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Inhibitor of DNA binding 4: a novel tumor suppressor gene in prostate cancer

Jason P.W. Carey
Clark Atlanta University

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ABSTRACT

BIOLOGICAL SCIENCES

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INHIBITOR OF DNA BINDING 4 (ID4):
A NOVEL TUMOR SUPPRESSOR GENE IN PROSTATE CANCER

Committee Chair: Dr. Jaideep Chaudhary

Dissertation dated December 2011

There are four isoforms of the Inhibitor of DNA-binding/differentiation proteins (Id1-4). Id1-3 are reportedly overexpressed in various cancer including colorectal, breast, lymphoma and prostate cancer. Id4 is expressed in the normal epithelium of the prostate gland and during early stage prostate cancer, however its expression is lost in advanced stage metastatic prostate cancer. In several cancers Id4 expression is lost through promoter hypermethylation a mechanism cancer cells utilize to silence tumor suppressor genes. We hypothesized that Id4 acts as a tumor suppressor in prostate cancer and its expression is downregulated through hypermethylation of the Id4 promoter. To test this hypothesis, we first observed the methylation status of the Id4 promoter in both androgen dependent and androgen independent prostate cancer cell lines. Then to observe the significance of Id4 in prostate cancer, we transfected a full length Id4 plasmid into the Id4 negative DU14 cell line to observe if Id4 may act as a tumor suppressor in prostate
cancer. Ectopic expression of Id4 reduced the proliferation rate of the prostate cancer cell line. Id4 expression resulted in an increase of endogenous apoptosis and an increased sensitivity to apoptotic inducing agents. Id4 expression initiated senescence at an increased rate of frequency in DU145+Id4 cells. Id4 inhibited progression of the cell cycle by upregulating key cell cycle regulatory genes p16, p21, p27 SKP2, E2F1. Exogenous Id4 expression resulted in the expression of a functional androgen receptor that was capable of nuclear translocation in response to androgen treatment and activating the AR target gene PSA. Id4 downregulates the expression of Id1 and Id3, two members of the Id family responsible for increasing prostate cancer progression. These results indicate that Id4 is a novel tumor suppressor gene in prostate cancer that is responsible for inducing senescence, apoptosis, cell cycle regulation and functional AR activity and that the loss of Id4 expression in prostate cancer progression is the results of hypermethylation of the Id4 promoter. Id4 may be a critical aspect in the transition from androgen dependent prostate cancer to androgen independence. Understanding regulation of the Id4 gene may provide a crucial target for prostate cancer drug therapy.
INHIBITOR OF DNA BINDING 4 (ID4):
A NOVEL TUMOR SUPPRESSOR GENE IN PROSTATE CANCER

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

BY
JASON PATRICK WILLIAM CAREY

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA
JULY 2011
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<td>ANOVA</td>
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<td>Analysis of Variance</td>
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<tr>
<td>ACS</td>
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<td>Advanced senescent cells</td>
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<td>AR</td>
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<td>Androgen Receptor</td>
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<td>ARE</td>
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<tr>
<td>Androgen Response Element</td>
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<tr>
<td>Bovine Calf Serum</td>
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<td>Benign Prostate Hyperplasia</td>
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<td>bHLH</td>
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<td>basic Helix-Loop-Helix</td>
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<td>basic fibroblast growth factor</td>
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<tr>
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<tr>
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<tr>
<td>Cyclic Adenosine Monophosphate</td>
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<td>Complementary Deoxyribonucleic Acid</td>
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<tr>
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<td>CYP11</td>
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<tr>
<td>Cytochrome P450 side chain cleavage</td>
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vii'
<table>
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<tr>
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<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PP</td>
<td>Post pubertal</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cells</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cells</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription – Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER ONE
INTRODUCTION

There are four isoforms of the Inhibitor of differentiation/DNA binding (Id) gene family (1). These genes are differentially expressed in various tissues and are required for normal development. These families of genes are dominant negative regulators of basic helix-loop-helix (bHLH) transcription factors. The basic helix-loop-helix domain is conserved from yeast to mammals (2). The bHLH families of proteins bind DNA as either homodimers or heterodimers and regulate transcription. Due to the fact that the Id proteins lack the DNA binding domain they negatively regulate the other classes of bHLH proteins that have the basic domain, and prevent them from binding to DNA or forming active homodimers (3). Id proteins are known regulators of the cell cycle in various tissue types (4).

The most familiar binding partners of the Id proteins are the E protein transcription factors. This family of proteins consists of E12/E47, E2-2, and HEB (2). E12 and E47 are spliced products of the E2A gene. E12/E47 is also responsible for regulating Id1 degradation (5). The following binding of Id proteins to E12/E47 help protect the E12/E47 protein from proteosome mediated degradation (6). Non-bHLH proteins are also known to bind Id proteins. These interactions; however appear to be specific to particular isoforms of Id proteins. One of these proteins includes the retinoblastoma protein Rb(7) that binds directly to Id2 and has been shown to bind Id1
also. Thus far Id3 or 4 have shown no interaction with Rb. The Id2-Rb interaction promotes cell cycle because Id2 sequesters Rb through direct interaction (3).

Id proteins are known to inhibit the differentiation of stem cells which is how their name was derived. Since these proteins were found to promote cell proliferation, hence it was not surprising that they were later shown to be over-expressed in many cancers (3). This is how they became associated with regulatory roles in key cancer processes. Hence, the role of Ids in growth and de-differentiation are key activities responsible for cellular transformation. Id1 and Id3 have been shown to be expressed in a number of different cancers including pancreatic cancer, colorectal cancer and prostate cancer (3, 8, 9). Id1 specifically has been associated with an altered steroid hormone response in human breast cancer cells and prostate cancer (10). Id1 has also been linked to the transformation of epithelial phenotypes to a more metastasis phenotype (11, 12). Loss of Id1 and Id3 has also been linked to inhibition of cancer progression (5). The Id proteins are incapable of directly activating oncogenic pathways but are seen as supporting oncogenes by facilitating the initiation of oncogenic proteins responsible for significant upregulation of key cancer pathways (13).

The Id genes (Id1, Id2, Id3 and Id4) are part of the broader basic helix loop helix family. The basic helix-loop-helix (bHLH) proteins are DNA binding proteins that regulate tissue-specific transcription within multiple cell lineages (14). Hetero- or homodimerization dependent DNA binding activity of class A bHLH proteins are regulated to a large part by the class D Id gene family (15). The Id proteins lack the DNA binding basic domain but have intact HLH domain (15, 16). This domain configuration allows the Id family to dimerize with bHLH transcription factors, but the lack of the basic domain
renders the Id-bHLH dimer transcriptionally inactive, as it fails to bind and regulate promoter activity of genes dependent on E-box (CANNTG) response element (16).

The four different isoforms of Ids (Id1, Id2, Id3 and Id4) have a highly conserved HLH domain but divergent N- and C- terminal domains. This sequence divergence may account for protein-specific interactions possibly resulting in differential functions of Id proteins (2, 3, 17). Although all Id proteins interact with E-proteins, isoform specific bHLH and non-bHLH interactions are known to occur. For example, interaction of a) Id2 directly with hypophosphorylated pRB protein family (4, 7) and polycystins (6) b) Id2 and Id4 with OLIG (class A bHLH (10)) c) Id1 and calcium/calmodulin-dependent serine protein kinase (CASK) (18) and d) Id1 and Id3 with v-ets erythroblastosis virus E26 oncogene homolog (Ets) (19)and Paired box transcription factor (Pax) homeodomain containing proteins (20). Consistent with gene specific interactions, the Id proteins also exhibit isoform specific functions such as modulation of breast cancer 1 early onset gene (BRCA1) promoter activity by Id4 (21, 22), localization of Id1 to the centrosomes (23) leading to accumulation of cells with abnormal centrosome number and induction of apoptosis by Id2 in myeloid precursors, osteosarcoma (8) and neuronal cells (9) by an HLH independent mechanism.

In general, Id proteins (Id1-3) promote cell proliferation in cancer (24-26). Consequently, the expression of Id proteins is generally high in proliferating cells that is down-regulated as a prerequisite for exit from the cell cycle during differentiation(27). Consistent with this observation, an increased expression of various Id isoforms has been detected in many cancers (1, 13, 28-34).
In comparison to Id1, Id2 and Id3, the function of Id4 is less understood and often conflicting. Both tumor promoting and tumor suppressor roles of Id4 have been reported in various cancers. Tumor suppressor roles of Id4, based on its loss of expression in association with promoter hypermethylation have been suggested in leukemia, (35) breast, colorectal (36, 37) and gastric cancers (38). The pro-tumor effect of Id4 is observed in bladder (39) and rat mammary gland carcinomas(40). Id4 is also the only Id gene that is deregulated by a t (6; 14) (p22; q32) chromosomal translocation in a B-cell acute lymphoblastic leukemia (41) and B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (42). The molecular mechanism responsible for the tumor promoter roles of Id4 could be consistent with other Id isoforms; however, the tumor suppressor role, unique to Id4 may involve distinct mechanisms that need to be understood. The likely switch between tumor suppressor and tumor promoter role of Id4 could be due to unique downstream effectors and activation (signaling) mechanisms or simply due to the presence/absence of unique Id4 interaction partner(s). We have demonstrated the mechanism that Id4 acts as a tumor suppressor through the use of novel tools and technologies developed in our laboratory.

Recently Id4 expression was also shown to be associated with increased metastasis in PCa that was largely based on the protein expression levels determined by the Santa Cruz antibody (43). In our lab, this antibody cross reacts with multiple bands in a western blot (data not shown) hence the data from this study needs to be re-evaluated using a mono-specific antibody. We have recently identified an Id4 antibody that reacts specifically with Id4 with negligible cross-reactivity (Aviva, Cat# ARP38058_T100). We propose to use this antibody for Id4 protein detection.
The expression of Id4 in prostate epithelial cells is particularly interesting. Id4 appears to be androgen regulated in normal prostate epithelial cells (44) and in the androgen sensitive prostate cancer cell line LNCaP (45). Id4 expression is undetectable or weakly expressed in androgen independent DU145 cells whereas its expression is observed (low) in PC3 prostate cancer cell lines (45). LNCaP prostate cancer cells are generally considered less tumorogenic and more differentiated as compared to highly tumorogenic DU145 and PC3 prostate cancer cells. These observations suggest that Id4 expression may be associated with the state of differentiation and tumorogenic potential of prostate epithelial cells. This hypothesis was examined in the present study by over-expressing Id4 in DU145 prostate cancer cells that are androgen receptor (AR) and Id4 negative. Our results, demonstrate that Id4 attenuates the cell cycle by promoting an S-phase arrest and inducing androgen receptor expression. Id4 expression is also significantly reduced in prostate cancer samples as determined through data mining.

Due to the conserved nature of Id proteins, the isoforms also exhibit specific functions such as modulation of BRCA1 promoter activity by Id4 (21), localization of Id1 to the centrosomes(23) leading to accumulation of cells with abnormal centrosome number and induction of apoptosis by Id2 in non-epithelial cells by an HLH independent mechanism (9). In general, Id proteins promote cell proliferation(24-26) by regulating the expression/activity of genes/proteins involved in cell cycle such as p16(46), p21(47) and Rb(4, 7). Consequently, the expression of Id proteins is generally high in proliferating cells but low in differentiated cells(27).

Previous studies have shown that Id1 and to a lesser extent Id2 are highly expressed and are strong prognostic markers of prostate cancer (48-50). Over
expression of Id1 alone can transform androgen sensitive prostate cancer cells (LNCaP) to androgen independence, protect from apoptosis through activation of NF-kB pathway and promote survival and invasion by regulating the expression of Epidermal Growth Factor Receptor (EGFR).

The loss of gene expression by siRNA based reverse genetics approaches have indicated that Id1 and Id3 have partial overlapping functions in terms of regulating proliferation in human prostate cancer cell lines (45). Id2, on the other hand is primarily involved in the apoptotic pathway (8). Collectively, these observations suggest that within the same cell system Id proteins target isoform specific pathways by regulating expression or function of unique downstream effector genes. Our results support the cell and isoform specific role of Id4 in prostate cancer cell lines and identifies novel downstream effectors that may explain some of their functions. In addition, we have demonstrated, for the first time that Id genes can function in the absence of their primary interacting partners the E-proteins.

To further clarify the expression of Id4 in PCa we searched for Id4 gene expression in publically available microarray databases. The consolidated oncomine database (51) was queried against Id4 and all prostate databases were analyzed. One notable observation in these studies is the high Id4 in BPH (BP) as compared to PCa (PC) suggesting that decreased Id4 is a cancer specific event. It is also noted that there is high normal (N), normal adjacent (NAP) and post pubertal (PP) Id4 gene expression that may signify the role of Id4 in maintaining normal prostate function (panels A and C) and 3) Low Id4 expression in PCa (metastatic PCa (MPC) < PCa (PC), panel D). Consolidated data from all these studies demonstrated that Id4 is significantly (T-values > 5 and low P-
values < E-6) down-regulated in PCa samples. What could be the molecular mechanisms leading to the down-regulation of Id4 in PCa? Therefore, we have investigated the potential mechanism responsible for addressing this query. An equally important question addressed in this investigation is the molecular mechanistic role of Id4 in PCa.

In the advanced stages of prostate cancer Id4 expression is diminished. Conversely in the advanced stages of prostate cancer Id1, Id2 and Id3 all show significantly higher expression level as compared to the normal prostate epithelial tissue. This data supports previous research suggesting Id1-3 may be linked to global cancer progression, including but not limited to the progression of prostate carcinogenesis. Also, the loss of Id4 expression follows the same pattern observed in various other cancers suggesting that loss of Id4 gene expression is linked to promoter hypermethylation (36, 38, 52, 53) suggesting the possible role of Id4 may play in prostate cancer progression in regards to and hormone regulation.

Studies suggest that Id4 may acts as a tumor suppressor; however direct evidence demonstrating this effect and its potential molecular mechanism is not known. Literature suggests that the loss of Id4 expression in a number of cancer cell lines is a direct result of promoter hypermethylation. The relationship between Id4, androgen receptor expression, and AR response genes may suggest a unique paradigm shift in Id biology, in regards to its tumor suppressor capacity. This warrants extensive research to explore the Id4 associated molecular events in relation to prostate cancer. The question still remains, what could be the potential mechanism of action of Id4? Possibly, Id4 may in act as an anti-Id (Id1, Id2 and Id3) molecule, at multiple levels. These levels may include transcriptional regulation (expression of bHLH regulated genes such as p16, p21, p27, E-
cadherin) and/or regulating the protein stability of key cell cycle genes. 1d4 may also act as a standalone tumor suppressor gene capable of regulating specific cellular mechanisms to illicit a tumor suppressive response, in cooperation with other tumor suppressor genes.

Therefore, we hypothesized that 1d4 plays a critical role in the advancement of prostate cancer from an androgen dependent to an androgen independent state by regulating key cancer specific pathways and acting as a putative tumor suppressor. To test this hypothesis, the following aims were investigated:

1. Determine the expression and correlation of 1d4 with androgen dependent to androgen independent prostate cancer progression
2. Determine the molecular mechanism of action of 1d4 in prostate cancer by investigating androgen regulated pathways.
3. Investigate the tumor suppressor role of 1d4 by studying cell cycle regulators, apoptotic activators, proliferation markers and metastasis related mechanisms, in prostate cancer cell lines.
4. Evaluate whether a correlation exists between 1d4 expression and response of prostate cancer cells to chemotherapeutic drugs and anti-androgens.
CHAPTER TWO

REVIEW OF LITERATURE

1. Prostate Development

The development of the male reproductive tract is dependent upon mesenchymal-epithelial interactions and fetal androgens (54). In the fetus, testosterone stimulates budding of prostate epithelium from the urogenital sinus which results in the production of the growth factors, sonic hedgehog signaling and activation of the underlying mesenchyme (55). During prostate development, when branching morphogenesis occurs the urogenital mesenchyme expresses high levels of the Androgen Receptor (AR). Paracrine growth factors from the mesenchyme are responsible for glandular morphogenesis and epithelial growth in the developing prostate. During maturation of the prostate AR is localized to the secretory cell layer (56). In the adult prostate, androgens act on the stromal and secretory epithelial cells.

A glandular epithelial and a fibromuscular stroma compartment constitute the structure of the normal adult prostate. Both compartments of the adult prostate have a slow rate of turnover with balanced levels of proliferation and cell death (57). Zonal anatomy of the prostate forms the basis for describing the location of neoplastic processes in the prostate. The glandular part of the prostate is composed of a large peripheral zone and a small central zone which constitutes ~95% of the gland. The transition zone and the periurethral gland constitute the remainder of the prostate. The
glands that constitute the central zone are morphologically distinct as a result of their embryonic origin from the Wolfman duct. Approximately 60-70% of prostatic cancers originate in the peripheral zone, and 10-20% in the transition zone however only 5% of cancers originate from the central zone. Benign prostate hyperplasia (BPH) develops mainly from the periurethral stroma and glands of the transition zone (58).

The androgen receptor is required in normal prostate development and function (59). Androgen action acts through an axis involving testicular synthesis of testosterone which is then converted by 5α-reductase to the more potent form 5α-dihydrotestosterone (DHT). Testosterone and DHT both bind to AR to induce transcriptional activity. The transcriptional activation of AR is modulated by coactivators and phosphorylation of AR in response to growth factors (60).

The prenatal development of the prostate is dependent upon androgens, specifically DHT. At about 10 week gestation, the ductal structure of the prostate arises from epithelial outgrowths of the urogenital sinus, and moves to the surrounding mesenchyme below the developing bladder. The 5α-reductase enzyme is present in the urogenital sinus before and during prostate development (61, 62). Absence of a functional 5α-reductase enzyme results in a small or undetectable prostate (63). The partial formation of a prostate in response to low levels of DHT suggests that a threshold of DHT is required for complete prostate morphogenesis (64). The initial prostate development is dependent upon functional AR and the prostate is absent in AR knockout mice, testicular feminized and individuals with complete androgen insensitivity (65). The formation of prostatic buds requires the presence of functional AR in the urogenital mesenchyme but not in the epithelia, suggesting that growth factors are secreted by the
mesenchyme and act upon the developing prostate epithelium (59). Prostate epithelium expresses AR in late fetal or early neonatal development when AR is implicated in the final morphogenesis of the prostate and the initiation of prostate secretory protein expression (66-69). The discussion began in the late 1960s, regarding the hypothesis that the androgen receptor might be necessary to mediate biological function of androgens, which resulted in numerous attempts to isolate the androgen receptor. Structural analysis of AR revealed that it contains four functional domains, similar to other members of the steroid receptor superfamily: a conserved DNA binding domain (DBD), a hinge region, a ligand-binding domain (LBD) and a less conserved amino acid terminal domain. AR also has two transcriptional activation functional domains (59).

After the development of the prostate, androgens continue to function by promoting the survival of secretory epithelia. This is the primary cell type thought to be transformed in prostate adenocarcinoma (70). In the normal prostate the rate of cell death is approximately 1-2% per day, which is balanced by a 1-2% rate of proliferation (71). The reduction of serum and DHT by castration results in a loss of 70% of the prostate secretory epithelial cells due to apoptosis, However, the basal epithelia and stromal cell populations are unaffected by castration (72). In the rat prostate, secretory epithelial cells show strong AR immunoreactivity but basal epithelial cells are AR negative (73). Normal epithelial function is dependent upon prostatic DHT levels. In humans the increase in proliferation in response to circulating androgens occurs mainly in the transitional zone of the prostate. This region is primarily affected in benign prostatic hypertrophy but rarely results in prostate carcinoma formation (74).
Epidemiologists have failed to find a correlation between elevated serum testosterone, DHT or adrenal androgens and prostate cancer risk (59). Castration also results in apoptosis of prostatic capillaries and constriction of larger blood vessels resulting in the reduction of blood flow to the prostate causing epithelial apoptosis. In the normal prostate homeostasis is modulated partly by paracrine growth factor regulation between epithelial and stromal cells.

2. **Prostate Cancer Disease and Progression**

The incidence of prostate cancer in the western world has a 10-fold increase versus Eastern Asian countries. Studies of immigrants in the western world allude to only partial correlation between genetic factors and prostate cancer incidence. Therefore, western diet is highly suggestive as being responsible for prostate cancer development. Current literature suggest that western diet may be low in protective factors i.e. micronutrients such as, vitamins like folate, phytoestrogens, antioxidants and lycopene. Epidemiological studies have discovered that major constituents of the western diet such as high fat, dairy, and red meat consumption share correlation with increased risk of prostate cancer.

Prostate cancer has become a major health problem in Western industrialized countries. The incidence of prostate cancer dramatically increases in the 7th decade of life. Prostate cancer can be an indolent, latent disease that will not result in clinical symptoms during the lifetime of an elderly patient. Conversely, prostate cancer can present in an aggressive form, spreading to the seminal vesicles, bladder and rectum and metastasizing to the lymph nodes, bone, lung and other organs (75).
Implementation of prostate cancer markers (prostate specific antigen (PSA) in diagnosis and current treatment regimens have allowed for the regression in the incidence of prostate cancer. An increasing number of prostate cancers are detected through elevated serum PSA levels. PSA screening has standardized a latent slow progressing disease and has helped in early detection of prostate cancer. Previously, many cases of prostate cancer were recognized by clinical symptoms or Digital Rectal Examination (DRE). This method of detection resulted in the discovery of advanced tumors that had extended beyond the organ capsule or had metastasized. Typically, PSA levels are detected at a range of 2.5-10 ng/mL in prostate cancer samples confirmed by histology of biopsies. Although PSA detection is very sensitive, it is an imperfect system for detecting prostate cancer (76).

Prostate cancers that develop in the peripheral zone are classified as adenocarcinomas. Prostatic Intraepithelial Neoplasia (PIN) is the earliest prostate cancer precursor, characterized by thickening of the epithelial layer and loss of basal and secretory layers. Although the loss of the basal layer is an important transition, prostate carcinoma cells carry basal and secretory cell markers (cytokeratins, AR and PSA). High grade PIN is an accepted precursor of carcinoma with an intact basal membrane. The proliferation rates of cells in PIN and prostate carcinomas are low. The following increase in cell number results from a decrease in cell apoptosis. One third of prostate carcinomas become locally invasive and spread beyond the tissue capsule and in some instances metastasizes to the lymph nodes, and distal organs (bone, liver and lung) (77).

Radical prostatectomy or radiation can cure organs-specific prostate cancers. Androgen ablation therapy by orchiectomy and androgen receptor antagonists can be
used to alleviate this metastatic disease. Early studies into prostate cancer development focused on androgen independence and insensitivity as the main issue into disease progression. In the 1940s it was discovered that androgen depletion by orchiectomy improved the symptoms of prostate cancer. In the 1990s, using colorectal cancer as a paradigm, it was proposed that human cancers are frequently caused by accumulation of multiple genetic alterations (point mutations and chromosomal alterations), that are responsible for inducing proto-oncogenes and inactivating tumor suppressor genes (78).

Prostate cancer is a hormone refractory disease, characterized heavily by first the induction of AR activity, and then by the loss of AR to an androgen independent status. Some evidence suggests a hereditary role of prostate cancer. Zinc phosphodiesterase ELAC protein 2 (ELAC2) and 2-5A-dependent ribonuclease (RNASEL) may share a role in heredity prostate cancer. Polymorphisms in genes related to hormone response, cell protection, DNA repair and nucleotide metabolism have all been suggested to play a role in prostate cancer progression.

Chromosomal alterations resulting in specific gene amplifications have been characterized in various cancers. The frequency of structural chromosomal alterations increase at advanced stages of prostate cancer. Several chromosomal regions are responsible for chromosomal alterations, including 5q, 6q and 17p (39). Tumor suppressor gene inactivation plays a crucial role in progression of several cancers. The most notable tumor suppressors which are inactivated, silenced or mutated in cancers are p53 and RB (79). Specifically, p53 and Phosphatase and tensin homolog (PTEN) are involved in progression of prostate carcinoma (80, 81). Chromosomal alterations and point mutations are responsible for functional inactivation of p53 in prostate cancer. Point
mutations have also been reported for PTEN that result in transcriptional reduction of expression. The Rb gene is located at 13q14, which is a chromosomal region frequently deleted in prostate carcinoma suggesting that tissue specific tumor suppressor genes play key roles in prostate carcinoma (82).

NKX3A is a candidate tumor suppressor gene expressed exclusively in the prostate. It is induced by androgens and stimulates the transcription of prostate specific genes. The inactivation of NKX3A in knockout mice inhibits maturation of the prostate and induces hyper proliferation. However, in prostate cancer, loss of NKX3A is not through promoter hypermethylation or mutations in the gene, a classical indicator of tumor suppressor inactivation.

Oncogenes play a crucial role in the development and progression of prostate cancer. MYC is a gene frequently overexpressed in many cancers, usually associated with increased gene copy numbers by chromosomal gains or amplifications. The anti-apoptotic protein Bcl-2 is overexpressed in about half of all prostate cancers, and even more frequently in androgen independent cases. In normal cells, the cell growth pathway initiated by MYC is inhibited by pro-apoptotic action of p14ARF1, p53 and Bax. MYC expression also leads to increased growth factor expression (EGFR, MET, FGFR2c, ET1A and ERBB2). Endothelins and local paracrine signals play a crucial role in bone metastasis of prostate carcinoma. Over expression of EGF is partially contributed to chromosomal gain, which plays a crucial role in prostate cancer metastasis (83).

Alterations in specific ‘cancer pathways’ that regulate various cellular processes including cell proliferation, differentiation, survival, senescence, and cell-cell interactions, are essential to the development of all cancers. Therefore, the progression of
prostate cancer requires the complex rearrangement of several cellular pathways for progression. These pathways include RAS mutations or transcription factor MYC induction. One specific cancer pathway is the WNT signaling pathway (84). In colon cancer WNT signaling stimulates proliferation and confers tissue stem cell phenotype. WNT signaling involves the induction of Cyclin D1 and MYC. Several cell cycle regulators also play a key role in this pathway including p16, RB and p53 (85). Also, downregulation of p27kip1, which is expressed in the normal prostate epithelium and retained in some carcinomas, is frequently observed in many cancers. The inhibitor of Cyclin dependent Kinases, p27kip1 coordinates cell cycle progression and helps to establish terminal differentiation. MYC stimulated phosphorylation of p27kip1 results in protein degradation allowing for the inactivation of a cell cycle checkpoint (86).

In the normal prostate, a careful balance between proliferation and apoptosis allows for a stable environment. Androgens assist in balancing this equilibrium. Androgen ablation is responsible for an influx in prostate gland apoptosis, particularly secretory epithelial cells. Similarly androgen ablation activates apoptosis in androgen dependent prostate cancer cells which is why androgen ablation is the primary treatment for early stage prostate cancers. Bcl-2 is an important component of prostate cancer progression and is not expressed in normal secretory epithelial cells but is upregulated in the epithelium starting with PIN. In androgen dependent cell line LNCaP, Bcl-2 expression is androgen-dependent. The growth factors that are upregulated in prostate cancer are also responsible for decreased apoptosis. FGF-7 decreases apoptosis and prolongs cell survival in prostate carcinoma cell by increasing Bcl-2 expression. Insulin
like Growth Factor-1 (IGF-1) activates PI3K/AKT pathway and stimulates the expression of BCL-like proteins and suppresses BAX (87, 88).

Immortalization of cells in prostate tumors is essential to escaping the limits of replicative senescence, which requires the inactivation of p53, Rb and the emergence of telomerase activity. To achieve this confirmation decreased cell adhesion, increased motility, altered interaction with the extracellular matrix and enhanced angiogenesis are all essential to this process. Inactivation of many tumor suppressor genes through promoter hypermethylation plays a contributory role to prostate cancer progression. Inactive chromatin tends to attract DNA methylation. ‘Global hypomethylation’ as it is known is closely related to tumor progression (89, 90). Hypomethylation is frequent in high-stage and lymph node positive carcinomas and ubiquitous in metastatic carcinomas recurring during androgen depletion therapy. Coincidentally, several chromatin proteins are differentially expressed in prostate cancer including coactivators and corepressors interacting with AR (91).

3. AR Role in Prostate Cancer Development

The activity of AR and AR modulators is an essential aspect of prostate cancer. Approximately 80-90% of prostate cancers are dependent upon androgens at its initial diagnosis (59). Androgen depletion is an essential part of many prostate cancer treatments, despite the frequent reoccurrence of prostate cancer post androgen ablation therapy. AR is expressed throughout prostate cancer progression and persists in the majority of patients with hormone refractory prostate cancer. The mechanisms involving progression of prostate cancer have been extensively studied and are seemingly involved in prostate carcinoma development. In prostate cancer the AR gene can be silenced
through promoter hypermethylation. Several growth factors, FGF7, EGF, and IL6 can activate the AR in a dependent and independent pathway. Somatic AR mutations can alter the receptor making it responsive to estrogens, progesterone and synthetic androgens. Most AR mutations derived during hormone refractory prostate cancer development are capable of transcriptional activity (59). Frequent amplification of AR gene is observed in prostate carcinomas growing under low levels of androgens resulting in increased sensitivity towards minimal levels of androgens (92). AR coactivators are also responsible for mediating affects of AR on chromatin structure and transcriptional initiation. The p53 gene is also responsible for repressing AR function. Loss of AR function is not a major cause of androgen ablation failure. Also, androgen independent prostate cancer cells do not have a significant growth or survival advantage (59). Studies suggest that it is the alterations of the normal androgen axis (AR coregulators and AR mutations) allowing AR to become transcriptionally active in response to ligands other than testosterone and DHT.

In the N-terminal transactivational domain of AR, polymorphic CAG, GGN repeats indicate a small correlation with risk of prostate cancer in relation to specific populations. Overall smaller repeats positively correlate with a slight increase in risk of disease progression. Many polymorphisms have been characterized in prostate cancer. These genes include, BRAC1, BRAC2, MTHFR, and GST. The exact significance of these polymorphisms in relation to disease progression is not clearly understood (59).

Low serum testosterone levels of men newly diagnosed with prostate cancer have shown a correlation with increased AR expression, increased capillary density within the tumor and higher Gleason score (93). High AR expression also correlates with lower
recurrence free survival and disease progression. After either chemical or surgical castration prostate cancer patients usually experience disease progression in a median of 12-18 months. Of the patients studied, 97% of patients presented with hormone refractory metastatic prostate cancer post exogenous androgen treatment which resulted in disease reoccurrence and unfavorable response (94). Therefore it has been established that AR expression is observed in primary prostate cancer and can be detected in both hormone sensitive and hormone refractory cancers (95). Additionally, AR expression also positively correlates with a greater degree of differentiation or lower Gleason score.

Clinical studies involving animal models have suggested that elevated AR expression can initiate the development of prostate cancer. However, AR expression in not necessarily associated with prostate cancer initiation and hormone refractory prostate cancers are not clonally selected from AR negative foci. Loss of AR gene expression is extremely rare and epigenetic silencing of AR expression by methylation has been observed in 8% of primary prostate cancers (96). Another possible explanation of loss of AR expression in some tumor cells is that the decrease in AR protein stability reduces protein levels of AR. Ubiquitination of AR is promoted by Akt kinase-mediated phosphorylation of the receptor correlating with reduced AR protein expression (59).

Androgen ablation by surgical castration results in 90-95% decrease in serum testosterone levels but intraprostatic DHT levels only decline by 50%. However this reduction still causes death of over 70% of normal prostate secretory epithelial cells. Also, prostate cancer cells that survive this treatment are overwhelmed with an abundance of DHT. Although androgen deprivation results in a dramatic loss in the population of prostate secretory cells, studies suggest that prostate cancer cells acquire a
relative resistance to androgen ablation-induced apoptosis early in transformation. Therefore a significant number of prostate tumors are resistant to androgen ablation-induced apoptosis at the time of treatment and the observed therapeutic benefit may be the result of a decreased proliferation rate in tumor cells. Antiapoptotic proteins Bcl-2, Bcl-x and mcl-1 have all been found to show increased expression in Prostatic Intraepithelial Neoplasia, suggesting resistance to apoptosis occurs early on (78, 97). Overexpression of Bcl-2 in LNCaP cells allows for cell growth in androgen depleted media and enhanced tumor formation in castrated male mice (98).

LNCaP cell growth is normally dependent upon androgens however LNCaP cell growth is inhibited at high concentrations (300nM) of DHT. LNCaP derivative cell lines that grow in androgen depleted media can also be inhibited by normal levels of DHT (1-10nM). Also, PC3 derivative cell lines have been shown to undergo growth arrest or apoptosis in the presence of physiological of androgen. It has been proposed that androgen dependent cells surviving androgen ablation adapt to low levels of androgens and eventually become androgen insensitive (99).

Prostate Specific Antigen is considered to be the most sensitive biochemical marker for monitoring the presence of prostatic disease and response to therapy. PSA is a glycoprotein that is a member of the kalikrein family of serine proteases (59). In the normal prostate PSA is secreted into the glandular ducts where it degrades high molecular weight proteins produced in seminal vesicles to prevent coagulation of the semen. During prostate cancer progression, PSA levels increase as a result of aberration of the normal prostate ductal structure by neoplastic epithelial cells. This allows PSA to be actively secreted into the extracellular space and enter the circulation. AR is the
primary regulator of PSA expression, by binding to three androgen response element-containing enhancer elements located within the PSA promoter region. Glucocorticoids, and progestins have also been shown to induce PSA expression (59). Another regulator of PSA expression is a transcription factor, GAGATA binding protein, which can affect androgen-mediated expression of PSA through binding to an alternative enhancer site in the PSA promoter. E-twenty six (Ets) transcription factors, epithelium-specific Ets factor 2 (ESE2), and prostate-derived Ets factor (PDEF) can also induce the transcription of a PSA reporter gene in AR negative cell line CV-1. There transcription factors may play a role in regulating PSA in prostate cancer.

4. Id proteins

Although the Helix-Loop-Helix (HLH) family consists of >200 members in various species with evolutionary relation from yeast to mammals (14), the Id gene family are a distinct subset of proteins in this gene family. The highly conserved HLH region comprises two aliphatic α helices 15-20 amino acid residues long, with a shorter intervening sequence varying in length. This HLH domain mediates homo- and heterodimerization, a necessary component of DNA binding. HLH proteins are generally characterized by their basic DNA binding domain, except for the Id gene family which lacks this basic DNA binding region. These HLH proteins HLH bind predominantly to an ‘E box’ sequence, but can also bind a ‘N box’ sequence (14). Id proteins function solely by negatively regulating the transcriptional activity of transcriptional regulators, primarily but not limited to the bHLH family of proteins. Therefore, Id proteins are dominant negative regulators of bHLH proteins (15, 100). The leucine zipper and homeodomain protein families are also capable of negatively regulating DNA binding of
bHLH proteins (101, 102). Id proteins were first characterized in the early 1990s. Since then they have demonstrated a strong relationship with a number of cellular processes during development. Id proteins are also highly expressed in the transition of normal cells into cancer cells. Some sciences suggest the expression of these proteins supports the theorem of cancer induction being a reemergence of the expression of developmental genes.

Normally, bHLH proteins positively regulate genes during cell differentiation; however this process is severely inhibited by Id proteins. In Drosophila, there is a single Id-like locus that encodes an Id-like gene (emc) (100). We have discussed the four Id isoforms that code four separate proteins Id1-4 (15, 103, 104). Although these proteins have been designated as negative regulators of differentiation, a wider more divers role has been revealed since their discovery over two decades ago (15, 29). These Id proteins range in size from 119 amino acids (Id3) to 199 residues (Id4). Id1 and Id3 display the highest sequence similarity, however Id proteins in general show remarkable divergence (29) in both structures and function via binding partners. The greatest homology of Id proteins are in the Helix-Loop-Helix region however, outside that region is where the greatest divergence occurs. Id1 was cloned in an interaction screen with MyoD as a novel HLH protein (15). Id1 showed an abrogate activation of a muscle specific enhancer through direct interaction with MyoD, E12 and E47. Id2 was cloned in a similar fashion (105). Id3 was identified as a part of the early response genes essential to development (103). The Id4 gene was isolated by PCR cloning using degenerate primers (13). The validated paradigm indicates Id proteins lack transcriptional activity due to the absence of a basic DNA binding domain. The amino acid residues in the loop region of the helix-
loop-helix protein and the adjacent protein sequence are critical determinants for binding specificity of bHLH dimerization. Id proteins show different expression patterns suggesting an isoform specific role for individual Id proteins. Id protein expression is high in developing tissue and proliferating cells but absent in terminally differentiated tissues (106, 107).

