The role of AP-1 proteins in the transforming growth factor-beta signaling pathway in prostate cancer

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ABSTRACT

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THE ROLE OF AP-1 PROTEINS IN THE TRANSFORMING GROWTH FACTOR-
BETA SIGNALING PATHWAY IN PROSTATE CANCER

Advisor: Dr. Shafiq Khan

Thesis dated December 2005

Transforming growth factor-beta (TGF-β) has a variety of roles including cell
growth inhibition, stimulation of proliferation, differentiation, apoptosis and extracellular
matrix (ECM) formation. TGF-β inhibits growth of the epithelial cells in the prostate
gland. The ability of TGF-β to inhibit cell growth enables it to act as a potent tumor
suppressor. Here, we show that TGF-β has different effects on proliferation in DU145
(TGF-β- responsive) and PC3M (TGF-β - resistant) prostate cancer cell lines. RT-PCR
analysis and Real-time PCR data have determined the varying levels of AP-1 family
members in both cell lines. In PC3M and DU145 cells, we have shown that TGF-β
exerts opposite effects on JunD, cFos and Fra2 expression, suggesting that AP-1 family
members may be involved in the differential effects of TGF-β in these cell lines. The
present observation confirms an intact inhibitory role of TGF-β on proliferation in
DU145 cells, and shows a non-inhibitory function in PC3M cell growth. In PC3M cells
TGF-β may exert effects leading to the metastatic nature of this cell line.
THE ROLE OF AP-1 PROTEINS IN THE TRANSFORMING GROWTH FACTOR-BETA SIGNALING PATHWAY IN PROSTATE CANCER

A THESIS
SUBMITTED TO THE FACULTY OF THE DEPARTMENT OF BIOLOGICAL SCIENCES OF CLARK ATLANTA UNIVERSITY IN FULLFILLMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE DEGREE

BY
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DEPARTMENT OF BIOLOGICAL SCIENCES

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CHAPTER I
INTRODUCTION

Prostate Cancer is the second leading cause of cancer death among men in the western world. The American Cancer Society estimates that 232,090 men will be diagnosed with prostate cancer and an estimated 30,350 will die of the disease in 2005. African-American men are especially at risk because they have the highest prostate cancer incidence and mortality rates in the world. The incidence rate is approximately 65% higher and mortality rate double that of Caucasian males, who have the second highest rate. During this year alone, 25,300 African-American men will be diagnosed and 6,100 African-American men will die from prostate cancer. As a result of the significant social and economic impact of prostate cancer, it is imperative that we better understand the mechanisms underlying the disease progression and develop specifically targeted therapeutics.

Prostate cancer progression is mediated by androgens, mainly testosterone, and growth factors, including epidermal growth factor (EGF), insulin-like growth factor (IGF), and transforming growth factor-beta (TGF-β). These growth factors and testosterone exert a synergistic effect on the growth, development, and function of the prostate (24). The growth factor this research is focused on is TGF-β, which is a 25 kilodalton dimeric polypeptide.
Transforming growth factor-beta (TGF-β1, TGF-β2 and TGF-β3) is a family of cytokines whose roles include a variety of cellular functions such as inhibition of proliferation, stimulation of proliferation, differentiation, apoptosis, and extracellular matrix formation (5). TGF-β effects vary depending on the type of cell and the state of the cell, however, in general terms, it inhibits proliferation of a wide variety of cell types, including the epithelial cells of the prostate. The ability of TGF-β to inhibit cell growth enables it to act as a potent tumor suppressor. It inhibits cell proliferation by causing cell cycle arrest at the G1 phase. There are certain prostate cancer epithelial cells that develop resistance to the inhibiting effects of TGF-β. Both TGF-β responsive and TGF-β resistant prostate cancer cells lines are available. These TGF-β resistant cell lines include PC3M and LNCap cells. In the DU145 cell line, the TGF-β signaling pathway is intact; therefore, inhibition of proliferation takes place in response to exogenous TGF-β. It has been hypothesized that TGF-β inhibits epithelial cell growth in DU145 acting through the Activating Protein-1 (AP-1) family of proteins.

