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Activator Protein-1 in Transforming Growth Factor-Beta Effects on Prostate Cancer Cell Proliferation, Migration, and Invasion

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ABSTRACT

BIOLOGICAL SCIENCES

BARRETT, CACHÉTNE S. X. B.S. CLARK ATLANTA UNIVERSITY, 2008

ACTIVATOR PROTEIN-1 IN TRANSFORMING GROWTH FACTOR-BETA EFFECTS ON PROSTATE CANCER CELL PROLIFERATION, MIGRATION, AND INVASION

Committee Chair: Shafiq Khan, Ph.D.

Dissertation dated May 2017

Activator Protein-1 (AP-1) family plays a central role in the transcriptional regulation of many genes that are associated with cell proliferation, migration, metastasis, and survival. Transforming growth factor beta (TGF-β) is a multifunctional regulatory cytokine that regulates many aspects of cellular function, including cellular proliferation, migration, and survival. This study investigated the role of FOS proteins in TGF-β signaling in prostate cancer cell proliferation, migration, and invasion. DU145 and PC3 prostate cancer cells were exposed to TGF-β1 at varying time and dosage, RT-PCR, western blot and immunofluorescence analyses were used to determine TGF-β1 effect on FOS mRNA and protein expression levels as well as FosB subcellular localization. Transient silencing of FOS protein was used to determine their role in cell proliferation, migration and invasion. Our data showed that FOS mRNA and proteins were
differentially expressed in human prostate epithelial (RWPE-1) and prostate cancer cell lines (LNCaP, DU145, and PC3). TGF-β1 induced the expression of FosB at both the mRNA and protein levels in DU145 and PC3 cells, whereas cFos and Fra1 were unaffected and Fra2 protein expression increased in PC3 cell only. Immunofluorescence analysis showed an increase in the accumulation of FosB protein in the nucleus of PC3 cells after treatment with exogenous TGF-β1. Selective knockdown of endogenous FosB by specific siRNA did not have any effect on cell proliferation in PC3 and DU145 cells. However, basal and TGF-β1-and EGF- induced cell migration was significantly reduced in DU145 and PC3 cells lacking endogenous FosB. TGF-β1- and EGF-induced cell invasion were also significantly decreased after FosB knockdown in PC3 cells. Transient silencing of Fra2 resulted in decrease in cell proliferation in PC3 cells whereas transient silencing of cFos resulted in an increase in cell number in PC3 cells. And lastly, TGF-β1 reduced FosB: cJun dimerization; cJun knockdown increased cell migration in PC3 cells and its overexpression decreased cell migration in DU145 cells. Our data suggest that FosB is required for migration and invasion in prostate cancer cells. We also conclude that TGF-β1 effect on prostate cancer cell migration and invasion may be mediated through the induction of FosB
ACTIVATOR PROTEIN-1 IN TRANSFORMING GROWTH FACTOR-BETA EFFECTS ON PROSTATE CANCER CELL PROLIFERATION, MIGRATION, AND INVASION

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

BY

CACHÉTNE SAMOIS X. BARRETT

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

MAY 2017
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<td>AMH</td>
<td>Anti-Müllerian Hormone</td>
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<td>ANOVA</td>
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<td>AP-1</td>
<td>Activator Protein 1</td>
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<td>ATF</td>
<td>Activating Transcription Factor</td>
</tr>
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<td>bp</td>
<td>base pair</td>
</tr>
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<td>BMPs</td>
<td>Bone Morphogenetic Proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>Basic Leucine Zipper Domain</td>
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<td>4’-6-Diamidino-2-phenylindole</td>
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<td>DNA Binding Domain</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Dinucleotide Triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>ERK1/2</td>
<td>Extra-cellular Signal Regulated Kinases</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>Acronym</td>
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<tr>
<td>Fra1</td>
<td>Fos-Like Antigen 1</td>
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<td>Fra2</td>
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<td>FOS</td>
<td>Finkel-Biskis-Jinkins murine osteogenic sarcoma virus</td>
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<td>GDF</td>
<td>Growth and Differentiation Factor</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine Triphosphate Hydrolytic Enzymes</td>
</tr>
<tr>
<td>HIPK2</td>
<td>Homeodomain Interacting Protein Kinase 2</td>
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<tr>
<td>IE</td>
<td>Immediate Early</td>
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<tr>
<td>IgG-HRP</td>
<td>Immunoglobulin Horseradish Peroxidase</td>
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<tr>
<td>JNK</td>
<td>JUN N-Terminal Kinases</td>
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<td>KSFM</td>
<td>Keratinocyte Serum Free Medium</td>
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<td>LAP</td>
<td>Latency-associated Peptide</td>
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<td>Leucine Zipper Domain</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated Protein Kinases</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MIS</td>
<td>Müllerian Inhibiting Substance</td>
</tr>
<tr>
<td>MMP1</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>MTS</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium inner salts</td>
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<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target of Rapamycin</td>
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<tr>
<td>NCBI</td>
<td>National Center for Bioinformatics</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor κB</td>
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NIH  National Institutions of Health
NLM  National Library of Medicine
PAI1  Plasminogen Activator Inhibitor 1
PBS  Phosphate Buffered Saline
PCa  Prostate Cancer
PCR  Polymerase Chain Reaction
PI3K  Phosphoinositide-3-Kinases
PSA  Prostate Specific Antigen
PTEN  Phosphatase and Tensin Homolog
PVDF  Polyvinylidene Difluoride
RAC1  Ras-Related C3 Botulinum Substrate 1
RT  Reverse Transcriptase
Rh  recombinant human
Rho  Rhodopsin
RNA  Ribonucleic Acid
RPMI  Roswell Park Memorial Institute
SARA  Smad Anchor for Receptor Activation
SDS-PAGE  Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Smad  Mothers against Decapentaplegic
SOX4  SRY-Related HMG-Box 4
TAD  Transactivation Domain
TβRI  Transforming Growth Factor-β Type I Receptor
TβRII  Transforming Growth Factor-β Type II Receptor
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
</tr>
<tr>
<td>TRE</td>
<td>TPA-Response Element</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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Background and Significance

Cancer is a key health issue across the world, causing substantial patient morbidity and mortality. Prostate cancer (PCa.) is the most common malignant disease in males and the second leading cause of cancer related deaths in men in developed countries. Prostate cancer accounts for 28% of cancer diagnoses and 10% of cancer deaths in men. Patients with localized prostate cancer have a relatively long-term survival due to great advances in surgical resection and adjuvant therapy. However, patients with advanced, especially metastatic bone disease are often associated with a poor prognosis. Studies have shown that around 30% of these patients will develop distant metastases within five years of diagnosis, even after radical surgery. Bone metastases occur in more than 80% of cases of advanced-stage prostate cancer, which confers a high level of morbidity, with a five-year survival rate of 25% and a median survival of approximately 40 months. Patient prognosis is tightly linked with metastatic dissemination of the disease to distant sites, with metastatic diseases accounting for a vast percentage of cancer patient mortality. A critical barrier for the successful prevention and treatment of recurrent prostate cancer is detection and
eradication of metastatic and therapy-resistant disease. While advances in this area have been made, the process of cancer metastasis and the factors governing cancer spread and establishment at secondary locations are still poorly understood.

With rare exceptions, the natural history of all types of tumors is known to progress from localized indolent stages to aggressive metastatic stages. Recent advancements in biomarker research have made significant progresses to help prediction of cancer progression and disease outcome. However, the molecular mechanisms behind tumor progression remain elusive. Transforming growth factor beta (TGF-β) is known to inhibit cell cycle in benign cells but promote progression and metastasis in cancer cells (Figure 1), a phenomenon known as TGF-β paradox. Although there are numerous articles with different approaches tackling this topic, to date, a logical explanation leading to TGF-β paradox remains elusive and is accepted as a scientific mystery.
Figure 1: The Role of TGF-β in Cancer; In normal and premalignant cells, TGFβ enforces homeostasis and suppresses tumor progression directly through cell-autonomous tumor-suppressive effects (cytostasis, differentiation, apoptosis) or indirectly through effects on the stroma (suppression of inflammation and stroma-derived mitogens). However, when cancer cells lose TGFβ tumor-suppressive responses, they can use TGFβ to their advantage to initiate immune evasion, growth factor production, differentiation into an invasive phenotype, and metastatic dissemination or to establish and expand metastatic colonies.

The TGF-β superfamily comprises TGF-β1–3, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), Nodal, activins/inhibins, Müllerian inhibiting substance (MIS)/anti-Müllerian hormone (AMH), and Lefty. These ligands were initially grouped accordingly to the functional roles observed following their
original identification. As it becomes clear that most ligands play multiple functions depending on cell type, developmental stage, or tissue conditions, they are now classified by sequence similarity and the downstream pathway they activate. Each family member has an overall basic structure, in which inactive forms are produced with an N-terminal secretion peptide and a large pro-peptide domain known as latency-associated peptide (LAP). Cleavage of the pro-peptide domain by pro-protein convertases releases a mature domain at the C-terminus, which eventually dimerizes. The pro-peptide domain has major regulatory roles. It influences protein stability and functions as chaperone during secretion, also mediating diffusion through interactions with the extracellular matrix and inhibiting the active peptide form even after cleavage. Secreted cytokines of the TGF-β family are found in all multicellular organisms and implicated in regulating fundamental cell behaviors such as proliferation, differentiation, migration and survival. Virtually, all types of cells produce and are sensitive to TGF-β superfamily members. TGF-β is a pleiotropic factor with several different roles in health and disease. As stated earlier, in cancer, TGF-β plays a paradoxical role, it represses epithelial tumor development in the early steps of tumorigenesis, while in advanced stages it can stimulate tumor progression. In epithelial cells, TGF-β has anti-proliferative and apoptotic roles which enable it to reverse local mitogenic stimulation in the pre-tumoral stage in the epithelium. During the advance of tumorigenesis, carcinoma cells acquire resistance to the proliferative inhibition and apoptosis induced by TGF-β. Several mechanisms have been described to explain the changes in the response of tumor cells to TGF-β1, including mutations in the machinery of TGF-β signaling.
Mammals express three genetically distinct isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) with high homology. The TGF-β1, β2, and β3 genes are located on chromosomes 19q13, 1q41, and 14q24, respectively. TGF-β initiates signaling by binding to cell-surface serine/threonine kinase receptors types I and II (TβRI and TβRII, respectively), which form a heteromeric complex in the presence of the dimerized ligand (Figure 2).

**Figure 2.** Schematic Diagram of TGF-β Signaling from Cell Membrane to the Nucleus
The arrows indicate signal flow and are color coded: orange for ligand and receptor activation, gray for Smad and receptor inactivation, green for Smad activation and formation of a transcriptional complex, and blue for Smad nucleocytoplasmic shuttling. Phosphate groups and ubiquitin are represented by green and red circles, respectively.

