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DR. WILL KOPACHIK

Major professor

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**FORKHEAD GENE EXPRESSION IN PROSTATE TISSUES  
AND CELL LINES**

**By**

**CONG DING**

**A THESIS**

**Submitted to  
Michigan State University  
In partial fulfillment of the requirements  
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## **ABSTRACT**

### **FORKHEAD GENE EXPRESSION IN PROSTATE TISSUES AND CELL LINES**

By

Cong Ding

Some members of the forkhead class of transcription factors may play a role in controlling the cell cycle or cell survival. The AFX forkhead subfamily might transcriptionally activate the p27<sup>Kip1</sup> gene. The FREAC-4 gene of the FREAC subfamily is related by sequence to the *qin*. We determined which subfamily members were expressed in the prostate by RT-PCR analysis using degenerate primers made to the conserved DNA-binding domain of the forkhead sequences. We used prostate cancer tissue, BPH tissue and prostatic cell lines (LNCaP, LNCaP C4-2B, DU-145, PC-3) as sources of the RNA. The sequence analysis of the cloned cDNAs showed no novel family members (if PCR generated mutations are discounted). Of five AFX subfamily members (AFX, FKHR, FKHL1, FKHRP1, FKHL1P1), only the first three were detected. For the FREAC subfamily, only FREAC-3 and FREAC-4 were detected. We designed sequence-specific primers to examine the expression of the individual family members by semi-quantitative RT-PCR in comparison to GAPDH expression. For the tissues, AFX expression was a minimum of 8 fold higher in prostatic cancer than in BPH, whereas FKHR and FKHL1 expression were lacking in the BPH. For the cell lines, all expressed AFX but PC-3 was unusual in that it alone had negligible expression of FKHR and FKHL1. The negligible expression of FKHR and FKHL1 correlated with the very low expression of p27<sup>Kip1</sup> in PC-3 cells and BPH. Thus, FKHR and/or FKHL1, but not AFX, may be responsible for p27<sup>Kip1</sup> expression in prostate cells.

**Dedicated to my parents and my husband  
For their endless love and support**

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## **LIST OF ABBREVIATIONS:**

<b>ALL</b>	<b>--acute lymphoblastic leukemia</b>
<b>AR</b>	<b>--androgen receptor</b>
<b>BPH</b>	<b>--benign prostate hyperplasia</b>
<b>CEF</b>	<b>--chicken embryo fibroblasts</b>
<b>CWH</b>	<b>--chicken winged helix</b>
<b>DBD</b>	<b>--DNA binding domain</b>
<b>DHT</b>	<b>--dihydrotestosterone</b>
<b>DTT</b>	<b>--dithiothreitol</b>
<b>GAPDH</b>	<b>--glyceraldehyde-3-phosphate dehydrogenase</b>
<b>HFH</b>	<b>--hepatocyte/forkhead homologue</b>
<b>HNF-3<math>\alpha</math></b>	<b>--hepatocyte nuclear factor-3<math>\alpha</math></b>
<b>PCa</b>	<b>--prostate cancer</b>
<b>PCR</b>	<b>--polymerase chain reaction</b>
<b>PI3K</b>	<b>--phosphatidylinositol 3-kinase</b>
<b>PKB</b>	<b>--protein kinase B</b>
<b>PSA</b>	<b>--prostate-specific antigen</b>
<b>RT</b>	<b>--reverse transcription</b>
<b>SAGE</b>	<b>--serial analysis of gene expression</b>

## **INTRODUCTION:**

### **Prostate Cancer and Androgen Response:**

Prostate cancer is the most commonly diagnosed human cancer and the second leading cause of cancer mortality (after lung cancer) among males in the United States (Greenlee et al. 2000). It is reported that in 2000, more than 180,000 American men have been diagnosed with prostate cancer and 38,000 will have died of the disease. The rate of prostate cancer (PCa) increases dramatically as men age. In men over the age of 55, PCa is responsible for nearly 4% of all deaths (Parker et al. 1997). An understanding of the molecular basis for prostate tumorigenesis is thus important.

The adult male prostate surrounds the neck of the bladder and prostatic urethra. Normal prostate is composed of tubuloalveolar glands embedded in fibromuscular stroma. The prostate can be divided into several zones: peripheral, central, transitional, and periurethral. About 70% of prostatic carcinomas develop from the peripheral zone (De Marzo et al. 1999). Normal prostate evolves atypical epithelial proliferations of the prostate gland. Benign prostate hyperplasia (BPH) is a benign disorder that develops predominantly in the transition zone of the prostate. BPH can be overgrowth or hyperplasia of both the epithelial and stromal compartments. Very large BPH glands are primarily rich in epithelial growth, but in some BPH glands the stromal elements are predominant.

Prostate homeostasis is controlled by balanced cell proliferation, cell survival, and cell death. Apoptosis is the process of programmed cell death in vertebrates that plays a central role in development and homeostasis. There is a close association between

programmed cell death (apoptosis) and prostate cancer initiation, progression, metastasis, and response to treatment. Prostate cancer cells require androgen for growth at early stages of development and later during puberty. The androgen dihydrotestosterone (DHT) can bind to the prostatic androgen receptor (AR). After binding DHT, the AR is released from cell membrane, then transferred into nucleus. AR can bind specific DNA sequences called androgen-responsive elements. The androgen-responsive elements are located in the promoter region of the androgen-responsive genes, such as prostate-specific antigen (PSA) (Sadar et al. 1999). Then the AR is able to induce the transcription of the androgen-responsive genes (Murtha et al. 1993).

The aim of androgen deprivation therapy is to reduce the androgen level in prostate cancer or interfere with the AR activity and thus reduce AR-mediated transcription. Deprivation of androgen function can lead to prostate cancer atrophy. However, during cancer progression, prostate cancer is composed of androgen-dependent and androgen-independent cells (Taplin et al. 1995). The androgen deprivation therapy fails when the androgen-independent cells keep growing even in very low androgen concentration. Failure to prevent androgen-independent progression is the main obstacle to improving the survival of this disease. Androgen deprivation eliminates most androgen-dependent cancer cells by inducing apoptosis but can rarely cure the patients due to the presence of androgen-independent cells and emergent of apoptosis-resistant clones (Kyprianou et al. 1990; Denmeade et al. 1996). Androgen-independent growth of prostate carcinoma is thought to mainly arise through mutations of AR. For example, mutations of AR in the steroid-binding domain could cause the loss of normal repression of AR transactivation in the absence of androgen. Some mutant AR will be constitutively

active (Castagnaro et al. 1993).

### **Forkhead Transcription Factor:**

Transcriptional factors other than steroid receptors can also control vertebrate organ and tissue development through binding to their target DNA sequences. Transcription factors are classified according to the structural motif involved in DNA binding. The forkhead/HNF-3 motif was detected in the *Drosophila* forkhead gene product and in rat hepatocyte nuclear factors 3 (HNF-3). The prototype gene coding for forkhead was discovered during developmental studies in *Drosophila*. The *Drosophila* forkhead gene is essential for the proper formation of terminal structures of the embryo. Forkhead refers to the phenotype of mutants that show homeotic transformations in the anterior and posterior gut with head-like structures at both ends of the embryo (Weigel et al. 1989). The prototypes of forkhead in mammals are hepatic nuclear factor (HNF) 3 $\alpha$ ,  $\beta$ , and  $\gamma$ . The HNF-3 protein family is hepatocyte enriched DNA-binding transcription factors in rodents.

Since the discovery of the winged-helix domain in the *Drosophila* forkhead and the rodent HNF-3 factors, approximately 90 members with homologues have been identified. They usually are a large family within a species. For instance there are 35 forkhead genes in the human genome. They are expressed in a wide range of organisms such as *Saccharomyces cerevisiae* (Bork et al. 1992), *Caenorhabditis elegans* (Miller et al. 1993), Zebra fish (Strahle et al. 1993), *Xenopus laevis* (Knochel et al. 1992), mouse (Kaestner et al. 1993), rat (Clevidence et al. 1993), and human (Pierrou et al. 1994; Kaufmann et al. 1996). Recently, in the domain-based comparative analysis by Venter *et.*

*al*, the forkhead domain was found in 36 proteins in *H. sapiens*, 16 proteins in *D. Melanogaster*, 6 proteins in *C. elegans* and 16 proteins in *S. cerevisiae* (Venter et al. 2001). All members of the family show high sequence homology within their 110 amino acid DNA-binding domain. Forkhead transcription factors are also called winged-helix proteins based on their three dimensional structure when bound to DNA. Winged helix domain is a variant of the helix-turn-helix motif as determined from the crystal structure of HNF-3 $\gamma$  (Clark et al. 1993). Three  $\alpha$ -helices (H1, H2 and H3) dominate the amino-terminal half of the protein and form a globular, three-helix cluster, whereas two wing (or loop)-like regions W1 and W2 are near the carboxyl-terminal end. The target DNA is recognized by the  $\alpha$ -helix H3 and the two wings W1 and W2. H3 helix makes contacts with the major groove of double-stranded DNA and the two adjacent loop structures stabilizes the contacts.

The forkhead family members have been shown to play key regulatory roles in embryonic development, differentiation, and tumorigenesis. For example, the HNF-3 proteins exert a profound role at all stages of embryogenesis beyond their role as a liver-specific transcription factor (Ang et al. 1993; Monaghan et al. 1993). In vertebrates, the forkhead proteins control a wide variety of developmental processes, including formation of the node and notochord, the development of the cerebral hemispheres and the differentiation of the gastrointestinal epithelium (Ang et al. 1994; Xuan et al. 1995; Kaestner et al. 1997). The expression of AR is regulated by Hepatocyte nuclear factor-3 $\alpha$  (HNF-3 $\alpha$ ). HNF-3 $\alpha$  is a member of the hepatocyte/forkhead homologue (HFH) family of transcription factors originally identified in the liver and expressed in the rodent prostate epithelial cells at a 14-20 fold higher level than the liver. The HNF-3 $\alpha$  consensus

sequence, VAWTRTTKRYTY, was found in regulatory sequences of the androgen receptor in a database search (Overdier et al. 1994) and HNF-3 $\alpha$  can repress gene transcription from the androgen receptor promoter in vitro (Olle and Kopachik, unpublished results).

### **Forkhead Transcription Factors and Tumorigenesis:**

Three forkhead family members, *qin* oncogene, AFX and FKHR, are linked to tumorigenic progression (Li et al. 1993; Parry et al. 1994; Fredericks et al. 1995). The first oncogene reported with winged helix structure was the avian sarcoma virus 31 (ASV 31) gene *qin*. ASV 31 is a highly oncogenic retrovirus found in a spontaneous connective tissue tumor of an adult chicken (Li et al. 1993). Retroviral oncogenes are derived from the genome of the host cell and are cellular growth-regulatory genes. They are mutated and inserted into the viral genome and expressed under control of viral regulatory sequences. Incorporation of a mutated cellular oncogene makes a retrovirus highly tumorigenic and capable of transforming cells in culture. The *qin* gene is a cell-derived insert in the genome of ASV 31 and determines the transforming activity of the retrovirus (Galili et al. 1993). The viral *qin* gene (*v-qin*) is the oncogene in the virus. Part of the viral *gag* and all of the *pol* region are missing and replaced by the *qin* insert that is expressed as a Gag-Qin fusion protein. The *v-qin* gene differs from its cellular counterpart *c-qin* by an N-terminal leader sequence, several amino acids substitutions and a C-terminal deletion. Both *c-qin* and *v-qin* stimulate the growth of chicken embryo fibroblasts (CEF) and induce transformed cell foci and colonies in soft agar culture (Li et al. 1997a). V-Qin is also highly tumorigenic in the animal in contrast to c-Qin, which

causes tumors in only a small fraction of the injected animals and only after a prolonged latent period (Li et al. 1997a).

Three cDNA clones "chicken winged helix (CWH) 1, 2, and 3" were isolated from a chicken embryonic cDNA library by low-stringency hybridization using the *c-qin* DNA binding domain as a probe (Freyaldenhoven et al. 1997a). The amino acid sequence of the DBD of CWH-1 is highly conserved in rat fork head factor HFH-B2, mouse fork head factor BF-2, and human fork head factor FREAC-4 (Freyaldenhoven et al. 1997b). Overexpression of wild type CWH-1 protein from the replication competent retroviral vector RCAS induces changes in morphology (Freyaldenhoven et al. 1997a), stimulates anchorage independent growth and causes increased saturation density of CEF. These results suggest that winged helix transcription factor CWH-1 has the potential to stimulate abnormal cell proliferation.