AP-1 proteins are a family of transcription factors that are classified as basic leucine zipper proteins (bZIP). They are called bZIP because the DNA binding structure consists of a region enriched in basic amino acids that is adjacent to a leucine zipper that is characterized by several leucine residues (12). The basic region binds DNA, whereas the leucine zipper mediates dimer formation with other AP-1 family members. There are seven members of the AP-1 family of transcription factors, JunD, cJun, JunB, cFos, bFos, Fra1 and Fra2.
Previous research done in S. Khan's lab shows the significant role that cJun and JunD play in TGF-β inhibitory effects on cell proliferation. DU145 cells respond to TGF-β by decreasing the protein levels of JunD, but the levels of cJun remain unchanged. Experiments have shown that when JunD levels decrease this allows cJun dimerization on the AP-1 site in the promoter region of TGF-β responsive genes. It is speculated that through this mechanism, TGF-β responsive genes are able to inhibit the proliferation of prostate epithelial cells. This hypothesis is supported by our previous findings. When cJun was over-expressed in DU145 cells, this caused a decrease in proliferation and enhanced inhibitory effects of TGF-β. However, when JunD was over-expressed in DU145 cells, an increased rate of proliferation was observed in these cells. This prior work suggests that TGF-β functions through c-Jun and JunD to inhibit cell proliferation by causing changes in specific gene expression.

Our preliminary studies (unpublished) indicate that TGF-β inhibits the expression of JunD in DU145 prostate cancer cells and this reduction is associated with inhibitory effects of TGF-β on cell proliferation. Therefore, we hypothesize that TGF-β responsive (DU145) and non-responsive (PC3M) cells have different levels of AP-1 proteins and that several members of the AP-1 family of proteins are involved in the inhibitory effects of TGF-β. To test the above hypothesis the following specific aims will be investigated:

1. To confirm the differences in the growth inhibitory response of DU145 and PC3M cells to TGF-β.
2. To determine which AP-1 family members are expressed in PC3M and DU145 cell lines.

3. To determine the expression pattern of AP-1 family members in these prostate cancer cell lines in response to TGF-β.

By carrying out these aims, a better understanding of the mechanisms involved in the activation of AP-1 proteins in prostate cancer cells in response to TGF-β will be achieved.
CHAPTER II

REVIEW OF THE LITERATURE

TGF-β Expression in Prostate Cancer

In the case of cancer cells, a shared characteristic is the production of growth promoters and the loss of function of tumor suppressors. In searching for specific features of prostate cancer, it was discovered that TGF-β levels are higher in advanced prostate cancers than in earlier stages of prostate cancer and in the normal prostate. It seems contradictory that malignant epithelial cells make high levels of TGF-β, seeing that it is such a potent growth inhibitor of epithelial cells (21). In vivo studies have shown that TGF-β1 actually enhances prostate tumor growth and metastasis (1). The mechanism by which prostate cancer cells, like PC3M, protect themselves from being growth-inhibited by TGF-β1 in vivo is not clear (11). Unlike PC3M, in the cell line DU145, the TGF-β functions as expected, therefore inhibition of epithelial cell growth takes place. It is apparent that the TGF-β receptors and signaling pathways for growth inhibition are intact in the DU145 cell line.

TGF-β Signaling and Cellular Response

TGF-β signaling is initiated by the binding of TGF-β to its receptor type II (TGFβ–RII) then the complex binds receptor type I (TGFβ–RI) serine/threonine kinase on the surface of the cell. This in turn causes RII to phosphorylate RI kinase domain
which then propagates the signal through the phosphorylation of the Smad proteins (19). Recently, there have been studies indicating that AP-1 proteins interact directly with Smad proteins to induce expression of TGF-β responsive genes (12). This signaling pathway is known as the Smad dependent pathway. When working through the Smad dependent pathway, AP-1 proteins along with Smads are transported into the nucleus where they bind to specific sites in the promoter region of specific TGF-β responsive genes. AP-1 proteins can also be involved in the Smad independent pathway. Jun terminal kinases (JNKs) cause AP-1 proteins to become phosphorylated which in turn increases or decreases gene expression of AP-1 target genes (6).