Binding of TGF-β to TβRII leads to the phosphorylation of TβRI, thus activating its kinase domain. When the receptor complex is activated, it phosphorylates and stimulates the cytoplasmic mediators, Smad2 and Smad3. The phosphorylation of
Smad2/3 releases them from the inner surface, where they are specifically retained by Smad anchor for receptor activation (SARA). Further on, Smad2/3 forms a heterotrimeric complex with the common Smad4, which is then translocated into the nucleus where, in collaboration with other transcription factors, it binds and regulates promoters of different target genes.\textsuperscript{28-29, 33} In addition to Smad signaling, TGF-\(\beta\)1 may activate other intracellular signaling pathways, called non-Smad pathways, such as mitogen-activated protein kinases (MAPK): ERK1,2, JNK and p38; PI3K (phosphoinositide 3-kinase)/AKT1/2 and mTOR, known as cell survival mediators; NF-\(\kappa\)B (nuclear factor \(\kappa\)B), Cyclooxygenase-2, and prostaglandins; and the small GTPase proteins Ras, Rho family (Rho, Rac1 and Cdc42), among others.\textsuperscript{29, 34-35}

TGF-\(\beta\) overproduction is a universal event in cancer cells and is a poor prognostic marker.\textsuperscript{29, 36-40} The mechanism, through which TGF-\(\beta\) regulates its own production, is different between benign and cancer cells. Under the normal physiological conditions, the level of TGF-\(\beta\) is tightly regulated within the microenvironment through a negative feedback loop to maintain a relatively constant level of TGF-\(\beta\). Too little or too much TGF-\(\beta\) will have an unfavorable consequence.\textsuperscript{29, 41-43} However, this principle does not apply to cancer. Cancer cells, especially the advanced cases, are capable of evading the immune surveillance program due to the well-known phenomenon of auto-induction of TGF-\(\beta\) by cancer cells,\textsuperscript{29} resulting in an elevated TGF-\(\beta\) in the microenvironment through a positive feedback loop.\textsuperscript{29, 44} As a result, there is an accumulation of TGF-\(\beta\) in the microenvironment, which further promotes tumor progression.\textsuperscript{29, 36, 39, 45} According to the current literature and experimental evidence, TGF-\(\beta\) is a potent ligand that regulates
carcinoma initiation, progression and metastasis through a broad and complex spectrum of interdependent interactions.\textsuperscript{30} The knowledge about the mechanisms involved in TGF-\(\beta\) signal transduction has allowed a better understanding of the disease pathogenicity as well as the identification of several molecular targets with great potential in therapeutic interventions.\textsuperscript{28}

It is also becoming apparent that TGF-\(\beta\) signaling intersects with several transcription factors and regulators, such as GL1, SOX4, Tieg3/Klf11, Id, and AP-1 proteins.\textsuperscript{46-50} Many studies have implicated Activator Protein-1 (AP-1) proteins in TGF-\(\beta\) signaling.\textsuperscript{50-52} Numerous studies have characterized the differential expression of specific genes in response to TGF-\(\beta\), revealing a common link in the ability of TGF-\(\beta\) to regulate many of these genes through the functions of the AP-1 family of transcription factors.\textsuperscript{53} The ability of TGF-\(\beta\) to induce the expression of several genes, including PAI-1, clusterin, monocyte chemoattractant protein-1 (JEyMCP-1), type I collagen, and TGF-\(\beta\) itself depends on specific AP-1 DNA-binding sites in the promoter regions of these genes.\textsuperscript{53-58} Furthermore, TGF-\(\beta\)-mediated transcriptional activation of several of these genes requires AP-1 proteins.\textsuperscript{53,54,56-58} Intriguingly, the expression of many AP-1 proteins themselves is induced as an early response to TGF-\(\beta\) in a cell type-specific manner.\textsuperscript{53,59-60} It has been demonstrated that this induced expression of particular AP-1 family members is involved in TGF-\(\beta\)-mediated regulation of subsequent target genes.\textsuperscript{53,58} In addition, genetic studies of TGF-\(\beta\) signaling in \textit{Drosophila melanogaster} reveal a direct overlap between AP-1 and TGF-\(\beta\) signaling and suggest an evolutionarily conserved convergence of these pathways.\textsuperscript{53,61} Together, these studies demonstrate a link between TGF-\(\beta\)
signaling and AP-1 proteins in the TGF-β-regulated expression of various genes. The molecular mechanisms responsible for the TGF-β-mediated transcriptional activation of these genes are just beginning to be elucidated.55

The AP-1 family consists of dimeric protein complexes composed of different Jun proteins (c-Jun, JunB, and JunD) and four FOS proteins (c-Fos, FosB, Fra1, and Fra2). These proteins form Jun-Jun homodimers and Jun-Fos heterodimers and bind to the 12-O-tetradecanoylphorbol-13-acetate response element, TGACTCA palindromic sequence, in the promoters of target genes.50, 62-63 AP-1 proteins have been shown to be involved in cell proliferation, inflammation, differentiation, apoptosis, wound healing, and carcinogenesis.50, 64-65 The transcription factor AP-1 converts extracellular signals into changes in the expression of specific target genes which harbor AP-1-binding site(s) in their promoter and enhancer regions. AP-1 proteins are certainly important participants and possibly determinant factors in the diverse mechanisms that contribute to the development of human cancers, although casual proof for these functions is yet to be established. The fact that AP-1 is positioned as a signal responsive transcription factor complex at the end of a large number of signaling cascades, makes it very likely that AP-1 components could provide the missing link between growth factor signaling and the cell cycle machinery.

c-Fos and c-Jun have been extensively characterized and studied following their identification as the original components of AP-1.66 Accordingly, a number of researchers assume that what 'has been mostly worked for Jun' (and Fos) 'serves as a useful paradigm for its other family members.66-67 However, as indicated by differences in the kinetic rates
of induction, modes of regulation and transactivation properties of each of these genes, this view can only hold true to a point. Ultimately, each FOS and JUN gene member should be investigated individually to gain a better understanding of the overall function of AP-1. Previous studies in our laboratory have elucidated the roles of JUN proteins in prostate cancer cell proliferation giving rise to this research project’s focus on understanding the individual roles of FOS family members in prostate cancer cell development and progression.

AP1 mediation of cellular response to growth factors is suggested by the observation that deregulated expression of certain members of the Fos and Jun families results in the neoplastic transformation of susceptible cells. Studies revealed that AP-1 DNA binding activity is not a single transcription factor but a dimer. Different AP-1 subunits display functional diversity in a cell-type specific manner and different subsets of AP-1 proteins have differing dimerization requirements. cJun, for example, can homo- and heterodimerize while cFos can only form heterodimers. These AP-1 dimers regulate a wide variety of cellular processes including the immune response, cell proliferation, apoptosis, and tumorigenesis. Different dimer compositions showed promoter-specific differences in activating transcription of reporter genes. The role of AP-1 proteins has been widely studied; however, discerning the distinct roles of individual dimer compositions remains challenging. This project arose from studies indicating that JunD plays an essential role in the proliferation of prostate cancer epithelial cancer cells. It has also been suggested by several studies that expression of AP-1 proteins is associated with a more aggressive clinical outcome in prostate cancer
patients.\textsuperscript{50, 74-75} Most of these studies have, however, focused on the expression and/or function of activated AP-1 complex containing c-Jun and c-Fos.\textsuperscript{50, 76} Consequently, the specific functions of individual AP-1 family members and various homo- and heterodimers in the regulation of specific cellular processes remain largely unknown.\textsuperscript{50} Studies also showed that Jun-Fos dimers that have similar DNA binding specificities can differ in transcriptional activity due to non-conserved domains located outside the bZIP region that can be regulated by phosphorylation. It is therefore plausible that AP-1 dimers of different composition execute specific cellular programs.\textsuperscript{73} With this in mind it is our desire to decipher the individual roles of the FOS family members in prostate cancer cell proliferation, migration and invasion and in the process determine which FOS family member could be the partner for JunD and is essential for prostate cancer cell proliferation.

cFos has been found to induce differentiation of certain cell types,\textsuperscript{66, 77} and to also transform cells following its overexpression and removal of part of its 3' untranslated region.\textsuperscript{66, 78} cFos may repress its own transcriptional activation and the activation of other genes such as Egr-1,\textsuperscript{66, 79} in addition to trans-activating the expression of genes like collagenase and Fra-1.\textsuperscript{66, 80-81} In oral cancer it has been observed that cFos/JunD heterodimer together showed higher transcriptional activity than JunD/JunD homodimers.\textsuperscript{73, 82} Therefore, it was speculated that JunD/JunD homodimer formation might prevent the precancerous cells entering into cancerous condition, but as soon as participation of cFos member takes place in AP-1 complex formation, the precancerous cells are pushed further in the aggressive cancerous condition.\textsuperscript{82} High cFos protein levels
significantly correlate with high MMP9 expression and both proteins are weakly associated with a positive nodal status in breast cancer patients.\textsuperscript{83-85} cFos expression also correlated with cyclin E which is another indicator of unfavorable outcome in breast cancer patients.\textsuperscript{85-86} In transient transfection experiments, cFos overexpression led to a weak (1.65–1.81-fold) stimulation of migration and invasion through matrigel membranes in MCF7 cells, whereas in MDA-MB231 cells, the invasive potential was non-significantly enhanced independent of cell migration.\textsuperscript{85} These data point to a stimulating effect of cFos on cell invasion and are in accordance with its oncogenic function observed in various cell systems.\textsuperscript{85, 87}

FosB expression is statistically associated with a well-differentiated, estrogen- and progesterone- receptor positive phenotype of breast cancer samples, FosB expression was associated with higher levels of the cell-cycle inhibitor Rb and low expression of the proliferation marker Ki67.\textsuperscript{85-86} FosB expression was statistically associated with the collagenase MMP1, which also correlates with a positive estrogen receptor status, and surprisingly, with a nodal-negative tumor type.\textsuperscript{85} In invasion assays with breast cancer cell lines, FosB had no significant influence on invasion of MDA-MB231 cells, whereas in MCF7 cells, the number of invasive cells was significantly increased after transient transfection with FosB expression vectors.\textsuperscript{85} Yet, the stimulating effect was even stronger in control inserts without matrigel membrane indicating that in MCF7 cells, FosB stimulates cell migration through the pores of the insert bottom, whereas the relative number of invasive cells is not increased.\textsuperscript{85} These data suggest that in mammary carcinomas, FosB is probably not involved in tumor invasion.\textsuperscript{85} Mice studies reveal that
mice lacking FosB develop normally but display a profound nurturing defect whereas, overexpression of ΔFosB (an alternative spliced form of FosB which lacks transactivation activity but binds JUN proteins and DNA with similar efficiency as FosB) interferes with normal cell differentiation.88

Like other AP-1 subunits, Fra1 has been recently linked to multiple cancers, including breast, bladder, colon and esophagus cancers and head and neck squamous cell carcinoma.70 Fra1 is highly expressed in many epithelial cancers including squamous cell carcinoma of the skin (cSCC) and head and neck squamous cell carcinoma (HNSCC). However, the functional importance and the mechanisms mediating Fra1 function in these cancers are not fully understood.70 While c-Jun was required for the expression of the G1/S phase cell cycle promoter CDK4, Fra1 was essential for AKT activation and AKT-dependent expression of CyclinB1, a molecule required for G2-M progression.70 Exogenous expression of a constitutively active form of AKT rescued cancer cell growth defect caused by Fra1-loss.70 Additionally, Fra1 knockdown markedly slowed cell adhesion and migration, and conversely expression of an active Fra1 mutant (Fra1DD) expedited these processes in a JNK/c-Jun-dependent manner.70 Fra1 only weakly transforms rat embryo fibroblasts and causes no overt morphological transformation of rat embryo fibroblasts.66, 81 The weak transforming potential of Fra-1 is due to a lack of a C-terminal transactivation domain,66 and so Fra-1 tends to repress the expression of IE genes,66, 79 particularly in combination with JunB and c-Jun.66, 89 However, when complexed with JunD, Fra-1 may stimulate AP-1-dependent transcription.66, 89 Fra-1 expression is strong in highly invasive cell lines like MDA-MB231, but negative or weak
in more differentiated breast cancer cell lines (MCF7, T47D).\textsuperscript{85} In clinical tumor tissues, only very low Fra-1 protein levels were found by Western blot analysis.\textsuperscript{85} Prior study on mammary carcinomas, showed Fra-1 expression was significantly associated with a poorly differentiated, estrogen-receptor negative phenotype and strong Ki67 and Cyclin E expression.\textsuperscript{85-86}

Invasion assays showed a significant increase of the invasive potential after forced Fra-2 overexpression in MDA-MB231, but not MCF7 cells. In clinical tumor tissue, total Fra-2 expression was significantly associated with high Cyclin D1 and Cyclin E expression.\textsuperscript{85-86} Fra-2 protein levels and expression of the more slowly migrating, phosphorylated Fra-2 bands correlated with high expression of MMP9, PAI-1 (both indicators of unfavorable outcome) and the PAI-1/uPA and PAI-1/tPA complexes.\textsuperscript{85} In addition, those same studies showed a significantly higher frequency of recurrence in patients with high levels of the phosphorylated Fra-2.\textsuperscript{85}

**Rationale**

Prostate cancer (PCa) is the most diagnosed cancer and the second leading cause of cancer death among men in the United States.\textsuperscript{90-91} TGF-\(\beta\) was originally described as being one of the most potent polypeptide growth inhibitors isolated from natural sources.\textsuperscript{92} TGF-\(\beta\) is a secreted cytokine that acts as a major anti-proliferative factor in the initial stages of prostate cancer, whereas in the advanced stages of prostate cancer, it acquires pro-oncogenic and pro-metastatic properties.\textsuperscript{50, 93-95} Deregulation of TGF-\(\beta\) expression or signaling has been implicated in the pathogenesis of a variety of diseases, including cancer. There is growing evidence that in the later stages of cancer
development, TGF-β is actively secreted by tumor cells and does not merely act as a bystander but rather contributes to the cell growth, invasion, and metastasis and decreases host-tumor immune response. Activator protein-1 (AP-1) was one of the first transcription factors to be identified, but its physiological functions are still being unraveled. AP-1 activity is induced by a plethora of physiological stimuli and environmental insults such as growth factors, cytokines, tumor-promoters and UV-irradiation. In turn, AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. Although AP-1 proteins share a high level of sequence and function homology, they exhibit distinct expression patterns and differ in their transcriptional and biological activities.