Id proteins are immediately induced in response to growth factor induction. Id proteins play an essential role in G1-S-phase transition and their expression is regulated in a cell cycle dependent manner (17, 24, 25, 103, 106-109). Id proteins hetero dimerize with several classes of bHLH proteins as well as non bHLH proteins. These proteins are ubiquitously expressed and have no nuclear cellular localization signal (15, 31).

Continually, the diverse role and functions of Id proteins in regulating several cellular processes including, cell cycle, proliferation, development, apoptosis and senescence, is slowly unraveling behind the veil of obscurity. The dominant negative regulatory role of Id proteins allows them to target diverse pathways with high efficacy. The exact importance of these non DNA binding transcription factors remains the emergent question to tumor biology and cancer progression.

5. Id Proteins in Development

Inhibitor of differentiation proteins have been recognized as regulators of epithelial cell growth, differentiation and invasion since 1999 (110). Id proteins are a small family of short proteins usually ranging from 13-20 kDa. They function as dominant negative regulators of basic helix-loop-helix (bHLH) transcription factors (15). Many organs and cell types have been shown to require Id proteins for normal development (111). During development cells proliferate rapidly and differentiate into
various living systems. Normal development is controlled by extracellular signals and genetic programming. During development Id proteins act as antagonists to other helix-loop helix transcription factor proteins. A major aspect of Id protein function is linked to cell cycle regulatory function (26). The four members of the Id family that have been identified are Id1-4 (15, 17, 103).

In Drosophila the emc (extra macrochaetae) locus is required for many developmental processes including sensory organ development and morphogenesis. Loss of emc expression is lethal in Drosophila, but the absence in gene expression through gain-of-function mutants has elucidated a role in commitment, differentiation and cell proliferation. EMC also hetero-dimerizes and antagonizes several bHLH proteins (29, 100). In Drosophila a balance must be obtained between EMC proteins and its bHLH targets to successfully regulate cell fate determination (112), more notably during Drosophila wing morphogenesis. Ras-signaling protein cooperates with EMC during cell proliferation but antagonizes its role during differentiation (113-115). A strong relationship between emc and genes of the Notch signaling pathway, have been extensively characterized. Notch signaling is an essential aspect of cellular response to developmental signals, however, the exact interaction between emc and notch have yet to be elucidated. This suggests a developmental relationship between Ids, Notch and Ras.

Studies have shown that bHLH transcription factors are of cellular differentiation in many tissue and cell types including, muscle and neuronal tissue (116, 117). The normal protocol of bHLH interaction involves the hetero-dimerization of a tissue specific bHLH and results in transcriptional activation and promotion of cell differentiation. Id proteins usually, de:er this function, differentiation, due to the absence of the basic
domain in these bHLH proteins. Id proteins usually dimerize with the ubiquitously expressed bHLH proteins (15). The use of gene targeted mice designed for overexpression/knockout studies have successfully highlighted the role of Id proteins in developmental and cancer biology (118-120). Loss-of-function studies provide insight into the regulatory roles of Id proteins in organ development (120). These studies have also been continued in vitro using culture systems (111) providing further insight into the molecular mechanism of action of Id proteins.

During development, all members of the Id family are expressed in many organs and tissues, with overlapping and distinct functions at various stages of development localized to specific regions (121-123). This reflects the importance of Id proteins in normal development and differentiation. Although Id1-3 have overlapping expression and functions during development, Id4 expression is uniquely distinct during embryogenesis (111, 122). It is also noted that tissues that undergo epithelial to mesenchymal transition, strongly express specific Id genes, particularly Id1 and Id2 (111) as observed in the salivary gland, kidney and lung (121, 123, 124). Although these genes are important in development it has not been proven that inactivation of a single Id gene is sufficient to cause fatal developmental defects. This may be due to the overlapping functions of Id proteins.

E12/47 is a gene spliced protein derived from the E2A gene. This bHLH protein is seen as the preferential binding partner of the Id gene family (5, 47, 118). The activity of these proteins is inhibited by the binding of the Id proteins specifically, Id1 and Id3 (111). E2A-null mice result in a significant increase in T-cell lymphoma, and an increase in neonatal death. When the Id1 activity is inhibited in the absence of E2A, the incidence
of neonatal death and T-cell lymphoma occurrence is severely decreased (118). However E2A/Id1 knockout mice still develop T-cell lymphomas, possibly due to a functionally redundant role of Id3 (47). These studies show that E2A/Id1 expressions are essential for T-cell development.

Id2 expression is an essential aspect of lymphatic organ development (119), proving that Id2 is essential for the normal development of mice. Id2 mice lack lymph nodes and Peyer's patches. Id1 and Id3 are essential for neurogenesis (125), angiogenesis (126) and tumor vascularization, supporting their role in both development and cancer biology. Loss of Id2 expression also negatively affects the development of the olfactory bulb. Id1/Id3 knockout mice present with severe brain deficiencies (125). Angiogenesis and invasion were the hallmark processes severely inhibited in these knockout mice. Id2 expression also appears in presumptive neurons which are undergoing maturation in the developing cerebellum and cerebral cortex (3, 127).

In the overexpression studies, constitutive expression of Id1 impaired mouse B cell development (111, 128). During B cell development Id gene function is downregulated upon initiation of differentiation. The generation of tissues such as cartilage, ganglia and cranial bone are all heavily dependent upon Id2 expression (129). Consequently, it has been shown that overexpression of Id1 in mice resulted in adenomas (130). Also, overexpression of Id2 draws a consistent link to inhibition of T-cell development (131). The overexpression of Id2 by a retrovirus induces the disappearance of ectoderm overlaying the neural tube which causes these cells to be converted into neural crest cells. Contrarily, Id overexpression in the mesenchyme has no effect. Therefore, Id2 has essential functions in neuronal cells, and mammalian embryos. Both
Id2 and Id4 show a similar expressing pattern in migrating post-mitotic neurons (127) which marks the divergent properties of the Id proteins, particularly the redundant functions of Id1 & Id3 compared to Id2 and Id4. Interestingly, the proliferative potential of Id genes can be neutralized by expression of the RB protein (1).

Studies have suggested that the subtle effects observed by the loss of the expression of a single Id gene, may be the result of functional redundancy of Id proteins, specifically Id1/Id3 (111). No direct link has been observed between loss of Id1/Id3 expression and induction of apoptosis in neuroblast cells. However, Id2 is an essential part of oligodendrocyte differentiation and loss of Id2 also decreases the proliferation rate in these cells. Interestingly, Id2 expression is located in the nuclease but translocates to the cytoplasm during differentiation induction (111).

BHLH transcription factors also play a pivotal role in the development of the immune system. Id proteins are important regulators of the immune system during development of lymphoid organs and NK cells. There are several genes involved in NK cell development and Id2 is a crucial aspect of this development that allows B and T cells to develop normally. For example, in Id2 -/- mice the number of NK cells was reduced by 90%. However, the NK cells developed in Id2 -/- mice were still functional. Studies show that Id proteins interact with Ets transcription family proteins and suppress their function. The Ets-Egr1-Id transactivation cascade is a paradox involving Id1-3 regulation. As a result, Ets proteins are direct upstream regulators of Egr1 and regulate the cascade. Therefore Id2 may be regulated by ETS-1 via the Egr1 in developing NK cells (111, 132). Id1 and Id3 are no longer expressed in the brain of adult animals but Id2 expression
persists in specific areas of the brain, particularly the cerebellum and the olfactory bulb (126).

Id4 specifically, is selectively expressed in the nervous system during development suggesting a very different role of Id4 compared to other Id proteins during development. Id4 -/- mice have a prominent phenotype. The brains of these mice are severely transformed and the hypothalamus is reduced in size and is disorganized. The lateral ventricles are also greatly enlarged by the absence of Id4 in these mice (111). Oligodendrocyte production is also severely reduced in the brain of these mice. The embryos of Id4 -/- mice do not differentiate in vivo (133), solidifying the essential function of Id4 in oligodendrocyte development. Id4 -/- neural precursor cells proliferate more slowly than their wild type counterpart. Id4 is an essential regulator of neural stem cell proliferation and fate determination. Id4 is also an intracellular timer in regards to oligodendrocyte differentiation. Oligodendrocyte precursor cells (OPCs) express all members of the Id family however, only Id4 shows differential expression depending on cell state. Retrovirus induced overexpression of Id4 in OPCs results in increased proliferation and impaired differentiation. Id4 -/- mice also suffer a frequent occurrence of embryonic lethality. Using ICC studies in OPCs Id4 is localized to the cytoplasm and no nuclear translocation has been observed (134). Id4, unlike the other Id proteins is localized exclusively in the CNE and peripheral nervous system. Its pattern of expression is also complimentary to Id1 and Id3 and found in regions undergoing neuronal maturation (3, 17, 122).

In developmental studies Id protein in vitro model systems were used to study inhibition of differentiation and stimulation of proliferation. As we have shown, Id
protein expression is crucial to the development of a number of organs and tissues. Id protein expression varies depending on the particular cell type. Id proteins can regulate bHLH transcription factors as either one-way inhibitors or bifurcation types. Inhibition of differentiation of neuroblast cells until the appropriate time point is one example of one way inhibition of Id proteins. Bifurcation inhibition is seen in NK cell development and although Id proteins are master regulators of bHLH transcription factors, Id proteins can regulate a wide variety of non bHLH transcription factors at the protein level including, MIDA1 (135), Pax 2, Pax 5, Pax 8 (20) Elk-1 (136), c-ETs-1 (137)and Retinoblastoma protein (4, 7). Verifying that Id proteins are essential to development of normal tissue. However, the impact of the expression of this family of proteins in cancer progression is still under investigation.

6. **Id proteins in Cancer**

Aside from cell fate determination, Id proteins are also key regulators of cell growth and proliferation. As a result Id protein expression is required for cell cycle progression in various cell models. Both quiescent (29) and senescent (34, 46, 138) cells express low levels of Id proteins. These cells are non-dividing, and the absence of Id expression (in particular Id1 and Id3) suggests a causative relationship between Id expression and cell cycle progression. When quiescent fibroblast cells undergo mitogenic stimulation, Id expression is induced within 1-2 hours as a part of an ‘early response gene’ cascade in. After an initial increases in Id1 expression, its expression is sustained throughout the G1 phase of the cell cycle and is upregulated as cells enter the S phase (25). Id proteins essentially regulate cell cycle function throughout multiple stages of the cell cycle by modulating the transcription of several known target genes. Id
proteins also interact with essential non-bHLH (pRB, p107, p130) cell cycle control genes (139). The involvement of Id2 and pRB and its “pocket proteins” p107 and p103 has been extensively characterized (4). There is also some evidence to suggest that Id4 shows involvement with pRB and the pocket proteins; however, Id1 and Id3 show no interaction with pRB highlighting the distinct regulatory roles of various Id proteins. Id1 also associates with MIDA1 (mouse ID associated protein 1). This gene is required for cell growth in erythroblastemia cells (140). In these cells Id1 association is essential for MIDA1 function in the cell cycle.

Id proteins have been shown to regulate a myriad of cell cycle genes including, p21^{Cip1/Waf1}, ERG1, and TCF proteins (see appendix figure 4). During the progression through the cell cycle, Id proteins downregulate the expression of early response genes, c-fos and erg-1, by antagonizing the SAP-1 and ELK-1. These ETS domain proteins are components of the ternary complex factor (TCF). Id1 alone has the ability to upregulate the expression of EGR-1 (141-143).

Another aspect of Id proteins transcriptionally regulating cell cycle control genes involves the interaction of Id proteins (specifically Id1) with E2A gene. E2A stimulates p21^{Cip1/Waf1} expression. Expression of Id1 leads to the repression of E2A resulting in a downregulation of p21^{Cip1/Waf1}. This results in the upregulation of the Cyclin A/E-CDK2 complex responsible for phosphorylation of pRB. Restoration of E2A encoded-E12/E47 can lead to growth arrest or apoptosis(47) (see appendix figure 5). This releases the E2F1-DP1 complex allowing progression through the S-phase and allowing the cells to continue through DNA synthesis. Id2, Id3 and Id4 are substrates for CDK2 dependent phosphorylation during late G1/early S-phase (25, 142). Id1 however is not a substrate
for CDK2 dependent phosphorylation. The phosphorylation of Id proteins, Id2 and Id3, alters their dimerization specificity thus affecting their ability to effectively regulate cell cycle progression. Id2/Id3 mutants lack the CDK2 phosphorylation sites and resulted in S phase arrest and cell death (144). The phosphorylation of Id proteins suggests a regulatory control of Id genes during late G1. However, the phosphorylation status of Id genes beyond G1 is still unknown. Deletion of E2-2 gene results in a high percentage of embryonic lethality (145, 146). E2-2-null mice suffer postnatal death within one week. HEB null mice die within two weeks after birth.

Class B bHLH transcription factors include MyoD, MRF4, Myf-5 and myogenin. These proteins have tissue specific profile expression patterns. Deletion of specific B class bHLH proteins is lethal in particular developmental stages. The leucine-zipper family of bHLH transcription factors includes Mad, Max and Mxi. In these proteins the leucine zipper domain is adjacent to the HLH-motif. This domain plays an integral role in carcinogenesis (147, 148). As indicative of their name the bHLH-PAS proteins contain a Per-Arnt-Sim (PAS) domain (see appendix figure 2). These proteins include AHR, Arnt and Sim. Although the etiology of these proteins is not clearly understood, these proteins have etched a role in transcriptional response to exposure to environmental toxins and hypoxia.

Id proteins are an essential component to the immortalization of fibroblast cell lines (109), particularly rodent fibroblast when transfected with Bcl-2 (29). The debate is still ongoing about the exact dominance of Id genes in cancer as either supporting oncogenes or dominant oncogenes. There is sufficient evidence to support both theorems. Id proteins show regulatory roles at a multitude of levels in the cell cycle through
transcriptional regulation of various classes of bHLH genes. Id1 can also immortalize human keratinocytes (149) by inactivating pRB function and activating telomerase activity. Id1 expression is also associated with mammary epithelial cell invasion of basement membranes (150, 151). Enforced Id1 expression is also associated with intestinal adenoma development (130).

Many tumor suppressor bHLH proteins are antagonized by Id gene activity, including E2A activation of p21\textsuperscript{Cip1/Waf1} induced growth arrest. E12/E47 restoration leads to growth arrest and apoptosis in the absence of Id genes (152). Although much of the Id bHLH interaction centers around Class A, E proteins (E2A, E2-2 AND HEB) and their tumor suppressor capabilities, which include Id inhibition of HEB knockout mice leads to T cell tumorigenesis (153, 154). E proteins are important regulators of cell growth and differentiation. Id proteins show an ability to block the tumor suppressor function of E2A encoded genes (which are the primary binding partners of Id genes). The E proteins have been shown to be growth suppressive only as hetero-dimers but not as tethered homo-dimers. This suggests that the inhibition of E proteins to form hetero-dimers with other bHLH proteins by Id proteins may cause growth promotion through an E protein homo-dimer complex (126, 155). The Id proteins are crucial to tumorigenesis induction, however, forced expression of MyoD and E47 resist the inhibitory effects of Id1 and Id3 (156, 157). Suggesting that although Id proteins may have a binding affinity for E proteins, E proteins may have other preferential binding partners.

Transcriptional targets of bHLH proteins include differentiation specific proteins as well as factors that induce cell cycle arrest most notably the Cyclin dependent Kinases (CDKs). Several E-box motifs have been identified in the promoter of p21\textsuperscript{Cip1/Waf1}.
p15\textsuperscript{INK4B} and p16\textsuperscript{INK4A} and are activated by bHLH transcription factors (13). Id proteins are one class of bHLH proteins that negatively regulate transcriptionally activity of bHLH proteins. OUT, MyoR and eHAND are also negative transcriptional regulators of bHLH proteins. Unlike Id proteins, these proteins possess the basic DNA binding domain however, they do not activate transcription through DNA binding hetero-dimers (13, 158, 159).

The other Id regulated cell cycle genes include MYC, Cyclin D, Cyclin E, and E2F1. Solitary ectopic expression of many of these genes plays a crucial role in apoptotic induction, and cell cycle regulation. Some of these proteins also display oncogenic properties, particular MYC. Some studies have suggested that Id proteins play a role in apoptosis induction (160). Id2 induced apoptosis is independent on bHLH dimerization, the typical characterization of Id proteins. The Id2 protein thus far, has shown the most divergent in function and capacity to interact with various non bHLH proteins. Particularly, Id2 induced apoptosis is linked to enhanced Bax protein expression (8) a known regulator of the apoptotic pathway.

A number of immortalized cell lines display increased Id mRNA (28) and is a key mediator of tumor cell biology, including but not limited to primary tumors. Elevated Id levels have been reported in various primary tumors including pancreatic tumors (161, 162), Astrocytic Tumors (163, 164), neural tumors of endothelial cells (125), breast carcinomas (110, 165), Colorectal adenocarcinomas (29) and multiple myelomas (29). The correlation among diverse tumor types has been extensively characterized, and the loss of Id expression in tumor cell lines suppresses cell growth (25, 161). These observations lead to a causative link between Id gene expression and tumorigenesis. The
exact reason for induction of Id gene expression in tumor biology is not completely understood. However, it is presumed that Id genes are regulated through upstream signaling pathways. Therefore, we pose the question, are Id proteins a causative indication of cancer progression, or an active induction of advance metastatic cancer machinery?

In primary breast cancer, Id1 expression is associated with invasion and more aggressive tumors (165) highlighting the link between Id and disease progression. Separate studies support the invasion of mammary epithelial cells on the basement membrane. Also, constitutive expression of Id1 in a non-aggressive breast cancer cell line induces a more aggressive phenotype. These studies highlight the invasive properties of Id1 specifically. In breast cancer, Id1 can also regulate steroid-hormone response growth of breast cancer cells. This correlation between cancer disease and hormone regulation can also be found in prostate cancer cells that gain androgen independence in response to Id1 expression (166), suggesting that Id1 is a marker for aggressive breast tumors. Mice lacking the (Id1+/− Id3+/+) showed significant regression in some tumor types. Id1/Id3 deficient mice show an inability to support metastasis and tumor growth (31).

Tumors of diverse histological origins share the same characteristics in Id deficient mice including, a defect in growth and metastasis, concomitant hemorrhage, necrosis and tumor regression. Tumors that range from neural precursor cells to infiltrating vascular endothelium cells share increased Id1 and Id3 expression (125). The regulation of growth and angiogenesis on tumor cells is highly dependent on intracellular levels of Id proteins. Id proteins remain high in tumor neovasculature (167) and drives
the “angiogenic switch” required for the progression from micro- to macro-metastases (168, 169). Therefore, it has been suggested that small molecule based inhibitors of Id proteins may pose a beneficial therapeutic drug treatment of human cancers (125, 165). However, the ability to generate Id targeting drugs is still under debate.

Several studies indicated how subtle changes in heterodimeric equilibrium between bHLH and Id proteins can cause severe changes in cell fate determination (153). Id proteins interact avidly with the four E proteins. Id proteins have distinct targets for their preferential E protein binding partner (107, 170). Crossing Id3-/- but not Id1-/- mice strains rescue post natal lethality of HEB-deficient mice. (153). Although Id proteins preferentially bind bHLH transcription factors and negatively regulate their ability to bind to DNA, they can also act as transcriptional activators. Id proteins fused to heterologous Gal4-DNA binding can activate Gal4-dependent transcription (171). This transcriptional induced expression is a forced profile it is unlikely a natural occurrence. However, Id proteins may bind bHLH proteins as a multicomponent transcriptional complex.

The list of non bHLH interaction partners of Id proteins has incrementally increased over the past decade. We have established the interaction between Id proteins and various cell cycle regulators (e.g. pRB, MIDA1, ETS transcription factor). Additionally, members of the paired homeobox family of transcription factors (PAX-2, PAX-5, PAX-8) associate in vitro and in vivo with Id1-Id3. Id proteins disrupt PAX-5 DNA binding and activation of PAX-responsive genes. ADD1, a bHLH-leucine-zipper transcription factor, also interacts with Id2 and Id3 (172). Although, present studies
elucidate the diverse promiscuity of Id protein interactions with various non-bHLH proteins, the in vivo role of Id-non bHLH interaction is still unknown.

Id gene expression responds to various cell surface, ligand-receptor interactions. However, the exact mechanism of these upstream signals that regulate Id expression is still unknown. Id regulation occurs at the post transcriptional level through cell cycle linked phosphorylation and ubiquitin-proteosome degradation of intracellular Id protein levels (171). Id proteins have rapid turnover with a half-life of 20-60 minutes (144). Of all the Id proteins, Id4 is the least sensitive Id-isoform to proteosome ubiquitination. However, evidence suggests that Id4 degradation may occur through an alternative pathway. When Id3 hetero-dimerizes with bHLH proteins it extends the half-life and enhances the degradation of its heterodimeric bHLH partner. Id proteins are more stable, and less susceptible to degradation by 26S proteosome pathway. Id protein function is also regulated by subcellular localization due to the fact that Id proteins do not possess nuclear localization signals. On the other hand, bHLH proteins possess efficient localization signals allowing efficient transcriptional activation. The lack of Id proteins with nuclear localization signals suggest, Id proteins require bHLH protein dimerization for nuclear sub cellular localization. Co-transfection of Id proteins with bHLH proteins leads to nuclear sequestering of Id proteins (126, 173, 174).

One essential hallmark of the role of Id proteins in cancer progression is the relationship between Id2's interactions with the retinoblastoma tumor suppressor protein (see appendix figure 4). By binding pRB, Id2 is seen as an essential negative regulator of a tumor suppressor genes linked to several cellular processes (e.g. Apoptosis, cell cycle, senescence, proliferation, invasion, cell division etc.)(1, 7, 175-184). In the cell cycle,
hypophosphorylated pRB acts as a repressor or E2F transcription factors at the G1 checkpoint. The pRB pathway is functionally compromised in almost every cancer (185). The tumor suppressor, pRB is targeted by various viral oncoproteins including the high-risk HPV E7 proteins. Similar to Id2, HPV E7 antagonizes the hypophosphorylated form of pRB and suppresses its growth suppressor activity. Id2 is highly expressed in neuroblastoma cell lines, as a result of N-myc gene amplification. Id2 expression can be regulated by myc through myc activated E-boxes in the Id2 promoter. This link is strengthened by the ability of Id2 to block the tumor suppressor function of pRB on c-myc (4). Animal experiments show that the lethal phenotype of RB-/- embryos are rescued by loss of Id2 gene expression (4). Although Id2 overexpression is not as abundant in tumors as either Id1 or Id3, the inhibitor role of Id2 on pRB supports the oncogenic characteristics of Id2.

Id1 overexpression has been detected in a large number of various cancer types and tumor derived cell lines. Id1 expression is also high in various primary human tumors including thyroid cancer, breast cancer, endometrial carcinoma, head and neck tumors, pancreatic carcinoma, esophageal squamous cell carcinoma, cervical cancer and early stage melanoma (162, 165, 186-192). In breast cancer Id1 expression is linked to the aggressive nature of the tumor, due to the correlation in frequency of Id1 overexpression displayed by infiltrating carcinomas versus non-infiltrating carcinomas. Id1 also induces detachment and invasion of murine mammary epithelial cells into an artificial basal membrane (193). There is an extensive link between Id1 expression and breast cancer progression involving increased metalloproteinase, and invasive enzymes during mammary gland involution. Supporting evidence suggests that Id1 has a role as an
oncogene in breast cancer development (151). The role of Id1 in cancer progression extends through many tumor types. An analysis of 20 tumor specimens suggested that Id1 expression might correlate with histological grade and invasiveness of the tumor (13). A study involving 89 specimens showed that increased Id1 expression in early stage cervical cancers was a marker for aggressive tumor growth and poor clinical prognosis. The current scientific paradigm is that Id1 may contribute to tumor formation by inhibition of cellular differentiation and promotion of proliferation and angiogenesis (13, 15, 27, 46, 150, 151).

Several tissue culture experiments support the oncogenic activity of Id1. Id1’s regulation of cellular proliferation may hinge upon its ability to regulate the activity of the retinoblastoma protein, pRB. In experiments using senescent human fibroblast cells, Id1 cooperated with pRB-binding deficient mice of SV40 large tumor antigen to reactivate DNA synthesis (46). This study began the work into the relationship with Id1 and cellular senescence. Id1 was also able to induce immortalization and dramatically increase the life span of human epithelial cells by inactivating p16INK4A and restricting cell growth of human keratinocytes. Id may down regulate p16INK4A through promoter E-box sequences. The role of Id1 protein expression levels have been extensively studied and correlates with the downregulation of p16INK4A via Id1 to regulate proliferation, cell cycle, senescence leading to early carcinogenesis (5, 34, 46, 136, 192).

Overall, most of the current literature supports Id3 as an overlapping redundant function to Id1. However, several studies have linked Id3 to human tumorigenesis. Id3 is overexpressed in several cancers including cervical, pancreatic, prostate and esophageal squamous cell carcinoma (5, 45, 194). Id3 may serve as a better histological marker in
cancer samples as lower grade cancers show decreased expression of Id3 and advanced metastatic cancers readily show increased Id3 expression (12, 107, 141, 145, 170). Id4 localization is not notably correlated with other Id protein expression though immunohistochemical studies or through cell lines experiments. However, a tumorogenic role of Id4 has been noted in breast cancer, where Id4 is a crucial regulator of the expression of the BRCA-1 tumor suppressor (22). Expression of the two proteins is inversely correlated suggesting Id4 may play a role in breast cancer development. However other studies have suggested an opposite of Id4 in breast cancer, particularly the promoter methylation loss of Id4 is a marker for tumor reoccurrence in human breast cancer (37).

Id proteins clearly posses oncogenic activities, though their various capacities to transform tumor growth and progression. Similar to the developmental characteristic of Id proteins, the cancer transforming properties are wide-spread and are involved in most cancers currently under investigation supporting the role of developmental genes as cancer inducers. As cancer involves an augmented proliferative profile, it is natural to observe the increased expression of Id proteins (members of the immediate early response genes) in this transition (31). Id proteins play critical role in differentiation and development and are necessary for cell to reach a mature state. Therefore, is the transition of normal cells to cancer cells, the attempt of mature cells to regain an immature (non-differentiated) status through the use of Id proteins? To support this theory, Id1 expression does correlate with a less differentiated phenotype in cervical cancer and head and neck tumors (13).
Cancer progression is a very complicated process involving the restructuring of several cellular pathways and the ability of these cancer cells to ascertain a mechanism allowing them to proliferating in an uncontrolled matter. One of the most important aspects of cancer progression is the ability of tumors to form new blood vessels, angiogenesis. This process involves the branching and sprouting of capillaries from primary vascular plexus. Signaling of VEGF and Tie-2 has been implicated in angiogenesis both during development and tumor formation (126). The ability of Id proteins to induce angiogenesis is an unglorified aspect of Id proteins' ability to induce cancer progression. This characteristic was first characterized in the forebrain during brain development (125, 126, 167, 195) then during the growth and metastasis in tumors in adults (126, 151, 196). The current studies that support Id1 and Id3 as crucial inducers of angiogenesis during cancer progression include, the co-expression of Id1 and Id3 both temporally and spatially during murine neurogenesis and angiogenesis (126). Loss of Id1 and Id3 leads to a decrease in VEGF expression (125). Angiogenesis in the brain and in tumors require invasion of avascular tissue by endothelial cells. Experiments with Id knockout mice show that the blood vessels of the mice lack the ability to branch and sprout with severely inhibited tumor progression (125). This may be due to a thickening of the extracellular matrix surrounding endothelial cells in the Id knockout mice. MMP2 and avb3 expression were also severely lacking in these blood vessels. Therefore, Id expression may be essential for MMP/integrin regulated angiogenesis.

Id gene expression is abundant in the blood vasculature infiltrating the cells during angiogenesis both in the brain and in tumors. In the mouse embryo, the formation of blood vessels coincides with expression of Id1 and Id3 at E10.5 (126, 197), while Id2
and ld4 are not detected. This detection of ld1 and ld3 continues throughout brain
development in a progressive nature. However their expression levels are downregulated
in the mature tissue, inferably due to the differentiated nature of mature blood vessels. In
contrast to high grade tumors the, no ld1 or ld3 expression was identified in the
vasculature of low grade tumors (126). Tumor angiogenesis requires mobilization of bone
marrow derived precursor cells that upregulate ld in response to VEGF suggesting a
significant distinction from embryonic angiogenesis. VEGF induces angiogenesis by
inducing proliferation, differentiation and chemotaxis of endothelial cells (198-200). ld1
and ld3 promote angiogenesis by inducing mobilization and recruitment of bone marrow
derived cells to tumor vasculature (126). Studies suggest that VEGF induced ld1 and ld3
may be essential to provide an environment suitable to facilitate mobilization of
endothelial precursor cells (CEPs) and hematopoietic precursors. Therefore, although ld
expression is a downstream effector of VEGF induction, its expression is essential to
endothelial induction in tumor angiogenesis.

7. The Role of Ids in Prostate Cancer Progression

Prostate cancer is hormone refractory disease characterized by the transition of
prostate cancer cells from an androgen dependent status to an androgen independent one.
The role of hormones, specifically androgens, is essential to prostate cancer development
and progression. It is has already been established that, elevated levels of ld proteins
have been reported in several malignancies including carcinoma, squamous cell
carcinoma, adeno carcinoma, neural tumors, melanoma, sarcoma, seminoma, and
leukemia (30, 42, 192, 201-208). Advanced metastatic prostate cancer results in a highly
proliferative cell with a poorly differentiated phenotype (48, 209). Therefore, the possible
correlation between Id protein expression and prostate cancer etiology was a logical deduction. Analysis of genetic alterations of Id genes in human tumors have revealed no genetic mutations associated with increased cancer risk (188). The exact mechanism of Id induction has not been elucidated; however several genes have displayed regulatory function of Id protein expression. Several oncogenes are also known regulators of Id expression, including MYC driven regulation of Id2 expression in neuroblastoma cells, B-catenenin may also regulate expression of Id2 expression in colon cancer progression. Gene promoter analysis of Id1 has revealed BMP transcription factor binding sites that allow Id1 induction by BMP-6 (210). Consequently studies have shown that elevated levels of Id1-3 protein has a corresponding effect on regulation of several mechanisms involved in prostate cancer progression that include, androgen independence induction, inhibition of apoptosis, inhibition of senescence and induction of proliferation/angiogenesis (5, 44-46, 211-213).

In normal prostate epithelium and benign prostate cancer, low expression levels of Id1-3 are observed with an inverse, high expression of Id4 (43). Histological tissue samples of advanced, high grade prostate cancer stain for high levels of Id1-3 with low/absent levels of Id4 (see appendix Table 4). Id4 expression is believed to be lost in metastatic prostate cancer through promoter hypermethylation as observed across other cancers (194).

The exact role of all the Id proteins in prostate cancer progression has not been uncovered although; studies are slowly indicating an essential role of Id proteins in this process. There may be some redundancy in Id1/Id3 function during prostate cancer progression although the localization pattern and isoform specific role of Id proteins in
various cancers suggest otherwise. Many of these molecular processes are crucial to prostate cancer progression and have been linked directly to Id1 overexpression, (see appendix figure 8) classifying Id1 as the most studied Id protein in prostate cancer. Id1 expression induces the transition from androgen dependent prostate cancer to androgen independent prostate cancer (214). Id1 is also responsible for induction of angiogenesis through activation by VEGF (215), while proliferation induction is achieved through stimulation of EGFR (214). The cell cycle regulator, p16INK4a is downregulated by Id1 inhibiting senescence (216) (see appendix figure 9). Many factors point to the necessity of unveiling the Id protein mechanism of action in prostate cancer, to better understand prostate cancer progression and resultantly discover viable therapeutic targets.

7.1 The role Id proteins in prostate cancer tissues specimens and cell lines

Id1-3 protein expression correlates with the progression of prostate cancer cells from an androgen dependent, properly differentiated phenotype to a more aggressive, androgen independent, poorly differentiated, invasive/metastatic phenotype (43) (see appendix figure 8). However, Id4 expression observes an inverse expression correlation profile compared to the other Id family members. The loss of Id4 expression in advanced androgen independent prostate cancer is suggested through promoter hypermethylation (194). Continuously, Id proteins are establishing a causative link during prostate cancer progression. From a clinical basis increased Id1 expression is associated with poor survival of prostate cancer patients, suggesting at the very least a molecular marker role of Id proteins in prostate cancer patient prognosis (209). This contributory/causative role of Id expression gains momentum after Id protein expression has been linked to PSA expression (217). Additionally, Id1-inactivation sensitizes prostate cancer cells to
chemotherapeutic drugs (218) highlighting the possible clinical applications and regulation of Id1 as a viable therapeutic target.

At the *in vitro* level, normal prostate cancer cell line and androgen dependent cell lines exhibit low levels of protein expression of Id1, Id2 and Id3 with a high expression of Id4 (194). In the classical androgen independent prostate cancer cell lines, DU145 and PC3, the levels of Id1, Id2 and Id3 are significantly higher than normal prostate epithelial cells (48). Id4 expression is absent in DU145 cells and expressed at very low levels in PC3 cells (194) mimicking the expression levels of these proteins observed during *in vivo* studies. In the terminally differentiated normal and prostatic intraepithelial neoplasia (PIN) tissue samples, Id1 and Id2 expressions are low and increase in expression as Gleason grade and de-differentiation in the tumor increases (48). The Gleason score-5 tissue sample had a mean protein expression of Id1 and Id2 protein expression at 3.5 and 3.82 respectively, compared to 1.49 and 1.62 of tissue samples with a Gleason score of 2. The mean protein expression of Id1 and Id2 in normal tissue was only 0.8 and 1.11 respectively (48).

**7.2 The role of Ids in Prostate Cancer Proliferation/Cell Survival/Apoptosis**

One reason Id proteins show a significant correlation with the transition from androgen dependent to androgen independent prostate cancer is referenced to their responsibility for regulating a number of developmental processes. Leading to the correlation of Id protein expression has being linked to an increased rate of proliferation in a number of cancers (13, 201, 219-224) resulting in a highly proliferative and metastatic state. This observation was confirmed when ectopic expression of Id1 in
LNCaP cells resulted in increased proliferation (212-216, 225-227) and upregulation of several metastatic mechanisms.

Id protein expression is characteristic of a highly proliferative state however normal regulation of Id proteins occurs during the cell cycle through phosphorylation during G1-S phase transition (1, 34). Overexpression of Id proteins in cancer cells causes an increased rate of proliferation with an inability of cells to properly regulate Id protein expression. The increased rate of proliferation of Id1 transfected LNCaP cells correlates with upregulation of a number of cellular regulatory protein including MAPK, VEGF (vasculogenesis and angiogenesis inducing factor), EGFR (cell surface growth factor), Egr-1 (transcriptional regulator), NF-κB (anti-apoptotic gene) and TGF-β (growth suppressor/promoter) (212-214, 225, 228). Whether Id1 is directly responsible for increased proliferation rate or whether one of the downstream effectors is directly responsible for increased proliferation in prostate cancer cells has yet to be determined. However, the induction of Id1 routinely results in the downregulation of CDK inhibitors p16\(^{INK4A}\) but not p21\(^{Cip1/Waf1}\) and p27\(^{kip1}\) (216, 229). These cell cycle regulators are essential to cell cycle progression and their increased expression suggests a shift in the proliferative profile of cancer cells.