The FREAC-4 gene is probably the human homologue of CWH-1. It was first obtained by a PCR-based strategy with primers designed from regions conserved between rat HNF-3 and *Drosophila* forkhead and a human cDNA template (Pierrou et al. 1994). Northern blot analysis by Pierrou *et al.* (1994) showed that FREAC-4 expressed in the human kidney and testis tissue but not in normal prostate tissue. Cotransfection experiments done by Ernstsson *et al.* (1996) demonstrated that FREAC-4 is regulated by two tumor suppressors, WT-1 and p53 (Ernstsson et al. 1996). FREAC-4 promoter contains a binding site capable of interacting with WT-1. A WT-1 expression- plasmid cotransfected with the FREAC-4-luc reporter construct induced a 3-fold increase in reporter gene activity. The tumor suppressor gene, p53, was found to repress the same reporter gene activity approximately 4-fold (Ernstsson et al. 1996).

Two additional forkhead genes, AFX and FKHR, were first identified at chromosomal breakpoints in human tumors. In one type of acute lymphoblastic leukemia (ALL), the t(X;11) translocation arises from the fusion of the Zn-finger transcription factor MLL/ALL1 gene on chromosome 11 to the AFX forkhead gene on chromosome X (Parry et al. 1994; Borkhardt et al. 1997). The AFX gene contributes a truncated forkhead domain and C-terminal sequences to the t(X;11) translocation. Similar to AFX, FKHR has also been implicated in oncogenesis as a result of its involvement in the chromosomal translocation t(2:13)(q35;q14) frequently found in human alveolar rhabdomyosarcomas (Galili et al. 1993; Davis et al. 1994). This translocation leads to the generation of a chimeric gene composed of PAX3, a member of the paired box transcription factor family, and FKHR. The resulting PAX-FKHR fusion protein contains an intact DNA-binding domain of PAX fused to the COOH-terminal half of the forkhead domain and the transactivation domain of FKHR. The PAX3-FKHR product exhibits potent transactivation activity when compared to wild-type PAX3. The fusion of FKHR and AFX with their corresponding partner genes PAX3 or MLL, respectively, occurs at identical amino acid positions. Previous studies have shown that members of the forkhead gene family can be subgrouped according to their degree of similarity within the DNA-binding forkhead domain (Kaufmann et al. 1996). The five human forkhead genes (FKHR, FKHL1, FKHL1P1, AFX, FKHRP1) that show striking similarity within the DBD can be grouped into a AFX subfamily (Galili et al. 1993; Shapiro et al. 1993; Davis et al. 1994; Anderson et al. 1998).

## **Forkhead Transcription Factors in Cell Signaling Pathway and Cell Cycle Control:**

Recent studies strongly suggest that AFX, FKHR and FKHL1 are downstream targets of the phosphatidylinositol 3-kinase (PI3K) pathway (Figure1). Akt (also called PKB) is the downstream target protein of PI3K. It is also a Ser/Thr protein kinase. Following insulin or insulin-like growth factor stimulation AFX, FKHR and FKHL1 can be phosphorylated by PKB. AFX, FKHR and FKHL1 contain multiple putative PKB phosphorylation sites that can be recognized by the amino acid sequence RXRXXS/T. Thus these transcription factors are potentially direct targets of insulin-activated PKB (Paradis et al. 1998; Ogg et al. 1998).

The activation of endogenous PKB can be impaired by PTEN/MMAC1 tumor suppressor gene. PTEN encodes a phospholipid phosphatase and acts a negative regulator of the PI3K pathway (Li et al. 1998). The PTEN gene, identified and mapped to chromosome 10q23, is implicated in causing a wide range of human cancers (Steck et al. 1997). A previous study showed the metastatic capabilities of rat prostate tumor cells can be significantly inhibited by the reintroduction of the 10q region (Nihei et al. 1995). PTEN homozygous deletion and point mutations were detected in prostate cancer cell lines and a set of prostate cancer samples. PTEN expression levels are reduced in prostate cancer xenografts derived from patients with advanced prostate cancer (Li et al. 1997b; Whang et al. 1998). Somatic mutations of PTEN are found in a number of human malignancies, and loss of expression, or mutational inactivation of PTEN, leads to the constitutive activation of PKB via enhanced phosphorylation of Thr-308 and Ser-473. Higher levels of PKB activation are observed in human prostate cancer cell lines and xenografts lacking PTEN expression when compared with PTEN positive prostate tumors

or normal prostate tissue (Wu et al. 1998). An antiapoptotic signal is provided by the induction of Akt activity (Kennedy et al. 1997). In addition, PTEN represses gene expression that can be rescued by Akt but not PI3-kinase (Li et al. 1998). It also can inhibit G1 phase progression in cells that lack PTEN (Ramaswamy et al. 1999).

AFX, FKHR, and FKHL1 are mammalian homologues of the *C. elegans* forkhead transcription factor *daf-16*. *Daf-16* is critical to the regulation of life span in *Caenorhabditis elegans*. *Daf-16* and its mammalian homologues AFX, FKHR and FKHL1 contain three putative phosphorylation sites for PKB. PKB also inhibits the translocation of AFX, FKHR and FKHL1 to the nucleus, and therefore inhibits their transcriptional activity. AFX transcriptional activity is inhibited by phosphorylation from PKB (Kops et al. 1999). FKHR's phosphorylation by PKB also negatively regulated its transcriptional activity (Nakae et al. 1999; Guo et al. 1999). Similarly, FKHL1 is phosphorylated by PKB resulting in suppression of transcriptional activity and promotion of cell survival (Brunet et al. 1999).

FKHL1 plays a role in apoptosis. When survival factors such as insulin-like growth factor 1 are present, Akt phosphorylates FKHL1 at T32 and S253. Akt phosphorylated FKHL1 binds to a scaffolding protein called 14-3-3 protein and remains in the cytoplasm (Brunet et al. 1999). The absence of survival factors leads to FKHL1 dephosphorylation. The dephosphorylated FKHL1 is translocated into nucleus and activates target gene transcription (Brunet et al. 1999). Within the nucleus, nonphosphorylated form of FKHL1 triggers apoptosis probably by induction of expression of genes and cell cycle blockage that are critical for cell death. . Furuyama *et al.* (2000) determined that the target genes of the four forkhead proteins share a core

consensus binding sequences, TTGTTAC (Furuyama et al. 2000). Thus any one of the three could potentially redundantly activate target gene transcription from P27<sup>KIP1</sup> for instance.

P27<sup>KIP1</sup> is cyclin-dependent kinase inhibitor. The p27<sup>KIP1</sup> gene was first identified in cells arrested in G<sub>1</sub>. The G<sub>1</sub> phase of the cell cycle is inhibited by two families of cyclin dependent kinase inhibitors (CKIs). The two CKI families, Cip/Kip and INK4, have previously been shown to inactivate the cyclin-Cdk holoenzyme complexes. Members of the Cip/Kip family include p21<sup>Cip/WAF1</sup>, p27<sup>KIP1</sup> and p57<sup>Kip2</sup>. The Cip/Kip inhibitors at high concentrations blocked cyclin D-, E-, and A-dependent kinase activities. P27<sup>KIP1</sup> preferentially binds to and inactivates the CyclinE/Cdk2 complexes therefore inhibiting entry into S-phase (Slingerland et al. 1994). Upregulation of p27<sup>KIP1</sup> is linked to cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> through its interaction with CDK-cyclin complexes (Toyoshima et al. 1994). p27<sup>KIP1</sup> levels decrease in response to mitogens and increase in quiescence. Increasing levels of p27<sup>KIP1</sup> serve as a barrier to G<sub>1</sub>-S transition and may promote cell cycle exit. Dijkers *et al.* (2000) showed cytokine-mediated proliferation and survival are regulated through downregulation of p27<sup>KIP1</sup> (Dijkers et al. 2000). p27<sup>KIP1</sup> is rarely mutated, but its reduced expression has been seen in several cancers including prostate. Recently, reduced p27<sup>KIP1</sup> has shown promise as a marker of malignant prostate cancer (Yang et al. 1998; Tsihlias et al. 1998; Cordon-Cardo et al. 1998; Lloyd et al. 1999). Decreased expression of p27<sup>KIP1</sup> has been correlated with androgen deprivation failure, aggressive metastases and poor prognosis (Yang et al. 1998). Cordon-Cardo *et al.* (1998) found low/absent p27<sup>KIP1</sup> expression in the majority of metastatic, androgen-independent tumors. Low p27<sup>KIP1</sup> may be used to identify androgen independent tumors

(Yang et al. 1998; Cote et al. 1998). Transcriptional induction of p27<sup>KIP1</sup> in murine pre-B-cell line Ba/F3 is regulated by the forkhead-related transcription factor FKHRL1 (Dijkers et al. 2000). Activation of FKHRL1 is sufficient to elevate p27<sup>KIP1</sup> mRNA and protein levels and induce apoptosis. The regulation of p27<sup>KIP1</sup> transcription by forkhead-related transcription factors may be a general mechanism of cell survival, proliferation or differentiation (Dijkers et al. 2000).

The objective of my project was to examine the expression of the forkhead genes involved in tumorigenesis to uncover potential roles in development of normal and neoplastic cells of the prostate. We investigated the expression of FREAC subfamily and AFX subfamily of forkhead factors in prostate cancer tissues. FREAC-4, AFX, FKHR and FKHRL1 were found to be expressed. The expression levels of the four forkhead genes were then detected in prostate cancer (PCa) tissues, benign prostate hyperplasia (BPH) tissues and prostate cancer cell lines.

## **METHODS:**

**Cells and tissues:** Human prostatic carcinoma cell line LNCaP was established from a metastatic lesion of human prostatic adenocarcinoma by Horoszewicz *et al* (1983) (Horoszewicz *et al.* 1983). LNCaP is an androgen-responsive cell line but the C4-2B subline is more tumorigenic, metastatic and androgen-independent than LNCaP cell line (Chen *et al.* 1998). DU-145 (metastasis to brain), and PC3 (metastasis to bone) are two androgen-independent prostate carcinoma cell lines; they were obtained from Simon Hayward (UCSF). Cells were grown in HEPES-buffered RPMI 1640 medium (GibcoBRL) containing 10% fetal bovine serum (Intergen), 100 µg/ml streptomycin (Sigma) and 100 U/ml of penicillin (Sigma) at 37°C. Every 3-4 days the cells were subcultured trypsinization (0.05% trypsin/ 1mM EDTA) for 5 min at 37°C, 5% CO<sub>2</sub> and neutralization with serum. The cells were then recovered by low speed centrifugation and one tenth of the cell suspension was added to T75 flasks. Prostatic carcinoma tissue and benign prostatic hyperplasia tissue were obtained from Dr. Wartinger (MSU Urology).

**RNA Isolation:** Total RNA were prepared from 0.2 g tissue or 2 T75 flasks of near-confluent LNCaP, LNCaP C4-2B, DU-145 and PC3 cell lines. The tissue samples were minced with a razor blade. Five ml of 4M guanidinium/0.1 mM β-mecaptoethanol solution was added into the minced tissue, and tissues were homogenized. Cells were collected directly into guanidinium/β-mecaptoethanol, and scraped into a 15 ml tube. The volume was made up to 13 ml with guanidinium solution and total RNA was isolated by the method of centrifugation through a cesium chloride cushion described by

Chirgwin *et al* (1979) (Chirgwin et al. 1979). The OD<sub>260</sub> and OD<sub>280</sub> were determined by spectrophotometric analysis (Spectronic 601, Milton Roy) and the concentration of the RNA was obtained by multiplying the OD<sub>260</sub> by 40 µg/ml. The quality and integrity of the RNA were checked by visual inspection of the 18S and 28S rRNAs in ethidium-bromide-stained agarose gels.

**DNase digestion:** Twenty µg RNA was digested by RQ1 DNase (Promega) to eliminate the contaminating genomic DNA. DNase digestion was performed in a final volume of 100 µl containing 5 mM Tris pH 7.5, 15 µg BSA, 100 µM MgCl<sub>2</sub>, and 2 units of RQ1 DNase. The reaction was incubated in a 37°C water bath for 15 minutes. RNA was recovered after phenol:chloroform extraction and ethanol precipitation.

**Reverse Transcription (RT):** First strand cDNA was made from 10 µg RNA using M-MLV Reverse Transcriptase (Promega). In order to eliminate any secondary structure, initial denaturation of 10 µg of RNA was performed in a total volume of 10 µl for 5 min at 80°C. RT was performed on tissue samples in a final volume of 80 µl containing 10 µg total RNA, 5 mM dithiothreitol (DTT), 200 ng random primers, 0.5 M Tris-HCl, pH 8.3, 75 mM KCl, 1 mM each of dATP, dCTP, dGTP, dTTP, 40 units of RNase inhibitor, and 400 units of Promega MML-V reverse transcriptase (Promega). The reaction was incubated at 37°C for 1 hour. A negative RT control was prepared using the same RNA but without the addition of reverse transcriptase in order to check for contaminating genomic DNA in the RNA sample. Extraneous bands were not found in the negative control lanes.