**Research Question**

Previous studies have shown different effects of TGF-β1 on proliferation of different prostate cancer cell lines; TGF-β inhibits proliferation of DU145 cells but has no effect on proliferation of PC3 cells in the presence of functional TGF-β receptors and Smad signaling, indicating differences in signaling mechanisms in two cell lines downstream of receptor-dependent Smad activation that are responsible for differential effects of TGF-β on cell proliferation. Other intracellular proteins influence TGF-β effects, and studies have shown that TGF-β signaling interacts with several transcription factors including AP-1. In prostate cancer, expression of AP-1 proteins has been associated with disease recurrence and more aggressive clinical outcome. Previous studies have shown that without JunD prostate cancer cells do not proliferate, those same studies indicated that JunB and cJun knockdown had no effect on cell
proliferation in prostate cancer cells; considering the fact that AP-1 proteins must function as dimers, neither JunB or cJun knockdown affected prostate cancer cell proliferation, and that JUN proteins are able to both homo and heterodimerize, the question becomes: which AP-1 protein could potentially be a partner for JunD and is required for prostate cancer cell proliferation to occur? In an attempt to answer this question there are some other primary queries that must be answered. Therefore, this project is designed to determine: 1) Are FOS family proteins expressed in prostate cancer cell lines and is their expression being regulated by TGF-β? 2) Does the presence of TGF-β influence FOS protein subcellular localization and dimerization? 3) Are FOS proteins involved in TGF-β effects on prostate cancer cell proliferation, migration, and invasion?

**Hypothesis**

With these questions in mind we hypothesize that JunD is essential for prostate cancer cell proliferation; therefore, without JunD, prostate cancer tumors would not develop. In addition, JunD does not work alone; it requires another AP-1 partner such as a member of JUN or FOS family to exert its effects on cancer cell proliferation and tumor development. JUN and FOS proteins share extensive homology within the leucine zipper and basic domains. However, despite their homology, these proteins display different transcriptional activity. Therefore, we also hypothesize that the FOS proteins contribute distinct functions towards the activity of the AP-1 heterodimers and that TGF-β1 could induce the expression of FOS proteins and these proteins could play an important role in prostate cancer cell proliferation, migration, and invasion.
Specific Aims

In an attempt to test the above hypotheses and answer the research questions discussed above, the following aims have been addressed:

**Specific Aim 1:** To determine the basal expression of FOS family members (FosB, cFos, Fra1, and Fra2) mRNA and protein in normal prostate epithelial cells and prostate cancer cells, and determine if their expression is regulated by TGF-β1.

**Rationale:** The transcription factor AP-1 is activated in response to an incredible array of stimuli, including mitogenic growth factors, inflammatory cytokines, growth factors of the TGF-β family, UV and ionizing irradiation, cellular stress, antigen binding, and neoplastic transformation.\(^{110}\) The AP-1 transcription factor consists of a large set of dimer combinations formed between the Jun, Fos and ATF families of proteins.\(^{111-113}\) AP-1 activity converts extracellular signals into changes in gene expression patterns through the binding of AP-1 dimers to specific target sequences located within the promoters and enhancers of target genes. These targets include genes important for regulating many biological processes including proliferation, differentiation, apoptosis and transformation.\(^{110, 112, 114-115}\) AP-1 activity functions in a hierarchy: dimerization of AP-1 proteins is required for DNA binding that in turn leads to transcriptional activity.\(^{112}\) TGF-β acts as a tumor suppressor in early stages of the tumor development, and then switches to a tumor promoter in later stages of tumor development\(^{52, 52-53}\) through undefined mechanisms. Many TGF-β1 regulated genes have AP-1 binding sites\(^{53-58}\) and AP-1 themselves are being regulated by TGF-β1.\(^{53, 59-60}\) Previous studies in our lab showed that all JUN family members are constitutively expressed in various prostate cell lines at the
mRNA level but exhibited differences in the levels of JUN proteins in various cell lines, indicating differential regulation of proteins in different cell lines. These results also showed that TGF-β induces a reduction in JunD protein levels in DU145 cells but not in PC3 cells. Because JunD is required for cell proliferation, these results suggest that reduction of JunD levels in DU145 cells in response to TGF-β may lead to reduction in cell proliferation in these cells. On the other hand, the lack of TGF-β effects on PC3 cell proliferation may be due to their resistance to TGF-β-induced reduction of JunD levels. Previous studies have revealed the response of JUN proteins to TGF-β1 stimulation and even demonstrated that JunD is essential for TGF-β1-induced effects on prostate cancer cell proliferation; however, it is still unclear whether FOS proteins are influenced by TGF-β1 and could be essential for TGF-β effects on prostate cancer cell growth and progression.

Experimental Design: The steady state expression of FOS mRNA and protein was determined using prostate and prostate cancer epithelial cells seeded in 10 cm dishes and allowed to grow for 48 hours; at which point the cells will be lysed and both RNA and protein will be extracted and analyzed for FOS mRNA and protein expression using reverse transcription polymer chain reaction (RT-PCR) and Western Blot respectively. Next, DU145 and PC3 prostate cancer cells lines will be seeded to 80% confluency in 6-welled plates and treated with 5ng/ml of exogenous TGF-β1 at varying time points (0-8 hours) as well as varying concentrations of TGF-β1 (0-10ng/ml) for four hours. The cells will be lysed and analyzed for mRNA and protein expression using RT-PCR and Western
Blot analyses. This should reveal the effects of TGF-β1 on FOS mRNA and protein expression.

**Specific Aim 2:** To determine the effects of FOS family members in TGF-β regulated cell proliferation, migration and invasion of prostate cancer cells.

**Rationale:** AP-1 subunits bind to a common DNA site, the AP-1-binding site. As the complexity of our knowledge of AP-1 factors has increased, our understanding of their physiological function has decreased.\(^{114}\) This trend, however, is beginning to be reversed due to the recent studies of gene-knockout mice and cell lines deficient in specific AP-1 components.\(^{114}\) Such studies suggest that different AP-1 factors may regulate different target genes and thus execute distinct biological functions.\(^{114}\) Also, the involvement of AP-1 factors in functions such as cell proliferation and survival has been made somewhat clearer as a result of such studies.\(^{114}\) In addition, there has been considerable progress in understanding some of the mechanisms and signaling pathways involved in the regulation of AP-1 activity.\(^{114}\) AP-1 activity is regulated in a given cell by a broad range of physiological and pathological stimuli, including cytokines, growth factors, stress signals and infections, as well as oncogenic stimuli.\(^{62}\) Regulation of net AP-1 activity can be achieved through changes in transcription of genes encoding AP-1 subunits, control of the stability of their mRNAs, posttranslational processing and turnover of pre-existing or newly synthesized AP-1 subunits, and specific interactions between AP-1 proteins and other transcription factors and cofactors.\(^{62}\) Previous studies in our lab using transfection of DU145 and PC3 prostate cancer cells with JunD siRNA caused a significant reduction in proliferation of both DU145 (82% inhibition, p < 0.05) and PC3 (71%
inhibition, *p* < 0.05) cells. On the other hand, knockdown of either c-Jun or JunB had no significant effect on cell proliferation in both cell lines. These results suggested that JunD is required for proliferation of both DU145 and PC3 cells. However, the specific roles of individual AP-1 family members in the development and progression of prostate cancer are still largely unknown.

**Experimental Design:** DU145 and PC3 cells lines will be seeded in 6-welled plates and grown to 80% confluency; the cells will be transiently transfected to knockdown the FOS proteins using siRNA specific for FosB, cFos, Fra1 and Fra2. The transfected cells will be used to perform proliferation, migration and invasion assays (MTT, transwell inserts and matrigel, respectively).

**Specific Aim 3:** To identify which AP-1 protein(s) dimerizes with JunD and is/are essential for regulating cell proliferation in prostate cancer cells.

**Rationale:** Recent studies using cells and mice deficient in individual AP-1 proteins have begun to shed light on their physiological functions in the control of cell proliferation, neoplastic transformation and apoptosis. The main characteristic of the AP-1 complexes in the cell is their heterogeneity in dimer composition. This heterogeneity is caused by the fact that multiple AP-1 sub-units can be expressed at the same time, including c-Jun, JunB, JunD, c-Fos, FosB, Fra1, Fra2, ATF2, ATFa and ATF3. The actual activities of JUN: FOS depend on the cell type, its differentiation state and the type of stimuli it has received. Earlier studies have shown that dimerization is a requirement for activation of AP-1 proteins and that AP-1 proteins form multiple homo- and heterodimers; and the composition of these dimers may dictate expression of specific
genes involved in specific biological responses. However, the specific roles of individual AP-1 family members in the development and progression of prostate cancer are still largely unknown. Few reports have shown the effects, if any, of TGF-β on AP-1 in prostate cancer.116-118

**Experimental Design:** DU145 and PC3 cells lines will be seeded in 6-welled plates and grown to 80% confluency; the cells will be transiently transfected to knockdown the FOS proteins using siRNA specific for FosB, cFos, Fra1 and Fra2. The transfected cells will be used to perform proliferation assays direct cell count, and MTT respectively.
Prostate and Prostate Cancer

The prostate, an androgen-regulated exocrine gland, is an integral part of the male reproductive system (Figure 3A) which has an essential function in sperm survival and motility.\textsuperscript{119} It is located immediately below the bladder and just in front of the bowel. Its main function is to produce fluid which protects and enriches sperm. In younger men, the prostate is about the size of a walnut (Figure 3B).
Figure 3: The Prostate A) Anatomy of the human prostate, B) Normal prostate compared to an enlarged prostate, C) Factors that promote prostate health, D) Factors that enhance prostate cancer risks

It is doughnut shaped as it surrounds the beginning of the urethra (the tube that conveys urine from the bladder to the penis). The nerves that control erections surround the prostate. Prostate cancer is usually one of the slower growing cancers. In the past, it was most frequently encountered in men over 70, and many of those men died of other causes before their prostate cancer could kill them. This led to the old saying “most men die with, not of, prostate cancer.” However, that is certainly not true today. Three developments have changed things considerably: 1) Men are living longer, giving the cancer more time to spread beyond the prostate, with potentially fatal consequences. 2) More men in their early sixties, fifties and even Forties are being diagnosed with prostate cancer. Earlier onset combined with the greater male life expectancy means those cancers have more time to spread and become life-threatening unless diagnosed and treated. 3) Prostate cancer in younger men often tends to be more aggressive and hence more life-threatening within a shorter time. Those diagnosed at a young age have a higher cause-specific mortality than men diagnosed at an older age, except those over age 80 years. Early-onset prostate cancer has a strong genetic component, which indicates that young men with prostate cancer could benefit from evaluation of genetic risk. Furthermore, although the majority of men with early-onset prostate cancer are diagnosed with low-risk disease, the extended life expectancy of these patients exposes them to long-term effects of treatment-related morbidities and to long-term risk of disease progression leading to death from prostate cancer.
Prostate cancer is the most diagnosed and the second leading cause of cancer deaths among American men (Figure 4).

According to American Cancer Society, 180,896 men will be diagnosed and 26,120 men will die of prostate cancer in US in 2016. Cancer statistics for 2016 indicates that apart from skin cancer prostate cancer is the most frequently diagnosed cancer in men. For reasons that remain unclear, the risk of prostate cancer is 70% higher in blacks than in non-Hispanic whites. With an estimated 26,120 deaths in 2016, prostate cancer is the second-leading cause of cancer death in men. Prostate cancer death rates have been decreasing since the early 1990s in men of all races/ethnicities, although they remain more than twice as high in blacks as in any other group. Overall, prostate cancer death rates decreased by 3.5% per year from 2003 to 2012 (American Cancer Society. Cancer Facts & Figures 2016. Atlanta: American Cancer Society; 2016). These declines are due to improvements in early detection and treatment. Early prostate cancer usually has no
symptoms. With more advanced disease, men may experience weak or interrupted urine flow; difficulty starting or stopping the urine flow; the need to urinate frequently, especially at night; blood in the urine; or pain or burning with urination. Advanced prostate cancer commonly spreads to the bones, which can cause pain in the hips, spine, ribs, or other areas. There are currently no practices that will guarantee prevention of prostate cancer; however, there are habits and life style practices that are encouraged as ways to reduce a man’s risk of acquiring the disease (Figure 3C). As well as some practices that could potentially increase a man’s risks of getting the disease (Figure 3D).