Id1 expression is essential to the MAPK-pathway-activated serum independent prostate cancer growth (225). In this model system Id1 induces phosphorylation of Raf-1 and MEK1/2, essential MAPK pathway components, and plays a crucial role in serum independent proliferation. The proliferation rates displayed a significant reduction in Id1-transfected cells in response to MEK1/2 inhibitor PD098059. MEK1/2 inhibition also
reverses Id1 G1-S phase transition and arrests Id1-transfected cells in the G1 phase of the cell cycle (225).

Interestingly, the E proteins (E2A, E2-2 and HEB) are absent in the less aggressive androgen dependent prostate cancer cell line, LNCaP. However, E-proteins are expressed abundantly in both androgen independent cell lines DU145 and PC3. Therefore, the tumor suppressor effect of E-proteins in DU145 and PC3 cells are negated by high Id1 expression levels. LNCaP with exogenous induction of Id1 may activate an alternative pathway in absence of the preferential binding partners of the Id family, the E proteins.

Coincidentally, Id1 promotes cell survival through the activation of NF-kB signaling pathway (212). LNCaP-Id1 transfected cells are resistant to TNFα induced apoptosis through inactivation of Bax and caspase 3. Increased NF-kB transactivation and nuclear translocalization of p65 and p50 proteins correlates with upregulated Bcl-xL and ICAM-1. Inhibition of Id1 expression in DU145 cells, through antisense oligonucleotides and retroviral constructs, reversed the nuclear transactivation of p65 and p50 proteins and sensitized cells to TNFα induced apoptosis (212). Id1 is exceedingly overexpressed in advanced stage prostate cancer cells with a resistance to apoptosis. Downregulation of Id1 expression may sensitize these cells to commercially available prostate cancer treatments (209).

Inhibition of Id1 and Id3 does not induce apoptosis in the prostate cancer cell lines LNCaP, DU145 or PC3 but does however inhibit proliferation and migration. This suggests that although Id1/Id3 downregulation is not enough to induce apoptosis in prostate cancer cell lines their respective expression level are essential to sustained
prostate cancer growth. However, inhibition of Id2 in LNCaP and PC3 cells does induced apoptosis, but not in DU145 cells (5) possibly due to the role of Id2 in binding pRB and inhibiting its function in the cell cycle due to the fact that both cell lines, PC3 and LNCaP have an intact RB gene. These studies support Id2 as an anti-apoptotic gene and suggest that the effects of Id2 are also independent of p53. Id2 expression resulted in the loss of SNAI2. Evidentially, Id2 is responsible for regulating SNAI2, which inhibits apoptosis in prostate cancer cells (5). Inhibition of Id1 and Id3 increases expression in p21Cip1/Waf1 in LNCaP cells (∼25 fold increase) and DU145 (>2 fold increase). Therefore it is evident that Id proteins play a regulatory role in the cell cycle in prostate cancer cells (5).

Id1 induced serum-independent proliferation in prostate cancer cells requires the inactivation of the p16INK4A gene. Exogenous Id1 expression in LNCaP cells resulted in increased DNA synthesis and a corresponding increase in S-phase induction observed by flow cytometry (216). Id1 expression increased proliferation by inducing phosphorylation of pRB and downregulating p16INK4A specifically in the absence of serum. The other CDKIs p21Cip1/Waf1 and p27kip1 showed no change in expression in response to Id1 induction, supporting Id1-p16INK4A specific regulation of the cell cycle, and senescence. The highly proliferative induction of Id1 allows of androgen independent growth of LNCaP cells (214).

Downregulation of Id2 in PC3 cells is responsible for reduced growth potential and invasiveness, suggesting Id2 may also regulate proliferation. The ability of cancer cells to proliferate unchecked by stimulatory agents is an essential aspect of the transition of prostate cancer cells to an androgen- independent, metastatic aggressive phenotype.
Id1 may play a role in regulating this process by activating serum/androgen-independent mechanisms to correspond with the apoptotic inhibitors responsible for evading cellular arrest/death mechanisms inhibitory of normal cell proliferation. The ability of cancer cells to escape cellular senescence is another crucial override function required by cancer progression. The strong relationship between Id1 and p16 expression in various cell models suggest that Id1 expression is an essential component of the ability of prostate cancer cells to bypass Hayflick's limit (34, 46, 230, 231).

7.3 Id protein regulated senescence in prostate cancer

Senescence is a highly specialized form of terminal differentiation induced by a variety of stimulatory factors including: - (1) alterations of telomere length and structure, (2) DNA damage (for example, oxidative stress), and (3) activation of oncogenes (232). The role of Id proteins during development, inhibiting differentiation, suggests a possible relationship between inactivation of Id proteins and cellular senescence. Initially, the stimulation of quiescent fibroblasts with serum/growth factors induces the expression of Id1, Id2 and Id3 (25, 103, 109). In normal cells, Id proteins are essential to G0-S phase transition and in the process inhibit the induction of cellular senescence. These Id proteins are responsible for coordinating cell growth and differentiation pathways.

In Id1-null primary mouse fibroblasts premature senescence is readily observed. Id1 has also been shown to facilitate the bypass of replicative senescence by directly inhibiting p16\textsuperscript{INK4a} expression in mouse and young human fibroblasts (46, 136, 230). The CDKIs p16\textsuperscript{INK4a} and p19\textsuperscript{ARF} are transcribed by the same gene however, p16\textsuperscript{INK4a} but not p19\textsuperscript{ARF} is upregulated in fibroblast cells that undergo senescence suggesting Id1 specific regulation of p16\textsuperscript{INK4A}. Senescence induction also results in the downregulation of cdk2
and cdk4 activity (46) critical mediators of the cell cycle. The downregulation of CDK4 is indicative of the cell cycle regulatory role of p16\textsuperscript{INK4A}. However, the primary role of CDK2 activity correlates to p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{kip1} cell cycle regulation not observed in Id1 transfected cells.

The loss of Id1 expression in normal epithelial cells sensitizes NPTX cells to TGF-β induced arrest with an upregulation of p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{kip1} (213). Therefore, Id1 expression may inhibit p21\textsuperscript{Cip1/Waf1} activation in prostate epithelial cells. Id1 regulates p16\textsuperscript{INK4a} transcriptional activity through E boxes within the promoter region (46). Therefore, Id1 may negatively regulate bHLH proteins that bind to the E-box of the p16\textsuperscript{INK4A} promoter. In various cancer cell models p16\textsuperscript{INK4a} expression has shown a highly specialized function during induction of senescence. Interestingly, Id1 and Id3 inhibition in LNCaP cells resulted in a significant increase in p16\textsuperscript{INK4A} expression. However, the inhibition of Id3 alone but not Id1 in DU145 cells resulted in increased p16\textsuperscript{INK4A} expression.

The inactivation of p16\textsuperscript{INK4A} is a common event in prostate cancer that is activated through epigenetic events and has been observed in several cell lines (216). The present paradigm suggests that in prostate cancer, senescence induction requires an intact p16\textsuperscript{INK4A} and pRB protein (216). S-aza-2'-deoxycytidine (5-aza-dC), treatment induces p16\textsuperscript{INK4a} expression, inhibits cell growth and induces senescence in the PPC-1 prostate cancer cell line. Induction of p16\textsuperscript{INK4A} inhibited cellular proliferation and was capable of inducing senescence. However, p16 induction in DU145 cells failed to induce senescence due to the non functioning pRB protein in DU145 cells. This study suggests that p16\textsuperscript{INK4A} senescence induction is dependent upon a functional pRB protein.
Therefore, p16<sup>INK4A</sup> suppressed growth in both normal prostate epithelial and cancer cells are dependent upon status of the pRB gene (213).

Use of Id1 antisense oligonucleotides in NPTX immortalized normal prostate epithelial cell resulted in moderate cellular senescent (3-5%) in antisense treated cells (213). Although the Id1 senescence pathway hinges upon the induction of p16<sup>INK4A</sup> expression, NPTX cells treated with anti-sense Id1 oligonucleotides induces senescence with inactivated RB and p53 genes. Studies have shown that RB pocket proteins p107 and p130 can replace RB function in RB compromised cells, suggesting the alternative senescence pathway in these cells (233). The prostate cancer cell model DU145 has retained its senescence programming observed by doxorubicin induced senescence in Ewald et al in the absence of a functional RB protein and in the presence of a mutant p16<sup>INK4A</sup>, DU145 cells still undergo senescence in the presence of Doxorubicin (234). However, in response to DNA damage DU145 cells are incapable of senescence induction as a result of a non functional RB protein (233). Senescence induction is a bonafide alternative to tumor suppression and may sensitize treatment resistant cancer cells to become more responsive to therapy.

**7.4 Id regulated Androgen Independence in Prostate Cancer**

The failure to effectively treat prostate cancer is largely due to the development of androgen independence, since androgen ablation therapy remains the only successful therapeutic treatment for this disease (235). The efficacy of androgen ablation and its responsibility for inducing androgen independence is still under debate. Due to the ability of Id proteins to stimulate serum-independent proliferation in prostate cancer cells, it is suggested they may also play a role in the progression of androgen independence (225).
Development of the prostate cancer disease has been characterized by the changes in the ratio of testosterone and estrogens, with advancing age as a primary risk factor (236). Although the etiology of the disease is generally characterized by the loss of androgens and the ability of prostate derived cells to proliferate without androgens as an essential regulator, a combination of testosterone and estradiol-17β have been able to induce prostate hyperplasia, dysplasia and adenocarcinoma in a noble rat system (236). Long term treatment of testosterone and estradiol-17β resulted in a high incidence of prostate cancer that correlated with an ~18 fold increase of Id1 mRNA when compared to cDNA of normal tissue samples as observed by microarray studies (236). Parallel to Id1 induction, 17 other genes were upregulated in response to prostate cancer progression in this biological system. Most notably these genes included MMP-7, Clusterin, c-met, IκB a-chain, c-crk c-akt and TNF receptor 1. Clusterin, MMP-7 and Id1 required further analysis into their specific role in progression of androgen independent prostate cancer.

Clusterin is an anti-apoptotic gene in prostate cancer that has been correlated with tumor grade in prostate cancer and precedes Id1 expression (236). Studies suggest Clusterin may be an early indicator of sex hormone-induced carcinogenesis as it can be induced by both testosterone and estrogen derivatives and is immunohistochemically present in both normal, low grade and high grade cancer samples in response to hormone treatment (236).

Studies show that MMP-7 (matrix metalloproteinase-7), a member of the metalloproteinase family of enzymes that are responsible for degrading extracellular matrix proteins. MMP-7 is also frequently upregulated in various tumor types including glioma, colon, esophageal, ovarian and prostate carcinoma (236-243). In DU145 prostate
cancer cells MMP-7 has been shown to enhance invasiveness in vitro and shows upregulation in response to estrogen treatment (236). This study suggests that due to its frequent testosterone-induced upregulation and the localization pattern, MMP-7 may be important in prostate cancer initiation and/or promotion simultaneously during Id1 induction (236).

Of the three genes analyzed (MMP-7, Clusterin and Id1), Id1 was the only protein to show preferential localization in correlation with disease progression. In the cDNA array studies Id1 displayed a ~18 fold increase in expression versus the control. Under RT-PCR conditions, the Testosterone + Estradiol treated samples displayed a 4-fold increase in expression over normal samples (236). No Id1 expression was observed in normal tissue samples, while Id1 levels were low in premalignant lesions. Id1 expression was found only in the malignant tissues and was undetectable in nonmalignant samples including controls. Id1 expression in these malignant tissue samples was localized in the cytoplasm, and displayed the strongest expression in the poorly differentiated tissue samples (236). In fact, the histopathological relationship between Id1 and tumor differentiation classification was obviously apparent. Id1 is upregulated during the progression of various cancers including the prostate (45, 166, 194, 214, 236).

To study the molecular involvement of Id1 in androgen independent prostate cancer progression, Ling et al studied the overexpression of stable Id1 vectors in low grade prostate cancer cell line LNCaP, to observe the transforming capabilities of Id1 in correlation to its downstream regulatory genes. Id1 overexpression reduced androgen stimulated growth and S-phase transition in transfected cells (214). The downstream effectors of exogenous Id1 induction were early response factor-1 (erg-1) which in turn
induced expression of epidermal growth factor receptor (EGF-R). Prostate specific antigen (PSA) an essential marker for prostate cancer progression and an essential downstream target of AR was also induced by exogenous Id1 expression. The ability of Id1 to induce PSA expression suggests a strong correlation between Id1 expression levels in relation to androgen independence. However, Zeilinski et al suggest that Id-1 expression inhibits PSA expression in prostate cancer cells (217), using microarray studies Id1 showed significant downregulation of PSA. This is contradictory to previous studies in LNCaP-Id1 transfected cells that stimulated induction of EGFR and PSA by Ling et al.

It is, however, apparent that Id1 is highly expressed in hormone refractory prostate cancer tissue samples and androgen independent cell lines DU145/PC3 (48). Ling et al suggest that EGFR is a primary downstream target of Id1, as EGFR has already been shown to induce androgen independence in prostate cancer cell lines. Use of antisense Id1 oligonucleotides successfully downregulates the expression of EGFR at both the mRNA and protein levels in DU145 cells (214). AR protein apparently remained unaffected by Id1 induction in LNCaP cells, suggesting that PSA response, in the absence of DHT, activated the translocation of AR to the androgen response elements located in the nucleus in an Id1 dependent manner. The promoter region of PSA contains NFk-B response elements suggesting a possible role of NF-κB in this pathway. Id1 upregulates NF-κB, therefore the resultant PSA upregulation through ectopic expression of Id1 in LNCaP cells is caused by Id1 induction of NF-κB. Inhibitors of NF-κB successfully blocks Id1 induced PSA expression in these cells (212, 214) confirming the role of NFk-B in this pathway.
Although, exogenous Id1 expression has demonstrated the ability to induce PSA expression, Xu et al showed that androgen-dependent prostate cancers positively regulates Id1 expression in response to androgen depravation (211). Serum starvation of LNCaP cells resulted in a 6 fold increase of Id1 mRNA expression. After ectopic induction of the androgen receptor in androgen independent (PC3) prostate cancer cells, treatment with DHT downregulated Id1 expression while flutamide (androgen receptor antagonist) increased Id1 expression. The regulation of Id1 by androgens via the androgen receptor suggests that Id1 and AR participate in a feedback loop mechanism and have a strong correlation between expression levels (211).

The ability of prostate cancer cells to proliferate in absence of androgens marks an essential transition for the disease that usually correlates with epithelial-mesenchymal transition (EMT), increased invasion/metastasis and unchecked proliferation. Therefore, the obvious role that Id proteins may play in this transition might provide a successful therapeutic target for a disease with limited treatment options. Id1 proteins are transcription factors with an ability to regulate growth and proliferation during development and cancer progression. Successful regulation of these genes may prove crucial to understanding overall cancer biology including prostate cancer specifically.

7.5 Id1 induced Angiogenesis in Prostate Cancer

Angiogenesis is an essential aspect of late stage cancer progression. The ability of metastatic tumors to form new blood vessels is essential to tumor growth and viability. Angiogenesis is a highly regulatory process during normal development, and Id proteins have already displayed an essential regulatory role in blood vessel development and characterization in embryogenesis (31, 124, 148, 174, 193, 244-251)
The relationship between Id proteins and angiogenesis has not been extensively characterized in prostate cancer. A contributory role of Id proteins during carcinogenesis is assumed due to their ability to form new blood vessels during development and their role in progression of several cancer types (126, 167, 197). A direct relationship with Id1 and vascular endothelial growth factor (VEGF) in prostate cancer has been observed (215). Id1 overexpression in LNCaP cells results in an upregulation of VEGF secretion (~40-80% increase in VEGF protein concentration). VEGF promoter activity also increases >2 fold in Id1 transfected LNCaP cells resulting in Id1 induced transcriptional activation of VEGF. VEGF activity may be induced through E-box elements within the promoter region. Intrinsically, native VEGF protein expression is relatively low in LNCaP cells compared to androgen independent DU145 cells. Inversely, treatment of DU145 cells with Id1 si-RNA results in downregulation of VEGF protein and mRNA expression and promoter activity (~90% decrease in promoter activity) compared with vector controls (215), solidifying the role of Id1 induced VEGF expression.

Media derived from Id1 expressing cells induces morphological changes, along with capillary tube formation human umbilical endothelial cells (HUVECs) (215) further strengthening the observation that secretory VEGF was able to promote proliferation and angiogenesis in HUVECs. Media from LNCaP-Id1-transfected cells induces an elongated morphology, with the formation of cell-cell networking of HUVECS. VEGF secretion was revealed to be the regulator of these processes, which was directly induced by Id1 expression (215). To further understand this process HUVEC cells treated with VEGF directly exhibited a similar phenotype as HUVEC cells treated with media from Id1 transfected cells. The secretory function of Id1 induced VEGF was diminished by VEGF
inhibitor Flk-1, which neutralizes the transforming capabilities of Id1 media on HUVEC cells. This suggests that Id1 induced angiogenesis in prostate cancer is the solitary result of secreted VEGF. Flk-1 also inhibits proliferation in HUVECs treated with Id1-transfectant media (215). Therefore, Id1 induction results in specialized activation of VEGF during angiogenesis and induced proliferation of HUVEC cells.

This study presents a specialized role of Id proteins, especially considering the absent DNA binding domain of Id proteins, resulting in specialized HLH dimerization as their preferential mode of transcription regulation. Id-1 induction in prostate cancer cells may be responsible for inducing autocrine signals to promote angiogenesis through activation of VEGF. In the clinical setting, Id-1 expression was observed in tumors of castrated mice with association of high VEGF levels (132, 252). Id proteins share some redundancy in function however, Id1-3 silencing studies in various prostate cancer cells resulted in no upregulation/downregulation of other Id proteins (5). VEGF expression has also been associated with prostate tumor progression from androgen-dependent to androgen-independent growth in an orthotopic prostate cancer animal model (253). Bone morphogenic protein (BMP) specific response elements are located in the Id1 promoter (254). BMPs are frequently upregulated in prostate cancer and BMP-6 overexpression induces Id1 in prostate cancer (210). Also, a separate study has associated BMP induction with upregulation of VEGF expression at both the protein and mRNA level (255). Therefore, upregulation of VEGF in prostate cancer may be the result of BMP activation of Id1, which also explains why bone metastasis is a common site of ectopic growth of prostate cancer.
Conversely, other Id proteins may also regulate other pro-angiogenic genes or VEGF directly. Id2 involvement in angiogenesis is possible, as Id2 suppression is a critical component of macroglial induced angiogenesis (244). Id2 expression is highly upregulated in angiogenesis during embryonic development, astrocytic tumor development and colon cancer (174, 193, 244, 256-259). Id2 has also shown a tight regulatory role during inhibition of the RB protein, which is an important factor in a number of angiogenic processes (260). In Kaposi’s sarcoma Id2 is responsible for directly inducing VEGF and down regulating pRB. Therefore, the evident role of Id2 in angiogenesis may be translated to prostate cancer progression. Id3 shares similar redundancy to Id1 in overlapping functions as the two protein share the most similar sequence homology of the Id gene family. As a result, Id3 may induce VEGF in a similar fashion to Id1 or in the absence of Id1. Id3 expression is a required factor for angiogenesis and vascularization of tumor xenografts (125). However, in follicular thyroid cancer, VEGF expression was responsible for inducing Id3, suggesting a possible feed-back-loop mechanism involving Id1, VEGF and Id3 (261). Id4 can also induce angiogenesis in human glioblastoma cells (262, 263). Recently, Id4 has shown a role in regulating pro-angiogenic factors, IL-8 and GRO-α, in mutant p53 breast cancer (223). Thus far no other Id proteins have been investigated for their role in angiogenesis in prostate cancer to a similar extent as Id1 but their etiology warrant further investigation.

7.6 Summary

It is apparent that Id proteins may play an essential role in prostate cancer progression. Id1 specifically, has been extensively studied and has shown a regulatory role in prostate cancer progression, drug resistance, and inhibition of senescence,
angiogenesis induction and cell proliferation stimulation. The roles of Id2, Id3 and Id4 are less clearly understood in prostate cancer. The strong relationship between Id1 and prostate cancer progression (to an androgen independent status) and the roles that all of the Id proteins play in the development of various cancers suggest that the responsibility of Id proteins in prostate cancer progression requires substantial investigation. The correlation/absence of Id4 expression in advanced stage prostate cancer suggest that Id4 may act as a tumor suppressor but it’s specific role in prostate cancer development is requires further study. Prostate cancer is a disease with a limited treatment options and Id proteins may provide an alternative to current medical limitations of prostate cancer treatment. 

8. **Id4 in Cancer: Tumor Suppressor or Tumor Promoter**

Inhibitor of differentiation/DNA-binding four (Id4) is the most mysterious and least understood member of the Id family of proteins. Although the identity between HLH regions of Id proteins is high, the remaining regions of Id proteins are not conserved (223). The N- and C-terminal fragments of Id proteins do not adopt a helical formation, except the 27-64 fragment of Id4 (264). This motif is dictated by the presence of an Ala-rich motif between residues 39 and 57. This structural feature may allow Id4 to exert unique functions opposed to the other Id protein family members. Congruent with this notion, Id proteins are known to bind various target proteins with different affinities (146, 195, 260, 265, 266). Id4 is the longest protein of the Id family at 161 amino acid residues long with a distinct HLH domain possibly allowing for atypical dimerization partners of the Id family (13).
Id4 is known to play a critical role during development. Depletion of Id4, in knockout mice, revealed that Id4 is essential for normal brain development and function (124, 134, 267). The loss of Id4 leads to severe birth defects, resulting in a smaller forebrain phenotype (268). Id4 expression is also essential to neural stem cell proliferation and differentiation (269). Specifically, Id4 regulates lateral expansion of the proliferative zone in the developing cortex and hippocampus (269, 270). Loss of Id4 expression in neuronal stem cells results in a significant reduction in proliferation in the ventricular zones of Id4-/- mice. During development, Id4 expression has been associated with localization of vascular sections of brain tissue (223, 263, 271).

As a member of the Id family, Id4 expression is frequently conflicting and atypical of other Id proteins during development and cancer progression. Histological localization of Id4 during cancer progression represents an inverse correlation to Id1, Id2 and Id3 in several types of cancers (7, 188, 194, 272-274). Id4 has an essential role during development indicative of the role Id proteins play as 'early response genes'. Atypical of Id proteins, differentiated cells express significant levels of Id4, dissimilar to expression levels of Id1, Id2 or Id3 (275). This increased Id4 expression is exhibited during premature differentiation of early cortical stem cells also (270, 276). This developmental observation is a previously undocumented characteristic of 'Inhibitor of differentiation' proteins. Id1, Id2 and Id3 are all responsible for delaying differentiation in stem cells (277). Additionally, Id proteins are essential regulators of the cell cycle and are responsible for G1-S phase transition; absence of Id4 results in a prolonged G1 phase of the cell cycle, suggesting a role of Id4 during G1-S phase transition (269). This aspect of development alludes to the uncharacteristic role of Id4 during development by
regulating the cell cycle. Id4’s role in the nervous system also indicates an essential role in oligodendrocyte development (278). Most recently, Id4 has been shown to directly bind OLIG1 and OLIG2 (278) key bHLH proteins responsible for regulating oligodendrocyte differentiation (10, 278, 279) in neural progenitor cells. Id4 also mediates the inhibitory effects of bone morphogenic protein 4 (BMP-4) on oligodendrocyte differentiation resulting in astrocytic differentiation (270, 279).

8.1 Tumor Promoting Role of Id4 in cancer

Developmental regulators are known to play a direct role in driving cancer cells toward a malignant phenotype (280-282). It is therefore postulated that the expression of developmental regulators may induce stem cell like properties to potential cancer cells. Enforced expression of Id4 in primary murine Ink4a/Arf-/- astrocytes drives a malignant transformation, consistent with the stem cell nature of mesenchymal cancer cells (283). Increased Id4 expression can also drive cells to a highly proliferative state through induction of Cyclin E and Jagged 1 in astrocytes (283). Id4 levels were found to have been increased in human glioblastoma multiform (GBM) (262) using microarray studies. Subsequent, investigation of Id4 protein expression in human GBM specimens displayed localization proximate to the vasculature (263, 283). This area of the brain shares a significant correlation with brain tumor stem cell formation.

Due to conflicting studies, the role of Id4 in breast cancer has arguments as both a tumor suppressor and a tumor promoter. In situ hybridization studies have shown that only estrogen receptor negative tissue specimens express Id4 (284). ER positive cells are negative for Id4 in normal epithelium and carcinoma. A negative correlation between Id4 expression and various breast cancer specific genes including BRCA1 and estrogen
receptor (285) has also been observed. This inverse correlation between Id4 expression and BRCA1 suggest that Id4 may act as a tumor promoter in sporadic breast cancer (285) due to the tumor suppressor role of BRCA1.

Berger et al showed that increased Id4 expression caused a decrease in BRCA1 expression and allowed for anchorage-independent growth of breast (SKBr3) and ovarian (PA-1) cancer cells (21). Inversely, Id4 depletion resulted in a large flat epithelial type. Id4 mRNA and protein levels are upregulated in rat mammary carcinomas compared with adenomas and human normal breast epithelium (40). Stimulation of ER positive breast cancer cells results in a reduction in overall Id4 expression (284). Other studies suggest the tumor promoting role of Id4 in breast cancer can be linked to the ability of mutated p53 to regulate Id4 at the promoter level through specific binding sites mediated by E2F1 and p65 (223). This study also suggest that Id4 overexpression through mutated p53 induction, can induce pro-angiogenic factors Gro-α and IL-8, suggesting a cytokine response prolife in response to Id4 induction in these cell lines (223). In a similar study observing Id4 expression in relation to p53 mutation, Id4 is highest in SKBr3(breast cancer) and MCF7 (breast cancer) and lowest in SW-480 (colon cancer) and HT29 (colon cancer) (268), supporting previous studies that show Id4 expression is low in colon cancer.

An attempt has also been made to link Id4 overexpression to chromosomal amplification in bladder cancer (39). Traditionally, invasive cancers are characterized by a high degree of chromosomal activity. As a result, specific regions of the chromosome tend to become over amplified. These regions include region 5p, 6p, 11q, and 20q (39, 286, 287) which usually contain amplified oncogenes. Id4 is located in a part of the
chromosome that is consistently amplified in bladder cancers which frequently results in indulgent copy number of approximate genes (SOX2, CDKAL, E2F3 and DEK). Microsatellites are the key regulators of the amplification of these genes in this chromosomal region. Id4 specifically has five microsatellites surrounding the gene at its chromosomal location, resulting in an investigation determining Id4 amplification/overexpression. Of the four genes amplified, CDKAL is the most commonly overexpressed gene from this amplified region. E2F2, a member of the E2F family of transcription factors, is overexpressed in high stage, high grade bladder cancer (39). There was also no significant relation between normal tissues versus tumorigenic tissue of Id4 expression. When compared to E2F2 (a repressor of the tumor suppressive role of E2F1) and DEK, known amplicons of this chromosomal region, Id4 showed no significant levels of overexpression. These studies suggest that, although Id4 was located in a typically amplified region of the chromosome in proximity to amplified oncogenes, there is no overexpression of Id4 in bladder cancer as observed in adjacent genes in the same amplified region (39).

8.2 Tumor suppressor role of Id4 via DNA methylation

Id proteins have shown a vast regulatory function across diverse cellular processes including cell cycle, senescence, apoptosis and tumorigenesis in cancer. Therefore, it is understandable to expect some similar mode of function in Id4 due to the highly conserved protein sequence within the HLH binding domain of Id proteins. Id4 shares similar homology to Id2, the Id protein with the highest characterized level of promiscuity of binding partners. Id2 has been known to bind both bHLH and non bHLH partners, therefore suggesting a possible similar role for Id4. The exact role of Id4 in
cancer has not been clarified. Studies support Id4 as both a tumor promoter (breast
cancer) and a tumor suppressor (prostate, colon, lymphoma etc.) gene in various cancers.
Presently, no studies have been able to determine any genetic mutation in Id4 or any of
the other Id proteins that illicit a genetic regulation of function. A number of studies have
reported reduced expression of Id4 through promoter hypermethylation during cancer
progression to a more malignant phenotype.

Gene silencing through promoter hypermethylation is a marquee characteristic of
tumor suppressor genes that is observed in Id4 due to the two CpG rich/island areas
within the promoter region. These CpG islands make Id4 highly susceptible to promoter
methylation by DNA methyl transferases. Reduced Id4 expression has been observed in
gastric and colorectal carcinomas, breast cancer, prostate cancer and Leukemia cell lines
(36, 38, 52, 53, 194, 273, 288) as a result of promoter hypermethylation. Several studies
not only extensively characterize the down regulation of Id4 expression through promoter
hypermethylation in many clinical samples and cell lines, but have also linked Id4
expression to cancer reoccurrence and poor prognosis in several cancers including breast
and colorectal cancer (36, 37, 273, 289).

A study by Noetzl et al found that the Id4 promoter was hypermethylated in 69%
of breast cancer samples leading to loss of Id4 expression at both the mRNA and protein
level (37). Demethylation of the breast cancer cell lines (MDA-MB231, BT20, MCF7
and T47D) resulted in clear Id4 re-expression. Id4 promoter methylation was also a
prognostic marker for unfavorable recurrence-free survival, and an increased risk for
lymph node metastasis (37). This work is supported by the work of Umeani et al who
postulate that Id4 promoter methylation increases risk of lymph node metastatic of T1 breast cancer (36).

Id4 shows a regulatory role during lymphocyte and lymphoma development (120) especially considering lymph node metastasis is a primary metastatic site of Id4 inactivated breast cancer. Studies into malignant lymphoma reveal that the Id4 gene is frequently methylated in lymphoid cell lines (100%) (52). In B-cell lymphoma, Id4 expression was reduced by promoter methylation in 95% of samples and 100% of follicular lymphoma samples. A strong relationship between Id4 methylation and Lymphoma stage, suggests that Id4 acts as a tumor suppressor in this system and is primarily inactivated by promoter hypermethylation (52, 290).

The methylation status of Id4 in 48 chronic myeloid leukemia patients was found to be 66% methylated in patients who were in chronic phase of the disease compared to the bone marrow of healthy individuals. (291, 292) There was a significant correlation with Id4 methylation status and disease progression. Methylation of the Id4 promoter increased as the disease progressed from ‘chronic phase’ to ‘accelerated phase’ to ‘blast crises.’ In a separate study Id4 methylation presented in 86% of acute leukemia patients, and 100% in leukemia-relapse patients strengthening the relationship between Id4 and re-occurrence free survival (53). The molecular mechanism of Id4 inactivation in both leukemia and lymphoma has not been extensively characterized. Therefore, the significance of Id4 inactivation through promoter hypermethylation is not entirely known.

The clinical status of the Id4 promoter has not been extensively investigated in prostate cancer; therefore the clinical significance of Id4 expression in prostate cancer is
unknown. Id1-3 are expressed throughout prostate cancer progression and increases as the cancer progresses from a normal epithelium to high grade metastatic cancer (45, 194). Yeun et al suggest Id4 may be a prognostic marker for prostate cancer progression due to the prevalence of Id4 expression in primary prostate cancer, correlating with increased risk of metastasis. However from a cell line aspect, as the hormone refractory disease progresses from an androgen-dependent status to androgen-dependent state Id4 expression is lost. The androgen independent cell lines DU145 and PC3 have absent/low expression levels of Id4 (194). Methylation specific PCR (MSP) studies have demonstrated that the DU145 cell line has a methylated Id4 promoter. Treatment with global de-methylation agent 5'-Aza-2-Deoxycytidine (5-AZA-CdR) in DU145 cells leads to Id4 expression as determined by western blot analysis and RT-PCR (194). Id4 is expressed in the normal epithelium and is significantly higher than in tissue extracted from prostate. Yuen et al also suggested that although Id4 may be a prognostic marker for prostate cancer due to the fact that Id4 expression is not observed in advanced stage prostate cancers specimens (43).

In colorectal carcinoma, Id4 promoter methylation is regulated by Cdc42. This may be a correlation implicative of many cancers with Id4 silenced expression. Cdc42, a member of Rho GTPases family is highly expressed in colorectal adenocarcinoma (272). Cdc42 is also responsible for rearrangement of actin cytoskeleton, membrane trafficking, cell-cycle progression, and transcriptional regulation, and is overexpressed in several tumors. Cdc42 overexpression resulted in downregulation of Id4 through promoter hypermethylation, suggesting that the promoter hypermethylation of Id4 in many cancers may be under molecular regulation of Cdc42 specifically.
8.3 The role of Id4 in cell cycle regulation

Id1, Id2 and Id3 have all been shown to regulate essential cell cycle components. Both Id1 and Id2 have shown direct regulatory function of tumor suppressor Rb (244, 260, 293). Id1 has also negatively regulated p16, p21, p27, p53 and E2F1 (4, 34, 166, 192, 294). With Id3 sharing some redundant regulation of these cell cycle genes. Id proteins expression, function and localization play integral roles in cell cycle control, specifically the G1-S phase transition (24, 171).

Id4 has also shown an ability to regulate the cell cycle. Ectopic expression of Id4 not only induces p53 in the DU145 prostate cancer cell line, but it also induces increased expression of p21, and p27 indicative of the decreased proliferation rate observed in this cell line (194). The resulting increase in p21, p27 expression did not induce a G1 arrest as seen by FACS analysis but did increase the frequency of sub-G0 events indicating an increase in apoptosis. A subsequent S-phase arrest was observed was observed in both DU145, and PC3 cells transfected with Id4 expression plasmids (194) suggesting a delay in S-G2 transition. An association with Id4 and S-phase has previously been observed in Id4 knockout mice during development, where loss of Id4 resulted in an inhibition of progression into S-phase (269). These results suggest that Id4 expression may play a crucial role in cells entering and exiting the S-phase.

Raji lymphoma cells treated with arsenic trioxide results in decreased cell growth of Raji cells, upon Id4 re-expression (295) followed by induction of apoptosis aG0/G1 arrest of the cell cycle. This observation correlates with the inability of Id4- absent progenitor cells to have successful G1-S transition. As stated above, knockdown of Id4
through promoter hypermethylation by Cdc42 has a distinct effect on cell cycle progression.

**8.4 The role of Id4 in regulating cancer growth potential**

Id4 has also show an ability to regulate the phenotype and molecular interactions of Id induction or Id knockdown. Cell proliferation is a typical response to Id protein induction or knockdown. Id1 induction in human fibroblasts increases proliferation and as a result inhibits differentiation in these cells (230, 296, 297). Id1 knockdown in human fibroblasts results in premature replicative senescence in human fibroblasts through an upregulation in CDK inhibitor p16Ink4a (46). Id1 knockdown severely inhibits the proliferation rate in these cells. Id4 also shares some control over proliferation/growth potential. In breast cancer cells where Id4 expression was induced through induction of mutant p53, increased proliferation of GBM cells was observed (263, 283, 298). This also resulted in an increase in colony formation in soft agar assays.

In prostate cancer cell lines, ectopic expression of Id4 had a significant correlation of the proliferative phenotype of these cells *in vitro* (194). Induction of Id4 results in an increase in the doubling time of these cells and, a decrease in the rate of proliferation. Contributory to the proliferation rate, Id4 induction also altered the morphological phenotype of the metastatic cell line and resulted in an increase in flat cell morphology with cells displaying an “epithelial-like” cell morphology attributed to increased cell-cell interaction between Id4 transfected cells (194). Id4 clearly has transforming cellular properties observed by other Id proteins.