**AP-1 Regulation of Gene Expression**

The AP-1 proteins are a family of transcription factors that are classified as basic leucine zipper proteins (24). The DNA binding structure consists of a region enriched in basic amino acids, and adjacent to this basic region is a leucine zipper that is characterized by several leucine residues (3). Whereas the basic region directly binds the DNA, the leucine zipper mediates dimer formation with other AP-1 family members.

The AP-1 dimers formed are comprised of Jun-Jun and Jun-Fos proteins. The Jun family is made up of cJun, JunD, and JunB, while the Fos family consists of FosB, cFos, Fra1, and Fra2 (20). The Fos family does not form homodimers (27), therefore AP-1 proteins can form 18 different dimeric combinations (26), including the Jun homodimers and Jun-Fos heterodimers. The Jun and Fos proteins can also dimerize
with other bZIP proteins including those from ATF, MAF, and CNC subfamilies which increases their combinatorial potential even more (8). The different combinations determine which genes are regulated (3). These dimers then bind to TPA-response element (TRE; TGACTCA) in the promoters of target genes and regulate transcription (2). AP-1 proteins convert extracellular signals into changes in the expression of specific target genes that have AP-1 binding sites in their promoter regions (7).

These proteins have been implicated in a large variety of biological processes including cell differentiation, proliferation, apoptosis and tumor suppression. AP-1 proteins control these various processes through their ability to regulate the expression and function of certain cell cycle regulators such as Cyclin D1 and p53 (18). AP-1 activity is modulated by interactions with other transcriptional regulators and is further controlled by upstream kinases that link AP-1 to various signal transduction pathways, including that of TGF-β (25). TGF-β effects, through AP-1 proteins, may involve both the Smad dependent and Smad independent pathway. In the Smad independent pathway, phosphorylation by JNK leads to changes in AP-1 dependent gene expression. Whereas, in the Smad dependent pathway, many of TGF-β dependent genes contain SBE sites as well as AP-1 sites, and both work in a cooperative fashion to control gene expression.

Previous studies have shown that AP-1 family of transcription factors are involved in TGF-β effects on cell proliferation (3). It has been shown that cJun is required for the inhibitory effects of TGF-β on target cells (5). Previous studies that have been
performed in our lab also confirm that AP-1 proteins are indeed involved in TGF-β effects on prostate cancer cells. Furthermore, research shows that this mechanism may involve the interaction between AP-1 and Smad proteins, as mentioned above (14).
After the media containing the various concentrations of TGF-β was removed, 1.0 uC/mL of $^3$H-thymidine was added and the incorporation of $^3$H-thymidine into DNA was determined after incubating for 4 hours. After this, $^3$H-hymidine was removed and 1mL of distilled water was added to each well. The cells were then sonicated and transferred to the MilliPore Vacuum System and filtered using DE-81, ion-exchange filters. The macromolecules, (including DNA) bind to these filters while free thymidine is removed. The filters were washed and collected in scintillation vials and counted using a liquid scintillation counter (10).

Specific Aim Two: To Determine which AP-1 Family Members are Expressed in PC3M and DU145 Cell Lines

Rationale: The purpose of this aim is to determine which of the seven members of the AP-1 family are present or absent in both DU145 and PC3M cells. The goal is to determine the mRNA expression levels of AP-1 family members and the effects of TGF-β on AP-1 mRNAs.