Early stage prostate cancer (Figure 5) which is localized in the prostate gland is treatable by surgery and radiation therapy and the prognosis in these patients is very good. Many of these treatments provide men with very little benefit in terms of life expectancy, but subject them to considerable harm. For instance, one in two is impotent, one in ten needs to wear pads because of urine leakage and one in ten has back passage problems. Currently, treating prostate cancer depends on: the stage of the cancer, the Gleason score, the level of prostate specific antigen (PSA) in the blood stream, the man’s age and general health, and the side effects of the treatments. According to the Prostate Cancer Foundation, treatments may include: image guided radiotherapy and intensity modulated radiotherapy, active surveillance, surgery, external beam radiotherapy, brachytherapy, hormone therapy, and high intensity focused ultrasound. Most of these treatments are specific for the localized cancer; once the cancer has escaped from the prostate, treatment becomes quite difficult and the patients’ quality of life is severely affected.
Figure 5: Stage I, Earliest stage, where the cancer is so small that it cannot be felt on rectal examination, but is discovered in a prostate biopsy or in prostate tissue that has been surgically removed to ‘unblock’ the flow of urine (as in a transurethral resection of the prostate – TURP). Stage II, The tumor can now be felt on rectal examination, but is still confined to the prostate gland and has not spread. Stage III, The tumor has spread outside the gland and may have invaded the seminal vesicles. Stage IV, The tumor has spread to involve surrounding tissues such as the rectum, bladder or muscles of the pelvis and lymph nodes.

Prostate cancers in later stages of disease metastasize (Figure 6) to other tissues and bone and pose a significant problem for treatment. Prostate cancer cells eventually break out of the prostate and invade distant parts of the body, particularly the bones and lymph nodes, producing secondary tumors, a process known as metastasis. Once the
cancer escapes from the prostate, treatment is possible, but “cure” as we know it becomes impossible. Death from prostate cancer results when cancer cells become metastatic after invading the lymph nodes and blood vessels and migrate to bone.\textsuperscript{122-123}

\textbf{Figure 6}: Transformed metastatic prostate cells escape from the prostate into blood vessels to eventually invade distant organs.

The most frequent sites of metastasis are lymph nodes and bone; 90% of patients who die of prostate cancer harbor bone metastases.\textsuperscript{124-125} Current treatments for metastatic disease are hormonal therapy and chemotherapy. Hormonal therapies are based on inhibition of biosynthesis and/or action of androgens.\textsuperscript{126-127} However, the cancer cells develop resistance to these treatments resulting in development of castration resistant or hormone refractory prostate cancers. There is no effective therapy for these cancers which are responsible for mortality in majority of patients.
Metastasis is the cause of most prostate cancer deaths,\textsuperscript{128} approximately 80\% of metastatic prostate cancers exhibit some degree of bone metastasis.\textsuperscript{129} The most typical locations of the metastases are pelvic lymphatic glands, bones and lungs, and very rarely it metastasizes into a testis.\textsuperscript{130} Bubendorf et al., in the series of 1589 patients with the prostate carcinoma, showed that 35\% of the patients had hematogenous metastases, mostly in bones (90\%), the lungs (46\%) and the liver (25\%), while the metastases in the testis were found only in 0.5\% of the cases.\textsuperscript{130-131}

\textit{Transforming Growth Factor Beta}

In mammals, TGF-β super family consists of over 30 structurally related proteins; these include 3 forms of TGF-β itself (TGF-β1, TGF-β2, and TGF-β3), 3 forms of Activins and over 20 Bone Morphogenetic Proteins (BMPs). These growth factors control a large range of cellular behavior\textsuperscript{132} including regulating cell growth, differentiation, and matrix production.\textsuperscript{133-135} TGF-β is a disulfide-linked homodimeric protein which is secreted as part of a complex consisting of two units of the large precursor segment of the TGF-β pro-polypeptide linked in a non-covalent association with the mature TGF-β dimer.\textsuperscript{136} This complex is "latent" in the sense that it does not bind to TGF-β receptors and therefore cannot exert any biological activities associated with TGF-β.\textsuperscript{136} The release under physiological conditions of active TGF-β from the latent complex may be a finely regulated event involving specific proteases.\textsuperscript{136}

The TGF-β isoforms is initially synthesized as a 75-kDa homodimer known as pro-TGF-β. Pro-TGF-β is then cleaved in the Golgi to form the mature TGF-β homodimer.\textsuperscript{137-138} These 25-kDa homodimers interact with latency-associated proteins to
form the small latent complex. In the endoplasmic reticulum, a single latent TGF-β binding protein forms a disulfide bond with the TGF-β homodimer to form the large latent complex, allowing for targeted export to the extracellular matrix. After export, the large latent complex interacts with fibronectin fibrils and heparin sulfate proteoglycans on the cell membrane. Eventually, the large latent complex localizes to fibrillin-rich microfibrils in the extracellular matrix, where it is stored until its activation; remaining biologically unavailable until its activation. Latent TGF-β is activated by several factors, including proteases, thrombospondin 1, reactive oxygen species, and integrins. These factors release mature TGF-β by freeing it from the microfibril-bound large latent complex. This occurs through liberation from latency-associated proteins, degradation of latent TGF-β binding protein, or modification of latent complex conformation.

The three mammalian isoforms of TGF-β are each encoded by different genes and share extensive homology (70-80% amino acid sequence identity). TGF-β signaling begins with the binding of activated TGF-β to specialized receptors on the cell membrane. There are three major classes of TGF-β receptor proteins (TGF-β receptors types I-III (abbreviated TβRI, TβRII, and TβRIII, respectively)). TβRI and TβRII are serine-threonine protein kinases. TβRII contains extra cellular ligand binding domain, and both TβRI/II contain a single transmembrane domain and (Figure 7) a cytoplasmic serine threonine kinase domain.
Figure 7: Schematic representation of TGF-β receptors

(A) TGF-β type I (TβR-I) and TGF-β type II (TβR-II) are single transmembrane protein serine/threonine kinases with two kinase inserts. The extracellular domains are rich in cysteine residues. The carboxyl terminal tail is shorter in the TβR-I compared to the TβRII. The glycine–serine rich (GS) domain, which regulates the receptor activation, and the L45 loop (an exposed nine-amino acid sequence between kinase subdomains IV and V), are only found in TβR-I. A comparison of amino acid sequences in L45 loop region between activin receptor-like kinase (ALK)1 and ALK5 is shown below. The two h strands (h4 and β5) that flank the L45 loop are shown as arrows.

(B) Endoglin and betaglycan (TGF-β type III receptors or TβR-III) are single transmembrane TGF-β accessory receptors that lack an enzymatic motif in their short intracellular domains. The percentages of identical amino acids in specific regions of the human endoglin and betaglycan are shown. Their cytoplasmic tails contain many serine and threonine residues and a putative PDZ domain at the last 3 Carboxy terminal residues. Proteolytic cleavage site and potential glycosaminoglycan (GAG) side chains that are rich in heparin sulfate and chondroitin sulfate are indicated. (This figure has been modified with permission from Miyazono et al.)

The kinase domains of the types I and II receptors share only 40% amino acid identity. TGF-β signal transduction requires the formation of a TβRI-TβRII heteromeric
complex. Once the ligand is activated, TGF-β signaling is mediated through SMAD and non-SMAD pathways to regulate transcription, translation, microRNA biogenesis, protein synthesis, and post-translational modifications. Although the downstream effects of TGF-β are heavily context dependent, its signaling is at least partially conserved in many cell types. In the canonical pathway, the TGF-β ligand binds to the TβRII that recruits the TβRI. These receptors dimerize and autophosphorylate serine/threonine residues, allowing for the phosphorylation of SMAD2 and SMAD3 by TβRI. The now activated SMAD proteins dissociate from the SMAD anchor for receptor activation (SARA) protein, hetero-oligomerize with SMAD4, and translocate to the nucleus, interacting with myriad transcriptional co-regulators and other factors to mediate target gene expression or repression. TβRIII (or betaglycan), a transmembrane proteoglycan that binds the TGF-β ligand, whose function is relatively unknown. Although TβRIII appears to lack a cytoplasmic signaling domain, it appears to have important roles in development, as well as in regulating TβRI and TβRII.

TGF-β also signals through a number of non-SMAD pathways, including p38 MAPK, p42/p44 MAPK, c-Src, m-TOR, RhoA, RAS, PI3K/Akt, protein phosphatase 2A (PP2A)/p70s6K, and JNK MAPK. Additionally, two studies have linked translational regulation to the cytostatic program governed by TGF-β. The first mechanism involves transcriptional activation of the translation-inhibiting protein eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) mediated by the SMAD signaling pathway, whereas the second relies on catalytic inactivation of the translation initiation factor eEF1A1 (eukaryotic elongation factor 1A1) by TβRI.
Both SMAD-dependent signaling and SMAD-independent signaling play multiple roles in homeostasis, particularly in the growth and plasticity of epithelial cells. SMAD-dependent TGF-β signaling induces growth arrest through a number of mechanisms, including control over various cyclin-dependent kinase inhibitors.\textsuperscript{138, 171-174} SMAD-independent mechanisms of TGF-β–induced apoptosis involve DAXX/HPK2 and transforming growth factor β associated kinase (TAK1)/TRAF6–dependent p38/JNK activation.\textsuperscript{138, 175-176}

**Transforming Growth Factor Beta (TGF-β) in Prostate Development and Function**

TGF-β signaling pathway is a key player in metazoan biology, and its misregulation can result in tumor development.\textsuperscript{171} TGF-β made its debut with the rise of the vertebrates. TGF-β evolved to regulate the expanding systems of epithelial and neural tissues, the immune system, and wound repair.\textsuperscript{171} Tied to these crucial regulatory roles of TGF-β are the serious consequences that result when this signaling pathway malfunctions, namely tumorigenesis.\textsuperscript{171} TGF-β superfamily was discovered in a hunt for autocrine factors secreted from cancer cells that promote transformation.\textsuperscript{22} However, it soon became clear that TGF-β and the related BMPs regulate diverse developmental and homeostatic processes and are mutated in numerous human diseases. Furthermore, TGF-β-superfamily members such as activins, Nodal, and growth differentiation factors (GDFs) were shown to control cell fate as a function of concentration, thus defining them as a key class of secreted morphogens.\textsuperscript{22} The actions of TGF-β are dependent on several factors including cell type, growth conditions, and the presence of other polypeptide growth factors.\textsuperscript{92} The TGF-β pathway has a complicated role in mediating the ability of
cells to participate negatively or positively in growth inhibition, proliferation, replication, invasion, metastasis, apoptosis, immune surveillance, and angiogenesis.

TGF-β-superfamily members are highly conserved across animals and comprise the largest family of secreted morphogens. TGF-β elicits context-dependent and cell specific effects that often appear conflicting, such as stimulation or inhibition of growth (Figure 8), apoptosis or differentiation. It is puzzling how such a diverse array of responses can result from binding of TGF-β ligand to a receptor complex that activates a seemingly straightforward signal transduction scheme dependent on shuttling SMAD transducer proteins from the receptor to the nucleus. This paradox is reflected in the clinic, where in early stage cancers, levels of TGF-β are positively associated with a favorable prognosis. Yet in advanced tumors, levels of TGF-β in the tumor
Figure 8: TGF-β in cancer progression: a.) Normally limits epithelial proliferation. b.) Loss of TGF-β inhibition leads to hyperplasia and supports transformation. c.) TGF-β growth response can enhances mesenchymal transition leading to invasion. d.) TGF-β suppresses T-cell response contributing to escape of immune recognition. e.) TGF-β displays angiogenic effects d.) TGF-β enhances extravasation and attachment of tumor cells to tissues of distant sites. e.) TGF-β promotes osteoclast response and bone remodeling in distant metastasis.

microenvironment are positively associated with tumor size, invasiveness, and dedifferentiation, making TGF-β a useful prognostic biomarker and predictor of recurrence after initial or failed therapy. 31, 138, 177-179

TGF-β expression has been studied in nearly all epithelial cancers including, prostate, breast, lung, colorectal, pancreatic, and skin cancers.31, 138 TGF-β acts on normal prostate epithelial cells and some prostate-cancer cells to inhibit proliferation and induce
apoptosis. TGF-β has also been shown to stimulate E-cadherin expression in prostate-cancer cells treated with an mTOR inhibitor. Prolonged incubation of cells with TGF-β results in the expression of actin-associated proteins (AAP’s), tropomyosin, and transgelin which promote the formation of stress fibers. Loss of TGF-β is also known to promote the formation of a less organized cytoskeleton. Studies have also shown that TGF-β can suppress or induce PTEN expression, depending on the Ras/ERK status. A Ras/ERK activated pathway facilitates TGF-β suppression of PTEN via a SMAD-4 independent signaling pathway however, when Ras/ERK is blocked, TGF-β induces PTEN expression through its classical SMAD-dependent pathway and stimulates a tumor suppressive response. Such a switch is echoed in pancreatic carcinoma cells, where both activated Ras and PI3K cause TGF-β to down regulate E-cadherin expression through a SMAD-independent pathway. In colorectal cancer higher TGF-β1 protein expression is associated with increasing T-stage and metastatic disease, indicating that TGF-β1 is of importance in tumor progression. Plasma TGF-β levels are markedly elevated in men with prostate cancer metastatic to regional lymph nodes and bone. In men without clinical or pathologic evidence of metastases, the preoperative plasma TGF-β level is a strong predictor of biochemical progression after surgery, presumably because of an association with occult metastatic disease present at the time of radical prostatectomy.

