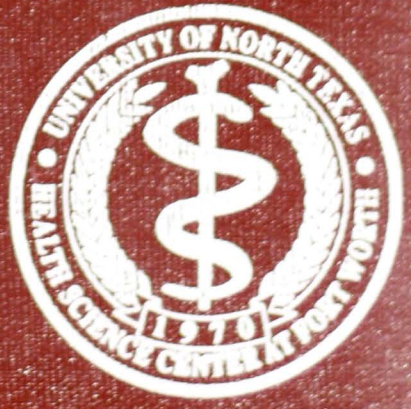
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
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Thomas, Rusha, HIF: A key survival factor for serum-deprived prostate cancer cells. Doctor of Philosophy (Molecular Biology and Immunology), May 2008, 134 pages, 47 illustrations, reference list, 105 titles.

The hypoxia-inducible factor (HIF) is central to hypoxic adaptation of tumors, and consists of an oxygen-labile HIF-1α and a constitutively expressed HIF-1β subunit. In specific aim 1, we report that prolonged serum deprivation is a potent inducer of HIF-1α in PC-3 and LNCaP prostate cancer (PCa) cells, despite normal oxygen conditions. In contrast, cells grown in the presence of serum did not upregulate HIF-1α protein levels. Moreover, HIF-1α protein increase during serum deprivation correlated with increased cell survival, while suppression of HIF-1α expression significantly decreased PCa cell viability. Our results further demonstrate that HIF-1α protein increase during serum deprivation is due to increased HIF-1α protein synthesis. First, there was no significant increase in HIF-1α mRNA. Secondly, cycloheximide, a protein synthesis inhibitor, prevented HIF-1α protein increase in serum-deprived PCa cells. Moreover, the expression of HIF-1α-target genes, VEGF and IGF-2, was concomitantly increased in serum-deprived PCa cells, while suppression of HIF-1α expression markedly inhibited their induction. Most interestingly, our study showed a significant decline in PCa cell survival following inhibition of IGF-2 activity. Taken together, our study demonstrates for the first time that PCa cells counteract the stress of prolonged serum deprivation by upregulating HIF-1α protein which increases IGF-2 expression to promote cell survival.

In specific aims 2 and 3, we investigated the molecular mechanism of autocrine regulation of HIF-1α, IGF-2 and cell survival in serum-deprived PC-3 and LNCaP PCa cells. We detected a time-dependent increase in Akt activation during serum deprivation, and inhibition of Akt activation attenuated the serum deprivation-mediated increase in HIF-1α and cell survival. Importantly, IGF-2 secretion significantly increased during serum deprivation, and was accompanied by increased activation of its receptor, insulin-like growth factor-I receptor (IGF-IR). Additionally, inhibition of IGF-2 activity markedly suppressed the serum deprivation-mediated increase in IGF-IR and Akt activation, HIF-1α expression, as well as its own transcription, suggesting autocrine regulation of HIF-1α expression via IGF-2. Reciprocal regulation of the IGF-2/IGF-IR system and PI3K-Akt pathway was further demonstrated by findings wherein Akt activation was prevented following suppression of IGF-IR expression, and IGF-IR activation was inhibited following PI3K inhibition. Lastly, HIF-1α suppression abolished the serum deprivation-mediated increase in Akt activation, and also resulted in higher IGF-IR protein levels indicating reduced IGF-IR activation. Collectively, our study demonstrates that a HIF-1α-dependent autocrine feedback loop upregulates HIF-1α, and thus promotes survival of normoxic, serum-deprived PCa cells.

HIF: A KEY SURVIVAL FACTOR FOR SERUM-DEPIVED PROSTATE CANCER CELLS

Rusha Thomas, B. D. S., M. S.

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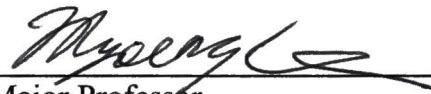

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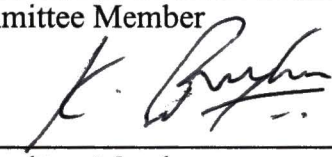
Committee Member



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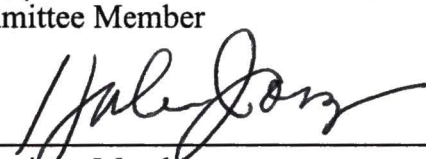

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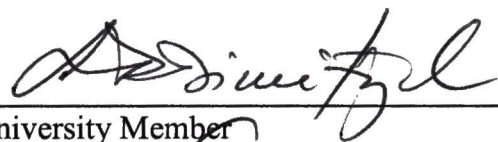

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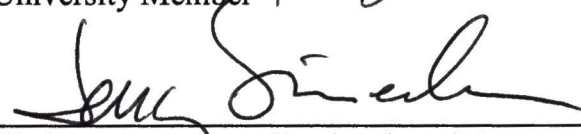

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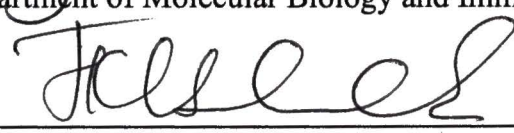

Dean, Graduate School of Biomedical Sciences

HIF: A KEY SURVIVAL FACTOR FOR SERUM-DEPIVED PROSTATE CANCER CELLS

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Rusha Thomas, B.D.S., M.S.

Fort Worth, Texas

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I would like to thank my family for their constant guidance, encouragement and prayers. Special thanks go out to my brother, Regi, for being a great source of encouragement and unconditional support in all my academic endeavors.

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LIST OF ABBREVIATIONS

HIF-1	Hypoxia inducible factor-1
PCa	Prostate Cancer
HRE	Hypoxia response element
VEGF	Vascular endothelial growth factor
IGF-2	Insulin-like growth factor-2
IGF-IR	Insulin-like growth factor-I receptor
IGFBP	Insulin-like growth factor binding protein
PI3K	Phosphatidylinositol 3-kinase
PGK	Phosphoglycerate kinase
MAPK	Mitogen-activated protein kinase
ERK	Extracellular-signal regulated kinase
mTOR	Mammalian target of rapamycin
ROS	Reactive oxygen species
EGFR	Epidermal growth factor receptor
eIF-4E-BP1	Eukaryotic translation initiation factor 4E binding protein-1

CHAPTER I

INTRODUCTION

1.1. Prostate Cancer

Prostate cancer is the most common non-cutaneous malignant neoplasm in men in Western countries [1], with more than 220,000 new cases diagnosed annually in the United States alone [2]. Moreover, these numbers are projected to increase annually as the ageing population expands [3]. Prostate cancer is also the second leading cause of cancer-related deaths in men in the United States, accounting for approximately 30,000 deaths per year in the United States [1].

The treatment options for clinically-localized prostate cancers include prostatectomy or radiation therapy, and are aimed at removing or destroying the cancerous cells that are still confined within the prostate capsule [4]. However, many patients are not cured by these therapies and their cancer recurs, or they are diagnosed after the cancer has spread [4]. Like the normal prostate, early-stage prostate cancers depend on androgens for growth and survival [4]. Androgens promote prostate cancer growth by stimulating proliferation and inhibiting apoptosis [4]. Thus, androgen ablation therapy, consisting of treatment with drugs that lower serum testosterone levels often in combination with competitive androgen-receptor antagonists, is commonly administered for the treatment of progressive prostate cancer [4]. Ultimately, however, prostate cancer

cells become independent of androgen requirements, and progress to a hormone-refractory state [3]. Thus, many men eventually fail androgen ablation therapy and die of recurrent androgen-independent prostate cancer (AI-PCa) [4]. AI-PCa is a lethal form of prostate cancer that progresses and metastasizes [4]. At present, there is no effective therapy for it [4]. Given the invasive nature of advanced prostate cancer, and the poor response of advanced prostate cancer to current therapies [3], it is crucial that the molecular basis of survival and progression of prostate cancer be well understood so that effective therapeutic strategies can be designed and implemented at the earliest stage.

1.2. The hypoxia-inducible factor (HIF)

HIF is a heterodimeric transcription factor consisting of a hypoxia-inducible HIF-1α subunit, and a constitutively-expressed HIF-1β subunit [5-8]. The HIF-1α-target gene products have been implicated in regulating several key aspects of cancer biology, such as tumor cell survival, cell proliferation, glucose metabolism, angiogenesis, and tumor invasion [9].

The degradation of HIF-1α is regulated predominantly by O_2–dependent mechanisms. Under normoxic conditions, HIF-1α protein is hydroxylated at two key proline residues by O_2–dependent HIF-1α-prolyl hydroxylases [10, 11]. This hydroxylation serves to target HIF-1α for proteasomal degradation [12]. However, under hypoxic conditions HIF-1α-prolyl hydroxylase is inactivated thereby resulting in the stabilization of HIF-1α [10, 13]. The stabilized HIF-1α subunit translocates to the nucleus where it dimerizes with HIF-1β subunit, and the dimer upregulates the expression of its

target genes by binding to hypoxia response elements located in the promoter/enhancer regions of these genes [14]. HIF-1α target gene products have been shown to regulate various processes involved in short-term and long-term tumor adaptation to hypoxia, such as glucose metabolism and angiogenesis [9]. A diagrammatic representation of the O_2–dependent regulation of HIF-1α protein stability is shown in diagram 1.

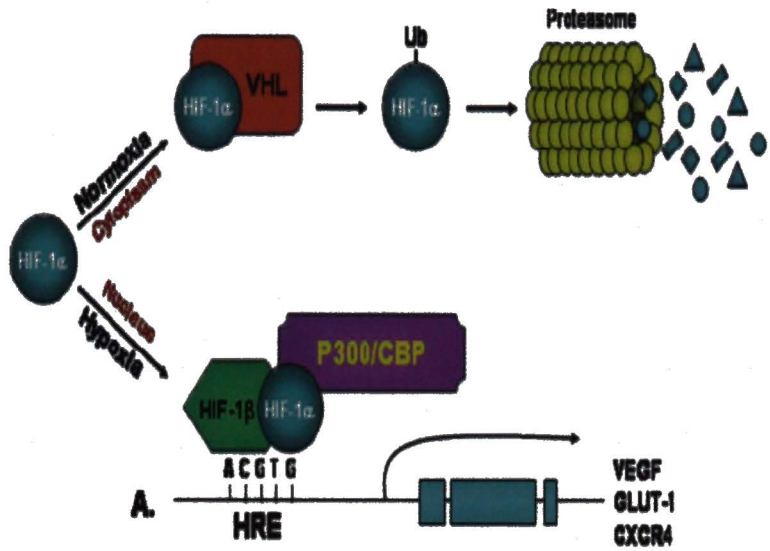


Diagram 1: Regulation of HIF-1α in mammalian cells. Under normoxic conditions, HIF-1α that is located in the cytoplasm is hydroxylated by an oxygen-sensitive enzyme. This hydroxylation targets HIF-1α for ubiquitination and degradation. However, during hypoxia, HIF-1α is not hydroxylated and degraded; instead, it is translocated to the nucleus where it dimerizes with HIF-1β. This complex then binds to hypoxic response elements (HREs) located in the promoter/enhancer regions of its target genes and activates the transcription of these genes [15].

In contrast to HIF-1α protein degradation, HIF-1α protein synthesis is regulated via O_2–independent mechanisms [9], in which protein synthesis is increased by the

activation of the phosphatidylinositol-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways [9]. The PI3K and MAPK pathways are activated following binding of growth factors, such as epidermal growth factor (EGF), to their cognate receptor tyrosine kinases [16]. The activated PI3K, in turn, activates downstream kinases, Akt and mammalian target of rapamycin (mTOR). In the MAPK pathway, MEK activates the downstream kinase, ERK. The activated ERK and mTOR phosphorylate and activate the ribosomal p70S6 kinase which, in turn, phosphorylates the ribosomal S6 protein. At the same time, the activated ERK and mTOR also phosphorylate and consequently inactivate the eukaryotic translation initiation factor 4E binding protein 1 (eIF-4E-BP1). Both these events, i.e. the activation of the ribosomal S6 protein and the inactivation of eIF-4E-BP1, increase the rate at which HIF-1α mRNAs are translated into protein [9, 17]. Diagram 2 depicts the mechanism by which the PI3K-Akt pathway regulates HIF-1α protein synthesis.

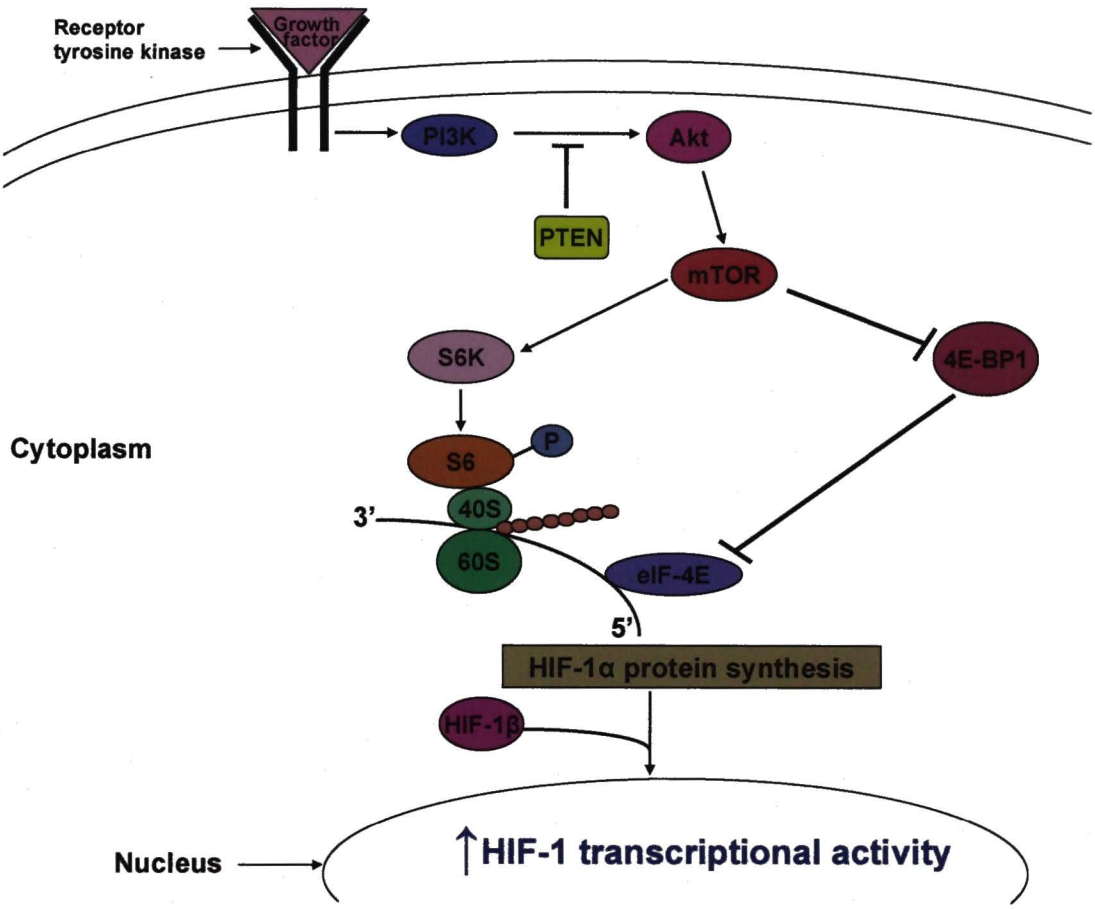


Diagram 2: Regulation of HIF-1α protein synthesis by the PI3K-Akt pathway. The PI3K pathway is activated by the binding of a growth factor to its cognate receptor tyrosine kinase. PI3K activates the downstream serine/threonine kinases, Akt and mTOR. mTOR phosphorylates p70 S6 kinase (S6K) which, in turn, phosphorylates the ribosomal S6 protein and the eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1). Both these events increase the rate at which HIF-1α mRNA is translated into protein [9].

1.3. HIF-1α and prostate cancer

High grade prostatic intraepithelial neoplasia (PIN) which is considered to be the precursor of a majority of invasive prostate adenocarcinoma express high levels of HIF-1α protein when compared to the respective normal epithelium, stromal cells, and benign prostatic hyperplasia [18]. HIF-1α expression has also been observed to increase as prostate tumors progressed from androgen-dependent to androgen-independent states [19]. Increased HIF-1α expression in PCa cells has been correlated with faster tumor growth and higher metastatic potential [20]. Angelloz-Nicoud *et al* reported that insulin-like growth factor-2 (IGF-2) is involved in the autocrine control of PC-3 cell proliferation via the insulin-like growth factor I receptor (IGF-IR) during serum deprivation [21]. As IGF-2 is a HIF-target gene [9], this finding points to a possible involvement of HIF in regulating serum-independent growth of PCa cells. Moreover, Feldser *et al* demonstrated that exposure of human embryonic kidney (HEK) 293 cells and mouse embryonic fibroblasts (MEFs) to insulin-like growth factor-1 (IGF-1) or IGF-2 induced HIF-1α protein expression [22]. These results suggest that there is complex cross-talk between the IGF system and HIF-1α [22].

1.4. Insulin-like growth factor system

Although androgens have been considered to be the primary growth factors for prostate epithelial cells, other non-androgenic growth factors have been reported to be involved in regulating the growth of PCas [23]. One of the survival tactics utilized by tumor cells, including several prostate cancer (PCa) cell types, is the overexpression of

certain growth factors and the constitutive activation of their cognate receptors [24-26]. One such growth factor family that has been implicated to have powerful mitogenic and anti-apoptotic effects in different cancer types including PCa is the insulin-like growth factor (IGF) system [23, 27, 28].

The IGF system consists of the IGF ligands, insulin-like growth factor -1 (IGF-1) and insulin-like growth factor-2 (IGF-2), cell surface receptors that mediate the biological effects of IGFs, namely insulin-like growth factor I receptor (IGF-IR) and insulin-like growth factor-2 receptor (IGF-IIR), as well as a family of IGF-binding proteins (IGFBPs) [27].

1.4A. The insulin-like growth factor receptors (IGFRs)

The effects of IGFs are mediated by their receptors IGF-IR and IGF-IIR. The IGF-IR is a member of the receptor tyrosine kinase family. Its molecular architecture comprises two identical α-subunits and two identical β-subunits [29]. Whereas the α-subunits are extracellular and responsible for ligand binding, the β-subunits span the plasma membrane and encompass an intracellular kinase domain devoted to the initiation of signal transduction cascades [29]. The peptides IGF-1 and IGF-2 are the cognate activating ligands of the IGF-IR [23, 29]. The binding of IGF-1 and IGF-2 to the extracellular domain induces conformational changes in the β-subunits that result in trans-autophosphorylation of specific tyrosine residues in the cytoplasmic tyrosine kinase domain of the β-subunits [23, 27, 29]. This converts the receptor from the unphosphorylated to the fully activated form [23, 27, 29]. Activation of the receptor

triggers, through docking and phosphorylation of signal transduction molecules, the initiation and activation of intracellular signal transduction cascades such as the mitogen activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase (PI3K) pathways [29]. In contrast to other tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR) family members, IGF-IR requires ligand binding to trigger the appropriate downstream pathways. IGF-IR overexpression alone is insufficient to cause receptor activation [23, 30].

IGF-IR is commonly overexpressed in many cancers, and IGF-IR function is implicated in many of the hallmarks of cancer such as self sufficiency in growth signals, evasion from apoptosis, tissue invasion and metastasis, as well as angiogenesis [29]. Interference with IGF-IR function results in inhibition of cancer cell proliferation, survival, anchgorage-independent growth *in-vitro*, inhibition of tumor growth and metastasis *in-vivo*, and sensitization of cancer cells to various chemotherapeutic and radiation regimens [29]. Thus, experimental evidence has demonstrated that IGF-IR could be a potential target for therapeutic intervention in cancer treatment.

The IGF-IIR is a monomer that has no intrinsic tyrosine kinase activity. IGF-IIR binds to and internalizes IGF-2 thereby reducing IGF-2 bioavailability for interaction with IGF-IR [23, 31]. Hence, IGF-IIR has been reported to possess tumor suppressor functions [31]. Loss of IGF-IIR is also correlated with increased IGF-2-initiated IGF-IR activation and increased proliferation [32, 33]. Thus, most of the physiological actions of IGF-2 are mediated through binding to IGF-IR [23]. Diagram 3 illustrates the mechanism of IGF-2 mediated activation of IGF-IR.

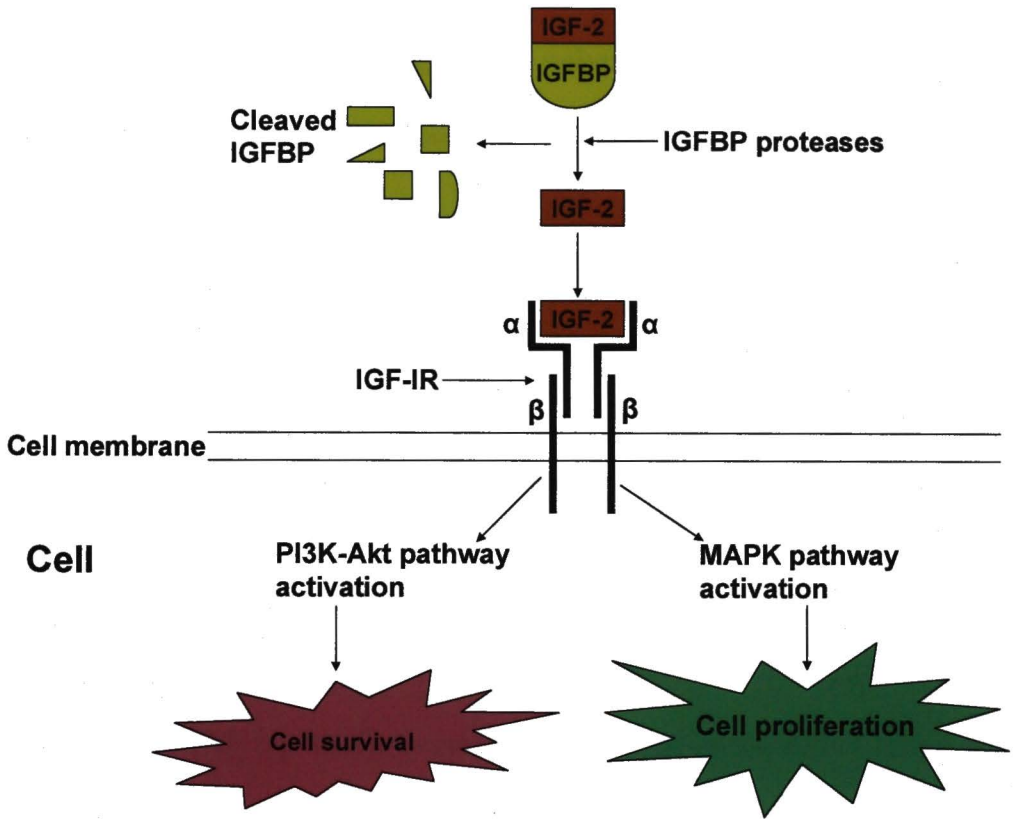


Diagram 3: **IGF-IR activation and downstream signaling.** IGF-IR is a tetrameric, trans-membrane, tyrosine kinase receptor. Following the degradation of IGFBPs by IGFBP proteases, IGF-2 interacts with IGF-IR. IGF-2 binding to the IGF-IR triggers the tyrosine kinase activity of the receptor, consequently stimulating downstream signaling cascades, including the PI3K-Akt and MAPK pathways that control cell survival and cell proliferation respectively [29, 31].