Experimental Design

RNA Isolation and RT-PCR

Total RNA was isolated from prostate cells using TRIzol (Life Technologies) followed by chloroform extraction and isopropanol precipitation. Cells were lysed by adding 1 mL of TRIzol to the sample and incubating at 15-30°C for 2-3 minutes. Chloroform was added (0.2 per mL of TRIzol) to the sample, and then it was centrifuged at 12,000g for 10 minutes at 8°C. RNA was removed from the aqueous
phase and transferred to a fresh tube. RNA was precipitated from the aqueous phase by adding isopropyl alcohol (0.5mL per 1mL of TRIzol). Samples were incubated at 30°C for 10 minutes and centrifuged at 12,000g for 10 minutes at 8°C. The supernatant was removed and the pellet, containing the RNA precipitate, was washed with 75% ethanol. The samples were mixed by vortexing and centrifuged at 7,500g for 5 minutes at 8°C. This step was repeated and all ethanol was removed. The RNA pellet sat for 10 minutes to ensure it was dry. The RNA pellet was redissolved in deionized distilled water and the concentration of total RNA was determined using a spectrophotometer.

Two micrograms of total RNA was reverse transcribed in a reaction mixture containing 10 mM dNTP, 400 U of M-MLV reverse transcriptase 1X RT buffer, 0.1mM DTT and 0.5μg of oligo dT. The reaction mixtures were incubated for 1 hour at 37°C. After incubation, the samples were heated at 65°C for 5 min to inactivate the enzyme. The cDNA samples were stored at -20°C. Samples of 1μl cDNA were added to separate PCR reaction mixtures (total 10 μl) containing 0.1 mM dNTPs, 0.5 U Taq DNA polymerase, PCR buffer with 3mM MgCl₂ and specific primer pairs. GAPDH and L19 primer pairs were used as internal controls. The samples went through PCR reaction in the Rapid Cycler thermal cycler. The PCR products were visualized on 2% agarose gels stained with ethidium bromide (28).
Specific Aim Three: To Determine Quantitative Differences in the Expression Pattern of AP-1 Family Members in Prostate Cancer Cells in Response to TGF-β

Rationale: The purpose of this aim is to determine how the expression levels of AP-1 family members from both cell lines change when treated with TGF-β. The goal is to determine whether mRNA expression is upregulated/downregulated as a result of TGF-β and to observe any differences in AP-1 mRNA expression between the cell lines as a result of TGF-β treatment.

Experimental Design

Real-Time PCR

Reverse transcriptase PCR is a semi-quantitative procedure therefore real-time PCR was employed because of the need to quantitate differences in mRNA expression. Gene-specific primers have been designed using Beacon Designer software (table 1). Primers were designed in the gene regions that were unique to the target genes, that is, not homologous to any other gene in GeneBank, and to span long introns when possible. Reverse transcription was performed for 1 h at 37°C in a reaction mixture containing 10 mM dNTP, 400 U of M-MLV reverse transcriptase 1X RT buffer, 0.1 mM DTT and 0.5 μg of oligo dT and was stopped by incubation at 65°C for 5 minutes. Real Time-PCR was performed using a master mix which consisted of RNAase free water and SYBR Green Supermix (BioRad Laboratories). The reaction conditions for Real-time PCR were the following: 40 cycles consisting of 15 s incubation at 95°C and 1
min incubation at 60°C on the I-Cycler IQ (BioRad, CA). Threshold cycle number (Ct) for each reaction was obtained at the default baseline settings of the instrument manufacturer. PCR reactions were run in duplicates to assess technical variability of the assay. Experiments were repeated four times starting with independent cell cultures and RNA isolation. Delta-delta Ct method was used for the calculation of relative gene expression change caused by TGF-β treatment for each target gene. L19 gene was used as internal standard gene to account for variability of input cell numbers, RNA isolation, and reverse transcription procedures (27).