Because perturbation of the TGF-β signaling network has a variety of tumorigenic effects, its mechanisms must be studied further to identify novel points of convergence with other pathways and maximize both the clinical efficacy and tumor specificity of
future therapies. Through investigation of the TGF-β pathway and its relationship with other oncogenic factors in the tumor microenvironment, additional strategic points of convergence can be identified and exploited as a means to prevent or reverse tumor progression. As was already discussed, prostate cancer remains an important clinical problem, with current therapies being far from adequate, so it is essential to develop new therapeutic approaches. Understanding of the integration of TGF-β pathway known to be involved in prostate cancer pathophysiology may be central to the development of improved pharmacological treatments. A dual role of TGF-β in cancer has long been noted, but its mechanistic basis, operating logic, and clinical relevance have remained elusive. What causes TGF-β signaling to be altered in cancer? What steps in tumor progression may benefit from a faulty TGF-β pathway? When does TGF-β act as a metastatic signal? And, most importantly, how can any of this knowledge be used to treat prostate cancer?

**Activator Protein-1 (AP-1)**

The primary control of eukaryotic gene expression occurs at the level of transcription where genes may be regulated in response to a specific signal or in a particular tissue-type. Transcriptional control of a gene involves the binding of regulatory proteins or transcription factors to short, *cis*-acting DNA sequence elements located within and near the promoter of a gene. Following the binding of transcription factors, the activity of RNA polymerase is modulated in either a positive or negative manner at the start site of transcription. There are several types of DNA sequence sites to which transcription factors will bind, these include: promoter elements situated close to or at the start site of transcription which are essential for activation or
significant levels of transcription; regulatory elements situated close to the general promoter region functioning in an orientation-independent manner, and enhancer/repressor elements located at a distance from the transcription start site which increase or decrease the rate of transcription.

**AP-1 Family members**

AP-1 is a collective term referring to dimeric transcription factors composed of JUN, FOS or ATF (activating transcription factor) subunits that bind to a common DNA site, the AP-1-binding site. The consensus binding site for AP-1 was identified as the palindromic sequence 5’ TGA/TCA 3’, which was found to be responsive to the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) referred to as the TPA-responsive element (TRE).

As the complexity of our knowledge of AP-1 factors has increased, our understanding of their physiological function has decreased. AP-1 is an important and well-studied transcription factor; the protein components of this transcription factor are encoded by a set of genes known as “immediate-early” (IE) genes. IE genes couple cytoplasmic biochemical changes, arising from the binding of stimulatory agents to cell-surface receptors to mediate specific cell responses. AP-1 was initially identified as a DNA-binding activity in HeLa cell extracts that bound to cis-elements within the promoter and enhancer sequences of the human metallothionein IIA gene and simian virus 40.

The transcription factor AP-1 was subsequently found to be comprised of protein dimers, containing the gene products of members of the *JUN* (JunD, cJUN, JUNB) and *FOS* (FosB, cFos, Fra1, Fra2) gene families. The JUN protein members form
homo- and heterodimeric complexes within their own gene family\textsuperscript{66, 207-208} and with the protein members of the \textit{FOS} gene family.\textsuperscript{66, 89, 209-211} Unlike the \textit{JUN} proteins, \textit{FOS} is unable to form homodimers and must heterodimerize with \textit{JUN} proteins in order to transcriptionally activate AP-1-containing promoter constructs in cells.\textsuperscript{66, 212-213}

\textit{Structure}

The AP-1 family of transcription factors is a basic leucine zipper (bZIP) dimeric protein complex of structurally and functionally related members of \textit{JUN}, \textit{FOS}, \textit{ATF} and musculoaponeurotic sarcoma (MAF) protein families. The basic region or DNA binding domain (DBD) of bZIP proteins contains positively charged amino acid residues required for DNA binding activity\textsuperscript{85} (Figure 9). The leucine-zipper domain (LZD), located immediately downstream of DBD, contains a heptad repeat of leucine residues. LZD mediates the dimerization of proteins, bringing two DBDs into juxtaposition, thereby facilitating the interaction of protein dimers with DNA. Although LZD and DBD are highly conserved among all AP-1 proteins, their amino (NH\textsubscript{2}) - and carboxy (COOH)-terminal regions are quite divergent.
Figure 9: Schematic diagram showing JUN and FOS molecular structure

A) The location of various modules is indicated. TAD, transcription-activating domain; LZD, leucine-zipper domain; DBD, DNA binding domain; N, amino terminus; C, carboxyl terminus.

B) LZD mediates the dimerization of proteins bringing two DBDs into juxtaposition, thereby facilitating the interaction of protein dimers with DNA. ATF, activation transcription factor; TRE, 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element; CRE, cyclic AMP-responsive element. Note that CRE has an extra base (underlined) compared with TRE.

The JUN proteins contain the transactivation domain (TAD) at their NH$_2$-terminal region, whereas FOS members, except Fra1 and Fra2, possess TADs at their NH$_2$- and COOH-terminal regions (Figure 9). In many tumors these non-transforming FOS proteins, especially Fra1 and Fra2, might be involved in the progression of many tumor types. The JUN gene family is similar to each other in their gene and protein
structure, particularly in the DNA-binding domain and the leucine zipper regions where there is 75% amino acid homology with the JUN Family.\textsuperscript{66, 215} The cFos protein is evolutionally well-conserved, as its protein sequence retains an overall homology of 97, 94 and 79% between rat and mouse, rat and human, and mouse and chicken cFos proteins, respectively.\textsuperscript{66, 216} In addition to \textit{cFos} other related genes have been identified. \textit{FosB} and its naturally truncated form \textit{ΔFosB} (missing the C-terminal 101 amino acids of FosB),\textsuperscript{217} \textit{Fos}-related antigen-1 (\textit{Fra-1}), and \textit{Fos}-related antigen-2 (\textit{Fra-2}), together with \textit{cFos} make up the \textit{FOS} gene family.\textsuperscript{66, 211, 217-220} These genes are structurally-related to \textit{cFos} in terms of having the same number of exons and introns; however, the size of the untranslated regions of the genes is variable. The amino acid sequence between each protein is also conserved, particularly in the basic DNA and leucine zipper regions.\textsuperscript{66, 219}

Similarly to \textit{cFos}, the protein products of other members of the \textit{FOS} gene family are unable to bind to DNA individually, and require dimerization with a JUN protein to form a functional AP-1 complex.\textsuperscript{66, 89, 210-211, 217} However, despite their homology, these proteins display different transcriptional activity.\textsuperscript{221}

\textit{Functions}

AP-1 is an inducible transcription factor,\textsuperscript{66} that may regulate different target genes and thus execute distinct biological functions.\textsuperscript{114} Various agents have been shown to induce the expression of the \textit{FOS} gene family; agents such as serum, growth factors, neurotransmitters, calcium, phorbol esters, metal ions, UV light and cAMP.\textsuperscript{66} The decision if a given AP-1 factor is positively or negatively regulating a specific target gene is made upon abundance of dimerization partners, dimer-composition, post-translational
In vitro studies have shown that JUN/FOS heterodimers are more stable and have a stronger DNA binding activity than JUN homodimers.\textsuperscript{85, 223} The reason for this binding specificity amongst bZIP families can be attributed to the amino acid combination between the leucine residue repeats within the leucine zipper region.\textsuperscript{66, 224} cFos has a highly acidic leucine zipper with a large net negative charge at neutral pH thus a homodimer formation would not be favored owing to general electrostatic repulsions between the monomer FOS proteins.\textsuperscript{66, 224} In addition, the acidic residues important for specificity are aligned along one face of the helix, causing intra-helical destabilization. The cJun leucine zipper has a more diffuse, net positive charge, allowing a JUN homodimer to form. However, the interaction of Jun proteins is not as stable as a FOS/JUN dimer. As the FOS and JUN monomers are of opposite charge, they may form a more stable heterodimer together as the interhelical component of the electrostatic destabilization is relieved.\textsuperscript{66, 224} Different FOS/JUN complexes also have different affinities for AP-1 sites, and this is partly attributed to different DNA sequences around the core AP-1 site.\textsuperscript{66, 223}

AP-1-regulated genes include important regulators of invasion, and metastasis, proliferation, differentiation and survival, genes associated with hypoxia and angiogenesis.\textsuperscript{87, 113, 115} Many oncogenic signaling pathways converge at the AP-1 transcription factor complex. The specific influence of a specific AP-1 protein on a promoter depends on the dimer partners, the promoter architecture as well as other transcription factors and co-activators acting on the promoter.\textsuperscript{113}
As stated earlier, DNA binding is a necessary prerequisite of transactivation; the expression of different proteins of the JUN and FOS family is crucial for the activation of downstream genes regulated by AP-1. AP-1 is known to control the expression of several target genes that regulate cell cycle (cyclin D, p16), differentiation (myogenin, involucrin), cell survival (Bcl-2, Bcl-xL, FasL), growth factors (VEGF), and cell adhesion (VCAM, EDAM-1) and angiogenesis/invasion (MMP’s, uPA, osteopontin, CD44). In the prostate, various members of the AP-1 family have been implicated in the actions of androgen receptors. Activation of cJun has been shown to play a role in the development of androgen independent prostate cancer; the overexpression of cJun has been shown to inhibit the expression and function of androgen receptor in human prostate cancer cells. The expression of interleukin-6, which increases in hormone refractory prostate cancer cell, is dependent on constitutive activation of Fra1 and JunD. Jun N-terminal Kinases (JNKs), which phosphorylate and activate AP-1 proteins have been shown to be involved in proliferation and tumor growth and survival of prostate cancer cells. These studies have underlined the importance of AP-1 proteins in the development and function of prostate cells and a change in the relative abundance and/or activities of specific proteins may be involved in development and maintenance of prostate cancers. However, in spite of these studies, the role of individual AP-1 family of transcription factors in prostate cancers growth, migration and invasion is far from being elucidated.
**Activator Protein-1 in TGF-β Signaling**

AP-1 transcription factors contribute to various TGF-β biological responses.\textsuperscript{230} The promoters of TGF-β1-responsive genes like plasminogen activator inhibitor-1 (PAI-1) and cJun contain AP-1 binding sites. Mutation of these AP-1 binding sites which impairs binding of the AP-1 complex inhibits transcriptional activation of these promoters by TGF-β1.\textsuperscript{231-232} SMAD proteins possess DNA-binding activity, but the SMAD4-RSMAD complexes must associate with additional DNA-binding cofactors in order to achieve binding with high affinity and selectivity to specific target genes. These Smad partners are drawn from various families of transcription factors, such as the forkhead, homeobox, zinc-finger, bHLH, and AP1 families.\textsuperscript{171, 233-234} These findings suggest that SMAD proteins and AP-1 complex synergize to activate the TGF-β1-responsive promoters.\textsuperscript{235} Naso et al 2003 showed that JunB is up-regulated by TGF-β-SMAD signaling and may contribute to the TGF-β-induced EMT and fibrotic responses.\textsuperscript{236} Recent studies indicate that SMAD-3 directly binds cJun and cFos of the AP-1 complex and that both SMAD-3 and SMAD-4 bind all three Jun proteins.\textsuperscript{53} Studies also show that TGF-β1 activates SMAD proteins and AP-1 complex (JunD: FosB) and that over-expression of their dominant negative forms inhibits TGF-β1 dependent apoptosis.\textsuperscript{235} Over expression of FosB enhances SMAD-dependent transcription of TGF-β1 responsive reporter. JunD: FosB recruits SMAD-3:SMAD-4 to from AP-1:SMAD complex that binds to the AP-1 binding site, 12-O-tetradecanoyl-13-acetate-responsive gene promoter element.\textsuperscript{235} These studies show that both SMAD proteins and AP-1 complexes play a critical role in TGF-β1-dependent apoptosis. Our data has shown that TGF-β1 transiently induces mRNA and protein expression levels of AP-1 components in DU145 and PC3 prostate cancer cell lines, but
the role of these inducted AP-1 components in TGF-β1 dependent growth, proliferation, migration, and invasion remains unknown.