1.4B. The insulin-like growth factors (IGFs)

The IGFs, IGF-1 and IGF-2, are single chain polypeptides that are derived from pre-propeptides [23]. IGF-1 is a 70 amino acid peptide and IGF-2 is a 67 amino acid peptide [23]. IGF-1 is mainly produced by the liver [23], whereas, IGF-2 is produced by most tissues in the body [23]. Serum levels of IGF-1 are age-dependent; they increase slowly from birth to pubertal peak and thereafter decline with age [23]. The serum concentration of IGF-2 peptide (400-600 ng/ml) is higher than that of IGF-1 (100-200 ng/ml) at all ages [23]. IGF-2 levels remain stable after puberty [23]. Thus, IGF-1 and IGF-2 peptides can act by autocrine, paracrine as well as endocrine mechanisms [23]. There is a lack of consensus regarding the influence of IGF-1 on the etiology of prostate cancer [34-40]. Chan *et al.* [37] reported an association between serum IGF-1 levels and the risk of prostate cancer. However, in other studies, a direct correlation between prostate cancer and serum IGF-1 could not be found [38, 39].

IGF-2 is commonly expressed by tumor cells and may act as an autocrine growth factor [27]. Increased serum levels of IGF-2 have been associated with an increased risk of developing breast, prostate, colon and lung cancer [27]. IGF-2 plays a critical role in the autocrine and paracrine stimulation of prostatic neoplasms. IGF-2 is involved in the autocrine control of proliferation of PC-3 and DU-145 androgen-independent prostate cancer cells [21, 36]. Furthermore, Tennant *et al* [41] reported that the expression of IGF-2 mRNA in prostate cancer specimens was 30% higher than in benign prostatic epithelium [40]. In other clinical studies, a significant correlation was found between elevated IGF-2 levels and clinicopathological parameters such as Gleason score and

extraprostatic invasion [42]. Nickerson *et al* [35] also found an up-regulation of mRNA levels for IGF-1 and IGF-2 in androgen-dependent LNCaP prostate cancers during progression to androgen-independent growth [40].

1.4C. The insulin-like growth factor binding proteins (IGFBPs) and IGFBP proteases

The biological activities of the IGF ligands are also modulated by a family of specific high-affinity IGFBPs, IGFBP-1, 2, 3, 4, 5 and 6 [27]. IGFBPs are found in the circulation and in the extra cellular fluids [23]. IGFBP-3 is the predominant IGFBP in serum [27]. Less than 5% of circulating IGF is free and most (more than 90%) of the circulating IGF is found in a ternary complex consisting of IGFBP-3 and another protein known as acid labile subunit (ALS) [23]. The remainder of the IGF in the circulation is bound to IGFBP-1, 2 or 4 [43]. Although most IGFBPs are synthesized in the liver, many other organs are capable of producing them [23].

IGFBPs have multiple functions that are essential in regulating the biological activities of IGFs [44] such as transporting IGFs, protecting IGFs from degradation and thus increasing half-lives of the circulating IGFs, and regulating the interactions between IGFs and IGF-IR. IGFs that are bound to the IGFBPs are prevented from interacting with IGF-IR [23].

Each of the IGFBPs is subjected to limited, and potentially regulated proteolysis by various proteases (IGFBP proteases) [27]. The IGFBP proteases are enzymes that cleave intact IGFBPs into smaller fragments thereby altering their abilities to bind the

IGFs [23]. Thus ligand-receptor interactions in the IGF system are subject to complex regulation as a result of the levels of IGFBPs, their expression profile, the degree of cell-surface association, and the extent of proteolysis [45].

1.5. The phosphatidylinositol 3-kinase signaling pathway

The phosphatidylinositol-3-kinase (PI3K) pathway has been implicated in cancer since its discovery as an enzymatic activity associated with viral oncoproteins 20 years ago [46]. However, in recent years it has become apparent that it is one of the most frequently targeted pathways in all sporadic human tumors, with estimates suggesting that mutation in one or another PI3K pathway component accounts for upto 30% of all human cancers [17, 46].

A survival strategy that is frequently utilized by cancer cells in response to various apoptotic stresses is the upregulation of PI3K signaling [47, 48]. Growth factor receptor tyrosine kinases engage PI3K, which is a heterodimer comprised of the p85 regulatory and p110 catalytic subunits [48]. At the cell membrane, PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP2) at the 3'position on its inositol ring and converts PIP2 to PIP3 [17]. The levels of PIP3 in the cell are strictly regulated by a 3' phosphatase, PTEN, which converts PIP3 back to PIP2 and thus shuts off PI3K signaling [49]. PIP3 recruits other downstream molecules, particularly the serine-threonine kinases, Akt and PDK1 [17]. At the cell membrane, Akt is activated by the phosphorylation of threonine 308 by PDK1 and serine 473 by a yet unknown kinase [17]. Akt in turn

regulates a wide range of target proteins that control cell proliferation, survival, growth and other processes [17].

Akt has emerged as a critical target of PI3K in human cancer [46]. Akt promotes cell survival via the phosphorylation of a diverse set of substrates. Akt phosphorylates and inactivates the pro-apoptotic protein BAD [17]. Following its phosphorylation, BAD is sequestered in the cytoplasm by 14-3-3 proteins, and is thus unable to heterodimerize with and inactivate the anti-apoptotic protein, Bcl-x_L [50]. Similarly, Akt-mediated phosphorylation of the FOXO transcription factors causes them to be retained and sequestered in the cytoplasm by association with 14-3-3 proteins [46, 50]. When dephosphorylated, the FOXO proteins migrate to the nucleus and drive the expression of pro-apoptotic genes such as FasL which activates the Fas-mediated cell-death cascade [50]. Thus, Akt-mediated phosphorylation of FOXO proteins inhibits the Fas-mediated cell-death cascade. Akt can also indirectly promote cell survival by various means. First, Akt can promote cell survival by indirectly activating the pro-survival NFκB pathway through the phosphorylation of I-κB kinase [51, 52]. Second, Akt can promote the degradation of the tumor suppressor protein, p53, via the phosphorylation and activation of p53's negative regulator, Mdm2 [53, 54]. In addition, increased PI3K-Akt activity has been associated with increased expression of hypoxia inducible factor (HIF)-1α [18], a key transcription factor that has been implicated in promoting cell survival under hypoxic stress [9].

Loss of the PTEN lipid phosphatase appears to be the most common mechanism of activation of the PI3K pathway in human cancers [46]. In nearly 50% of prostate

tumors, the PI3K-Akt pathway is constitutively upregulated due to mutations in the tumor suppressor, PTEN, which functions as a negative regulator of PI3K [55-58]. Thus, by enhancing the activation of Akt, a downstream target of PI3K, cancer cells are able to raise the apoptosis threshold in response to apoptotic stimuli such as trophic factor withdrawal [59].

1.6. Summary

Our preliminary studies in PC-3 and LNCaP PCa cells revealed that HIF-1α protein levels increase as the duration of serum deprivation increases, despite normal oxygen conditions. In addition, the increase in HIF-1α protein levels during serum deprivation correlated with increased cell survival.

Although it is well known that HIF plays a key role in tumor adaptation to hypoxia, to date, there have not been any studies on the significance of HIF upregulation in normoxic PCa cells during prolonged serum deprivation. This is especially important with regard to PCa, as most prostate tumors have been shown to overexpress HIF-1α. Hence, this project investigated the role of HIF-1α in cell survival during stress of prolonged serum deprivation, and also elucidated the molecular mechanism of HIF-1α upregulation in normoxic, serum-deprived PCa cells.

1.7. Significance of this study

Hypoxia, low nutrient environments, and exogenous growth factor depletion are among the most important challenges that any rapidly growing solid tumor must

surmount in order to survive, grow and metastasize. Tumor cells can be deprived of serum growth factors following treatments such as radiotherapy or anti-angiogenic therapy, as these treatment strategies frequently disrupt tumor vasculature [60, 61]. Tumors can also outgrow their supply of exogenous growth factors during the course of their progression to advanced states. Diffusible growth factors have the ability to deliver survival and mitogenic signals to cells by binding to their cognate receptors and activating several downstream effectors of the activated growth factor receptors. Thus, limitations in growth factor availability and/or signaling lead to cell death [50, 62, 63]. Despite the necessity of growth factors for the maintenance of normal cell viability, many tumors have developed adaptive mechanisms to circumvent this requirement for exogenous growth factors. These adaptive mechanisms are important for tumors to continue to grow and invade surrounding and distant tissues.

Approximately one-third of early PCas are treated nowadays with a radiotherapy modality [64]. A recent study showed that patients undergoing surgery for clinically localized PCa may have a cancer specific survival advantage when compared to those undergoing radiation therapy [65]. Persistent PCa following radiation therapy results in cancer related-death in at least 27% of patients within 5 years of exhibiting a rising prostate specific antigen (PSA) level [66]. In addition, clinical trials of anti-angiogenic agents such as recombinant anti-VEGF monoclonal antibody, and SU-5416, a VEGF receptor 2 inhibitor, have not shown significant disease modifying effects in PCa, as assessed by changes in PSA levels [67, 68]. Assessment of serum PSA levels has been widely used both for initial diagnosis of PCa and for monitoring of response to treatment

[69]. Thus, these studies demonstrate that PCa cells have the ability to adapt to micro-environmental stresses arising following treatments such as radiotherapy or anti-angiogenic therapy.

To date, little is known about the mechanisms by which PCa cells sense exogenous growth factor depletion in their microenvironment, or the signal transduction pathways involved in mounting an anti-apoptotic response to the stress of prolonged serum deprivation. As the stress of serum deprivation mimics, to some extent, the tumor micro-environmental condition arising following radiotherapy or anti-angiogenic therapy, the insights gained from this study would immensely help in understanding the anti-apoptotic response mounted by cancer cells, and would also help in planning suitable therapeutic strategies for more effective inhibition of survival of PCa cells.

CHAPTER II

MATERIALS AND METHODS

Reagents-Primary antibodies to IGF-IRβ and total-Akt were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-β-actin and anti-PTEN antibodies were from Sigma (St. Louis, MO), phospho-tyrosine, phospho-Akt and phospho-Erk1/2 antibodies were from Cell Signaling Technology (Danvers, MA). HIF-1α primary antibody was from Novus Biologicals (Littleton, CO). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies, M-PER mammalian protein extraction reagent and Supersignal West Femto Chemiluminescence substrate were from Pierce (Rockford, IL). Protein G-agarose beads, trypan blue and BCA assay reagents were obtained from Sigma. Dual Luciferase reporter assay system, RNase A, oligo dT primers, random primers, dNTPs and reverse transcriptase were from Promega (Madison, WI). Lipofectamine 2000 transfection reagent was from Invitrogen (Carlsbad, CA). HIF-1α siRNA and IGF-IR siRNA were purchased from Dharmacon (Lafayette, CO). Propidium iodide was obtained from Roche. VEGF and IGF-2 neutralizing antibodies, and VEGF ELISA kit were obtained from R&D Systems (Minneapolis, MN). IGF-2 ELISA kit was from Diagnostic Systems Laboratories (Webster, TX). LY294002 and cycloheximide were obtained from Calbiochem (San Diego, CA). 4–20% gradient SDS polyacrylamide gels were obtained from Lonza (Allendale, NJ).

Plasmid constructs- pGL3-6xHRE-Luc was a generous gift from Dr. Peter RatCliffe (University of Oxford) [70], and contained six copies of the hypoxia response element (HRE) from the erythropoietin gene promoter linked to the thymidine kinase basal promoter and firefly luciferase gene. pRL-TK (Promega) was used as transfection efficiency control and contained the thymidine kinase promoter linked to Renilla luciferase gene. pCEP-HIF-1α expression vector expressing full-length HIF-1α was from ATCC, and PTEN expression vector expressing wild-type PTEN was from Dr. Ramon Parsons (University of Columbia) [71].

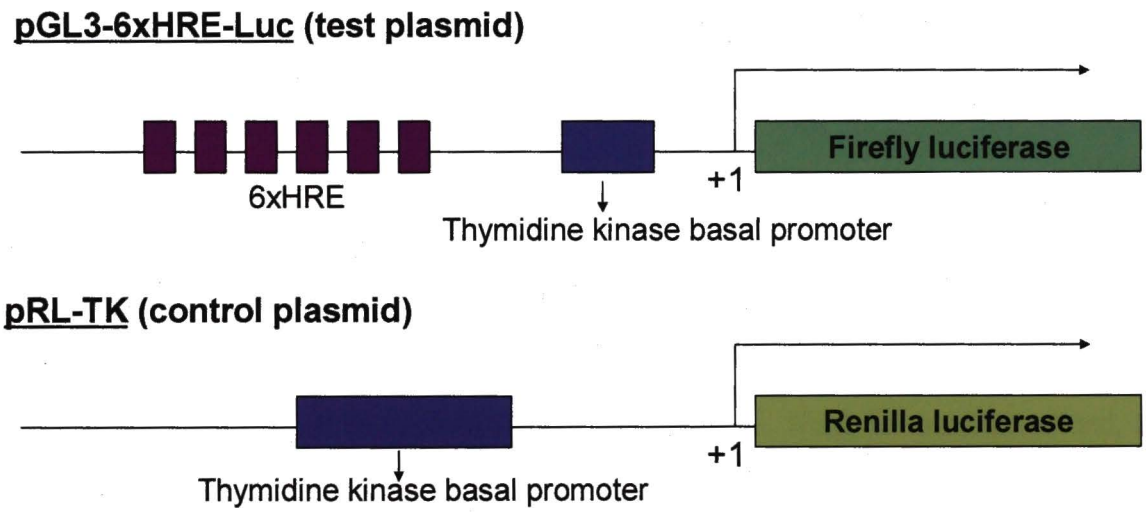


Diagram 4: Plasmid constructs used in the dual luciferase reporter assay.

Tumor cell lines and culture- The androgen non-responsive PC-3, and the androgen responsive LNCaP cell lines were obtained from ATCC (Manassas, VA). PC-3 cells were cultured in F-12K Nutrient Mixture (Kaighn's Modification), and LNCaP cells in RPMI-1640 medium (Invitrogen/Gibco) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate and 100 units/ml penicillin G sodium. All cell cultures were

maintained in a humidified 5% CO_2 incubator at 37°C, and routinely passaged when approximately 80% confluent.

Establishment of serum-deprived conditions-PCa cells were grown to 70-80% confluency in medium containing 10% FBS (complete medium). On day 0, the cells were first washed with serum-free (SF) medium and fresh SF medium was added. The cells were then incubated under normoxic conditions for up to 4 days.

Luciferase reporter gene assay- PC-3 and LNCaP cells were grown in 48-well plates in complete medium without antibiotics until 90% confluent. The cells were then transiently transfected with pGL3-6xHRE-Luc test plasmid along with pRL-TK-Luc as internal control using Lipofectamine 2000 transfection reagent. At 4 h post-transfection, medium was replaced with fresh complete medium and incubation continued. At 24 h post-transfection, i.e. on day 0, day 0 serum-free cells were harvested, and for the day 1 serum-free analysis, cells were washed with SF medium, fresh SF medium added, and cells incubated under normoxic conditions for a further period of 24 h. Cells were harvested for the dual luciferase assay to determine HRE-mediated transcriptional activity (procedure is as described in the protocol provided by the manufacturer-Promega). The firefly luciferase expression from pGL3-6xHRE-Luc, and renilla luciferase expression from pRL-TK were measured sequentially from a single sample, in a TD-20/20 Luminometer (Turner Designs) according to the Dual- Luciferase Reporter System protocol (Promega). The activity of pGL3-6xHRE-Luc was normalized to the

activity of the pRL-TK internal control, and represented as relative luciferase activity on a bar graph.

Whole cell lysate preparation and immunoblot analysis-PC-3 and LNCaP cells were harvested by scraping, and then washed in ice-cold PBS and lysed in M-PER reagent supplemented with protease inhibitors leupeptin (5 μg/ml), pepstatin (1 μg/ml) and aprotinin (1.7 μg/ml), and phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (2 mM). After a 15-min incubation on ice, lysates were cleared by centrifugation at 16,000 rpm, at 4 °C. The resulting lysates were stored at -80 °C until they were used for western blot analysis. Protein concentration was measured by bicinchoninic acid (BCA) assay. Whole cell lysates (30–80 μg of protein) were separated on a 4–20% gradient SDS polyacrylamide gel under reducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in T-PBS, pH 7.5 (PBS with 0.1% Tween-20) with 5% non-fat dry milk. After washing, the blot was incubated with primary antibody for 1 h at room temperature (RT) or overnight in the case of phospho antibodies. The blot was washed 3 times in T-PBS and incubated with HRP-coupled secondary antibody for 1 h at RT. After extensive washing, the bands were detected using the SuperSignal West Femto Maximum Sensitivity Substrate System. The blots were stripped and sequentially probed for HIF-1α, phospho-Akt, total-Akt, PTEN and β-actin. β-actin served as the loading control.

VEGF and IGF-2 ELISA-Cell culture supernates of the serum-deprived PC-3 and LNCaP cells were collected at each time point and centrifuged to remove cellular debris. The supernates were subsequently analyzed for VEGF and IGF-2 protein levels by ELISA using the VEGF and IGF-2 ELISA kits provided by the manufacturers, R&D Systems and Diagnostic Systems Laboratory respectively. The procedure for the IGF-2 ELISA is essentially as described in the protocol. Briefly, the pre-treated culture supernatates were added to wells of an IGF-2 microplate coated with an antibody against IGF-2 and incubated for 2 h at room temperature. The plate was washed 5 times with wash buffer (provided in the kit). Horseradish peroxidase-conjugated anti-IGF-2 antibody solution (IGF-2 conjugate) was added, and the plate was incubated for 30 minutes at room temperature. The plate was then washed and color reagent added. Color development was stopped after 10 minutes by the addition of stop solution and the absorbance was measured in an Elx808IU Ultra Microplate Reader (BIO-TEK Instruments, Inc.) at a wavelength of 450 nm and reference wavelength of 630 nm. The IGF-2 concentration in each well was calculated using the equation of standard curve and normalized according to the cell number in each well and expressed as nanogram per 10^6 cells. Cell number was determined by trypan blue assay.

The procedure for the VEGF ELISA is as follows. Cell culture supernatants were added to wells of VEGF microplate coated with a monoclonal antibody against VEGF and incubated for 2 h at room temperature. The plate was washed 3 times with wash buffer (provided in the kit). VEGF conjugate was added, and the plate was incubated for 2 h at room temperature. The plate was then washed and color reagent added. Color

development was stopped by the addition of stop solution and the absorbance was measured at a wavelength of 450 nm and reference wavelength of 630 nm. The VEGF concentration in each well was calculated using the equation of standard curve and normalized according to the cell number in each well and expressed as picogram per 10^6 cells. Cell number was determined by trypan blue assay.

VEGF and IGF-2 neutralization study- PC-3 and LNCaP cells were grown in serum-free medium containing VEGF or IGF-2 neutralizing antibody, or isotype-matched control antibody for 2 days (in the case of IGF-2 neutralizing antibody) or 3 days (in the case of VEGF neutralizing antibody) followed by harvest and subsequent analysis. The concentration of neutralizing antibody used in the experiments was optimized based on the amount of protein secreted by the cell lines and also the neutralization $dose_{50}$ (ND_{50}) of the antibody as recommended by the manufacturer of the antibodies (R&D Systems).

Immunoprecipitation- After the indicated treatments, cells were lysed in lysis buffer containing 1% Triton X-100, 50 mM Tris (pH 7.4), 300 mM NaCl, 5 mM EDTA supplemented with protease inhibitors (1 mM PMSF and 2 μg/ml leupeptin) and phosphatase inhibitors (50 mM sodium fluoride and 2 mM sodium orthovanadate). IGF-IR protein was immunoprecipitated from the cell lysates with IGF-IR antibody conjugated to protein G-agarose beads. The samples were boiled in 2x SDS sample buffer, and the beads were removed by centrifugation. Proteins were electrophoretically separated on 4-20% gradient gels, transferred to PVDF membranes for immunoblot

analysis. Blots were probed for phospho tyrosine, and subsequently stripped and reprobed for IGF-IR.

Transient transfection- PCa cells were transfected with plasmids expressing wild-type PTEN (provided by Dr. Ramon Parsons, University of Columbia) [71] or the control pCMV empty vector using Lipofectamine 2000 transfection reagent as per the protocol provided by the manufacturer (Invitrogen). 24 h post-transfection, on day 0, cells were washed with SF medium and fresh SF medium was added. Cells were then grown under normoxic, serum-free conditions for the indicated time periods, followed by harvest and cell lysis.

PC-3 cells were transfected with pCEP-HIF-1α expression vector which expresses full length HIF-1α (ATCC) or the control pCMV empty vector using Lipofectamine 2000 transfection reagent as per the protocol provided by the manufacturer (Invitrogen). Cells were grown in serum-containing medium under normoxic conditions for 1 day post-transfection. Cells were then harvested and lysed for immunoblot analysis.

RNA isolation and RT-PCR-Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA (2 μg) isolated from cells was reverse transcribed to cDNA using oligo-dT and random primers. The cDNA was amplified by PCR using the following specific primers:

HIF-1α: forward 5'-CTCAAAGTCGGACAGCCTCA -3'

reverse 5'-CCCTGCAGTAGGTTTCTGCT -3'

Vascular endothelial growth factor(VEGF):forward 5'-GGGCAGAATCATCACGAA-3'

reverse 5'-ACATTTACACGTCTGCGG -3'

IGF-2: forward 5'-AGTCGATGCTGGTGCTTCTCA -3'

reverse 5'-GTGGGCGGGGTCTTGGGTGGGTAG-3'

b-actin: forward 5'-TGTGATGGTGGGAATGGGTCA-3'

reverse 5'-TTTGATGTCACGCACGATTTCC-3'.

IGF-IR: forward 5'-ATTGAGGAGGTCACAGAGAAC-3'

reverse 5'-TTCATATCCTGTTT TGGCCTG-3'

The PCR cycling parameters were as follows:

VEGF: 95 °C for 15 min, 94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min; 32 cycles.

IGF-2: 95 °C for 15 min, 94 °C for 45 s, 54 °C for 45 s, 72 °C for 1 min; 34 cycles.

HIF-1α: 95 °C for 15 min, 94 °C for 45 s, 53 °C for 45 s, 72 °C for 1 min; 29 cycles.

β-actin: 95 °C for 15 min, 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min; 30 cycles.

IGF-IR: 95 °C for 15 min, 94 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min; 40 cycles.

The amplified products were visualized on 1% agarose gels.

Real-time PCR analysis- 2 μl samples of cDNA were quantified by real-time PCR using primer pairs for VEGF, HIF-1α or β-actin, SYBR Green, and lyophilized PCR Master Mix (Taka Ra, Cepheid) according to the manufacturer's instructions. Real-time PCR was performed using the Cepheid SmartCycler system. β-actin served as the internal control. Relative quantitation of VEGF or HIF-1α was done by the comparative cycle

threshold (comparative C_T) method, also known as the $2^{-\Delta\Delta Ct}$ method. In this method, the ΔC_T value for each treatment sample was obtained by subtracting the C_T of the β-actin internal control from the C_T of the target gene i.e. VEGF or HIF-1α. Thereafter, $\Delta\Delta C_T$ was obtained using the following equation: $\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ reference}$. Fold change or relative abundance of VEGF or HIF-1α expression was then calculated by substituting the $\Delta\Delta C_T$ value in the formula $2^{-\Delta\Delta Ct}$, and represented on a bar graph.

Cell cycle analysis-The PCa cells were harvested on day 0, 1, 2, 3 and 4, washed with PBS and fixed in 70% ethanol overnight at -20 °C. The fixed cells were rehydrated by washing with PBS and resuspended in 1 ml of propidium iodide (PI) staining solution (20 μg/ml PI and 20 μg/ml RNase A in PBS) for 1 h at RT. Samples were then analyzed by flow cytometry using the Beckman Coulter Cytomics FC 500 Flow Cytometer.