**Design of primers:** Primers used are listed in Table 1. For FREAC subfamily expression, we used comparisons among the FREAC-3, FREAC-4, HFH-2, HNF-3 $\gamma$  motifs to design degenerate primers corresponding to conserved residues within the DNA-binding domain. We synthesized a sense primer corresponding to the KPPYSYI amino acid sequence and an antisense primer corresponding to REKFPW amino acid sequence. For the AFX/FKHR subfamily, we designed degenerate primers according to the conserved residues within the DBD of five AFX subfamily members (AFX, FKHR, FKHRL1, FKHRL1P1, and FKHRP1). We synthesized a sense primer corresponding to the AWGNQSY amino acid sequence and an antisense primer corresponding to WKNSIRH amino acid sequence. The sense and antisense primers contain *Hind III* and *Sal I* restriction sites at the 5' end, respectively. We also designed oligonucleotide primers based on the published human cDNA sequences FREAC-4, AFX, FKHR, FKHRL1 and GAPDH (Galili et al. 1993; Ernstsson et al. 1996; Borkhardt et al. 1997; Anderson et al. 1998). Expected sizes of the PCR products are as follows: FREAC and AFX subfamily: 180bp; FREAC-4, 311bp; AFX, 221bp; FKHR, 437bp; FKHRL1, 193bp; p27KIP1, 469bp; GAPDH, 452bp. The primers were synthesized by Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility.

**Polymerase Chain Reaction (PCR) of degenerate primers:** For the amplification of DBD sequences of FREAC and AFX subfamily, a PCR reaction mixture containing a final concentration of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 0.4  $\mu$ M each of sense and antisense primers, 4.0 units Taq polymerase in a final volume

of 100 µl. All samples were heat treated for 4 minutes at 94°C in a thermocycler for an initial denaturation, and then amplified for 40 cycles at 94°C for 1 min; 48°C for 2 min; and 72°C for 1 min. The samples were finally incubated at 72°C for 15 min for a final extension, and then stored at 4°C.

**DNA cloning:** The PCR amplification products made using degenerate primers were separated by 1.8% agarose gel electrophoresis. No PCR products were obtained in the negative RT samples that did not contain the reverse transcriptase. PCR products were isolated from agarose blocks with a Qiagen kit II (Qiagen). The isolated PCR products were subcloned into *Hind III / Sal I* cut Bluescript KS plasmids and then transformed into *E.coli* DH 5α competent cells (Stratagene). Bacterial colonies with inserts were selected by white/blue selection.

White colonies or light blue colonies were transferred into 20 µl water in a 500 µl microfuge tube, and heated in a boiling water bath for 10 min. The cell suspension was clarified by 2 min centrifugation. One µl of the supernatant was used in a 25 µl final volume PCR reaction. Primers T7 and T3 of the Bluescript KS plasmid were used in the PCR amplification. The 140 bp PCR product was obtained from the intact Bluescript KS plasmid and served as a reference. The plasmids containing the 180 bp DNA insertion have an expected size of 320 bp. Contrary to the expectation, the white colonies did not, but the light blue colonies did have the insertion.

The light blue colonies were cultured in 5 ml SOB broth containing 500 µg ampicillin overnight in a 37°C degree shaking incubator. Plasmid DNA was purified with the Wizard plasmid preparation kit (Promega).

**Sequencing Analysis:** The nucleotide sequence of the DNA insertions was determined by the dideoxynucleotide chain-termination method (Dalphin et al. 1997) with the Sequenase™\* Version 2.0 DNA sequencing kit (United States Biochemical). Template DNA was purified with a Wizard Kit (Promega) and annealed to synthetic oligonucleotide primer T7 of the Bluescript KS plasmid. The primer was extended using 10 nM of dGTP, dCTP, dTTP, 6.25 µCi <sup>35</sup>S labeled dATP, 3.25 units T7 DNA polymerase, and 0.625 units of pyrophosphatase. The elongation was terminated by adding 80 nM dideoxynucleotide ddA, ddG, ddT and ddC. The reactions were terminated by the addition of 48 nM Ethylenediamine Tetraacetic Acid (EDTA) and 16% formamide, denatured by heating and separated by a 6% acrylamide gel electrophoresis (50 watts constant power).

**Semiquantitative RT-PCR:** PCR was performed in 50 µl reactions with a final concentration of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 0.4 µM each of sense and antisense primers, and 2 units of Taq polymerase. The reactions were incubated in a thermal cycler for 31 cycles (FREAC-4, FKHR and FKHRL1), 32 cycles (AFX), 30 cycles (p27<sup>KIP1</sup>), or 23 cycles glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The forkhead genes expression were normalized to the house keeping gene GAPDH. Results were expressed as the ratio of forkhead:GAPDH.

PCR reaction parameters: for FREAC-4, 94°C 60s, 52°C 60s and 72°C 60s; for AFX, 94°C 60s, 60°C 90s and 72°C 90s; for FKHR, 94°C 60s, 55°C 60s and 72°C 60s; for FKHRL1, 94°C 60s, 60°C 60s and 72°C 60s; for p27<sup>KIP1</sup>, 94°C 60s, 62°C 120s and

72°C 180s; for GAPDH, 94°C 60s, 60°C 60s and 72°C 60s. PCR was completed by a final extension at 72°C for 10 min, and then stored at 4°C. Fifteen microlitres of the PCR products were separated on 1.8% agarose gels and stained with 0.2 µg/ml ethidium bromide for 20 minutes under 80 volts. The PCR product was visualized with UV light. Photographic documentation of the gels was performed using Type 667 black and white Polaroid films. The correct size of the PCR products was determined by the use of a standard DNA marker, and the sequences were confirmed by manual sequencing analysis. The polaroid images were scanned with a HP ScanJet IIC Scanner. Scans were saved as 8-bit TIFF files and imported into image analyzing software, UN-SCAN-IT (Silk scientific) for analysis. A standard baseline for samples was used and magnification of the image and width of the measurement window were constant. Areas of FREAC-4, AFX, FKHR, and FKHL1 DNA bands measured in square pixels were normalized to the area of GAPDH bands.

**Data analysis:** Each RNA sample was analyzed by RT-PCR 3 times, and the average intensity of the gel image of the PCR products obtained was taken. Data were expressed as means  $\pm$  SD. Values were compared using student's *t*-test, and considered statistically significant when the *p* value was less than 0.05.

**SAGE analysis:** SAGE provides a quantitative profile of the mRNAs expressed in a tissue or cell line by generating short-sequence tags that identify specific mRNAs (Velculescu et al. 1995). The short sequence tags (~10bp) used in SAGE are isolated from mRNA at a defined position (directly 3'-adjacent to the 3'-most restriction site for a

particular restriction enzyme). The short tags are long enough to uniquely identify the corresponding transcript in database searches. The frequency of each tag directly reflects transcript abundance. Basically, the SAGE technique measures not the expression level of a gene, but quantifies a "tag" which represents the transcription product of a gene. Thus, SAGE results in an accurate picture of gene expression at both the qualitative and the quantitative level. The use of SAGE in expression profiling has been demonstrated in analysis of expression profiles in normal versus cancer cells (Zhang et al. 1997).

## **RESULTS:**

### **Expression of FREAC forkhead gene subfamily DNA binding domain:**

To detect the expression of the FREAC forkhead gene subfamily, we designed degenerate primers according to the conserved DNA binding domain (DBD) sequences of four forkhead genes (FREAC-4, HFH-2, HNF-3 $\gamma$  and FREAC-3). The degenerate primers were used in a polymerase chain reaction (PCR) to amplify forkhead family members from cDNA pools prepared from human prostatic carcinoma (PCa) tissue, benign prostate hyperplasia (BPH) tissue and LNCaP cell line. The PCR products of the degenerate primers amplification were cloned and sequenced.

Among the fourteen forkhead DBD sequences we obtained, twelve of them were FREAC-4 or FREAC-4-like, and two were FREAC-3 (Figure 2). Because every clone had a different flanking oligonucleotide primer sequence (Figure 3), we will only consider the sequence between the primers. The differences in the flanking amino acid sequences (KPPYSYI and REKFP AW) probably result from mismatched oligonucleotides at the low annealing temperature of 48°C. The FREAC-4 DBD sequence was found in PCa #2,3,9,10,15, 17, BPH #21, 38 and LNCaP #26. PCa samples #13, #16 and BPH #26 have one amino acid different from the published FREAC-4 sequence: a serine (S-coded by 'tcc' in PC #13) replaced a proline (P-coded by 'ccc'); a valine (V-coded by 'gtg' in PC #16 and BPH #26) replaced a Leucine (L-coded by 'ctg'). Valine and leucine are conservative changes, resulting from a single base change. Of fourteen sequences only PC #12 and BPH #7 are similar to FREAC-3 DBD sequence. Thus, only sequences most similar to FREAC-4 and FREAC-3 are found using these

primers. No sequences similar or identical to HFH-2 and HNF-3 $\gamma$  were found in this small group.

#### **Expression of FREAC-4 in PCa and BPH tissues and LNCaP cell line:**

Previous studies have shown that FREAC-4 is not expressed in normal human prostate tissue (Pierrou et al. 1994). Therefore it is important to know if increased FREAC-4 expression occurs in prostate cancer tissues and cell lines.

To detect the expression of FREAC-4 in PCa and BPH tissues, and LNCaP cell line samples, we designed FREAC-4 primers based on unique human FREAC-4 cDNA sequence (GenBank™/EBI Data Bank, accession number U59832.) (Ernstsson et al. 1996). The FREAC-4 cDNA products derived from RT-PCR analysis using these primers in human PCa, BPH tissues and LNCaP cell lines are shown in Figure 4. The length of the amplified FREAC-4 cDNA fragment, 311 bp, was as predicted. The sequence analysis of the fragment showed it to be identical to the published human FREAC-4 cDNA sequence from position 1693-2003 (GenBank™/EBI Data Bank, accession number U59832) (Ernstsson et al. 1996). The 293 cell line derived from human embryonic kidney known to express FREAC-4 (Ernstsson et al. 1996), was used as a positive control. A minus-RT control was prepared for all tissues and cell lines, but in all cases of reverse transcriptase deletion, no band was obtained on the ethidium bromide stained agarose gel after PCR amplification (Fig 4).

Although these results confirm the expression of FREAC-4 mRNA in PCa, BPH and LNCaP, we could not conclude that the apparent differences in expression were real in Figure 4 because no precaution was taken to be ensure that the amplification was

linear. Next we used a semi-quantitative method to examine the differential expression level of FREAC-4 between PCa tissues and BPH tissue, as well as among four prostate cancer cell lines, LNCaP, LNCaP C4-2B, DU-145 and PC-3.

#### **FREAC-4 mRNA level in prostate cancer tissues and cell lines.**

The RT-PCR method was employed in a semiquantitative manner (Horikoshi et al. 1992) to study the expression of FREAC-4 mRNA relative to the expression of the housekeeping gene GAPDH (Zentella et al. 1991).

To establish a semiquantitative RT-PCR method, two preliminary experiments were carried out. A constant amount of cDNA was amplified for a variable number of cycles to determine the number of cycles within the linear range. The amount of amplified DNA was plotted as a function of the number of PCR cycles in Figure 5. The linear amplification for FREAC-4 is within 20-40 PCR cycles, whereas the linear amplification for GAPDH is within 20-30 PCR cycles. Therefore thirty cycles were used for FREAC-4, and twenty-three cycles were used for GAPDH. Next, different amounts of cDNA were amplified for 30 cycles of PCR. Figure 6 shows the linear amplification of FREAC-4 PCR product as a function of the amount of template RNA. In the range of 4 to 16  $\mu$ l (equivalent to 0.2 to 0.8  $\mu$ g of total RNA), a linear correlation was observed between the amount of amplified product and cDNA input. Therefore 8  $\mu$ l of cDNA mixture (equivalent to 0.4  $\mu$ g of total RNA) were used for RT-PCR in this study. These preliminary experiments were also carried out to detect expression from other genes (AFX, FKHR, FKHL1, and p27<sup>KIP1</sup>) in the subsequent experiments to determine the

number of cycles and amount of cDNA to be used in PCR amplification (results not shown).

All subsequent analyses used scanned photographs of ethidium bromide stained gels to obtain an intensity value of the FREAC-4 band to be normalized to the intensity value of a GAPDH PCR band (Figures 7 and 8).

