumor cell and its distant new home in other vital organs and tissues. This invasive
difficulty is mirrored by the fact that less than 0.05% of circulating tumor cells are actually able to become stable secondary site metastases. Each of these hurdles involved in metastasis requires numerous specific cell signaling and molecular interactions by the cancer cell itself, the surrounding extracellular matrix (ECM) and stromal cells. This can occur via cell-ECM contact, cell-to-cell contact or by secreted factors such as matrix metalloproteinases (MMPs) and transforming growth factor-β.

2.4 Transforming Growth Factor-β

One cytokine that has been the focus of cancer metastasis studies is transforming growth factor-β (TGFβ). Originally discovered in the conditioned media of sarcoma virus transformed mouse fibroblasts, TGFβ has been shown to be a secreted protein that is involved in the regulation of many cellular processes and has been implicated as a factor in cancer formation and progression. It is part of the TGFβ super family which includes TGFβ ligands, bone morphogenic proteins (BMP’s), activins and nodals. The TGF-β ligands are pleiotropic cytokines that play an integral role in numerous cellular functions that include differentiation, proliferation, motility and extracellular matrix (ECM) production in many normal cell types and can act as a potent anti-tumor agent. However, in diseases such as cancer, TGFβ can also act as a tumor promoter by its role in stimulating angiogenesis, epithelial to mesenchymal transformation (EMT), or promoting the degradation of ECM; all of which aid in invasion and metastasis. The exact mechanisms by which TGFβ switches from tumor suppressing to tumor promoting cytokine are currently under investigation.
2.4.1 TGFβ Isoforms

As seen in many gene arrays and oncomine data sets, the TGFβ isoforms, TGFβ1, TGFβ2 and TGFβ3, are expressed throughout many mammalian tissues. All three of these cytokines are secreted in the ECM in a latent form (L-TGFβ) and become biologically active when cleavage or conformational change occurs by acidic microenvironment and factors such as plasmin and matrix metalloproteinases (MMPs). Of the three isoforms, TGFβ1 is the most commonly studied and tends to be ubiquitously expressed among cell lines and in diseased tissues such as cancer. Until recently, TGFβ2 and TGFβ3 have been thought to carry out the same biological functions as TGFβ1. However, to assume all 3 isoforms perform the same functions in cells by default can be misleading, since TGFβ3 is structurally and biologically unique compared to other isoforms. TGFβ3 shares an 86% similarity to TGFβ1, while sharing a 91% similarity to TGFβ2 (Figure 3). Though TGFβ2 and TGFβ3 share the highest similarity, TGFβ2 binding to its Receptor II requires a co-receptor, whereas TGFβ1 and TGFβ3 bind in the same manner, and through different residues than TGFβ2. Other observations indicate TGFβ1 and TGFβ3 ligand/receptor complexes differ in that the complex may engage signaling pathways differently, leading to different biological activities. Nuclear Magnetic Resonance (NMR) data shows that the active domain of TGFβ1 is structurally ordered, whereas TGFβ3 is structurally disordered, suggesting that TGFβ3 can adopt a more “open” state which may allow for a more open receptor/ligand complex. This strengthens the idea that different isoforms may engage multiple pathways in different ways regardless of its structural similarities. TGFβ1 is most commonly studied isoform in many biological systems, whereas TGFβ3 studies are
considerably less. TGFβ1 null mice exhibit vasculogenic defects and result in death of approximately 50% of the null embryos by day ten, giving the ligand an important role in fetal development. TGFβ2 also tends to be ubiquitously expressed among tissues, however null mice exhibit abnormalities in cardiopulmonary, urogenital, neural and skeletal systems. TGFβ3 null mice experience 100% mortality due to a cleft palate and the inability to suckle effectively. The differences in biological features that TGFβ isoform null mice exhibit is a clear isoform specificity in fetal development. The differential role that these three isoforms have on tumor progression and metastasis is still unclear.

2.4.2 Signaling Pathway

In the canonical TGFβ pathway, there are 3 types of receptors, Type I, II and III of which receptor I and II are serine/threonine kinases. There are at least five known mammalian type II receptors: transforming growth factor β receptor II (TGFβRII), activin receptor II and IIB(ActRII, ActRIIB), bone morphogenic protein receptor II (BMPRII), and anti-müllerian hormone receptor 2 (AMHRII) and seven type I anaplastic lymphoma kinase receptors (ALKs 1–7) that can interact through a variety of combinations, though in most cells, the ligands signal through the TGFβRII-ALK 5 complex. The type III receptor, which is considered an accessory receptor, includes β-glycan and endoglin (CD105). Once TGFβ ligands become biologically active, they bind to the membrane-bound Type II receptor. It is at this time that TGFβ downstream target Smad proteins begin recruitment to the receptor complex. The Smad protein family consists of 8 members including Smads 1, 2, 3, 5, 8 (receptor-activated Smads, R-Smads), Smad 4 (common-mediator Smads, Co-Smads) and Smad 6 and 7 (inhibitory Smads, I-Smads).
A majority of the Smad proteins are widely expressed in many cell types and are important for all TGFβ activity. Once ligand binding to Type II receptor is complete, Type II recruits and phosphorylates the Type I receptor to form an active ligand-receptor complex, leading to downstream phosphorylation of Smad2/3, substrates for ALKs 4, 5 and 7. Smad2/3 forms a complex with the common mediator Smad4, which can then translocate and accumulate in the nucleus, where it can carry out gene regulation via binding to gene promoters and regulating transcription (Figure 4).

2.4.3 TGFβ isoforms in normal development

The TGFβ isoforms are multifunctional cytokines that inhibit cellular proliferation of many cell types, including those from epithelial origin and play important roles in embryonic development and tissue homeostasis. TGFβ is heavily involved in extracellular matrix synthesis and modulation of the immune response including wound repair processes and inflammatory reactions. It is the inappropriate functioning and changes in expression of these cytokines that has been implicated in several diseases including carcinogenesis. Because all three isoforms require activation before they can exert biological activity, it is thought that this activation is a crucial regulatory step in controlling their affects. Multiple gene knockout and overexpression studies suggest that precise regulation of each isoform is essential for survival. The TGFβ signaling pathway has both important extracellular and intracellular phases, all of which can lead to modifying the expression of specific sets of target genes. As seen in multiple gene expression profiles and studies, TGFβ can act as a tumor suppressor, targeting genes that regulate cell proliferation such as p21, E2F-1 and c-myc and apoptosis such as Fas and Bcl-xL. As a tumor promoter, however, TGFβ not only reverses its tumor-
suppressive effects on genes involved with proliferation and apoptosis, but also targets genes involved in angiogenesis such as VEGF, MMP-2, MMP-9, as well as immune response suppressor genes in multiple cell types and diseased tissues.  

2.4.4 TGFβ isoforms in cancer

TGFβ1 has been shown to be a potent tumor suppressor in many cells. However, it has also been shown in later stages of the disease that the presence of TGFβ1 is required for disease progression, giving it a tumor-promoting role. In early stages of tumorigenesis, TGFβ acts in a similar manner as it does in normal epithelial cells, continuing as an influential anti-proliferative ligand via inhibition of cell growth, induction of apoptosis and cell cycle arrest. During the latter stages of the disease, however, TGFβ can achieve tumor-promoting properties. Though the main hallmarks of cancer are increased mutations in multiple proteins and pathways, TGFβ tends to keep its functionality as well as an increase in its expression. This increase in expression of ligands and receptors can lead to production of an environment that promotes tumor invasion and metastasis including remodeling and/or degradation of the ECM due to increased production of MMPs. Unfortunately, a majority of studies performed to determine the role of TGFβ in tumor progression have focused solely on the TGFβ1 isoform. Observations of TGFβ3 in cancer progression have been scarce and indecisive. One study that gives TGFβ3 a possible role in cancer metastasis showed that TGFβ3 increased the invasiveness of endometrial carcinoma cells which was PI3K-dependent. TGFβ3 expression levels have been reported in several different cancers from clinical studies. In colon carcinomas biopsies, the expression of TGFβ3 was uniform across tumor tissue stages as well as normal tissue samples, suggesting it is unlikely that the
isoform plays a role in its’ progression. In breast carcinomas, however, clinical studies reveal an increase in TGFβ3 expression, correlating with a decrease in overall survival. In prostate cancer, there is very little known about the expression levels of the three isoforms, specifically TGFβ3 in numerous cell lines and clinical tissue samples. One study of 14 prostate adenocarcinomas showed low to no expression of the TGFβ3 isoform, with only 3 of the 14 showing any expression at all. Another study, however, indicates TGFβ3 expression increases 2 fold in prostate tumors. Other studies indicate a down regulation of TGFβ3 in prostate carcinomas compared to normal tissue, suggesting that increased expression of the isoform is correlated with suppression of prostate cancer. With such contradicting evidence, it is important to explore the role of these isoforms in established prostate cell lines.