Assessment of cellular morphological changes-Cellular morphology was evaluated using phase-contrast microscopy, and photographs were captured with a computer-imaging system (Olympus Q-Color 3RTV camera and Adobe Photoshop for image anaylsis).

Trypan blue dye exclusion assay-After the cells were incubated for the stipulated time periods, they were washed with PBS, detached with trypsin-EDTA, neutralized with complete medium, centrifuged and re-suspended in PBS. An aliquot of cell suspension was diluted with 0.4% trypan blue, pipetted onto a hemocytometer and counted under a

microscope at 40X magnification. Live cells excluded the dye, whereas dead cells admitted the dye and consequently stained intensely with trypan blue. The number of viable cells for each experimental condition was counted and represented on a bar graph.

siRNA transfection- PC-3 cells that were 40-50% confluent were transfected with 100 nM HIF-1α or IGF-IR siRNA or a negative control siRNA (Dharmacon) using Lipofectamine 2000 transfection reagent. 24 h following transfection, cells were washed once with SF medium, and fresh SF medium was added. Cells were then incubated under normoxic conditions for the indicated time periods prior to analysis. The siRNA transfection procedure for LNCaP cells was similar to that for the PC-3 cells, except that the LNCaP cells were transfected with 100 nM siRNAs for 2 consecutive days prior to serum deprivation.

Data Interpretation and Statistical Analysis- Most experiments were performed at least 2 separate times independently, and samples were assayed in triplicate. Mean values and standard deviation values are reported wherever possible. All generated data were tested for statistical significance by one-way ANOVA with Tukey post-test using the Graph-Pad Instat software (Version 3). Data were considered statistically significant only if $p<0.05$.

CHAPTER III

CENTRAL HYPOTHESIS AND SPECIFIC AIMS

The hypoxia-inducible factor (HIF) transcription factor is central to most adaptation responses of tumors to hypoxia, and consists of an oxygen-labile HIF-1α subunit and a constitutively expressed HIF-1β subunit. Our initial studies in PC-3 and LNCaP PCa cells revealed that HIF-1α is also induced under conditions of prolonged serum deprivation, despite normal oxygen conditions. Moreover, this increase in HIF-1α protein levels during serum deprivation correlated with increased cell survival. A previous study showed that serum-deprived PC-3 cells produced insulin-like growth factor-2 (IGF-2), which in turn activated the IGF-IR and promoted cell proliferation in an autocrine manner [21]. Zhao *et al* recently demonstrated that serum-deprived PC-3 PCa cells were more susceptible to apoptosis when IGF-IR synthesis was inhibited following overexpression of PTEN [72]. As PTEN is a negative regulator of PI3K activity, this finding suggests that IGF-IR and PI3K play important roles in PCa cell survival. In addition, activation of the PI3K-Akt pathway has also been shown to increase the rate of HIF-1α protein synthesis [16].

The data from our initial experiments, as well as observations from previous studies [16, 21, 72] led us to our hypothesis that **the IGF-2/ IGF-IR system, via the PI3K-Akt pathway, upregulates HIF-1α expression during prolonged serum**

deprivation, and the serum deprivation-mediated increase in HIF-1α promotes normoxic PCa cell survival during this stress.

Our hypothesis was tested by pursuing the following 3 specific aims:

Specific Aim 1: Determine if HIF-1α upregulation promotes the survival of normoxic PCa cells during stress of prolonged serum deprivation.

Specific Aim 2: Investigate if activation of the PI3K/Akt pathway mediates the upregulation of HIF-1α protein in normoxic, serum-deprived PCa cells.

Specific Aim 3: Determine if IGF-IR/IGF-2 system plays a role in the upregulation of HIF-1α in normoxic PCa cells during prolonged serum deprivation.

CHAPTER IV

SPECIFIC AIM 1

Specific Aim 1: Determine if HIF-1α upregulation promotes the survival of PCa cells during stress of prolonged serum deprivation.

(A) Rationale

IGF-2 has been reported to be involved in the serum-independent proliferation of PC-3 cells [21]. As IGF-2 is a HIF-target gene [9, 22], this observation points to a possible role of HIF-1α in inhibiting apoptosis and promoting PCa cell survival. Hence, we hypothesized that the upregulation of HIF-1α during prolonged serum deprivation will promote the survival of PCa cells during this stress situation.

(B) Results

Prostate cancer cells upregulate HIF-1α protein levels under normoxic, serum-deprived conditions

To study the effect of prolonged serum deprivation on HIF-1α protein expression in normoxic PCa cells, PCa cells were grown in serum-free (SF) medium for 1-4 days, followed by HIF-1α protein analysis. We detected a significant, time-dependent increase in HIF-1α protein levels in the serum-deprived PCa cells, despite normal O_2 conditions (Fig. 1A). We observed a 4.2-fold increase in HIF-1α protein levels in PC-

3 cells (Fig. 1B, top panel), and a 6.3-fold increase in LNCaP cells by day 4 of serum deprivation (Fig. 1B, bottom panel), when compared to HIF-1α protein levels in PCa cells grown in the presence of serum, i.e. day 0 cells.

To determine if the increase in HIF-1α protein levels in the normoxic, serum deprived PCa cells was mainly in response to serum deprivation, we assessed HIF-1α protein levels in PCa cells of a similar confluency that were grown in the presence or absence of serum for 3 days under normoxic conditions. In contrast to the increase in HIF-1α protein levels in serum-deprived PCa cells, we did not observe a significant increase in HIF-1α protein levels in PCa cells that were grown in the presence of serum for the same time duration (Fig. 1C). This result indicated that the normoxic PCa cells upregulated HIF-1α protein levels mainly in response to serum deprivation.

Serum-deprived PCa cells upregulate HIF-1α protein levels by increasing HIF-1α protein synthesis

To gain insight into the mode of upregulation of HIF-1α protein during prolonged serum deprivation, we first assessed HIF-1α mRNA levels by RT-PCR analyses to determine if the increase in HIF-1α protein was due to increased transcription of HIF-1α mRNA. We did not observe a significant increase in HIF-1α mRNA levels during the entire duration of serum deprivation in either the PC-3 or LNCaP cells (Fig. 2A), suggesting that the increase in HIF-1α protein during serum deprivation was not due to increased levels of HIF-1α mRNA.

We next assessed the effect of prolonged serum deprivation on HIF-1α protein synthesis. PCa cells were serum deprived for 2 days, and then treated with 10 μM cyloheximide (CHX), a protein synthesis inhibitor, for 0, 4, 8 and 24 h followed by cell lysate preparation. Vehicle-treated PCa cells served as control. As shown in Figs. 2B and 2C, CHX treatment abolished the serum deprivation-induced increase in HIF-1α protein when compared to that in the vehicle-treated cells (Fig. 2B and 2C). Furthermore, in order to confirm if 10 μM CHX was effective in inhibiting protein synthesis in PCa cells during serum deprivation, we assessed protein levels of phosphoglycerate kinase (PGK), a house-keeping gene, following vehicle and CHX treatment (Fig. 2D). We detected a 2.7-fold increase in PGK levels after 24 h of vehicle treatment when compared to that after 0 h of vehicle treatment (Fig. 2D). In contrast, 10 μM CHX resulted in a marked decrease in PGK protein synthesis (Fig. 2D). We detected only a 1.2-fold increase in PGK levels after 24 h of CHX treatment when compared to that after 0 h of CHX treatment (Fig. 2D), thereby demonstrating that the concentration of CHX used in our experiment was effective in inhibiting protein synthesis in serum-deprived PCa cells. Thus, taken together, our results suggested that the increase in HIF-1α protein levels during serum deprivation is most likely due to an increase in HIF-1α protein synthesis.

Increase in HIF-1α protein promotes PCa cell survival during serum deprivation

PCa cells have been known to survive prolonged serum deprivation [21, 73]. As HIF-1α has been attributed with pro-survival roles during hypoxia [9], we

investigated if the increase in HIF-1α protein levels correlated with PCa cell survival during serum deprivation.

First, trypan blue dye exclusion assay revealed a modest increase in cell number during this stress (Fig. 3A, top and bottom panels). We determined a 1.9- and 1.4-fold increase in viable PC-3 and LNCaP cell numbers respectively by day 4 of serum deprivation when compared to the number of viable cells on day 0. The number of viable cells on day 0 was set at 1 (Fig. 3A, top and bottom panels).

Secondly, we employed flow cytometric analysis to determine cell-cycle distribution of the serum-deprived PCa cells. Interestingly, although a large percentage of the cells were in the G_0 phase of the cell cycle by day 4 of serum deprivation, 78.9% for PC-3 (Fig. 3B, top-right panel) and 76.8% for LNCaP (Fig. 3C, top-right panel), an appreciable percentage of G2-M phase PCa cells were still undergoing mitosis during serum deprivation, 13.3% for PC-3 (Fig. 3B, bottom-right panel) and 9.2% for LNCaP (Fig. 3C, bottom-right panel). Moreover, we did not detect a marked increase in the number of apoptotic (Sub-G_0) PCa cells during serum deprivation when compared to that on day 0 (Fig. 3B and 3C, top-left panels).

Thirdly, we assessed morphology of the serum-deprived PCa cells. The PCa cells did not exhibit any obvious morphological changes characteristic of apoptosis such as reduction in cell volume or membrane blebbing. (Fig. 3D, top and bottom panels).

Lastly, to assess the effect of serum deprivation-mediated increase in HIF-1α on cell survival, we suppressed HIF-1α expression by transient transfection with HIF-1α-siRNA, and determined viability of the serum-deprived PCa cells by the trypan blue

dye exclusion assay. As shown in Fig. 4A, HIF-1α suppression significantly reduced viable PC-3 (Fig. 4A, top panel) and LNCaP (Fig. 4A, bottom panel) cell numbers by 32.4% and 23.5% respectively when compared to that following control siRNA transfection. HIF-1α suppression was confirmed by RT-PCR analyses (Fig. 4B, left and right panels). Thus, our results demonstrated that the serum deprivation-mediated increase in HIF-1α protein is a major contributor of PCa cell survival.

Effect of HIF-1α protein upregulation on HRE-mediated transcription, and expression of HIF-1α-target genes, VEGF and IGF-2.

HIF-1α's pro-survival role under hypoxic conditions has been attributed to increased transcription of pro-survival HIF-1α–target genes such as vascular endothelial growth factor (VEGF) and insulin-like growth factor-2 (IGF-2) [9, 74]. We thus investigated transcriptional activity of HIF-1α, and expression of its target genes, VEGF and IGF-2, in normoxic, serum-deprived PCa cells.

We performed a luciferase reporter gene assay to determine the transcriptional activity of the serum deprivation-induced HIF-1α protein. We observed a 1.7- and 2-fold increase in HRE-mediated luciferase reporter gene activity in serum-deprived PC-3 and LNCaP cells respectively when compared to that in the control cells (Fig. 5A, top and bottom panels). As HIF-1α upregulates the expression of its target genes by binding to HREs found in the promoter regions of these genes, our luciferase assay results suggested that the HIF-1α induced during serum deprivation was transcriptionally active.

We next investigated the expression of HIF-1α-target genes, VEGF and IGF-2. RT-PCR analyses of the serum-deprived PCa cells revealed a time-dependent increase in the mRNA expression of VEGF as well as IGF-2 (Fig. 5B) during serum deprivation. We observed a marked increase in the mRNA levels of the $VEGF_{189}$ and $VEGF_{165}$ isoforms by day 4 of serum deprivation in the PC-3 cells (Fig. 5B, top left panel), whereas the LNCaP cells exhibited a modest increase in VEGF mRNA levels by day 4 (Fig. 5B, top right panel). We also observed an increase in IGF-2 mRNA levels in the serum-deprived PCa cells. Although the PC-3 cells exhibited an initial decrease in IGF-2 mRNA levels on day 1 of serum deprivation when compared to that on day 0, the IGF-2 mRNA levels continued to increase during the entire period of serum deprivation (Fig. 5B, bottom-left panel). The LNCaP cells exhibited a marked increase in IGF-2 mRNA levels during the entire period of serum deprivation (Fig. 5B, bottom-right panel). Furthermore, real-time PCR analyses revealed a 2.6- and 24.9-fold increase in VEGF mRNA levels in day 2 and day 4 serum-deprived PC-3 cells respectively when compared to that on day 0 (Fig. 5C, top panel). Real-time PCR analyses of VEGF expression in LNCaP cells showed a 1.7- and 1.8-fold increase in VEGF mRNA levels by day 2 and day 4 of serum deprivation respectively when compared to that on day 0 (Fig. 5C, bottom panel).

To assess if the increase in VEGF and IGF-2 mRNA levels during serum deprivation was due to HIF-1α protein upregulation, we suppressed HIF-1α expression by transient transfection with siRNA, and then determined VEGF and IGF-2 mRNA levels in serum-deprived PCa cells by RT-PCR analyses. As shown in

Fig. 5D, HIF-1α suppression markedly decreased IGF-2 mRNA levels in serum-deprived PC-3 and LNCaP PCa cells when compared to that in the control siRNA-transfected cells. HIF-1α suppression resulted in a marked decline in the mRNA levels of $VEGF_{189}$ and $VEGF_{165}$ isoforms in serum-deprived PC-3 cells when compared to that in the control (Fig. 5D, left panel). On the other hand, HIF-1α suppression brought about a modest decrease in the mRNA levels of $VEGF_{189}$ isoform, but no significant effect on the $VEGF_{165}$ isoform when compared to that in the control (Fig. 5D, right panel). We also assessed the effect of suppressing HIF-1α expression on VEGF mRNA levels in serum-deprived PC-3 and LNCaP cells by Real-time PCR analysis (Fig. 5E). As shown in Fig. 5E, HIF-1α silencing reduced VEGF mRNA levels in day 2 serum-deprived PC-3 and LNCaP cells by 60% (Fig. 5E, top panel) and 30% (Fig. 5E, bottom panel) respectively when compared to that in the control siRNA-transfected cells. Collectively, our results demonstrated that the increase in VEGF and IGF-2 mRNA levels in serum-deprived PCa cells is largely mediated by HIF-1α.

IGF-2 neutralizing antibody significantly inhibits survival of serum-deprived PCa cells

As VEGF and IGF-2 are known to promote survival of cancer cells under various stresses [21, 74, 75], we investigated if blocking the activity of these growth factors would inhibit PCa cell survival during serum deprivation. Although the serum-deprived PC-3 cells significantly increased their production of VEGF protein by 2.7-

and 5.7-fold on day 3 and day 4 SF respectively when compared to that on day 0 (Fig. 6A, top panel), inhibition of VEGF activity via use of VEGF neutralizing antibody during serum deprivation did not have any inhibitory effect on PCa cell survival, regardless of the dose of the VEGF neutralizing antibody used in the experiment (Fig. 6A, bottom panel). The concentration of neutralizing antibody used in the neutralization experiments was optimized based on the amount of protein secreted by the cell lines and also the neutralization $dose_{50}$ (ND_{50}) of the antibody as recommended by the manufacturer (R&D Systems). Serum-deprived PC-3 cells exhibited a modest increase in IGF-2 protein secretion, ranging from 2.3-fold on day 1 SF to 1.4-fold on day 4 SF, when compared to that on day 0 (Fig. 6B, top panel). Although there was only a modest increase in IGF-2 protein secretion, viability of serum-deprived PC-3 cells in the presence of IGF-2 neutralizing antibody reduced by 39.4% when compared to that following control-antibody treatment (Fig. 6B, bottom panel). Thus, our results suggest that despite the absence of exogenous growth factors, the IGF-2 secreted by the serum-deprived PCa cells might be acting in an autocrine manner to promote cell survival.

(C) Figures:

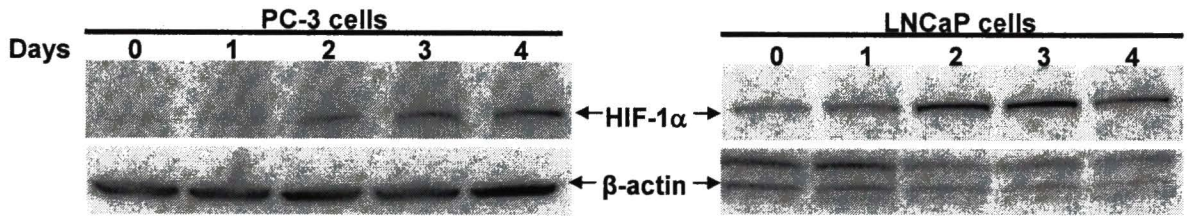
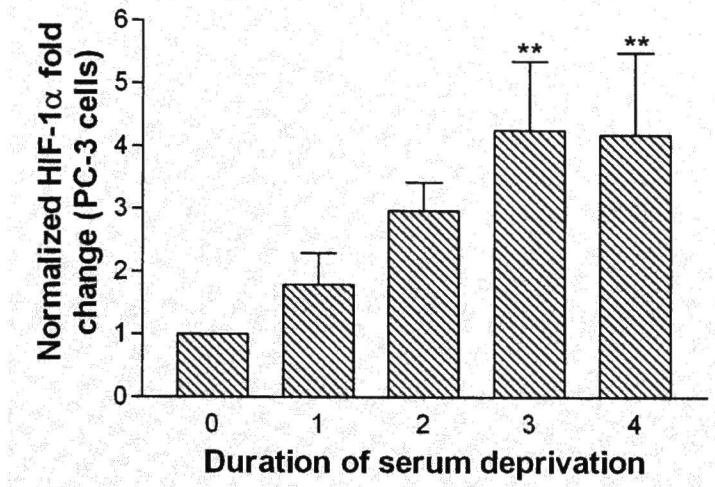


Figure 1A: Prostate cancer cells upregulate HIF-1α protein levels under normoxic, serum-deprived conditions. PCa cells were cultured in serum-free medium for 0-4 days. Cells were harvested at the indicated time points, and whole cell lysates were analyzed for HIF-1α protein by western blot analysis. The blots were stripped and reprobed for β-actin, which served as a loading control. The western blot is representative of 1 of 3 experiments.



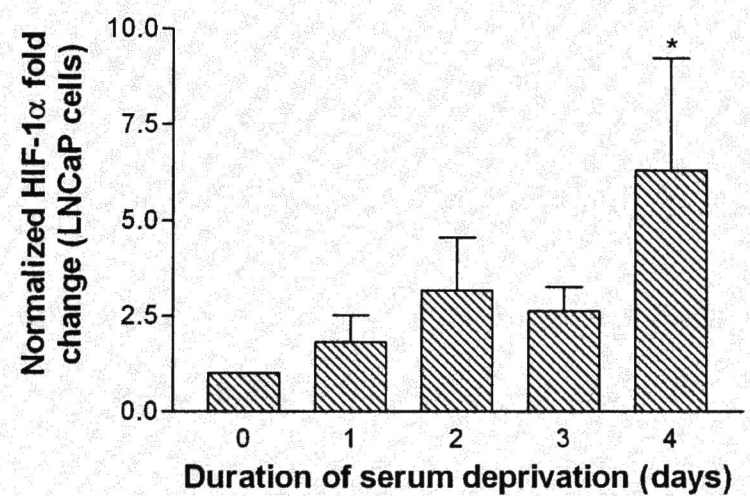


Figure 1B: Prostate cancer cells upregulate HIF-1α protein levels under normoxic, serum-deprived conditions. In PC-3 cells (top panel) and LNCaP cells (bottom panel), the intensity of the HIF-1α protein band at each time point was normalized to that of the β-actin band at the same time point and expressed as a fold-change, with HIF-1α expression on day 0 set at 1. Data are the means ± S.D. (n=3). * indicates $p<0.05$ and ** indicates $p<0.01$ versus day 0.

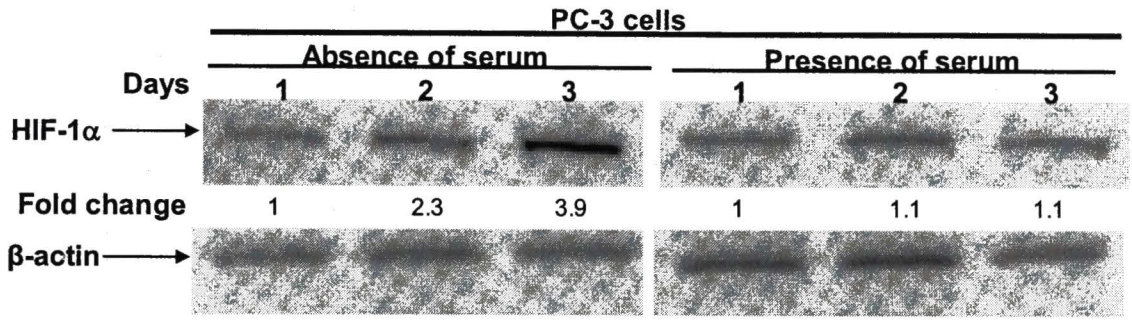


Figure 1C: Prostate cancer cells upregulate HIF-1α protein levels under normoxic, serum-deprived conditions, but not in the presence of serum. PC-3 cells were grown to 50% confluency in complete medium (medium +10%FBS), the medium was then replaced with fresh serum-free medium or fresh complete medium and cells were cultured for upto 3 days. Whole cell lysates were prepared at the indicated time points and assessed for HIF-1α protein by western blot analysis as described in (A). β-actin served as a loading control. HIF-1α expression in each sample was normalized to that of β-actin in the same sample, and HIF-1α fold change in the absence or presence of serum was expressed relative to the HIF-1α protein levels on day 1 serum free or day 1 complete medium respectively, which was set at 1.

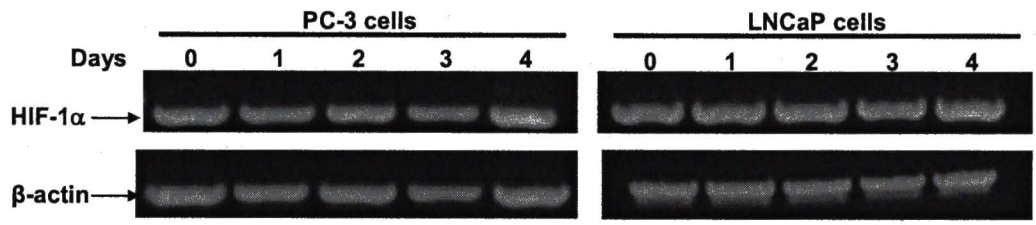


Figure 2A: No significant change in HIF-1α mRNA levels during serum deprivation. PCa cells were cultured in serum-free medium for 0-4 days. Total RNA was isolated from the cells at the indicated time points, and HIF-1α mRNA levels in PC-3 cells (left panel) and LNCaP cells (right panel) were assessed by RT-PCR analyses. β-actin served as a control.

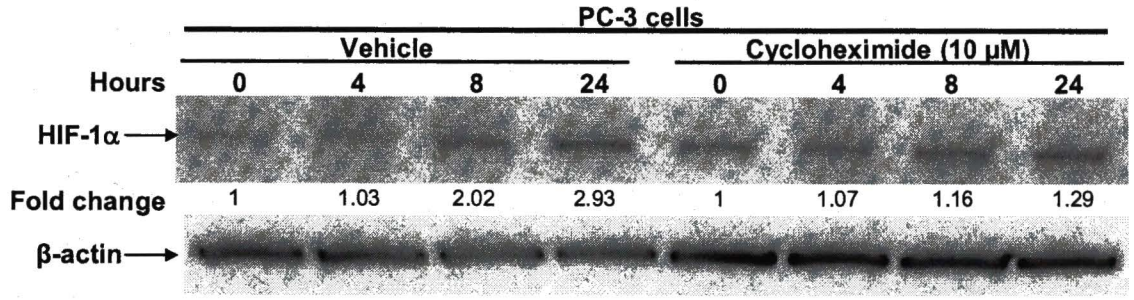


Figure 2B: Serum-deprived PCa cells upregulate HIF-1α protein levels by increasing HIF-1α protein synthesis. PC-3 PCa cells were cultured for 2 days in serum-free medium, and then treated with 10 μM cycloheximide or vehicle (ethanol) for 0, 4, 8 and 24 h followed by lysate preparation. Whole cell lysates were analyzed for HIF-1α protein expression by western blot analysis as described in materials and methods. β-actin served as a loading control. HIF-1α expression in each sample was normalized to that of β-actin in the same sample, and expressed as fold change, with HIF-1α expression after 0 h vehicle or 0 h cycloheximide treatment set at 1. The blot is representative of 1 of 3 experiments.