STATISTICAL ANALYSIS

Data were expressed as mean ± SE. Statistical differences were analyzed using Student’s t-test. Differences were considered as statistically significant at the p< 0.05 level.
Table 1. Gene Primer Sequences for AP-1 Family Members

<table>
<thead>
<tr>
<th>Accession (RefSeq #)</th>
<th>AP-1 members</th>
<th>Product Length, bp</th>
<th>Sense Primer</th>
<th>Anti-sense Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>J04111</td>
<td>c-Jun</td>
<td>242</td>
<td>TGGAAACGACCTTCTATGACGA</td>
<td>GTTGCTGGACTGGATTATCAGG</td>
</tr>
<tr>
<td>NM 002239</td>
<td>JunB</td>
<td>129</td>
<td>TACCACGACGACTCATACACA</td>
<td>CGCTTTGAGACTCCGGTAGG</td>
</tr>
<tr>
<td>NM 005354</td>
<td>JunD</td>
<td>322</td>
<td>CGGCAGCATGATGAAGAAGGA</td>
<td>GTGTAATCCTCCAGGGCCCTTG</td>
</tr>
<tr>
<td>NM 006732</td>
<td>FosB</td>
<td>245</td>
<td>GAAATGCCCCGATTCTTCGT</td>
<td>CTGGTAGTCCCGCTGGTGGA</td>
</tr>
<tr>
<td>NM 005252</td>
<td>c-fos</td>
<td>72</td>
<td>CTACCACTCAAGCCAGACT</td>
<td>AGGTCCGTGCAAGATCCT</td>
</tr>
<tr>
<td>NM 005253</td>
<td>Fra-2</td>
<td>150</td>
<td>CTGCTGGATCAAGTGCTTTC</td>
<td>GATTCAACAGACAACCGAATGG</td>
</tr>
<tr>
<td>NM 005438</td>
<td>Fra-1</td>
<td>220</td>
<td>TCTTCACCTACCCACGCACTC</td>
<td>GCTGGAGTTGGATGTTGGGATAC</td>
</tr>
</tbody>
</table>

Note: These primer sequences were designed for the AP-1 family members using Beacon Designer 4.01 (Premier Biosoft International) according to the manufacturer's preset defaults following GenBank cross-homology analysis. Primers within each gene-specific pair were chosen to anchor proteins of long introns when possible.
CHAPTER IV

RESULTS

TGF-β Inhibits DNA Synthesis in DU145 Cells

In normal epithelial cells, TGF-β inhibits growth and DNA synthesis. We examined TGF-β effects in the DU145 cell line using $^3$H-Thymidine-incorporation procedure. Cells were treated with 0 ng/mL (control), 0.1 ng/mL, 1.0 ng/mL and 10.0 ng/mL of TGF-β for 18 hours (fig. 1). TGF-β in all tested concentrations inhibited DNA synthesis in DU145 cells, with a maximum effective concentration between 1 and 10 ng/mL of TGF-β. Data presented here demonstrate that growth of DU145 cells was inhibited by exogenous TGF-β in a concentration-dependent manner.
Fig. 1. Rate of DNA synthesis was determined using $[^3H]$Thymidine-Incorporation. DU145 cells were treated with 0.1 ng/mL, 1.0 ng/mL, and 10 ng/mL of TGF-β for 18 hours, (control, no TGF-β). Cells were sonicated and filtered through the DE-81 filters and $[^3H]$Thymidine Incorporation into DNA was determined.

TGF-β Does Not Inhibit DNA Synthesis in PC3M cells

Under the aforementioned conditions, TGFβ response was examined in PC3M cells. We chose a concentration of 10.0 ng/mL of TGFβ for treatment of PC3M cells as it was most effective in DU145 cells. PC3M cells treated with 10.0 ng/mL of TGF-β exhibited no inhibition of DNA synthesis (fig. 2).
Fig. 2. Rate of DNA synthesis was determined using [3H]Thymidine-Incorporation. PC3M cells were treated with 10 ng/ml of TGF-β for 18 hours (control, no TGF-β).