**8.5 Molecular Interactions suggesting a correlation between Id4 and p53 expression**
The roles of p53 in various aspects of the cell cycle have been extensively characterized. The TP53 gene is a classical tumor suppressor that is mutated in more than half of all human cancers (299). Most p53 alterations are missense mutations within the DNA binding domain of the translated protein (300) altering binding affinity for partner proteins. Mutant p53 has established a gain-of-function activity, which promotes tumorigenesis in absence of wild type p53. Mutant p53 also induces cellular resistance to anticancer treatments, and contributes to genomic instability by abrogating the mitotic spindle checkpoint. Depletion of mutant p53 facilitates DNA damage induced cell death (301). Mutant p53 mice have a higher incidence of solid tumors, more readily than p53 knockout mice due to the fact that the mutant p53 proteins are full length proteins with a longer half life than wild-type p53 protein (302).

*In vitro and in vivo* studies highlight the transforming capabilities of mutant p53 on cell cultures and tumorogenic mice (80). Mutant p53 is believed to bind to and sequester proteins whose function is required for anti tumor effects such as apoptosis and growth inhibition. One example of p53 gain-of-function involves p53 mutant’s interaction with p63 and p73; impairing the transcriptional activity and apoptotic induction of these two proteins (301, 303). Mutant p53 also binds to DNA and transcriptionally regulates the expression of oncogenes. Mutant p53 is also capable of large transcription competent complexes that regulate the expression of target genes (223, 299, 302, 304).

Fortemaggi* et al* have extensively characterized the molecular interaction between Id4 and mutant p53 in promoting neo-angiogenesis in breast cancer (223). In this study, using microarray profiles, they found that mutant p53-R175H upregulated Id4 expression
in vivo (223). Various cancer cell lines displayed upregulation of Id4 in response to mutant p53 protein. Wild-type p53 was unable to regulate the mRNA expression of Id alone however mutant p53 is recruited in complex with transcription factor p65 and E2F1 to the Id4 promoter suggesting a tight regulatory role of p53 in conjunction with E2F1 in regulating the Id4 promoter. Mutant p53 is incapable of binding to the Id4 promoter on its own but was only capable of inducing transcriptional activation of the Id4 gene in the presence of either p65 or E2F1 (223). This suggests that the regulatory role of mutant p53 on Id4 is limited by the expression of these ‘accessory’ proteins. E2F1 has been shown to have classical tumor suppressor capabilities, including induction of apoptosis though an E2F1 dependent mechanism. The direct interaction between mutant p53 and E2F1 on the Id4 promoter supports the “inhibition of tumor suppressor” function of mutant p53 in cancer (223).

Treatment of endothelial cells with media derived from mutant p53/Id4 expressing cells resulted in an increase in proliferation, resulting in the activation of secreted cytokines in Id4 expressing cells. As a result of Id4 overexpression in SKBr3 cells, pro-angiogenic factors IL8 and GRO-α were induced along with several other cytokines. Of the 60 human cytokines studied 9 cytokines were induced including SGP130, IL2R-a, sTNF-RII and IL-6R (223). Of the cytokines observed, only IL8 and GRO-α had any pro angiogenic properties. Conversely, no cytokines observed were downregulated. The overexpression of Id4 allowed for the stabilization of IL8 and GRO-α mRNAs in the presence of Actinomycin D, an inhibitor of transcription. IL8 and GRO-α also formed stabilization complexes with Id4 through direct interaction. This observation supports the ability of Id proteins to bind non bHLH proteins (e.g. Id2
interaction with pRB), and supports the role of Id4 as a possibly more promiscuous member of the Id gene family. One of the more interesting observations was that other angiogenesis regulators such as vascular endothelial growth factor B (VEGFB) and thrombospondin-1 (THBS-1) showed no regulation in response to Id4 overexpression suggesting a specific molecular upregulation of Id4 on IL-8 and GRO-α (268).

In a separate study, Dell’Orso et al provide evidence strengthening the relationship between mutant p53 induction of Id4, by observing the induction of Id4 via mutant p53 following anticancer treatments (268). Id4 expression was increased in response to Adriamycin and cisplatin treatments on mutant p53/Id4 expressing cell lines. Knockdown of mutant p53 abrogated this response. This study indicated that in response to DNA damage mutant p53 in complex with E2F1 and p300 bind to the CDE element closest to the transcriptional start site, on the Id4 promoter, to achieve “mutant p53 associated” chemoresistance. In response to proliferation, p53 binds to both CDE elements and an NF-kB binding site. Stimulation of tumor neoangiogenesis is stimulated through the expression of IL-8 and GRO-α (223). A strong relationship between Id protein regulation by p53 has already been established. In particular, Id1 expression is inhibited by DNA damaging agents’ captothecin and Adriamycin. Wild-type p53 represses Id1 by inducing DEC1, which in turn binds to the Id1 promoter and represses its transcription (305). This study shows that the role of mutant p53 in breast cancer cells may be to induce the pro-angiogenic effects elicited during DNA damage (268). Therefore, in response to DNA damage mutant p53 induces Id4 as a cell survival response mechanism. These studies also highlight that wild-type p53 has no regulatory control of Id4 but however, mutant p53 can transcriptionally regulate Id4.
In 186 specimens from patients with breast cancer revealed that 44% of tissue specimens expressed Id4. Of these samples, Id4 was expressed in 60% of p53+ tumors and only 38% in p53- tumors, strengthening the relationship between Id4 and p53. In the presence of HER2 subtype among this sample size, Id4 is expression is 80% in p53+ tumors versus 40% in p53- tumors. HER2 overexpressing breast cancer subtype presents with frequent TP53 mutation similar to cancers which possess a basal phenotype. In correlation with this observation, data mining from the oncomine database revealed, that high levels of Id4 transcript are present in basal-like versus non-basal breast cancers in various studies (268).

It should be noted that mutant p53 is also capable of inhibiting the expression of Id2 in an opposite fashion. The Id2 promoter region is also under regulation by mutant p53, and not wild-type p53, through direct binding of mutant p53 to the promoter of Id2 in MIA PaCa-2 pancreatic cancer cell line (304). However in this these cells knockdown of mutant p53 (R248W) resulted in increased Id2 expression. Conversely, endogenous Id2 was found to be inhibited by exogenous mutant p53 in p53-null HTC116 cell line (304).

Further strengthening the relationship between Id4 and p53, studies in prostate cancer by Carey et al shown that ectopic Id4 expression in the prostate cancer cell line DU145 has lead to increased p53 expression and has results in a tumor suppressor effect of Id4 (194). In this study increased p53 expression at the mRNA level results in decreased proliferation, and upregulation of the cell cycle control regulators p21^{Cip1/Waf1} and p53. Furthermore, in prostate cancer Id4 expression is highest in normal and low grade prostatic tumors and cell lines (LNCaP) and reduced/absent in High grade tumors.
and metastatic cell lines (DU145, PC3) (5). However, DU145 cells contain a mutant p53 with two missense mutations (P223L, and V274F) (306, 307) that shows now regulatory function over Id4 expression.

In the study by Fortemaggi et al, mutant p53 regulation of Id4 expression was dependent on the type of mutation, with R273H representing the highest regulatory function over Id4 expression (223). This may explain the divergence in mutant p53 expression of Id4 in prostate cancer. Also, Id4 expression is reduced in the DU145 cell line due to hypermethylation of the promoter, therefore suggesting that any pro-oncogenic effect that may be exerted by mutant p53 regulation of the Id4 promoter in prostate cancer is severely inhibited. However, the overall expression and localization pattern of Id4 in prostate cancer point to a tumor suppressor effect and correlates with the hypothesis stated by Dell'Orso et al that Id4 functions as a tumor suppressor in ER+ breast tumors where it is frequently inactivated by promoter hypermethylation. However, Id4 displays oncogenic activity in breast cancer cells expressing mutant p53, which are mainly ER- (268).

8.6 Defining the relationship between Id4 and BRCA1

Id4 is seen as a tumor promoter in breast cancer through a relationship between mutated p53 leading to Id4 expression. Id4 expression also shows a negative correlation to BRCA1, a known tumor suppressor gene in breast cancer (21). Loss of Id4 expression leads to BRCA1 upregulation in mammary tumorigenesis (21). The same study highlighted the negative relationship between Id4 expression and ER-α expression. Treatment of BRCA1 positive, breast cancer cell lines with estradiol, results in a decrease in Id4 expression suggesting a feed-back loop mechanism of action between BRCA1 and
Id4 (22, 308). BRCA1 can also upregulate Id4 in normal mammary cell division, establishing a regulatory loop between BRCA1 and ID4 (22).

8.7 Identifying Id4 as a key hormone regulatory protein

Id4 regulation at the molecular level has not been extensively characterized possibly due to the fact that Id4 is a negative regulator of bHLH proteins and its exact mechanism of transcriptional regulation is not entirely understood. Interestingly, Id4 has shown a correlation pattern with two hormone regulatory genes (AR and ER) in both breast cancer and prostate cancer. In breast cancer Id4 has demonstrates an inverse correlation with ER mRNA expression in sporadic breast tumors (285). Also, in a study of several tumor specimens Id4 mRNA was seen lowest in samples expressing high levels of ER mRNA. Also, treatment of ER+ breast cancer cells with estradiol resulted in decreased expression of Id4 (22). Conversely, Ectopic Id4 expression in (androgen independent) prostate cancer cell line DU145, resulted in induced expression of AR, and downstream transcriptional target PSA (194). In clinical specimens of prostate cancer tissue specimens, Id4 expression correlates with loss of AR expression; further strengthening the relationship between Id4 and AR expression. In normal prostate epithelial cells and low grade prostate cancer cell line (LNCaP), both Id4 and androgen receptor are expressed. Conversely, in metastatic prostate cancer cell lines DU145 and PC3, Id4 expression is absent or severely diminished (194). Studies have shown that this decrease in Id4 expression is the result of hypermethylation of the Id4 promoter. Conversely, as Id4 expression decreases, prostate cancer progresses to a metastatic phenotype as Id1-3 expression levels increase (5). This is an observation supported by Id4 knockout mice that show increased expression of Id1, Id2 and Id3 in stem cells (269).
The highest Id4 expression is observed in the testes and the brain, kidney and prostate(39). Therefore, it is not surprising Id4 expression can be regulated by both androgens and estrogens. To support the organ specific relationship between Id4 and AR, Id4 expression is found only in the androgen positive cell line LNCaP but absent in DU145 and PC3. Treatment of normal prostate epithelial cells with testosterone and hepatocyte growth factor induces Id4 expression and promotes epithelial cell differentiation (275). Treatment of Sertoli cells with the gonadotropin FSH resulted in decreased expression of Id4, while increasing the expression of Id1, Id2 and Id3 (309). The extensive relationship between Id4 downregulation by estradiol induction in breast cancer has extensively been discussed previously. Therefore, Id4 may be a hormone regulatory protein in cancers involving hormone induction. Most hormone receptors share structural similarities and as a result, Id4 may play more discerning roles across the hormone ligand paradigm pertaining to other hormone receptors (androgen, estrogen, and progesterone and glucocorticoid receptor)

8.8 Summary

Inhibitor of differentiation 4 is at present the least understood/characterized member of the Id gene family. It is evident that Id4 shows an evident expression divergence from the other Id family members both during development and cancer progression. Id4 has apparently etched an essential role in brain development and function and is an essential regulator of angiogenesis. The exact role of Id4 during cancer progression remains unclear. Although Id proteins are seen as proto oncogenes, Id4 has shown an inhibitory role of cancer progression analogous to Id1-3. Most cancers suggest a direct correlation between loss of Id4 expression through promoter hypermethylation
and progression of selective cancers including, prostate, lymphoma, colon carcinoma and leukemia. A very convincing argument has been made for Id4 to act as an inducer of breast cancer progression whether it is through its inverse correlation with both BRCA1 and ER, or the regulation of Id4 expression through gain-of-function p53 mutation resulting in pro-angiogenic factor stimulation. However, a negative correlation between loss of Id4 expression through promoter hypermethylation and re-occurrence free survival has also been established in breast cancer models. Therefore, the exact role of d4 in Breast cancer, as either a tumor promoter or a tumor suppressor remains unknown.

The Epstein bar virus' ability to negatively regulate Id4 suggests that it may possibly susceptible to virus induced inactivation, resulting in new implications for Id4 regulation (310). Whether Id4 is a classic tumor suppressor gene or a tumor promoter is not conclusive at this point. Id4’s functional role may stimulate both processes depending on the molecular environment. Thus far, it is conclusive that Id4 is a different kind of Id protein and the inactivation of Id4 through promoter hypermethylation does suggest that Id4 may in fact act as a classical tumor suppressor gene. However, analysis into the molecular activation/inactivation of Id4 in cancer progression is necessary to clarify this controversy.
CHAPTER THREE
MATERIALS AND METHODS

Cell Lines and Cell Culture

Human prostate cancer cell lines DU145 and LNCaP were obtained from American Type Culture Collection (ATCC, Rockville, MD). The DU145 cells were cultured in F12-BCS-A (Ham's F12 (Gibco, Carlsbad, CA) medium containing 10% Bovine Calf Serum (Hyclone) with appropriate antibiotics (pen/strep, fungizone, and gentamycin). LNCaP cells were cultured in RPMI-10% Fetal Calf Serum (FCS) and antibiotics. The normal human prostate epithelial cells (PrEC) were obtained from Cambrex (Baltimore, MD) and were cultured in PrEGM (Cambrex, Baltimore, MD) for approximately 10-15 doublings. Cells were cultured at 37°C in a fully humidified atmosphere containing 5% CO₂.

5'-Aza-2-Deoxycytidine (5-AZA-CdR) Treatment

The DU145 cells were cultured in F12-BCS-A containing 4uM of 5-AZA-Cdr (4uM). The media with freshly added 5-AZA-Cdr was changed every 24 hours for 96 hours before harvesting the cells for RNA. The gene expression was analyzed before and after 5-AZA-Cdr treatment on the reverse transcribed RNA using gene specific primers.
Plasmids and transfections

The full length human Id4 cloned into pCMV vector (pCMV-Id4) was a generous gift from Dr. Mark Israel. The pCMV-Id4 vector and pCMV vector alone was transfected in sub-confluent (60%) DU145 cells grown in six well plates using TransIT-prostate transfection reagent cocktail (10 μl TransIT-prostate reagent, 5 μl prostate boost reagent (Mirus Bio) and 2 μg pCMV-Id4 DNA in 200ul of serum free media). The culture media was changed once after an overnight incubation with the cocktail. Forty-eight hours after transfection, the cells with incorporated pCMV-Id4 were selected by incubation in fresh media containing 350 μg/ml G418 (Invitrogen) for one week with media change every 2 days. Following this selection cycle, the transfected cells were passaged once in F12-BCS-A and then re-exposed to F12-BCS-A with 350 μg of G418/ml for an additional week (second G418 selection). This approach ensured the survival of only transfected cells. Simultaneous experiments were also performed in which cells were transfected with no DNA (control, parental) or with pCMV DNA (transfection control). The G418 selection procedure described above resulted in no surviving DU145 parental cells. The cells were grown to confluence (80%), trypsinized (0.25 % v/v trypsin and 0.03 % w/v EDTA in calcium- and magnesium-free phosphate buffered saline), counted, and plated at a 1:2 dilution in new 100-mm plates.

RNA preparation:

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) as described previously (45). The final RNA pellet was re-suspended in diethylpyrocarbonate (DEPC)-treated H2O at a concentration of 1 mg/ml and stored at -80°C until analysis.

Proliferation assay:
DU145 cells were cultured at sub-confluent densities (growth permissive, 40%) in 24 well plates (40% confluency) and serum starved for 48 hours. The cells were then treated with 10% BCS for 20 h followed by a 6h incubation with [3H] thymidine. Counts per min (cpm) of [3H] thymidine incorporated into DNA were determined and normalized to the total DNA per well. Total DNA content was determined by SYBR green fluorescent assay (311).

**Gene Expression**

RNA (2 µg) was reverse transcribed in a final volume of 25 µl as per standard protocols (RT-Mix: 20 U RNAout (Invitrogen, Carlsbad, CA); 1.25 mM each of dNTP’s; 250ng oligo dT (Promega, Madison, WI), 10 mM dithiothreitol, and 200 U MMLV reverse transcriptase (Invitrogen) in the MMLV first-strand synthesis buffer (Invitrogen)). The RNA was denatured for 10 min at 65°C, and then cooled on ice before addition of RT mix and enzyme. The reverse transcriptase reaction was carried out at 42°C for 1 h.

Each PCR reaction was performed with 250 pg reverse-transcribed DNA using published protocols (45). The possible contamination of RNA with DNA was distinguished by performing the RT reaction without MMLV reverse transcriptase enzyme (-MMLV RT). The PCR-based amplification reactions were carried out in duplicate on each reverse-transcribed RNA sample using gene specific primer sequences as indicated in respective figure legends. Simultaneous PCR reactions were also carried out using primers designed to β-actin to monitor the qualitative and quantitative efficiency of the RT-PCR reactions. The identity of each of the corresponding PCR products was size and sequence/restriction digest confirmed.
Real-Time PCR

Reverse transcribed RNA from DU145, DU145-Id4 and DU145-pCMV cells were used for real time quantitative gene expression analysis based on the TaqMan chemistry. The validated probes for Id4, β-actin (control), E-cadherin (CDH1), p21 (CDKN1A) and p53 were obtained from Applied Biosystems. Amplification of target sequences was detected with ABI7900HT sequence detection system (Applied Biosystems, Foster City, CA) and analyzed with ABI prism software (Applied Biosystems). All PCR reactions were performed in a final volume of 50 μl according to the manufacturer’s instructions (Applied Biosystems). The cycle threshold (Ct) was used to calculate relative amounts of target RNA.

Immunocytochemistry

The cells lines were cultured (60% confluency) in 8 chamber slides (Lab-Tek) at 37°C as described above. After 2 days in culture, the media was removed and the adherent cells were washed once with PBS. The cells were then fixed in cold methanol (20°C) for 1 hour and stored at -20°C. The fixed adherent cells on slides were equilibrated at 4°C before further processing. The slides were incubated in 3% Bovine Serum Albumin (BSA) in PBS for 1 hour at 4°C in order to block non-specific sites. The slides were then incubated with anti-Androgen Receptor-antibody (1:300 PG21, Millipore) or anti-actin antibody (1:50, H-196, Santa Cruz) for 1 hour at 4°C. After primary antibody reaction, the cells were washed 3 times in PBS for 5 minutes each and then incubated with Alexa Fluor 594 anti-rabbit IgG (1:10, Invitrogen) at 4°C for 1 hour. The slides were then rinsed for 5x5 minutes in PBS, counter stained with DAPI (VWR), post fixed with 3.7% formalin in PBS for 15 minutes and mounted with Glycerol. Cells were
examined using a Fluorescent Microscope (Zeiss) and images captured via AxiomCam/AxioVision (v 4.4).

**Western Blot Analysis:**

The prostate cancer cell lines were cultured on 150 mm plates in their respective media. Cells \(5 \times 10^6\) were dissociated off the plate by adding ice cold PBS and scrapping with a cell scraper. Total cellular protein was prepared as described previously (45).

20µg of total protein was separated by 4-20% SDS-polyacrylamide gel (BioRad) and subsequently blotted onto a nitrocellulose membrane (BioRad). The blotted nitrocellulose membrane was subjected to western blot analysis using protein specific antibodies (Id4: Aviva ARP32335_T100) as mentioned above. After washing with 1x PBS with 0.5% Tween 20, the membranes were incubated with a secondary antibody against anti-rabbit IgG (Pierce Rockford, IL) and the signal was visualized using the Super Signal West Dura Extended Duration Substrate (Pierce).

**Flow Cytometric Analysis of Cell Cycle Progression and Apoptosis**

The cells were cultured in 24 well plates to a sub confluent density. After the culture the cells were collected by trypsinization and washed with phosphate buffered saline. The cells were then fixed with 70% ethanol and stored at -20°C overnight. The following day, cells were washed twice with ice cold phosphate buffered saline PBS/FCS (10%) followed by a final wash in 1X PBS. The cells were then finally resuspended 1ml of PBS (1X) containing 50µg/ml RNase A, 0.1% TritonX-100 and 1mM EDTA and then incubated at 37°C for 30 minutes. Finally, 20µg/ml of propidium iodide was added.

Data acquisition and analysis were performed on a BD FACSscan flow cytometer (FACS core, Morehouse School of Medicine, Atlanta, GA). The cell cycle profiles were then
analyzed using BD Cellquest Pro (for apoptosis, Sub G0) and MODFIT software cell cycle analysis. At least 10,000 cells in each sample were analyzed to obtain a measurable signal. All measurements were performed using the same instrument settings.

The fraction of cells in Sub G0 phase was used to detect apoptotic cells. An increase in Sub G0 cells is due to loss of fragmented DNA as a result of apoptosis from permeabilised cells (ethanol fixed) due to DNA fragmentation. The cells stained with a DNA intercalating dye like propidium iodide, results in a DNA profile representing cells in G1, S-phase and G2M phase while the apoptotic cells are represented by a sub G0/G1 population seen to the left of the G0/G1 peak (312-314).

**Statistical analysis:**

The ΔΔCt method (Applied Biosystems User Bulletin2; ABI PRISM 7700 detection system) was used for relative quantification of gene expression. The Ct values of the target genes from triplicate PCR reactions were normalized to the levels of β-actin (endogenous control) from the same cDNA preparations. The average Ct for each gene was calculated by subtracting the Ct of the sample RNA from that of the control RNA. This value or ΔCt reflected the relative expression of the treated sample compared with the control and became the exponent in the calculation for amplification $2^{\Delta \Delta \text{Ct}} = 2^{\Delta \text{Ct}_{(\text{control})} - \Delta \text{Ct}_{(\text{sample})}}$, the equivalent to the fold change in expression. A statistically significant difference between various treatments and/or cell lines was determined by student's t-test.

**DNA Methylation Analysis**

The methylation status of the Id4 promoter region was analyzed using methylation-specific PCR (MSP) (315). This assay entails the initial modification of
genomic DNA by sodium bisulfite, converting all un-methylated cytosines to uracils, but leaving the methylated cytosines unchanged. Subsequently, the DNA region of interest was amplified in two separate reactions with primer pairs specific for either the methylated or the un-methylated sequence.

Genomic DNA was isolated using DNeasy kit (Qiagen). Approximately 1 μg of DNA was sodium bisulfite-modified and subjected to MSP as described previously (315). MSP primers that specifically recognized un-methylated Id4 sequence were 5'-GGT AGT TGG ATT TTT TGT TTT TTA GTA TT-3' (sense) and 5'- AAC TAT ATT TAT AAA ACC ATA CAC CCC A-3' (antisense); primers specific for the methylated Id4 sequence were 5'- TAG TCG GAT TTT TCG TTT TTT AGT ATC-3' (sense) and 5'- CTA TAT TTA TAA AAC CGT ACG CCC CG-3' (antisense). The primers for the U reaction cover the bases -194 until -166 and -60 until -33, and the primers for the M reaction cover the bases -192 until -166 and -60 until -35 (relative to the transcription start site). Reactions were hot-started at 95°C for 5 min and held at 80°C before addition of 0.625 U of Taq polymerase (Sigma, St. Louis, MO). Temperature conditions for PCR were as follows: 35 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, followed by 1 cycle of 72°C for 5 min. Normal DNA from peripheral blood was treated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA) in order to generate a positive control for methylated alleles of Id4 (316). PCR products were separated on 2.5 % agarose gels and visualized by ethidium bromide staining.

**Apoptosis Assay**

DU145/DU145+Id4/LNCaP/PC3 cells were seeded in 10 cm² cell culture dishes at a density of 10⁶ cells/plate in RPMI 1640 supplemented with 5% FBS for 48 hours.
The medium was replaced with phenol red-free RPMI 1640 containing 5% CSS for 48 hours. The cells were then treated either with or without: 10ng/mL of Actinomycin D (for 6hrs) or 25nM Doxorubicin (for 72hrs). The cells were harvested and an assessment of apoptosis was carried out using the Vybrant® Apoptosis Assay Kit (Molecular Probes, Eugene, OR) according to the instructions provided by the manufacturer. The kit uses recombinant Annexin V conjugated to Alexa Fluor 488 dye to detect phosphatidylserine on the surface of apoptotic cells which can be measured by flow cytometry. The kit also uses propidium iodide for detection of dead cells. Samples were kept at room temperature and analyzed immediately on a C6 Flow Cytometer with CFlow Plus Software (AccuriCytometers Inc., Ann Arbor, MI).

**Statistical Analysis**

All results were confirmed in at least three independent experiments using different cell preparations. Students “t” test and ANOVA were employed to assess if there were significant differences between different cell lines and treatment groups.
1. Status of Id4 in Prostate Cancer Cell Lines

In vitro prostate cancer cell lines are used to mimic various stages of prostate cancer progression. In order to understand the mechanistic profile of Id4 in the prostate cancer we chose a cell line that was Id4 negative, DU145. Using an Id4+pc3DNA 3.1 plasmid we transfected the Id4 gene into the DU145 cell line and observed the results. Id4 expression is low in a number of malignant, metastatic cancer cell lines including, MCF-7, HL-60 and the prostate cancer cell line DU145 (268) (see appendix Table 5). Initially, we screened various prostate cancer cell lines to observe the level of Id4 gene expression (see appendix Figure 12). As expected the less metastatic, androgen dependent cell line LNCaP showed the highest expression among the prostate cancer cell lines. Id4 mRNA expression decreased as the cell lines advanced to a more metastatic state with DU145 showing very little to negligible expression of Id4 under normal conditions.

The metastatic prostate cancer cell line derived from bone showed some level of Id4 expression but the PC3 derivative cell line PC3M showed a decreased expression level compared to PC3. The PC3M cell line is both more metastatic and more invasive than PC3 (210, 317). A constructed pc3DNA 3.1+Id4 plasmid was transfected into the cell line and resulted in a significant upregulation of Id4 mRNA. One interesting
observation is that the more aggressive LNCaP derivative prostate cancer cell lines, C33 and C81, showed decreased expression of Id4. C-33 and C-81 cells were originally derived from the lymph nodes of a patient with metastatic prostate cancer, with C-81 having more migratory and invasive properties including an ability to grow in androgen depleted media (318). This suggests that there may be a direct correlation with androgen receptor independence and Id4 expression. DU145 and PC3, the more aggressive androgen independent cell lines, both had negligible mRNA expression at 30 cycles in the PCR, but showed increased expression at 35 cycles.

Many studies have suggested that loss of Id4 expression is lost through promoter hypermethylation (36, 38, 52, 53, 288, 295), a key characteristic of many tumor suppressor genes. Various studies have highlighted the relationship between Id4 promoter hypermethylation in advanced stage, breast cancer, colorectal cancer and pancreatic cancer (36, 38, 52, 273, 288, 319). Therefore, we have attempted to link the loss of Id4 expression in advanced prostate cancer to promoter hypermethylation of the Id4 promoter. To understand the methylation status of Id4, DU145 cells were treated with a demethylation agent, 5-aza-2'-deoxycytidine (5A2) (320) (see appendix figure 13). 5A2, is responsible for reversing the methylation status of all methylated genes within a given cell system. Post treatment with 5A2, Id4 expression was regained, and was comparable to the androgen independent cell line LNCaP suggesting Id4 expression is lost through Id4 promoter methylation. To further understand the methylation status of the Id4 promoter, methylation specific primers were created to determine whether the Id4 gene promoter was under methylation. LNCaP cells were used as a negative control because they show significant Id4 expression and have an unmethylated Id4 promoter. The
DU145 cell line which shows no expression for Id4, displayed a methylated Id4 promoter using methylation specific primers. The DU145+Id4 cell line also showed a partially unmethylated Id4 promoter, suggesting that the Id4 gene may play a role in the methylation programming of the cell and consequentially the methylation status of its own promoter region. The fibroblastic cell line, BJ, which shows Id4 gene expression, has an unmethylated Id4 promoter. These results suggest that loss of Id4 expression in advanced prostate cancer cell lines is attributed to promoter hypermethylation.

2. **Morphological changes associated with Id4 overexpression**

   One of the initial observations of ectopic expression of Id4 in DU145 cell line was the drastic changes in morphology when compared to the untransfected and empty vector cell line. DU145 cells are an aggressive, highly proliferative cell line with a slightly mesenchymal phenotype. The initial observation of the DU145+Id4 cell line was the “clustered” growth formation of the cell line not usually associated with the DU145 cell line (see appendix Figure 13). These cells displayed a less mesenchymal morphological phenotype and assumed a normal epithelial phenotype. The dividing DU145+Id4 cells displayed decreased capability of disassociating itself from progenitor cells and instead resulted in close cell-cell interaction. Under close inspection of this transfected cell line at 200x magnification, there were more subtle intracellular morphological changes displayed. The DU145+Id4 cell line cells showed a flattened phenotype with an increased cellular diameter. These cells, with and increased diameter, also displayed small visible spherical macromolecules located within the cytoplasm of the cell (See appendix Figure 15). These macromolecules suggest a significant change in the molecular machinery within the DU145+Id4 transfected cell line. Review of the literature suggests that these
macromolecules appear to be lipofuscin molecules, a subcellular molecule indicative of lysozomal digestion and is considered to be an aging pigment and may correlate with increased lysozomal activity within the cell.

Our laboratory uses Ham’s F-12 media supplemented with Bovine Calf Serum (BCS) to successfully propagate the androgen independent cell line DU145 as opposed to Fetal Bovine Serum (FBS). Normal DU145 Cells show no preferential growth response or phenotypical change to FBS as opposed to BCS as they can grow in androgen depleted media. However, the transfected DU145+Id4 cell line displayed two different growth morphological changes between the two types of serum (see appendix figure 16). In BCS supplemented media, DU145+Id4 cells displayed a decreased mesenchymal phenotype and a clustered epithelial like growth pattern. In FBS supplemented media the transfected cell line displayed a more disassociated intracellular interaction as opposed to the cells supplemented with BCS serum. The drastic responses to different types of media suggest that there may be key components (i.e. growth factors, steroid hormone supplements) that affect the growth potential of this prostate cancer, Id4 transfected cell line. Literature suggests that one of the main differences between the two types of media is the difference in steroid hormone levels. FBS serum has much higher levels of androgens as opposed to BCS serum suggesting Id4 transfected cells may be responding to the androgens levels in the serum.

One of the major characteristic of cancer cells, specifically highly metastatic cancer cells, is their ability to continue to proliferate for a significantly long time, bypassing all apoptotic and cell maintenance machinery responsible for either curtailing excessive proliferation or initiating apoptosis. The DU145 cell line is an example of a
highly metastatic cell line that can be passaged to an infinite degree without little change in the metastatic ability of the cell line. However, the DU145+Id4 transfected cell line displayed a distinct characteristic not associated with the normal DU145 cell line. We conducted a number of passaging studies and observed that the DU145+Id4 transfected cell line seemed to reach an end point in the number of passages the cell line could attain. This number appeared to be approximately forty passages. At approximately the 40th passage of the DU145+Id4 cell line post transfection with the pc3DN3.1+Id4 plasmid, the cells stopped dividing. These cells also exhibited mass apoptosis with no viable cells remaining within the culture medium (see appendix Figure 17). Before this final termination process was initiated there were visible signs of necrotic cells within the media of freshly supplemented Sub-40 transfected cells (see appendix Figure 16B). The significant presence of these necrotic cells at passage 40 of DU145+Id4 transfected cells when compared to the Sub-25 cell line, suggests an initiation in the apoptotic molecular machinery at this time point previously abated in the earlier passage numbers. This may be the result of an ‘endpoint limit’ initiated by the expression of the Id4 gene.

Upon realizing that the transfected DU145+Id4 cell line showed morphological changes along with an age associated apoptosis initiation we wanted to understand; whether or not this cell line was more susceptible to environmental changes? And what was the ability of these cancer cells to continue to proliferate in a nutrient deprived environment? Therefore a series of serum deprivation studies were carried out. The DU145 and DU145+Id4 were seeded in normal Ham’s F-12 media along with FBS serum for 48 hours to allow for normal growth. Post 48 hours, the FBS media was removed from the cancer cells and the cell were propagated in Ham’s F-12 media only. The DU145 cells
became confluent after 10 days in serum deprived media (see appendix Figure 18). Upon passaging after the second doubling the normal DU145 cancer cells showed an inability to reattach to the surface of the flask without FBS supplemented media. The DU145+Id4 transfected cells showed a significant reduction in the rate of proliferation. After 10 days these cells showed significantly decreased proliferation but were not dying.

We continued to propagate these cells in a serum deprived environment. After 63 days there was an increase in the number of cells in the flask. However, the cells did not become confluent and as a result were not passaged. Along with a decreased proliferation rate, the transfected starved cells also showed an inability to disassociate from progenitor cells, suggesting a role of specific growth factors during late stage mitosis and migration in DU145+Id4 transfected cells. The cells were also growing on top of one another. Additionally the ‘new’ cells (recently divided) were of a smaller phenotype and displayed an inability to adhere to the surface of the flask. This suggests that although the DU145+Id4 cells were able to grow new cells, the newly formed cells were unable to attach to the cell surface without properly conditioned media. The cells were continually propagated in serum deprived media and upon day 101 were observed. After 101 days the cells were still not confluent and as a result they were not passaged. The remaining cells that were observed after 101 days displayed a unique characteristic. There were a number of cells with a significantly increased diameter approximately 10-15 times larger than a normal DU145+Id4 cell (see appendix Figure 18). There is evidence to suggest that these larger cells were multinucleated cells. This observation suggests that although overexpression of Id4 in these cells may allow them to escape apoptosis in an androgen deprived environment and survive for an extended length of time, these cells had a
decreased doubling time. This series of experiments suggest that in a nutrient deprived environment the expression of Id4 allows the cells to enter a quiescent non proliferative state until nutrients become available and require specific factors for normal growth and migration indicative of a normal cancer cell.

To understand the proliferative potential of DU145+Id4 transfected cells compared to normal DU145 cells the doubling time was observed. DU145+Id4 cells showed an increase doubling time of five days. DU145+Id4 cells needed 9 days to become confluent as opposed to approximately 3 days by DU145 cells. A proliferation assay showed that the proliferation rate of DU145 cells had been significantly decreased by the overexpression of the Id4 gene (see appendix Figure 19). This statistical data along with the observation of the morphological changes and the increased doubling time highlight Id4 as a growth and proliferative resistant gene in prostate cancer cells. Therefore, loss of Id4 through promoter hypermethylation may trigger the increased growth/proliferative potential in advanced metastatic prostate cancer cells.

3. Status of Id genes in Id4 Transfected Cell Line

Previous studies have shown a non redundant function of Id proteins on other Id expression in prostate epithelial cells (45). Therefore there was no reason to believe that Id4 expression should have any effect on the expression of Id1-3. However, Id4 expression caused a downregulation in Id1 and 3 specifically (see appendix Figure 20A). Both genes showed significant downregulation at the mRNA level by RT-PCR (see appendix Figure 19). Id1 and Id3 are promoters of proliferation/metastasis in various cancer biosystems including prostate cancer. Therefore, the ability of Id4 to downregulate the expression of these proteins may directly affect both the proliferation rate of these
cells and their metastatic potential. Id2 mRNA displayed a slight upregulation in response to Id4 ectopic expression (see appendix Figure 21). It is unclear the affect this may have on these prostate cancer cells however, Id2 has been associated with the apoptotic pathway (5). In another experiment, Id4 shRNA was transfected into the Id4 positive, androgen dependent, prostate cancer cell line LNCaP (see appendix Figure 20B). Several Id4 shRNA constructs were used and this resulted in the downregulation of Id1, Id2 and Id3 proportional to the downregulation of Id4 by the shRNA plasmids. This suggests that some regulatory mechanism of Id4 may occur. The regulatory role of Id4 over other Id proteins remains unclear as loss of Id4 expression did not result in overexpression of Id1 as the inverse observation was observed in DU145+Id4 transfected cells. Also regulation of Id proteins typically consists of phosphorylation at the end of the G1 phase of the cell cycle. Few transcription factors have been known to regulate Id expression, more notably MYC induced Id1 expression. Therefore, the loss in Id1 expression in DU145+Id4 may be the result of reduce proliferation induced by exogenous Id4 expression and not direct inhibition of Id1 by Id4. Another Viable mechanism is direct Id4-Id gene interaction, through yeast two hybridization Id4 has been shown to bind to Id3 (321), suggesting direct inhibition of Id3 at the protein level. The androgen receptor in this cell line was also significantly downregulated by Id4 knockdown. Whether this knockdown was a direct result of Id4 downregulation or a result of Id1 reduction is unclear (322).