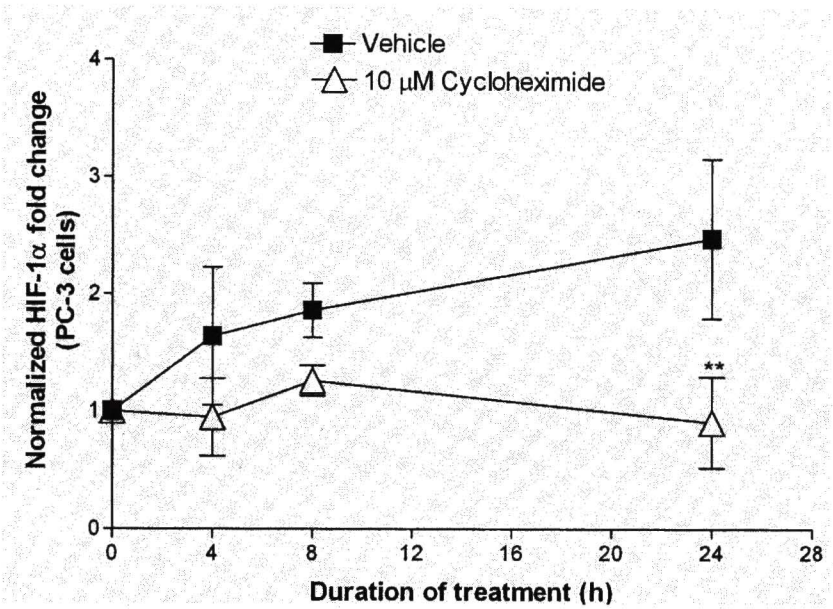


Figure 2C: Serum-deprived PCa cells upregulate HIF-1α protein levels by increasing HIF-1α protein synthesis. The western blot results of normalized HIF-1α fold change in the vehicle- and cycloheximide-treated PC-3 PCa cells were summarized on a line graph. Data are the means ± S.D. (n=3). ** indicates $p<0.01$ versus 24 h vehicle-treated control cells.

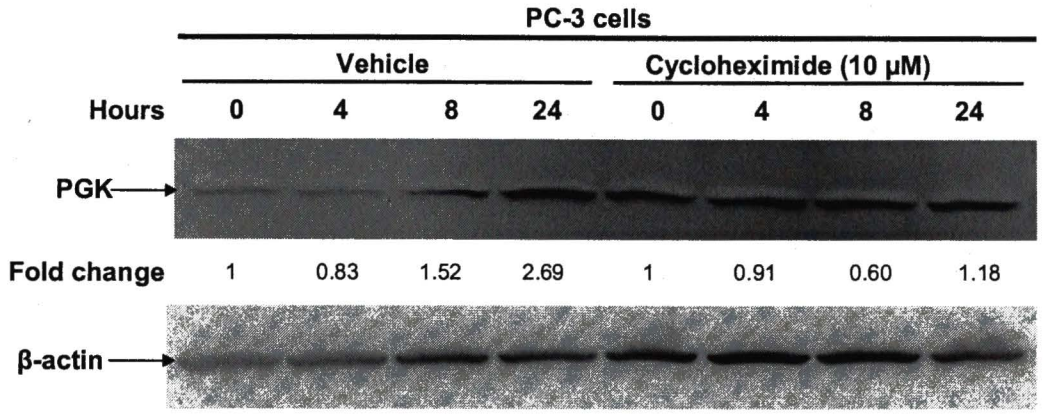
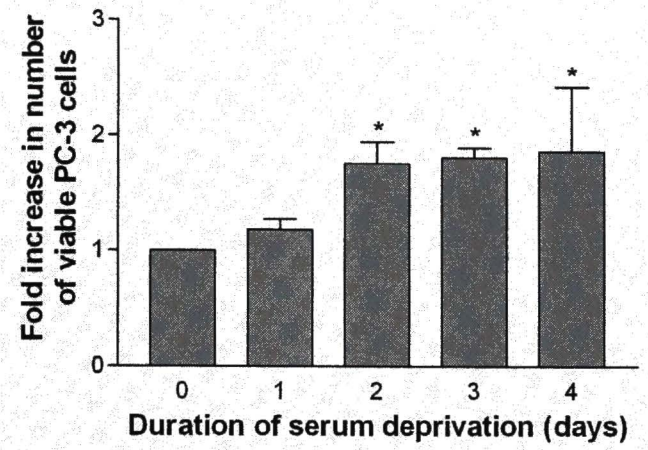


Figure 2D: PGK served as a positive control to confirm efficacy of cycloheximide in inhibiting protein synthesis during serum deprivation. Same as (B), except that whole cell lysates were analyzed for PGK protein expression by western blot analysis. β-actin served as a loading control. PGK expression in each sample was normalized to that of β-actin in the same sample, and expressed as fold change, with PGK expression after 0 h vehicle or 0 h cycloheximide treatment set at 1.



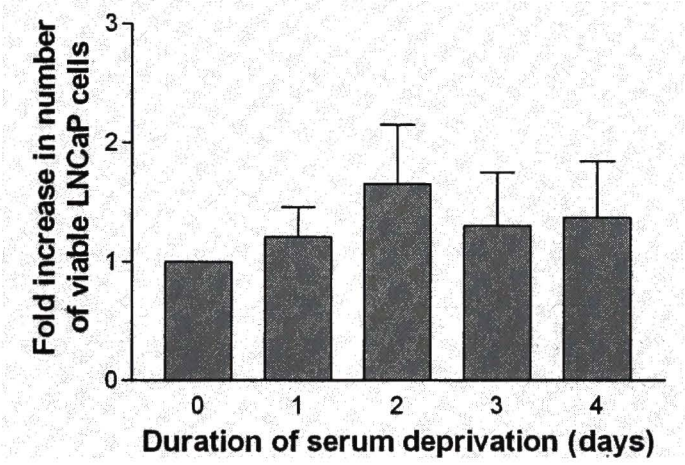
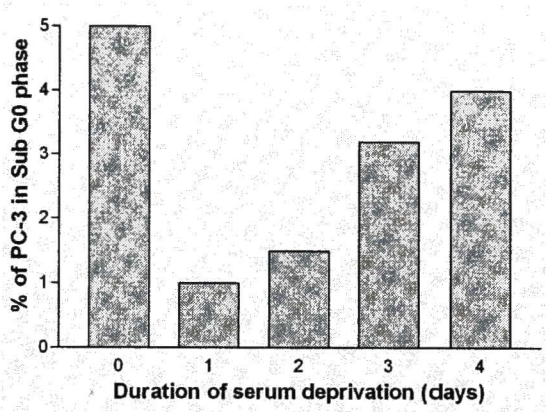
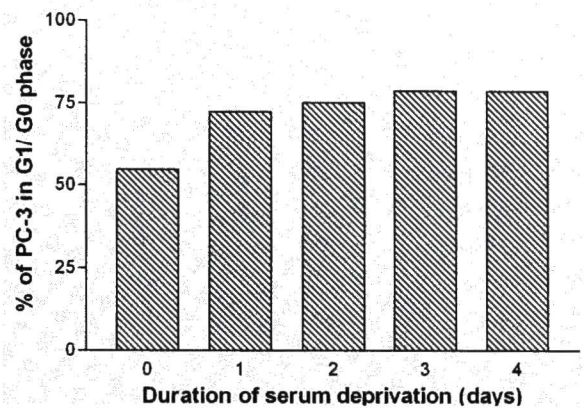
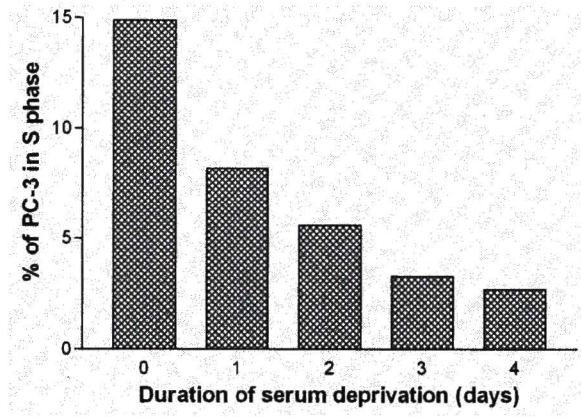


Figure 3A: Increase in HIF-1α protein levels correlates with PCa cell survival during serum deprivation. PCa cells were cultured in serum-free medium for 0-4 days. Cells were trypsinized at the indicated time points, and live cell numbers determined by trypan blue dye exclusion assay. Viable PC-3 (top panel) and LNCaP (bottom panel) cell numbers were then expressed as fold increase, with viable cell number on day 0 set at 1. Data are the means ± S.D. of triplicate wells/experiment, and each experiment was repeated at least twice. * indicates $p<0.05$ versus day 0.







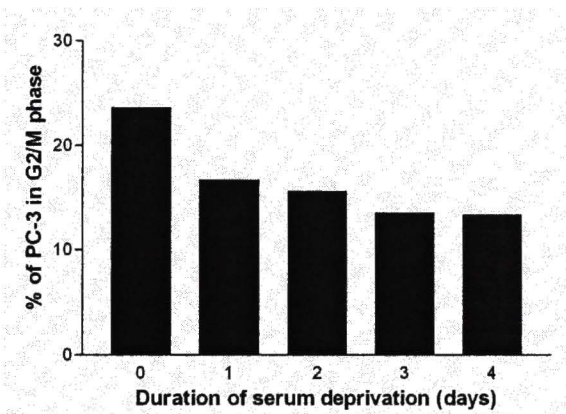


Figure 3B: Cell-cycle analysis of serum-deprived PC-3 cells. PC-3 cells were harvested after culture in serum-free medium for 0-4 days, and cell cycle distribution was assessed by flow cytometry according to the procedure described in Materials and Methods.

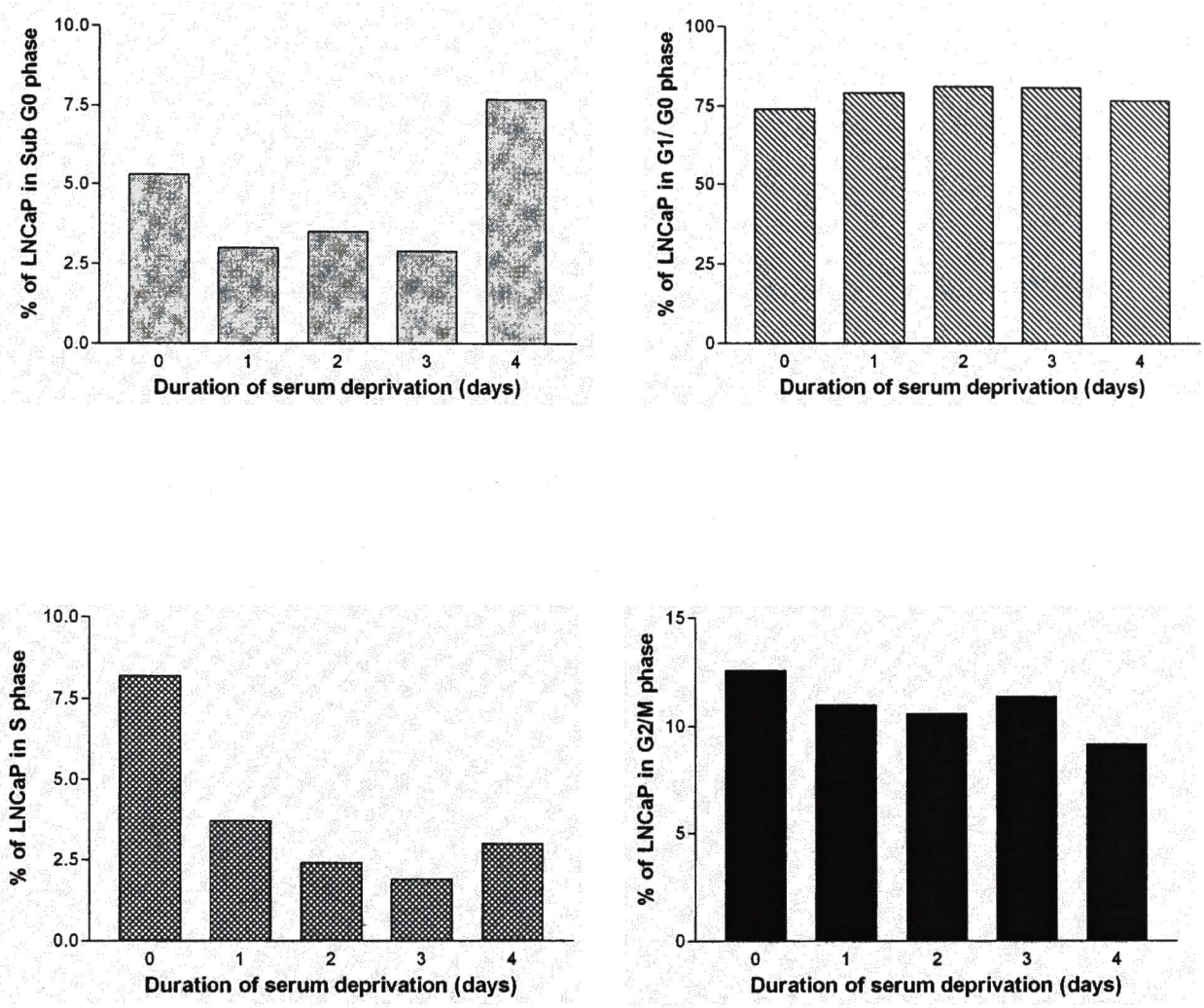


Figure 3C: Cell-cycle analysis of serum-deprived LNCaP cells. LNCaP cells were harvested after culture in serum-free medium for 0-4 days, and cell cycle distribution was assessed by flow cytometry according to the procedure described in Materials and Methods.

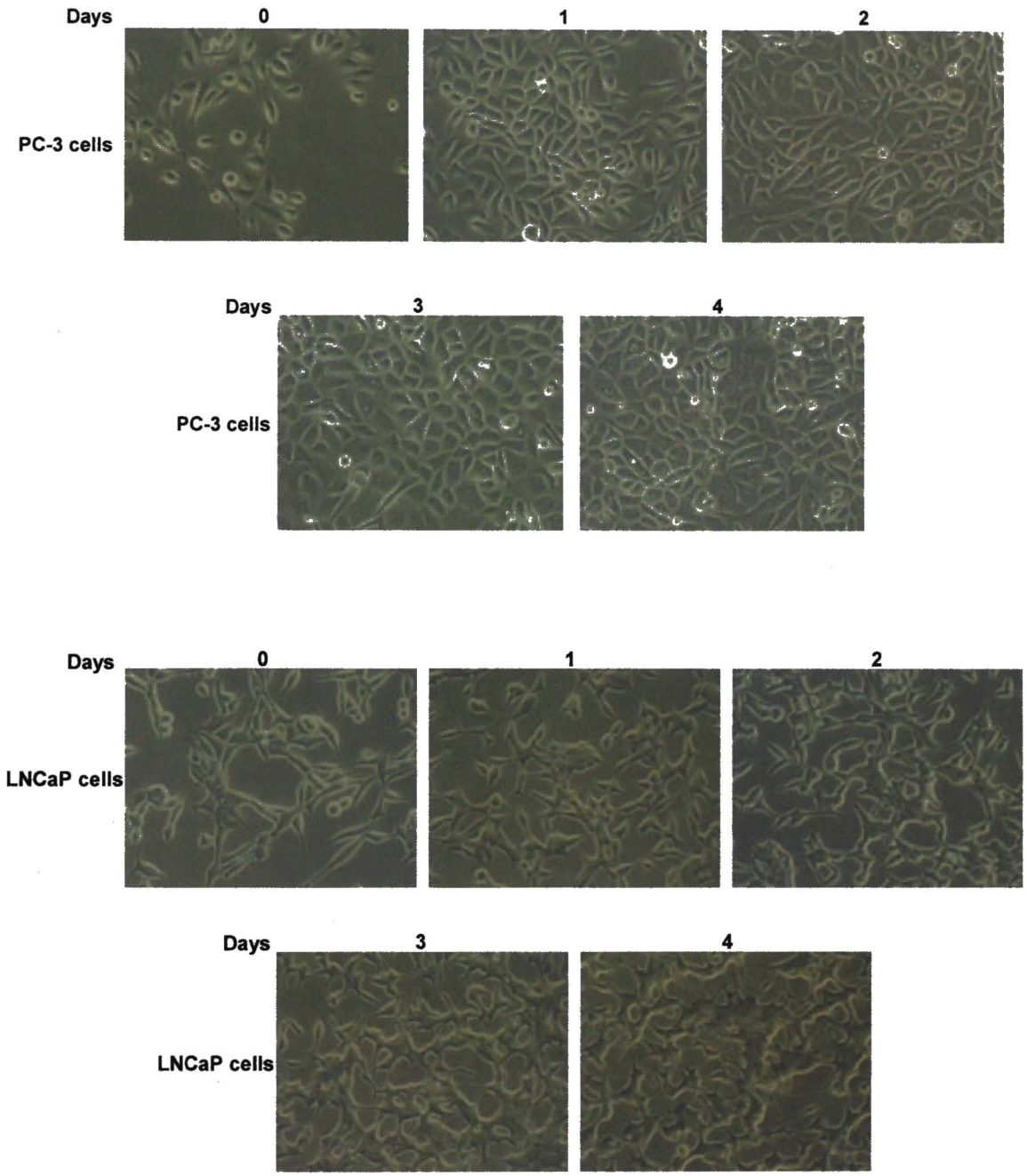


Figure 3D: Cellular morphology of serum-deprived PC-3 and LNCaP cells. PCa cells were cultured in serum-free medium for 0-4 days. Pictures of cellular morphology were taken at the indicated time points for the PC-3 (top panel) and LNCaP cells (bottom panel).

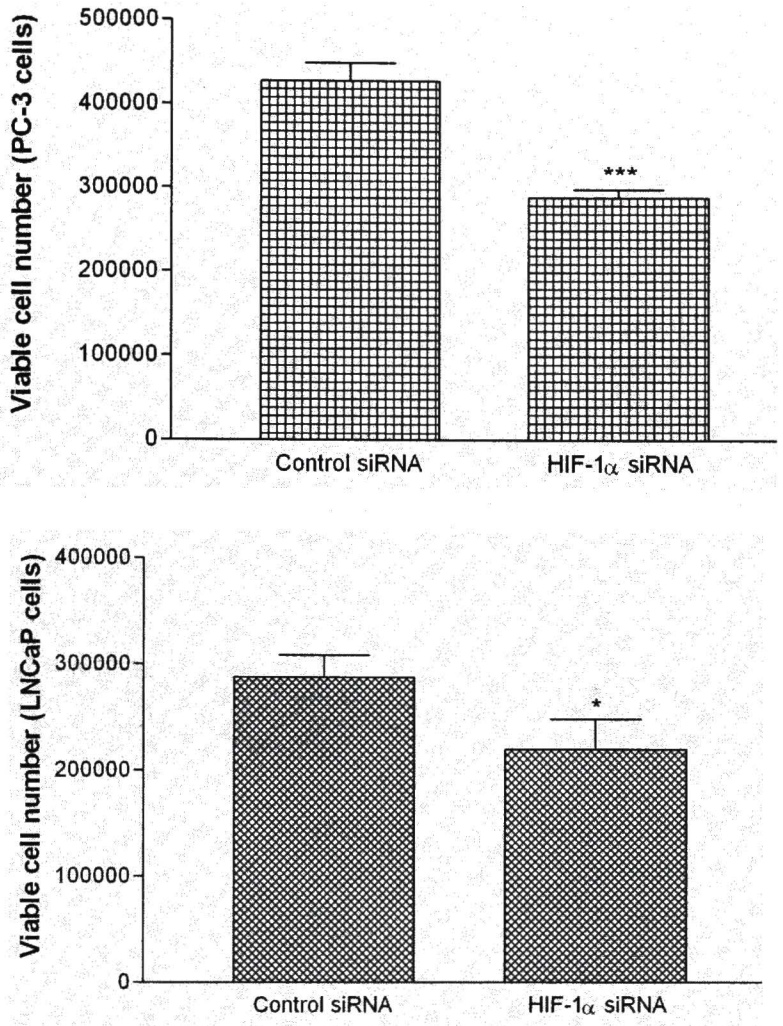


Figure 4A: HIF-1α plays a major role in promoting PCa cell survival during serum deprivation. PC-3 (top panel) and LNCaP (bottom panel) cells were transfected with HIF-1α siRNA or control siRNA, and then cultured for 2 days in serum-free medium under normoxic conditions. Subsequently, cells were trypsinized and live cell numbers determined by trypan blue dye exclusion assay. Cell numbers were represented on bar graphs. Data are the means ± S.D. of triplicate wells/experiment, and each experiment was repeated at least twice. * indicates $p<0.05$, and *** indicates $p<0.001$ versus control siRNA-transfected cells.

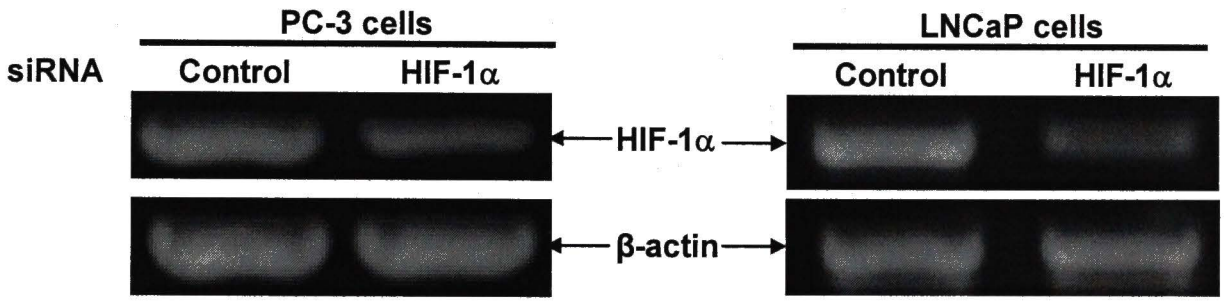
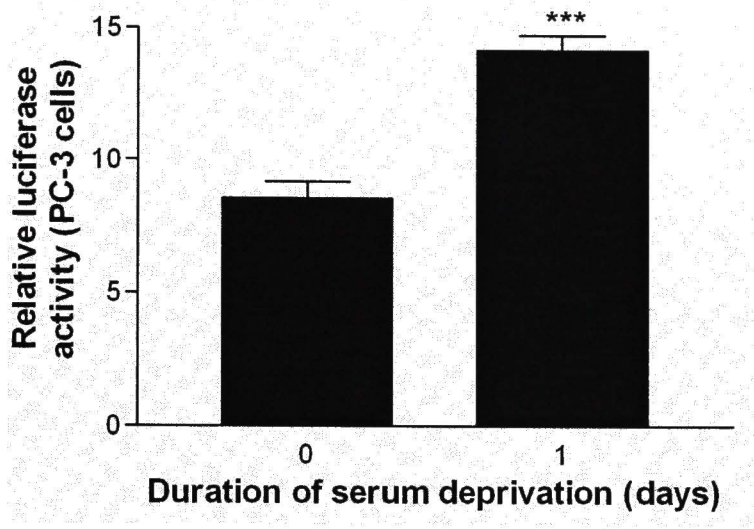


Figure 4B: HIF-1α expression was suppressed with siRNA. HIF-1α expression in the siRNA-transfected PC-3 (left panel) and LNCaP cells (right panel) was determined by RT-PCR analyses. β-actin served as the control.