2.5 The Phosphatidylinositol 3-kinase (PI3-Kinase) pathway

There has been a great deal of recent evidence that also suggests the role of many non-traditional pathways involved in TGFβ mediated invasion and metastasis. One such pathway, the phosphatidylinositol 3-kinase (PI3-Kinase) pathway, has been shown to have an integral part in many cellular processes including cell proliferation, growth and survival in both normal and diseased tissues. In the classical pathway, PI3-Kinases can be divided based on their functions and structure into 3 classes: Class I (IA and IB), Class II, Class III, with Class I being the most commonly studied and understood in mammalian cells. PI3K is most notably activated by receptor tyrosine kinases (RTK’s) and G-protein coupled receptors (GPCR’s), which are stimulated by ligands such as growth factors and hormones. Activation of PI3-Kinase by upstream receptors leads to downstream activation of the pathway, beginning with subsequent conversion of
phosphatidylinositol-4,5 bisphosphate (PIP2) to phosphatidylinositol-3,4,5 bisphosphate (PIP3) via phosphorylation. Once phosphorylated, PIP3 can bind to phosphoinositide-dependant kinase 1 (PDK1) and AKT/protein kinase B. This leads to membrane recruitment of AKT and phosphorylation of AKT at sites Thr 308 by PDK1 and Ser 473 by PDK2 (Figure 5). Once full activation of AKT occurs, it can move to the nucleus and/or cytoplasm where it can activate or inhibit numerous targets that are involved in metabolism, cell survival, and cell cycle progression, making AKT the most important downstream target of the PI3-Kinase pathway. As mentioned earlier, it has been shown that TGFβ can activate PI3K, as shown by increased phosphorylation of AKT and this activation of AKT appears to be Smad2/3 independent. The PI3-Kinase pathway has also been implicated as a contributing pathway to TGFβ induced EMT as well as fibroblast proliferation and morphological transformation, all precursors to invasion and metastasis. This evidence indicates the multiple roles TGFβ plays in the activation or suppression of the PI3-Kinase pathway. Whether or not TGFβ isoforms play a role in invasion and metastasis, and if this role is Smad-dependent or independent, remains unclear. In Van Themsche, Mathieu et al. 2007, they were able to show that only TGFβ3 could increase the invasiveness of the endometrial carcinoma cell lines and that this increased invasion was triggered via TGFβ3 activation of PI3-Kinase and AKT. The activation of PI3-Kinase/AKT led to a Smad-dependent up-regulation of XIAP and induction of MMP-9. There is very little, if any information available on the isoform-specific roles of TGFβ and PI3-Kinase pathway in prostate cancer.

One important antagonist of the PI3-Kinase pathway is Phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN is known to be an important factor
in maintaining homeostasis and keeping the PI3-Kinase pathway in check. In the PI3-Kinase pathway, PTEN hydrolyzes PIP3 to PIP2, antagonizing any downstream activity including phosphorylation of AKT. 132 A PTEN mutation leads to an inactive form, allowing high activation of the PI3-Kinase pathway and subsequently AKT. 125, 133 Mutations of the PI3-Kinase pathway and loss of PTEN are common in many types of cancers and is associated with tumor progression. 31 It is thought that this mutation, along with overexpression of PI3-Kinase and activating mutations of the p110α subunit of the PI3-Kinase protein lead to the initiation and progression of tumorigenesis. 134-137

2.6 TGFβ and the PI3-Kinase Pathway: A link?

There are numerous findings to suggest a role for TGFβ in PI3-Kinase signaling. It has been shown that TGFβ can activate PI3-Kinase, as shown by increased phosphorylation of AKT, however the exact mechanism by which it does so is still elusive. 32-36 In an effort to elucidate how TGFβ activates the PI3-Kinase pathway, studies have shown that this activation of AKT can be independent of Smad2/3 and that both TGFβRI and TGFβRII kinase activity can associate with the p85 subunit of the PI3-Kinase protein, thus activating the pathway. 138-142 There is also evidence to suggest a physical interaction between Smad3 and AKT. 143, 144 Formation of this complex is inhibited by TGFβ stimulation allowing for Smad3 phosphorylation, Smad4 binding and translocation to the nucleus. 143, 144 These findings suggest some interaction between the TGFβ pathway and the PI3-Kinase/Akt pathway that may be tightly regulated in normal cell types and differentially active in diseased cells. Multiple studies have also linked the PI3-Kinase pathway as a contributing pathway to TGFβ induced EMT as well as fibroblast proliferation and morphological transformation. 36 Whether or not TGFβ
isoforms play a differential role in invasion and metastasis, and act through non-smad pathways such as PI3-Kinase, is still unclear.

2.7 The Mitogen-Activated Protein Kinase (MAPK) Pathways

Other non-Smad pathway that has been implicated in the progression of cancer are the mitogen activated protein kinase (MAP-Kinase) pathways. MAP-Kinases are Serine/Threonine kinases that affect a wide range of cellular responses. Multiple MAP-Kinase pathways are expressed in all mammalian cells and regulate gene expression, motility and apoptosis. There are 14 known MAP-Kinases that are arranged in several conventional groups including ERK1 and 2 (extracellular signal-regulated kinases 1 and 2), JNK1, 2 and 3 (c-Jun amino (N)-terminal kinases 1, 2 and 3), p38 isoforms, and ERK5. These conventional MAPKs consist of a cascade of three conserved, sequentially acting Serine/Threonine kinases. MAPKK Kinase (MAPKKK) is activated via extracellular stimuli leading to the activation of MAPK kinase (MAPKK) and finally MAP-Kinase (MAPK). ERK1 and 2 are the most notably studied and defined of the MAP-Kinases and is known to be activated in response to growth factors including EGF, PDGF and in response to insulin. ERK1 and 2 MAP-Kinase model contains MAPKKKs (A-Raf, B-Raf, Raf-1, Mos and TPL-2), MAPKKs (MEK1 and MEK2), and the MAPKs (ERK1 and ERK2) (Figure 6). This cascade tends to be activated via receptor tyrosine kinase (RTK) activity. PD98059, an inhibitor of MEK1 and 2 in the ERK signaling cascade, interact with the inactive unphosphorylated MEK1 and MEK2, leading to inhibition of its activity. Upon extracellular activation of the ERK1 and 2 MAP-Kinase pathway, ERK1 and 2 can
accumulate in the nucleus and activate a number of substrates associated with cell proliferation, differentiation and survival.\textsuperscript{155}

The ERK1 and ERK2 pathway is often up-regulated in human tumors leading increased proliferation, angiogenesis and metastasis.\textsuperscript{156} TGFβ is known to signal through the MAP-Kinase pathways.\textsuperscript{105,138} Rapid activation by TGFβ of ERK MAP-Kinase has been observed in many cell types including epithelial, breast cancer cells and fibroblasts whereas other studies suggest a delayed activation by TGFβ occurring hours after the initial stimulation.\textsuperscript{146,157-159} Though the exact mechanisms by which TGFβ activates ERK are still under investigation, studies suggest a role of the TGFβ receptors. TGFβ receptors are defined as Serine/Threonine kinases, however they do contain several tyrosine phosphorylation sites that undergo both autophosphorylation and activation upon ligand binding, allowing for the recruitment of downstream effectors known to activate MAP-Kinases.\textsuperscript{160-162} ERK activation has also been shown to be a necessary non-smad pathway involved in TGFβ induced EMT through both Smad-dependent and Smad-independent effects, specifically in late stage tumor progression including loss of the ECM and metastasis.\textsuperscript{163-166} Little is known, however, about the effects of specific TGFβ isoforms on the activation of the MAP-Kinase pathways, specifically ERK1 and 2.
CHAPTER 3
MATERIALS AND METHODS

Chemicals and Reagents

Recombinant human TGFβ1 and TGFβ3 were purchased from R & D systems (Minneapolis, MN). Inhibitors of TGFβRI (SB431542) and Smad3 (SIS3) were purchased from Tocris Bioscience (Ellisville, MO) and EMD Biosciences (Gibbstown, NJ), respectively. Specific inhibitors of PI3-Kinase (LY294002) and MAP-Kinase MEK1/2 (PD98059) were purchased from Sigma-Aldrich (St. Louis, MO). The antibodies against pAKT^{ser473}, AKT (PAN), pSmad2, pSmad3, Smad2/3 and pERK (phospho p44/42 ERK1/2) were purchased from Cell Signaling Technology (Beverly, MA). Total-ERK1/2 antibody was purchased from Promega (Madison, WI). Antibodies against TGFβ1, 2 and 3 were purchased from Peprotech (Rocky Hill, NJ). Anti-β-Actin (clone AC-15) antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-rabbit IgG HRP was purchased from Bio Source (Camarillo, CA) and Anti-mouse IgG HRP was obtained from Promega (Madison, WI). Matrigel and invasion inserts were purchased from BD Biosciences (Bedford, MA). The HEMA 3 stat dye was purchased from Fisher (Pittsburgh, PA).