A direct link between Id1 expression and androgen receptor expression has already been established, the regulatory role of Id4 is still under investigation. Regulatory control over proliferation and metastasis is a hallmark aspect of androgen dependent prostate cancer progression. These experiments show a strong correlation between Id
expression and other Id protein regulation which is an atypical characteristic of the Id protein family. This alone has the ability to support Id4 as a tumor suppressor gene. The strong inverse correlation of Id4 expression leading to the downregulation of Id1 and Id3 specifically may allow for the inhibition of prostate cancer progression before Id4 methylation occurs in advanced stage prostate cancer.

4. The role of Id4 in cell cycle regulation

A marquee characteristic of tumor suppressor genes is their ability o influence the cell cycle through the regulation of key cell cycle control genes usually leading to an arrest or induction of an apoptotic pathway (1). Cyclin Dependent Kinases (CDKs) are proteins responsible for inhibiting the Kinases responsible for phosphorylating key proteins involved in cell cycle events (e.g. pRB) (176). Two of the main CDKs are p21 and p27. These proteins are known tumor suppressor genes and play a role in several aspects of the cell cycle (323). In the G1 phase of the cell cycle both of these proteins are responsible for inhibiting CDK2 which is a protein responsible for phosphorylating pRB and promoting G1 to S-phase transition (324). The ectopic expression of Id4 in the DU145 cell line causes a significant increase in the expression of both p21 and p27 (see appendix Figure 22). P27 showed the most significant change in expression. Therefore, the overexpression of Id4 resulted in the increase in expression of these two key cell cycle/tumor suppressor genes. However, the exact effect of the over expression of these G1 checkpoint proteins is unclear as DU145 lacks a functional pRB, and overexpression of p21 nor p27 can change its genetic status (325)

After observing the upregulation of p21 and p27 at the transcript level, further understanding of the role of these proteins in DU145+Id4 cells is needed to understand
the significance of their upregulation. Immunocytochemistry studies confirmed that p21
expression is increased in DU145+Id4 cells compared to DU145 cells. DU145+Id4 cells
show a sub nuclear localization of p21 protein, which is the expected localization site of a
cell cycle regulatory gene (see appendix Figure 23A). Specific populations of
DU145+Id4 cells express some p21 in the cytoplasm (see appendix Figure 23 D)
however the phenotype of these cells is a large with an increases diameter in the nucleus,
suggesting a senescent phenotype. The localization of p21 in the nucleus suggests that
p21 is actively acting as a cell cycle regulator in the nucleus of DU145+Id4 cells. DU145
cells also express p21 strictly in the nucleus. The expression level of p27 was also
increased in the DU145+Id4 transfected cell line. However, the localization of p27 in the
transfected cell line was mostly in the cytoplasmic region and void of nuclear localization
in the transfected cell line. This is an unexpected result due to the cell cycle regulatory
role of p27, which requires activity of the protein almost exclusively in the nucleus to
retain its regulatory function.

Studies have linked cytoplasmic p27 to an oncogenic induced effect in various
cancer cell types. Translocation of p27 from the nucleus to the cytoplasm is indicative of
p27 phosphorylation. The main purpose of p27 phosphorylation is the prerequisite for
protein degradation through ubiquitination. Therefore, it is observed that p27 expression
is highly cytoplasmic in DU145+Id4 with little to no expression in the normal DU145
cell line (see appendix Figure 24). Despite the high expression of p27 in this cell line the
cytoplasmic activity would allow the transfected cell to abate the expected G1 arrest
associated with increased p27 expression, and bypass the G1 checkpoint, allowing the
cell to continue on to the S-phase stage of the cell cycle.
In most cells the induction of p21/p27 results in a G1-phase arrest due to the inability of CDK to phosphorylate the retinoblastoma protein which is an essential aspect of the cell cycle (79). However, in DU145 cells the pRB gene is mutated and under hyperphosphorylation status.

Using FACS analysis we observed that although both p21 and p27 showed significant expression when compared to the normal DU145 cell line, there was not a G1-phase arrest observed (see appendix Figure 25). The lack of a G1-phase arrest is not surprising considering that the cytoplasmic localization of p27 protein in DU145+1d4 cells. The previously observed decrease in proliferation is not attributed to the increased expression of p21 and p27 causing a G1 arrest in these cells through inhibition of pRB activity. However, FACs analysis did show that there was an S-phase increase of 36.5% in the DU145+1d4 cell line, between the 48 hour starved cell line and the cell line treated for 48 hours with serum supplemented media. The normal DU145 cell line only displayed a nominal increase of 14% in the S-phase from 11% to 25%, which is expected after the 48 hour deprivation preceding treatment (see appendix Figure 24B). This data suggest that 1d4 is capable of initiating an S-phase arrest not observed in the normal DU145 cell line under normal conditions. These observations support the role of 1d4 as a essential regulator of S-phase entry in 1d4 knockout mice (269) As a result of the S-phase arrest observed in the DU145+1d4 cell line, there was a sub sequential decrease in both the G1 and G2/M phases of the cycle, 29% and 8% respectively. This observation leads the investigation of 1d4 activity in the cell cycle to the aspect of regulating specific S-phase components.
To confirm the S-phase arrest in DU145+Id4 cells was attributed to the ectopic expression of the Id4 gene another metastatic prostate cancer cell line PC3 was transfected with the Id4 gene and FACS analysis was conducted (see appendix Figure 25B). The results show that although there was an S-phase increase it was not as dramatic as the increase seen in the DU145 transfected cell line. However, when both cell lines were normalized by the serum deprived cell line as a control, both cell lines, DU145+Id4 and PC3+Id4, showed almost exactly the same approximate increase in s-phase expression of 1.8 times (1.83 and 1.81 respectively) (see appendix Figure 25F). The lack of the significant increase in the S-phase of PC3+Id4 as observed in the DU145+Id4 cell line may be due to the lack of specific cell cycle control genes such as p53. This data does suggest that Id4 is responsible for causing an S-phase regression previously not associated with Id4.

The main proteins responsible for phosphorylating p27 before ubiquitination are CDK2 and cyclin E. It is the phosphorylation of p27 that allows it to be degraded by an SCF complex with a crucial constituent consisting of the SKP2 protein. Studies have already highlighted the role Id4 plays in activating cyclin E expression (283). Therefore, to observe if there was a relationship between SKP2 expression and possible cytoplasmic localization of p27 protein we first did RT-PCR studies to observe the expression of SKP2 in DU145+Id4 transfected cells. The ectopic expression of Id4 leads to the upregulation of SKP2 (see appendix Figure 26), a correlation supported by recent studies in Hodgkin lymphoma where Id4 and SKP2 expression display similar expression correlation. Previously, we observed p27 located in the cytoplasm of Id4 transfected cells. Therefore, we wanted to observe the localization of SKP2 protein in Id4 transfected
cells. SKP2 was located either exclusively in the cytoplasm or perinuclear, suggesting a similar localization pattern as p27 expression (see appendix Figure 27). SKP2 is also responsible for degrading another essential cell cycle protein E2F1. E2F1, an essential cell cycle progression protein, is responsible for activating S-phase linked genes to initiate DNA synthesis, and propagate cellular proliferation of the cancer cell. Studies have shown a dual nature of E2F1 to function as a tumor suppressor (through p53 dependent/independent apoptosis incution) and tumor promoter (activation of oncogenes involved in propagating invasion and metastasis).

E2F1 is essential to the progression of the cell to transition through the S-phase. Therefore, the possible degradation of E2F1 would allow for the extended S-phase arrest observed in DU145+1d4 transfected cells. Using ICC studies we observed the expression of E2F1 in the transfected cell line. Ectopically expressing 1d4 cells have a nuclear localization of E2F1 mostly (see appendix Figure 27). However cells that retain a large flat phenotype possessed colocalization of Skp2 and E2F1 in the cytoplasm suggesting a SKP2-E2F1 degradation mechanism. E2F1 is located primarily in the nucleus and functions as a transcription factor throughout cell cycle progression. SKP2 is located in the cytoplasm although there is some overlap of both proteins. In DU145, interestingly, SKP2 expression is localized mainly in the nucleus. SKP2 is not known to act as a transcription factor and its protein ubiquitination mechanism is cytoplasmic dependent. It can be inferred that in DU145 cells E2F1 is not under any regulatory action by SKP2 and is active in the nucleus as a transcription factor to promote the cell cycle. Therefore it is inconclusive whether or not E2F1 degradation through SKP2 protein is responsible for the S-phase arrest in DU145+1d4 cells.
5. **Androgen Receptor Regulation by Ectopic Id4 expression**

In prostate cancer, the progression from an androgen dependent to an androgen independent state usually marks the transition to a highly aggressive, more metastatic form of the disease. The regulation of the androgen receptor is a key component of prostate cancer treatment. In the early stages of the disease the preferential clinical treatment is androgen ablation therapy either through radical prostatectomy or through chemical castration using AR antagonist responsible for inhibiting circular levels of androgens in the blood (326). The LNCaP cell line is used in vitro to mimic androgen dependent prostate cancer; with a mutated AR receptor with the T877A AR mutation which has the AR gene transcriptionally turned on (327). Whereas, DU145 and PC3 are the accepted androgen independent prostate cancer cell lines, with a transcriptionally inactive AR (328).

Our previous studies showed that silencing Id4 in LNCaP cells resulted in the downregulation of the AR receptor. After overexpressing Id4 in DU145 cells, we see an activation of AR at both the mRNA transcript level and the protein level (194) (see appendix Figure 28). This is a significant observation in an androgen independent prostate cancer cell line. This suggests that Id4 may be responsible for directly regulating the expression of AR. The induction of Id4 resulted in a ~50 fold increase in androgen receptor expression. Due to the ability of DU145 cells to proliferate in absence of a functional androgen receptor, the ability of this cell line to maintain a functional response to AR induction is questionable. Therefore, we observed the expression of downstream AR target genes specifically prostate specific Antigen (PSA). As a result we noted that the expression of PSA was induced in the ectopically expressing Id4 cell line, upon
treatment of this cell line with DHT the potent metabolically active form of testosterone (see appendix Figure 29). PSA was upregulated in the DU145+1d4 cell line but showed no response in normal DU145 cells suggesting that 1d4 is directly responsible for this induction of PSA. Treatment of DU145 cells with DHT also resulted in the induced expression of AR, but no PSA response. This suggests that, in the androgen independent state of DU145 cells, 1d4 may play a crucial role in activating a functional androgen receptor.

Previously, we observed a correlation between ectopic 1d4 expression and p21/p27 expression. Understandably, p21/p27 expression is an essential checkpoint in the transition from G1-S-phase in most cells. Also, the AR is a proliferation associated gene, which in androgen dependent prostate cancer is responsible for promoting proliferation. Therefore, because p21 and p27 are highly expressed in DU145+1d4 cells under normal conditions we wanted to see how the expression of these genes were affected by DHT treatment in the DU145+1d4 cells with a functional AR. As a result, both p21 and p27 were downregulated upon treatment with DHT. At 6 hours both p21 and p27 expression had significantly decreased and although p21 expression reemerges by the 12th hour of treatment, p27 expression is still significantly downregulated at 12 hours. An interesting note is that the expression of DHT had an adverse effect on p21 and p27 expression. This suggests two things; firstly that the transfected cell line has a different response to androgens as the normal DU145 cell line, and that DHT may play a role in regulating the cell cycle of DU145 despite the inactive androgen receptor.

To further understand the profound relationship between 1d4 and AR, we wanted to observe AR response in the cell using Immunocytochemistry. In an androgen
responsive cell line, such as LNCaP treated with DHT, DHT passes through the cell membrane and directly into the cytoplasm. In the cytoplasm DHT binds to the androgen receptor displacing the HSP protein (59). Upon ligand binding of the androgen receptor the complex is then translocated to the nucleus. In the nucleus AR coactivators assist in allowing AR to bind to the promoter of key AR target genes at the androgen response element (ARE). After binding to the ARE, transcriptional target genes are upregulated.

Using ICC first we serum starve the cells for 48 hrs in RPMI only media. Post starvation in serum deprived media, we treated the cells with media supplemented with either charcoal stripped media (csFBS), DHT, or Casodex (CSD), the androgen receptor antagonist, for 12 hours. The purpose of this study was to confirm the transcriptional activity of AR in DU145+1d4 cells through translocation of the AR to the nucleus upon treatment with DHT. In serum deprived media DU145+1d4 cells show considerably less expression of AR (see appendix Figure 29). Also the AR expression is apparently mostly cytoplasmic, suggesting none/little nuclear activity. Treatment of DU145+1d4 cells with csFBS showed no differential change in expression of AR. Although there was some minimal nuclear staining, most of the AR protein was located in the cytoplasm of the cell suggesting no activity in response to charcoal stripped FBS. Treatment of the DU145+1d4 cell line with DHT resulted in an increased expression of the Androgen Receptor. It was also noted that in the DHT treated cells the expression of the AR protein was both cytoplasmic and nuclear. In these studies the DHT treated cells displayed the highest nuclear staining observed amongst the treated samples. Upon treatment with DHT in DU145+1d4 cells, DHT binds to the Androgen Receptor and is translocated to the nucleus suggesting not only a functional androgen receptor but also a function AR response
network. This element of the study was necessary to prove that DU145 cells have retained the machinery necessary for AR response. In prostate cancer sometimes the androgen receptor can become mutated to allow for hyper activity of the androgen receptor, where multiple ligands are able to bind and activate the AR receptor including the antagonist Casodex. Therefore, we wanted to see if Casodex would antagonize or activate the androgen receptor in DU145+Id4 cells. After treating the Id4 expressing cells with Casodex for 12 hours we noticed that although AR expression was higher than the androgen starved cells, the location of androgen receptor was almost exclusively cytoplasmic. The nucleus of the DU145+Id4 cell was almost completely devoid of AR expression. This experiment verifies that the androgen receptor in these cells is functional and that Casodex is incapable of activating the androgen receptor. Instead, Casodex in these cells acts as an antagonist as seen in other androgen dependent cell lines.

To get a full understanding of the roles charcoal stripped FBS, DHT and Casodex play prostate cancer we also conducted ICC on the advance, androgen independent prostate cancer cell lines DU145 and PC3 (see appendix Figure 31 &33). We also used the androgen dependent prostate cancer cell line LNCaP as a positive control for functional AR activity (see appendix Figure 32). In the DU145 cells, there was minimal expression of AR in the starved and csFBS treated cells as expect. The DHT treated cell line did show an increase in AR expression, however, this expression did not result in the translocation of AR to the nucleus in DU145 cells. There was no nuclear staining of AR. This suggests that there is an essential Id4 related component of the AR translocation-function pathway responsible for allowing AR to be transcriptionally active upon DHT treatment. Casodex did not have the same effect on AR treatment as did DHT. Treatment
of Casodex did not fully activate the AR response machinery; however there was some nuclear staining of AR. This may be the result of a mutated AR. However because our previous PSA studies showed no downstream expression of AR this therefore suggest that this nuclear staining of AR upon Casodex is superficial at best.

LNCaP cells treated with Casodex displayed the same AR ICC profile as seem with DU145+Id4 cells. Treatment of LNCaP cells with DHT did result in AR being translocated to the nucleus as expected. Treatment with Casodex resulted in a decreased expression of AR, however there was some residual nuclear translocation in response to Casodex. This response is supported by literature that states LNCaP cells have a mutated androgen receptor with the ability to bind non DHT/Testosterone ligand that may activate the androgen receptor. Serum starvation and treatment of LNCaP cells treated with charcoal stripped FBS resulted in decreased expression of AR with minimal nuclear staining.

PC3 cells were unresponsive to androgen treatment. This has been well documented, and is the result of PC3 cells having an inactive androgen receptor. PC3 cells did show an increase in androgen receptor however these cells were lacking AR nuclear localization as seen in either LNCaP or DU145+Id4 cells. Both the starved and charcoal stripped PC3 cells showed significantly decreased expression of AR. The PC3 cells treated with Casodex, displayed the same inflection of nuclear staining previously observed in DU145 cells treated with Casodex. The exact nature of this sub nuclear, specifically located AR expression is not completely understood. But it is understood that the expression of the AR protein in these specific locations does not result in downstream
AR target genes of functional AR activity. The expression of these nuclear specific AR proteins may suggest another role of Casodex treatment in PC3 cells.

Androgen receptor expression and function is an important part of prostate cancer growth and progression. The ability of ectopic Id4 expression to induce a functional androgen receptor has great significance of the ability of Id4 to act as a tumor suppressor. This also has great implications that the role Id4 may play in prostate cancer progression and that role may be the result of Id4 methylation. The ability of the Casodex to inhibit translocation of the AR to the nucleus in DU145+Id4 cells, may allow for new therapeutic targets targeting a functional Id4-AR regulatory network.

To further understand the role androgen receptor response plays in DU145Id4 cells, we observed the transition of these cells through the cell cycle in the presence of DHT in comparison to the control DU145 cell line. We have already discovered a relationship between ectopic Id4 expression and cell cycle control genes. If Id4 is responsible for regulating the cell cycle then the cell may show a change in cell cycle response due to treatment with androgen receptor agonists and antagonists. Downregulation of p21 and p27 in response to DHT treatment suggest that DHT treated cells are prone to bypass cellular checkpoints to initiate a quicker cell cycle response. The cells were serum deprived for 48h hours before DHT treatment. The normal cell line showed a normal progression through the cell cycle when treated with DHT, suggesting that the DU145 cells were cycling normally (see appendix Figure 34). This cell line showed a peak of S-phase at 24hrs a: approximately 50% which had regressed to 23% by the 48 hour mark. This expression level shows a similar tri-phasic profile as observed previously in figure 7 with a slightly increased G1-G0 accumulation from 50% to 64%.
This suggests that DU145 cells may have a slightly different response to DHT as opposed to serum.

The DU145+1d4 cells line displayed a very interesting cell cycle profile once exposed to androgens. Initially the cells had a very high G1-G0 phase accumulation and that accumulation in G1 continued throughout the 48hrs in response to DHT (see appendix Figure 35). After 48 hours 74% of the cells were in G1-G0 phase of the cell cycle. Our proliferation assay showed an increase in proliferation so we know this is not the result of a G1 arrest occurring. This therefore suggest that the cells are proliferating at a higher rate and bypassing though the checkpoints of the cell cycle designed to slow down/halt the proliferation of the cell. This suggests that these DU145+1d4 cells are responding to DHT in the same manner expected as normal androgen dependent prostate cancer cells. This promotes the notion that Id4 allows the cells to proliferate through the cell cycle in an androgen dependent fashion.

In the normal prostate and androgen dependent prostate cancer cells, AR has a regulator control over proliferation. Therefore was wanted to observe the effect that androgen and anti-androgen treatment had on DU145+1d4 cells that have a functional ER. We observed that DU145+1d4 cells had a 3 fold increase in a rate of proliferation in response to androgens (see appendix Figure 36.. Anti-androgen treatment with Casodex resulted in a decrease in proliferation in both DU145 and DU145+1d4 cells. These results show that DU145_Id4 cells respond to androgen treatment. DU145+1d4 cells also proliferated slower in androgen deprived media.

6. Id4 Induction of Cellular Senescence in Prostate Cancer cell line
Cellular senescence is a dramatic alteration of cellular replicative programming. Replicative senescence is indicative of an individual cell attaining its limitation within replication, by the shortening of telomeres. Normal cells reach an endpoint, characterized as Hayflick's limit (see appendix Figure 5), and lose the ability to divide further. Cancer cells are characterized as cells that have an unlimited dividing potential and have bypassed the ability to undergo cellular senescence. Recent studies have focused on synthetic compounds and treatments designed at inducing cellular senescence in cancer cells. The ability to induce senescence in cancer cells suggests a significant regression in the most devastating characteristic of cancer cell behavior, uncontrolled proliferation.

Senescent cells are characterized by a long flatten elongated shape, usually at least twice the size of the normal replicative cell (see appendix Figure 37). Senescent cells express a high amount of β-galactosidase. This enzyme is responsible for converting β-galactosidase to monosaccharides. This enzyme is accumulated in the lysozomes in senescent cells. Therefore the senescent assay used is responsible for staining the β-galactosidase in the lysozomes of senescent cells. The stain results in an accumulation of the dark green pigment in the senescent cells, which highlights the presence and localization of β-galactosidase in these cells. There was a clear disparity in the presence of cells with β-galactosidase staining in DU145+1d4 cells versus normal DU145 control cells (see appendix figure 38). There were a number of cells with some β-galactosidase staining but had clearly not taking the phenotypic cellular structure of a senescent cell. These cells were characterized as moderately senescent. Cells with a high accumulation of β-galactosidase staining were characterized as advanced cellular senescent cells.
To quantify the presence of senescent cells, images were taken under the light microscope at 200x magnification. The cells were seeded in 24 well plates and each treatment was done in triplicate. After treatment with the senescence assay images from 5 random fields in each well were taken for the greatest statistical significance. In each field of image, first the total number of cells was counted. Then the total number of advanced senescent cells (ACS) was counted, then the numbers of moderately cellular (MCS) senescent cells were counted. The sum of each of the senescent characteristics was taken and divided by the total number of cells in the field to obtain the percentage of either moderately or advanced senescent cells. Cells were starved for 48 hours then treated with charcoal stripped FBS, normal FBS, DHT and Casodex. Because we know the DU145+Id4 cells have a unique ability to respond to androgen, we wanted to know if that might also affect their ability to undergo senescence (see appendix Figure 39).

In the cells that were starved for 48 hrs, the DU145 cells showed 0% ACS and 10% MCS, compared to DU145+Id4 which had 12% of the cells undergoing ACS and 68% of the cells with MCS. In the cells that were treated with charcoal treated FBS, the DU145 cells had 3% with ACS, and 14% with MCS. The DU145+Id4 cells had 18% of the cells with ACS, and 63% of the cells with MCS. In the cells treated with normal FBS, the DU145 cell line had no cells with ACS and 14% of the cells with MCS. In the DU145+Id4 cell line 30% of the cells had ACS, with 62% of the cells with MCS. In the cells treated with DHT, the DU145 cell line had 8% of the cells with ACS, and 46% of the cells with MCS. In the DU145 Id4 cell line 34% of the cells had ACS, and 66% of the cells with MCS. In the cells treated with the androgen antagonist Casodex, the dU145 cell
line had 3% of the cells with ACS, and 23% of the cells with MCS. In the DU145+Id4 cell line, 64% of the cells had ACS and 87% of the cells had MCS.

These results clearly indicate that DU145+Id4 cells have a higher propensity for cellular senescence than normal DU145 cells under normal conditions as indicated by their response to starved treatment and normal FBs treatment. There is also a correlation between androgen receptor activity and cellular senescence in the DU145+Id4 cells. The cells treated with Casodex, the DHT antagonist, exhibited the highest percentage of cells with advanced cellular senescence as indicated by the assay (see appendix Figure 40). This suggests that inhibition of the AR in these cells drives the cells to undergo cellular senescence as the proliferation is inhibited. Another interesting observation was the fact that normal DU145 cells displayed an increase in both moderate and advanced cellular senescence in response to DHT suggesting that although these cells do not have a functional AR there may still be some evolutionary relationship between DHT treatment and cellular senescence. DU145+Id4 cells treated with DHT also showed an increased affinity for cellular senescence as opposed to normal FBS treatment. This is also an interesting observation whose implications are not fully understood.

The molecular mechanisms behind cellular senescence are not totally understood. The phenotypical profile of senescent cells is visible in by their flat elongated shape, and is assisted by the use of β-galactosidase staining. Although the exact genes responsible for senescence are not entirely understood, there are several genes which show a relationship with influencing the cellular senescent programming. Therefore, to get an understanding of the role of senescence in these cells we decided to look at some of these genes in our Id4 transfected DU145 cell line. These genes include Vimentin, p16, p21,
p27 and p53. We will discuss p53 expression further in this study. In our DU145+Id4 cell line all of these genes showed an upregulation over the normal DU145 cell line supporting the data displayed by our senescence assay suggesting that these cells have the molecular programming to undergo cellular senescence (see appendix Figure 41). We have already looked extensively at p21 and p27. Two of the major contributors to cellular senescence are p16 and Vimentin. Vimentin is responsible for altering the cellular structure of the cell and initiating the flat elongated shape observed by senescent cells and we see that Vimentin is significantly upregulated in our senescent cells. P16 is another G1 cell cycle regulator that has shown a significant correlation between p16 expression and cellular senescence. However, in the DU145 cell line p16 has been characterized as mutated which has posed a problem for inducing cellular senescence. However in these DU145+Id4 cells, they have found a way to bypass the mutation association of p16 and induce cellular senescence at a significant level. Therefore to understand the role p16 plays in these cells we wanted to look at its localization using ICC. After performing ICC experiments on DU145 and DU145+Id4, we noted that p16 had a very similar localization as p27 in DU145+Id4 where the expression of p16 was highly localized to the cytoplasm of the cell (see appendix Figure 42). This is very atypical of a cell cycle regulator as p16 expression should be localized to the nucleus specifically. The applicable translation of p16 cytoplasmic localization in correlation of senescence induction is not entirely understood but it does pose an intriguing questions. “What is p16 doing in the cytoplasm of these highly senescent cells?”

7. Id4, an inducer of apoptosis in DU145 prostate cancer cells
One of the main characteristics of a tumor suppressor is its ability to induce apoptosis in a cancer cell. Advanced cancer cells have adapted their molecular programming to evade apoptosis and override checkpoints designed to inhibit excessive proliferation in normal cells. As these cells become immortalized they switch off certain mechanisms and override the apoptotic pathway to an extent. In this experiment the primary goal was to ascertain how influential the Id4 gene was at inducing apoptosis in the advanced prostate cancer cell line DU145. This cell line is known to be very resistant to apoptosis. Several of its transcriptional machinery components are either mutated or non functional. The first step of understanding the role Id4 plays in apoptosis in DU145 was to use an apoptosis assay to observe the level of apoptosis in comparison to the normal DU145 cell line. An Annexin V apoptosis assay was used to stain live DU145 and DU145+Id4 cells. Annexin V is a dye conjugate that will fluoresce under apoptosis in a green fluorochrome. Under normal conditions Annexin V will only bind to the surface of a normal "live" cell and cannot penetrate the cell membrane barrier and will lightly stain the surface of the "healthy" non apoptotic cell. However, if the cell is undergoing apoptosis, then Annexin V can penetrate the cell membrane and will highly fluoresce a green pigment. The Annexin V stain in this assay is used along with Propidium Iodide which binds to DNA. However, in live cells PI cannot penetrate the cell membrane barrier and therefore in a non apoptotic cell the PI will not stain the cell. Only in an apoptotic cell with a deteriorated cell membrane can PI transverse the cell membrane and stain the DNA content in the nucleus. In DU145+Id4 cells there was an increased staining of both Annexin V and PI compared to DU145 cells when observed under the fluorescent microscope (see appendix Figure 44).
Using a flow cytometer we were able to quantify the cells that were non-apoptotic, undergoing apoptosis and already dead. Cells with little to no Annexin V staining and no PI staining were non-apoptotic (see Appendix figure 45). Cells with some Annexin V stain but no PI staining were cells undergoing apoptosis, and cells with both Annexin V and PI staining were either dead or dying cells. To also get an understanding of the susceptibility of these cells to apoptosis we conducted these experiments using the Annexin V staining both in the presence and the absence of Actinomycin D, an apoptotic inducing agent. Cells were either grown in the presence of Actinomycin D for 6 hrs at a concentration of 10nM, or without the presence of Actinomycin D for 6 hrs (see appendix Table 6). Using flow cytometry we observed the percentage of cells either dying or undergoing apoptosis (see table 1). Under normal conditions 73% of DU145 cells were non-apoptotic and viable with little to no Annexin V staining. In DU145+Id4 cells only 34% of the cells were initially non-apoptotic and showed no signs of Annexin V staining. Only 24% of normal Du145 cells exhibited higher levels of Annexin V staining and were thus apoptotic. In comparison, 60% of DU145+Id4 cells exhibited significant Annexin V staining. Only 3% of DU145 cells were either dead or dying compared to 7% of DU145+Id4 cells which had high Propidium Iodide staining. These results indicate that even under normal conditions, without and apoptotic inducing agent, DU145+Id4 cells are more readily apoptotic and dying at a faster rate. This data supports our earlier images seen with the fluorescence microscope and our earlier cell morphology images showing necrotic cells.

In the cells treated with Actinomycin D for 6 hrs, 23% of the Normal DU145 cells exhibited little to now Annexin V staining, which was similar to the amount of DU145
+Id4 cells, 20%, which showed little to no Annexin V staining. When treated with Actinomycin D the normal DU145 cells also had a higher percentage of cells showing apoptosis, 69 %, when compared to 57% of the DU145+Id4 cells. However, the greatest difference between the two cell lines was seen in the percentage of cells with high levels of PI staining. Actinomycin D caused only 9% of normal DU145 cells to either be dead or dying. Whereas, 23% of DU145+Id4 cells were either dead or dying after a 6 hr treatment with Actinomycin D. This suggest that DU145+Id4 cells are more susceptible to apoptosis than DU145 Cells due to a more than 3 fold increase in the percentage of dead cells after treatment with Actinomycin D. There was a decrease in the number of cells undergoing apoptosis when compared to the normal DU145 cell line. This discrepancy may be due to Actinomycin D being a target of DNA synthesis. DU145+Id4 cells already have an inhibited S-phase progression. This may result in an initial inhibition of DNA synthesis before Actinomycin D treatment. This data also suggests that cells expressing Id4, even advanced prostate cancer cells, are more susceptible to therapeutic treatment that Id4 absent cells.

In order for there to be an increase in apoptosis under normal conditions several molecular mechanisms must have been altered by the overexpression of Id4. One of the main regulators of the apoptotic pathway is p53. This gene is a regulator of apoptosis, senescence, cell cycle and a number of other key events in the cellular programming. In our transfected cell line all of these cellular processes have been altered. Therefore, it is only natural that we investigate the expression profile of p53 in these cells. At the mRNA level p53 expression showed a significant increase of the normal DU145 cell line by
Real-Time PCR. There was a 17 fold increase of p53 expression as detected by real-time PCR (see appendix Figure 47).

Another key tumor suppressor gene that was regulated by the ectopic expression of Id4 was E-cadherin. E-cadherin is a key cell to cell interaction protein. At the mRNA level E-cadherin expression increased over two fold. The over expression of E-cadherin explains the increased epithelial morphology observed in our DU145+Id4 cells. It also explains the decreased proliferation of DU145+Id4 cells (see appendix Figure 47). E-cadherin is another tumor suppressor gene and its increased expression supports the hypothesis of Id4 as a tumor suppressor. At the protein level, there was an increase in p53 expression by DU145+Id4 cells. This suggests that ectopic expression of Id4 resulted in a post transcriptional modification of the p53 protein and may have lead to protein instability.

It is noted that the p53 gene is mutated in DU145+Id4 cells and has been shown to act as an oncogene. Therefore, to get a better understanding of the mechanism of p53 function we utilized ICC to understand the localization and function of p53 in the Id4 expressing cells, mutated or not. In the normal DU145 cells, p53 expression was found primarily in the nucleus. Suggesting any activity p53 may have as a cell cycle regulator or transcription factor was localized in the nucleus. Mutated p53 does attribute a gain-of-function that allows it to act as a transcription factor to regulate specific oncogenes. Therefore, it is possible mutated p53 in normal DU145 cells is acting as a proto-oncogene. In the DU145+Id4 cell line there is a significant localization of p53 protein in the nucleus as well as in the cytoplasm. This supports the ability of p53 to retain some of its transcriptional activity despite being mutated in DU145 cells. The localization of p53
in the nucleus not only supports 1d4 as a tumor suppressor gene, but also offers an explanation for the increased apoptosis in DU145+1d4 cells. Cytoplasmic staining of p53 has been linked to increased apoptosis. In the nucleus p53 may induces apoptosis through a transcriptional mechanism. In the cytoplasm p53 has been linked to direct induction of apoptosis through the mitochondria. In the cytoplasm p53 can induce apoptosis through MOMP and inhibit autophagy. Functional activity of p53 protein may also suggest why there is an increased susceptibility of DU145+1d4 cells to undergo apoptosis in the presence of therapeutic agents. This makes understanding 1d4 expression an essential part of finding a therapeutic target for advanced prostate cancer.
CHAPTER FIVE

DISCUSSION

The role of Id4 in cancer progression is not entirely understood. However, Id4 expression is essential to normal brain development and function (269, 276). Also loss of Id4 correlates with increased expression of other Id proteins in its absence (1, 269). The role of Id4 in cancer progression is even less understood. The significant correlation with Id proteins (Id1-3) and cancer progression has been extensively studied and verified (1, 26, 27, 30, 126, 151, 167, 193, 201), and the cornucopia of mechanisms that Id proteins regulate provide scientist with an excellent target for drug development that can provide great hope for cancer treatment. Initially, Id proteins were classified as bHLH transcription factors with the ability to regulate transcription of many genes (30, 31). However your classical bHLH transcription factor requires DNA recognition sites for binding and hetero/homo-dimerization partners (117, 329, 330). Id proteins have neither. These characteristics however make Id protein activation more influential and effective in their role as transcription factors.

The absence of a basic domain designates Id proteins as the ‘regulators of the transcription regulators.’ It also allows Id proteins the ability to regulate many more types of proteins, and are not limited by a specific DNA recognition site. Therefore, the ability to regulate Id proteins in cancer may provide for a more effective drug target by simultaneously regulating multiple cellular processes (i.e. Angiogenesis proliferation,
apoptosis, cell survival, senescence etc.) discussed by several authors (13, 29, 34). The potential for Id drug targets is extended by the ability of Id proteins to directly interact with specific proteins (e.g. RB, ETS). This further extends the mechanism of inactivation of Id cellular disruption by utilizing effective drug inhibition of Id interaction. The mechanism of action of Id proteins also provides for selective drug development. Due to the fact that Id proteins bind specific target, there are options to tailor drug treatment to these targets. By determining the specific bHLH/non-bHLH targets that Id proteins inhibit, drugs developed to disrupt Id-inhibition of these specific bHLH proteins may provide an effective drug treatment option. Also, drugs may be developed around the specific bHLH targets that Id proteins influence. An example of this is; if Id proteins inhibit a specific bHLH protein, per ser E2A, and E2A is responsible for inhibiting proliferation by binding to the promoter region of ‘gene X’. Then drugs can be developed to stimulate gene-x directly and bypass the Id inhibition mechanism. Therefore, it is essential we elucidate the Id inhibition mechanisms by discovering the vast network of Id interaction proteins that are both non-bHLH and bHLH alike. These discoveries may provide a fortified surge in the war against cancer.