The semiquantitative RT-PCR analysis of FREAC-4 gene expression level in human PCa and BPH tissues shows some variation in expression of FREAC-4. These differences, however, were not statistically significant.

In contrast, there were interesting differences in the FREAC-4 gene expression in prostate cancer cell lines LNCaP, LNCaP C4-2B, DU-145 and PC-3 (Figure 8). Cell line C4-2B shows 2.2-fold lower FREAC-4 expression than LNCaP, and DU-145 has 1.5-fold higher FREAC-4 expression than LNCaP. Surprisingly no FREAC-4 expression was found in the PC-3 cell line. The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs PC-3; C4-2B vs PC-3; DU-145 vs PC-3; and C4-2B vs DU-145.

#### **Expression of AFX forkhead gene subfamily DNA binding domain:**

AFX and FKHR forkhead transcription factors have been shown to be involved in tumorigenesis (Galili et al. 1993; Shapiro et al. 1993; Parry et al. 1994; Borkhardt et al. 1997). Five human forkhead genes (FKHR, FKHL1, FKHL1P1, AFX, FKHRP1) that show sequence similarity have been found and grouped into a AFX subfamily (Anderson et al. 1998).

To detect the role of AFX subfamily members in prostate tumorigenesis, we examined the expression of the AFX subfamily genes in prostate cancer tissue. Degenerate primers were designed according to the conserved DBD sequences of the five subfamily members. The degenerate primers were used in PCR to amplify the subfamily members from cDNA pools prepared from the same human PCa tissues used previously. The PCR products were purified, subcloned and sequenced. Among the nineteen forkhead DBD sequences we obtained, eleven of them are FKHRL1 DBD, five are AFX DBD and three are FKHR DBD (Figure 9). DBD sequences of FKHR subfamily members FKHRL1P1 and FKHRP1 were not found.

#### **AFX, FKHR and FKHRL1 expression in prostate cancer tissues and cell lines:**

To investigate the differential expression of the three AFX subfamily forkhead genes which were found in PC tissue, we used the same semiquantitative RT-PCR method as we did for FREAC-4 to compare the mRNA levels between PCa tissues and BPH tissue, as well as between cell lines LNCaP, LNCaP C4-2B, DU-145, and PC3.

We designed unique AFX, FKHR, and FKHRL1 primers based on the sequences of human cDNA to amplify the three forkhead genes in those tissues and cell lines (Table 1). The length of the amplified AFX, FKHR, and FKHRL1 cDNA fragments are 211 bp, 469 bp, and 193 bp, respectively. The sequence analysis of the fragments showed them to be identical to the revised published human AFX cDNA sequence, position 566-787 (GenBank™/EBI Data Bank, accession number GI=1418758) (Borkhardt et al. 1997); FKHR cDNA sequence, position 581-1028 (GenBank™/EBI Data Bank, accession

number GI=435422) (Galili et al. 1993); FKHL1 cDNA sequence, position 1598-1790 (GenBank™/EBI Data Bank, accession number GI=2895493) (Anderson et al. 1998).

The semiquantitative RT-PCR method was used to study the expression of AFX, FKHR, and FKHL1 mRNA relative to the expression of the housekeeping gene GAPDH mRNA. To use this method, we first determined the linear amplification range for the three forkhead genes as a function of PCR cycles and the amount of template cDNA (cDNA from all tissues and cell lines were used). The number of PCR cycles used for AFX, FKHR, and FKHL1 amplification was 32, 31, and 31, respectively. Four micro liters of cDNA (made from 0.2 µg) was used in this RT-PCR study.

The semiquantitative RT-PCR analyse of AFX, FKHR, and FKHL1 gene expression levels in human PCa tissues and cell lines are shown in Figures 10-15.

The RT-PCR analysis revealed an unequal distribution of AFX, FKHR and FKHL1 between PCa tissues and BPH tissue. There was a 7.2-9.7 fold higher expression of AFX in PCa tissues than in BPH tissue. The differences between PCa tissues and BPH tissue are statistically significant ( $p < 0.05$ ). For cell lines LNCaP and PC-3, the AFX expression is approximately equal but 2 fold lower than in C4-2B and DU-145. The difference between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs DU-145; C4-2B vs PC-3; and DU-145 vs PC-3.

FKHR and FKHL1 show similar patterns to that of AFX in that they were undetectable or barely detectable in BPH tissue and PC-3 cell lines (Figure 13). Once again, cell line C4-2B and DU-145 showed higher FKHR expression level than LNCaP (1.5-fold and 3.1-fold, respectively). The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs PC-3, C4-2B vs PC-3; and DU-145 vs PC-3.

For FKHL1, C4-2B shows higher expression than LNCaP and DU-145 (1.2-fold and 1.8-fold, respectively). The differences between the following groups are statistically significant ( $p < 0.05$ ) (Figure 15): LNCaP vs PC-3, C4-2B vs PC-3; and DU-145 vs PC-3.

#### **Expression of p27<sup>KIP1</sup> in PCa, BPH and cell lines:**

As previously mentioned, AFX, FKHR and FKHL1 forkhead genes are part of the PI3K pathway for the inhibition of apoptosis. Furthermore, constitutively active AFX and FKHR induces p27<sup>KIP1</sup> transcription. Is just the constitutively active AFX that induces p27 or can the wild type do it as well? In order to investigate the potential role of these forkhead genes in alteration of apoptosis and cell cycle regulation in prostate cancer, we examined the expression level of p27<sup>KIP1</sup> in PC tissues and cell lines. The expectation was that because all three forkhead proteins can bind to the same promoter sequences any one would be sufficient for expression of the p27<sup>KIP1</sup> gene transcription.

The semiquantitative RT-PCR method was applied to study the expression level of p27<sup>KIP1</sup> in PCa and BPH tissues and cell lines (Figures 16,17). Interestingly, the PC-3 cells and BPH tissue that have negligible FKHR and FKHL1 also have negligible p27<sup>KIP1</sup> expression. The highest p27<sup>KIP1</sup> expression level is in the LNCaP cell line where it is 2.5 and 2.0 fold higher than in C4-2B and DU-145, respectively. The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs PC-3, C4-2B vs PC-3; and DU-145 vs PC-3 (Figure 17).

### **SAGE Analysis of Forkhead Factors:**

We also used the serial analysis of gene expression (SAGE) database to study the gene expression profile of the forkhead transcription factors and downstream target genes. The SAGE expression profiles of several forkhead transcription factors are shown in Figure 18. The data was obtained from human transcriptome map web site, (<http://bioinfo.amc.sara.nl>). The total number of SAGE tags for each library is: 136968 for normal prostate tissue; 133493 for primary prostate tissue; 399291 for prostate tumor tissue; and 265798 for prostate cancer cell line LNCaP. The tag numbers shown on Figure 18 are counts per 100,000 tags.

The SAGE data showed that AFX, FKHR, FKHL1 and p27<sup>KIP1</sup> genes are detected in prostate tumor tissue (Figure 18). This is in agreement with our results that these genes are expressed in PCa tissues using RT-PCR (Figures 10, 12, 14, 16). SAGE data are also useful for the information on expression of these genes in normal prostate tissue, since our RT-PCR analysis lacks a normal group. Only AFX and p27<sup>KIP1</sup> are detected in normal tissue in SAGE data, but not FKHR or FKHL1, despite their expression in PCa tissue,

The expression of FREAC-4 is different between SAGE analysis (Figure 18) and RT-PCR results (Figures 7, 8). In all prostate tissues and cells examined there is FREAC-4 expression by RT-PCR analysis but not by SAGE analysis.

The SAGE analysis also showed expression information for two other forkhead factors, FREAc-2 and HFH-11A (Figure 18). Both FREAC-2 and HFH-11A were expressed in an interesting pattern of increasing amounts in the transition from normal to PCa to LNCaP.

## DISCUSSION:

The forkhead group is very large, with 35 human genes found in the Human Genome Project analysis (Venter et al. 2001). Therefore, we approached our objective of examining the expression of the forkhead genes to uncover potential roles in neoplastic cells of the prostate by concentrating on members most likely to have an effect on prostate tumorigenesis. We analyzed two subgroups of the family (the FREAC-4 group that is related to *qin* and AFX group that is implicated in promotion of apoptosis and cell cycle arrest) in an effort to find those that may play a role in prostate tumorigenesis.

Several different chromosomal deletions or rearrangements have been found in prostate cancer cells (Bova et al. 1996): including 1) loss of sequences within the short arm of chromosomes 8; 2) loss of sequences within the long arm of chromosome 13q; and 3) gain of sequences within the long arm of chromosomes 8 and X, particularly in advanced disease. The chromosome loci of the forkhead factors and p27<sup>KIP1</sup> are: AFX, Xq13.1; FKHR, 13q14.1; FKHL1, 6q21; FREAC-4, 5q12-q13; p27<sup>KIP1</sup>, 12p13.1-p12. Loss of sequence within the long arm of chromosome 13 might result in the loss of forkhead factor FKHR (13q14.1). The loss of FKHR gene could account for the lower amount of RT-PCR products in BPH tissue and PC-3 cell line since losses of gene result in losses of transcription. My results show FREAC-4 is expressed in prostate cancer tissues and cell lines in contrast to the absence of FREAC-4 expression in normal prostate tissue demonstrated by Pierrou *et al.* (1994) (Pierrou et al. 1994). The detection of FREAC-4 expression in prostate cancer tissues and cell lines could result from an increase in copy number of chromosome 5q since higher copy number could result in higher transcription level. A number of rearrangements have been identified in LNCaP,

DU-145 and PC-3 cell lines, both androgen-insensitive cell lines DU-145 and PC-3 exhibited a higher number of chromosome rearrangements than the androgen-sensitive cell line LNCaP. PC-3 exhibited rearrangement of chromosome 1, 2q, 4, 5, 6q, 8, 10, 11, 12q, 14, and 15, but the breakpoints of these rearrangements could not be ascertained. The high frequency of rearrangement may be a result of increased genetic instability caused by deficient cell cycle control or DNA repair.

The sequencing and RT-PCR analysis used in this project would not detect some types of mutations. The primers used for AFX PCR amplification are located in the N-terminal portion of AFX gene, so the primers could not be used to detect the expression of fusion gene MLL-1/AFX in which the C-terminus of AFX fused to N-terminus of MLL-1 gene. FKHR primers crossed the fusion point of PAX-3 gene and FKHR gene, so the FKHR primers could not detect the expression of fusion genes PAX-3/FKHR either, and so maybe there is a fusion in BPH or PC-3 and that is why we did not see a FKHR band.. There are also point mutations and deletions in the N-terminal of *qin* gene, which RT-PCR analysis would miss if FREAC-4 has the same kind of mutations and deletion. We did find amplified sequences have differences when compare to FREAC sequences (Figure 2), but the differences are most likely resulted from PCR artifacts or primers mismatches (Figure 3). This will be discussed in detail later. Also if the designed primers happened on the mutation region, the mutation would perhaps prevent binding of the primer to the template DNA, so the detection of the gene would be missed.

We used sequencing and RT-PCR in the search for expression level of several forkhead transcription factors, because elevated or otherwise inappropriate expression of transcription factors has been implicated in tumorigenesis. We designed degenerate

primers based on the FREAC-4 subgroup of forkhead transcription factors. Fourteen forkhead DBD sequences (all either FREAC-3 or 4) were found using PCa tissue cDNA. All of the 14 sequences had different flanking oligonucleotide primer sequences (Figure 3). The nucleotide differences in the flanking primer region might simply be due to mismatched oligonucleotides at the low annealing temperature of 48°C, so these differences from published sequences are probably artifacts and not from novel sequences. There are three sequences that have only one nucleotide different from FREAC-4 gene. The changes include a serine (coded by 'tcc') to a proline (coded by 'ccc') and from a valine (coded by 'gtg') to a leucine (coded by 'ctg'). Although the serine to proline, valine and leucine are conservative changes, there are no other nucleotide changes in the three sequences. Therefore these changes most likely result from mutation during PCR amplification. The absence of novel forkhead DBD sequences meant that we could concentrate on FREAC-3 and 4. It is interesting that FREAC-4 had not previously been reported in the prostate (Pierrou *et al.* 1994). The absence of FREAC-4, however, could perhaps be explained by our use of RT-PCR in contrast to the less sensitive northern blotting technique used or because the previous study used normal prostate tissue in contrast to the PCa tissue we used (Pierrou *et al.* 1994).