Cell Lines and Cell Culture

Prostate cell lines LNCaP (prostate carcinoma), DU145 (prostate carcinoma), PC3 (prostate adenocarcinoma), RWPE1 (epithelial cells from histologically normal adult human prostate), RWPE2 (immortalized epithelial cells were derived from RWPE-1 cells...
by transformation with Ki-ras using the Kirsten murine sarcoma virus (Ki-MuSV)), PZ-HPV7 (immortalized epithelial cells from histologically normal adult human prostate) and MvILu (mink lung epithelial cells) were acquired from American Type Cell Culture Collection (Rockville, MD). PC3M cells (metastatic derivative of PC3) were kindly provided by Dr. Girish Shah (University of Louisiana). All Cells were cultured in recommended growth medium at 37°C with 5% CO2 and 100% humidity. DU145, PC3, PC3M and MvILu cells were cultured in Eagle's minimum essential medium (MEM) with Earle's salts with 0.1 mM of the essential amino acid supplements as described previously.\textsuperscript{167,168} LNCaP cells were maintained in RPMI 1640 containing 4 mM glutamine and 50 μg/ml gentamycin. Both MEM and RPMI media (Mediatech, Herndon, VA) were supplemented with 5% fetal bovine serum (FBS; HyClone, South Logan, Utah). RWPE1, RWPE2 and PZ-HPV7 were cultured in keratinocyte serum free medium (KSFM) containing 50 μg/ml gentamycin (Invitrogen) and with 0.05 mg/ml bovine pituitary extract and 5ng/ml EGF.

**RNA isolation, cDNA Synthesis and RT-PCR**

For RT-PCR analysis, cells were seeded (5 x 10\textsuperscript{5}/well) into 6-well plates overnight. Total RNA was isolated from the cells using TRIzol (Invitrogen) followed by chloroform extraction and isopropanol precipitation and quantified by optical density reading at 260 nm. OD\textsubscript{260}/OD\textsubscript{280} ratios were used for quality assessment of RNA preparations. Total RNA (2 μg) were reverse transcribed as previously described.\textsuperscript{169} RT-PCR reactions were performed on I-Cycler IQ (BioRad, Hercules, CA). RT-PCR reactions were performed according to procedures described previously.\textsuperscript{167} Gene encoding ribosomal protein L-19 was used as an internal control. All gene-specific
primers were designed with the assistance of the computer program Beacon Designer 5.0 (PremierBiosoft International, Palo Alto, CA) which was set to exclude regions of cross-homology against the non-redundant set of human genes from GenBank, EMBL, and DDBJ database sequences) and to exclude regions of significant secondary RNA structure. When possible, primer sets were designed to span long introns to avoid amplification of genomic DNA. Sequences of all primers used in RT-PCR experiments are shown in table 1. The PCR products were visualized on 1-2% agarose gels stained with ethidium bromide or on 4% SFR agarose gels (Amresco, Solon, OH). Analyses of PCR products were carried out in three independent experiments using different cell preparations.

**TGFβ Treatments**

To determine the effects of TGFβ isoforms on phosphorylation of AKT, Smad2, Smad3 and the phosphorylation of ERK, DU145, PC3, and LNCaP cells were cultured in 6 well plates (5x10^5 cells/well) in 5%FBS/MEM and allowed to attach overnight. Cells were serum starved for 2 hours and incubated with or without TGFβRI inhibitor (SB431542 : 5μM), Smad3 Inhibitor (SIS3 : 3μM), PI3-Kinase inhibitor (LY294002 : 10 μM) or MAP-Kinase MEK1/2 (PD98059 : 25μM) for 30 minutes. Phosphatase inhibitor (SodiumVanadate; 200 μM) was added 10 minutes prior to the treatment. Cells were then treated with TGFβ1 or TGFβ3 (5ng/ml) over various time points. Cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1%Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Sodium Vanadate, 1μg/ml leupeptin and 1X protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein concentrations were determined by the Lowry HS assay
using the Bio-Rad DC Protein Assay kit (Bio-Rad) according to the instructions provided by the manufacturer.

**Western Blot Analyses**

Cell lysates were mixed with Laemmli’s buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 10% glycerol) and individual samples (25-50 μg proteins) were subjected to SDS-PAGE in 8 or 10% gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked for 1 hr in TBST (50 mM Tris, pH 7.5, containing 0.15 M NaCl, 0.05% Tween 20) containing 5% fat free skim milk. The blots were then incubated with appropriate dilutions of specific primary antibodies overnight at 4°C in TBST containing 5% bovine serum albumin (BSA). After washing, blots were incubated with anti-rabbit or anti-mouse immunoglobulins coupled to horseradish peroxidase (dilution 1:10,000) in blocking buffer (TBST with 5% milk) for 1h and washed in TBST for 1hr. The blots were developed in SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) or ECL Prime (Amersham) for 3 minutes, exposed to an X-ray film and visualized by autoradiography. Western blots for AKT (Pan), Smad2/3, ERK1/2 and β-actin were carried out in parallel as loading controls. The relative intensities of specific protein bands were determined by QuantityOne image analysis software.

**Scratch Wound Assay**

The migratory properties of DU145 PC3, and LNCaP cells were measured using a scratch wound assay. Cells were plated in 6-well plates \((5 \times 10^5 \text{ cells/well})\) in MEM with 5% FBS and cultured overnight. Before treatment, wells were scratched down the middle
with a 200 µl pipette tip. Culture media were replaced with MEM containing 0.1%FBS and cells were treated with TGFβ1 or TGFβ3 (1 ng/ml) with or without specific inhibitors of TGFβRI (SB431542: 5µM), Smad3 (SIS3: 3µM), PI3-Kinase (LY294002: 10 µM) or MAP-Kinase (PD98059: 25µM). Cells were allowed to migrate across the scratch for 48h. Images of the scratch area were recorded at three random spots at 0 and 48h. The migrating cells were counted using a standard size field for each image. Statistical analysis was performed using students t-test (n=3) with SigmaPlot Analysis Software.

**Invasion Assay**

The invasive properties of DU145 and PC3 were measured using the BD BioCoat Matrigel Invasion inserts. Inserts (BD Biosciences) were coated with 50 µl of a 1:4 Matrigel/Medium dilution (BD Biosciences) and allowed to solidify at 37°C for 1 hr. Cells were resuspended (5x10^4 cells/ml) in MEM with .1% FBS and 500 µl of cell suspension was added to each insert. Cells were treated with or without specific inhibitors of TGFβRI (SB431542: 5µM), Smad3 (SIS3: 3µM) or PI3-Kinase (LY294002: 10 µM) followed by TGFβ1 or TGFβ3 (.1, 1 or 10 ng/ml) and allowed to invade through a porous membrane coated with Matrigel at 37°C for 48h. Matrigel and non-invading cells were removed via scrubbing. Invading cells on the membrane were fixed in 3.7% paraformaldehyde and stained using the HEMA3 stain set (Fisher). Pictures were taken in five different fields for average number of invading cells to be determined. Statistical analyses were performed using students t-test (n=4) with SigmaPlot Analysis Software.

**TGFβ Bioassay**

MvILu mink lung epithelial cells were used as target cells for the detection of any differences in recombinant TGFβ isoform bioactivity using CellTiter 96 Non-Radioactive
Cell Proliferation Assay (Promega).\textsuperscript{170,171} MvILu were seeded at $5 \times 10^3$ on 96-well plates in DMEM containing 5% FBS. After allowing cells to attach overnight, medium was replaced with fresh DMEM + 5% FBS and recombinant TGF\(\beta\)1 or TGF\(\beta\)3 was added at 1 and 10 ng/ml and incubated for 48 hours at 37°C. After 48 hrs, medium was again replaced with DMEM + 5% FBS and 15 ul Dye Solution and incubated at 37°C in the dark for 4 hrs. Following 4 hr incubation, Solubilization/Stop Solution was added and incubated for 1 hr. Absorbance was read at 570 nm using a 96-well plate reader. Statistical analysis was performed using student t-test (n=3) with SigmaPlot Analysis Software.

**MTT Assay**

PZ-HPV7, DU145 and PC3 cells were used as target cells for the detection of any differences in the cell viability after treatment with recombinant TGF\(\beta\) isoforms using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega).\textsuperscript{170,171} Cells were seeded ($5 \times 10^3$ cells/well) in 96-well plates in appropriate media. After allowing cells to attach overnight, media was replaced with fresh media and recombinant TGF\(\beta\)1 or TGF\(\beta\)3 were added at the concentration of 1 and 10 ng/ml and incubated for 48h at 37°C. After 48h, media was again replaced with media containing 15 \(\mu\)l Dye Solution and incubated at 37°C in the dark for 4h. Following 4h incubation, solubilization/stop solution was added and incubated for 1h. Absorbance was read at 570 nm using a 96-well plate reader. Three independent experiments were carried out with similar results. Statistical analysis was performed using student t-test (n=3) with SigmaPlot Analysis Software.
CHAPTER 4

RESULTS

4.1 Gene Expression of TGFβ isoforms, Receptors and Smad proteins in prostate cell lines

Gene expression of TGFβ isoforms and receptors in prostate cells was determined by RT-PCR across several established cell lines, (table 2) with L-19 used as a control (Fig. 7). TGFβ1 mRNA was expressed across all seven cell lines (PZ-HPV7, RWPE1, RWPE2, DU145, PC3, PC3M, LNCaP cells); however the expression was substantially lower in LNCaP cells. TGFβ2 mRNA was detected in six of the seven cell lines with low expression in PZ-HPV7, RWPE1 and RWPE2 cells. A slight increase was detected in cancer cell lines DU145, PC3 and PC3M, however there was no detectable expression of TGFβ2 in LNCaP cells.