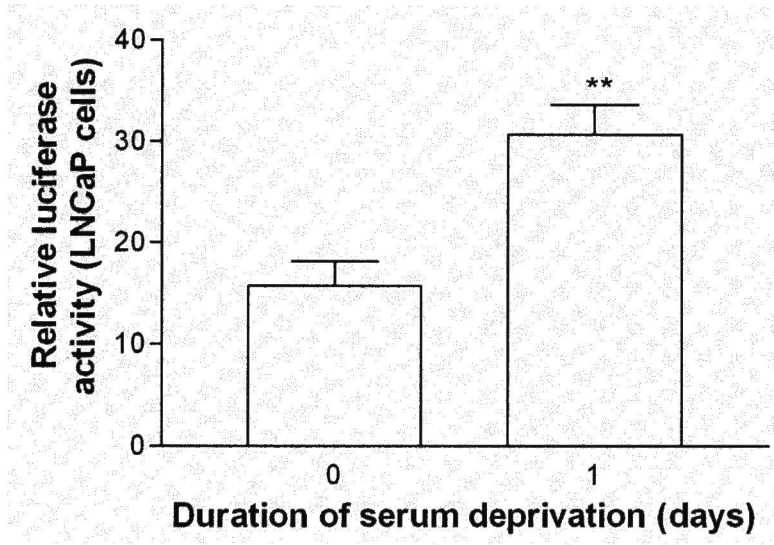


Figure 5A: Effect of HIF-1α protein upregulation on HRE-mediated transcription during serum deprivation. Firefly luciferase activity from pGL3-6xHRE-Luc was measured in control (day 0) or serum-deprived cells (day 1), and normalized to renilla luciferase activity from pRL-TK and represented as relative luciferase activity on a bar graph. Data are the means ± S.D. of triplicate wells/experiment. ** indicates $p<0.01$ and *** indicates $p<0.001$ versus day 0 relative luciferase activity.

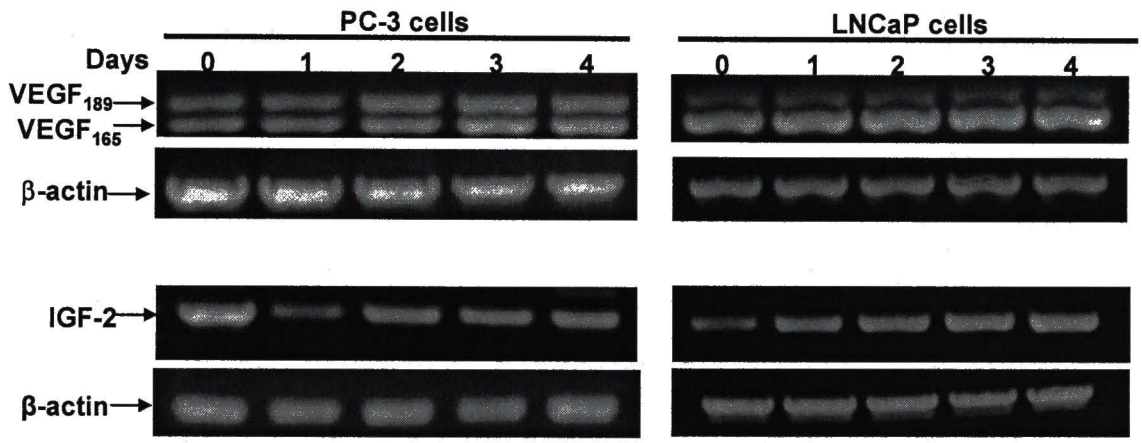
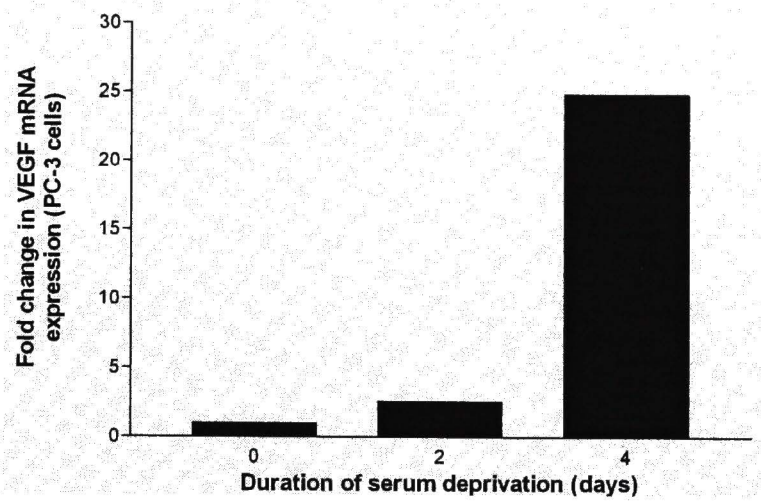


Figure 5B: Effect of HIF-1α protein upregulation on mRNA expression of HIF-1α-target genes, VEGF and IGF-2, during serum deprivation was assessed by RT-PCR. PCa cells were cultured in serum-free medium for 0-4 days. Total RNA was isolated from the cells at each time point, and VEGF and IGF-2 mRNA levels in PC-3 cells (left panel) and LNCaP cells (right panel) were assessed by RT-PCR analyses. β-actin served as the control.



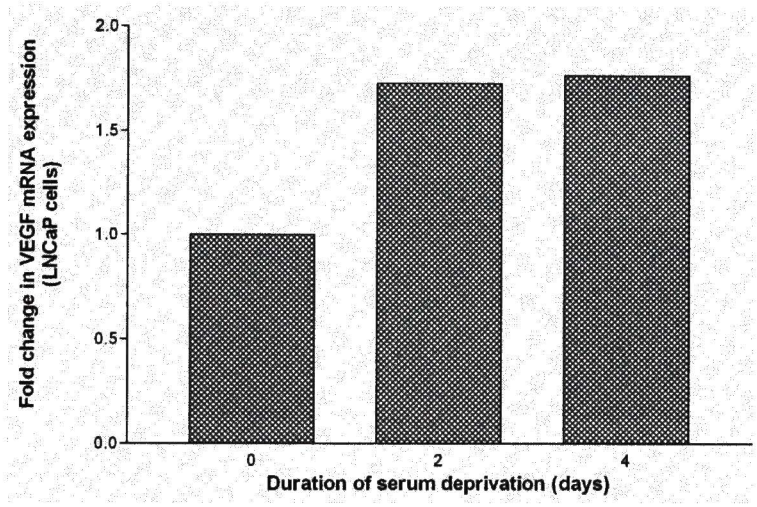


Figure 5C: Effect of serum deprivation-mediated increase in HIF-1α protein on mRNA expression of HIF-1α-target gene, VEGF, was assessed by real-time PCR. Same as (B), except that VEGF mRNA levels in PC-3 cells (top panel) and LNCaP cells (bottom panel) were assessed by Real-time PCR analyses. β-actin served as the control. VEGF mRNA expression in each sample was normalized to that of β-actin in the same sample, and expressed as fold change, with VEGF expression on day 0 SF set at 1.

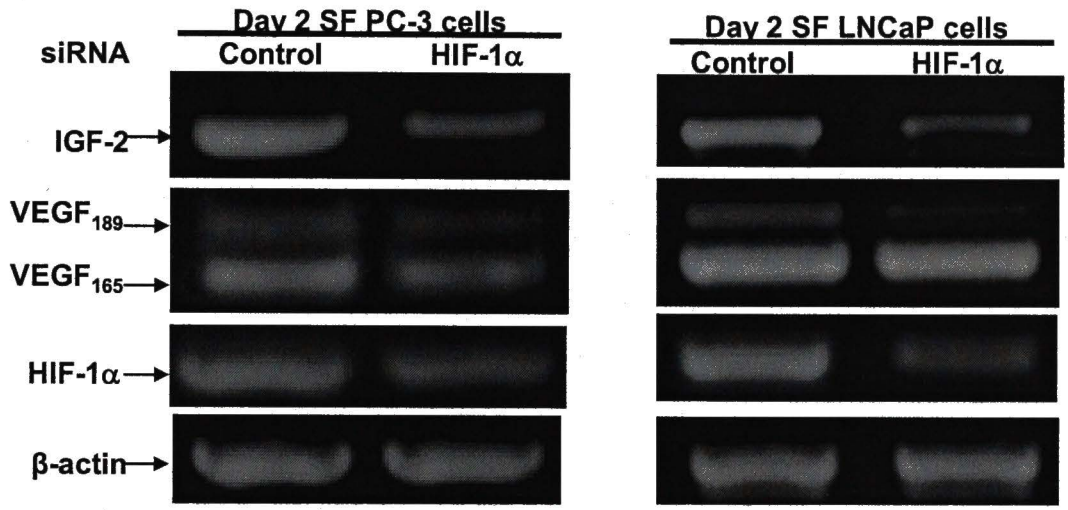
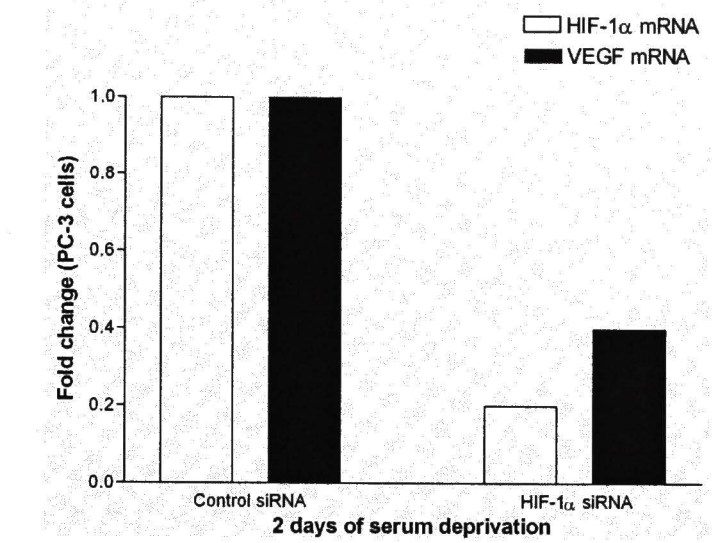


Figure 5D: Effect of HIF-1α suppression on mRNA expression of HIF-1α-target genes, VEGF and IGF-2, during serum deprivation was assessed by RT-PCR. PC-3 and LNCaP cells were transfected with HIF-1α siRNA or control siRNA, and then cultured for 2 days in serum-free medium under normoxic conditions. IGF-2, VEGF and HIF-1α mRNA levels in the control siRNA and HIF-1α siRNA-transfected PC-3 (left panel) and LNCaP cells (right panel) were assessed by RT-PCR analyses. β-actin served as the control.



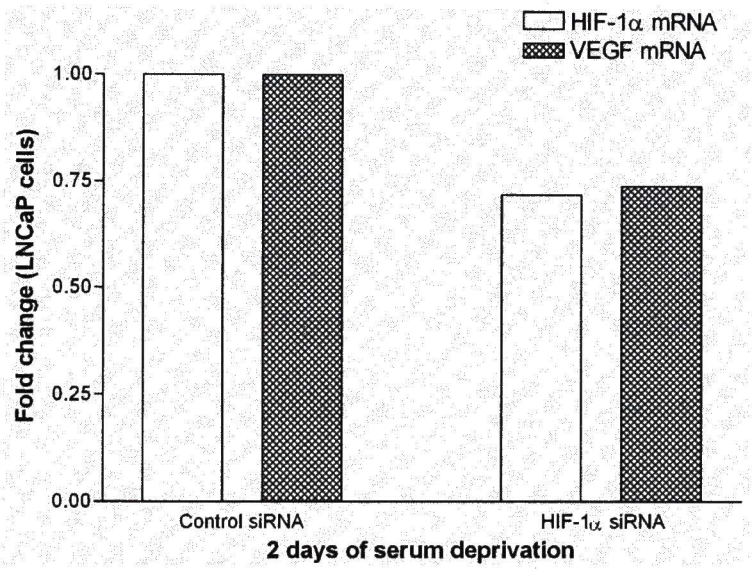
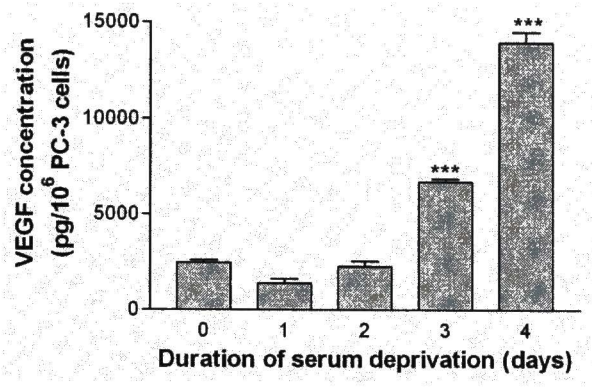


Figure 5E: Effect of HIF-1α suppression on mRNA expression of HIF-1α-target gene, VEGF, during serum deprivation was assessed by real-time PCR. Same as (D), except that VEGF and HIF-1α mRNA in the control and HIF-1α siRNA-transfected PC-3 (top panel) and LNCaP cells (bottom panel) were assessed by Real-time PCR analyses. β-actin served as control. VEGF and HIF-1α mRNA expression in each sample was normalized to that of β-actin in the same sample, and expressed as fold change, with VEGF and HIF-1α expression in the control siRNA-transfected cells set at 1.



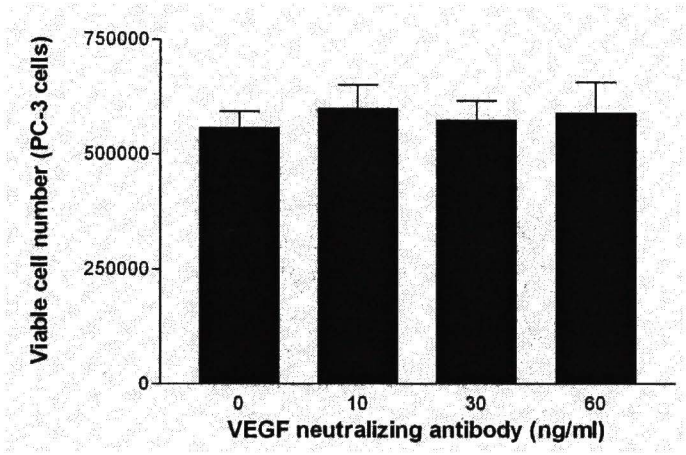
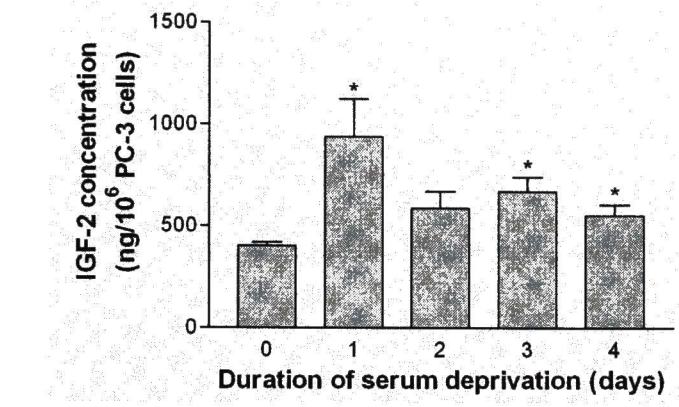


Figure 6A: Despite a significant increase in VEGF protein expression, VEGF inhibition does not inhibit survival of serum-deprived PCa cells. (Top panel) PC-3 cells were cultured in serum-free medium for 0-4 days. Cell-culture supernates were collected at the indicated time points, and assessed for VEGF protein levels by ELISA. Data are the means ± S.D. of triplicate wells/experiment, and each experiment was repeated twice. *** indicates $p<0.001$ versus day 0 IGF-2 protein levels. (Bottom panel) PC-3 cells were cultured for 3 days in serum-free medium containing 0, 10, 30 or 60 ng/ml VEGF neutralizing antibody. Subsequently, live cell numbers were determined by trypan blue dye exclusion assay. Data are the means ± S.D. (n=3).



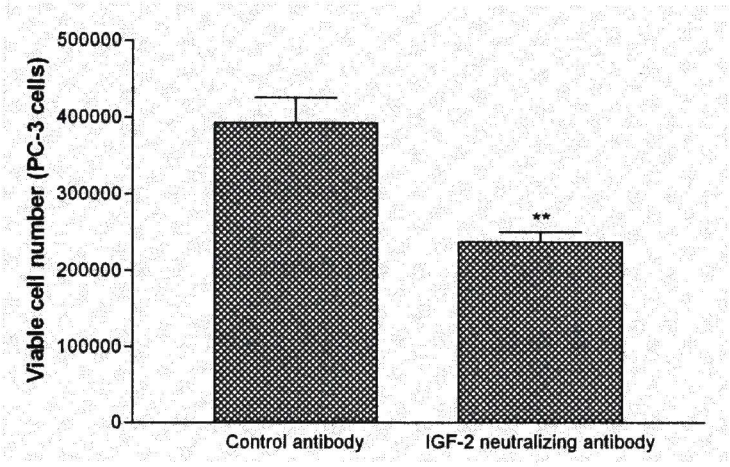


Figure 6B: IGF-2 neutralizing antibody significantly inhibits survival of serum-deprived PCa cells. (Top panel) PC-3 cells were cultured in serum-free medium for 0-4 days. Cell-culture supernates were collected at the indicated time points, and assessed for IGF-2 protein by ELISA. Data are the means ± S.D. of triplicate wells/experiment, and each experiment was repeated twice. * indicates $p<0.05$ versus day 0 IGF-2 protein. (Bottom panel) PC-3 cells were cultured in serum-free medium for 2 days in the presence of 3.3 μg/ml IGF-2 neutralizing antibody or isotype-matched control antibody. Thereafter, cells were trypsinized, and live cell numbers determined by trypan blue dye exclusion assay. Data are the means ± S.D. of triplicate wells/experiment, and each experiment was repeated twice. ** indicates $p<0.01$ versus control antibody treatment.

(D) Discussion

As the name implies, hypoxia inducible factor-1α (HIF-1α), is upregulated during hypoxia, and promotes cell survival under hypoxic stress [9, 76]. Our study demonstrates for the first time that prolonged serum growth factor deprivation is a potent inducer of HIF-1α protein in normoxic PCa cells, and that HIF-1α plays a key role in promoting PCa cell survival under this apoptotic stress. Our study further shows that the increase in HIF-1α protein during serum deprivation occurs at the translational level, and that the HIF-1α protein is transcriptionally active and upregulates the expression of its target genes, VEGF and IGF-2.

Our study presents several lines of evidence to prove that upregulation of HIF-1α protein in serum-deprived PCa cells is mainly due to increased HIF-1α protein synthesis and not due to reduced degradation. First, a steady, time-dependent increase in HIF-1α protein levels was detected during serum deprivation (Fig. 1B, top and bottom panels). If the increase in HIF-1α protein were due to reduced degradation, then HIF-1α protein levels would remain constant during the entire course of serum deprivation, without further increase. Second, CHX, a protein synthesis inhibitor, abolished an increase in HIF-1α protein levels in the serum-deprived PCa cells, indicating that continuous protein synthesis is required for HIF-1α protein increase. Thus, our data strongly argues that the increase in HIF-1α protein levels in serum-deprived PCa cells is most likely due to increased HIF-1α protein synthesis.

It should be noted that serum deprivation-induced HIF-1α upregulation occurs in androgen-independent PC-3 cells as well as in the androgen-dependent LNCaP cells.

This result suggests that the upregulation of HIF-1α during prolonged serum growth factor deprivation is independent of the androgen-dependent status of the PCa cells. However, both the PC-3 and LNCaP cells lack a functional PTEN, a tumor suppressor and a negative regulator of the phosphotidylinositol-3-kinase (PI3K) signaling pathway [57]. This finding suggests that there is constitutive activation of PI3K signaling in the PC-3 and LNCaP cells. Studies show that the activation of the PI3K pathway increases HIF-1α protein synthesis by increasing the rate of HIF-1α mRNA translation in an O_2–independent manner [9, 17]. Our observation is further supported by the finding that in PCa cells where PTEN is inactivated, there is increased HIF-1α-mediated gene expression leading to increased tumor growth and tumor vascularity when compared to cells expressing wild-type PTEN [77]. In addition, more recently, another study demonstrated that overexpression of PTEN in PTEN-deficient PC-3 PCa cells inhibited cell proliferation and increased serum deprivation-induced apoptosis [72]. In light of these observations, our results suggest that PTEN deficiency may be a strong stimulus for PCa cells to upregulate HIF-1α protein expression during stress of serum deprivation. As loss-of-function mutations and deletions of the PTEN gene are found in nearly 50% of PCas [57, 71, 78], the results from our study imply that upregulation of HIF-1α during serum deprivation may be a common occurrence in prostate tumors as an attempt to resist apoptosis due to prolonged serum growth factor deprivation.

Furthermore, siRNA-mediated suppression of HIF-1α expression in PC-3 and LNCaP cells during serum deprivation led to a significant decline in cell survival

(Fig. 4A). Studies have demonstrated the pro-survival role of HIF-1α during serum growth factor deprivation of renal clear cell carcinoma (RCC) cells [79, 80]. RCC cells that are deficient in VHL, a key protein involved in HIF-1α degradation, exhibit high levels of HIF-1α protein and are able to proliferate even in the absence of exogenous growth factors [79, 80]. In contrast, inhibition of endogenous HIF-1α function by overexpression of dominant negative HIF-1α abolished the ability of VHL-deficient RCC cells for serum-independent growth [80]. Therefore, these observations as well as our current results point to an essential role of HIF-1α in promoting cancer cell survival during stress of prolonged serum growth factor deprivation.

Surprisingly, although the serum-deprived PC-3 cells exhibited a marked increase in VEGF mRNA levels (Figs. 5B and 5C), correlating with HIF-1α protein levels, there was only a modest increase in VEGF mRNA levels in the serum-deprived LNCaP cells (Figs. 5B and 5C). This differential regulation of VEGF expression in serum-deprived PC-3 and LNCaP cells is further reflected in the effect of siRNA-mediated suppression of HIF-1α expression on VEGF mRNA levels (Figs. 5D and 5E). HIF-1α suppression resulted in a marked decline in the mRNA levels of $VEGF_{189}$ and $VEGF_{165}$ isoforms in serum-deprived PC-3 cells when compared to that in the control (Fig. 5D). In contrast, HIF-1α suppression brought about a modest decrease in the mRNA levels of $VEGF_{189}$ isoform, but no significant effect on the $VEGF_{165}$ isoform when compared to that in the control (Fig. 5D). It is not known as to why PC-3 and LNCaP cells differ in their expression of VEGF despite the

observation that both cell lines have elevated levels of HIF-1α protein during serum deprivation. One possible explanation is that HIF-1α may not have a major role in the regulation of VEGF expression in LNCaP cells. A recent study by Mizukami *et al* reported that the expression of VEGF in hypoxic colon cancer cells is regulated through HIF-dependent and HIF-independent pathways [81]. Thus, it is likely that the differential regulation of VEGF expression in different cell lines may be due to cell type-specific characteristics of the cell lines. Nonetheless, as HIF-1α suppression markedly reduced mRNA expression of HIF-1α-target genes, VEGF and IGF-2, in the serum-deprived PC-3 cells, and IGF-2 in serum-deprived LNCaP cells (Figs. 5D and 5E), our results indicate that the increase in VEGF and IGF-2 mRNA expression during serum deprivation is largely mediated by HIF-1α.