We concentrated on detecting FREAC-4 expression because of its relatedness to known oncogenic versions of *qin* (Freyaldenhoven *et al.* 1997b). Semiquantitative RT-PCR was used to examine FREAC-4 mRNA levels in human PC and BPH tissues and cell lines. Alterations in expression were not found in tumorigenic PCa versus non-tumorigenic BPH tissue. Histologically, PCa is classical adenocarcinoma and made of epithelial cells, whereas BPH has a variable composition ranging from a highly epithelial

but usually a fibromuscular type largely devoid of epithelial components. Unfortunately, we did not have the histological information of the PCa and BPH tissue used to determine the cellular composition. If we assume that the PCa was predominately epithelial and the BPH predominately stromal then the equal expression between the PCa and BPH could mean that epithelial and stromal cell types express FREAC-4 equally or that the increased FREAC-4 mRNA levels are not characteristic of malignant prostate growth. Elevated *c-qin* levels, however, can transform chicken embryo fibroblasts into tumorigenic cells (Freyaldenhoven et al. 1997a). Unfortunately, we didn't have normal prostate tissues available to see if normal cells have low FREAC-4 and elevated FREAC-4 is associated with both benign and malignant growth. However, in the SAGE analysis no FREAC-4 was detected in normal prostate tissue, or PCa tissue and LNCaP cell line for that matter. The SAGE results suggest that the FREAC-4 expression is extremely low because it can not be detected in over 100,000 tags (<http://bioinfo.amc.sara.nl>). Another explanation relevant to tissues is that perhaps the expression is low because only a few cells of many in the tissue express the FREAC-4. Maybe only cycling cells express it or only basal but not luminal epithelial cells do. The possibility of only certain cells expressing a gene can be addressed by ISH. FREAC-4 expression level comparison was also done among prostate cell lines LNCaP, LNCaP C4-2B, DU-145 and PC-3. Cell line LNCaP C4-2B showed lower (2.2-fold) FREAC-4 expression than LNCaP and PC-3 did not have FREAC-4 expression. This suggests that FREAC-4 expression is downregulated with prostate cancer progression since C4-2B is amore tumorigenic, metastatic and androgen-independent subline of LNCaP (Chen et al. 1998), whereas PC-3 is even more highly metastatic and androgen-independent. The absence of FREAC-4 expression in PC-3 cell

line is very interesting since it correlates with the similar FKHR and FKHL1 forkhead gene expression characteristics in PC-3 cell line in our studies. Since the three forkhead genes are located in different chromosomes (FKHR in 13q, FKHL1 in 6q and FREAC-4 in 5q), the correlation of expression loss with chromosome loss in the PC-3 cell line for the three forkhead genes can be discounted.

The second subgroup to be analyzed was the AFX forkhead subfamily in prostate cancer tissue. Five members in this subfamily are known but only FKHL1/AFX/FKHR (not FKHL1P1 or FKHRP1) were found in PCa. We therefore could concentrate on these three and their proposed role in promoting apoptosis via the PI3K pathway. Our expectation was that decreases in transcription of one or more of the three forkhead factors would aid oncogenesis.

Semiquantitative RT-PCR analysis showed that AFX levels are at least 7-fold lower in BPH versus PCa tissues. The FKHR and FKHL1 expression patterns are similar in that they are undetectable or barely detectable in BPH tissue, whereas they are equally expressed among the three prostate cancer tissues. Interestingly, the PC-3 cell line, in contrast to all other cell lines and PCa tissues, lacks FKHR and FKHL1 mRNA expression. The PC-3 cell line is more resistant to apoptosis than LNCaP (Liu et al. 1998; Sintich et al. 1999; de la et al. 1999). Possibly the resistance is due to lack of FKHR and FKHL1. It would be interesting to see if increased apoptosis could be restored in PC-3 cells transfected to express FKHR or FKHL1. Several apoptosis proteins are expressed differently among cell lines: PC-3 cells are p53-null whereas LNCaP cells express wild-type P53 and DU-145 cells express mutant P53; PC-3 cells express more anti-apoptotic protein Bcl-2 than LNCaP and DU-145 cells; PC-3 cells also lack pro-apoptotic factor

Bak (Tang et al. 1998). It is conceivable that the decreased expression of FKHR and FKHL1 is more important than AFX decreased expression in prostate cell tumorigenesis.

P27<sup>KIP1</sup> is a target of AFX and FKHL1 forkhead transcription factors in osteosarcoma cells U87MG and murine pre-B-cell line Ba/F3, respectively (Medema et al. 2000; Dijkers et al. 2000). The Cyclin E/Cdk2 inhibitor p27<sup>KIP1</sup> is involved in cell cycle control by acting as a barrier to G<sub>1</sub>-S transition. P27<sup>KIP1</sup> preferentially binds to and inactivates the Cyclin E/Cdk2 complexes, therefore inhibiting entry into S-phase (Slingerland et al. 1994). BPH, prostatic intraepithelial neoplasia (PIN), and PCa all exhibit decreased levels of p27<sup>KIP1</sup> (Yang et al. 1998). Normal human prostate tissue exhibited high levels of p27<sup>KIP1</sup> mRNA in both epithelial cells and stromal cells (Yang et al. 1998). However, p27<sup>KIP1</sup> mRNA was almost undetectable in epithelial and stromal cells of BPH lesions (Cordon-Cardo et al. 1998). In contrast to BPH, PCa was found to contain abundant p27<sup>KIP1</sup> mRNA (Cordon-Cardo et al. 1998). Our results similarly show that the mRNA expression of p27<sup>KIP1</sup> was abundant in PCa tissues but undetectable in BPH tissue. Interestingly, the lack P27KIP1 correlates with the lack of FKHL1 and very low levels of FKHR mRNA in BPH.

BPH and PCa have been suggested to share a common origin because they commonly coexist and are both androgen dependent (Scher et al. 1995; Oesterling 1996). However, since BPH occurs in the periurethral transitional region of the human prostate, and 70% of PCa arises from the peripheral zone (De Marzo et al. 1999), the relationship between BPH and PCa remains unclear. It is likely that prostate cancers arise from prostatic intraepithelial neoplasia (PIN) instead of BPH. PIN shares similar genetic

lesions to prostate cancer (Haggman et al. 1997; Qian et al. 1999). The results on forkhead gene expression certainly are consistent with the expected different origins of BPH and Pca.

The active non-phosphorylated forms of FKHL1 and AFX may affect cell cycle regulation by inducing the expression of p27<sup>KIP1</sup>. As found for FKHR and FKHL1, PC-3 cells in contrast to all other cell lines and PCa tissues, lack p27<sup>KIP1</sup> expression. The very low levels of FKHR and FKHL1 that were found in this study could explain the lowered p27KIP1 in PC-3. It was not unexpected that p27KIP1 mRNA should be low in PC-3 cell line because it is more invasive and metastatic than LNCaP and low levels correlate with more advanced cancers (Medema et al. 2000). Furthermore these data suggest that perhaps FKHR and/or FKHL1 but not AFX is capable of increasing the expression of the p27<sup>KIP1</sup> gene transcription in prostate cancer cells. This hypothesis would be testable by transfection of the FKHL1 or FKHR gene into PC-3 resulting in increased P27<sup>KIP1</sup> levels.

The results presented here provide an initial analysis of the role of forkhead proteins in prostate tumorigenesis. Our survey of forkhead gene expression indicates where efforts can now be directed to analyze the function of this important class of transcriptional regulators in prostate cancer.

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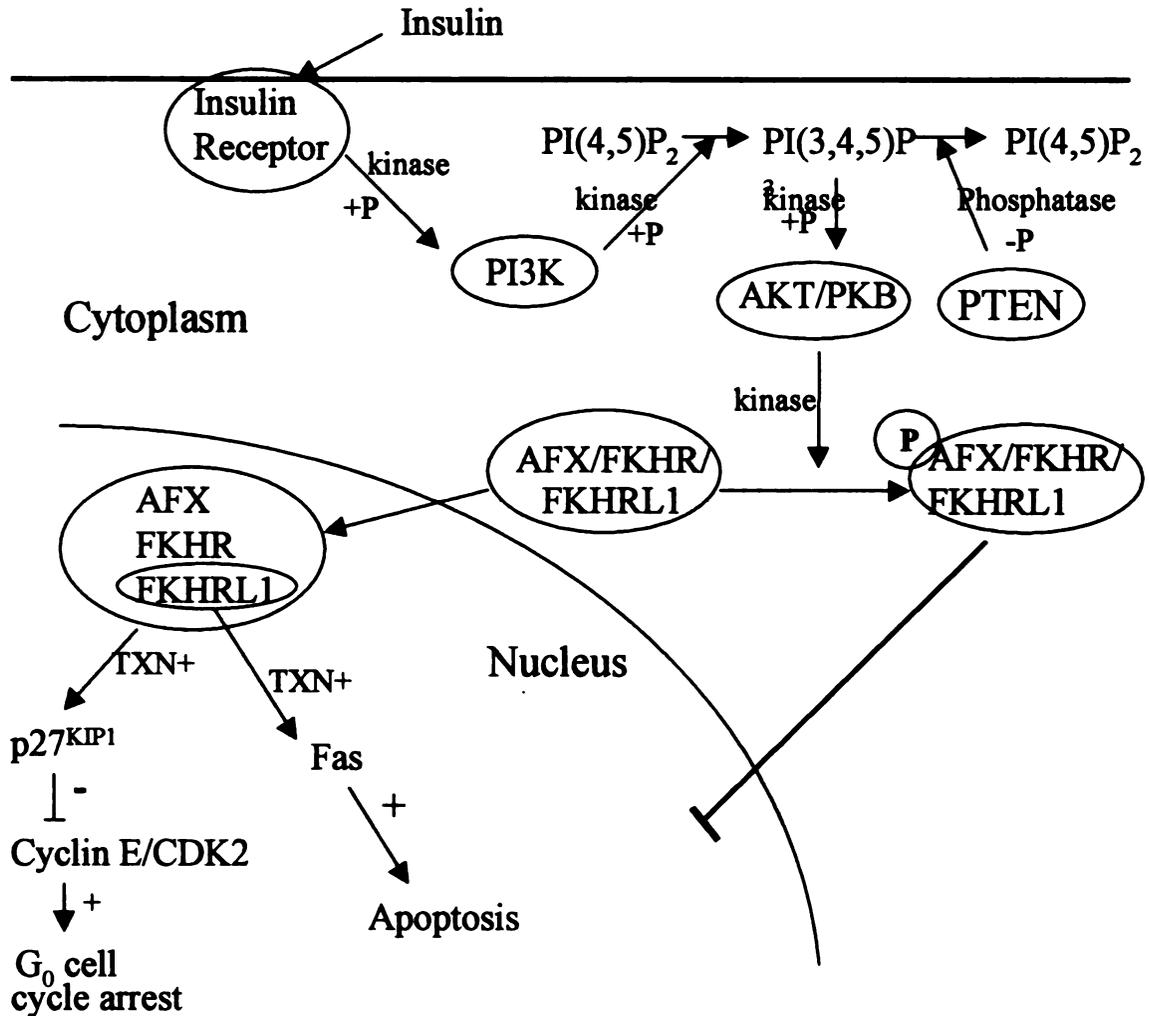
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**Table 1. PCR primers**

<b>Gene</b>	<b>Sequence</b>	<b>Size of product (bp)</b>
<b>FREAC subfamily</b>	<b>sense</b>	<b>ARGCCRCCCTAYTCTSTAYWYC</b>
	<b>antisense</b>	<b>GCCAGGSSCSKGSARCTSTCCC</b>
<b>AFX subfamily</b>	<b>sense</b>	<b>GCSTGGGMAAYCWGTCMTA</b>
	<b>antisense</b>	<b>RTGRCGRATYGARTTCTTCCA</b>
<b>AFX</b>	<b>sense</b>	<b>CGTACTGTACCCTACTTCAAGGACA</b>
	<b>antisense</b>	<b>CGGAGCAGCTTGCTGCTGCTATCCATGGAGG</b>
<b>FKHR</b>	<b>sense</b>	<b>AGAGCGTGCCCTACTTCAA</b>
	<b>antisense</b>	<b>ATCATCCTGTTCGGTCATA</b>
<b>FKHRL1</b>	<b>sense</b>	<b>ATGAGGGAACTGGCAAGAG</b>
	<b>antisense</b>	<b>GAGAGCTGGAGGGACTGT</b>
<b>p27KIP1</b>	<b>sense</b>	<b>CCTCTTCGGCCCGGTGGAC</b>
	<b>antisense</b>	<b>TCTGCTCCACAGAACCGGC</b>
<b>FREAC-4</b>	<b>sense</b>	<b>TGCGAATTCTCGGACAAA</b>
	<b>antisense</b>	<b>AAAAGAATTGGCGTGGC</b>
<b>GAPDH</b>	<b>sense</b>	<b>ACCACAGTCCATGCCATCAC</b>
	<b>antisense</b>	<b>TCCACCACCCTGTTGCTGTA</b>

(R=A and G, Y=C and T, S=G and C, W=A and T, K=G and T)



**Figure 1. Forkhead genes and PI3K signaling pathway.** Insulin activates PI3-kinase via autophosphorylation of the insulin receptor. PI3K produces phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P<sub>3</sub>) that acts as a second messenger to recruit AKT to the plasma membrane. AKT becomes phosphorylated on T308 and S473 by PIP(3, 4, 5)P<sub>3</sub>. PTEN is a lipid phosphatase which can degrade PIP<sub>3</sub>, thereby negatively regulating AKT activation. AKT can phosphorylate and inactive forkhead transcription factors (AFX, FKHR, and FKHL1), which results in forkhead retention in the cytoplasm. Thus, phosphorylation of forkhead factors by AKT suppresses transcription of their target genes, p27<sup>KIP1</sup> and Fas. Upregulation of p27<sup>KIP1</sup> is linked to cell cycle arrest in G<sub>0</sub> through its inhibition of cyclin E/CDK2 complexes. Fas ligand gene promotes apoptosis.