TGFβ3 expression was relatively low in normal prostate epithelial cell line PZ-HPV7 and in LNCaP cells, and was slightly higher in RWPE1 and RWPE2 cells, as well as in DU145 cells. The highest expression of TGFβ3 was found in PC3 and PC3M cells, both metastatic prostate cancer cell lines.

TGFβRI mRNA was expressed throughout all seven cell lines, with LNCaP cells again having the lowest expression. TGFβRII was expressed in PZ-HPV7, RWPE1, RWPE2, DU145, PC3 and PC3M cells; however it was not detected in LNCaP cells. TGFβRIII was detected at low levels in PZ-HPV7, RWPE1, RWPE2, DU145, PC3, and PC3M cells, however it was not detected in LNCaP cells. The mRNA expression of
Smad2 and Smad3 (R-Smads), Smad4 (Co-Smad) and Smad7 (I-Smad) were detected in all seven cell lines.

4.2 Protein Expression of TGFβ isoforms and TGFβRII in prostate cell lines

Western blot analysis was performed to determine relative protein abundance of TGFβ isoforms (TGFβ1, 2 and 3) in total cell lysates from PZ-HPV7, RWPE1, RWPE2, DU145, PC3 and PC3M cells (Fig. 8) and for TGFβRII in cancer cell lines LNCaP, DU145 and PC3 cells (Fig. 9). All cells were cultured under standard growth conditions. TGFβ1 (50kd precursor protein) was expressed across all cell lines, while TGFβ2 (25kd mature protein) was detected only in PC3 and PC3M cells. TGFβ3 (50kd precursor protein) was detected in all cell lines, with high levels in metastatic cell lines DU145, PC3 and PC3M cells. TGFβRII protein was detected in DU145 and PC3 cells, however it was absent in LNCaP cells. LNCaP cells were included in future assays as a negative control to rule out non-specific effects of TGFβ isoforms as LNCaP cells do not contain mRNA or protein for signaling through TGFβRII.

These studies showed an increase in both gene and protein expression of TGFβ3 in metastatic cell lines DU145 and PC3 cells indicating a possible autocrine role of this isoform in migration and invasion. Therefore, for further studies, DU145 and PC3 cell lines were selected to determine migratory and invasive potential in response to exogenous TGFβ1 and TGFβ3. Both DU145 and PC3 cells express all of the TGFβ ligands, receptors and Smad proteins.

4.3 Effects of TGFβ isoforms on proliferation of Mink Lung Cells

In order to rule out bioactivity differences in the recombinant TGFβ isoform
proteins used for treatments, a standard Mink Lung Cell (MV1Lu) bioassay was performed to determine dose dependant and isoform specific effects of recombinant TGFB1 and TGFB3 on cell growth. Figure 10 shows that even though both TGFB1 and TGFB3 significantly inhibited Mv1Lu cell proliferation at 1, 1 and 10 ng/ml, none of the treatments were statistically different from each other, suggesting both recombinant isoforms have similar bioactivity at different dilutions.

4.4 Effects of TGFB isoforms on proliferation of prostate cell lines

TGFB is known to inhibit proliferation in normal prostate epithelial cells. The effects of TGFB1 and TGFB3 on the proliferation of immortalized normal epithelial (PZ-HPV7) cells were determined using MTT assay in order to rule out possible differences in the biopotencies of recombinant proteins. Figure 11 shows that both TGFB1 and TGFB3 significantly inhibited PZ-HPV7 cell proliferation at both 1 and 10 ng/ml, with no differences in the potencies of the two isoforms. We also studied the effects of the two isoforms on proliferation of DU145 cells. As shown in figure 12, both isoforms inhibited growth of these cells at 1 and 10 ng/ml and again, showed no differences in potencies. PC3 cells did not respond to the growth inhibitory effects of either isoform (Fig.13).

This proliferation data, along with the gene and protein expression data strengthen the need to use DU145 and PC3 cells as a possible model for the mechanistic switch of TGFB from tumor-suppressor to tumor-promoter and to help elucidate the possible signaling differences between the TGFB isoforms.

4.5 Differential effects of TGFB isoforms on migration of prostate cancer cells

The effects of TGFB1 and TGFB3 on migration of metastatic prostate cancer cell lines PC3, DU145 and LNCaP were determined using a scratch wound assay. PC3,
DU145 and LNCaP cells were treated with either TGFβ isoform and allowed to migrate according to established procedures. As shown in Figure 14, both TGFβ isoforms caused an increase in the migration of PC3 cells. However, TGFβ3 effects were 2.1 fold (n=3) higher than those of TGFβ1 on number of migrating cells (See Fig. 23; P < 0.01). Both TGFβ isoforms had no effects on the migration of DU145 and LNCaP cells under similar experimental conditions (Figure 15 and 16, respectively).

4.6 Differential effects of TGFβ isoforms on invasion of prostate cancer cells

As shown in Figure 17, both TGFβ1 and TGFβ3 increased invasiveness of PC3 cells. TGFβ1 had a significant effect on invasion at 1ng/ml (P < 0.05), which declined at both 1ng/ml and 10ng/ml doses. However, TGFβ3 caused significantly higher invasion of PC3 cells compared to TGFβ1 at 1ng/ml (P < 0.05) and 10ng/ml (P < 0.01) doses (Fig. 18). Both TGFβ isoforms had no effect on invasiveness of DU145 cells under identical experimental conditions (Fig. 19).

These results, along with similar migration data, suggest a differential role of TGFβ3 versus the ubiquitously expressed TGFβ1. In order to determine how these isoforms are differentially increasing both migration and invasion, it is important to determine what role, if any, the canonical TGFβ pathway plays in these results.

4.7 The role of TGFβRI and Smad3 in TGFβ isoform induced migration and invasion

To determine whether TGFβ1 and TGFβ3 effects on cell migration and invasion are mediated by classical TGFβ signaling pathway, PC3 cells were treated with TGFβ1 or TGFβ3 in the presence of specific inhibitors of TGFβRI. As shown in Figure 20, TGFβRI inhibitor (SB431542) blocked TGFβ1 and TGFβ3 induced migratory effects on PC3
cells. Smad3 Inhibitor (SIS3) also blocked migration of PC3 cells induced by TGFβ isoforms as well (Fig. 21). The migrating cells were counted using a standard size field for each image. Statistical analysis was performed using students t-test (n=3) with SigmaPlot Analysis Software (Fig. 23).

TGFβRI inhibitor (SB431542) and Smad3 Inhibitor (SIS3) also blocked the effects of TGFβ isoforms on invasive behavior of PC3 cells (Fig. 24 and 25, respectively). Pictures were taken in five different fields for average number of invading cells to be determined. Statistical analyses were performed using students t-test (n=4) with SigmaPlot Analysis Software (Fig. 27).

Based on these results, we determined that even though the TGFβ isoforms act differentially on cells during the migration and invasion process, both isoforms require a minimum of the canonical TGFβ pathway machinery including the TGFβRI and the Smad3 protein. This suggests that any differential activity of TGFβ1 or TGFβ3 may be occurring via separate pathways that are traditionally involved in the metastatic cascade leading to migration and invasion. One such pathway that we deemed necessary to explore is the PI3-Kinase pathway.

4.8 Differential effects of TGFβ isoforms on migration are mediated by PI3-Kinase pathway

To determine if TGFβ isoform-induced migration is mediated via the PI3-Kinase pathway, scratch wound assays were performed in the presence or absence of a specific PI3-Kinase inhibitor (LY294002: 10 μM). PC3 Cells were treated with TGFβ1 or TGFβ3 (1ng/ml) and allowed to migrate across a scratch for 48h. Pictures were taken at 0hr and
48hr and number of migrating cells was determined by counting. As shown in Figure 22, PI3-Kinase inhibitor (LY294002) blocked both TGFβ1 and TGFβ3 induced migratory behavior in PC3 cells. Statistical analysis was performed using students t-test (n=3) with SigmaPlot Analysis Software (Fig. 23).

4.9 Differential effects of TGFβ isoforms on invasion are mediated by PI3-Kinase pathway

Similar to previous migration studies, invasion assays were performed to determine if TGFβ isoform-induced invasion is mediated via the PI3-Kinase pathway as well. Invasion assays were performed in the presence or absence of a specific PI3-Kinase inhibitor (LY294002: 10 μM). PC3 Cells were treated with TGFβ1 or TGFβ3 (1ng/ml) and allowed to invade a matrigel-coated insert for 48h. Pictures were taken at 0hr and 48hr and number of invading cells was determined via staining. As shown in Figure 26, PI3-Kinase inhibitor (LY294002) also blocked the effects of both isoforms on the invasion of PC3 cells. Statistical analysis was performed using students t-test (n=4) with SigmaPlot Analysis Software (Fig. 27).