Id proteins are a very specific type of bHLH transcription factor, with no nuclear signals, no DNA binding domain and no ability to directly regulate transcription. However, their role in both development and cancer progression is quite evident (1, 31). Unlike many “oncogenes” that are activated through mutations or genetic regulating mechanisms, no such occurrences have been discovered thus far in Id proteins. This is a profound observation, due to the frequency of genetic alterations, in cancer cells that become mutated to an extent where they lose/reverse normal gene function (e.g. p53,
pRB). And although Id proteins are an essential component to the cell cycle, they are regulated through phosphorylation dependent mechanisms, indicative of classical ‘Cyclin/CDK’ cell cycle regulation (1). Therefore the role of these proteins in cancer progression is both understandable and justified.

Most of the Id proteins (Id1-3) have been extensively studied and their role in cancer progression has been adequately verified. However, Id4 poses the most mysterious role in cancer progression. As the Id protein with the longest protein sequence and the most divergent protein structure, a significant role is indicated. Initially, as Id4 was one of the last Id proteins to be characterized by scientists, an attempt was made to assimilate Id4 into the stigma of Id protein “pro-oncogene” role in cancer progression (43). However, studies suggest Id4 may provide the most influential/divergent role of all the Id proteins (17) due to a different expression pattern. Thus far Id4 is the only Id protein observed to undergo epigenetic regulation, through promoter hypermethylation observed in various cancer specimens suggesting a continuous role of Id4 in universal cancer progression. Id4 is the only Id protein highly expressed in differentiated tissue (43), suggesting a very divergent role of this “Inhibitor of differentiation” protein as the namesake suggests. Therefore, these observations alone strengthen the designation of Id4 a tumor suppressor in cancer.

There is a general lack of investigation into the method of Id protein interaction, in prostate cancer when compared to frequency of study of Id proteins in other cancers. With Id4 being the least studied Id protein, this results in an obscure understanding of the role of Id4 in prostate cancer. Despite the fact that several studies have characterized Id4 as a global tumor suppressor gene (52, 194, 291), its role in prostate cancer may be more
specific, and insightful into androgen-dependent to androgen-independent progression.
The most significant Id4 expression is observed in the brain, which is indicative of the role Id4 plays in normal brain development (269). The second greatest Id4 expression is observed in the testis, an organ whose growth and development is govern by the role that androgens play in its development. The other organ with androgen stipulated regulation of development is the prostate, suggesting a significant correlation between Id4 expression and prostate development and conversely prostate cancer progression. Therefore, we can further extrapolate that the prostate is more prone to cancer progression than the testis as the prostate is an organ with higher epithelial content than the testis, as the epithelial cell layer is the most frequent site of origin for many cancer types. Epithelial cancers (Carcinomas) are the most frequent type of cancers, with 85% of all cancers being carcinomas.

Our investigations of the role of Id4 in prostate cancer progression are among the few research endeavors to understand this role of this protein in the prostate. However, our studies suggest a very strong indication that Id4 acts as a tumor suppressor in prostate cancer development. Initially, it was observed that Id4 expression is absent in late stage and metastatic prostate cancer tissue sample, when the other Id proteins show significant expression in metastatic prostate cancer tissue samples (37). Through our studies we have observed that Id4 is downregulated in prostate cancer through promoter hypermethylation in advanced stage prostate cancer. Also some evidence suggest that Id4 may be able responsible for regulating the methylation programming of its own promoter, and possibly the methylation status of the promoter region of other genes. Id4 is also an evident regulator of cellular senescence, in prostate cancer, a key cellular replication-
limiting checkpoint. Cancer cells are able to bypass the limitation and proceed to proliferate uncontrollably. Id4 is also a regulator of the growth potential of prostate cancer cells and has a role in attenuating the proliferative potential of prostate cancer cells. Id4 induction can also induce apoptosis in cancer cells that lack a conventional apoptotic pathway.

As an Id protein, it is not surprising to observe cell cycle regulatory aspects of Id4 induction in cancer cells that attempt to limit the progression through the cell cycle in an Id4-S phase specific manner. However, the two most surprisingly observation of Id4 as a prostate cancer specific tumor suppressor is: (1) the ability of Id4 to regulate other Id genes, specifically Id1 and Id3 at both the transcript and protein level (data not shown); (2) the ability of Id4 to regulate the AR receptor in an androgen independent cell line, with an androgen receptor that is hypermethylated. Our studies into the role of Id4 are novel for their ability to provide transparency into a vague understanding of Id protein regulation/interaction. Through our investigation into Id4 molecular mechanisms we may provide a profound understanding of prostate cancer progression from an androgen-dependent to androgen-independent mechanism.

1. Id4 expression and methylation status in prostate cancer

Id gene expression is lost during prostate cancer progression, using a panel of in vitro prostate cancer cell lines we observe that the loss of Id4 expression is accelerated as the prostate cancer cell lines become more aggressive, invasive and androgen independent. These in vitro cell lines mimic Id4 loss of expression in prostate cancer progression similarly. The normal cell lines PrEC and BJ cells have higher id4 mRNA expression. Two significant observations were observed, firstly the androgen dependent
cell line LNCaP shows minimal Id4 expression. However the LNCaP derivative cell lines, C-33 and C81 show decreased Id4 expression. Id4 promoter studies show that C-33 and C-81 have a methylated Id4 promoter (data not shown) resulting in downregulation of Id4 expression. These studies support both the observation of loss of Id4 expression in prostate cancer cell lines through promoter hypermethylation. Also it is observed that PC3 expresses some low levels of Id4 expression, however the efficacy in PC3 cells may be limited by the litany of tumor suppressor genes down regulated in this cell line (i.e. p53 and RB, p16, p27 etc). Therefore in the PC3 cell line Id4 activation may be abated by the absence downstream target. However it is observed that, the PC3M cell line, a more metastatic invasive derivative in the PC3 cell line shows a decrease in Id4 expression when compared the Pc3 cell line. The observations verify the presence of Id4 in our transfected DU145+Id4 cell line and absent in the DU145 untransfected cell line, verifying the presence of Id4 in our studies.

Epigenetic downregulation of tumor suppressor genes through promoter hypermethylation is one mechanism that cancer cells utilize to evade destruction and continue to proliferate unchecked. Several tumor suppressor genes are silenced in prostate cancer (90, 331). The Id4 promoter region has two distinct CpG island rich regions that are susceptible to DNA hypermethylation (273, 289). Id4 has been shown to be downregulated in several cancers through promoter hypermethylation. Id4 hypermethylation is also associated with poor prognosis and cancer reoccurrence. Treatment with 5-aza-cytidine results in Id4 re-expression. In our androgen-independent prostate cancer cell line DU145, the Id4 is also hypermethylated, both in DU145 and PC3 cells. The interesting observation is the reoccurrence of a partially unmethylated Id4
promoter in DU145+ld4 cells. This suggests that the exogenous expression of ld4 is responsible for the demethylation its own promoter. These results also implicate that ld4 is capable of controlling methylation specific genes (i.e. DNMT’s). Therefore the expression of ld4 may be responsible for the demethylation of other tumor suppressor genes in prostate cancer.

One gene that is methylated in prostate cancer that has significant implications in prostate cancer is the Androgen Receptor (59). The androgen receptor is methylated and expression lost in approximately 8% or primary tumors and unknown in hormone refractory prostate cancer. However 20-30% of all androgen independent prostate cancers have loss of androgen receptor expression. Therefore, ld4 may act as a tumor suppressor by abating the methylation programming of the androgen receptor and subsequent loss of ld4 expression may result in AR promoter hypermethylation. The loss of ld4 in prostate cancer through promoter hypermethylation strengthens its role as a tumor suppressor gene in prostate cancer. Also ld4 is the only known ld gene that is epigenetically regulated in cancer that we presently know of.

2. The significance of ld4 morphological changes and phenotypical changes

The ability to rearrange the cellular structure and growth pattern of cells is a hallmark characteristic of tumor suppressor genes. The first observed changes of ld4 induction into the androgen-independent prostate cancer cell line DU145 were the phenotypical changes. The initial observation was that the cell diameter of cells changed dramatically in response to ld4 induction suggesting that ld4 is responsible for key changes in cell structure and alteration of structural proteins such as microtubules and actin filaments. ld4 may be responsible for regulating the expression and localization
patterns of these proteins in an attempt to retain a differentiated phenotype, and turn off the mesenchymal, cancer-induced changes to the cell.

In later studies, it was observed that the senescent phenotype observed in doxorubicin induced DU145+ld4 cells led to changes in Vimentin and E-cadherin protein expression. Vimentin appeared to be involved in the displaced, flattened shape observed in DU145+ld4 senescent cells with concentrations at the poles of the cells with an increased diameter. E-cadherin, a known tumor suppressor gene and protein responsible for cell-cell interaction, observed an alteration in the localization pattern in DU145+ld4 cells with an emphasis in E-cadherin expression surrounding the nuclei of polyploidy cells, and not around/within the cell membrane. Therefore, the emphasis of E-cadherin in the large cells with an increased diameter suggests a causative role for E-cadherin in obtaining this phenotype. Contrary to the tumor suppressor role of ld4, some studies suggest that an increase in cell size can be the result of EMT activation, leading to a more aggressive phenotype. However, the presence of β-galactosidase in the large DU145+ld4 cells supports the tumor suppressor role of ld4 by classifying these cells as senescent cells, which have attained a replicative endpoint.

Another major observation in DU45+ld4 transfected cells was the presence of cells that were either had an enlarged nucleus or were multinucleated. As stated above one of the first observations were the increase in the cell diameter of DU145+ld4 prostate cancer cells. Using Immuno-fluorescent imaging it was also observed that many of these cells had an enlarged nucleus, or were multinucleated with cells containing 3+ nuclei within one cell. Several cells with 10+ nuclei were also observed. There are several possible explanations for multinucleated cells. Mutinucleation is most often observed in
cancer cells treated with DNA damaging agents which result in a fusion of the nucleus resulting in polyploid cells. Also, the presence of many nuclei within one cell suggest a problem in cell cycle exit upon completion of mitosis, with an inability of the cell to divide properly through an error in the cytokinesis/mitosis exit mechanism. However the exact designation multinucleated cells play in cancer cells, whether it is a pro-oncogenic/tumor suppressor role is unclear. It is however evident that DU145+1d4 prostate cancer cells readily become multinucleated in response to 1d4 induction.

Several cellular changes were observed in the type of growth induced in DU145+1d4 cells when compared to control (DU145) cells. There was a severe reduction in the rate of proliferation, when observed under the microscope; this may be attributed to the morphological characteristics observed in 1d4 transfected cells. 1d4 transfected cells observed a more “epithelial-like” phenotype, congregating in clusters, which may be attributed to the increased E-cadherin expression at both the mRNA level and the protein level. In several instances, DU145+1d4 cells grew with closes cell-to-cell interaction and in many instances exhibited growth directly on top of other cells, which is a very divergent phenotype from a prostate cancer cell line, known for its migratory properties. This suggested that the migratory abilities of this prostate cancer cell line had been severely reduced in response to 1d4 induction. These studies were later supported with a scratch wound assay that observed wound closure within 72hrs in untransfected DU145 cells, where DU145+1d4 cells had a significantly reduced migratory capacity and extended the wound closure time to more than 6 days. Beyond a 72hr time point it is unclear whether the wound closure rate can be attributed to migration or general proliferation which is reduced in DU145+1d4 cells when compare to untransfected
DU145 cells. Therefore, it is concluded that DU145+1d4 cells have a significantly
decreased rate of migration that may be attributed to a combination of increased E-
cadherin expression resulting in strengthened intercellular interactions and a decreased
rate of proliferation. Although no EMT markers were directly observed, these studies
support the role of 1d4 in maintaining an epithelial like phenotype by possibly reducing
EMT transition in these cancer cells.

The DU145+1d4 cells also observed a change in response to environmental
stimuli that were not observed in untransfected DU145 cells. DU145+1d4 observed
preferential growth patterns in response to the type of serum the cancer cells were grown
in. The initial studies were observed in DU145+1d4 cells propagated in RPMI media
supplemented with Bovine Calf Serum (BCS). However, a change in supply, initiated a
different growth profile of DU145+1d4 cells. Upon growth of DU145+1d4 cell in RPMI
media supplemented with Fetal Bovine Serum (FBS), the close cell to cell interactions
were relaxed and resulted in a more dispersed growth pattern of DU145+1d4 cells.
Therefore, it is obvious that DU145+1d4 cell responds differently to constituents within
the 2 types of serum. The exact constituents within the two types of serum are unknown
however, one of the main constituents are the levels of hormones, particularly androgens,
within these two types of serum. FBS is known to contain higher levels of androgens that
BCS serum, suggesting a possible stimulation by androgens of DU145+1d4 cells that was
later supported by migration studies (scratch wound assay) in DU145+1d4 that showed a
higher migratory pattern with a more elongated structural phenotype in RPMI +charcoal
stripped FBS supplemented with 10nM DHT, when compared to cells propagated in
RPMI media supplemented with CSFBS alone or with RPMI+CSFBS+25uM Casodex.
This suggests that DHT can induce a more migratory phenotype in DU145+ld4 cells. DU145 cells showed no preferential growth pattern in response to BCS serum versus FBS serum or any differential migratory patterns observed in response to CSFBS, DHT or Casodex. Therefore, these responses to serum stimuli are entirely attributed to induction of ld4 expression.

At first, the exhibition of the spherical cytoplasmic structures observed solely in DU145+ld4 cells was a quandary, as to their purpose and function. However, review of literature suggests that these organelle structures are ‘lipofuscin’ molecules. Lipofuscin is the term given to finely granular yellow-brown pigments granules composed of lipid containing residues of lysozomal digestion. Lipofuscin is considered a sign of cellular ageing similar to cellular senescence. Coincidentally a relationship has been established between senescent cells and lipofuscin accumulation. Lipofuscin molecules have been observed in prostate cancer cell line (PPC-1) treated with 5-aza-dC resulting in induction of senescence and accumulation of lipofuscin molecules (325). Most, DU145+ld4 cells express levels of these lipofuscin molecules, with fully senescent cells displaying the highest level of these molecules. Therefore the fact that many DU145+ld4 cells express these lipofuscin molecules suggests that DU145+ld4 cells have a high propensity to become senescent. Also, ld4 may be a crucial regulator of cellular senescence, through a positive induction pathway, inverse to the inhibitory role ld1 plays in replicative senescence in developing fibroblast and cancer cells.

The ability of the DU145+ld4 to maintain 101 days of serum deprived media support the ability of the ld4 transfected cells to undergo senescence. Senescence is a state similar quiescence resulting in an attenuation of the proliferative profile of cells and
is seen as an alternative to apoptosis in cancer cells incapable of a conventional apoptotic pathway. Therefore, the explanation with DU145+1d4 cells that survived for 101 days without any serum in the media, and no observed cell death suggest that these cells may have undergone senescence in the absence of serum stimulation. Although no specific senescence studies were done to support these assumptions, the DU145+1d4 cells did observe the classic senescence phenotype with the diameter of cells greatly increasing beyond “normal” parameters. However, it can also be debated whether these cells were senescent or rather quiescent in nature, with an ability to exit the cell cycle with adequate stimulation factors, allowing the cells to escape the cell cycle arrest. If this is possible, this may present a problem for cancer cells with 1d4 allowing the cells to survive in serum deprived environment for an extended periods of time with an ability to regain normal proliferatory function upon stimulation by adequate growth factors. However, it is apparent that although the proliferation rate of DU145 cells is limited by the absence of androgens, these cells were still able to become confluent over a period of 10 days. Suggesting at the very least, a very different growth profile in the response to the absence of serum/growth factors between 1d4 transfected and untransfected cells. One of the major factors responsible for allowing serum independent growth is Id1 in prostate cancer cells (216, 225). Id1 inhibits senescence through inactivation of p16 and induction of the MAPK signaling pathway. The downregulation of Id1 in DU145+1d4 transfected cells may be responsible for the induced senescence through p16 upregulation and inhibition of the MAPK signaling pathway through downregulation of Id1.

One of the major components of replicative induced senescence is the shortening of telomeres. Cancer cells have found a way to bypass the shortening of telomeres and
bypass replicative senescence. However, the role of these telomeres represents a finite doubling potential of normal cells that reaches an end point. A similar occurrence is observed in our DU145+Id4 transfected cells. DU145+Id4 cells achieve a passage associated cell death end point. Propagation of DU145+Id4 cells by several individuals, on several occasions, observed an increase in cell death, at approximately passage 40 (sub 40). Cells at approximately passage 40 were grown in the same cell culture conditions (RPMI+10% FBS in 5% CO2) resulted in differences in the cellular phenotype, with an elevation of necrotic cells in the media of DU145+Id4 (Sub 40) eventually resulting in a massive cell death. DU145+Id4 (Sub 25) grown under the same conditions did not observe similar results. Therefore, the Induction of Id4 may be responsible for initiating a time dependent cell death mechanism. The exact reason for this cell death mechanism is not known. It is known that DU145 prostate cancer cells have a litany of mutations, indicative of most cancer cells that wreak havoc on the molecular programming of the cells. The induction of Id4 may be responsible for initially repairing any DNA damage within the cell. When the cell is beyond repair the apoptotic programming is then initiated in these cells. Senescence studies suggest that Sub 36+ DU145+Id4 cells are no more prone to senescence than earlier subtypes (Data not shown). Suggesting that this time dependent apoptotic mechanism is separate pathway from the senescence induction pathway.

The list of morphological and phenotypical changes in response to Id4 induction suggests an alteration of a myriad of underlying cellular processes resulting in an entirely different cell line in response to exogenous Id4 expression. This change generally supports the role of Id4 as a tumor suppressor and suggests that the loss of Id4 through
promoter hypermethylation has great implications on cell morphology and the ability of the cell to respond to various stimuli in its environment. To further understand these changes in relation to cancer progression, more studies in EMT transition, hormone response and age-related apoptosis induction are necessary.

3. **Id4 a major regulator of cell cycle progression**

The regulation of the cell cycle is an expected function of Id4 expression. Id genes have displayed a propensity to regulate several aspects of the cell cycle. Id proteins themselves are necessary for normal G1-S transition in proliferating cells and are regulated through phosphorylation status of Id proteins. Id proteins have a short half-life of approximately 20-30 minutes in the cell, however this half-life can be extensively lengthened upon binding of bHLH proteins, most notable the E2A transcript variants E12 and E47. Induction of Id1 expression in LNCaP cells results in an increased G1 phase. Id2 can bind directly to the retinoblastoma protein an essential regulator of the cell cycle. RB is responsible for regulating a number of processes within the cell cycle including multiple checkpoints responsible for inhibiting uncontrolled proliferation in cancer cells. This protein, pRB is mutated and inactivated through hyperphosphorylation in various types of cancer. RB is also an essential mediator of the replicative induction senescence pathway, however other RB “pocket proteins” have shown some redundancy of pRB in its absence/inactivation. Id1 is suggested to also directly bind the RB protein. To stimulate proliferation, Id proteins readily downregulate/inactivate several cell cycle inhibitor/tumor suppressors responsible for regulating the cell cycle (i.e. p16, p21, p27). Id1 is an inhibitor of TGF-β cell cycle arrest in normal prostate cells by inhibiting its induction of p21 and p27.
The upregulation of p21 and p27 in DU145+1d4 cell lines suggest that 1d4 is actively involved in regulating the cell cycle in a fashion dissimilar form other 1d proteins. However the upregulation of p21 and p27 did not result in a G1 arrest in DU145+1d4 prostate cancer cells. This may be partially due to the status of pRB in the DU145 cell line. The pRB protein is hypermethylated in the DU145 prostate cancer cell line. Therefore the CDK inhibitory role of p21 and p27 is incomplete/ineffective in eliciting a cell cycle arrest in DU145 cells. Under normal conditions upregulation of either p21 or p27 will result in the inhibition of CDK2-Cyclin E complex responsible for phosphorylating the pRB protein and disassociating pRB from the pRB-E2F1-DPI complex. However the hyperphosphorylated status inhibits this attenuation by p21/p27. Despite the increase of p21 in DU145+1d4 cells compared to untransfected DU145 cells, it is still expressed in normal DU145 cells and the mRNA level. ICC studies of p21 in both normal DU145 and DU145+1d4 cells shows a significant nuclear expression of p21 in both cell lines suggesting activity as a cell cycle regulatory protein. P21 is a downstream target of the p53 gene, suggesting that upregulation of p53 in DU145+1d4 cell line may be the result of increased p53 observed though RT-PCR. Coincidentally in the enlarged cells with a senescent phenotype, p21 shows some cytoplasmic staining. The exact implications of cytoplasmic staining of p21 are unclear; however cytoplasmic p21 expression has been linked to poor prognosis in breast cancer patients. Therefore, normal p21 inhibition of CDK2-Cyclin E is disrupted somehow.

In DU145 cells, p27 is not expressed or expressed at low amounts. However, ectopic expression of 1d4 results in the upregulation of p27. However, using ICC studies we have observed that p27 expression is largely cytoplasmic in DU145+1d4 cells. This
observation supports the FACS analysis data that show DU145+Id4 cells do not undergo a G1 arrest in response to p27 overexpression. The expression of p27 correlated with the cytoplasmic regulation of SKP2 in DU145+Id4 cells. Although co-localization studies were not conducted to observe the co-localization of SKP2 and p27 together, the resultant ICC experiment exhibited cytoplasmic staining of both p27 and SKP2 proteins. SKP is a known post-translational regulator of p27. Upregulation of SKP2 in DU145+Id4 may correlate with p27 protein ubiquitination through an SCF complex, of which SKP2 is the main constituent. The degradation of p27 through an SKP cascade requires the phosphorylation of p27 by CK2 and Cyclin E. The subsequent phosphorylation of p27 by CDK2-Cyclin E complex results in the shuttling of p27 from the nucleus to the cytoplasm for SKP2 mediated degradation. Therefore, it is safe to assume that in DU145+Id4 cell Id4 is responsible for upregulation of both p27 and SKP2 resulting in the degradation of p27 through the SKP-SCF complex. Cytoplasmic p27 may also be linked to a pro-oncogenic role in cancer cells, suggesting that cytoplasmic p27 is oncogenic and cooperates with the Ras pathway. However, the pro-oncogenic effects of p27 are dependent upon the specific phosphorylation sites of p27. In DU145+Id4 cells it is unclear what p27 upregulation and cytoplasmic activity is indicative of. It is apparent that neither p21/p27 upregulation does not result in a G1 arrest in DU145+Id4 cells. Therefore, ectopic Id4 expression results in upregulation of these critical cell cycle regulatory genes; however its exact role in regulating these proteins is uncertain as it cannot be determined whether the response to p21/p27 upregulation (i.e. SKP2 upregulation) is a result of Id4 expression or a response to Id4 stimulation of p21 and p27.
Recent studies in Hodgkin lymphoma have ascertained a correlation between SKP2 and Id4 expression that supports our findings in this study. SKP2 is generally accepted as a tumor promoting gene, which is responsible for degrading key cell cycle genes through the SCF complex. Therefore the upregulated expression of SKP2 does not favor the case for Id4 as a tumor suppressor gene. However, SKP2 induction may be a byproduct of Id4 overexpression and a mechanism to delay Id4 tumor suppressor effects in cancer cells. SKP2 is responsible for regulating another cell cycle gene, E2F1. The correlation between E2F1 and Id4 expression has gained recent ground lately as studies have shown that the Id4 promoter contains E2F1 recognition sites that allow E2F1 to bind to the promoter of Id4 and regulate its transcription. (223) In this study we suggest that Id4 may be a regulator of E2F1 and therefore the subsequent regulation of the Id4 promoter by E2F1 may be the result of a feedback loop mechanism in Id4. E2F1 has shown both tumor suppressor and oncogenic functions. E2F1 is responsible for initiation of an apoptotic pathway in both a p53-dependent and p53 independent manner. Therefore our observed intrinsic increased apoptosis observed in DU145+Id4 cells may be the result of E2F1 induced expression, in the absence of a mutated p53 gene. SKP2 and E2F1 are co-localized in DU145+Id4 cells. In DU145 this co-localization is not observed as SKP2 is mostly nuclear, suggesting it is inactive as a protein degradation complex in DU145 cells In DU145+Id4 cells E2F1 is localized in both the nucleus and the cytoplasm. In the enlarged ‘senescent –like’ cells E2F1 localization is mostly cytoplasmic with some nuclear stating. In DU145+Id4 cells that do not appear senescent like and are probably proliferating normally, E2F1 expression is almost exclusively nuclear. Therefore Id4 may also be responsible for the degradation of E2F1 in
DU145+1d4 cells specifically in senescent cells. However, in proliferating DU145+1d4 cells E2F1 may be acting like a cell cycle gene in the nucleus and activating S-phase specific genes. The fact that 1d4 has an E2F1 binding site in its promoter suggest that 1d4 may be an S-phase specific gene initiated through activation by E2F1 during the S-phase. The correlation between 1d4 and E2F1 is strengthened by their cell cycle associations. 1d4 has been established as a crucial cell cycle control gene. 1d4 expression is essential to G1-S phase transition. Neural progenitor cells with knockout 1d4 result in a prolonged G1 phase, whereas the induction of 1d4 results in an increased S-phase in 1d4 expressing cells. E2F1 is an essential S-phase associated gene. Disassociation of E2F1 from the pRB complex is essential to the transition from the G1 phase of the cell cycle to the S-phase, where E2F1 is responsible for initiation transcription of S-phase specific genes. Therefore, it is observed that both 1d4 and E2F1 are G1-S phase transition essential genes. The E2F1 DNA binding sites in the 1d4 promoter may result in 1d4 upregulation throughout the S-phase.

Id4 plays an essential role in cell cycle progression in particular the S-phase of the cell cycle. We have observed that DU145+1d4 cells display a decreased rate of proliferation and an increased doubling time. This decrease in proliferation may be the result of the S-phase arrest observed in DU145+1d4 cells. As stated previously both 1d4 and E2F1 are S-phase specific genes. This role of 1d4 is supported by the accumulation of cells in the S-phase in both DU145+1d4 and PC3+1d4 transfected cells. Ectopic expression of 1d4 in both transfected cell lines resulted in an almost 2-fold increase in S-phase expression in both cell lines, strengthening the role 1d4 plays in the S-phase of the cell cycle.
Another CDK inhibitor responsible for inhibiting the phosphorylation status of pRB is p16, which is also upregulated in the DU14+ld4 cell line. However the p16 gene is mutated in the DU145 cell line, which makes its status in cell cycle regulation unclear. Also upregulation of p16 should result in an increase in G1 phase of the cell cycle, which did not occur. Generally, p16 is responsible for inhibiting a different CDK-Cyclin complex than p21 and p27. The upregulation of p16 is responsible for inhibiting the CDK4/6-Cyclin D complex also responsible for phosphorylating RB. Transfection of a function p16 gene in DU145 does not correct the RB inefficiency in DU145 cells. To achieve p16 induced senescence a functional pRB gene is required. However drug induced senescence has been observed in DU145 cells in the presence of both mutated pRB and p16 genes suggesting an alternative mechanism for senescence induction, which ld4 may be responsible for inducing.

4. Id4 AR response

Prostate cancer is designated as a hormone refractory disease largely dependent upon the androgen receptor to regulate proliferation, growth, apoptosis and various other cellular processes in both the normal prostate cells and androgen-dependent prostate cancer cells. The androgen receptor interaction network is dependent upon a number of different factors including the AR coactivators, phosphorylation of the androgen receptor, promoter regulation etc. It is evident that regulation of the androgen receptor is crucial to understanding prostate cancer progression. Most therapeutic treatments are centered around inhibition of the androgen receptor activity in prostate cancer cells. However not a lot is known about exactly how prostate cancer cells become androgen independent. Several theories have been proposed that include the generation of androgen independent
prostate cancer cells from prostate cancer stem cells. Therefore the observation of
induced Id4 expression in DU145+Id4 cells presents a significant role of Id4 as a tumor suppressor in prostate cancer. This observation is even more significant in relation to the status of the androgen receptor in the DU145 cell line. In the DU145 cell line AR expression is silenced through promoter hypermethylation, suggesting re-expression in DU145+Id4 cells is largely dependent upon the ability of Id4 to regulate the promoter methylation status of AR similar to the observed regulation of the methylation status of its own promoter.

The initial observation of an induced androgen receptor in DU145+Id4 transfected cells provides an explanation for the possible mechanism of AR downregulation in prostate cancer cell lines in vitro and suggests a mechanism of androgen-independent prostate cancer progression. Although the ectopic expression of Id4 resulted in the upregulated expression of the AR in the DU145+Id4 cell line, the question then remained; was the mechanism of functional AR activity retained in DU145 through induction of Id4 alone?

Many factors are responsible for traditional AR activation and downstream activation of AR response genes including PSA. Treatment of DU145+Id4 cells resulted in upregulated expression of the prostate specific antigen. The DU145 cell line is known to not produce PSA, which is a characteristic verified by our experiments of treating the untransfected DU145 cell line with 10nM DHT resulted in no PSA expression. DHT treatment did however result in the expression of the androgen receptor at the mRNA level. This was an interesting observation, but not totally obscure as PC3 cells have show reintroduced expression of AR due to the low level of expression of AR in PC3 cells.
Also the androgen receptor in PC3 cells is not methylated. The upregulation of PSA clearly indicates that DU145+ld4 cells not only expressed a functional androgen receptor but that this expression also resulted in the upregulation of the prostate specific antigen. Therefore it is evident that DU145+ld4 cells possess the mechanistic programming for androgen receptor induction of AR response genes.

To determine if this downstream PSA expression was exclusively due to AR activation within the nucleus we used ICC studies to observe AR expression in DU145+ld4 cells in response to various stimuli. DU145+ld4 cells treated with DHT resulted in the translocation of AR to the nucleus of the cell, presumably to bind to the ARE (androgen response elements) of AR response genes and induce expression. To support our observations of an intact functional androgen receptor interaction network in DU14+ld4 cells, cells were treated with Casodex the known AR antagonist. The DU145+id4 cells were also treated with just RPMI media+10%CSFBS and also with RPMI alone. The fact that DHT was the only stimulant to initiate androgen receptor translocation from the nucleus to the cytoplasm suggests that loss of ld4 expression may be a crucial aspect of failed anti-androgen treatment and the induction of ld4 may provide a therapeutic target for late stage hormone refractory prostate cancer. The only cell line to show a similar mechanistic profile to DU145+ld4 cell in response to androgen and anti-androgen treatment was the LNCaP cell line which displayed increased nuclear activity of AR in response to AR. However less nuclear expression was observed in Casodex treated LNCaP cells. A decreased localization of AR expression within the nucleus was observed in LNCaP cells treated with RPMI media supplemented with CSFBS or RPMI media alone. Therefore, both DU145+ld4 and LNCaP cells respond to androgen
treatment in a similar fashion. Androgen independent cell lines DU145 and PC3 displayed minimal nuclear stating of AR in response to androgens. Therefore, DU145 and DU145+Id4 cells responds differently to androgen treatment. These results indicate that Id4 is an essential component to AR nuclear activity in prostate cancer cells. Also, both PC3 and DU145 are incapable of translocating androgens to the nucleus, suggesting an absence of an essential AR-Id4 associated component in these androgen-independent cell lines.

Migration studies performed on DU145+Id4 cells in response to DHT and Casodex support the finding that Id4 induced AR is inhibited by AR antagonist Casodex. A scratch wound assay performed in the presence of Casodex inhibited cellular migration of DU145+Id4 cells more than in DHT treated cells. However, both DHT and Casodex treated cells migrated at a slower rate than untransfected DU145 cells. This suggests that although Id4 may induce functional AR activity, the Id4 induced cell line has significantly reduced migratory properties when compared to the untransfected DU145 cell line. Also DU145 cells displayed no differential migratory preference to DHT or Casodex treatments, which supports the androgen-independent growth pattern of DU145 cells.

The proliferation rate of DU145 cells was not stimulated by androgens in the media; however treatment of DU145 cells with anti-androgens did result in some inhibition in proliferation. DU145+Id4 cells displayed a 3-fold increase in proliferation over normal DU145+Id4 cells cultured in RPMI+CSFBS media alone. These results suggest that DU145+Id4 cells proliferate at a higher rate in response to androgens, a characteristic observed in both normal prostate cells and androgen-dependent prostate
cancer cells. These results therefore suggest that DU145+ld4 cells grow preferentially in an androgen dependent fashion.

Treatment of DU145+ld4 cells with DHT results in increased proliferation. From a cell cycle regulation aspect, this increase in proliferation is sparked by the downregulation of p21 and p27 in DU145+ld4 cells. The downregulation of these cell cycle control genes are the result of AR activity in the cell cycle. AR is known to downregulated cell cycle control genes to facilitate progression through the cell cycle therefore the downregulation of p21 and p27 in DU145+ld4 cells in response to androgens is an essential aspect of cell cycle progression in response to AR activity. FACS analysis showed that DU145+ld4 cells display a highly proliferative cell profile, opposite from normal DU145 cells which progress through the cell cycle in a normal fashion with no alteration in cell cycle progression in response to androgens. Although DU145+ld4 cells show a sustained G1-S phase cell cycle percentage, the increase in the rate of proliferation suggest that this is not a cell cycle arrest mechanism but instead a highly proliferative profile of a cancer cell. Also, the increased S-phase observed in DU145+ld4 cells grown in serum for 48hrs is no longer observed in DU145+ld4 after 48hr treatment with DHT. This suggests that the induction of DHT bypasses the S-phase associated slowdown induced by ectopic Id4 expression. In fact the DU145+ld4 cells spend very little time in either S-G2/M phase of the cell cycle in response to androgen treatment.

Interestingly, DU145 cells did show some preferential changes in p21, p27 expression in androgen independent cell line DU145. In an opposing fashion to DU145+ld4 cells, DU145 cells showed an upregulation of p21 and p27 in response to
DHT treatment. As no G1 cell cycle arrest was observed in these cells in response to DHT treatment the exact reason and mechanism of the upregulation of both p21 and p27 in untransfected DU145 cells in response to androgen treatment remains unclear. However, studies have suggested that DHT can be converted into a -dil form known as 5alpha-androstane-3alpha, 17beta-diol and 5alpha-androstane-3beta, 17beta-diol. These hormones can be synthesized from the potent androgen DHT by the enzyme 3 β-HSD that is expressed by DU145 cells. These -dil forms of DHT can bind the Estrogen receptor. DU145 possesses an activated ER-α receptor and the 5alpha-androstane-3beta, 17beta-diol form of DHT has been known to bind the estrogen receptor. Therefore, activation of this compound in DU145 in response to DHT may be responsible for p21, p27 induction in these cells.

Prostate cells are largely dependent upon the secretion of androgens to regulated proliferation and apoptosis. Normal prostate cancer cells posses an equilibrium between proliferation/generation of new cells/apoptosis induction of old cells. Androgen deprivation therapy in androgen dependent prostate cancer cells results in a significant induction of apoptosis. Therefore, we conducted an apoptosis assay on DU145+1d4 prostate cancer cells in the presence of CSFBS alone, DHT and Casodex. Cells treated with CSFBS alone showed the highest percentage of cell death, cells treated with both androgens and Casodex displayed no significant differences in dead cells, apoptotic cells or Viable (non apoptotic) cells. This therefore suggest that DU145+1d4 cells do not show an increase in apoptosis in response to androgen deprivation therapy, even though these cells do show decreased migration and proliferation to anti-androgens. The lack of apoptosis in Casodex treated DU145+1d4 cell may be the result of the androgen
dependent status of DU145 cells or may be the result of an ineffective apoptotic pathway in DU145 cells riddled with mutations and alterations in apoptotic pathway genes. However, a significant correlation has been observed between anti-androgen treatment and senescence induction in DU145+Id4 cells. Treatment of DU145+Id4 cells with anti-androgens results in a significant increase in senescence suggesting that although DU145+Id4 cell may not undergo apoptosis in response to anti-androgen treatment, an alternative mechanism in DU145+Id4 cells results in the induction of senescence. Therefore, DU145+Id4 cells respond differently to anti-androgen, which have a negative effect on proliferation in DU145+Id4 cells by inducing senescence. The same induction level of senescence is not observed in DU145+Id4 cell treated with DHT or untransfected DU145 treated with Casodex. The mechanism of action in DU145+Id4 suggest that anti-androgens result in, a possible upregulation of possibly p16, and an accumulated G1 arrest resulting in an increase in senescence in DU145+Id4, Casodex treated cells. These results suggest that even in the absence of a functional apoptotic pathway Id4 expressing cells are capable of an alternative mechanism of inhibition of proliferation in response to loss of androgens to the androgen dependent DU145+Id4 cell line.