AP-1 Family Members Expressed in PC3M and in DU145 Cells

PC3M and DU145 cells were treated with TGF-β for 4 hours. Media were then removed and total RNA was isolated. Total cellular RNA was used in semi-quantitative RT-PCR to detect mRNA for JunD, cJun, JunB, FosB, cFos, Fra1 and Fra2 AP-1 family members. In order to successfully isolate and purify this RNA, specific optimization techniques were employed. Total RNA was isolated from 10 mm plates of DU145 and PC3M cells using the appropriate amounts of TRIzol followed by chloroform extraction and isopropanol precipitation. Appropriate controls were used to ensure that there was no degradation or contamination of RNA. These included no template control, which checked for cross contamination and no RT controls which checked for the presence of genomic DNA (data not shown). In both DU145 and PC3M cell lines, all AP-1 family
mRNAs were detected (fig. 3). RT-PCR results shown in figure 3 indicate significant quantitative variation in the amounts of amplification products among different AP-1 genes and between cell lines in both control and TGF-β treated cells. This suggested that TGF-β has different effects on the expression levels of the AP-1 family members in PC3M and in DU145. However, the more quantitative method to determine gene expression, Real-Time PCR, was employed to measure differences in AP-1 gene expression.
Fig. 3. Detection of mRNA expression for JunD, cJun, JunB, FosB, cFos, Fra1 and Fra2 AP-1 family members. RT-PCR was performed on total RNA isolated from TGF-β-treated (+) and non-treated (-) DU145 and PC3M cells. Samples were ran on 2% agarose gels and visualized using ethidium bromide staining followed by imaging on BioRad Imaging station.
TGF-β -Induced Changes in AP-1 Gene Expression in PC3M Cells

Expression levels of AP-1 family members in PC3M cells were determined using real time RT-PCR method. Gene-specific primers were designed using Beacon Designer software (table 1). Primers were designed in the gene regions that were unique to the target genes (not homologous to any other gene in GeneBank) and to span long introns, when possible. The conditions for RT-PCR were optimized as described in the Materials and Methods section and reverse transcription was performed. Threshold cycle number (Ct) for each reaction was obtained at the default baseline settings of the instrument manufacturer. PCR reactions were run in duplicates to access technical variability of the assay. Calculations of the fold changes caused by TGF-β treatments were done as follows: Ct cycle numbers were first averaged between two technical duplicates and then input into the following equation (27):

$$ R = 2^{-\Delta Ct_{\text{treated}} - \Delta Ct_{\text{control}}} $$

Where R is ratio of expression in treated over untreated samples

$\Delta Ct_{\text{treated}} = Ct_{\text{target gene}} - Ct_{\text{std.gene in treated samples}}$

$\Delta Ct_{\text{control}} = Ct_{\text{target gene}} - Ct_{\text{std.gene in untreated samples}}$

The fold differences were averaged from three independent experiments and plotted with standard errors of the mean (SEM) as shown in figure 4.

The results (fig. 4) indicate that TGF-β significantly upregulated Fra2 and FosB and significantly downregulated JunD and Fra1 mRNAs (indicated by asterisks). Results shown previously indicated that TGF-β had no inhibitory effects on PC3M cells at the
level of DNA synthesis. However, data presented here indicates that TGF-β does influence the expression of AP-1 family members in PC3M cells at the transcriptional level.

Fig. 4. Real-time PCR analysis of gene expression in TGF-β treated PC3M cells. Cells were treated with 10ng/ml of TGF-β for 4 hours. Total RNA was isolated using Trizol as described in Materials and Methods. Reverse transcription was performed with 20 ug of RNA using random primers. Samples containing 1ug of cDNA were subjected to PCR using gene-specific primer pairs and SYBR-Green containing master mix (BioRad). Detection in real time was done on the IQ ICycler (BioRad, Inc). Level of gene expression in untreated cells was assumed to be 1, fold differences in gene expression due to TGF-β treatment are shown.