Interestingly, although there was a significant, time-dependent increase in VEGF (Fig. 6A, top panel) and IGF-2 protein secretion (Fig. 6B, top panel) during serum deprivation, a significant decline in PCa cell survival was achieved by the inhibition of IGF-2 activity, and not by the inhibition of VEGF activity (Figs. 6B, bottom panel and 6A, bottom panel respectively). Thus, our observation suggests that IGF-2 may act in an autocrine manner to promote PCa cell survival during serum deprivation. Although VEGF did not have any direct pro-survival effects on serum-deprived PCa cells, we cannot exclude the possibility that the VEGF secreted by the PCa cells may indirectly promote the survival of PCa cells within tumors due to their well-established roles in promoting prostate cancer angiogenesis [82-84].

The observation of increased expression of HIF-1α-target gene products, VEGF and IGF-2, has pointed to the possible involvement of a HIF-1α-dependent autocrine growth factor loop in HIF-1α induction during serum deprivation. Studies conducted under Specific Aims 2 and 3 have elucidated the precise signal transduction pathway involved in HIF-1α upregulation.

Taken together, the data presented in this Specific Aim demonstrate that HIF-1α is a key survival factor for serum growth factor-deprived PCa cells, and thus our study provides a strong rationale for the therapeutic targeting of HIF-1α under both normoxic and hypoxic conditions for more effective inhibition of PCa cell survival and proliferation.

CHAPTER V

SPECIFIC AIMS 2 AND 3

Specific Aim 2: Investigate if activation of the PI3K-Akt pathway mediates the upregulation of HIF-1α protein in serum-deprived PCa cells.

(A) Rationale

PI3K-Akt signaling is known to promote cancer cell survival under various apoptotic stresses [72]. Increased PI3K activity in PCa cells has been associated with increased expression of HIF-1α [16]. Moreover, since the PC-3 and LNCaP PCa cells are deficient in PTEN, a negative regulator of PI3K activity, the uninhibited activation of PI3K could result in a marked increase in HIF-1α protein expression by increasing the rate of HIF-1α mRNA translation to protein [9].

We hypothesized that activation of the PI3K pathway upregulates HIF-1α protein expression in PCa cells during prolonged serum deprivation.

(B) Results

Activation of PI3K-Akt pathway mediates HIF-1α protein increase and cell survival in response to serum deprivation

In addition to playing a pivotal role in cell survival under various apoptotic stresses [85-88], the PI3K-Akt pathway also regulates HIF-1α protein synthesis

following growth factor stimulation of cells [16, 89, 90]. We, therefore, investigated involvement of the PI3K-Akt pathway in the upregulation of HIF-1α protein levels during serum deprivation. As shown in Fig. 7, there was a time-dependent increase in levels of phosphorylated-Akt in PC-3 as well as LNCaP cells during serum deprivation when compared to that on day 0. This result suggested that Akt was activated in serum-deprived PCa cells.

Akt is a downstream effector of PI3K. Thus, we investigated whether Akt activation was dependent on PI3K activity. We cultured PCa cells for 2 days in serum-free (SF) medium containing 20 μM LY294002, a PI3K-specific inhibitor, or vehicle (DMSO), and then assessed Akt activation. Treatment with LY294002 markedly attenuated the serum deprivation-mediated increase in Akt phosphorylation (Fig. 8A), suggesting that PI3K mediated Akt activation. Moreover, LY294002 abolished the serum deprivation-mediated increase in HIF-1α protein levels in PC-3 as well as LNCaP cells when compared to that in the vehicle-treated control cells (Fig. 8A). In addition, treatment with LY294002 markedly inhibited PCa cell survival during serum deprivation. We observed a significant, dose-dependent decrease in viable PC-3 and LNCaP cell number following treatment with LY294002 when compared to that following vehicle treatment (Fig. 8B, top and bottom panels). We also detected a significant, time-dependent decline in viable PC-3 cell number when cells were serum deprived for up to 4 days in the presence of 10 μM LY294002 (Fig. 8C), when compared to that in the presence of vehicle at the same time point. These

results suggested that serum deprivation activates the PI3K-Akt signaling pathway, which in turn, increases HIF-1α protein.

Overexpression of wild-type PTEN prevents upregulation of HIF-1α protein, and inhibits cell survival during serum deprivation

PTEN is a tumor suppressor and a negative regulator of PI3K activity [91]. As PC-3 and LNCaP cells are deficient in a functional PTEN [57], PI3K is constitutively active in these cells. Therefore, to further confirm PI3K-Akt involvement in HIF-1α upregulation during serum deprivation, we transiently overexpressed wild-type PTEN in the PTEN-deficient PC-3 cells, and then assessed HIF-1α protein expression. Overexpression of wild-type PTEN (Fig. 9A, right panels) decreased Akt activation as evidenced by a reduction in levels of phosphorylated-Akt when compared to that in the empty vector-transfected control cells (Fig. 9A, left panels). Moreover, overexpression of PTEN also markedly reduced HIF-1α protein expression during the entire course of serum deprivation when compared to that in the empty vector-transfected control cells (Fig. 9A). Additionally, the wild-type PTEN transfected PC-3 and LNCaP cells exhibited cellular morphological features characteristic of apoptosis such as rounding of cells when compared to that following empty vector transfection (Fig. 9B). There was also a marked increase in the number of floating cells following PTEN transfection relative to that following empty vector transfection. Thus, these results further showed that PI3K-Akt activation increases HIF-1α protein expression and cell survival during serum deprivation.

Effect of serum deprivation on MAPK activation in PCa cells

In addition to the PI3K pathway, the MAPK pathway has also been implicated in upregulating HIF-1α protein synthesis [89]. We, therefore, investigated activation of the MAPK pathway in serum-deprived PCa cells. We detected a modest increase in levels of phosphorylated-Erk1/2 (p42 and p44 MAPK) in PC-3 cells during serum deprivation when compared to that on day 0 (Fig. 10, left panel). On the other hand, there was no detectable increase in levels of phosphorylated-Erk1/2 in serum-deprived LNCaP cells when compared to that on day 0 (Fig. 10, right panel).

(C) Figures

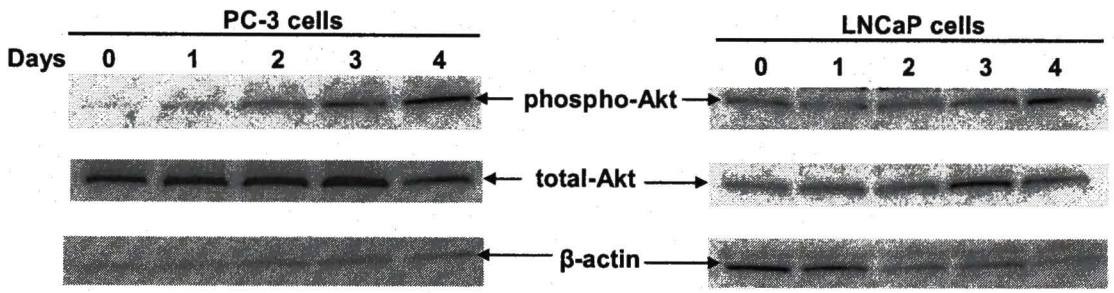


Figure 7: PCa cells increase Akt activation in response to serum deprivation. PC-3 and LNCaP cells were cultured in serum-free medium for 0-4 days. Cells were harvested at the indicated time points. Whole cell lysates were prepared and analyzed for phospho-Akt by western blot analysis. The blots were sequentially stripped and reprobed with antibodies against total-Akt and β-actin in order to detect total-Akt and β-actin protein levels respectively. β-actin served as a loading control. The western blots are representative of 1 of 3 experiments.

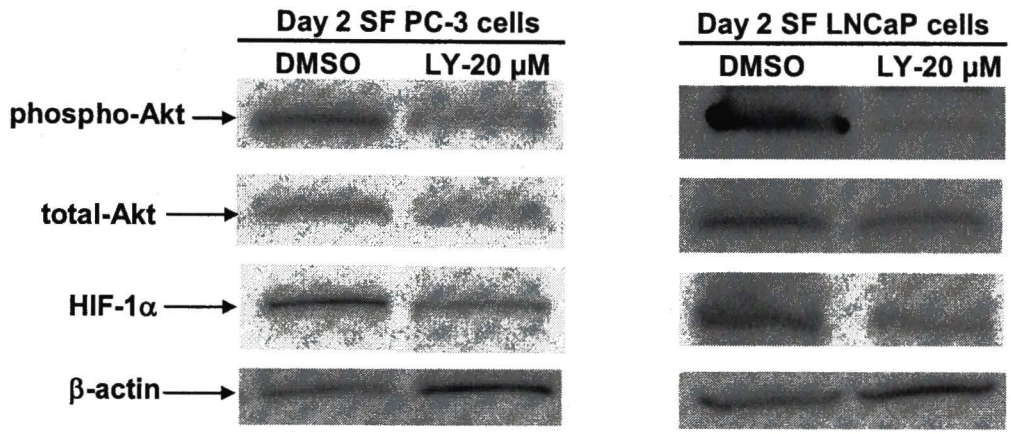
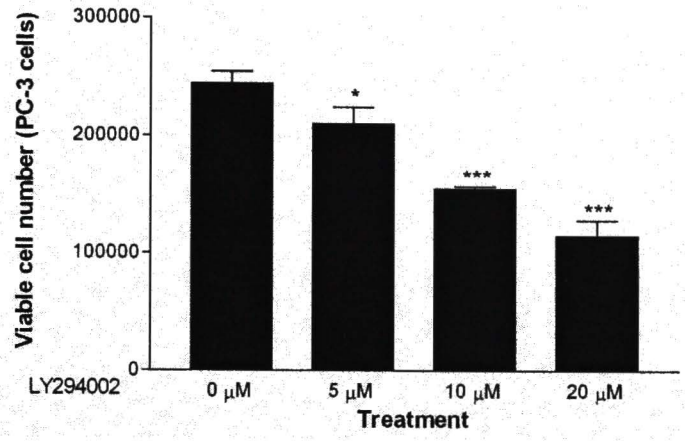


Figure 8A: Inhibition of PI3K activity attenuates serum deprivation-mediated increase in HIF-1α protein. PCa cells were cultured in serum-free medium for 2 days in the presence of 20 μM LY294002 (LY-20 μM) or vehicle (DMSO). Thereafter, cells were harvested, and whole cell lysates were prepared. HIF-1α protein levels in the PC-3 (left panel) and LNCaP (right panel) cells were assessed by western blot analysis. The blots were sequentially stripped and re-probed for phospho-Akt, total-Akt and β-actin. β-actin served as a loading control. The western blots are representative of 1 of 3 experiments.



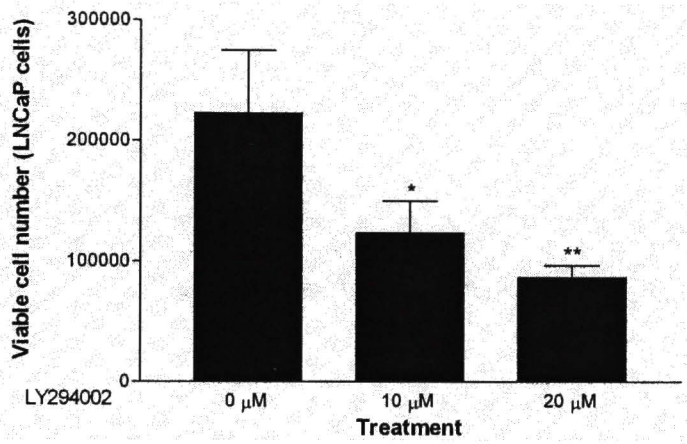


Figure 8B: Inhibition of PI3K activity decreases cell survival during serum deprivation. PC-3 (top panel) and LNCaP (bottom panel) cells were cultured in serum-free medium, under normoxic conditions, for 2 days in the presence of 0-20 μM LY294002. Subsequently, cells were trypsinized and live cell numbers determined by trypan blue dye exclusion assay. Cell numbers were represented on bar graphs. Data are the means ± S.D. of triplicate wells/experiment, and each experiment was repeated at least twice. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ versus vehicle-treated control.

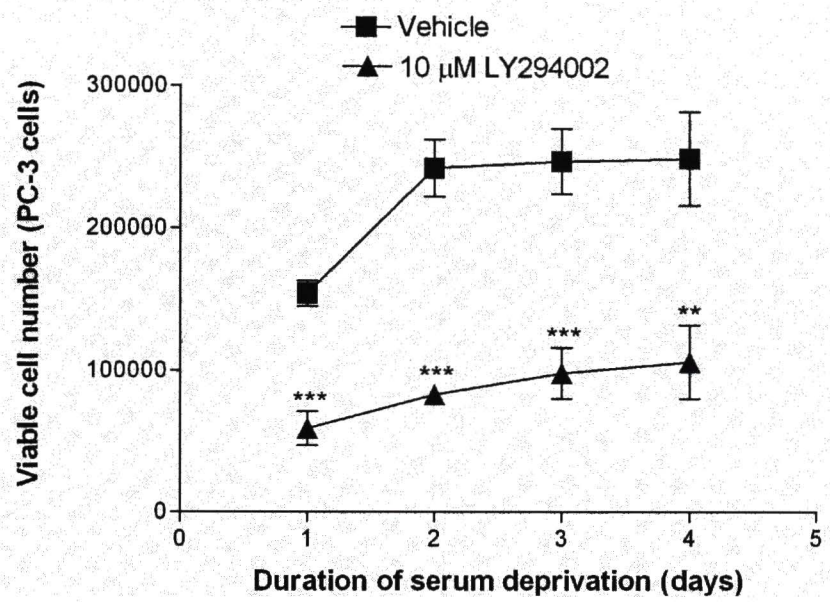


Figure 8C: Inhibition of PI3K activity decreases cell survival during serum deprivation. PC-3 cells were cultured in serum-free medium for 1-4 days in the presence of 10 μM LY294002 or vehicle (DMSO). Then, cells were trypsinized and live cell numbers determined by trypan blue dye exclusion assay. Cell numbers were represented on a line graph. Data are the means ± S.D. of triplicate wells/experiment, and the experiment was repeated twice. ** indicates $p<0.01$ and *** indicates $p<0.001$ versus vehicle-treated control on day 1, day 2, day 3 and day 4 respectively.

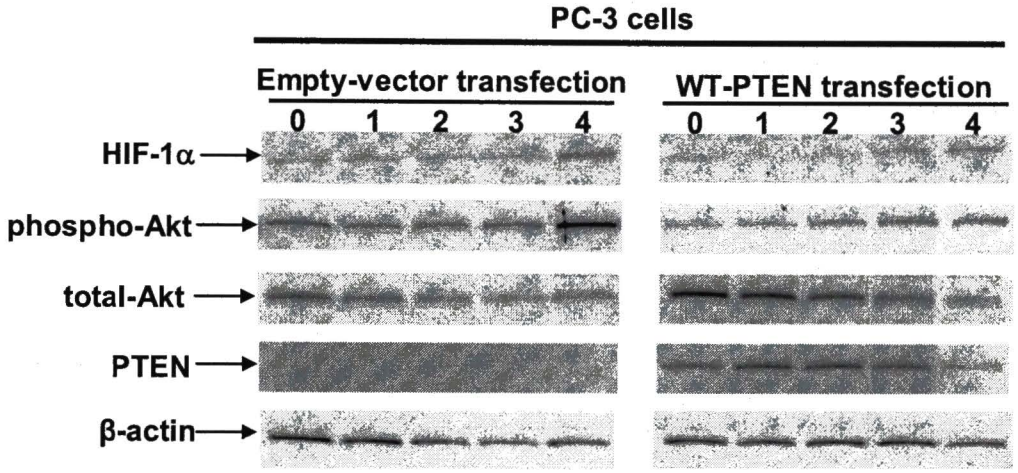


Figure 9A: Overexpression of wild-type PTEN prevents upregulation of HIF-1α protein during serum deprivation. PC-3 cells were transiently transfected with wild-type (WT) PTEN expression vector or empty vector, and then cultured in serum-free medium for 0-4 days. Cells were harvested at the indicated time points, and whole cell lysates were prepared and analyzed for HIF-1α protein by western blot analysis. The blots were sequentially stripped and re-probed for phospho-Akt, total-Akt and β-actin. β-actin served as a loading control. The western blots are representative of 1 of 2 experiments.

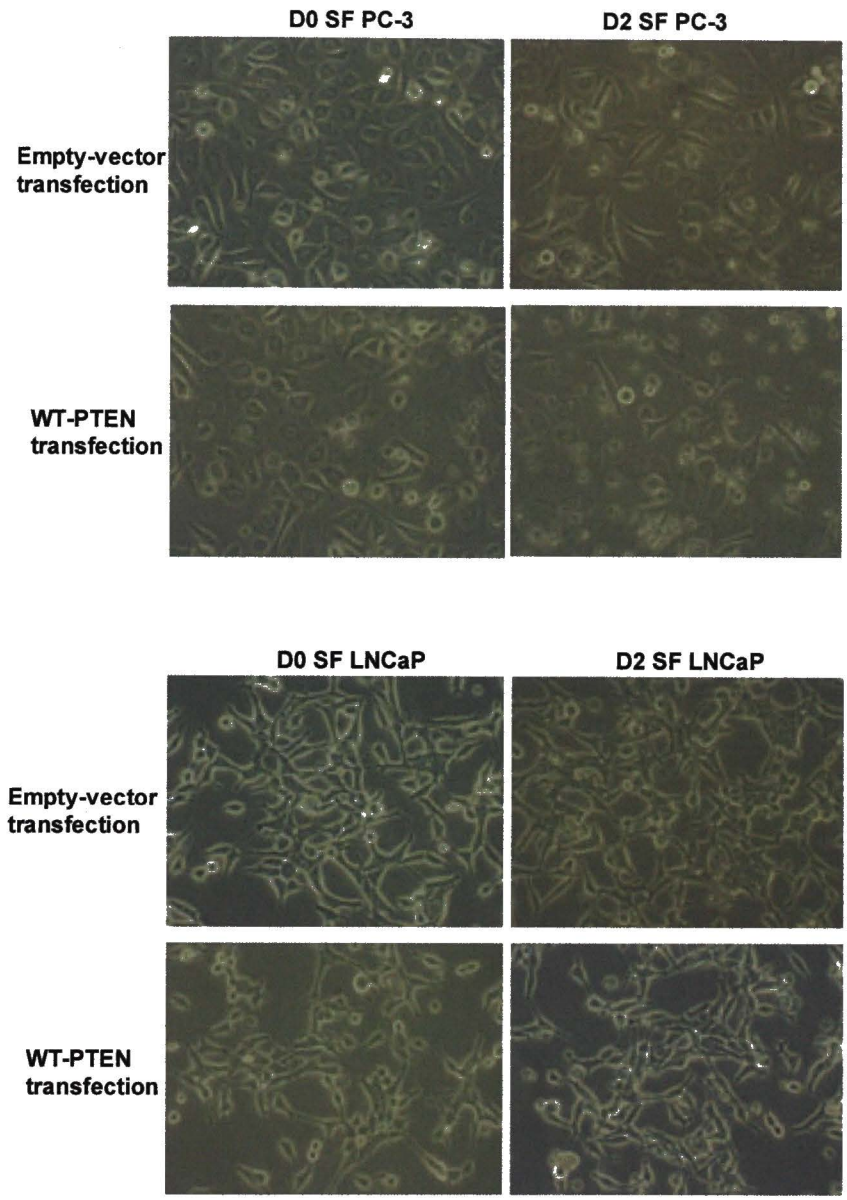


Figure 9B: Overexpression of wild-type PTEN inhibits PCa cell survival during serum deprivation. PCa cells were transfected as described in (A) and cultured in serum-free medium for up to 2 days under normoxic conditions. Pictures of PC-3 (top panel) and LNCaP (bottom panel) cellular morphology were taken on day 0 (D0) and day 2 (D2) of serum deprivation.

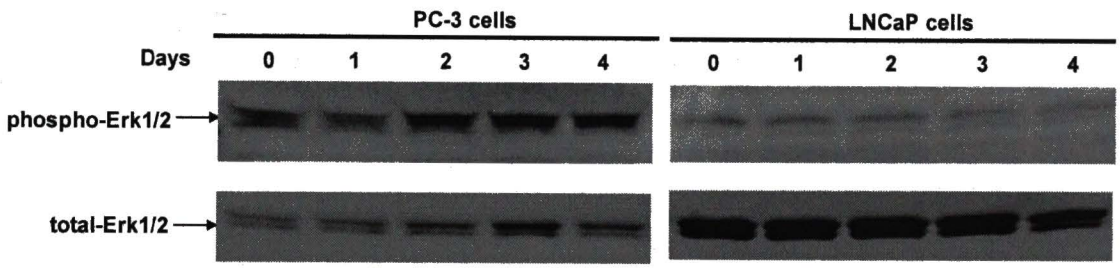


Figure 10: Modest to no activation of Erk 1/2 in serum-deprived PC-3 and LNCaP cells respectively. PC-3 and LNCaP cells were cultured in serum-free medium for 0-4 days. Cells were harvested at the indicated time points. Whole cell lysates were prepared and analyzed for phospho-Erk1/2 by western blot analysis. The blots were stripped and reprobed for total-Erk1/2.

Specific Aim 3: Determine if IGF-IR plays a role in the upregulation of HIF-1α in PCa cells during prolonged serum deprivation.

(A) Rationale

Signaling pathways originating from the IGF-IR affect several aspects of PCa biology including cancer cell survival and proliferation [72]. IGF-2 secreted by serum-deprived PC-3 PCa cells has been reported to regulate the proliferation of these cells via the IGF-IR [21]. HIF-1α expression was increased in normoxic human embryonic kidney (HEK) 293 cells following exposure to exogenous IGF-2 [22]. Although IGF-IR has been associated with protection from serum deprivation-induced apoptosis, to date, there have not been any studies on its role in the upregulation of HIF-1α in serum-deprived normoxic PCa cells.

We hypothesized that IGF-IR activation signals the upregulation of HIF-1α protein during prolonged serum deprivation.

(B) Results

IGF-2 protein expression is associated with increased activation of IGF-IR during serum deprivation

In specific aim 1, we demonstrated a marked HIF-1α-mediated increase in IGF-2 mRNA expression in serum-deprived PCa cells. As shown in Fig. 11, ELISAs revealed a significant increase in IGF-2 protein levels in the cell culture supernatants of serum-deprived PC-3 (Fig. 11, top panel) and LNCaP (Fig. 11, bottom panel) cells when compared to that on day 0.

As IGF-2's pro-survival effects are mediated via the IGF-IR [23], we next assessed the activation of IGF-IR by probing immunoprecipitated IGF-IR for phospho-tyrosine by immunoblotting [92, 93]. We observed a marked, time-dependent increase in the activation of IGF-IR during serum deprivation in the PC-3 (Fig. 12A, left panel) and LNCaP (Fig. 12A, right panel) cells when compared to that on day 0. Although the LNCaP cells exhibit low basal levels of IGF-IR when compared to that in the PC-3 cells, they demonstrated a robust activation of the IGF-IR during the entire course of serum deprivation (Fig. 12A, right panel). We also observed a marked decrease in levels of the immunoprecipitated IGF-IR following serum deprivation when compared to that on day 0. This decrease in IGF-IR protein levels was also confirmed by assessing total IGF-IR levels in whole cell lysates. We detected a dramatic decrease in total IGF-IR protein levels by day 3 and day 4 of serum deprivation in PC-3 cells when compared to that on day 0 (Fig. 12B). IGF-IR protein levels in immunoblots of LNCaP whole cell lysates were too low to assess differences between IGF-IR protein levels in the presence or absence of serum (Fig. 12B). Since we utilized almost 20 times more protein for the LNCaP immunoprecipitation experiments (1.5 mg) when compared to that used for whole cell lysate analysis (75 μg), we were able to detect a marked reduction in IGF-IR protein levels during the entire course of serum deprivation when compared to that on day 0 (Fig. 12A, right panel). Our results are consistent with previous studies which report that following interaction of IGF-IR with its cognate ligand, the receptor is

internalized and degraded, thus accounting for the reduced levels of IGF-IR following its activation [94-96].