Apparently, Id4 expression is essential AR nuclear activity. Studies within our lab show direct Id4-AR interaction in prostate cancer cells. Id for may be acting on several different aspect of the AR pathway to illicit a functional response mechanism (see appendix 50) Previous studies have demonstrated that AR treatment in sertoli cells results in an increased expression of Id4, therefore suggesting a possible feed-back-loop mechanism between Id4 and AR. Id proteins have previously shown a relationship through regulation of AR in prostate cancer cells. Most notable AR has been shown to
negatively regulate Id1 expression. Therefore the relationships between Id proteins and AR have been previously discussed. However the novel observation of AR regulation though Id4 expression initiates a considerable new paradigm for androgen-dependent to androgen-independent prostate cancer progression with Id4 as the crucial mediator of this progression. Id4 may be the missing factor in effectively understanding AR activity within prostate cancer cells. It is therefore possible to generate a synthetic form of Id4 may be capable of inducing the same effect (inducing AR expression) in prostate cancer cells resulting in the possible option of treating androgen-independent prostate cancer cells with a combination of a synthetic Id4 compound and anti-androgen therapy.

5. Id4 induced senescence

In addition to apoptosis, therapy-mediated premature senescence has been identified as another drug-responsive program that impacts the outcome of cancer therapy. Senescence is a state of non-dividing that cancer cells undergo in response to stimuli, whether it be drug induce, oncogene induced or molecular programming, through telomere end point shortening. One of the major regulators of senescence is the CDK inhibitor p16 which is responsible for inducing senescence in a regulatory manner through a pathway involving an intact pRB gene. DU145 cells do not readily undergo senescence, due to a mutated p16 gene and a hyperphosphorylated pRB gene. Therefore, the induction of senescence in DU145+Id4 cells in the absence of any DNA damaging agent or therapeutic treatments presents a correlation with replicative senescence and Id4 expression.

A solid relationship between Id proteins and p16 has been well established in prostate cancer. Id1 is an inhibitor of replicative senescence and upregulation of Id1
results in downregulation/suppression of p16INK4a in LNCaP prostate cancer cells and immortalized normal prostate epithelial cells. Id1 and Id3 have both shown regulatory function over p16 and as result senescence. Silencing of Id1 and Id3 in LNCaP cells results in a significant increase in p16 mRNA expression (5). Although, silencing of only Id3 in DU145 results in an increase in p16 expression. Id2 silencing in DU145 cells results in downregulation of p16 expression (5). Therefore, Id1 and Id3 may both inhibit p16 expression in a cell line with wild-type p16 and pRB genes. Our results suggest that Id4 may be the antithesis protein to Id1 and Id3 in response to cellular senescence. The induction of exogenous Id4 in DU145 results in a dramatic increase in cellular senescence and SA-b-galactosidase staining, suggesting that Id4 expression is responsible for the induction of senescence. The induction of Id4 expression also correlates with the downregulation of Id1; therefore it is uncertain whether the upregulation of Id4 in DU145+Id4 cells directly initiates a senescent pathway or whether induction of Id4 results in the downregulation of Id1 and the disruption of Id1-inhibition of senescence.

The exact mechanism of senescence induction in DU145+Id4 cells is unclear. Without any treatment DU145+Id4 cells. Even in normal serum the levels of advanced cellular senescence were approximately 30% compared to 0% in untransfected DU145 cells. Although Id1 is a known repressor of the p16 gene, in DU145 cells p16 is mutated therefore it can be assumed that the induction of senescence in DU145+Id4 cells is through a pathway not involving p16 or hyperphosphorylated pRB. DU145 cells have been shown to undergo senescence in response to DNA damaging irradiation, through a p53 dependent pathway that bypasses the hyperphosphorylated pRB and utilizes the RB-like "pocket proteins" p107 and p130 to induce cellular senescence. The genes involved
in this pathway share similarity to the genes induced in our experiment including p27, SKP2. This study suggests that p107 and p130 cooperate to induce cellular senescence in response to irradiated DNA damage. Another interesting observation in this study suggest that loss of p130 alone in DU145+1d4 cells is enough to induce cellular senescence either with or without irradiated DNA damage. DU145 cells also undergo drug induced senescence when introduced to low levels of doxorubicin, approximately 25nM. Therefore, although the traditional senescence pathway in DU145 cells is mutated, there is an alternative pathway of inducing cellular senescence in DU145 cells. However, this induction is usually in response to DNA damage or drug induction and not response to a single gene. Even transfections of either wild-type p16 or RB into DU145 cells did not induce cellular senescence. The question then remains what pathway does 1d4 utilize to induce cellular senescence in DU145+1d4 cells?

Previously, we discussed that the presence of lipofuscin molecule in cells has a strong correlation with senescence. Post transfection of ectopic 1d4, the cell diameter of transfected cells increases greatly. Coincidently, DU145+1d4 cells experience frequent multi-nucleation, in the cells with the ‘senescent-like’ phenotype (large diameter with flattened body). DU145+1d4 also display a propensity of undergoing senescence in response to types of serum used. DU145+1d4 cells treated in the presence or absence of serum and in the presence of androgens, DHT or anti-androgens Casodex. Cells treated with Casodex showed the greatest propensity for undergoing senescence suggesting a relationship between inhibition of AR activity and induction of senescence. Previously we discussed the androgen dependent growth of DU145+1d4 cells, these results strengthen the relationship between androgen dependent growth and 1d4 expression.
Blockade of this androgen dependent growth in DU45+1d4 cells resulted in a total inhibition of the cell cycle and forcing a high percentage of cells to undergo cellular senescence in the absence of nuclear androgens. The levels of advanced cellular senescence increased to 64% in response to Casodex treatment. We also observed whether cells treated with androgens underwent apoptosis along with senescence. Our studies show that DU145+1d4 cells treated with DHT, Casodex and just RPMI with charcoal stripped FBS for 7 days did not result in increased apoptosis. In fact the highest percentage of cell death was DU145+1d4 cells cultured in RPMI+CSFBS alone. This suggest that DHT inhibits apoptosis in DU145+1d4 cells but Casodex nor DHT does not induce apoptosis, instead DU145+1d4 cells undergo senescence in response to inhibition of AR activity.

In DU145, cells a concentration of 25nM is considered a low concentration and results in cellular senescence instead of apoptosis. In DU145 cells a concentration of >250nM for greater than a 40hr period are required to induce cell death. As our DU145+1d4 cells undergo senescence in the absence of any DNA damaging agents, we wanted to determine if introduction of low levels of Doxorubicin were sufficient to induce apoptosis in these cells. Correctly, DU145+1d4 cells undergo apoptosis at a higher rate (9% dead cells, 10% apoptotic) in response to 25nM doxorubicin treatment when compared to untransfected DU145 cells that show more resistance to apoptosis (6% dead cells, 15% apoptotic) in response to 25nM doxorubicin treatment. These results suggest that DU145+1d4 are more sensitive to doxorubicin treatment and even low level of doxorubicin increase apoptosis, as well as senescence.
Initially, we observed an increase in a number of senescence associated genes in DU145+1d4 cells, including p16, Vimentin, p21, p27, SKP2 and SKP2. We have discussed the molecular mechanisms of most of these genes except p16 and Vimentin. As discussed previously p16 is mutated in DU145 cells therefore the significance of the observed increase in p16 expression is unclear. Also untransfected DU145 cells also express substantial level of this mutated p16 gene. Therefore, we wanted to observe the localized protein expression of p16 in DU145+1d4 cells. Using ICC studies we observed that DU145+1d4 cells had significantly high levels of p16 when compared to DU145 untransfected cells which had almost exclusively nuclear staining of p16. Cytoplasmic localization of p16 is usually regarded as nonspecific as p16 expression as a cell cycle inhibitor requires nuclear localization. Nuclear factors are all produced in the cytoplasm and transferred to the nucleus; therefore they all may exhibit cytoplasmic staining theoretically. It has been suggested that cytoplasmic staining of p16 in cancer may represent a method of p16 inactivation. Studies show that cytoplasmic accumulation of p16 can be used for identification of a subset of breast tumors with increased proliferation and poor prognosis in breast cancer patients. However, this ideology goes against the increased senescence observed in DU145+1d4 cells. One possible rationale is that although 1d4 expression results in the upregulation of p16, a specific protein such as CDK may bind p16 in the cytoplasm and prevent its nuclear shuttling.

We also observe the expression of Vimentin, a gene associated with epithelial to mesenchymal transition, is also highly upregulated in DU145+1d4 cells. Another function of Vimentin is its involvement in obtaining the senescent phenotype in the large, flattened cells. Overexpression of Vimentin in human fibroblast cells results in a ‘senescent-like’
phenotype. The expression of Vimentin is an essential component of cells undergoing senescence. The upregulated mRNA Vimentin expression was also correlated with the upregulated protein expression of Vimentin (data not shown). Cells were treated in the presence or absence of doxorubicin to observe the localization of senescence of Vimentin in both DU145+Id4 cells and untransfected DU145 cells. DU145 observed no changes in Vimentin expression when cultured in FBS or 25nM doxorubicin for 72hrs. However DU145+Id4 cells observed a differential expression pattern of Vimentin in response to Vimentin. DU145+Id4 cells cultured in FBS displayed more peri-nuclear staining of Vimentin. DU145+Id4 cells treated with 25nM doxorubicin results in polar expression of Vimentin, displaying the role of Vimentin in senescent cells. Therefore DU145+Id4 cells upregulate the expression of Vimentin to achieve cellular senescence.

E-cadherin is another protein that is upregulated at both the mRNA an protein level in DU145+Id4 cells. E-cadherin displays a more than 5 fold increase in mRNA expression in DU145+Id4 cells. E-cadherin is an important tumor suppressor gene however in DU145 E-cadherin may play a role in senescence induction. Using ICC studies we observed that senescent cells expressed high level of E-cadherin however this expression was localized in the intracellular portion of the cytoplasm and away from the cell surface. Although E-cadherin expression existed in pockets within the senescent cells, E-cadherin expression was only localized in a cytoplasmic/globular fashion in multinucleated cells. Increased E-cadherin expression has been shown to cause multinucleated cell expression. It is possible that in DU145+Id4 cells increased E-cadherin expression localized around the nucleus prevents nuclear separation of DU145+Id4 cells at the end of mitosis resulting in multinucleated cells that undergo
senescence due to genetic instability. These observations may present a novel method of senescence induction associated with multi-nucleated cellular phenotype. The question also remains, is this event a tumor suppressive role of exogenous Id4 expression or is it indicative of the alterations in the apoptotic pathway in DU145 cells?

6. **Ectopic Id4 expression sensitizes cancer cells to apoptosis**

Cancer cells genetically regulate many constituents of the apoptotic pathway to inhibit apoptosis and increase cell survival. Induction of apoptosis in normal cells usually involves the induction of p53 a major apoptotic pathway gene. However, p53 is mutated in more than half of all cancers. The DU145 cell line is no exception and has a p53 gene with two mutated alleles. Initially we observed an increase in necrosis in DU145+Id4 cells in response to the induction of Id4. Id4 also apparently resulted in the induction of a massive cell death mechanism of at sub 40. Therefore, it was natural to assume that DU145+Id4 cells may undergo apoptosis more readily than DU145 untransfected cells. DU145 cells are immortalized cancer cells and can undergo an infinite number of mitotic divisions. In the initial cell cycle experiments, there was an increase in the sub G0 from 10% to approximately 35%. The sub G0 phase of the cell cycle is indicative of apoptotic cells that have undergone cellular degradation. Therefore the increase in Sub G0 events suggests that DU145+Id4 cells undergo apoptosis more readily than DU145 cells.

To confirm these observations, we conducted an apoptosis assay. The assay initially determined that DU145+Id4 cells undergo apoptosis more readily than untransfected DU145 cells as observed by Immunocytochemistry and flow cytometry. Under normal conditions DU145+Id4 cells undergo more than twice the amount of apoptosis. Also twice the amount of dead cells was observed. To observe the sensitivity
of DU145+Id4 cells to apoptotic inducing agents, DU145+Id4/DU145 cells were treated with Actinomycin D for 6 hrs. The treatment resulted in a 3 fold increase in the percentage of dead cell in DU145+Id4 cells treated with Actinomycin D. Actinomycin D inhibits transcription by binding DNA at the transcription initiation complex and preventing elongation by RNA polymerase. The significantly extended S-phase accumulation observed in DU145+Id4 cells suggest the reason for Actinomycin D efficacy. As stated previously, DU145+Id4 cells were also more susceptible to apoptosis induced by low levels of doxorubicin. Therefore, we can say Id4 is responsible for inducing apoptosis in a number of different ways in DU145+Id4 cells and are susceptible to several types of apoptotic inducing agents. DU145+Id4 cells undergo a programmed cell death at sub 40, have a higher percentage of apoptosis in cells cultured in normal serum (approximately 35%) and are more susceptible to apoptosis induced by Actinomycin D and doxorubicin.

Although the p53 gene is mutated in DU145, ectopic expression of Id4 in these cells resulted in a >17 fold increase of p53 mRNA expression. It has been observed that mutant p53 is capable of binding to the promoter of Id4 through a p53-E2F1-p300 complex in breast cancer cells, and upregulating the expression of Id4. Wild-type p53 showed no regulatory function over Id4 through activation of its promoter (223). However we have shown that Id4 possesses regulator function over p53 expression. Therefore, the previously mentioned mutant p53 regulation of Id4 may be the result of an Id4-p53 feedback loop mechanism. Although we observed an increased p53 mRNA expression and increased apoptosis we did not know the exact role p53 protein played in DU145+Id4 cells. ICC expression displayed an increase of p53 in the nucleus of
DU145Id4 cells. Cytoplasmic p53 has been linked to induction of apoptosis through a separate p53 dependent mechanism. Localization of p53 in the nucleus results in p53-transcription induced apoptosis. In the cytoplasm, p53 has the ability to directly or indirectly activate Bax resulting in cytochrome c release from the mitochondria. Cytoplasmic p53 can also displace pro-apoptotic Bid from BCL-xl resulting in an indirect activation of Bax. Cytoplasmic p53 can also repress autophagy in p53 deficient cells suggesting how serum deprived Id4 cells are able to survive 101 days of serum deprivation. Therefore, although p53 may be mutated in DU145+Id4 cells preventing its binding to DNA in DU145+Id4 cells, cytoplasmic p53 may be responsible for initiating an alternative apoptotic pathway through cytoplasmic p53 localization.

7. **Id4 regulator of Id genes**

Inhibitors of differentiation/DNA binding proteins have shown a regulatory function in both development and cancer progression. Id4 has been observed as a sequence/structurally different Id protein that posses an inverse expression profile compared to other Id proteins both during development and in cancer progression. Exogenous expression of Id4 in DU145 cells resulted in the downregulation of specifically Id and Id3. The expression profile observation and the direct downregulation of Id1 and Id3 suggest that Id4 may be the universal regulator of Id proteins (or at least Id1 and Id3). Xenopus studies have shown that Id4 can bind directly to Id3 which may negatively regulate its activity within the cell. Therefore one mechanism by which Id4 may inhibit oncogenesis in DU145+Id4 cells is by binding to Id3 and negatively regulating its expression. Recent Immunoprecipitation studies in our lab have demonstrated that Id4 can also bind Id1 directly, and may possibly negatively regulate
Id1. The ability of Id4 to bind both Id1 and Id3 may be due to their sequence homology. The downregulation of Id1 and Id3 in the DU145+Id4 transfected cell line alone can explain a number of our observations, including decreased proliferation, upregulation of p16 expression and an increase in senescence. The upregulation of p21 and p27 in the cell cycle and the increased apoptosis observed can also be explained by d1 downregulation. Id1 is also responsible for decreasing apoptosis and increasing cell survival by upregulating NF-κB, therefore Id1 reduced expression may be responsible for increased apoptosis.

Not many proteins have shown an ability to regulate Id proteins. C-myc is in a known inducer of Id1 expression. Id4 may act as a neutralizer to Id1/Id3 expression both during development and cancer progression. Id1-3 expression is an essential component to cell cycle progression in the G1-S phase transition. Therefore, Id1 may be responsible for guarding the cell from uncontrolled proliferation induced by Id expression, by inhibiting the proliferative effects of Id proteins on the cell cycle. In early stage prostate cancer where Id4 is expressed, low levels of Id1/Id3 does not result in an invasive metastatic phenotype of prostate cancer cells. In early stage prostate cancer Id4 may act as a neutralizer, by minimizing the levels of Id1/Id3 and negating the pro-oncogenic effect they have on cancer progression. The loss of Id4 expression through promoter hypermethylation may then allow Id proteins (Id1-3) to become activated within the cell resulting in cancer progression. The ability of Id4 to negatively regulated Id proteins represents a significant tumor suppressor role, with very few proteins showing an ability to negatively regulate Id1-3 protein expression. Id4 may also provide insight into finding a therapeutic target for Id proteins (Id1-3). Proteomic analysis of Id4-Id1/3 interaction
may present an understanding if Id protein interaction/dimerization that can be inhibited through therapeutic means. Nullification of Id proteins though synthetic compounds may present a significant breakthrough in the battle against cancer progression. Many scientists believe that cancer induction is largely the result of developmental genes being re-initiated, and resulting in the uncontrolled proliferation/angiogenesis and inhibition of apoptosis in cancer cells. Therefore the significant roles that Id proteins play in cancer progression are expected considering the significant role Id proteins play during normal development. Id4 may be the key to regulating this network of proteins.

Our results strongly point to Id4 as a tumor suppressor in prostate cancer. Ectopic induction of Id4 in androgen-independent DU145 cell line resulted in an increase in a number of tumor suppressor pathways. Ectopic expression of Id4 increased expression of tumor suppressor genes (p21, p27, p53, and E-cadherin). Id4 was capable of inducing senescence in a cell absent of many of the known necessary components for senescence induction. Therefore, Id4 expression is an essential component of senescence induction. Id4 expression resulted in a number of significant cellular changes both phenotypical and morphological. The transfected DU145+Id4 became an entirely different cell line, with an enlarged nucleus and often resulted in multinucleated cells. Id4 may also share a relationship with telomere shortening, or apoptosis induced cell death in response to DNA damage. The ability of Id4 to induce cell death in the absence of apoptotic inducing agents suggests a strong role of Id4 to drive genetically instable DU145 toward cell death. Therefore, we suggest that Id4 is a novel tumor suppressor gene and has a novel ability to regulate several different mechanisms in the cell cycle to induce its tumor suppressive role and inhibit prostate cancer progression.
CHAPTER FIVE
CONCLUSION

Cancer is a disease that has plagued the human race almost since the beginning of time. Surprisingly, very few advancements have been made in the war against cancer along the way to eradicate its existence. Even with our advanced knowledge and medical prowess, at the most all we have done is slow down the progression of a disease that is capable of adapting to every obstacle we hurl at it. Prostate cancer is no exception to the rule in the fight against cancer. Prostate cancer, a deadly disease is a major killer of men in the U.S. In 2010 approximately 217,000 men were diagnosed with prostate cancer and resulting in 32,000 deaths. These statistics translate into an incidence of 1 in 6 men will be diagnosed with prostate cancer in his lifetime. Prostate cancer is also an age associated disease, showing a strong correlation of incidence with age progression.

As a hormone refractor disease, prostate cancer signaled by the ability of prostate cancer cells to grow in a manner dependent upon androgen receptor activity and then transition to an androgen independent method of growth as the cancer progresses. The loss of androgen receptor activity in regulating prostate epithelial cells marks a crucial step in the progression of the disease to a metastatic invasive phenotype from a localized restricted one. The androgen receptor itself plays a crucial role in regulating growth, proliferation and apoptosis in normal epithelial cells and retains its significance throughout prostate cancer progression.
Inhibitor of differentiation/DNA binding proteins plays an essential role during normal development. The first investigations into the role of Id proteins play during development were initiated almost two decades ago. The ability of Id proteins to regulate development is also the key characteristic to their role as important players in cancer progression. Over the past two decades several attempts have been implemented to understand the exact role of Id proteins play in cancer development and utilize this information to formulate successful drug treatments centered on these genes. Although we have observed the substantial extent of Id protein involvement in various types of cancer we have not totally unveiled their entire role in cancer development as it requires more vigilance.

The silencing of tumor suppressor genes in cancer, specifically prostate cancer is an important checkpoint in cancer progression. Through our studies we have demonstrated that d4 may be an influential tumor suppressor gene in prostate cancer. Previous studies have demonstrated the clinical correlation of the absence of Id4 expression in various cancers including colorectal, lymphoma, and leukemia and breast cancer. These studies along show that Id4 is downregulated through a promoter hypermethylation programming event often associated with tumor suppressor genes. However, the fact that Id4 is derived from a family of proteins that are known oncogenes does require trepidation in labeling Id4 as a tumor suppressor gene. Although many studies have focused on the methodology of Id4 downregulation in cancer progression, very few studies have attempted to understand the role of Id4 plays in prostate cancer. Our research is one of the first studies to attempt to understand the significant role Id4 plays in prostate cancer progression. The roles of other Id proteins, specifically Id1 and Id3
have been extensively studied in prostate cancer, and as a result we can assuredly say that similar to the expression of Id proteins (Id1-3), the evolution of prostate cancer progression to an aggressive, invasive, metastatic hormone refractory status intimately involves the expression of Id proteins.

In this study, we have attempted to answer the question; what is the role of Id4 in normal prostate cancer progression? In order to answer this question, we have attempted to understand the molecular mechanisms involved in loss of Id4 expression in prostate cancer. By utilizing a ‘reverse engineered’ approach and reintroducing Id4 into a system that has attempted to silence its expression we have attempted to achieve a better understanding of the role Id4 plays in prostate cancer progression. As a result the evidence does suggest that Id4 is a tumor suppressor in prostate cancer.

The ability of Id4 to regulate several pathways suggests an influential role of Id4 even in normal prostate epithelial cells, which abundantly express Id4. The multi-platformed response of Id4 expression in prostate cancer cells also speaks to the promiscuous nature of Id proteins. As these proteins have no specific DNA binding domain and cannot bind to DNA directly they negatively regulate the transcription of several genes by regulating their ability of other bHLH proteins to bind to DNA. This unique feature of Id proteins transcends a limited scope observed by other bHLH proteins. Initially, it was though that Id proteins were only capable of binding bHLH proteins and that they have no ability to bind to DNA directly. Recently, studies have shown that Id proteins are quite capable of binding non bHLH proteins such as pRB and ETs. Also at least one study has suggested that Id4 is capable of stabilizing the RNA of
IL-6 and Gro-α through direct binding. Therefore, the extent of what we possibly know about how Id proteins function is sufficiently adequate and incomplete.

The ability of Id to regulate the expression of the androgen receptor in prostate cancer or even in normal prostate epithelial cells suggest that Id4 is both crucial to normal prostate development and also to prostate cancer progression. The loss of the androgen receptor is a key event in prostate cancer progression and although scientist may not know exactly why or how androgen independent prostate cancer arises, consensus states that androgen-independent prostate cancer marks the transition to a more malignant phenotype with unfavorable prognosis. Therefore Id4 may provide the key to deciphering the cryptic transition that is prostate cancer progression.

Inhibition of apoptosis in any cancer, including prostate cancer is essential to the ability of cancer cells to proliferate unchecked. Cancer genes are incredibly unstable largely due to the vast mutations and genetic regulatory mechanisms responsible for achieving this status. Id4 is a crucial mediator of this apoptotic pathway and even in the presence of a genome that is genetically altered to a point beyond recognition, Id4 is able to induce apoptosis without any stimulation from apoptotic inducing agent and in the presence of key mutated genes (p53, pRB) in the apoptotic pathway. These studies provide a lot of hope and promise to the role that Id4 plays in the progression of all cancers that have Id4 silenced through promoter hypermethylation. The ability to attain regulatory function in cancer cells is the primary goal of many research studies, to develop more effective drug treatments. Therefore, the loss of Id4 expression may be the crucial factor allowing cells to become highly drug resistant.
In our study, we observed that Id4 played regulator role during cell cycle progression, induction of senescence and even decreased proliferation. All these observations point toward Id4 being a tumor suppressor. However, Id4 itself is a novel protein with an obscure understanding of its function. Although we have attempted to understand the role Id4 plays in prostate cancer, in reality we are only beginning to scratch the surface of understanding the complex role Id4 may play whether as a tumor suppressor or tumor promoting gene. The ability of Id4 to act as a tumor suppressor may be totally context depending on its environment. Microarray studies tell us that Id4 is highly expressed in the brain, testes and prostate. Knockout studies in mice also show that Id4 is important for normal brain development. Therefore it is understandable that Id4 may play a crucial role in initiating transformation of astrocytes and glial cells in a neuronal cancer system. Although, Id4 expression may be a crucial factor in neuronal development, once the brain is fully developed it is quite possible that Id4 expression is both unnecessary and counterintuitive to organ physiology resulting in cancer progression.

Id4 has also been suggested as a tumor promoting gene in breast cancer but several studies have also supported Id4 as a tumor suppressor gene as well. Many have these studies have observed the clinical expression of Id4 protein in relation to breast cancer tissue and prognosis, only recently have studies delved into the exact mechanism Id4 may be eliciting a tumor suppressor/promoter role in breast cancer. These studies have suggested that normal breast tissue and breast cancer cells with a wild-type p53 gene Id4 may in fact act as a tumor suppressor gene. However in the presence of a mutant p53, Id4 then acts as an oncogene. Personally my review of these studies have led me to
believe that in breast cancer mutant p53 is acting as an oncogene and has found an ability to alter the tumor suppressor effects of Id4 and curtail its expression toward an oncogenic stimulation. One of the major observations of mutant p53 induction of Id4 is the resistance to apoptosis in the presence of DNA damage. This may be due to the ability of Id4 to initiate a DNA repair mechanism, and as a result apoptosis is inhibited. Although the evidence to support these claims has not been conduct at present this is my theory that awaits to be validated.

Although we may designate Id4 as a tumor suppressor gene, we have no idea of exactly how Id4 is able to initiate various cellular processes. Several questions thus arise; what are the target proteins that Id4 bind to? Is there a crucial bHLH protein that Id4 is responsible for downregulating in order to initiate the myriad of cellular processes that have been associated with tumor suppression? Or does Id4 directly bind to other non bHLH proteins as well as traditional bHLH proteins to initiate these cellular responses? Is Id4 negatively regulating the expression of other Id genes, and as a result influencing the cell into obtaining a less metastatic phenotype? Although we may not have the answer to these questions it is obvious that Id4 expression in cancer warrants further investigation. At the very least we can say that the loss of Id4 expression is just as influential as the induction of Id1-3 expression.

As scientists we attempt to classify genes into single categories in an attempt to label them as having one specific role. However, in the cell a single gene may have a multitude of roles and Id4 may be one of these genes. Id4 may neither be an apoptosis associate gene, neither a cell cycle regulatory gene nor a senescence associated gene. The role of Id4 in the cell may be all of the above depending on the signals received from the
cell. Although we do not know the extent of which Id4 may regulate cancer progression our studies lead us to believe that in prostate cancer Id4 is a crucial tumor suppressor gene with the ability to regulate many functions in the cell cycle and may serve as an excellent therapeutic drugs target, eventually.

In conclusion it is quite evident that cancer is a complex disease, involving the regulation of thousands of genes whether it is at the transcriptional level, the translational level or even epigenetic regulation. As scientist we attempt to understand cancer one gene at a time, however for us to truly understand cancer we have to understand the entire process and also understand that cancer is not just a disease but it almost a human entity in itself. Consequently, the premier characteristic that allows human beings to be human and survive over time is our ability to adapt to our environment a trait that is evident even in cancer.
APPENDIX

Figure 1
Figure 1: Id sequence and protein structure comparison. (A) Id proteins are ranked in terms of protein sequence. Id2 shares closest sequence similarity to Id4 and Id1 and Id3 are closely related in regards to protein sequence homology (B) Protein structure shows that Id1 and Id4 are most dissimilar protein sequences, Id1 and Id3 sharing the most similar protein sequence. (C) Observation of protein length characterizes Id4, Id1, Id2 then Id3 from longest amino acid sequence to shortest. Id proteins share a conserved region within the loop region of Id proteins.
**Figure 2**

![Diagram of HLH protein structure families](image)

**Figure 2:** Schematic structure of different HLH protein structure families. The basic DNA binding region (b), leucine zipper (LZ), and PAS domains that bind the HLH regions (are not shown).
Figure 3: Negative regulation of bHLH proteins by Id1 proteins. (A & B) Id proteins negatively regulate E proteins by inhibiting binding of bHLH proteins to the consensus E-box sequence (CNNTG), by binding to the HLH domain of E proteins. Class A and Class B bHLH proteins are preferential binding partners.
Figure 4: Id proteins regulate various aspects of the cell cycle. Id proteins are key regulators of the G1 phase of the cell cycle and regulate various cell cycle regulators including p21CIP1, pRB and of bHLH proteins involved in cell cycle regulation.
**Figure 5**

**Figure 5: Id proteins regulation of cell cycle control gene RB.** Id proteins are key regulators of the G1 phase of the cell cycle and regulate various cell cycle regulators specifically RB, a crucial cell cycle gene.
Table 1: Characteristics of the four Human Id Proteins. Id1-4 are ubiquitously expressed and inhibit several different cellular processes. Id proteins are located on various chromosomes and some Id proteins share redundant functions.
Table 2

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<tr>
<td>Id1-.</td>
<td>No major abnormalities</td>
<td>9,54</td>
</tr>
<tr>
<td>Id2-.</td>
<td>No lymph nodes and Peyer’s patches</td>
<td>85,86</td>
</tr>
<tr>
<td></td>
<td>Defects in the differentiation of natural killer cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Defects in lactation and formation of the mammary gland</td>
<td></td>
</tr>
<tr>
<td>Id2/Rb-.</td>
<td>Embryos survive to term with minimal or no defects in neurogenesis and haematopoiesis, but die at birth from severe reduction of muscle tissue, whereas Rb-/- is embryonic lethal.</td>
<td>42</td>
</tr>
<tr>
<td>Id3-</td>
<td>Impaired immune response including defective B-cell proliferation and severely perturbed H-Y TCR-mediated negative selection</td>
<td>87,88</td>
</tr>
<tr>
<td>Id1/Id3-</td>
<td>Premature maturation of neurons with elevated levels of CDK-inhibitors and vascular malformation in the CNS</td>
<td>54</td>
</tr>
<tr>
<td>Id1+/Id3-.</td>
<td>Impaired tumor neoangiogenesis and tumor growth.</td>
<td>54</td>
</tr>
</tbody>
</table>

Table 2: Results of Id Gene Ablation in Mice. Studies have demonstrated the importance of Id proteins during development in mice and the phenotypic observations of Id ablation studies.
Figure 6: Model of cellular senescence. Human cells have at least two barriers to cancer. One is the Hayflick’s limit which is induced by a variety of stimuli. The other is telomere limit which is induced by telomere shortening. PDL: populations doubling limit.
Figure 7

PC3

DU145

LNCaP

PrEC

Genomic Instability
Survival
Metastasis

AR Differentiation
Apoptosis

MPC

PC

PIN

BPH
Figure 7: Experimental/Clinical model of prostate cancer progression. In both the clinical and experimental model systems prostate cancer progression is marked with a regression of Androgen Receptor activity, differentiation and apoptosis. As prostate cancer progresses, cell survival, invasion/metastasis and genomic instability are remarkably increased. (BPH = Benign Prostatic Hyperplasia, PIN = Prostatic Intraepithelial Neoplasia, PC = Prostate Cancer (Gleason grade 3), MPC = Metastatic Prostate Cancer (Gleason grade 5)).
Table 3: List of experimental cell lines. List of prostate cancer cell lines (with source of origin and initial reference) that are used in this experiment. Each cell line has its own unique properties that correlated with the *in vivo* transition of prostate cancer progression.

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>PROFILE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ (Fibroblast)</td>
<td>Established from skin taken from normal foreskin With a long lifespan in comparison with other normal human fibroblast cell lines.</td>
<td>Bodnar AG, et al. 1998</td>
</tr>
<tr>
<td>PrEC (Normal prostate Epithelial)</td>
<td>Propagated normal primary prostate epithelial cells</td>
<td></td>
</tr>
<tr>
<td>LNCaP (Prostate Cancer)</td>
<td>LNCaP clone FGC was isolated in from a needle aspiration biopsy of the left suprachlavicular lymph node of a 50-year-old Caucasian male (blood type B+) with confirmed diagnosis of metastatic prostate carcinoma.</td>
<td>J.S. Horoszewicz, et al. 1977</td>
</tr>
<tr>
<td>C33 (Prostate Cancer)</td>
<td>Androgen sensitive LNCaP derivative cell line, with low migration and invasion</td>
<td></td>
</tr>
<tr>
<td>C81 (Prostate Cancer)</td>
<td>Androgen insensitive LNCaP derivative cell line, with high migration and invasion</td>
<td></td>
</tr>
<tr>
<td>DU145 (Prostate Cancer)</td>
<td>Anrogen insensitive prostate cancer cell line derived the brain of a 69 year old male caucasion with advanced metastatic prostate cancer.</td>
<td>Papsidero LD, et al. 1978</td>
</tr>
<tr>
<td>PC3 (Prostate Cancer)</td>
<td>Anrogen insensitive prostate cancer cell line derived from the bone of a 62 year old male caucasion with stage IV metastatic prostate cancer.</td>
<td>Kaighn ME, et al. 1979</td>
</tr>
<tr>
<td>PC3M (Prostate Cancer)</td>
<td>Anrogen insensitive PC3 derivative cell line, with higher metastatic and invasive properties</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8: Id-prostate cancer pathways. In prostate cancer Id proteins stimulate several cellular processes that stimulate prostate cancer progression. Id-induced stimulation of proliferation, cell survival, metastasis/invasion and angiogenesis are crucial to prostate cancer progression. Also, the inhibition of senescence is another crucial aspect of Id-induced prostate cancer progression.
Figure 9: The molecular switch in vitro from androgen responsive (LNCaP) to androgen insensitive (DU145). The transition from androgen sensitive prostate cancer to androgen insensitive prostate cancer requires the activation of multiple Id genes (Id1-3), increased proliferation, metastasis, invasion and EMT markers. Conversely, Id4 expression is downregulated coinciding with the repression or tumor suppressor genes, cell cycle regulators and apoptosis.
Table 4: *Id* gene relative expression in prostate cancer cell lines. *Id* genes share a correlation with prostate cancer progression. *Id1*, *Id2* and *Id3* increase in expression as prostate cancer increases to a more invasive/metastatic phenotype. *Id4* expression decreases as the prostate cancer cell lines become more invasive. *Id4* expression also correlates with AR expression.