TGF-β -Induced Changes in AP-1 Gene Expression in DU145 Cells

This Real-time PCR procedure was performed under the same conditions as the previous experiment to determine the specific mRNA expression pattern of AP-1 family members in DU145 cells. The results depicted in figure 5 show that TGF-β exerted some
effect on AP-1 gene expression levels; however, due to high experiment-to-experiment variability, all of the fold data differences were statistically not significant.

Fig. 5. Real-time PCR analysis of gene expression in TGF-β treated DU145 cells. Cells were treated with 10ng/ml of TGF-β for 4 hours.

Comparison of Expression Pattern of AP-1 Genes in TGF-β-Treated DU145 and PC3M Cells

The results depicted in figure 6 compare the effects of TGF-β on mRNA expression levels of the AP-1 family members in DU145 and PC3M cells. These results indicate that exogenous TGF-β altered mRNA expression levels of the AP-1 family members in both cell lines.
Fig. 6. Real-time PCR analysis of gene expression in TGF-β - treated DU145 and PC3M cells. Both cells were treated with 10ng/ml of TGF-β for 4 hours.
CHAPTER V
DISCUSSION

TGF-β has a myriad of functions, including cellular proliferation, differentiation, and function in a wide variety of target cells. However, as it pertains to the prostate gland, the well known effect of TGF-β is to inhibit growth of prostate epithelial cells (26). In response to preliminary studies (unpublished) that investigated the role of JunD in TGF-β inhibitory effects, we decided to examine all seven members of the AP-1 family. The primary objective of this project was to investigate which of the AP-1 family members are expressed and how their expression changes in response to treatment with TGF-β in the DU145 cell line and in the PC3M cell line. There is little information in the current literature on AP-1 proteins as it pertains to the TGF-β signaling pathway in prostate cancer cells. As a result, much of this research was preliminary effort in order to achieve a better understanding of the manner in which AP-1 proteins play a role in TGF-β signaling in prostate epithelial cells.

The parent line of PC3M is PC3 and literature confirms that PC3 cells contain TGF-β receptors (9). Therefore, the PC3M cells should have been responding to the inhibitory effects of TGF-β, as does the parent line. Conversely, under conditions used in the laboratory, PC3M cells did not respond to this effect, so we initially hypothesized that perhaps the TGF-β signaling was impaired in this cell line. However, this research suggests that TGF-β signaling is active in PC3M cells, but the effect is not inhibitory.
The present findings confirm that TGF-β inhibits DNA synthesis in DU145 cells. As a result this line was considered to be TGF-β responsive (11). However, under the conditions that we used in the laboratory, TGF-β had no inhibitory effects on DNA synthesis in PC3M cells, so this line was classified as TGF-β unresponsive. The PC3M cell line is a metastasized line derived from liver metastasis of PC3 transplanted prostate tumor in nude mice. Unlike the parent line, PC3M is very aggressive and highly invasive (1). There have been few studies on the PC3M line, but many studies on the parent line PC3 showed opposite results than those reported in the present study. That is, that PC3 cells are growth inhibited by TGF-β (11). We conclude that the differences in TGF-β response are due to the metastatic nature of the PC3M line in contrast to that of the parent PC3 line or due to the different experimental conditions used in the different studies (17).

After confirming that there was a difference in the growth response after treatment with TGF-β, the next step was to determine whether or not the AP-1 members had some effect on the TGF-β inhibitory response. To do this we first performed Reverse Transcriptase-PCR to examine which of the AP-1 family members were expressed in both cell lines. We confirmed that all the AP-1 family members were expressed in both DU145 and PC3M cells. Moreover, there were differences in the expression levels of the mRNAs in the individual lines in response to TGF-β. In DU145 cells the RT-PCR results showed that there was an increase in JunD, JunB, and cJun expression levels and a decrease in Fra2 expression levels after being treated
with TGF-β for 4 hours. Under the same conditions, the levels of Fra1, cFos and FosB were similar in the control and TGF-β treated samples.