**Figure 2. Alignment of sequences obtained aligned to the DBD of known HFH family proteins.**

<b>FREAC-4</b>	<b>KPPYSY</b>	<b>IALITM</b>	<b>AILQSP</b>	<b>KKRLTL</b>	<b>SEICEF</b>	<b>ISGRFP</b>	<b>YREKFL</b>	<b>PAW</b>
PC #3	KPPYSY	SALITM	AILQSP	KKRLTL	SEICEF	ISGRFP	YREKFL	QAW
PC #17	RPPYSY	SALITM	AILQSP	KKRLTL	SEICEF	ISG?FP	YREKFL	PG?
PC #10	RPPYSY	FALITM	AILQSP	KKRL?L	SEICEF	ISGRFP	YRDKL	LAW
PC #9	RPPYSY	FALITM	AILQSP	KKRLTL	SEICEF	ISGRFP	YREKFL	QGW
PC #15	RPPYSY	FALITM	AILQSP	KKRLTL	SEICEF	ISG?FP	YREKFL	QGW
BPH #21	KPPYSY	SALITM	AILQSP	KKRLTL	SEICEF	ISGRFP	YREKFL	QAW
BPH #38	????Y	SALITM	AILQSP	KKRLTL	SEICEF	ISGRFP	YREKFL	EGW
LNCaP #26	RPPYSY	FALITM	AILQSP	KKRLTL	SEICEF	ISGRFP	YRDKF	HAW
PC #13	?PPYSY	FALITM	AILQSP	KKRLTL	SEICEF	ISGRFS	YREKFL	HAW
PC #2	WPPYSY	FARITM	AILQSP	KKRLTL	SEICEF	ISGRFP	YRYKL	QAW
PC #16	??PYSY	VALITM	AILQSP	KKRLTV	SEICEF	ISGRFP	YRDKL	HGW
BPH #2	??PYSY	SALITM	AILQSP	KKRLTL	SEICEF	VSGRFP	YREKFL	QGW
<b>FREAC-3</b>	<b>KPPYSY</b>	<b>IALITM</b>	<b>AIQNAP</b>	<b>DKKITL</b>	<b>NGIYQF</b>	<b>IMDRFP</b>	<b>FYRDNK</b>	<b>QGW</b>
PC #12	KPPYSY	TALITM	AIQNAP	DKKITL	NGIYQF	IMDRFP	FYREKFL	QGW
BPH #7	KPPYSY	FALITM	AIQNAP	DKKITL	NGIYQF	IMDRFP	FYRDKL	PAW

**Figure 3. DNA Sequences of the primers used:**

Sense primer: 5' ARGCCRCCTAYTCSTAYWYC  
**FREAC-4:** 5' **AAGCCGCCCTACTCGTATATC**  


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PC #3: 5' AGGCCGCCCTACTCCTATTCC  
PC #17: 5' AGGCCGCCCTATTCCCTATTCC  
PC #10: 5' AGGCCGCCCTATTCCCTACTTC  
PC #9: 5' AGGCCACCCTATTCCCTACTTC  
PC #15: 5' AGGCCGCCCTACTCCTACTTC  
PC #13: 5' ??GCCGCCCTATTTCGTATTCC  
PC #2: 5' TGGCCGCCCTACTCCTATTTC  
PC #16: 5' ?????GCCCTACTCGTATGTC  
BPH #21: 5' AAGCCGCCCTATTTCGTACTCC  
BPH #26: 5' ??????CCCTACTCGTACTCC  
BPH #38: 5' ?????????????????TATTCC  
LNCap #26: 5' AGGCCGCCCTATTCCCTATTTC  
**FREAC-3:** 5' **AAGCCGCCCTATAGCTACATC**  


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PC #12: 5' AAGCCGCCCTATTTCCTACACC  
BPH #7: 5' AAGCCGCCCTATTTCGTACTTC

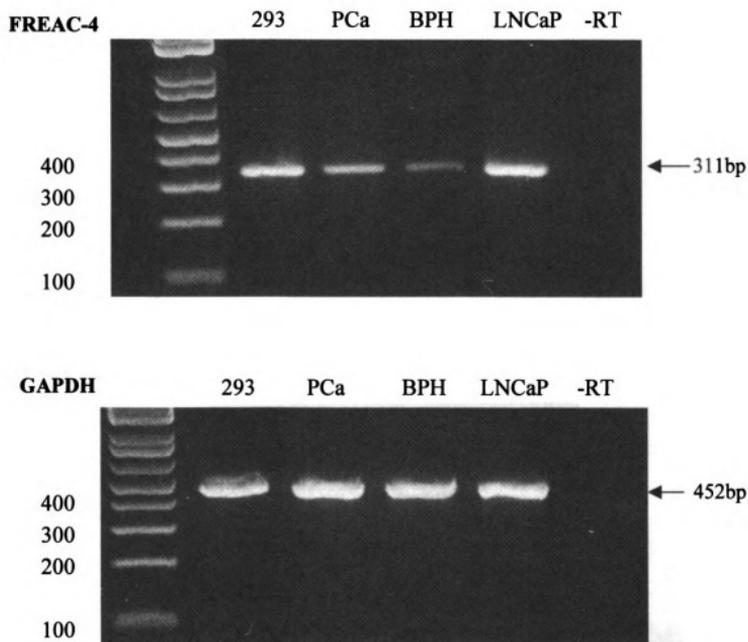
Antisense primer: 5' GGGASAAGYTSCMSGSCTGGC  
**FREAC-4:** 5' **GGGAGAAGTTCCTCCGCTGGC**  


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PC #3: 5' GGGAGAAGCTCCACGGCTGGC  
PC #17: 5' GGGAGAAGCTGCCCGCT???  
PC #10: 5' GGGACAAGCTGCTCGCCTGGC  
PC #9: 5' GGGAGAAGTTGCAGGGCTGGC  
PC #15: 5' GGGAGAAGCTCCAGGGCTGGC  
PC #13: 5' GGGAGAAGCTCCACGCCTGGC  
PC #2: 5' GGGACAAGCTGCAGGCCTGGC  
PC #16: 5' GGGACAAGCTGCACGGCTGGC  
BPH #21: 5' GGGAGAAGCTGCAGGCCTGGC  
BPH #26: 5' GGGAGAAGCTCCAGGGCTGGC  
BPH #38: 5' GGGAGAAGTTCGAGGGCTGGC  
LNCaP #26: 5' GGGACAAGTTCACGCCTGGC  
**FREAC-3:** 5' **GGGACAACAAGCAGGGCTGGC**  


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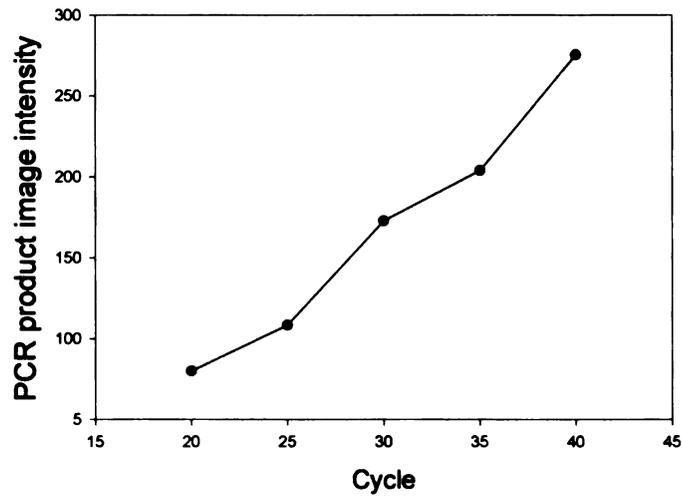
PC #12: 5' GGGAGAAGCTCCAGGGNTGGC  
BPH #7: 5' GGGACAAGCTGCCGGCCTGGC



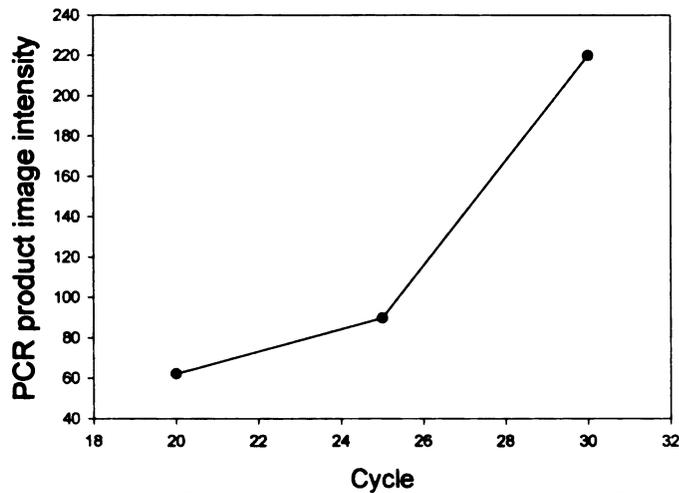
**Figure 4. RT-PCR analysis of the FREAC-4 expression in human PCa (prostatic cancer tissue), BPH (benign prostatic hyperplasia tissue) and prostatic cancer cell line LNCaP.** Images of agarose gels stained with ethidium bromide show electrophoretic bands corresponding to the cDNA amplification products derived from PCR amplification using FREAC-4 primers or GAPDH primers. Size of the molecular marker is shown on the left of the pictures. 293 is a kidney cell line used as a positive control for FREAC-4 expression. GAPDH is a housekeeping gene used as a control for RNA loading and integrity. No band was found in the reverse transcriptase (-RT) control.