**AP-1 Proteins in TGF-β signaling in Cancers and Prostate Cancer**

Jun family members (c-Jun, JunB, and JunD) were expressed at different levels and responded differentially to TGF-β treatment. TGF-β effects on JunD protein levels, but not mRNA levels, correlated with its effects on cell proliferation. TGF-β induced significant reduction in JunD protein in RWPE-1 and DU145 cells but not in PC3 cells. Selective knockdown of JunD expression using siRNA in DU145 and PC3 cells resulted in significant reduction in cell proliferation, and forced overexpression of JunD increased the proliferation rate.\(^{50}\) Previously published work in our lab shows that overexpression of c-Jun and JunB decreased the proliferation rate in DU145 cells. Further studies showed that down-regulation of JunD in response to TGF-β treatment is mediated via the proteasomal degradation pathway.\(^{50}\) Thus concluding, specific Jun family members exert differential effects on proliferation in prostate cancer cells in response to TGF-β, and inhibition of cell proliferation by TGF-β requires degradation of JunD protein.\(^{50}\)
CHAPTER 3
MATERIALS AND METHODS

**Chemical and Reagents**

Recombinant human TGF-β1 (Catalog # HEK293 100-21) was purchased from Peprotech (Rocky Hill, NJ). The antibodies against FosB (Catalog# 2251S), cFos (Catalog # SC-52), Fra1 (Catalog # SC-605), and Fra2 (Catalog # SC-604) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The anti-β-Actin (Catalog # A5441) antibody was purchased from Sigma-Aldrich (St. Louis, MO). The anti-mouse and anti-rabbit immunoglobulins coupled with horseradish peroxidase (IgG-HRP) were obtained from Promega (Madison, WI). Cell lysis buffer was purchased from Cell Signaling (Danvers, MA). TRIzol was purchased from Invitrogen (Carlsbad, CA).

**Human Prostate Cell Lines and Treatments**

All cell lines were obtained from American Type Culture Collection. These include immortalized prostate luminal epithelial cell line (RWPE1) and prostate cancer cell lines (LNCaP, DU145, and PC3).

**Expression of FOS family members:** Prostate cells (RWPE1, LNCaP, DU145 and PC3) were cultured using established procedures. To determine the basal expression of Fos mRNA and protein levels, RWPE1, LNCaP, DU145 and PC3 cells were seeded at
a density 1.0 X 10^6 cells/dish in a 10 cm petri dish in the appropriate growth media and cultured at 37°C for 48 h. After 48 h, the cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mm NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin, and 1 X protease inhibitor cocktail (Calbiochem, San Diego, California). Protein concentrations were determined by the Lowry HS assay using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA.) according to the instructions provided by the manufacturer. The total RNAs were isolated from parallel experiments and were also used for RT-PCR as described below. To determine the effects of TGF-β1 on FosB, cFos, Fra1, and Fra2 expression, prostate cancer cells were seeded in six-well plates at a density of 3.0 X 10^5 cells per well. Before each experiment DU145 and PC3 cells were incubated with media supplemented with 1% serum for 2 h followed by treatment with different doses of TGF-β1 (0, 1, 5, 10 ng/mL) for specific time periods. RNA and proteins were isolated and quantified.

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

Total RNAs were isolated from prostate cells using TRIzol (Invitrogen, Carlsbad, CA.) and the resulting RNA samples were quantified by optical density reading at 260 nm as described previously. Total RNAs (2 µg) were reverse transcribed in a 50 µl reaction mixture containing 0.5 mM dNTP (Fisher Scientific, Pittsburgh, PA), 0.5 mM dithiotreitol (Bio-Rad, Hercules, CA), 0.5 µg of oligo dT, and 400 U of M-MLV Reverse Transcriptase (Promega, Madison, WI.) at 37°C for 1.5 h. The reaction was terminated by heating the samples at 65°C for 5 min and subsequently cooled to 4°C. PCR was
performed to detect mRNA levels of FosB1, FosB2, cFos, Fra1, Fra2, and L-19. The PCR mixture was composed of 0.1 mM deoxynucleotide triphosphates, 0.5 U Taq DNA polymerase, 10X PCR Buffer with 3 mM MgCl$_2$ and 25 pM of the specific primers in a total volume of 15 µL. Primer information and the size of specific amplicons for individual genes are shown in Table 1. L-19 (a ribosomal protein) was used as a loading control. RNA samples processed without RT and PCR amplified were used as negative controls. Amplification was performed at 1, initial denaturant 94°C for 2 min; 2, 94°C for 15s; 3, 58°C for 15s; 4, 72°C for 30s, 5, repeating steps 2 and 4 for 35 cycles for FosB1, FosB2, cFos, Fra1, Fra2, and L-19; 5, final extension 72°C for 2 min. The PCR products were separated on 1.0-2.0% agarose gels, and viewed under UV.

**Western Blot Analyses**

Total cellular proteins were prepared from different prostate cell lines and were analyzed by Western blot as described previously. Briefly, cell lysates were mixed with Laemmeli’s buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% glycerol). Individual samples containing 30–35 µg protein were subjected to SDS-PAGE in 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). After blocking the membranes with 5% fat free milk in TBST (50 mM Tris, pH 7.5, containing 0.15 M NaCl, and 0.05% Tween–20) (cFos, Fra1, Fra2) or 5% bovine serum albumin (BSA) in TBST (FosB), for 1 h at room temperature, the membranes were incubated with appropriate dilutions of specific primary antibodies (1:500 for Fos proteins and 1:5000 for β-actin) overnight at 4°C. After washing, the blots were incubated with secondary anti-rabbit (for FOS proteins) and anti-mouse (for β-actin) IgG HRPs (1:20,000) for 1 h. The blots were developed in ECL mixture (Thermo Fisher
Scientific Inc., Rockford, IL), and placed inside the Syngene PXi/PXi Touch darkroom imaging (high resolution, multi-application image analysis systems) (Frederick, MD) according to the manufacturer’s directions and the density of specific protein bands were determined using ImageJ image processing and analysis software and values were normalized using β-actin.

**Immunofluorescence of FosB**

PC3 cells were seeded at a density of 8.0 x 10^4 cells/well into six-well plates containing sterile glass coverslips. The cells were incubated at 37°C and allowed to attach for 48 h. The media were replaced with fresh media containing 1% FBS for 2 h before treatment with TGF-β1 (10 ng/ml) for 4 h. At the end of the treatment, media were aspirated and cells were fixed in 3.7% paraformaldehyde for 20 minutes at room temperature. The fixative was aspirated and cells were rinsed three times with 1.0 ml 1X PBS and permeabilized using 0.1% Triton at room temperature. The cover slips were transferred to an aluminum wrapped 20 cm Petri dish and outlined using a hydrophobic marker. The cells on the cover slips were blocked in blocking buffer containing 10% normal goat serum in 1X PBS for 1 h. Blocking solution was aspirated and primary antibody (1:1000) for FosB was added overnight at 4°C in 1X PBS with 2% normal goat serum. After washing 5 times in 1X PBS for 10 minutes each, secondary antibody containing green fluorochrome (Alexa Fluor 488 goat anti-rabbit IgG, Life Technologies, Carlsbad, CA) was added at room temperature for 1 h in 1X PBS (light sensitive). After washing, DAPI (3 µg/ml) was added to cells for 20 minutes at room temperature to stain the nuclei. The cells were rinsed and the cover slips were then mounted on slides. Slides were kept at room temperature in the dark for 2 h before viewing under inverted
florescence microscope. Images were captured using 40 X magnification with an Axiovision camera of a Carl Zeiss zoom inverted florescence microscope (Carl Zeiss, Thornwood, NY).

**Transfections**

*Transfection with FosB siRNA*

To knockdown endogenous FosB expression, DU145 and PC3 cells were seeded in six-well plates at the density 1.5 X 10^5 cells per well in 1.0 mL antibiotic-free normal growth medium supplemented with 5% FBS. The cells were cultured at 37°C to 60-80% confluence. Control (scrambled) and FosB specific siRNAs were transfected into DU145 and PC3 cells according to the manufacturers’ instructions. Briefly, transfection complex were mixed together in a 1:1 ratio (2.5 µL for FosB) of siRNA to transfection reagent in 200 µL of antibiotic-free normal growth medium. The mixture was allowed to incubate at room temperature in the dark for 20 minutes. During this time the cells were washed once with 1 mL of siRNA transfection medium, after which the antibiotic-free medium was mixed with 1% FBS and added to the transfection reagent mixture. The transfection reagent siRNA duplex was overlaid onto the washed cells, and the cells were incubated overnight. The medium containing the transfection complex was replaced with complete medium containing 5% FBS and incubated for 48 h. Cells were trypsinized (0.25% Trypsin/ 2.21 mM EDTA) for 1 minute and trypsin was neutralized with 3.0 mL of complete medium. Cells were centrifuged at 1000 RPM 4°C for 5 minutes and re-plated for biological assays. Western blot analyses were used to confirm FosB protein knockdown.
Transfection with cFos, Fra1 and Fra2 siRNAs

To knockdown endogenous cFos, Fra1 and Fra2 expression, DU145 and PC3 cells were seeded in six-well plates at the density $1.5 \times 10^5$ cells per well in 1.0 mL antibiotic-free normal growth medium supplemented with 5% FBS. The cells were cultured at 37°C to 60-80% confluence. Control (scrambled) and cFos, Fra1 and Fra2 specific siRNAs were transfected into DU145 and PC3 cells according to the manufacturers’ instructions. Briefly, transfection complex were mixed together in a 1:1 ratio (6 µL for cFos, Fra1 and Fra2) of siRNA to transfection reagent in 200 µL of antibiotic-free normal growth medium. The mixture was allowed to incubate at room temperature in the dark for 20 minutes cells were treated as previously described. After being transiently transfected to silence cFos, Fra1 and Fra2 DU145 and PC3 cells were used in cell proliferation and migration assays.

Transfection with cJun siRNAs

siRNA was used to knockdown endogenous cJun expression, PC3 cells were seeded in six-well plates at the density $1.5 \times 10^5$ cells per well in 1.0 mL antibiotic-free normal growth medium supplemented with 5% FBS. The cells were cultured at 37°C to 60-80% confluence. Control (scrambled) and cJun specific siRNAs were transfected into PC3 cells according to the manufacturers’ instructions. Briefly, transfection complex were mixed together in a 1:1 ratio (6 µL for cJun) of siRNA to transfection reagent in 200 µL of antibiotic-free normal growth medium. The mixture was allowed to incubate at room temperature in the dark for 20 minutes cells were treated as previously described. After being transiently transfected to silence cJun, PC3 cells were used in cell migration assays.
Cell Proliferation Assays

MTT Assays

After transient transfection, the cells were counted and seeded into 96-well plates at a density of $5 \times 10^3$ cells/well and treated with 10 ng/mL of TGF-β1 in the presence of 1% FBS for 72 hours. Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. MTT assays were performed using Cell Titer 96 Non-radioactive Cell proliferation assay (Promega, Madison, WI) following the manufacturer’s instructions.

MTS Assays

After transient transfection, the cells were counted and seeded into 96-well plates at a density of $5 \times 10^3$ cells/well and treated with 10 ng/mL of TGF-β1 in the presence of 1% FBS for 72 hours. Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium inner salts (MTS) assay. MTS assays were performed using Cell Titer 96 AQ ueous One Solution Cell proliferation assay (Promega, Madison, WI) following the manufacturer’s instructions.