Inhibition of IGF-2 activity attenuates the serum deprivation-mediated increase in HIF-1α protein

As signal transduction pathways that are downstream of IGF-IR have been known to regulate HIF-1α protein synthesis [89], we next investigated the effect of inhibiting IGF-2 activity on HIF-1α protein expression during serum deprivation. IGF-2 neutralizing antibody was shown to inhibit IGF-2 activity in studies using colon and colorectal cancer cells [97, 98]. Thus, we employed IGF-2 neutralizing antibody to inhibit IGF-2's biological activity during serum deprivation. As shown in Fig. 13, treatment with IGF-2 neutralizing antibody markedly attenuated the serum deprivation-mediated increase in HIF-1α protein levels in PC-3 (Fig. 13, left panel) as well as LNCaP cells (Fig. 13, right panel). These results suggested that IGF-2 mediated the upregulation of HIF-1α protein expression during serum deprivation.

Inhibition of IGF-2 activity prevents the serum deprivation-mediated increase in IGF-IR and Akt activation, and also inhibits its own transcription

Our experiments thus far demonstrated involvement of IGF-2 (Fig. 13) and PI3K-Akt pathway (Figs. 8A and 9A, Specific Aim 2) in HIF-1α upregulation during serum deprivation. As IGF-2 is a HIF-1α-target gene product [9], our observations pointed to the possible involvement of a HIF-1α→IGF-2→IGF-IR→PI3K→Akt→HIF-1α

signaling loop in HIF-1α upregulation during serum deprivation. We, therefore, investigated the effect of inhibiting IGF-2 activity on IGF-IR and Akt activation, as well as transcription of IGF-2 itself during serum deprivation.

We first assessed the effect of inhibiting IGF-2 activity on IGF-IR activation. PCa cells were cultured in serum-free medium for 2 days in the presence of IGF-2 neutralizing antibody or isotype-matched control antibody. IGF-IR was immunoprecipitated from whole cell lysates, and activated IGF-IR was detected by probing the immunoprecipitated IGF-IR for phospho-tyrosine by immunobloting. Treatment with IGF-2 neutralizing antibody attenuated the serum deprivation-mediated increase in IGF-IR activation in the PC-3 and LNCaP cells when compared to that in the control antibody-treated cells (Fig. 14A). Moreover, treatment with IGF-2 neutralizing antibody also resulted in higher total-IGF-IR levels when compared to that in the control antibody-treated cells (Fig. 14A). These results demonstrated that the IGF-2 neutralizing antibody used in this study was effective in blocking IGF-2 activity, and thus inhibiting IGF-IR activation.

In addition, IGF-2 neutralizing antibody attenuated the serum deprivation-mediated increase in Akt activation in the PC-3 (Fig. 14B, left panel) and LNCaP (Fig. 14B, right panel) cells when compared to that in the control antibody-treated cells.

Moreover, most importantly, treatment with IGF-2 neutralizing antibody attenuated the serum deprivation-mediated increase in HIF-1α protein levels in PC-3 and LNCaP cells (shown in Fig. 13), as well as resulted in a marked decrease in its

own mRNA levels in PC-3 cells, when compared to that following control antibody treatment (Fig. 14C, top panel). Treatment with IGF-2 neutralizing antibody did not elicit a decrease in IGF-2 mRNA levels in serum-deprived LNCaP cells when compared to that in the control antibody-treated cells (Fig. 14C, bottom-left panel). However, we detected an appreciable decrease in IGF-2 mRNA levels when IGF-IR expression was suppressed by IGF-IR siRNA when compared to that in the control siRNA-transfected LNCaP cells (Fig. 14C, bottom-right panel). IGF-IR silencing was confirmed by RT-PCR analysis. These results suggested that the IGF-2 which is secreted by serum-deprived PCa cells might act in an autocrine manner to upregulate HIF-1α protein as well as its own transcription via IGF-IR and the downstream PI3K-Akt pathway.

Reciprocal regulation of the IGF-2/IGF-IR system and PI3K-Akt pathway during serum deprivation

Our results indicated that activation of the IGF-2/IGF-IR system mediates the activation of the PI3K-Akt pathway during serum deprivation. Therefore, we further assessed reciprocal regulation of IGF-IR and PI3K activity during serum deprivation. As shown in Fig. 15A, suppression of IGF-IR expression led to a decline in Akt activation when compared to that in the control siRNA-transfected PC-3 cells (Fig. 15A).

Moreover, inhibition of PI3K activity with 20 μM LY294002 resulted in approximately 3-fold and 2-fold higher IGF-IR protein levels in PC-3 (Fig. 15B, left

panel) and LNCaP cells (Fig. 15B, right panel) respectively when compared to that in the vehicle-treated control cells. Since IGF-IR activation was accompanied by a decrease in total IGF-IR protein levels, higher total IGF-IR protein levels suggested that there was less internalization/degradation of the receptor in LY294002-treated cells, most likely due to decreased receptor activation.

Suppression of HIF-1α expression attenuates Akt activation during serum deprivation, and reverses serum deprivation-mediated decrease in IGF-IR protein levels

In specific aim 1, we demonstrated that HIF-1α silencing led to IGF-2 downregulation during serum deprivation. Moreover, inhibition of IGF-2 activity decreased HIF-1α expression (Fig. 13), suggesting an autocrine feedback loop involving HIF-1α and IGF-2. Therefore, we further investigated HIF-1α's role in the autocrine feedback loop during serum deprivation. We first assessed the effect of suppressing HIF-1α expression on Akt activation during serum deprivation. The serum deprivation-mediated increase in Akt activation was attenuated following suppression of HIF-1α expression in PC-3 and LNCaP cells when compared to that in the control siRNA-transfected cells (Fig. 16A).

Next, as we detected that the decrease in total IGF-IR protein levels during serum deprivation correlated with HIF-1α upregulation (Fig. 16B), we investigated the effect of HIF-1α suppression on IGF-IR protein levels in serum-deprived PCa cells. HIF-1α suppression by siRNA resulted in higher IGF-IR protein levels in PC-3 (Fig.

16C, left panel) and LNCaP (Fig. 16C, right panel) cells when compared to that in the control siRNA-transfected cells, suggesting that there was less degradation of the receptor following HIF-1α suppression, most likely due to decreased receptor activation.

Moreover, transient overexpression of HIF-1α led to a marked increase in Akt activation in PC-3 cells cultured in the presence of serum (complete medium) under normoxic conditions when compared to that in the empty vector-transfected cells (Fig. 16D, top and bottom panels). This result suggested that the increase in HIF-1α protein promotes Akt activation in PCa cells.

(C) Figures:

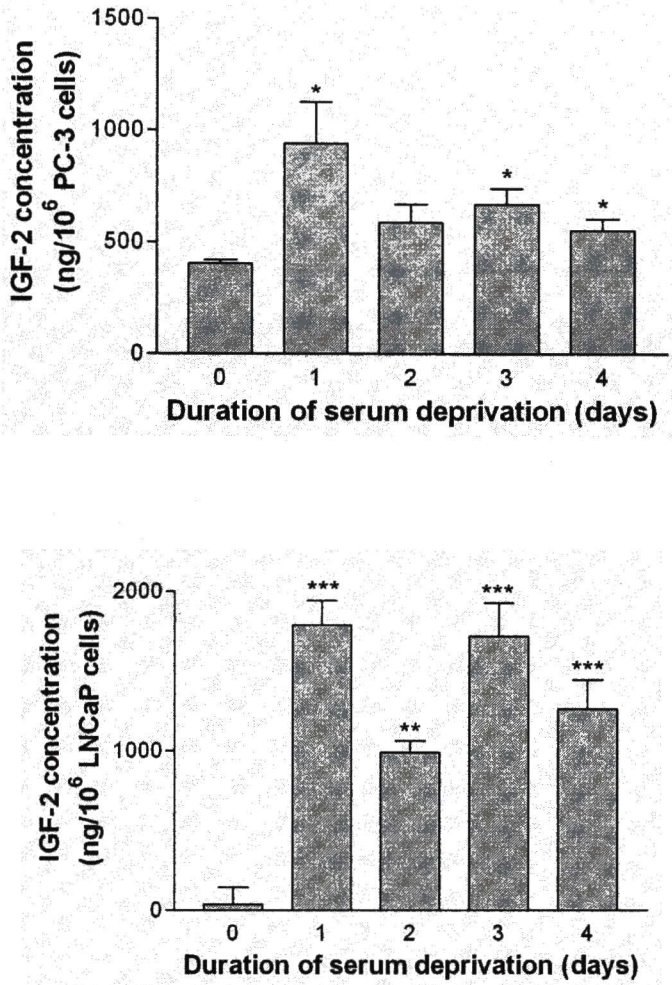


Figure 11: Serum-deprived PCa cells upregulate IGF-2 protein expression. PC-3 (top panel) and LNCaP (bottom panel) cells were cultured in serum-free medium for 0-4 days. Cell-culture supernates were collected at the indicated time points, and assessed for IGF-2 protein levels by ELISA. Data are the means ± S.D. of triplicate wells/experiment, and each experiment was repeated twice. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ versus day 0 IGF-2 protein levels.

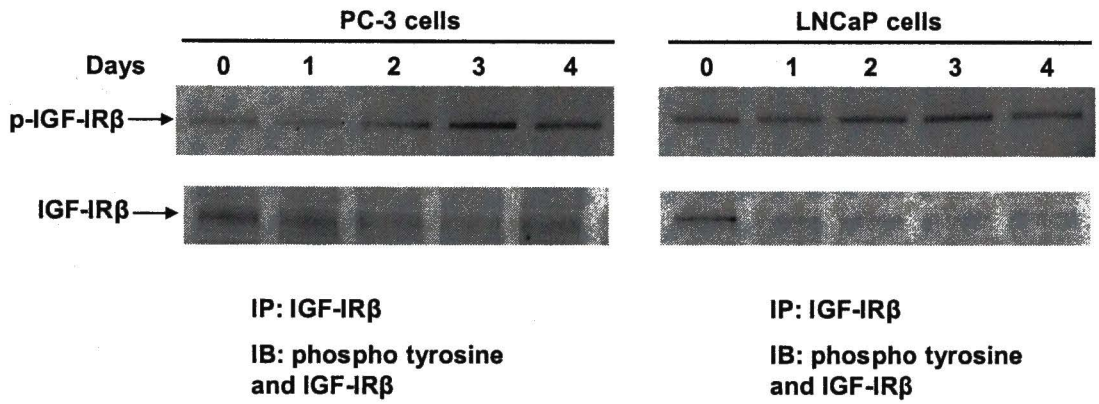


Figure 12A: Increased activation of IGF-IR during serum deprivation. PC-3 (left panel) and LNCaP (right panel) cells were cultured in serum-free medium for 0-4 days. Cells were harvested at the indicated time points, and whole cell lysates were prepared. IGF-IRβ was immunoprecipitated from the lysates and resolved on 4-20% gradient gels for western blot analysis. Phosphorylated IGF-IRβ was detected by probing the blots for phospho-tyrosine. Blots were subsequently stripped and reprobed for IGF-IRβ. The western blots are representative of 1 of 3 experiments.

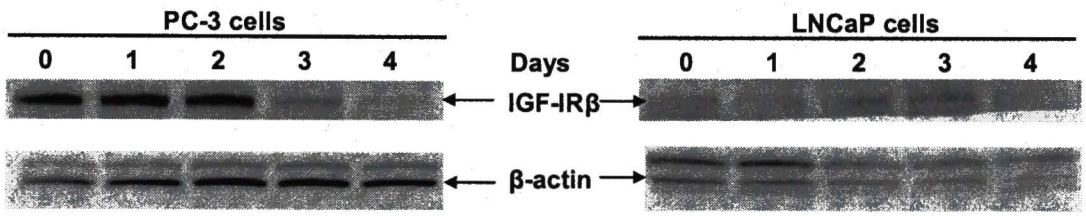


Figure 12B: Decrease in total IGF-IR levels during serum deprivation. PCa cells were harvested after culture in serum-free medium, under normoxic conditions for 0-4 days. Whole cell lysates were prepared at the indicated time points, and analyzed for IGF-IRβ by western blot analysis. The blots were stripped and reprobed for β-actin, which served as a loading control. The western blots are representative of 1 of 3 experiments.

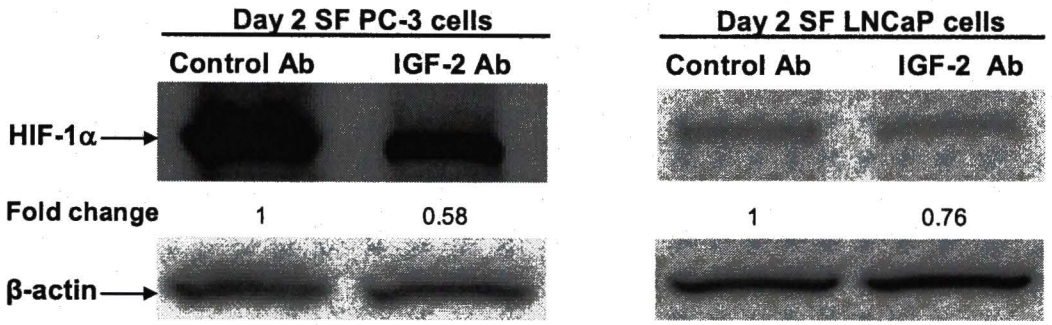


Figure 13: Attenuation of serum deprivation-mediated increase in HIF-1α protein following inhibition of IGF-2 activity. PC-3 (left panel) and LNCaP cells (right panel) were cultured in serum-free medium for 2 days in the presence of 3.3 μg/ml IGF-2 neutralizing antibody (IGF-2 Ab) or isotype-matched control antibody (Control Ab), and whole cell lysates were prepared. HIF-1α protein was assessed by western blot analysis. The blots were stripped and reprobed for β-actin, which served as a loading control. The western blots are representative of 1 of 3 experiments.

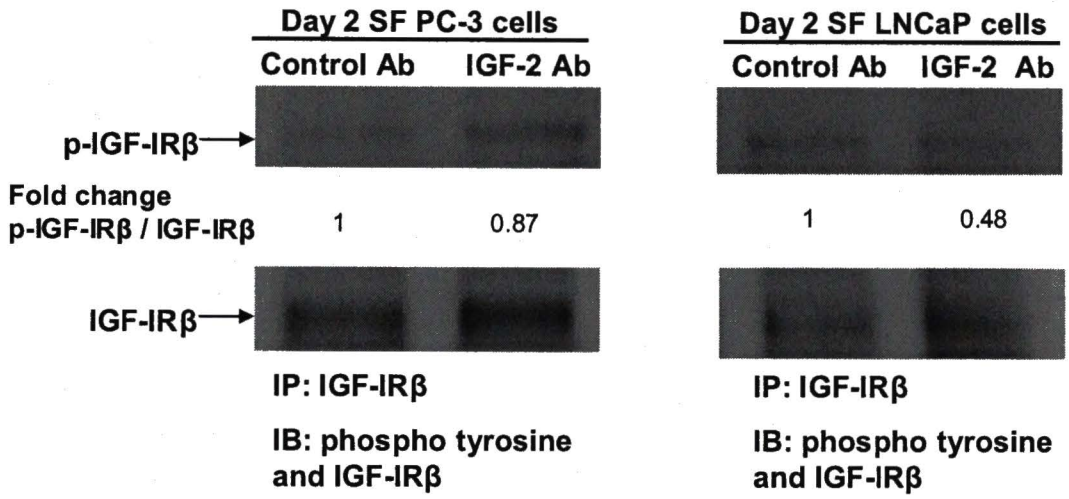


Figure 14A: Autocrine regulation of IGF-IR activation during serum deprivation. PC-3 (left panel) and LNCaP cells (right panel) were cultured in serum-free medium for 2 days in the presence of 3.3 μg/ml IGF-2 neutralizing antibody (IGF-2 Ab) or isotype-matched control antibody (Control Ab), and whole cell lysates were prepared. Phosphorylated-IGF-IRβ was detected as described in Fig. 12A.

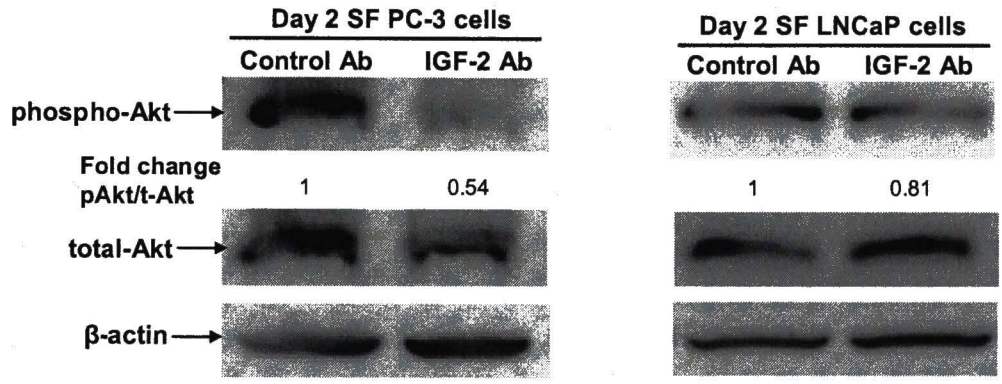


Figure 14B: Autocrine regulation of Akt activation during serum deprivation. PC-3 (left panel) and LNCaP cells (right panel) were cultured in serum-free medium for 2 days in the presence of 3.3 μg/ml IGF-2 neutralizing antibody (IGF-2 Ab) or isotype-matched control antibody (Control Ab), and whole cell lysates were prepared. Phospho-Akt was assessed by western blot analysis. The blots were sequentially stripped and reprobed for total Akt and β-actin respectively. β-actin served as a loading control. The western blots are representative of 1 of 2 experiments.

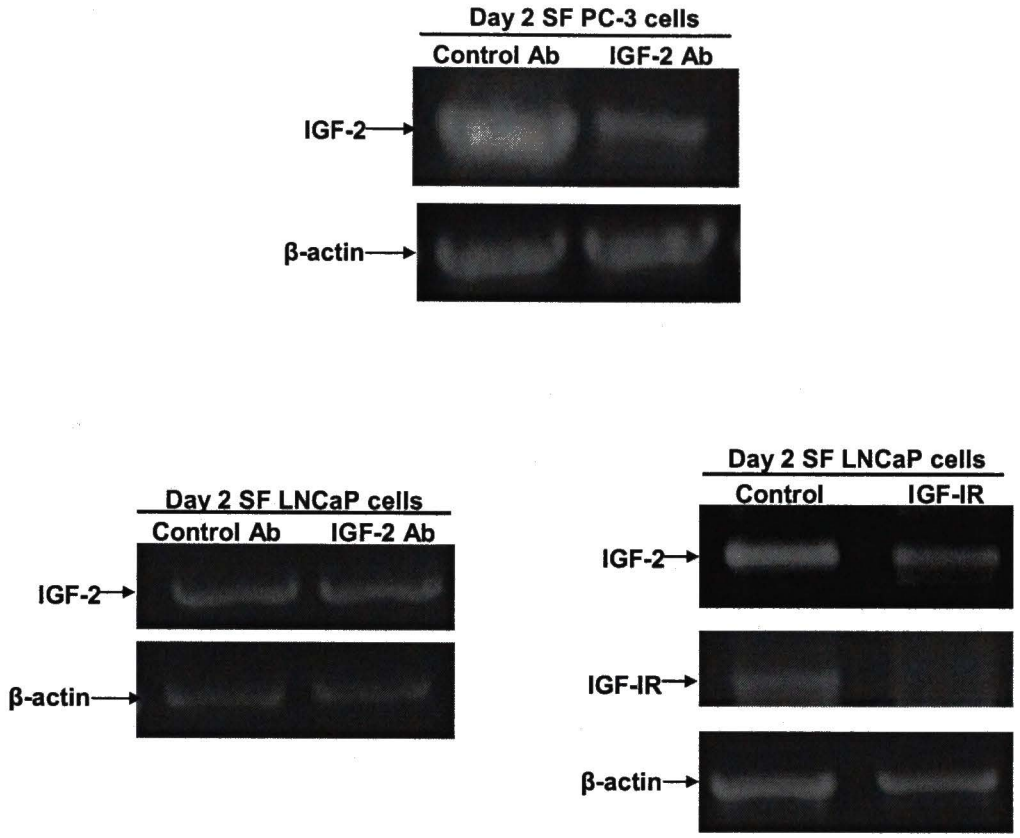


Figure 14C: Autocrine regulation of IGF-2 mRNA levels in serum-deprived PCa cells. PC-3 (top panel) and LNCaP cells (bottom-left panel) were cultured in serum-free medium for 2 days in the presence of 3.3 μg/ml IGF-2 neutralizing antibody (IGF-2 Ab) or isotype-matched control antibody (Control Ab) Total RNA was isolated from the PC-3 and LNCaP cells, and IGF-2 mRNA levels were assessed by RT-PCR analysis. β-actin served as the control. (Bottom-right panel) LNCaP cells were transfected with IGF-IR siRNA or control siRNA, and then cultured for 2 days in serum-free medium under normoxic conditions. IGF-2 and IGF-IR mRNA levels in the control and IGF-IR siRNA-transfected LNCaP cells were assessed by RT-PCR analyses. β-actin served as the control.

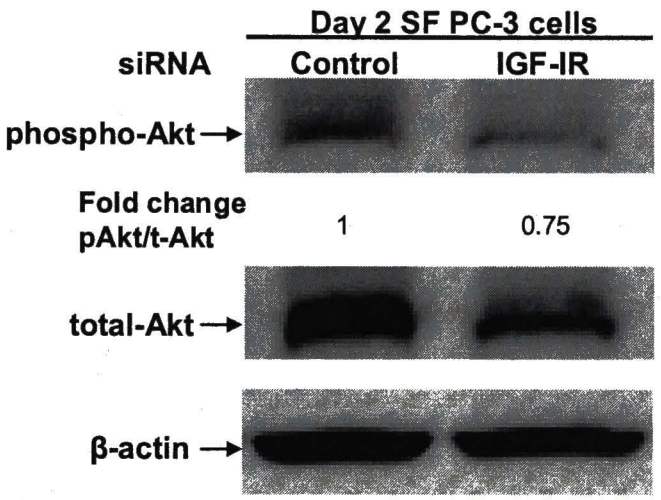


Figure 15A: Reciprocal regulation of the IGF-2/IGF-IR system and PI3K-Akt pathway during serum deprivation. PC-3 cells were transfected with IGF-IR siRNA or control siRNA, and then cultured for 2 days in serum-free medium under normoxic conditions. Subsequently, cells were harvested, and whole cell lysates were prepared and analyzed for phospho-Akt by western blot analysis. The blots were sequentially stripped and reprobed for total-Akt and β-actin respectively. β-actin served as a loading control. The western blot is representative of 1 of 2 experiments.