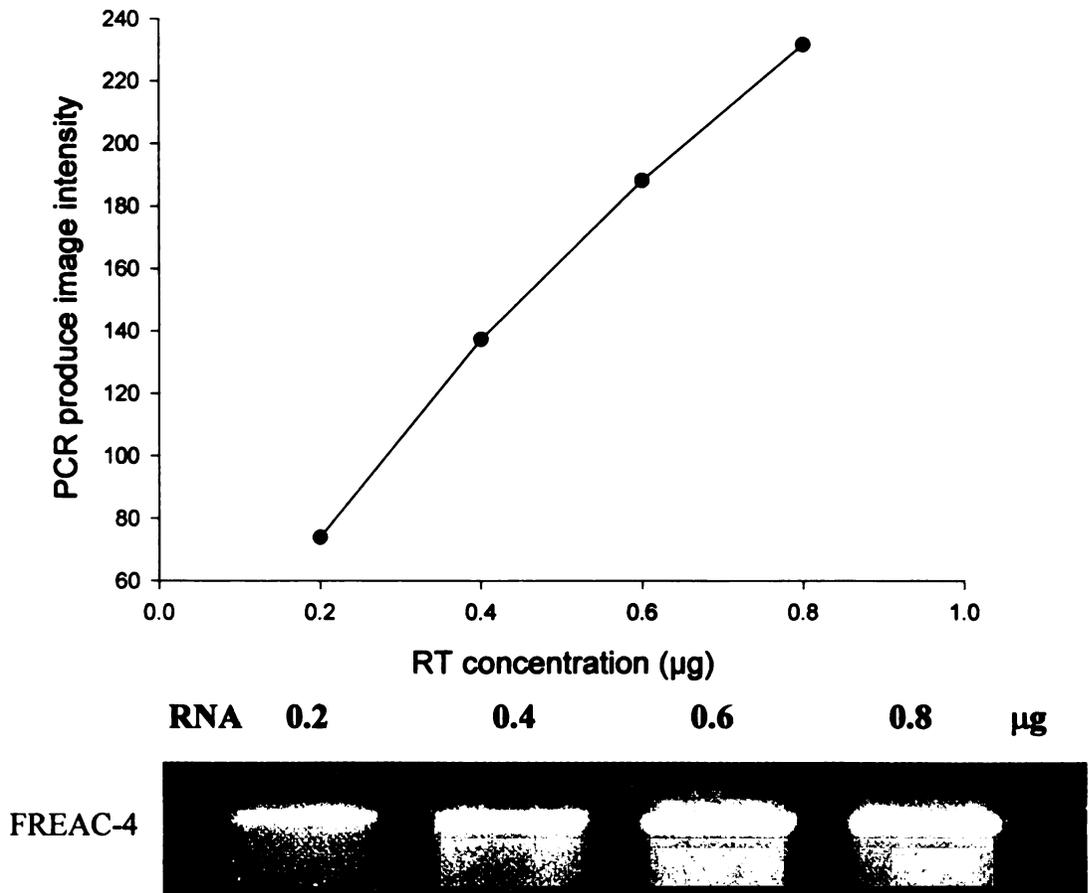
### A. FREAC-4



### B. GAPDH

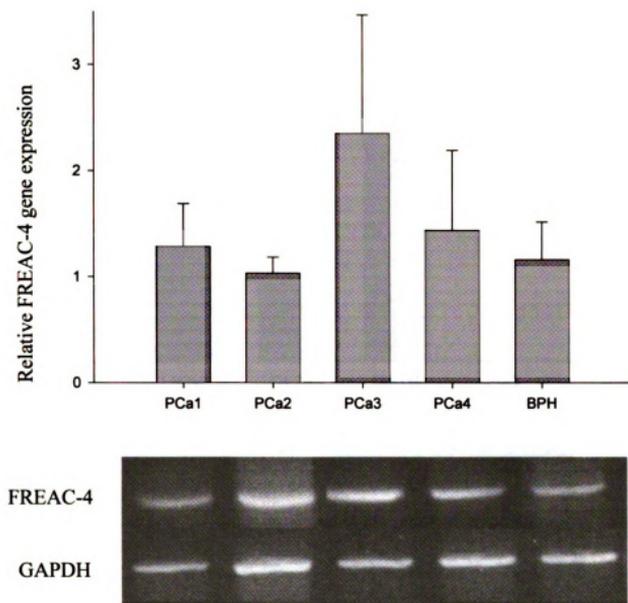


**Figure 5. PCR products as a function of PCR cycles for both Freac-4 and GAPDH cDNAs. Total RNA was reverse transcribed. The RT reaction was used to: (A) amplify Freac-4 cDNA for 20-40 PCR cycles; (B) amplify GAPDH cDNA for 20-30 PCR cycles.**



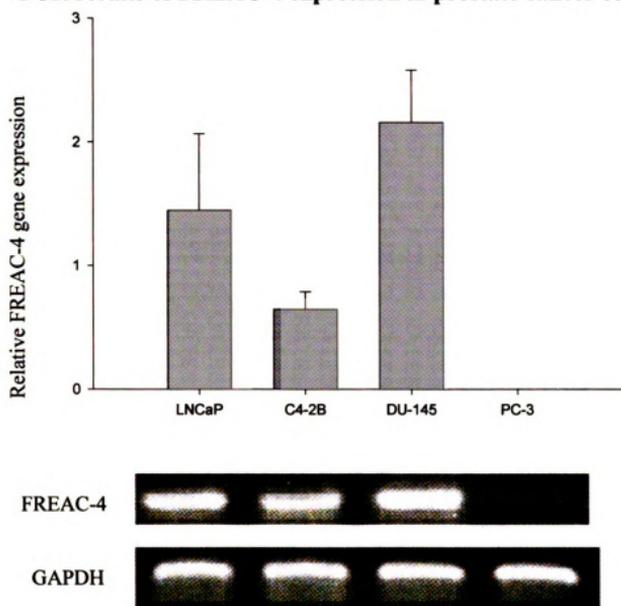
**Figure 6. PCR products as a function of the amount of template RNA.** Different amounts of FREAC-4 cDNAs (equivalent to 0.2-0.8 µg of total RNA) were amplified for 30 cycles. The PCR products were plotted as a function of total RNA made into cDNA.

## PCR results of FREAC-4 expression in PCa tissues and BPH tissue



**Figure 7. Semi-quantitative RT-PCR analysis of FREAC-4 gene expression in human PCa tissues (PCa1, PCa2, PCa3) and BPH tissue.** Images of agarose gels stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using FREAC-4 primers and GAPDH primers. Eight  $\mu$ l of cDNA was used for FREAC-4 amplification, and 4  $\mu$ l of cDNA used for GAPDH amplification. The relative expression of FREAC-4 for each tissue specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. No statistically significant differences were found between the samples.

### PCR results of FREAC-4 expression in prostate cancer cell lines



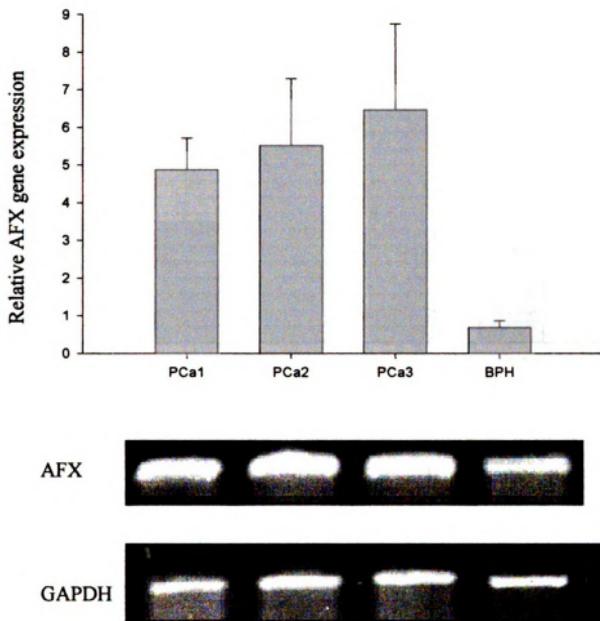
**Figure 8. Semi-quantitative RT-PCR analysis of FREAC-4 gene expression in human prostate cancer cell lines (LNCaP, C4-2B, DU-145 and PC-3).**

Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using FREAC-4 primers and GAPDH primers. Four  $\mu$ l of cDNA was used for FREAC-4 amplification. The relative expression of FREAC-4 for each cell line specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs PC-3; C4-2B vs PC-3; DU-145 vs PC-3; and C4-2B vs DU-145.

Figure 9. Alignment of DBD sequences to known AFX/FKHR subfamily members

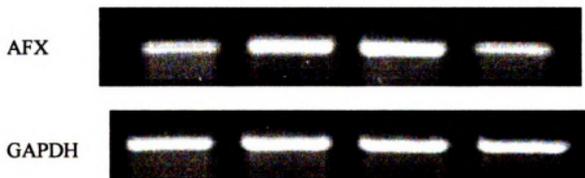
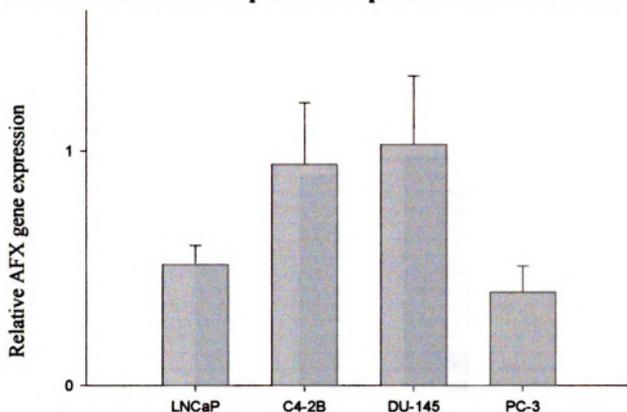
<b>FKHRL1</b>	<b>WGNLSY</b>	<b>ADLIT</b>	<b>RAIES</b>	<b>SPDKR</b>	<b>LTLSQ</b>	<b>IYEM</b>	<b>VRVVP</b>	<b>YFKDK</b>	<b>GDSNS</b>	<b>SAGWK</b>	<b>NSIRH</b>
PC #5	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NS???
PC #9	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #10	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #11	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #12	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #20	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NS???
PC #6	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #21	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #22	WGNLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #17	??NOSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #8	??NLSY	ADLIT	RAIES	SPDKR	LTLSQ	IYEM	VRVVP	YFKDK	GDTNS	SAGWK	NSIRH
<b>AFX</b>	<b>WGNQSY</b>	<b>AELIS</b>	<b>QAIES</b>	<b>APEKR</b>	<b>LTLAQ</b>	<b>IYEM</b>	<b>VRTVP</b>	<b>YFKDK</b>	<b>GDSNS</b>	<b>SAGWK</b>	<b>NSIRH</b>
PC #14	WGNQSY	AELIS	QAIES	APEKR	LTLAQ	IYEM	VRTVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #7	???LSY	AELIS	QAIES	APEKR	LTLAQ	IYEM	VRTVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #13	WGNLSY	AELIS	QAIES	APEKR	LTLAQ	IYEM	VRTVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #19	????SY	AELIS	QAIES	APEKR	LTLAQ	IYEM	VRTVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #23	WGNLSY	AELIS	QAIES	APEKR	LTLAQ	IYEM	VRTVP	YFKDK	GDSNS	SAGWK	NSIRH
<b>FKHR</b>	<b>WGNLSY</b>	<b>ADLIT</b>	<b>KAIES</b>	<b>SAEKR</b>	<b>LTLSQ</b>	<b>IYEM</b>	<b>VKSVP</b>	<b>YFKDK</b>	<b>GDSNS</b>	<b>SAGWK</b>	<b>NSIRH</b>
PC #24	WGNLSY	ADLIT	KAIES	SAEKR	LTLSQ	IYEM	VKSVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #2	WGNLSY	ADLIT	KAIES	SAEKR	LTLSQ	IYGM	VKSVP	YFKDK	GDSNS	SAGWK	NSIRH
PC #15	?GNLSY	ADLIT	KAIES	SAEKR	LTLSQ	IYEM	VKSVP	YFKDK	GDS??	?????	?????

## PCR results of AFX expression in PCa tissues and BPH tissue



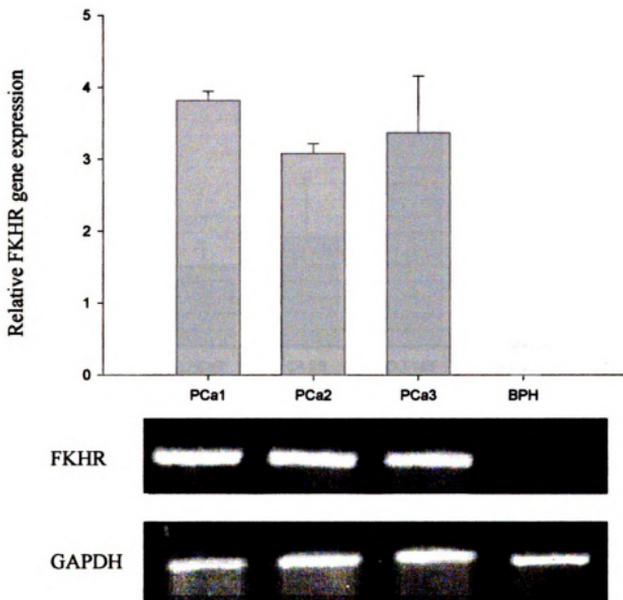
**Figure 10. Semi-quantitative RT-PCR analysis of AFX gene expression in human PCa tissues and BPH tissue.** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using AFX primers and GAPDH primers. Four  $\mu$ l of cDNA was used for AFX amplification. The relative expression of AFX for each tissue specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The difference between PCa tissues and BPH tissue are statistically significant ( $p < 0.05$ ).

### PCR results of AFX expression in prostate cancer cell lines



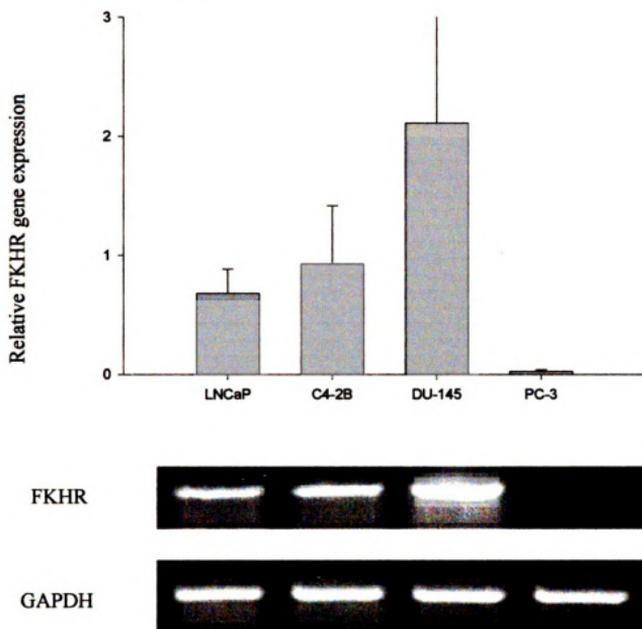
**Figure 11. Semi-quantitative RT-PCR analysis of AFX gene expression in human prostate cancer cell lines (LNCaP, C4-2B, DU-145, and PC-3).** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using AFX primers and GAPDH primers. The relative expression of AFX for each cell line specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs DU-145; C4-2B vs PC-3; and DU-145 vs PC-3.