4.10 Differential effects of TGFβ isoforms on activation of PI3-Kinase pathway

To determine what effects TGFβ isoforms have on the PI3-Kinase pathway and phosphorylation of AKT (pAKT), we first wanted to determine levels basal pAKT^{ser473} in our panel of prostate cell lines. As shown in Figure 28, our normal prostate epithelial cell lines show no detectable levels of pAKT^{ser473}, however both PC3 and LNCaP show detectable pAKT^{ser473}, with basal phosphorylation higher in LNCaP. It is important to note that though DU145 is considered a metastatic cell line, it indeed has no detectable phosphorylation of AKT under these normal conditions. This data again demonstrates
why the DU145 and PC3 cell lines are an excellent model to determine the differential role of TGFβ isoforms on the PI3-Kinase pathway. PTEN, a negative regulator of the PI3-Kinase pathway, is active in the DU145 cell line, but is mutated and biologically inactive in PC3 cells and absent from LNCaP cells altogether. It is these differences in the cell lines that will help to elucidate what effect and/or association, if any, the TGFβ isoforms have with PI3-Kinase pathway and its downstream target pAKT^ser473.

To determine if TGFβ isoforms differentially activate the PI3-Kinase pathway and phosphorylation of AKT (pAKT), PC3 cells were treated with either TGFβ1 or TGFβ3 (5ng/ml) for specific time points and probed for pAKT^ser473, a downstream target of PI3-Kinase. Both TGFβ isoforms induced an increase in the phosphorylation of AKT^ser473 over several time points in PC3 cells. TGFβ3 was more effective than TGFβ1 in increasing pAKT^ser473 (Fig. 29). Based on the most significant time points, we then compared TGFβ isoform induced pAKT^ser473 in DU145, PC3 and LNCaP cells 15 and 60 min after treatment. TGFβ isoforms again induced an increase in the phosphorylation of AKT^ser473 in PC3 cells (Fig. 30). Once again, TGFβ3 was more effective at inducing the phosphorylation of AKT^ser473 with a significant (3.7 fold; n = 3) increase at 60 min as determined by densitometry using β-actin as a control (Fig. 31). However, neither TGFβ isoform increased pAKT^ser473 levels in DU145 (Fig. 32) or LNCaP cells (Fig. 33) at both time points. Once again, DU145 cells showed no detectable phosphorylation of AKT even upon treatment with TGFβ isoforms, whereas LNCaP cells showed detectable, yet unchanged levels of pAKT^ser473.
4.11 The role of TGFβRI, Smad3 and PI3-Kinase on TGFβ isoform-induced phosphorylation of AKT\textsuperscript{ser473}

To determine whether TGFβ1 and TGFβ3 induced pAKT\textsuperscript{ser473} are mediated by TGFβRI, Smad3 or PI3-Kinase pathway, PC3 cells were treated with a TGFβRI inhibitor (SB431542), Smad3 inhibitor (SIS3) or PI3-Kinase inhibitor (LY294002) followed by TGFβ1 or TGFβ3 (5ng/ml). Cell lysates were collected and analyzed for pAKT\textsuperscript{ser473}, Total AKT (pan) and β-actin. TGFβRI inhibitor (SB431542) blocked TGFβ-induced pAKT\textsuperscript{ser473} (Fig. 34) as did Smad3 inhibitor (SIS3) (Fig. 35). PI3-Kinase inhibitor (LY294002) also blocked basal and TGFβ-induced accumulation of pAKT\textsuperscript{ser473} in PC3 cells (Fig. 36).

4.12 The role of TGFβRI, Smad3 and PI3-Kinase on TGFβ isoform-induced phosphorylation of Smad 2 and Smad 3

To determine any TGFβ isoform-specific effects on the phosphorylation of Smad 2 and Smad 3, we again treated PC3 cells with either TGFβ1 or TGFβ3 (5ng/ml) for 60 min. As shown in figure 37, both TGFβ1 and TGFβ3 induce the phosphorylation of Smad 2 and Smad 3 and at similar intensities. We also looked at the phosphorylation of Smad 2 and Smad 3 upon treatment with the same inhibitors. TGFβRI inhibitor SB431542 blocked the effects of both TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 (Figure 38). Smad 3 inhibitor SIS3 blocked effects of both isoforms on Smad3 phosphorylation without affecting the phosphorylation of Smad2 (Figure 39). PI3-Kinase inhibitor LY294002 did not influence Smad2 or Smad3 phosphorylation in response to both isoforms (Figure 40).
4.13 The effects of TGFβ isoforms on the activation of the Mitogen-Activated Protein Kinase extracellular signal-regulated kinases 1/2 (ERK1/2)

In order to determine any TGFβ isoform-specific differential activation of the MAP-Kinase ERK1/2, we looked at the phosphorylation of ERK1/2 (p44/42) in response to TGFβ treatment. TGFβ has been shown to activate the MAP-Kinase pathway via phosphorylation of ERK1/2.\(^{162}\) As seen in figure 41, TGFβ1 induces a slight increase in the phosphorylation of ERK2 (p42) whereas TGFβ3 does not. We do note that in PC3 cells under these conditions, we only see phosphorylation of ERK at the p42 position, however when EGF is added, we see phosphorylation at both p44/p42 sites. When an inhibitor of MAP-Kinase (PD98059) is added, all phosphorylation of ERK1/2 is blocked as expected. Both Total ERK1/2 and β-actin were used as loading controls.

To determine whether the ERK/MAP-Kinase pathway and the PI3-Kinase pathway have any crosstalk upon activation by treatment with TGFβ isoforms, we again treated PC3 cells with an inhibitor of ERK/MAP-Kinase MEK1 and 2 (PD98059) followed by TGFβ1 or TGFβ3 and probed for the pAKT\(^{\text{ser473}}\) antibody. As seen in figure 42, blocking the MEK1/2 did not affect the TGFβ isoform-induced phosphorylation of AKT, suggesting that there is no cross-talk or requirement for ERK/MAP-Kinase for TGFβ-induced activation of the PI3-Kinase pathway.

4.14 The role of the Mitogen-Activated Protein Kinase pathway in TGFβ isoform-induced migration

Since the ERK/MAP-Kinase pathway has been implicated in the progression of cancer in multiple biological processes, we wanted to determine whether having an active ERK/MAP-Kinase was necessary for cell motility at both a basal and TGFβ induced
level. We found that by blocking the MAP-Kinase pathway with an inhibitor of
ERK/MAP-Kinase MEK1/2 (PD98059), we inhibit all migration of PC3 cells with and
without the presence of TGFβ1 or TGFβ3 (Figure 43).
CHAPTER 5
DISCUSSION

This study is the first of its kind to systematically determine the expression of all TGFβ isoforms, receptors and Smads simultaneously in several prostate cell lines. Though previous studies have shown the presence of TGFβ isoforms and machinery in different cancers including prostate tissues and cell lines, none have looked at the relative mRNA and protein expression levels in numerous prostate cell lines under the same experimental conditions. We have shown that TGFβ1 is ubiquitously expressed at both the mRNA and protein level in both normal and metastatic prostate cell lines. This suggests that any differential effects seen in the normal versus metastatic cell lines may be due to changes in expression of other TGFβ isoforms. Alternatively, post translation activation may play a role in changing effects of TGFβ during different stages of prostate cancer. We show an increase of both TGFβ2 and TGFβ3 in metastatic prostate cell lines, however TGFβ2 protein expression changes were only seen in PC3 cells. The steadiest change was seen in TGFβ3 expression, with it increasing consistently in the more metastatic cell lines. This may indicate a specific role of TGFβ3 in the metastatic phenotype. This differential role for this isoform is supported by similar high expression of TGFβ3 in breast carcinomas correlating with decreased overall survival rate \(^{114,115}\) and that TGFβ3 increased invasiveness of endometrial carcinoma cells. \(^{27,114,115}\)

Based on our expression data, we deemed it important to focus on two of the isoforms, TGFβ1 and the lesser studied TGFβ3. As mentioned earlier, TGFβ3 shares less
similarity to TGFβ1 then TGFβ2 does, however, TGFβ1 and TGFβ3 share similar binding affinities to the receptors, whereas TGFβ2 binds with 100-1000 times lower affinity and at different residues with the assistance of a co-receptor (TGFβRIII or β-Glycan).  

We first compared the biological activities of human recombinant TGFβ1 and TGFβ3 protein using a standard TGFβ bioactivity assay. Mink lung epithelial cells (Mv1Lu) are a widely used model for evaluation of the effects of exogenous TGFβ both in transcriptional and growth inhibitor assays. We show that both TGFβ1 and TGFβ3 inhibit cell growth of Mv1Lu cells at the same rate at multiple concentrations. In previous work from our lab, we have shown that TGFβ1 acts as a growth inhibitor in both our normal prostate epithelial PZ-HPV7 cells and our metastatic prostate cancer DU145 cells, whereas TGFβ loses its ability to inhibit growth in metastatic cell lines PC3 and TGFβRII null LNCaP. This suggests that though DU145 is a metastatic cancer cell line, it may still contain the ability for TGFβ to act as a tumor-suppressor in the sense of inhibiting proliferation. Our PC3 cell line, however, no longer responds to TGFβ inhibitory effects on proliferation, effectively suggesting TGFβ is now a tumor promoter in these cells. This led us to use both the DU145 and PC3 cell lines as a possible model for the elusive TGFβ “switch” from tumor suppressor to tumor promoter.