In the DU145 cell line, there were changes in the expression of specific AP-1 members after treatment with TGF-β. Therefore, further experimentation was needed to distinguish which of the AP-1 proteins may be directly involved in TGF-β inhibitory effect in this line.

In PC3M cell line, RT-PCR results indicated that when TGF-β was added there was a slight increase in the expression levels of cJun and the other AP-1 family members had similar expression levels in both the control sample and the TGF-β-treated sample. These results indicate that even though TGF-β did not inhibit DNA synthesis in the PC3M line, it is exerting some effects on expression of AP-1 proteins. Therefore, TGF-β signaling is intact in these cells and through AP-1 family members, TGF-β may be exerting one of its other functions in PC3M cells. In order to confirm and quantify these RT-PCR findings in both cell lines, the more precise method of gene expression, Real-Time PCR, was employed to measure AP-1 family gene expression.

The Real-Time PCR results clearly indicate that TGF-β had an effect on both of the cell lines. In PC3M cells, TGF-β significantly upregulated Fra2 and FosB and significantly downregulated JunD and Fra1. This result is evidence that not only is the TGF-β signaling intact in PC3M cells, but that TGF-β has an alternative role, one that is distinct from the inhibition of cell proliferation (26).

The data generated in this study indicated that TGF-β had an effect on the AP-1 expression levels in DU145 cells. There was an increasing trend of JunD, JunB, cJun
and FosB expression levels and a decreasing trend in Fra1 and JunD in response to TGF-β.

The results for this experiment alone were inconclusive because the Real-Time PCR results for DU145 were variable. Even though both PC3M and DU145 experiments were done under the same conditions at the same time, the results for DU145 were not consistent. This variability could have been due to a number of factors, including the biological nature of DU145 cell line. However, we compiled data from the most reproducible experiments and compared both of the cell lines.

When examining both lines simultaneously, we noticed several AP-1 family members had opposing expression profiles after the addition of TGF-β, namely JunD, cFos and Fra2. In DU145, expression levels of JunD were increased, while expression levels of cFos and Fra2 were decreased as a result of TGF-β treatment. Whereas in PC3M cells, expression levels of JunD were decreased and there was an increase in levels of cFos and Fra2. The findings presented here strongly suggest that the TGF-β signaling pathway is intact in both cells lines. The results are also indicative of AP-1 family members being involved in the TGF-β signaling pathway in DU145 as well as PC3M. These results also suggest that TGF-β induces separate and opposing effects on AP-1 which result in differential biological responses in PC3M and DU145 cells. We know that TGF-β receptors are present in both lines, so this varying of function may be due to some differences in the post-receptor pathways in PC3M cells. This difference can perhaps be accredited to the fact that the PC3M cell line is metastasized and highly aggressive whereas DU145 cell line is not.
In summary, the goal of this work was to attain a better understanding of the role of specific members of the AP-1 proteins that aid TGF-β in the inhibition of cellular proliferation. In the case of PC3M cells, we confirmed that TGF-β has a role other than cell inhibition, which may be due to the fact that PC3M is a highly aggressive, metastatic line. In the DU145 cells we simply confirmed what is already known, that TGF-β inhibits cell proliferation. In both cells lines our data strongly suggests that AP-1 family members are involved in both TGF-β inhibitory function in the DU145 and its alternative function in PC3M cells. Further studies must be carried out in order to confirm exactly the alternative function of TGF-β in PC3M cells. Further studies in S.Khan’s laboratory, along with the findings presented here, will be able to clarify the exact mechanism by which AP-1 proteins interact with TGF-β in order to perform its various functions. Once this can be elucidated, it will lead to therapeutically targeting AP-1 family members in TGF-β mediated processes in prostate cancer development and progression.
REFERENCES