## PCR results of FKHR expression in PCa tissues and BPH tissue



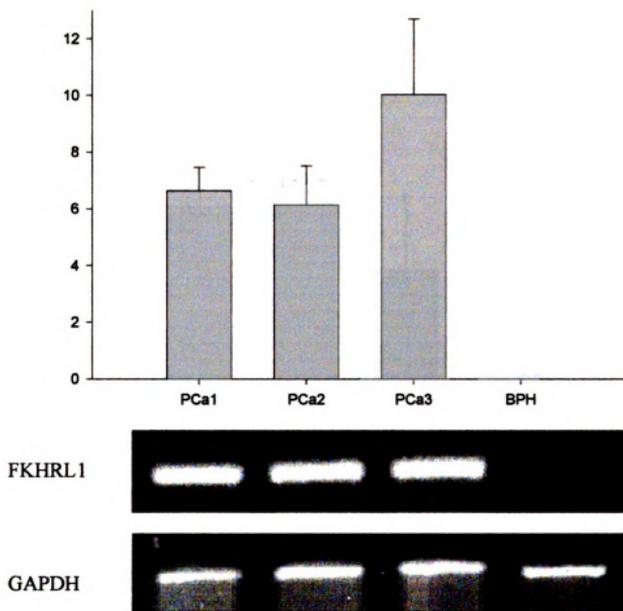
**Figure 12. Semi-quantitative RT-PCR analysis of FKHR gene expression in human PCa tissues and BPH tissue.** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using FKHR primers and GAPDH primers. Four  $\mu$ l of cDNA was used for FKHR amplification. The relative expression of FKHR for each tissue specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The difference between PCa tissues and BPH tissue are statistically significant ( $p < 0.05$ ).

### PCR results of FKHR expression in prostate cancer cell lines



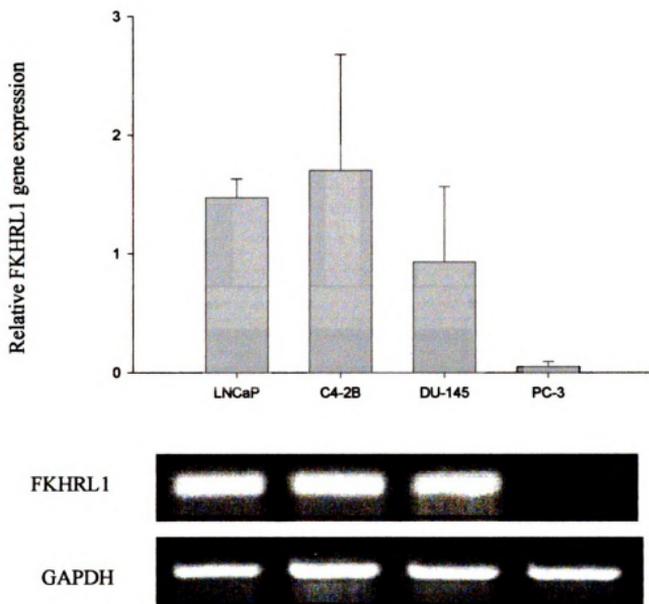
**Figure 13. Semi-quantitative RT-PCR analysis of FKHR gene expression in human prostate cancer cell lines (LNCaP, C4-2B, DU-145, and PC-3).** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using FKHR primers and GAPDH primers. The relative expression of FKHR for each cell line specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs PC-3, C4-2B vs PC-3; and DU-145 vs PC-3.

## PCR results of FKHL1 expression in PCa tissues and BPH tissue



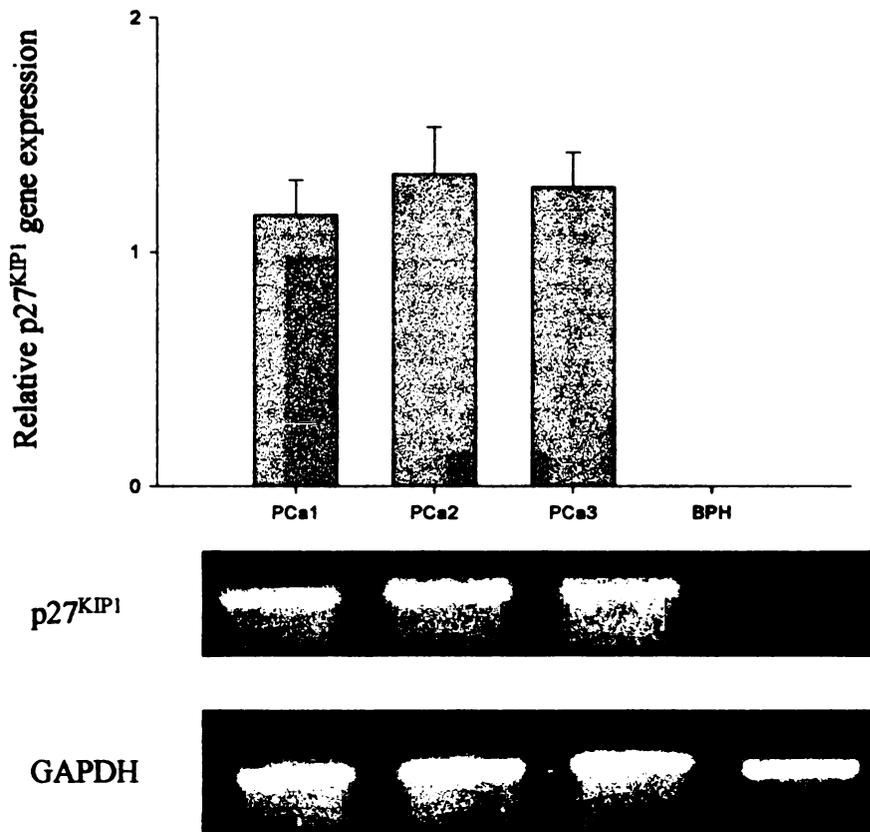
**Figure 14. Semi-quantitative RT-PCR analysis of FKHL1 gene expression in human PCa tissues and BPH tissue.** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using FKHL1 primers and GAPDH primers. Four  $\mu$ l of cDNA was used for FKHL1 amplification. The relative expression of FKHL1 for each tissue specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The difference between PCa tissues and BPH tissue are statistically significant ( $p < 0.05$ ).

## PCR results of FKHL1 expression in prostate cancer cell lines



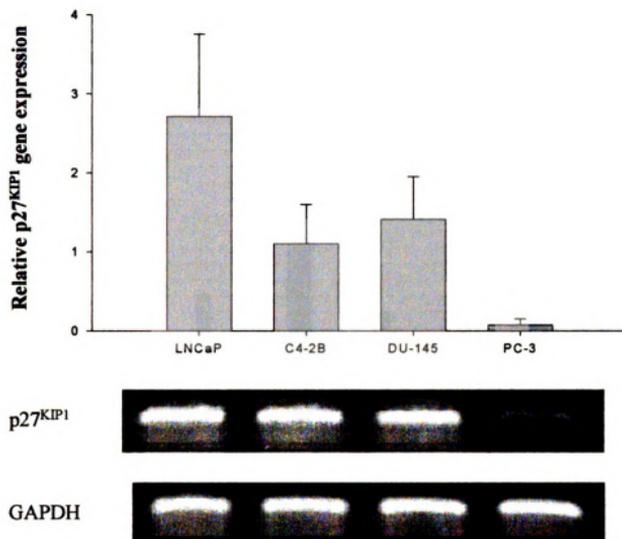
**Figure 15. Semi-quantitative RT-PCR analysis of FKHL1 gene expression in human prostate cancer cell lines (LNCaP, C4-2B, DU-145, and PC-3).** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using FKHL1 primers and GAPDH primers. The relative expression of FKHL1 for each cell line specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs PC-3, C4-2B vs PC-3; and DU-145 vs PC-3.

## PCR results of p27<sup>KIP1</sup> expression in PCa tissues and BPH tissue



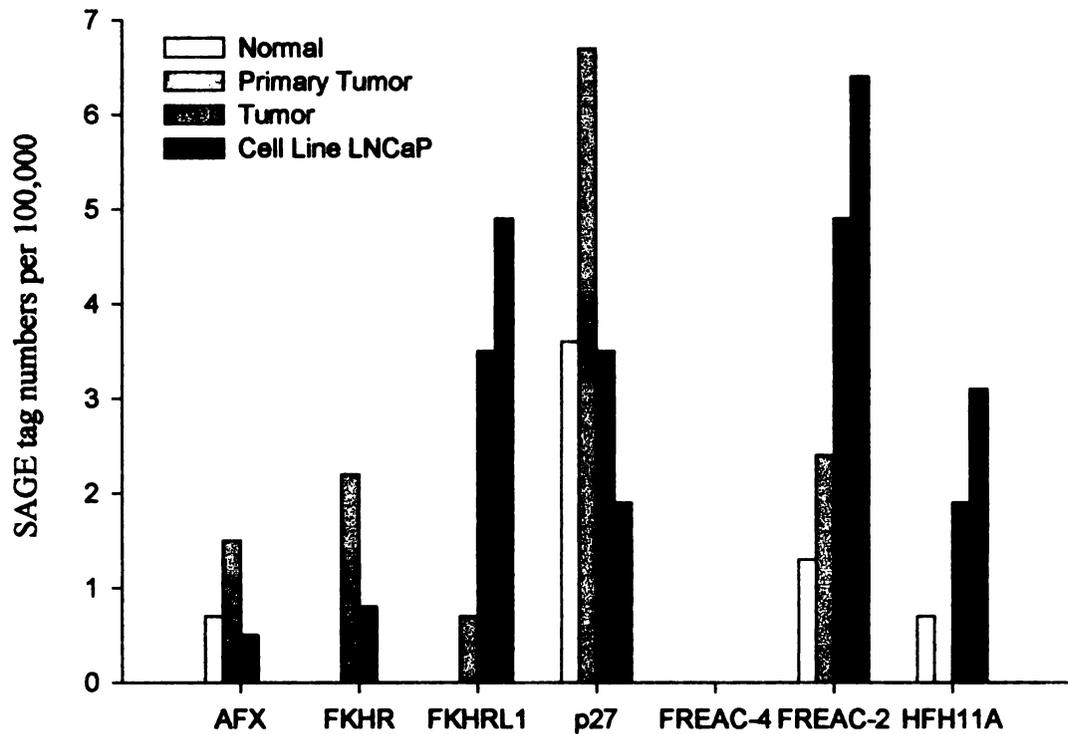
**Figure 16. Semi-quantitative RT-PCR analysis of p27<sup>KIP1</sup> gene expression in human PCa tissues and BPH tissue.** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using p27<sup>KIP1</sup> primers and GAPDH primers. Four  $\mu$ l of cDNA was used for p27<sup>KIP1</sup> amplification. The relative expression of p27<sup>KIP1</sup> for each tissue specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The difference between PCa tissues and BPH tissue are statistically significant ( $p < 0.05$ ).

## PCR results of p27<sup>KIP1</sup> expression in prostate cancer cell lines



**Figure 17. Semi-quantitative RT-PCR analysis of p27<sup>KIP1</sup> gene expression in human prostate cancer cell lines (LNCaP, C4-2B, DU-145, and PC-3).** Images of agarose gel stained with ethidium bromide show bands corresponding to the cDNA amplification products derived from PCR amplification using p27<sup>KIP1</sup> primers and GAPDH primers. The relative expression of p27<sup>KIP1</sup> for each cell line specimen was normalized to housekeeping gene GAPDH to control for RNA loading. Three experiments were done for each sample with results expressed as the mean  $\pm$ SD. The differences between the following groups are statistically significant ( $p < 0.05$ ): LNCaP vs PC-3, C4-2B vs PC-3; and DU-145 vs PC-3.

**SAGE analysis of the expression profile of forkhead transcription factors and p27<sup>KIP1</sup>**



**Figure 18. Expression profiles by SAGE analysis.** The expression levels in the four libraries are normalized per 100,000 and are shown by gray scale bars.

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