Total Cell Number Assays

After DU145 and PC3 prostate cancer cells were transiently transfected to silence the Fos family proteins (cFos, Fra1 and Fra2) cells were trypsinized using 400 µl of trypsin and neutralized using 3000 µl of MEM and centrifuged at 5000 rpm, 4°C for 5 minutes to remove trypsin. The cells were then re-suspended in 1000 µl MEM in a 1.5 µl Eppendorf tubes as previously described and counted using the hemocytometer. Media was gently pipetted to ensure the cells are evenly distributed. Before the cells have a chance to settle, 20 µl of cell suspension was removed using a sterile pipette and placed
into both chambers on the hemocytometer glass slide underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. An Oxioskop 2 Plus Zeiss light microscope was used to focus on the grid lines of the hemocytometer with a 10X objective. Cells were then counted using a hand tally counter. A counting system was employed whereby cells were only counted when they are set within a square or on the right-hand or bottom boundary line. The hemocytometer was moved to the next set of 16 corner squares and carry on counting until all 4 sets of 16 corners are counted. This was done twice for each treatment allowing a total of 8 sets of 16 squares per treatment. The average of each set of 16 corner squares were taken and multiplied by 10,000 (10^4). Each treatment was done at least three times using different cell preparation each time and average displayed in a representative figure that showed the number of cells represented on the Y axis and varying treatments on the X axis.

**Cell Migration Assays**

After the transfections, *in vitro* cell migration assays were performed using 24-well transwell inserts (8 µm) as previously described.²³⁻²⁴ Chemoattractant solutions were made by diluting TGF-β1 (10 ng/ml) or EGF (10 ng/ml) into MEM for DU145 and PC3 cells supplemented with 0.2% BSA. The results were expressed as migration index defined as: the average number of cells per field for test substance/the average number of cells per field for the medium control. The experiments were conducted at least three times using independent cell preparations.

**Cell Invasion Assays**

After transfection, the invasive behavior of PC3 cells was measured using the BD BioCoat Matrigel Invasion inserts.⁴⁹ Cell culture inserts (VWR International, Bridgeport,
NJ) were coated with 50 µl of 1:4 Matrigel/Medium dilutions (BD Sciences, San Jose, CA) and allowed to solidify at 37°C for 1 h. Cells were resuspended (5.0 X 10⁴ cells/ml) in MEM and 0.1% FBS and 500 µl of cell suspension was added to each insert. Chemoattractant solutions were made as described above with TGF-β1 and EGF into MEM supplemented with 0.1% FBS. Matrigel and non-invading cells were removed by scrubbing. Invading cells in the membrane were fixed in 3.7% paraformaldehyde and stained with DAPI. Pictures were taken from five different fields for average number of invading cells to be determined. The results were expressed as an invasive index defined as: the average number of cells per field for test substance/the average number of cells per field for the medium control. The experiments were conducted at least three times using independent cell preparations.

**Co-immunoprecipitation**

3.0 X 10⁶ PC3 cells were plated in 10 cm petri dish with total volume of 10 ml MEM supplemented with 5% FBS and allowed to incubate overnight at 37°C. The MEM was then aspirated and replaced with fresh MEM supplemented with 1% FBS for 2 hours before treatment. The cells were then treated with 10 ng/ml of TGF-β1 for 4 hours and then harvested under non-denaturing conditions. Briefly, MEM was removed and cells were rinsed once with ice cold 1X PBS. The PBS was removed and replaced with ice cold cell lysis buffer (containing 20 mM Tris-HCl (pH 7.5), 150 mm NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/mL leupeptin, and 1 X protease inhibitor cocktail (Calbiochem, San Diego, California)) and placed on ice for 10 minutes. The cells were then scraped off the plates and transferred to a 2.0 ml micro-centrifuge tube. The lysates
were then sonicated three times 5 seconds each whilst still on ice. Cells suspension was then centrifuged for 10 minutes at 14,000 X g, 4°C and the supernatant was transferred to a new tube. The cell lysates were pre cleaned to reduce nonspecific binding of Protein by combining 400 µl of cell lysates with 40 µl of protein A sepharose beads and incubation at 4°C for 45 minutes without rotation shaker. The cells lysates were then spun for 10 minutes at 4°C and the supernatant transferred to a fresh tube. FosB primary antibody was added (1:50 dilution) to 400 µl of the cell lysates and incubated with gentle rocking overnight at 4°C. 40 µl of protein A sepharose beads were added to cell lysates and incubated overnight at 4°C with gentle shaking. Tubes containing cell lysate and protein A were then centrifuged at 4°C for 30 seconds. The supernatants were transferred to fresh tubes. The pellets were washed 5 times with 500 µl of 1X cell lysis buffer and kept on ice during washes. The pellets were re-suspended in 40 µl of 2X SDS sample loading buffer, vortexed and the micro centrifuged for 30 seconds at 4°C. The samples were then heated 95-100°C for 5 minutes and centrifuged for 1 minute at 14,000 X g. After which the samples were loaded on SDS-PAGE gel and analyzed using Western Blot.

**Statistical analysis**

All experiments were repeated at least three times using a different cell preparation. ANOVA, Duncan’s modified multiple range and Student-Newman-Keuls tests were employed to assess the significance of differences between treatment groups.
CHAPTER 4
RESULTS

Expression of FOS family members in prostate cancer cell lines

We initially screened four human prostate cell lines, RWPE1 (normal prostate epithelial cells), LNCaP, DU145 and PC3 (prostate cancer cell lines) for expression of FOS family members. Levels of mRNA for FosB, cFos, Fra1 and Fra2 were determined using RT-PCR, with L-19 used as control (Figure 10A). Fra2 mRNA was robust in all four cell lines, Fra1 and FosB mRNA were highly expressed in all cell lines except in LNCaP, which also had very low mRNA levels of cFos ΔFosB was highly expressed in all cells with only moderate expression in RWPE1 cells (Figure 10A). Western blot analyses was performed to determine the relative protein abundance of FosB, cFos, Fra1 and Fra2 (Figure 10B). Fra1 protein levels were very low in all prostate cancer cell lines (LNCaP, DU145, and PC3) with slightly higher levels in RWPE1 cells (Figure 10B). Fra2 protein levels were relatively high in RWPE1, DU145 and PC3 but were not detectable in LNCaP cells. cFos showed moderate expression in RWPE1, DU145 and PC3 cells but was very low in LNCaP cells. FosB and ΔFosB protein levels were high in RWPE1 and PC3 cells but low to moderate in LNCaP and DU145 cells respectively (Figure 10B) shown below.
Figure 10: FOS family basal expression

A. Total RNA’s were isolated and semi quantitative RT-PCR was performed to determine the mRNA levels of FosB, cFos, Fra1, and Fra2 in prostate cells. L-19 was used as an internal control.

B. Western blot analysis of FosB, cFos, Fra1, and Fra2 in prostate cells. β-actin was used as a loading control.

Figure 10: FOS family basal expression- Steady state mRNA levels of FOS (FosB, cFos, Fra1, and Fra2) mRNA and protein expression. A, Total RNA’s were isolated and semi quantitative RT-PCR was performed to determine the mRNA levels of FosB, cFos, Fra1, and Fra2 in prostate cells. L-19 was used as an internal control. B, western blot analysis of FosB, cFos, Fra1, and Fra2 in prostate cells. β-actin was used as a loading control.
TGF-β1 effects on FOS protein expression and nuclear accumulation in prostate cancer cells

DU145 and PC3 cells were treated with exogenous TGF-β1 (10 ng/ml) for 0, 1, 2, and 8 h. TGF-β1 induced an increase in the mRNA levels of FosB (P<0.05) in both cell lines but had little effect on the mRNA levels of the other FOS family members (Figure 11A). At the protein levels, TGF-β1 induced an increase in the levels of FosB (P<0.05) protein in both cell lines and an increase in Fra2 (P<0.05) protein levels was observed only in PC3 cells. TGF-β1 had no effect on the levels of cFos and Fra1 in both cell lines (Figure 11B). Spot densitometry analysis confirmed TGF-β1 effects on FosB and Fra2 protein levels in DU145 and PC3 cells in a time-dependent manner (Figure 11C). TGF-β1 induction of FosB was dose dependent (Figure 11D). Immunofluorescence of FosB in PC3 cells showed that treatment with TGF-β1 induced increased expression and nuclear localization of FosB (Figure 11E). Inhibition of TGF-β receptors as well as Smad3 abrogated TGF-β1 ability to increase FosB protein expression (Figure 11F); however inhibitors against PI3K and MAPK had no effect on TGF-β1 ability to increase FosB protein expression (Figure 11F).
Figure 11: TGF-β1 on FOS Family mRNA and protein expression

A. RT-PCR analysis of FosB, cFos, Fra1, and Fra2 mRNA levels in DU145 and PC3 prostate cancer cells after exposure to exogenous TGF-β (10 ng/mL) for different times.

B. Western blot analysis of FosB, the FosB antibody used recognizes both full length FosB (higher molecular weight band) and ΔFosB (lower molecular weight band), cFos, Fra1, and Fra2 protein levels in DU145 and PC3 prostate cancer cells after exposure to exogenous TGF-β1 (10 ng/mL) for different times.

C. Band density analysis of FosB and Fra2 in DU145 and PC3 cells after treatment with TGF-β1 for 2 and 8 hours. Each band density was normalized by density of β-actin bands. Each bar represents the Mean ± SD from 3 independent experiments. “a” and “b” denote significant differences (P<0.05) from untreated controls.
Figure 11: D, The dose dependent effects of TGF-β1 on expression of FosB; Western blot analysis of FosB in prostate cancer cells DU145 and PC3 after treatment with varying concentrations of exogenous TGF-β1 (0, 1, 5, 10 ng/mL) for 4 hours. E, Immunofluorescence, TGF-β1 activation of FosB in PC3. Cells were treated with exogenous TGF-β1 (10 ng/mL) for 0, and 4 hours. F, DU145 and PC3 cells were treated with inhibitors for TGF-βRI/II, Smad3, PI3K and MAPK.

FosB knockdown on TGF-β1-mediated cell proliferation, migration and invasion

Next we determined the role of FosB in TGF-β1-induced cell proliferation, migration and invasion in prostate cancer cells. A transient knock down of FosB was carried out using siRNA specific to FosB, followed by a MTT proliferation (Figure 12A, B), trans-well inserts migration assays (Figure 13A, B) and Matrigel invasion assays (Figure 14). Knock down of FosB had no effect on cell proliferation in DU145 and PC3 cells (Figure 12A, B) however; there was a significant decrease (P<0.05) in cell migration (DU145, PC3) and cell invasion (PC3: P<0.05) in response to TGF-β1 and
EGF in these cells (Figure 13A, B and Figure 14). Our data also suggests that FosB knockdown reduced both TGF-β1-and EGF- induced cell invasion but had no significant effect on the basal invasive potential of these cells (Figure 14).

**Figure 12:** The effect of FosB knock down on TGF-β-induced prostate cancer cell proliferation

- **A.** DU145 and (A) PC3 (B) cells were transfected with siRNA to transiently silence FosB followed by an in vitro proliferation assay.

**Figure 12:** Effects of FosB knock down on TGF-β1-induced cell proliferation- DU145 and (A) PC3 (B) cells were transfected with siRNA to transiently silence FosB followed by an in vitro proliferation assay.
Figure 13: Effects of FosB knock down on cell migration - Prostate cancer cells DU145 (A) and PC3 (B) were pretreated with siRNA against FosB for 72 hours. Western blots were used to confirm knock down of endogenous FosB (inserts). DU145 and PC3 cells were pretreated with siRNA against FosB, followed by treatment with 10 ng/mL of exogenous TGF-β1 and 10 ng/ml EGF migratory behavior were measured using transwell insert migration assay. Each bar represents Mean ± SEM from three independent experiments. Different letters designate statistically significant (P<0.05) differences among different treatments.
**Figure 14:** Effects of FosB knock down on cell invasion - PC3 cells were pretreated with siRNA against FosB, followed by treatment with 10 ng/mL of exogenous TGF-β1 and 10 ng/ml EGF invasive behavior were measured using and Matrigel in vitro invasion assay. Insert shows western blot used to confirm FosB knock down. Each bar represents Mean ± SEM from three independent experiments. Different letters designate statistically significant (P<0.05) differences among different treatments.

**FOS Family Members role in Prostate Cancer Cell Growth and Proliferation**

After finding out that FosB increased protein expression in DU145 and PC3 prostate cancer cells in response to TGF-β1 stimulation had no effect on prostate cancer cell proliferation; the other FOS family members (cFos, Fra1, and Fra2) were transiently silenced using siRNA specific to each family. This was followed by cell count and MTS proliferation assay. Our results indicated that transiently silencing cFos and Fra1 had no effect on cell number in DU145 prostate cancer cells (Figure 15A). Our data also showed
that cFos knock-down increased (P<0.05) cell number in PC3 cells and Fra1 knock down had no effect on cell number in these cells (Figure 15B). Our data showed that TGF-β1 increases the expression of Fra2 (Figure 11B, C) protein expression in PC3 prostate cancer cells only. In order to determine the role of this increased Fra2 protein expression Fra2 was transiently silenced in both DU145 and PC3 prostate cancer cells followed by cell count and MTS proliferations assays. The cell count data (not shown) indicated that Fra2 knock-down in PC3 cells results in decrease in cell number, MTS data supported the data seen by cell counting showing that Fra2 knock-down in PC3 cells resulted in decreased (P<0.05) cell proliferation (Figure 16B). On the other hand knock-down of Fra2 had no effect on cell count or cell proliferation in DU145 cells in the presence or absence of TGF-β1 (Figure 16A). Our data also showed that transiently knocking down FOS family members had no effect on cell morphology in PC3 prostate cancer cells (Figure 17).
Figure 15: The effect of cFos and Fra1 knockdown on prostate cancer cell number

A.