With this model, we first compared the effects of the exogenous recombinant TGFβ1 and TGFβ3 proteins on the growth of the prostate cell lines PZ-HPV7, DU145 and PC3. Again, we show that not only do both TGFβ isoforms inhibit proliferation of the normal epithelial PZ-HPV7 cells and the metastatic DU145 cells, but they do so at the same rate at both 1 and 10ng/ml doses. PC3 cell growth was not inhibited by either
isoform. This not only strengthens the use of DU145 and PC3 cells as a “switch” model, but shows that both isoforms have similar biological activity on proliferation of cells in multiple normal and metastatic cell lines. Any differences seen between TGFβ1 and TGFβ3 are not due to differential potencies on growth inhibition.

When comparing the effects of TGFβ1 and TGFβ3 on the motility and invasive abilities of three metastatic prostate cancer cell lines, DU145, PC3 and LNCaP cells, it is important to note that both DU145 and PC3 cells express TGFβ1 and TGFβ3 as well as all receptors and Smad proteins, whereas LNCaP lacks the TGFβRII, a necessary receptor for TGFβ signaling. However, while both TGFβ isoforms induced migratory and invasive behavior in PC3 cells, DU145 and LNCaP cells were unresponsive to both isoforms under identical experimental conditions. Of the two isoforms that induced migration and invasion of PC3 cells, TGFβ3 was much more potent in doing so. The differential and more potent effects of TGFβ3 on invasive behavior of prostate cancer cells are similar to those previously reported on TGFβ- induced invasiveness of endometrial carcinoma cells.

Again, these differences do not appear to be a consequence of differential bioactivities of the two recombinant proteins, since no differences were observed in the effects of the two isoforms on cell proliferation.

In both in vitro migration and invasion, cells require the ability to move across a gradient, whether it be on a plastic surface (scratch wound assay) or in an insert with a pourous membrane and an extracellular matrix (invasion assay). For invasion, however, not only do the cells need the ability to move, but to degrade or displace the components of an extracellular matrix (ECM) such as Matrigel, which mimics the in vivo ECM. There have been multiple studies showing the isoform-specific differences in mammalian
embryos versus adult mammals in regards to wound healing, a process that requires both growth and motility of surrounding epithelial cells. In these studies, it is shown that mammalian embryos had wound healing abilities with no scarring while exhibiting high amounts of TGFβ3 and that the addition of exogenous TGFβ3 reduced scarring in both adult mice and in man. The role of TGFβ3 in wound healing, specifically whether TGFβ3 induces required motility or other processes such as cytoskeleton formation or angiogenesis, is still elusive. As for the process of invasion, the extracellular matrix (ECM) is an important determinant of cellular behavior including regulation of cellular adhesion, migration, and proliferation. TGFβ has long been known to play diverse roles in both the production and degradation of the ECM, however little is known about the isoform-specificity of this control. Taken together, these studies suggest that isoform-specific functions such as TGFβ3 acting in a more potent manner then TGFβ1 in both migration and invasion, may be doing so at numerous biological levels and affecting multiple processes and pathways.

Prior studies have revealed that TGFβ isoform-specific effects can be both dependent or independent of Smad signaling depending on the context in which it is studied. Using small molecule inhibitors, we wanted to determine whether TGFβ isoform-induced migration and invasion in PC3 cells was TGFβRI or Smad3 dependent. SB431542, a small molecule TGFβRI inhibitor, is a well characterized inhibitor of activin receptor-like kinase (ALK5) as well ALK4 and ALK7, which are highly similar in parts of their structure but does not affect other ALK family members. It is known to inhibit TGFβ signaling via competitive ATP binding of the serine/threonine kinase which, in turn, inhibits Smad3 phosphorylation and has no effect on non-Smad pathways
such as the MAP-kinase pathway.\textsuperscript{183} Another small molecule inhibitor of TGFβ activity is SIS3, characterized as a specific inhibitor of TGF-β1/ALK-5 induced phosphorylation of Smad3 and interaction of Smad3 and Smad 4.\textsuperscript{184} It is also known not to affect Smad 2 phosphorylation, expression of other Smads and activation of multiple MAP-Kinase pathway components; however the exact mechanism by which it is a selective inhibitor of Smad 3 alone is not clear. Our study indicates that the TGFβ isoform-specific increase of migration and invasion is both TGFβRI and Smad3 dependent in PC3 cells. Any isoform-specific activity between TGFβ1 and TGFβ3 requires activation of the canonical TGFβ pathway receptor I and the phosphorylation of Smad 3 before it can induce migration and invasion. This suggests that the differential activity of TGFβ1 and TGFβ3 on migration and invasion may lie in the activation of a different pathway, specifically a pathway that is well known to be involved in cancer metastasis and can be activated by TGFβ. This led us to look at the PI3-Kinase and MAP-Kinase pathways.

The PI3-Kinase pathway has been shown to play an essential role in the migration and invasion of many cell types. Previous studies indicate that TGFβ can activate PI3-Kinase, as shown by increased phosphorylation of AKT, leading to increased invasion of cancer cells.\textsuperscript{27,32-36} In keeping with these studies, our results indicate that the PI3-Kinase pathway is essential for both TGFβ1 and TGFβ3 induced migration and invasion. LY294002 is a PI3-Kinase inhibitor of the p110α, p110δ and p110β subunits which inactivate PI3-Kinase leading to decreased phosphorylation of AKT\textsuperscript{Ser473}.\textsuperscript{185,186} Though it is a commonly used PI3-Kinase inhibitor, it is known to also have a broad inhibitory profile across different classes of PI3-Kinases as well as PI3-Kinase-independent effects such as inhibition of Ca\textsuperscript{2+} signaling and transcription factors such as NF-kB.\textsuperscript{185,187,188}
Pharmacological inhibition of PI3-Kinase via LY294002 blocked TGFβ isoform-induced movement of PC3 cells indicating PI3-Kinase activation is required for TGFβ induced migration and invasion. To support this assumption, both TGFβ isoforms also caused an increase in the phosphorylation of AKT in PC3 cells, with TGFβ3 being more potent than TGFβ1. This TGFβ isoform-induced phosphorylation of AKT was TGFβRI, Smad3 and PI3-Kinase dependent. We also show that not only do both isoforms induced phosphorylation of Smad 2 and Smad 3, but that this phosphorylation is indeed TGFβRI and Smad3 dependent. Inhibition of the PI3-Kinase pathway did not affect TGFβ isoform induced phosphorylation of both Smads.

Other non-Smad pathway that has been implicated in the progression of multiple cancer types are the mitogen-activated protein kinase (MAP-Kinase) pathways. MAPKs are protein Serine/Threonine kinases that are involved in wide range of cellular responses including motility and apoptosis. Of the multiple MAP-Kinases that have been characterized, we focused on the conventional extracellular signal-regulated kinases 1/2 (ERK1/2). ERK1 and ERK2 are activated in response to multiple stimuli including insulin and by growth factors such as epidermal growth factor (EGF) and TGFβ, although the exact mechanism by which TGFβ activates MAP-kinase pathways is still unresolved. TGFβ1 slightly induced the phosphorylation of ERK2 (p42) whereas TGFβ3 did not. When an inhibitor of ERK/ MAP-Kinase MEK1/2 (PD98059) was added, all phosphorylation of ERK1/2 was blocked. Inhibiting the ERK/MAP-Kinase pathway also did not affect the TGFβ isoform-induced phosphorylation of AKT, suggesting that there is no cross-talk or requirement for ERK/MAP-Kinase for TGFβ-induced activation of the PI3-Kinase pathway. However, blocking the ERK/MAP-Kinase
pathway inhibited all migration of PC3 cells with and without the presence of TGFβ1 or TGFβ3. Taken together, our data shows that the TGFβ isoform effects on increased migration and invasion requires both the canonical TGFβ pathway and the PI3-Kinase pathway, though the activation of the PI3-Kinase pathway is not necessary for TGFβ induced phosphorylation of Smads. Motility of these cells, with or without exogenous TGFβ isoform-induced movement, also requires an active ERK/MAP-Kinase pathway.

These results, along with previous studies, confirm a novel isoform-specific role of TGFβ in the migration and invasion of the metastatic PC3 prostate cancer cells which are dependent on the activation of the PI3-Kinase pathway. We did not see similar effects of TGFβ isoforms on activation of PI3-kinase and AKT phosphorylation in DU145 and LNCaP cells, indicating that lack of this response may be responsible for lack of TGFβ effects on migration of these cells. It is tempting to speculate that the ability of TGFβ to activate PI3-kinase signaling may be pre-requisite for the induction of invasive behavior in more advanced stages of prostate cancers and may represent the “switch” from anti-tumorigenic to tumor-promoter role of TGFβ.