<table>
<thead>
<tr>
<th></th>
<th><strong>PrEC</strong></th>
<th><strong>LNCaP</strong></th>
<th><strong>DU145/PC3</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Id1</em></td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>Id2</em></td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Id3</em></td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Id4</em></td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>
Cell Cycle

Angiogenesis

Cell Survival

TGF-β induced growth arrest

Serum Independent Growth

Androgen Independent Cell growth

Senescence

AR

G1

G0

S

G2

M

Id1

TGF-β

BMP6

VEGF

NF-κB

EGFR

MAPK

SSA
Figure 10: **Id1 interaction network in Prostate Cancer.** Id1 regulates several crucial processes in prostate cancer. Id1 induction results in VEGF secretion leading to angiogenesis. Id1 also induces both VEGF and EGFR to induce androgen/serum-independent growth in prostate cancer cell lines (LNCaP). In normal prostate epithelial cells Id1 blocks TGF-β induced cell cycle arrest through induction of p21CIP1/p27KIP1. Id1 expression inhibits p16INK4A activation of senescence and G1 arrest. Id1 can be induced by BMP6 though BMP recognition sites in the Id1 promoter. Also AR may possess some regulatory function over Id1, due to PSA downregulation in response to Id1 expression.
Table 5

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ID1</th>
<th>ID2</th>
<th>ID3</th>
<th>ID4</th>
<th>% cells with n&gt;2 centrosomes</th>
<th>% mitoses</th>
<th>% abnormal mitoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>C33A</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>11.2 (±1.8)</td>
<td>6.8 (±4.5)</td>
<td>1.3 (±0.9)</td>
</tr>
<tr>
<td>JURKAT</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>10.5 (±1.1)</td>
<td>3.3 (±0.5)</td>
<td>0.9 (±0.5)</td>
</tr>
<tr>
<td>T47D</td>
<td>(+)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>9.7 (±1.8)</td>
<td>5.5 (±1.7)</td>
<td>1.2 (±0.2)</td>
</tr>
<tr>
<td>HeLa</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>9.3 (±0.9)</td>
<td>6.9 (±0.2)</td>
<td>0.8 (±0.2)</td>
</tr>
<tr>
<td>Caski</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.8 (±1.1)</td>
<td>11.0 (±4.7)</td>
<td>3.8 (±1.2)</td>
</tr>
<tr>
<td>HaCat</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>8.7 (±1.9)</td>
<td>4.2 (±1.6)</td>
<td>1.7 (±0.1)</td>
</tr>
<tr>
<td>HCT-15</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>8.3 (±3.3)</td>
<td>1.2 (±1.6)</td>
<td>0.3 (±0.6)</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>6.6 (±2.0)</td>
<td>3.2 (±0.2)</td>
<td>1.2 (±0.2)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>6.5 (±2.3)</td>
<td>4.3 (±1.4)</td>
<td>0.5 (±0.2)</td>
</tr>
<tr>
<td>SiHa</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>6.5 (±0.4)</td>
<td>5.3 (±1.4)</td>
<td>1.5 (±0.2)</td>
</tr>
<tr>
<td>293T</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>6.5 (±1.9)</td>
<td>5.3 (±0.4)</td>
<td>1.2 (±0.2)</td>
</tr>
<tr>
<td>H-2171</td>
<td>+</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>6.0 (±0.5)</td>
<td>8.0 (±1.0)</td>
<td>1.2 (±0.2)</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>5.8 (±1.8)</td>
<td>2.5 (±0.7)</td>
<td>0.7 (±0.7)</td>
</tr>
<tr>
<td>HL-60</td>
<td>(+)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>5.3 (±1.2)</td>
<td>6.0 (±1.0)</td>
<td>0.7 (±0.2)</td>
</tr>
<tr>
<td>U937</td>
<td>(+)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>5.2 (±0.9)</td>
<td>7.3 (±3.3)</td>
<td>1.1 (±1.2)</td>
</tr>
<tr>
<td>U2OS</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>4.9 (±1.1)</td>
<td>3.3 (±0.5)</td>
<td>1.2 (±0.7)</td>
</tr>
<tr>
<td>NCI-H295</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>4.7 (±0.4)</td>
<td>3.0 (±0.4)</td>
<td>0.3 (±0.5)</td>
</tr>
<tr>
<td>KASUMI-1</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2.9 (±0.2)</td>
<td>2.3 (±1.4)</td>
<td>0.7 (±0.9)</td>
</tr>
</tbody>
</table>

Table 5: Id1-4 expression in various cancer cell lines. Id4 expression is absent in various cancer cell lines including breast, colorectal, and lymphoma cancers. Therefore loss of Id4 expression may be crucial to cancer progression across various cell lineages.
Figure 11

<table>
<thead>
<tr>
<th>Class</th>
<th>N</th>
<th>PC</th>
<th>BP</th>
<th>PC</th>
<th>NAP</th>
<th>NP</th>
<th>PP</th>
<th>PC</th>
<th>AE</th>
<th>BP</th>
<th>PIN</th>
<th>PC</th>
<th>MPC</th>
<th>T-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>41</td>
<td>62</td>
<td>6</td>
<td>7</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>25</td>
<td>5</td>
<td>22</td>
<td>13</td>
<td>30</td>
<td>19</td>
<td>10.82</td>
<td>1.1E-17</td>
</tr>
<tr>
<td></td>
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<td>11.01</td>
<td>2.9E-7</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>5.822</td>
<td>7.1E-6</td>
</tr>
</tbody>
</table>

Correlation: -0.584

References:

1. [46]
2. [47]
3. [48]
4. [49]
Figure 11: Id4 expression in prostate cancer as determined from publically deposited microarray databases. The Oncomine database was queried against Id4 and all prostate databases were analyzed. The box and whisker plots from four of these representative studies is shown. Consolidated data from all these studies demonstrated that Id4 is significantly (T-values > 5 and low P-values < E-6) down-regulated in prostate cancer samples. Abbreviations: n: No. of samples in each analysis, PC: Prostate Cancer (Red), MPC: Metastatic prostate cancer (Yellow), NAP: Normal adjacent prostate (Blue), NP: Normal Prostate (Blue), PP: Post-pubertal prostate (Blue), BP: Benign prostatic hyperplasia (Blue), PIN: prostate intra-epithelial neoplasia (green).
Figure 12: RT-PCR Id4 expression in various prostate cancer cell lines. Using Id4 PCR primers Id4 gene expression was detected in several prostate cancer cell lines. At 30 cycles Id4 expression was very low across most cell lines. After running at 35 cycles Id4 expression was detected in several cell lines specifically LNCaP, DU145+Id4 and PC3. This experiment correlates with the low expression of Id4 in more metastatic cell lines.
Figure 13

A

B

<table>
<thead>
<tr>
<th>BJ</th>
<th>DU145</th>
<th>LNCaP</th>
<th>DU145+Id4</th>
<th>N</th>
<th>IVD</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
</tr>
</tbody>
</table>

Western Blot

RT-PCR
Figure 13: (A) Id4 expression is generally absent in DU145 cells but present in androgen receptor positive LNCaP cells. Treatment of DU145 cells with global de-methylation agent 5'-Aza-2-Deoxycytidine (5-AZA-CdR) leads to Id4 expression as determined by western blot analysis (upper panel) and reverse transcriptase polymerase chain reaction (RT-PCR, lower panel). Beta-actin was used as a loading and RT-PCR control. The primer pairs used for amplification were previously described. (B) Analysis of Id4 methylation in human fibroblasts (BJ) and the prostate cancer cell lines DU145, DU145-Id4 (DU145 cells with constitutively expressed Id4) and LNCaP by methylation specific PCR (MSP). The presence of a PCR band in lanes marked "M" indicates a methylated gene sequence, the presence of a PCR band in lanes marked "U" indicates an un-methylated gene sequence. Normal peripheral blood cells (N), in vitro methylated DNA (IVD) and water served as controls.
Figure 14: Cell Morphology changes in DU145+Id4 cell line(x100). (A , B)
DU145+Id4 cells posses a colony formation morphology more indicative of epithelial cell types and also adhere to one another more readily than DU145 cells.(C,D) DU145 cells appear more mesenchymal with long spindle formations and are highly metastatic and grow aggressively, without growth factors in the serum.
Figure 15: DU145+Id4 has different cytoplasmic molecular structure than DU145 (x200). (B) Appearance of macro molecules in DU145+Id4 cell lines suggest a specific cell morphology not observed in untransfected (A) DU145 cells. Therefore the molecular machinery of DU145+Id4 cells have been altered by the expression of ectopic Id4 resulting in an accumulation of ‘Lipofuscin-like’ molecules indicative of cellular aging. Cells with accumulation of molecules also appear with large flattened phenotype, with a separate phenotype than cells without the accumulation of ‘Lipofuscin like’ molecules.
Figure 16: DU145+Id4 cells respond differently to FBS versus BCS serum (x100). In FBS serum (A) DU145 cells are more migratory and disassociate more readily from proximal cells. In BCS serum (B) DU145+Id4 share high cell-cell interactions and disassociate less readily than in the presence of FBS serum suggesting lower migration capacity.
Figure 17: Time-line induced apoptosis in DU145+Id4 cells (x100). At sub 25 (A), DU145+Id4 cells appear to be morphologically health and proliferate at a slow but steady rate. At approximately sub 40 (B), necrotic cells appear very frequently resulting in an end to DU145+Id4 propagation beyond any more passages through massive cell death.
Figure 18: DU145 & DU145+Id4 cells display different morphological responses to serum deprived conditions (x100). (A) DU145 cells cultured in serum free media for 10 Days and become confluent. (B&C) DU145 cells cultured for 69 days in serum free media results in cells with a large diameter, along with cells growing on top of one another with a close cell-cell interaction network without cells becoming confluent. (D) Cells grown in serum free media for 101 days results in large aggregates of cells without a migratory/invasive phenotype. Cells become largely quiescent and have a problem attaching to the surface of the flask. DU145+Id4 cells do not become confluent and are in cable of initiating new cell growth in serum free media. Although DU145+Id4 cells did not become confluent they did not die after 101 days of serum free media and by the observance of a large number of cells free floating in the media may suggest anchorage independent growth.
Figure 19

The figure shows a bar chart comparing the doubling time (days) of different cell lines: DU145, DU145-CMV, and DU145-Id4. The error bars indicate the variability in the data. Additionally, the chart includes a vertical axis for CPM/ugDNA ranging from 0 to 9000 CPM/ugDNA.
Figure 19: Doubling time (in days, White bars) and rate of proliferation (Black bars) of DU145, DU145-CMV and DU145-1d4 cells line in culture. All the cell lines have matched passages. The DU145 cells were either transfected with no plasmid (DU145), CMV vector alone (DU145-CMV/EV) or pCMV-1d4 (DU145-1d4). White Bars: The average doubling time of DU145-1d4 cells as compared to DU145 and DU145-CMV. The data is an average cell count from four passages. The doubling time was calculated by counting days required for the number of plated cells (usually at 40% confluence) to reach confluence (80%). For DU145-1d4 cells, this average is from passage 13–16. A highly significant increase in the doubling time (approximately 2.5 fold) (P < 0.001) suggests that 1d4 over-expression leads to a decrease in proliferation.

Black Bars: 3H thymidine incorporation assay. 3H thymidine incorporation (CPM) was evaluated as a measure of rate of proliferation. The counts per minute (CPM) of incorporated 3H-thymidine was normalized to total DNA. The data represents CPM/ug DNA of cell lines at passages 14 and 15. The data is represented as mean ± SEM of three experiments performed in triplicates. *: P < 0.001.
Figure 20: RT-PCR on Id gene expression in Id4 overexpression/silencing cell lines.

(A) DU145 cells transfected with pc3DNA3.1+Id4 plasmid were observed for Id4 overexpression and corresponding Id1-3 expression. cDNA of LNCaP cells transfected with various Id4-shRNA constructs for knockdown of Id4 were studied for Id4 genes expression and Id1-3 expression. The following primers were used: Id1: Id2: Id3: Id4: AR: β-actin: (A
**Figure 21**

**Semi-Quantitative Id mRNA RT-PCR expression**

![Bar chart showing mRNA expression levels of Id1, Id2, and Id3 in different cell lines.]

**Figure 21: Semi-Quantitative Id mRNA RT-PCR expression.** The expression levels of Id1-3 in DU145, DU145+Id4, and LNCaP were measured by densitometry analysis of RT-PCR expression. Id4 expression resulted in downregulation of Id1 and Id3 with a slight upregulation of Id2 expression.
Figure 22

A

DU145  DU145-ID4  PrEC

p21

p27

β-actin

B

Relative Expression/β-actin

DU145  DU145-ID4  PrEC

p27  p21
Figure 22: RT-PCR and semi-quantitative expression levels of cyclin dependent kinase inhibitors p27 and p21 in DU145, DU145-Id4 and PrEC (normal prostate epithelial) cells. (B) Semi-quantitative analysis of RT-PCR results shown in (A). The intensity of each p27 and p21 band was normalized to constitutively expressed beta actin. The RT-PCR data and semi-quantitative analysis (expressed as mean ± SEM) is representative of 3 different RT and corresponding PCR reactions (***: P < 0.001, *: P < 0.05 as DU145). The following primers were used: p27 (CDKN1A): Forward 5'-TCA AAC GTG CGA GTG TCT AA and Reverse 5'-ACG TTT GAC GTC TTC TGA GG, p21: Forward 5'-CGA CTG TGA TGC GCT AAT G and Reverse 5'-TTA GGG CCT TCT GGA GA, β-actin: Forward-5' AGA AAA TCT GGC ACC ACA CC, Reverse-5' GGG GTG TTG AAG GTC TCA AA.
Figure 23

**MERGED**

**DAPI**

**P21**

**DU145+144**

**DU145**
Figure 23: Fluorescence immuno-cytochemistry demonstrating expression and localization of p21 (CDKN1A) at the cellular level. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The green fluorescence demonstrates p21 (panels A, C, D, E). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, E, C, F). The green (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigen (p21). P21 expression was upregulated in DU145+ld4 cells compared to DU145 cells (Panel A & D). P21 was localized primarily to the nucleus in DU145 (panel D, AR+ve control) and DU145-ld4 (Panel A) with some cytoplasmic staining of p21 in DU145+ld4 cells (Panel A) Panels A, B, C: DU145-ld4, Panels D, E, F: DU145. The anti-p21 antibodies were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 24
Figure 25: Fluorescence immuno-cytochemistry demonstrating expression and localization of p27 (CDKN1A) at the cellular level. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The red fluorescence demonstrates p27 (panels A, C, D, E). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, E, C, F). The green (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigen (p21). P27 expression was upregulated in DU145+Id4 cells compared to DU145 cells (Panel A & D). P27 was localized primarily to the nucleus in DU145 (panel D). In DU145-Id4 (Panel A) p27 was localized almost exclusively to the cytoplasm. (Panel A) (Panels A, B, C: DU145-Id4), (Panels D, E, F: DU145). The anti-p27 antibody was highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 25

A

DU145
G0-G1 = 50%
S = 25%
G2/M = 25%

B

DU145-Id4
G0-G1 = 40.5%
S = 46%
G2/M = 14%

C

PC3
PC3-Id4
Id4
Actin

D

PC3
G0-G1 = 64%
S = 9.5%
G2/M = 26.8%

E

PC3-Id4
G0-G1 = 42.5%
S = 17.6%
G2/M = 40%

F

DU-Id4/DU
PC3-Id4/PC3
Figure 25: FACS analysis of cell cycle parameters in DU145, DU145-Id4, PC3 and PC3-Id4 cell lines. The cells were serum starved for 24 hours in order to synchronize the cell cycle. The cells were then serum stimulated (10% BCS) in order to determine the effect of Id4 on cell cycle. A and D: Serum treated DU145 and PC3 cells respectively, B and E: Serum treated DU145-Id4 and PC3-Id4 cells respectively. The cell cycle analysis and the fraction of cells in each phase (G0-G1, S and G2/M, indicated as % cells) were determined using ModFit cell cycle analysis software. The data is representative of triplicate experiments. Of note is the number of cells (scale) represented on the Y-axis. C: Reverse Transcriptase polymerase chain reaction demonstrating the expression of Id4 in PC3 (lane 1) and PC3 cells stably transfected with Id4 expression plasmid (Lane 2). Weak Id4 expression was observed in PC3 cells whereas significantly higher expression was observed in PC3-Id4 cells (representative of 3 different PCR reactions). F: The ratio of cells in each phase. The ratio was calculated by dividing the % cells in Id4 expressing cells by parental cells in each phase of cell cycle. This calculation was used to normalize and compare the data between two cell lines. The error bars represent standard error of mean calculated from three different experiments (*: P > 0.05 and ***: P > 0.001).
Figure 26: RT-PCR SKP expression in DU145+Id4 transfected cells. In DU145+Id4 transfected cells the expression of SKP is upregulated versus DU145+Id4. The following primers were used: Forward: Reverse:
Figure 27

DU145

A SKP2

B DAPI

C E2F1

D MERGED

DU145+Id4

E SKP2

F DAPI

G E2F1

H MERGED
Figure 27 Fluorescence immuno-cytochemistry demonstrating expression and localization of SKP2 and E2F1 at the cellular level. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The green fluorescence demonstrates E2F1 (panels C, D, G, H), the red fluorescence demonstrates SKP2 (panels A, D, E, H). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, D, F, H). The green (antigen specific), red (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigens (SKP2 & E2F1). SKP2 expression was upregulated in DU145+ld4 cells compared to DU145 cells (Panel A & D). SKP2 was localized primarily to the nucleus in DU145 (panel A) cells although some cytoplasmic staining was observed in DU145 cells. In DU145-ld4 cells (Panel E) SKP2 was localized almost exclusively to the cytoplasm (Panel A). E2F1 expression was upregulated in DU145+ld4 cells compared to DU145 cells (Panel C & G). E2F1 was localized primarily to the nucleus in DU145 (panel A) cells with some peri-nuclear staining. In DU145-ld4 (Panel G) E2F1 was localized in both the nucleus and the cytoplasm. Cytoplasmic staining was mostly observed in larger cells. In DU145 cells there was some co-localization of SKP2 and E2F1 although it was not frequent. In DU145+ld4 cells SKP2 and E2F1 displayed co-localization more frequently and displayed the highest co-localization in the larger cells where SKP2 was mostly cytoplasmic. Panels A, B, C, D: DU145, Panels E, F, G, H: DU145+ld4. The anti-SKP2, anti-E2F1 were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 28
Figure 28: (A) Western blot analysis of Androgen receptor expression analysis (Upper Panel). Lower Panel: Expression of androgen receptor (AR) and E2A (E12/E47) bHLH transcription factor by RT-PCR. The gain of androgen receptor expression in DU145-ld4 cells as compared to DU145 cells at the transcript and protein level is evident. The total RNA and protein was purified from DU145-1d4 at passage 28 and analyzed for the expression studies. As controls, the parental, mock transfected DU145 cells (AR -ve) and LNCaP (AR +ve) cell lines were used. The expression of beta-actin was used as loading and RT-PCR control. The data is representative of at least three different RT-PCR reactions and Western blot analysis. The following primers were used:

E2A F-5' CAC CAG CCC TCA TGC ACA ACC, R-5' CTC CAA CCA CAC CTG ACA C and androgen receptor (AR): F-5' ATG GTG AGC AGA GTG CCC TA and R-5' GTG GTG CTG GAA GCC TCT CCT. (B) Real time PCR analysis, performed on the same batch of reverse transcribed RNA used in panel A confirms the RT-PCR data. The fold change in AR expression is normalized to beta-actin (3 different RT reactions) and calculated by the Δ Ct method as described in materials and methods section (*** P < 0.001).
Figure 29

A

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<th>PSA</th>
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<td>Du145+LD4 6hr DHT</td>
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<td>Du145+LD4 12hr DHT</td>
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<td>LnCap (normal)</td>
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B

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<td>Du145 (DHT 12hr)</td>
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Figure 29: AR-PSA RT-PCR expression in DU145+ld4 transfected cells compared to untransfected control (DU145). DU145 and DU145+ld4 cells are starved for 48hrs in serum deprived media and then treated with 10nM DHT for 0h, 6hr and 12hr. RNA extracted/isolated and cDNA preparation is explained in materials and methods. (A) In DU145 & DU145+ld4 transfected cells the expression of AR is upregulated in response to DHT. PSA expressions is induced in DU145+ld4 transfected cells and not control (DU145) cells. (B) p21 & p27 expression is induced in DU145+ld4 transfected cells and not control (DU145) cells. The following primers were used: androgen receptor (AR): Forward-5' ATGGTGAGCAGAGTGCCCTA and Reverse-5' GTGGTGCTGGAA GCC TCT CCT. Prostate Specific Antigen (PSA Froward: Reverse:
Figure 30

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Figure 30: Fluorescence immuno-cytochemistry demonstrating expression and localization of AR at the cellular level in DU145+Id4 cells. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The red fluorescence demonstrates AR (panels A, D, G, J, C, F, I, J). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, C, E, F, H, I, K, J). The red (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigens (AR). AR expression was upregulated in DU145+Id4 cells in response to androgen (DHT) (Panel G-I) and anti-androgen (CSD) (Panel J-L) compared to DU145+Id4 cells deprived of serum (Starve) (Panel A-C) of in charcoal stripped media (Panel A-C & D-F). AR was localized primarily to the cytoplasm in Starved and Charcoal stripped and Casodex treated cells (panel A, D, J) with minimal nuclear staining. In DU145-Id4 treated with DHT (Panel G) AR was localized almost exclusively to the nucleus with some residual cytoplasmic activity. The anti-AR, were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 31

<table>
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<tr>
<td>CSD</td>
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Figure 31: Fluorescence immuno-cytochemistry demonstrating expression and localization of AR at the cellular level in DU145 cells. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The red fluorescence demonstrates AR (panels A, D, G, J, C, F, I, J). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, C, E, F, H, I, K, J). The red (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigens (AR). AR expression was slightly upregulated in DU145 cells in response to androgen (DHT) (Panel G-I) and compared to DU145 cells deprived of serum (Starve) (Panel A-C) of in charcoal stripped media (Panel A-C & D-F). AR was localized primarily to the cytoplasm in all treated cells Starved and Charcoal stripped and Casodex and DHT treated cells (panel A-L) with minimal nuclear staining. The most nuclear staining was observed in DU145 cells treated with CSD (panel J, L). In DU145 treated with DHT (Panel G) although AR was slightly upregulated AR was localized almost exclusively to the cytoplasm with almost no nuclear activity. The anti-AR, were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 32

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<tr>
<td><strong>CSD</strong></td>
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Figure 32: Fluorescence immuno-cytochemistry demonstrating expression and localization of AR at the cellular level in LNCaP cells. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The red fluorescence demonstrates AR (panels A, D, G, J, C, F, I, J). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, C, E, F, H, I, K, J). The red (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigens (AR). AR expression was upregulated in LNCaP cells in response to androgen (DHT) (Panel G-I) and anti-androgen (CSD) (Panel J-L) compared to LNCaP cells deprived of serum (Starve) (Panel A-C) or in charcoal stripped media (Panel A-C & D-F). AR was localized primarily to the cytoplasm in Starved and Charcoal stripped and Casodex treated cells (panel A, D, J) with minimal nuclear staining. In LNCaP treated with DHT (Panel G) AR was localized almost exclusively to the nucleus with some residual cytoplasmic activity. The anti-AR, were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (x400) are representative of at least 3 different experiments performed in replicates.
Figure 33

<table>
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Figure 33: Fluorescence immuno-cytochemistry demonstrating expression and localization of AR at the cellular level in PC3 cells. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The red fluorescence demonstrates AR (panels A, D, G, J, C, F, I, J). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, C, E, F, H, I, K, J). The red (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigens (AR). AR expression was slightly upregulated in PC3 cells in response to androgen (DHT) (Panel G-I) and compared to PC3 cells deprived of serum (Starve) (Panel A-C) of in charcoal stripped media (Panel A-C & D-F). AR was localized primarily to the cytoplasm in all treated cells Starved and Charcoal stripped and Casodex and DHT treated cells (panel A-L) with minimal nuclear staining. The most nuclear staining was observed in PC3 cells treated with CSD (panel J, L). In PC3 treated with DHT (Panel G) although AR was slightly upregulated AR was localized almost exclusively to the cytoplasm with almost no nuclear activity. The anti-AR, were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 34

FACS Analysis of DU145 Treated with 10nM DHT

DHT Treatment
- G0-G1
- S
- G2-M
Figure 34: Graph of FACS analysis of cell cycle parameters in DU145 cell line treated with 10nM DHT over 48hrs. The cells were serum starved for 24 hours in order to synchronize the cell cycle. The cells were then DHT-stimulated (10nM DHT in RPMI media with 10% FBS) in order to determine the effect of DHT on the cell cycle for 0, 6, 12, 24 and 48hrs. Cells were extracted and prepared for FACS analysis by method described in ‘materials and methods’. The cell cycle analysis and the fraction of cells in each phase (G0-G1, S and G2/M, indicated as % cells on the Y axis) were determined using ModFit cell cycle analysis software. The data is representative of triplicate experiments.
Figure 35

FACS Analysis of DU145+Id4 Treated with 10nM DHT

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<th>G2-M</th>
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<td>81</td>
<td>74.4</td>
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<tr>
<td>6 hr</td>
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<tr>
<td>48 hr</td>
<td>9.7</td>
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Figure 35: Graph of FACS analysis of cell cycle parameters in DU145+Id4 cell line treated with 10nM DHT over 48hrs. The cells were serum starved for 24 hours in order to synchronize the cell cycle. The cells were then DHT-stimulated (10nM DHT in RPMI media with 10% FBS) in order to determine the effect of DHT on the cell cycle for 0, 6, 12, 24 and 48hrs. Cells were extracted and prepared for FACS analysis by method described in ‘materials and methods’. The cell cycle analysis and the fraction of cells in each phase (G0-G1, S and G2/M, indicated as % cells on the Y axis) were determined using ModFit cell cycle analysis software. The data is representative of triplicate experiments.
Figure 36: Proliferation assay of 48hr DHT/CSD treated DU145 & DU145+1d4 cells.

Cells were starved in serum deprived media for 48hrs and then treated for 48hrs with Charcoal Stripped Fetal Bovine Serum (CSFBS), DHT (1nM DHT in RPMI+10%CSFBS), or CSD (50uM Casodex in RPMI+10%CSFBS). After treatment cells were incubated using CYQUANT NF cell proliferation assay kit and fluorescence was measured.
Figure 37: DU145+Id4 cells undergo senescence in response to ectopic Id4 expression (x200). A large number of DU145+Id4 cells exhibit a senescent phenotype (Increased cell diameter, with increased cell surface and flattened shape. Cells were fixed and stained with SA-β-Gal assay to measure senescence. SA-β-Galactosidase (Green Pigment) staining represents the SA-β-Galactosidase activity in the cell. Cells are observed under normal phase (A) and phase contrast (B) to display the percentage of senescent cells.
Figure 38: SA-β-Galactosidase assay in DU145 (A & C) & DU145+Id4 (B & D) cells (x200). Cells were cultured under appropriate conditions and treated with SA-β-Galactosidase (MBC senescence detection kit) enzyme stains cells containing the β-Galactosidase enzyme a green pigment. Cells with some green pigment were labeled “moderate” senescent cells and Cells with significant green pigment accumulation and a “senescent” phenotype were labeled as “advanced” senescent cells.
Moderate Cellular Senescence

![Bar chart showing percentage of total cell # for different conditions: STARVE, cFBS, FBS, DHT, CSD. The percentages are 68%, 63%, 62%, 66%, and 23% respectively. The legend indicates DU145 and DUID4 colors.](image-url)
Figure 39: Percentage of moderate cellular senescent DU145/DU145+1d4 cells in various media. Cells were treated for 24hrs in media supplemented with either serum deprived (Starve), charcoal stripped serum (CSFBS), DHT (10nM DHT in RPMI+10%CSFBS), or Casodex (25nM Casodex in RPMI+10%CSFBS). Cells were plated then fixed with a fixative solution then stained with the β-galactosidase and allowed to incubate overnight to observe the percentage of cells that had moderate advanced levels of cellular senescence. Moderate senescent cells are characterized as seen in figure 35. The DU145 cell line had very low levels of moderate senescence, excluding the 44% of senescent DU145 in DHT supplemented media. The DU145+1d4 cell line had a higher average of moderate senescent cells with the highest average of senescent cells seen in cells supplemented in Casodex media.
Figure 40

Advanced Cellular Senescence

Starve cFBS FBS DHT CSD

DU145 0% 3% 8% 3% 64%

DU145 12% 30% 34%

DUID4 0% 18%
Figure 40: Figure 36 Percentage of moderate cellular senescent DU145/DU145+1d4 cells in various media. Cells were treated for 24hrs in media supplemented with either serum deprived (Starve), charcoal stripped serum (CSFBS), DHT (10nM DHT in RPMI+10%CSFBS), or Casodex (25nM Casodex in RPMI+10%CSFBS). Cells were plated then fixed with a fixative solution then stained with the β-galactosidase and allowed to incubate over night to observe the percentage of cells that had moderate advanced levels of cellular senescence. Moderate senescent cells are characterized as seen in figure 35. The DU145 cell line had very low levels of moderate senescence, excluding the 44% of senescent DU145 in DHT supplemented media. The DU145+1d4 cell line had a higher average of moderate senescent cells with the highest average of senescent cells seen in cells supplemented in Casodex media.
Figure 41: RT-PCR on senescence markers in prostate cell lines. Several genes are associated with the induction of senescence. These genes include Vimentin, p16, p27 and p21. The expression of these genes was observed by RT-PCR in several prostate cancer cell lines. The expression levels were normalized by b-actin. The following primers were used: SKP2, VIM (Vimentin), p21, p27, p16.
Figure 42: Fluorescence immuno-cytochemistry demonstrating expression and localization of p16 (CDKN1A) at the cellular level. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The red fluorescence demonstrates p21 (panels A, C, D, E). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, E, C, F). The green (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigen (p21). P21 expression was upregulated in DU145+1d4 cells compared to DU145 cells (Panel A & D). P21 was localized primarily to the nucleus in DU145 (panel D, AR+ve control) and DU145-1d4 (Panel A) with some cytoplasmic staining of p21 in DU145+1d4 cells (Panel A) Panels A, B, C: DU145-1d4, Panels D, E, F: DU145. The anti-AR and anti-β actin antibodies were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 43: Sub G0 events in increases in DU145+1d4 cells. Indicative of increased apoptosis in DU145+1d4 cells the sub G0 events increase dramatically in DU145+1d4 cells versus DU145 cells. FACS analysis was conducted as described in the materials and methods section.
Figure 44: Immuno-fluorescence apoptosis of DU145 & DU145+Id4 cells (x100).

Cells were grown on chamber slides treated with Annexin V 488 apoptosis assay kit as described in ‘materials and methods’ and apoptosis was observed under the fluorescence microscope. Green pigment (Annexin V) stains for apoptosis in live cells. Red pigment (Propidium Iodide) stains for ‘dead cells’. DU145+Id4 cells had a higher ratio of both green and red pigments suggesting a higher rate of apoptosis.
Figure 45: Immuno-fluorescence apoptosis of DU145 & DU145+Id4 cells (x400).

Cells were grown on chamber slides treated with Annexin V 488 apoptosis assay kit as described in ‘materials and methods’ and apoptosis was observed under the fluorescence microscope. Green pigment (Annexin V) stains for apoptosis in live cells. Red pigment (Propidium Iodide) stains for ‘dead cells’. DU145+Id4 cells had a higher ratio of both green and red pigments suggesting a higher rate of apoptosis.
Table 6

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<tr>
<td>Viable Cells</td>
<td>73%</td>
<td>34%</td>
</tr>
<tr>
<td>Apoptotic cells</td>
<td>24%</td>
<td>60%</td>
</tr>
<tr>
<td>Dead cells</td>
<td>3%</td>
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Table 6: Apoptosis assay for DU145/DU145+Id4 treated with Actinomycin D. Cells were either grown in normal media (RPMI+10%FBS) or treated with normal media supplemented with Actinomycin D at 100ng/mL for 6hrs then harvested and apoptosis assay was performed (Annexin V apoptosis assay kit). The DU145+Id4 has a higher basal level of apoptosis Table 1 shows the comparison of apoptosis of both Normal DU145 cells and Du145+Id4 cells treated with Actinomycin D. Treatment with Actinomycin D resulted in a ~2 fold increase in apoptotic cells in DU145+Id4 treated cells.
Figure 46

[Image of flow cytometry plots showing Annexin V 488 and propidium iodide staining for different cell lines]
Figure 46: Doxorubicin induced apoptosis in DU145+Id4 compared to DU145, LNCaP and PC3 cells. All four cell lines were treated with 25nM Doxorubicin for 72 hrs and an apoptosis assay (Annexin V 488/PI apoptosis assay kit #2) was performed. Cells were counted on a flow cytometer to observe dead, apoptotic and viable cells. Annexin V 488 is along the X axis, Propidium Iodide concentration is along the Y axis. Therefore, cells (dead cells) with highest stains for both Annexin V 488 and Propidium Iodide aggregate in the top right hand corner of the graph.
### Table 7

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<td>3.9</td>
<td>87.8</td>
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</table>

Table 7: Apoptotic assay of Prostate cancer cells treated with 50nM Doxorubicin 72hrs. DU145, DU145+Id4, LNCaP and PC3 cells were treated with media (RPMI+10%FBS) supplemented with 50nM Doxorubicin for 72hrs. After treatment apoptosis assay was performed as described in the material and method. Cells were associated into three main groups, Viable cells, apoptotic cells, and Dead cells.
Figure 47: Real-time RT-PCR expression of p53 and cdh1 in DU145+Id4 transfected cells compared to untransfected control (DU145). In DU145+Id4 p53 has a ~17 fold increase over control (DU145). In DU145+Id4 cells cdh1 has a ~2 fold increase in expression over control.
Figure 48: Fluorescence immuno-cytochemistry demonstrating expression and localization of p53 at the cellular level. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The green fluorescence demonstrates p53 (panels A, C, D, E). The cells were counterstained with DAPI (blue) to reveal the nucleus (panels B, E, C, F). The green (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigen (p53). P53 expression was upregulated in DU145+Id4 cells compared to DU145 cells (Panel A & D). P53 was localized primarily to the nucleus in DU145 (panel D, AR+ve control) and DU145-Id4 (Panel A) with some cytoplasmic staining of p53 in DU145+Id4 cells (Panel A) Panels A, B, C: DU145-Id4, Panels D, E, F: DU145. The anti-p53 antibodies were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 49

DU145
50nM DOXO
DU145+Id4
FBS

E-cadherin  DAPI  MERGED
Figure 49: Fluorescence immuno-cytochemistry demonstrating expression and localization of E-cadherin cells treated with in the presence and absence of Doxorubicin at the cellular level. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The green fluorescence demonstrates Ecadherin expression. The cells were counterstained with DAPI (blue) to reveal the nucleus. The green (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigen (p21). Ecadherin expression was upregulated in DU145+Id4 cells compared to DU145 cells. E-cadherin was localized primarily to the nucleus in DU145 and DU145-Id4 with some cytoplasmic staining of E-cadherin in DU145+Id4 cells. The anti-E-cadherin antibodies were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (×400) are representative of at least 3 different experiments performed in replicates.
Figure 49: Fluorescence immuno-cytochemistry demonstrating expression and localization of Vimentin cells treated with in the presence and absence of Doxorubicin at the cellular level. The cells were processed for immuno-cytochemistry as described in the materials and methods section. The red fluorescence demonstrates Vimentin expression. The cells were counterstained with DAPI (blue) to reveal the nucleus. The red (antigen specific) and blue (nucleus) images were superimposed to demonstrate cellular localization of the antigen (p21). Vimentin expression was upregulated in DU145+ld4 cells compared to DU145 cells. Vimentin was localized primarily to the nucleus in DU145 and DU145-ld4 with some cytoplasmic staining of Vimentin in DU145+ld4 cells. The anti-Vimentin antibodies were highly specific and displayed no non-specific binding as determined by western blot analysis. The photomicrographs (x400) are representative of at least 3 different experiments performed in replicates.
Figure 51 Possible mechanism of Id4 interaction within the AR pathway. AR can be induced by several different proteins in the AR pathway. Several proteins are essential for AR induction including AR coregulators, MAPK, PI3K, and AKT. It is possible that Id4 may be responsible in inducing AR activity though regulation of specific AR coregulators or through activation of AR stimulatory proteins.
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