B.

Figure 15: A) DU145 cells were transfected with siRNA to transiently silence cFos and Fra1 followed by an in vitro proliferation assay. Insert western blot image confirming cFos and Fra1 knock down. B) PC3 cells were transfected with siRNA to transiently silence cFos and Fra1 followed by an in vitro proliferation assay. Different letters designate statistically significant (P<0.05) differences among different treatments. Insert western blot image confirming cFos and Fra1 knock down.
Figure 16: A) DU145 cells exposed to siRNA specific for Fra2 followed by stimulation with exogenous TGF-β1 (10ng/ml) for 72 hours. Inserts show western blot analysis confirming Fra2 knock down and 96 well plate layout of treated cells. B) PC3 cells exposed to siRNA specific for Fra2 followed by stimulation with exogenous TGF-β1 (10ng/ml) for 72 hours. Inserts show western blot analysis confirming Fra2 knock down and 96 well plate layout of treated cells. Different letters designate statistically significant (P<0.05) differences among different treatments.
Figure 17: PC3 cells were transiently transfected to silence FosB, cFos, Fra1, and Fra2, morphology images were obtained using Ziess microscope at X10 magnification.

TGF-β1 Effect on AP-1 dimerization

In an attempt to determine which JUN protein could function as FosB dimer partner responsible for its effects on in cell migration; PC3 prostate cancer cells were seeded at a density of 3 million cells per 10 cm dish and stimulated with TGF-β1 for 4 hours, the cells were lysed with cell lysis buffer as described previously and lysates used to perform a co-immunoprecipitation assay to determine if FosB dimerization with JUN proteins was regulated by the presence of TGF-β1. Our data showed that FosB dimerization with JunD is minimal and not influenced by TGF-β1 (Figure 18). The effect of TGF-β1 on FosB: JunB dimerization is currently being investigated. The most
interesting finding is that TGF-β1 reduced FosB: cJun dimerization (Figure 18). This led to an interest in determining whether cJun has a role in prostate cancer cell migration.

**Figure 18:** The effect of TGF-β1 on FosB:cJun Dimerization

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**Figure 18:** PC3 cells were stimulated with TGF-β1 followed by co-immunoprecipitation assay to determine the effect of TGF-β1 stimulation on FosB dimerization with JUN proteins.

**The Role of cJun Protein in Prostate Cancer Cell Migration**

Our data showed that FosB is essential for basal, TGF-β1-and EGF-induced cell migration to occur. Previous studies have shown that FOS proteins must function as dimers more specifically heterodimers and that dimerization is a prerequisite for nuclear translocation and thus activation of AP-1 dimer complex \(^7^1\). This finding as well as our data indicating that TGF-β1 stimulation decreases cJun: FosB dimerization led to a new found interest in the role of cJun in FosB effects, specifically in TGF-β1 signaling and prostate cancer cell migration. cJun was transiently silenced in PC3 cells using siRNA
specific for cJun followed by transwell migration assay. Our data showed that cJun knock-down led to significant increase (P<0.05) in basal, TGF-β1 and EGF-induced cell migration (Figure 19). Using DU145 prostate cancer cells that were stably transfected in our lab to over-express cJun; we determined that over-expression of cJun leads to a significant decrease (P<0.05) in basal cell migration (Figure 20). We are currently working on transiently over-expressing cJun in PC3 prostate cancer which will be followed by cell migration assays, as well knocking down JunB and JunD and determining their effects if any on prostate cancer cell migration.

**Figure 19:** The effect of cJun knockdown on prostate cancer cell migration

![Western Blot Image](image)

**Figure 19:** PC3 cells transiently transfected to knock down cJun and stimulated with 10 ng/ml of TGF-β1 and EGF followed by transwell migration assay, insert showing western blot image confirming cJun knock down. Different letters designate statistically significant (P<0.05) differences among different treatments.
Figure 20: DU145 cells stably transfected to over express cJun and stimulated with 10 ng/ml of EGF followed by transwell migration assay. Different letters designate statistically significant (P<0.05) differences among different treatments.
CHAPTER 5
DISCUSSION

In this study, we demonstrate for the first time the role of FOS transcription factors in migration and invasion of prostate epithelial cancer cells and the role of cJun in prostate cancer cell migration. To determine the role of FOS proteins in prostate cancer cell proliferation migration and invasion, we performed two types of experiments: first, the effect of TGF-β1 on the Fos family expression levels were determined by western blot analysis using different doses and varying times of exposure to TGF-β1; second, FOS knock-down was used to determine their roles in TGF-β-regulated prostate cancer cell proliferation, migration, and invasion. The key findings in this study are that 1) TGF-β1 induces and increased expression of FosB in prostate epithelial cancer cells, 2) FosB is essential for migration and invasion to occur in prostate cancer cells, and 3) FosB is required for TGF-β1-and EGF-induced cell migration and invasion, 4) TGF-β1 induces increased protein expression of Fra2 in PC3 cells only, 5) Fra2 is necessary for basal and TGF-β1 induced cell proliferation in PC3 cells, 6) TGF-β1 decreases FosB : cJun dimerization, 7) cJun over expression inhibits prostate cancer cell migration, 8) cJun
Knockdown increases cell migration. AP-1 family of transcription factors are a part of the complex immediate early genomic response of a variety of cells to transmembrane signaling agents. Additional complexity results from the variety of possible Jun dimers and the JUN-FOS heterodimers and from potential dimer formation between JUN or FOS and other leucine zipper proteins. JUN and FOS proteins share extensive homology within the leucine zipper and basic domains. However, despite their homology, these proteins display different transcriptional activity. The FOS proteins contribute distinct functions towards the activity of the AP-1 heterodimers. For example, c-Fos can both activate and repress transcription, the full-length Fos B is a transcriptional activator and a naturally occurring short form of FosB inhibits AP-1 transactivation. The Fos-related antigens, Fra-1 and Fra-2 lack functional transactivation domains and are poor transcriptional activators. We believed that an alteration in the composition of AP-1 either directly or indirectly regulates cell growth and motility, which in turn pushes the normal cell into pre-malignant or malignant state. Therefore we analyzed the effect of TGF-β1 on Fos mRNA and protein expression in both normal as well as different prostate cancer cell lines. The most interesting observation was an immediate increase in FosB expression both at the mRNA and the protein levels in prostate cancer cells as well as increased Fra2 protein expression in PC3 cells only.

TGF-β super family signaling is well known as a key regulator of many biological processes including differential effects on cell proliferation and migration in prostate cancer cells. These differential effects of TGF-β during different stages of cancer progression presumably depend on selective loss or acquisition of specific
intracellular signals that are required to elicit different biological responses to TGF-β. A loss of TGF-β receptors and/or Smad proteins has been shown to result in TGF-β resistance in cancer cells. However, most cancer cells retain classical TGF-β signaling components throughout cancer progression but modify or recruit additional signaling pathways to exert novel or different biological effects. Our data shows that TGF-β1 increases FosB expression in prostate cancer cells, which, in turn, mediates its effects on migration and invasion but does not play a role in TGF-β1 effects on cell proliferation. Thus TGF-β1 induction of FosB may represent a shift in intracellular signaling involved in the escape from inhibition of proliferation to the stimulation of more migratory and invasive behavior in advanced stages of prostate cancer. TGF-β1 reduces the dimer formation between cJun and FosB; our data demonstrates that the presence of cJun contributes to inhibition of cell migration; this information could shed light on the dual role of TGF-β1 prostate cancer cell progression, a role that could involve the regulation of AP-1 dimer formation as least in the case of cell migration.

While essential to normal development and homeostasis, the process of cellular migration is also a trait essential for metastasis. Enhanced migration is key across the metastatic cascade and is involved in the initial scattering of cells and migration from the primary tumor. Numerous proteins and pathways have been implicated in altering the migratory potentials of cancer cells and therefore their aggressive nature. Given its essential role in cancer progression, treatments that inhibit cell migration or such proteins/pathways involved in enhancing cellular motility represent an attractive strategy for controlling metastatic dissemination. Because Fra1 and Fra2 exhibit a lack of trans-
activating domain as seen in cFos and FosB, they might exert inhibitory functions on tumor growth. Yet recent data point to a positive effect of Fra1, and partly Fra2, on tumor progression in many tumor types. Our data suggests that Fra2 plays an important role in cell proliferation of aggressive prostate cancer cells but does not have the same effect on prostate cancer cells in the early stages of development. In contrast to the bulk of data on the function of cFos and Fra1, far less is known about the role of FosB and its smaller splice variant ΔFosB which is often expressed more strongly than Fra1 in clinical cancer tissues. Although, the FOS family of proteins has been extensively studied as immediate early genes, the role of FosB in cancer cell proliferation and migration and invasion has not been previously investigated. There are also no studies demonstrating the role of cJun in prostate cancer cell migration. Our data shows that transient silencing of FosB with or without the presence of TGF-β1 has no effect on prostate cancer cell proliferation but significantly reduces cell migration and invasion. Numerous studies have demonstrated that TGF-β1 induces the migration and invasion of prostate cancer cells; however, we show in this study that TGF-β1 is unable to induce prostate cancer cell migration and invasion without FosB. The data also suggests that epidermal growth factor (EGF), a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types is unable to induce migration and invasion in prostate cancer cells in the absence of FosB; further confirming that FosB does indeed have a major role in migration and invasion of prostate cancer cells. Thus the differences in migratory and invasive behavior observed in different stages of prostate cancer progression can be due to AP-1 (specifically FosB) activation. FosB may have a
role in the aggressive phenotype observed in prostate cancer, thus inhibition of FosB activity may serve as a therapeutic tool in the management of prostate cancer.

TGF-β1 is known to switch from being a tumor suppressor in early stage prostate cancer to becoming a tumor promoter in the later stages of the disease. Our data suggests that TGF-β1 is unable to induce and increase in cell proliferation without the presence of Fra2 in PC3 cells which is used in this study to represent an advanced stage prostate cancer. This further supports the idea of AP-1 proteins playing essential roles in TGF-β1 induced prostate cancer cell progression.

Our data also suggests that TGF-β1 is able to reduce dimer formation between cJun which we have shown to be necessary for inhibition of prostate cancer cell migration and FosB which we also shown to be essential for prostate cancer cell migration to occur. This study has further implicated that targeting AP-1 proteins could serve as an alternative therapeutic target in treating prostate cancer.
In conclusion, our results obtained using human prostate cancer cell lines suggest that the transcription factors FosB, Fra2 and cJun may be important regulators of TGF-β1 effects on proliferation, migration and invasion in human prostate cancer cells. The functions of AP-1 proteins have been known to be modulated in four major ways: 1) changes in protein expression, 2) variations in dimer partners, 3) changes in subcellular localization and 4) changes in phosphorylation. Our data indicates that TGF-β1 is able to regulate AP-1 by varying their expression, subcellular localization and dimerization. This was seen as an increase in FosB and Fra2 protein expression, an increase in FosB nuclear localization, and a decrease in FosB: cJun dimerization as a result of prostate cancer cell stimulation with TGF-β1. Though still elusive, these studies may help to further understand TGF-β1 dual role in prostate cancer progression. Further studies are needed to decipher the Jun protein partner that is influenced by the presence of TGF-β1 to bind to FosB leading eventually to cell migration and invasion. Also, since TGF-β1 does not increase cell proliferation in PC3 cells it still remains unclear as to the specific role of Fra2 protein increase in PC3 cells stimulated with TGF-β1, and how this increased
expression contribute to PC3 cells being insensitive to the growth inhibitory effects of TGF-β1.

Further studies are also needed to completely decipher the role of cJun in FosB/TGF-β1 induced cell migration. Further study of the roles of FosB, Fra2 and cJun in prostate cancer carcinogenesis, especially in vivo, will be of great importance and will probably open new perspectives for therapy.
### APPENDIX A

#### Table 1

**Table 1: Gene Specific primers used for RT-PCR amplification**

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