There are multiple studies on the effect of TGFβ-PI3-Kinase activity on both normal and diseased tissues such as cancer. It is clear that TGFβ rapidly activates the PI3-Kinase pathway and its downstream target, AKT in multiple cell types.\textsuperscript{32,34,138} TGFβRII has been shown to be associated with p85, the regulatory subunit of PI3-Kinase, and that an association of the TGFβRI with p85 is required for the activation of PI3-Kinase stimulation by TGFβ.\textsuperscript{141} Additionally, inhibition of TGFβRI activity using a small molecule inhibitor prevented TGFβ-induced activation of AKT by PI3-Kinase.\textsuperscript{32,33} Unfortunately, the exact mechanism by which TGFβ isoforms differentially activate AKT
and induce motility and invasion is still unknown. One possible contributor in differential effects of TGFβ on activation of PI3-Kinase in DU145, LNCaP and PC3 cells may be the Phosphatase and Tensin Homolog (PTEN), which inhibits PI3-kinase dependent phosphorylation of AKT. PTEN has been shown to be mutated in many cancers which can lead to higher basal levels of pAKT<sub>ser473</sub> and increased survival of cells. 31, 125, 133 PTEN is active in the DU145 cell line, but is mutated and biologically inactive in PC3 cells and absent from LNCaP cells altogether. 172-175 Although LNCaP cells have higher basal levels of pAKT, we did not observe any effects of TGFβ on pAKT<sub>ser473</sub>, due to lack of TGFβ signaling in these cells. It is therefore possible that a PTEN mutation leading to loss of AKT regulation, along with the ability of ligand-activated TGFβ receptors to activate the PI3-Kinase pathway through the TGFβR-p85 association can play a role in TGFβ effects on activation of PI3-kinase in prostate cancer cells and that TGFβ- dependent invasive and metastatic behavior may be more pronounced in patients where the tumor cells harbor PTEN mutations. This suggests that PTEN and, ultimately, the PI3-Kinase pathway may have a role in the TGFβ isoform-induced migration and invasion and the TGFβ “switch” from anti- to pro-tumorigenic cytokine in prostate cancer. As for the differences in activity of TGFβ1 and TGFβ3, very little is known and it is recently becoming apparent that these ligands are expressed and operate in a differential manner depending on the context of the cell. Elucidating the mechanism by which these isoforms act in a differential manner shall be the focus of future TGFβ isoform studies.
CHAPTER 6
CONCLUSION

In conclusion, these results, along with previous studies, confirm an isoform-specific role of TGFβ in the migration and invasion of the metastatic PC3 prostate cancer cell line. These effects are mediated by both TGFβRI and Smad3 dependent activation of the PI3-Kinase pathway. We did not see the same effects of TGFβ isoforms in the metastatic DU145 or LNCaP cell line, suggesting a cell-line model of a TGFβ “switch” from anti-tumorigenic to tumor-promoter, possibly through the activation of the PI3-Kinase pathway. One possible contributor to TGFβ differential effects and activation of PI3-Kinase in this model is Phosphatase and Tensin Homolog (PTEN), which assists in the regulation of the phosphorylation of AKT. PTEN has been shown to be mutated in many cancers which can lead to higher basal levels of pAKT^{ser473} and increased survival of cells. PTEN is active in the DU145 cell line, but is mutated in PC3 cells. We show that basal pAKT^{ser473} levels are undetectable in DU145 under these experimental conditions, even with the addition of TGFβ isoforms. This suggests PTEN and, ultimately, the PI3-Kinase pathway may have a role in the TGFβ isoform-induced motility and the TGFβ “switch” from anti to pro-tumorigenic in the DU145/PC3 cell line model.
**APPENDIX**

Figure 1

Leading New Cancer Cases and Deaths – 2012 Estimates

<table>
<thead>
<tr>
<th>Leading New Cancer Cases</th>
<th>Estimated Deaths</th>
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<tr>
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<td><strong>Female</strong></td>
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<tr>
<td>Prostate</td>
<td>Breast</td>
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<tr>
<td>241,740 (25%)</td>
<td>226,870 (29%)</td>
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<tr>
<td>Lung &amp; bronchus</td>
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<td>116,470 (14%)</td>
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<td>Colon &amp; rectum</td>
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<tr>
<td>73,420 (9%)</td>
<td>70,040 (9%)</td>
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<tr>
<td>Urinary bladder</td>
<td>Uterine corpus</td>
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<td>47,130 (6%)</td>
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<td>Melanoma of the skin</td>
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<td>Kidney &amp; renal pelvis</td>
<td>Non-Hodgkin lymphoma</td>
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<tr>
<td>40,250 (5%)</td>
<td>12,040 (4%)</td>
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<tr>
<td>Non-Hodgkin lymphoma</td>
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<td>38,160 (4%)</td>
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<td>Oral cavity &amp; pharynx</td>
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<td>22,090 (3%)</td>
<td>21,830 (3%)</td>
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<tr>
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<td>848,170 (100%)</td>
<td>790,740 (100%)</td>
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</tbody>
</table>

*Includes basal and squamous cell skin cancers and in situ carcinomas except urinary bladder

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Figure 1: 2012 Estimates for Leading Cancer Cases and Deaths in the United States. (American Cancer Society 2012)
Figure 2: The Metastatic Cascade (Scheel et al., 2012)
Figure 3: Amino acid sequence alignments of human TGFβ isoforms (Laverty et al. 2009)

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**:**.****.****:*******;****** ***** **:**: * **:**
Figure 4: The Transforming Growth Factor Signaling Pathway (Expert Reviews in Molecular Medicine 2003)
Figure 5: The PI3-Kinase Pathway (Cell Signaling)
Figure 6: The Mitogen-Activated Protein Kinase (MAPK) Cascades. (Cell Signaling)
Table 1: The Origins of the Cell Lines Used

<table>
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<tr>
<th>Cell line</th>
<th>Origin</th>
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<tr>
<td>PZ-HPV7</td>
<td>epithelial cells from histologically normal adult human prostate</td>
</tr>
<tr>
<td>RWPE1</td>
<td>epithelial cells from histologically normal adult human prostate</td>
</tr>
<tr>
<td>RWPE2</td>
<td>epithelial cells were derived from RWPE-1 cells by transformation with Ki-ras using the Kirsten murine sarcoma virus (Ki-MuSV)</td>
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<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>
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Table 2. Gene-specific primers used for RT-PCR amplification.

<table>
<thead>
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<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
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Figure 7: mRNA expression of TGFβ isoforms and signaling components in prostate cell lines. Semi-quantitative RT-PCR was performed using RNA from PZ-HPV7, RWPE1, RWPE2, DU145, PC3, PC3M, and LNCaP cells to determine relative mRNA levels of TGFβ isoforms (TGFβ1, 2 and 3), TGFβ receptors I, II and III and Smads 2, 3, 4 and 7. The mRNA levels in all cell line samples were normalized against L-19.
Figure 8: Protein expression of TGFβ isoforms in prostate cell lines. Western blot analysis was performed to probe for TGFβ isoforms (TGFβ1, 2 and 3) in total cell lysates from PZ-HPV7, RWPE1, RWPE2, DU145, PC3 and PC3M cells.
Figure 9: Protein Expression of the TGFβRII in Prostate Cancer Cell lines. Western blot analysis was performed to determine relative protein levels of TGFβRII in LNCaP, DU145 and PC3 cells. Western blots for β-actin were performed as protein loading controls.
Figure 10: Mink Lung Cell (MV1Lu) TGFβ Bioactivity Assay. Mv1Lu mink lung epithelial cells were used as target cells for the detection of any differences in recombinant TGFβ isoform bioactivity using Cell Proliferation Assay. Statistical analysis was performed using student t-test (n=3) with SigmaPlot Analysis Software.
Figure 11: Dose dependent effects of TGFβ1 and TGFβ3 on proliferation of PZ-HPV7 prostate epithelial cells. Each bar represents Mean ± SD from a representative experiment of three independent experiments.
Figure 12: Dose dependent effects of TGFβ1 and TGFβ3 on proliferation of DU145 prostate cancer cells. Each bar represents Mean ± SD from a representative experiment of three independent experiments.
Figure 13: Dose dependent effects of TGFβ1 and TGFβ3 on proliferation of PC3 prostate cancer cells. Each bar represents Mean ± SD from a representative experiment of three independent experiments.
Figure 14: Effects of TGFβ1 and TGFβ3 on migration of PC3 prostate cancer cells. The migratory properties of PC3 were measured using a scratch wound assay. Cells were cultured overnight and scratched with a 200μl pipette tip. Culture media were replaced with MEM or RPMI (containing 0.1%FBS) and the cells were treated with TGFβ1 or TGFβ3 (1ng/ml) for 48h. Images of the scratch area were recorded at three random spots at 0 and 48h and statistical analysis performed. Pictures were taken at 10x magnification.
Figure 15: Effects of TGFβ1 and TGFβ3 on migration of DU145 prostate cancer cells. The migratory properties of DU145 were measured using a scratch wound assay. Cells were cultured overnight and scratched with a 200μl pipette tip. Culture media were replaced with MEM or RPMI (containing 0.1%FBS) and the cells were treated with TGFβ1 or TGFβ3 (1ng/ml) for 48h. Images of the scratch area were recorded at three random spots at 0 and 48h. Pictures were taken at 10x magnification.
Figure 16: Effects of TGFβ1 and TGFβ3 on migration of LNCaP prostate cancer cells. The migratory properties of LNCaP were measured using a scratch wound assay. Cells were cultured overnight and scratched with a 200μl pipette tip. Culture media were replaced with MEM or RPMI (containing 0.1%FBS) and the cells were treated with TGFβ1 or TGFβ3 (1ng/ml) for 48h. Images of the scratch area were recorded at three random spots at 0 and 48h. Pictures were taken at 10x magnification.
Figure 17: The invasive properties of PC3 cells were measured using the BD BioCoat Matrigel Invasion inserts. Cells were treated with different concentrations of TGFβ1 or TGFβ3 and allowed to invade through a porous membrane coated with Matrigel for 48h. Pictures were taken in five different fields for average number of invading cells to be determined. Pictures were taken at 10x magnification.
Figure 18: Number of invading PC3 cells through Matrigel after treatment with TGFβ1 or TGFβ3 for 48h. Each bar represents Mean ± SEM from 4 independent experiments. *Significantly different (P<0.05; Students 't' test) from appropriate controls.
Table 19: The invasive properties of DU145 cells were measured using the BD BioCoat Matrigel Invasion inserts. Cells were treated with different concentrations of TGFβ1 or TGFβ3 and allowed to invade through a porous membrane coated with Matrigel for 48h. Pictures were taken in five different fields for average number of invading cells to be determined. Pictures were taken at 10x magnification.