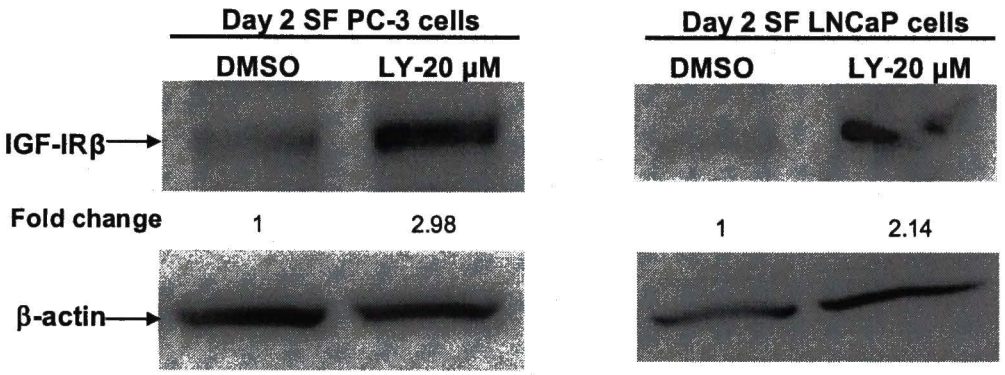


Figure 15B: Reciprocal regulation of the IGF-2/IGF-IR system and PI3K-Akt pathway during serum deprivation. PCa cells were cultured in serum-free medium for 2 days in the presence of 20 μM LY294002 (LY-20 μM) or vehicle (DMSO). Thereafter, cells were harvested, and whole cell lysates were prepared. IGF-IRβ protein levels in the PC-3 (left panel) and LNCaP (right panel) cells were assessed by western blot analysis. The blots were stripped and re-probed for β-actin, which served as a loading control. The western blots are representative of 1 of 2 experiments.

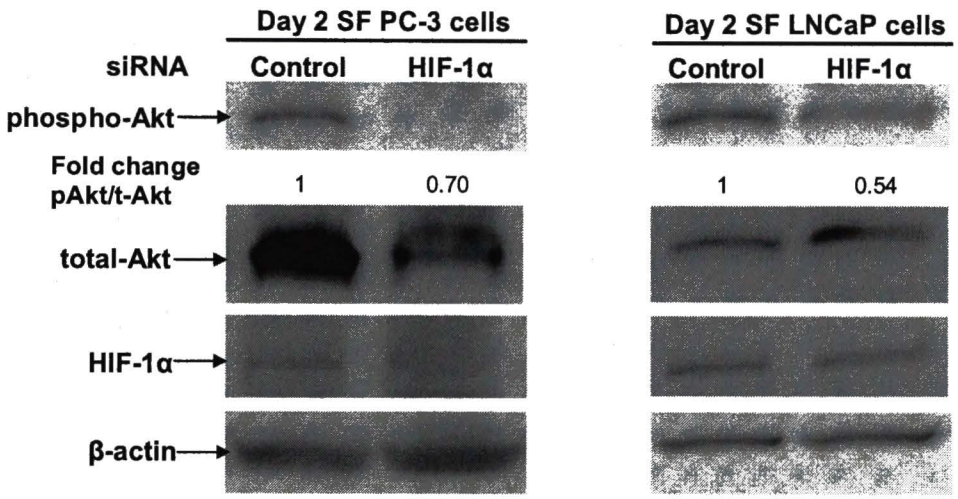


Figure 16A: HIF-1α regulates Akt activation during serum deprivation. PC-3 cells (left panel) and LNCaP cells (right panel) were transfected with HIF-1α siRNA or control siRNA, and then cultured for 2 days in serum-free medium under normoxic conditions. Subsequently, cells were harvested, and whole cell lysates were prepared and analyzed for phospho-Akt by western blot analysis. The blots were sequentially stripped and reprobed for total Akt, HIF-1α and β-actin respectively. β-actin served as a loading control. The western blots are representative of 1 of 2 experiments.

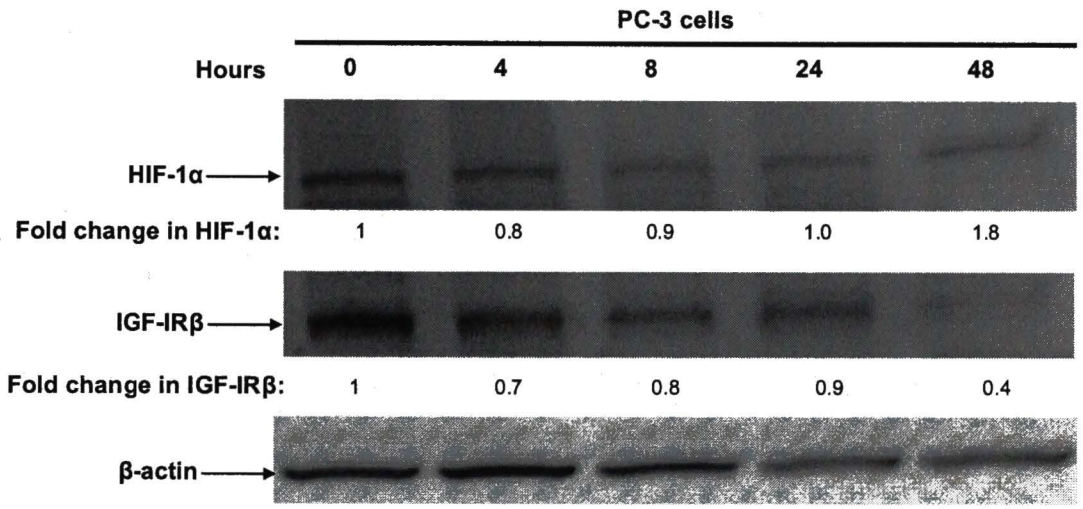


Figure 16B: Increase in HIF-1α protein is associated with a decrease in total IGF-IR protein levels during serum deprivation. PC-3 cells were cultured in serum-free medium for 0, 4, 8, 24 and 48 h. Cells were harvested at the indicated time points, and whole cell lysates were prepared and analyzed for HIF-1α expression by western blot analysis. The blots were sequentially stripped and reprobed for IGF-IR and β-actin respectively. β-actin served as a loading control.

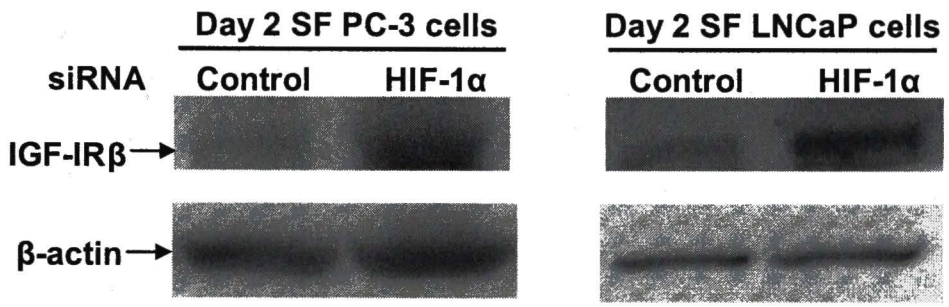


Figure 16C: Effect of HIF-1α suppression on IGF-IR protein levels during serum deprivation. PC-3 (left panel) and LNCaP cells (right panel) were transfected and treated as described in (16A). Thereafter, whole cell lysates were analyzed for IGF-IRβ protein levels by western blot analysis. The blots were stripped and re-probed for β-actin, which served as a loading control. The western blots are representative of 1 of 2 experiments.

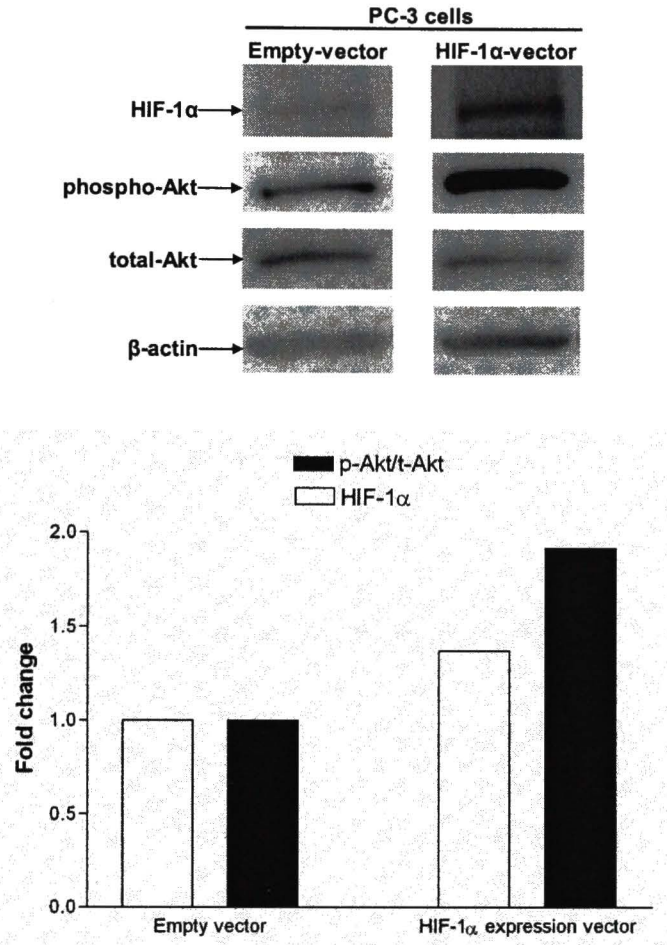


Figure 16D: HIF-1α overexpression increases Akt activation in PCa cells. PC-3 cells (top panel) were transiently transfected with HIF-1α expression vector or empty vector, and then cultured under normoxic conditions for 1 day in complete medium (medium containing serum). Cells were subsequently harvested, and whole cell lysates were prepared and analyzed for HIF-1α protein by western blot analysis. The blots were sequentially stripped and re-probed for phospho-Akt, total-Akt and β-actin. β-actin served as a loading control. (Bottom panel) Immunoblot results of the ratio of HIF-1α to β-actin, and the ratio of phospho-Akt to total-Akt in the empty vector-transfected and HIF-1α vector-transfected cells were represented on a bar graph.

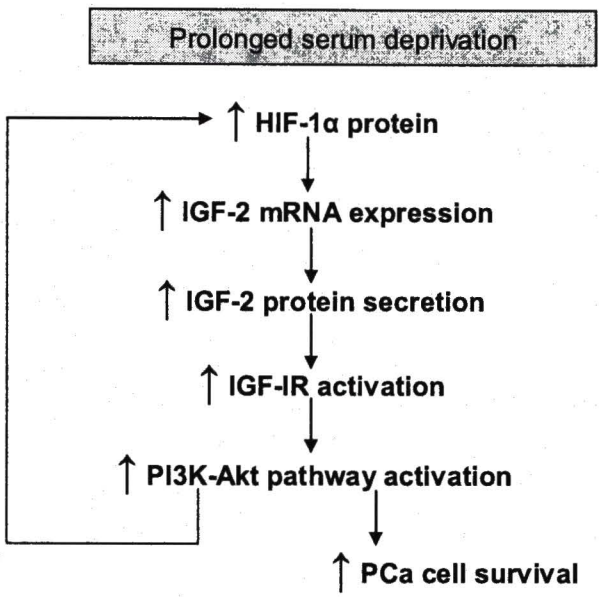


Figure 17: HIF-1α-dependent autocrine, pro-survival feedback loop during serum deprivation. Pre-existing HIF-1α protein in the PCa cells facilitates the expression of its target gene, IGF-2. The secreted IGF-2 protein binds to, and activates IGF-IR on the serum-deprived PCa cells, which consequently activates the downstream, pro-survival PI3K-Akt pathway. Activation of the PI3K-Akt pathway promotes PCa cell survival, and also markedly upregulates HIF-1α protein expression, thereby resulting in increased IGF-2 expression and maintenance of the pro-survival, autocrine feedback loop.

(D) Discussion

As tumors can be deprived of serum during the course of their progression to more advanced states, and also following treatment strategies which disrupt tumor vasculature such as radiotherapy and anti-angiogenic therapy, adaptation to the stress of prolonged serum growth factor deprivation is crucial for tumor survival, growth and metastasis. In this study, we report for the first time the existence of a pro-survival, HIF-1α/IGF-2/IGF-IR/PI3K/HIF-1α autocrine feedback loop which upregulates HIF-1α expression in normoxic, serum-deprived PCa cells. Several lines of evidence support the existence of this novel autocrine feedback loop illustrated in Fig. 17. First, HIF-1α upregulated IGF-2 mRNA expression during serum deprivation, and this increase in IGF-2 expression was prevented by siRNA-mediated suppression of HIF-1α (specific aim1). Reciprocal regulation of HIF-1α and IGF-2 during serum deprivation was also demonstrated by the finding that HIF-1α suppression reduced IGF-IR activation (Fig. 16C). Furthermore, inhibition of IGF-2 activity with neutralizing antibody effectively prevented HIF-1α upregulation (Fig. 13) as well as its own expression during serum deprivation (Fig. 14C). Suppression of IGF-IR expression also abolished the serum deprivation-mediated increase in IGF-2 mRNA expression (Fig. 14C). Second, cross-talk between IGF-2 and Akt during serum deprivation was demonstrated by findings where inhibition of IGF-2 activity with IGF-2 neutralizing antibody attenuated IGF-IR activation (Fig. 14A), as well as Akt activation (Fig. 14B). Additionally, Akt activation during serum deprivation was also prevented by siRNA-mediated suppression of IGF-IR expression (Fig. 15A).

Moreover, LY-294002-mediated inhibition of PI3K activity resulted in higher IGF-IR protein levels when compared to that in the vehicle-treated control (Fig. 15B). IGF-IR activation was accompanied by a reduction in total IGF-IR protein levels due to increased IGF-IR internalization and degradation. Thus, higher IGF-IR levels resulted from decreased receptor activation. Third, prolonged serum deprivation activates Akt (Fig. 7), and inhibition of Akt by overexpression of wild-type PTEN as well as LY294002 treatment attenuated the serum deprivation-mediated increase in HIF-1α protein (Figs. 9A and 8A respectively) and cell survival (Figs. 9B, 8B and 8C). Evidence that HIF-1α mediated the activation of Akt during serum deprivation is found in the observation wherein suppression of HIF-1α expression markedly inhibited the serum deprivation-mediated increase in Akt activation (Fig. 16A). Furthermore, overexpression of HIF-1α markedly increased Akt activation in the PCa cells (16D).

HIF-1α has been known to upregulate the expression of a vast array of genes that play crucial roles in promoting cell survival and proliferation under hypoxic stress [9]. One such HIF-1α-target gene product, IGF-2, has been implicated in promoting cell survival under apoptotic stress [21, 75]. We observed a marked increase in IGF-2 protein secretion by day 1 of serum deprivation (Fig. 11). This observation can be explained, in part, by the fact that IGF-2 bioavailability and activity are modulated by high-affinity IGF binding proteins (IGFBPs) [23, 27]. Increased IGF-2 activity is seen following cleavage of the IGFBPs by specific proteases [99]. Studies have reported an increase in IGFBP degradation during serum deprivation [21, 100] thereby

facilitating increased interaction of IGF-2 with its receptor, IGF-IR. Moreover, IGFs have also been reported to activate specific proteases that cleave IGFBPs and thus increase their own bioavailability for interaction with IGF-IR. Thus, it is likely that the IGF-2 secreted by the PCa cells during the very early stages of serum deprivation is bound to cell surface-associated or extracellular IGFBPs, and is released due to increased cleavage of IGFBPs during prolonged serum deprivation.

Inhibition of IGF-2 activity by treatment with IGF-2 neutralizing antibody markedly attenuated its own transcription in serum-deprived PC-3 cells (Fig. 14C). However, we did not detect a similar attenuation of IGF-2 mRNA levels in LNCaP cells (Fig. 14C, bottom-left panel). On the other hand, siRNA-mediated suppression of IGF-IR expression markedly decreased IGF-2 mRNA levels in the LNCaP cells (Fig. 14C, bottom-right panel). Kimura *et al* demonstrated that although IGF-2 is the predominant IGF-IR ligand produced by the PC-3 and LNCaP cells, LNCaP cells also produce small amounts of IGF-1, the other activating ligand of IGF-IR [101]. In contrast, PC-3 cells do not produce measureable amounts of IGF-1 [21, 101]. Thus, a possible explanation for the differential response of serum-deprived PC-3 and LNCaP cells to inhibition of IGF-2 activity via IGF-2 neutralizing antibody could be that LNCaP cells also produce trace amounts of IGF-1, and may thus continue to activate the IGF-IR even though IGF-2 activity is inhibited.

Although PC-3 and LNCaP cells markedly vary in their basal IGF-IR protein expression levels, both cell types exhibit robust activation of the IGF-IR during serum deprivation. This observation is also supported by studies which report that in

contrast to other tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR) family members, overexpression of IGF-IR alone is insufficient to cause receptor activation, but IGF-IR requires ligand binding to trigger the appropriate downstream pathways [23, 30]. Furthermore, inhibition of IGF-2 activity not only inhibited IGF-IR activation, but also prevented the serum deprivation-mediated increase in HIF-1α expression, and the subsequent transcription of IGF-2. Thus, our observations underscore the importance of IGF-IR activation and signaling for HIF-1α upregulation and cell survival during prolonged serum deprivation.

Our findings support the notion that the decrease in IGF-IR levels during serum deprivation is due to increased activation, and consequently increased internalization and degradation of IGF-IR. First, treatment with IGF-2 neutralizing antibody decreased receptor activation, and simultaneously resulted in higher total IGF-IR protein levels when compared to that in the control antibody-treated cells (Fig. 14A). Second, LY294002-mediated inhibition of PI3K activity resulted in markedly higher total IGF-IR protein levels when compared to that in the vehicle-treated control cells (Fig. 15B). Third, siRNA-mediated suppression of HIF-1α resulted in higher IGF-IR protein levels when compared to that in the control siRNA-transfected cells during serum deprivation (Fig. 16C). Taken together, our results suggest that the total IGF-IR protein levels are low during serum deprivation due to increased HIF-1α-dependent expression of IGF-2, and consequently increased activation and degradation of the receptor. Therefore, any blockade of the HIF-1α-dependent

autocrine feedback loop will result in decreased receptor activation, and hence elevated IGF-IR protein levels.

As the PC-3 and LNCaP cells are deficient in PTEN [57], a negative regulator of the PI3K-Akt pathway, we first investigated involvement of the PI3K-Akt pathway in HIF-1α upregulation and cell survival during prolonged serum deprivation. In addition to observing marked increase in Akt activation during serum deprivation (Fig. 7), we also detected significant inhibition in cell survival as well as attenuation of serum deprivation-mediated increase in HIF-1α expression following LY294002-mediated inhibition of PI3K activity (Fig. 8). We also investigated MAPK activation to assess if the MAPK pathway also contributed to HIF-1α upregulation during serum deprivation (Fig. 10). Although the PC-3 cells exhibited a modest activation of Erk1/2 during serum deprivation, Erk1/2 was not activated in the serum-deprived LNCaP cells (Fig. 10). As the MAPK pathway has been known to promote cell proliferation, the modest activation of the MAPK pathway in serum-deprived PC-3 cells may account for the modest increase in PC-3 cell number during serum deprivation (specific aim 1). Similarly, the lack of MAPK activation in serum-deprived LNCaP cells could account for the observation wherein we did not detect a significant increase in LNCaP cell number during serum deprivation (specific aim 1). Thus, although we cannot completely rule out the possibility that the MAPK pathway may play a role in HIF-1α upregulation in serum-deprived PC-3 cells, our data lead us to conclude that the PI3K-Akt pathway is the predominant signaling pathway that

upregulates HIF-1α protein, and consequently promotes PCa cell survival during serum deprivation.

At present, the initial stimulus that leads to HIF-1α upregulation during serum deprivation is still unclear. Low, but detectable levels of HIF-1α protein are present in the normoxic day 0 PC-3 and LNCaP PCa cells as was demonstrated in Specific Aim 1. Thus, it is possible that the pre-existing, low levels of HIF-1α that are found in PCa cells during the initial stages of serum deprivation facilitate the production and secretion of low levels of IGF-2, which then act in an autocrine manner to amplify HIF-1α protein synthesis in the later stages of serum deprivation. Other factors such as reactive oxygen species (ROS) may also contribute to the initial upregulation of HIF-1α protein during serum deprivation. PCa cells have been reported to spontaneously produce ROS [102]. As ROS promotes HIF-1α expression in PCa cells [102, 103], it is possible that initially the serum-deprivation mediated increase in ROS [104, 105] may promote HIF-1α expression, which in turn upregulates IGF-2 expression to further increase HIF-1α protein levels and thus maintain the HIF-1α-IGF-2-IGF-IR-PI3K autocrine feedback loop.

HIF-1α's significance in promoting PCa cell survival during serum deprivation could be two-fold. First, HIF-1α could directly promote cell survival by activating the pro-survival PI3K-Akt pathway via increased expression of IGF-2 as was demonstrated in this study. Second, in specific aim 1 we demonstrated an increased HIF-1α–dependent expression of VEGF during serum deprivation. Although VEGF did not have any direct pro-survival effects on serum-deprived PCa cells *in-vitro*,

given the importance of VEGF in promoting tumor angiogenesis [82-84], it is possible that the VEGF secreted by the PCa cells may indirectly promote survival of PCa cells within tumors by promoting new blood vessel formation. Future *in-vivo* studies will help define the predominant mechanism by which HIF-1α mediates cell survival during serum deprivation.

In conclusion, our study has identified a novel mechanism by which normoxic PCa cells upregulate HIF-1α protein synthesis, and consequently promote cell survival during stress of prolonged serum deprivation. As illustrated in Fig. 17, our results demonstrate that increased HIF-1α protein levels during serum deprivation result in increased IGF-2 expression and subsequent activation of IGF-IR, leading to increased activation of the PI3K-Akt pathway and thus further upregulation of HIF-1α protein levels. As the stress of serum deprivation mimics, to some extent, the tumor micro-environmental condition arising following certain treatment strategies, and as HIF-1α is commonly overexpressed in PCa cells [18, 19], our study provides a strong rationale for the therapeutic targeting of HIF-1α under normoxic as well as hypoxic conditions, in combination with other chemotherapeutic drugs that disrupt tumor vasculature in order to improve their therapeutic efficacy.

CHAPTER VI

CONCLUSION AND FUTURE DIRECTIONS

Our study has identified serum deprivation to be a novel factor that contributes to HIF-1α protein upregulation in normoxic PCa cells. Furthermore, our study has demonstrated, for the first time, the existence of an autocrine HIF-1α- and IGF-2-dependent feedback loop that upregulates HIF-1α protein expression and thus promotes survival of serum-deprived PCa cells. As HIF-1α is frequently overexpressed in PCa cells, and given the pivotal role of HIF-1α in promoting PCa cell survival, our results explain, at least in part, how prostate tumors survive and continue to grow under serum-deprived conditions that arise following different treatment strategies that are known to disrupt tumor vasculature.

The ability of serum deprivation to upregulate HIF-1α is particularly interesting as HIF-1α is involved in the regulation of several other pathways implicated in tumor progression [9]. Thus, it would be interesting to conduct future studies directed at investigating the effect of serum deprivation-mediated increase in HIF-1α protein on PCa invasion, angiogenesis and drug resistance.

As our study demonstrated an increased expression of VEGF, a key angiogenic factor, by serum-deprived PCa cells, it will be beneficial to assess the *in-vivo* effect of VEGF upregulation on tumor survival and progression following strategies that disrupt

tumor vasculature. Furthermore, as tumor cells are frequently deprived of serum growth factors following treatment strategies such as radiotherapy and anti-angiogenic therapy, it will be beneficial to conduct *in-vivo* studies to ascertain the effect of selectively inhibiting HIF-1α on response to radiotherapy and anti-angiogenic therapy.

Given HIF-1α's critical role in promoting survival of PCa cells, it would be an excellent target for novel anti-cancer drug development. Moreover, as HIF-1α is rapidly degraded in normal cells, it is dispensable for normal cells under normal cellular oxygen conditions. Thus, the approach of using HIF-1α inhibitors may also have the added benefit of preferentially targeting cancer cells. In conclusion, our study has provided a strong rationale for the therapeutic targeting of HIF-1α under normoxic as well as hypoxic conditions, either alone or in combination with other chemotherapeutic drugs, for a more effective inhibition of survival and progression of PCa.

CHAPTER VII

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