<table>
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<th>Control</th>
<th>TGFβ1 (1ng/ml)</th>
<th>TGFβ3 (1ng/ml)</th>
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<td>DU145</td>
<td></td>
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Figure 20: The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on migration of PC3 cells in the presence of inhibitor of TGFβRI (SB431542). Scratch wound assays were performed following treatment with TGFβ isoforms for 48h in the presence or absence of specific inhibitors. Pictures were taken at 10x magnification.
Figure 21: The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on migration of PC3 cells in the presence of inhibitor of Smad3 (SIS3). Scratch wound assays were performed following treatment with TGFβ isoforms for 48h in the presence or absence of specific inhibitors. Pictures were taken at 10x magnification.
Figure 22: The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on migration of PC3 cells in the presence of inhibitor of TGF P13-kinase (LY294002). Scratch wound assays were performed following treatment with TGFβ isoforms for 48h in the presence or absence of specific inhibitors. Pictures were taken at 10x magnification.
Figure 23: Number of migrating PC3 cells after various treatments. Each bar represents Mean ± SEM from three independent experiments. a: statistically significant (p<0.05) from untreated controls, b: statistically significant (p<0.05) from TGFβ1 treated cells, c: statistically significant (p<0.05) from TGFβ3 treated cells.
Figure 24: The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on invasion of PC3 cells in the presence of inhibitor of TGFβRI (SB431542). Invasion assays were performed following treatment with TGFβ isoforms for 48h in the presence or absence of specific inhibitors. Pictures were taken at 10x magnification.
Figure 25: The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on invasion of PC3 cells in the presence of inhibitor of Smad3 (SIS3). Invasion assays were performed following treatment with TGFβ isoforms for 48h in the presence or absence of specific inhibitors. Pictures were taken at 10x magnification.
Figure 26: The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on invasion of PC3 cells in the presence of inhibitor of TGF PI3-kinase (LY294002). Invasion assays were performed following treatment with TGFβ isoforms for 48h in the presence or absence of specific inhibitors. Pictures were taken at 10x magnification.
Figure 27: Number of invading PC3 cells after various treatments. Each bar represents Mean ± SEM from three independent experiments. a: statistically significant (p<0.05) from untreated controls, b: statistically significant (p<0.05) from TGFβ1 treated cells, c: statistically significant (p<0.05) from TGFβ3 treated cells.
Figure 28: Comparative expression of phosphorylation of AKT$^{\text{S473}}$ in prostate cell lines. Total (PAN) AKT and β-actin were used as loading controls. Data is presented from a representative experiment.
Figure 29: Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT ser473 in PC3 cells after treatment with the two isoforms over multiple time points. Total (PAN) AKT and β-actin were used as loading controls. Data is presented from a representative experiment.
Figure 30: Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT<sup>ser473</sup> in PC3 cells after treatment with the two isoforms for 15 and 60 min. Total (PAN) AKT and β-actin were used as loading controls.
Figure 31: Band density analysis of pAKT<sup>ser473</sup> in PC3 cells after treatment with TGFβ isoforms for 15 or 60 min. Each band density was normalized by density of β-actin bands. Each bar represents Mean ± SE from three independent experiments. *Significantly different (p<0.05) from untreated controls.
Figure 32: Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT

DU145 cells

Control TGFβ1 TGFβ3 IGF-1

pAKT

AKT

β-actin

Figure 32: Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT\textsuperscript{ser473} in DU145 cells after treatment with the two isoforms for 15 and 60 min. Total (PAN) AKT and β-actin were used as loading controls.
Figure 33: Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT$\text{ser}^{473}$ in LNCaP cells after treatment with the two isoforms for 15 and 60 min. Total (PAN) AKT and β-actin were used as loading controls.
Figure 34: Effects of TGFβ1 and TGFβ3 on phosphorylation of AKT\textsuperscript{ser473}, in PC3 cells in the presence of inhibitor of TGFβRI (SB431542). PC3 cells were incubated with specific inhibitor for 30 min followed by treatment with TGFβ1 or TGFβ3 (5 ng/ml) for 60 min. Total (PAN) AKT and β-actin were used as loading controls.
Figure 35: Effects of TGFβ1 and TGFβ3 on phosphorylation of AKT \textsuperscript{ser473} in PC3 cells in the presence of inhibitor of Smad3 (SIS3). PC3 cells were incubated with specific inhibitor for 30 min followed by treatment with TGFβ1 or TGFβ3 (5 ng/ml) for 60 min. Total (PAN) AKT and β-actin were used as loading controls.
Figure 36: Effects of TGFβ1 and TGFβ3 on phosphorylation of AKT
in PC3 cells in the presence of inhibitor PI3-Kinase inhibitor (LY294002). PC3 cells were incubated with specific inhibitors for 30 min
followed by treatment with TGFβ1 or TGFβ3 (5 ng/ml) for 60 min. Cells treated with EGF (5 ng/ml) were
used as positive controls. Total (PAN) AKT and β-actin were used as loading controls.
Figure 37: Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells. PC3 cells were incubated with TGFβ1 or TGFβ3 (5 ng/ml) for 60 min. Total Smad2/3 and β-actin were used as loading controls.
Figure 38: Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells in the presence of inhibitor of TGFβRI (SB431542). PC3 cells were incubated with specific inhibitor for 30 min followed by treatment with TGFβ1 or TGFβ3 (5 ng/ml) for 60 min. Total Smad2/3 and β-actin were used as loading controls.
Figure 39: Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells in the presence of inhibitor of Smad3 (SIS3). PC3 cells were incubated with specific inhibitor for 30 min followed by treatment with TGFβ1 or TGFβ3 (5 ng/ml) for 60 min. Total Smad2/3 and β-actin were used as loading controls.
Figure 40: Effects of TGFβ1 and TGFβ3 on phosphorylation of Smad2 and Smad3 in PC3 cells in the presence of inhibitor of PI3-Kinase inhibitor (LY294002). PC3 cells were incubated with specific inhibitor for 30 min followed by treatment with TGFβ1 or TGFβ3 (5 ng/ml) for 60m. Total Smad2/3 and β-actin were used as loading controls.
Figure 41: The effects of TGFβ1 or TGFβ3 on the phosphorylation of ERK, a downstream target of the Mitogen-Activated Protein Kinase Pathway. PC3 cells were treated with TGFβ1 or TGFβ3 (5ng/ml) for 60 min in the presence or absence of MAPK inhibitor PD98059 and probed for pERK. Total ERK and β-actin were used as loading controls.
Figure 42: The effects of MAPK inhibitor PD98059 on the TGFβ1 or TGFβ3 induced phosphorylation of AKT<sup>Thr473</sup> in PC3 cells. PC3 cells were incubated with specific inhibitor for 30 min followed by treatment with TGFβ1 or TGFβ3 (5 ng/ml) for 60 min. Total (PAN) AKT and β-actin were used as loading controls.
Figure 43: The effect of TGFβ1 (1 ng/ml) and TGFβ3 (1 ng/ml) on migration of PC3 cells in the presence of inhibitor of MAPK (PD98059). Scratch wound assays were performed following treatment with TGFβ isoforms for 48h in the presence or absence of specific inhibitors. Pictures were taken at 10x magnification.
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