ANDROGEN REGULATED GENES IN PROSTATE CANCER CELLS

by

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Abstract

Prostate cancer is a leading cause of cancer death in Canadian men. Prostate tumors initially depend on androgens for survival and androgen ablation is an effective therapy. Prostate cancer adapts to the androgen depleted environment and begins to grow in an androgen independent (AI) form. Androgens exert their effects through the action of the androgen receptor (AR), a ligand dependent transcription factor. AR is important for cell survival and appears central to the progression of prostate cancer to AI. The hypothesis driving the present studies is that androgen regulated genes are reactivated during prostate cancer progression to AI and as such, identification of novel androgen regulated genes will provide new targets for which therapeutics can be directed. To discover novel androgen regulated genes, cultured prostate cells were treated with androgens and an expression profile generated using a microarray screen. Validation of the microarray experiment revealed the upregulation of genes involved in cell survival (GADD45G), development of drug resistance (MRP4) and protection against oxidative stress (NFE2L2), while a pro-apoptotic gene (BARD1) was downregulated. Probing a tissue microarray, GADD45G was found upregulated in AI prostate cancer. *In vitro* studies revealed that GADD45G is important for cell survival and acts as a control point for several critical intracellular signaling cascades. One surprising result was that while GADD45G mRNA levels increase with androgens, protein levels decrease. A miRNA, hsa-miR-326 was found to be responsible for inhibition of GADD45G translation and this inhibition could be relieved under stress conditions such as treatment with high levels of taxol. Clusterin, a gene involved in apoptosis, has two isoforms resulting from alternate transcriptional start sites and

these were found to be differentially regulated by androgens. Initial transcripts of both isoforms encode an anti-apoptotic protein; however Isoform 1 can be spliced into a pro-apoptotic form, while Isoform 2 cannot. Isoform 1 was found to be repressed by androgens while Isoform 2 is upregulated, consistent with androgens being cytoprotective in prostate cells. The work presented here highlights the importance of androgen regulated genes in prostate cancer survival and identifies GADD45G and clusterin as being potential targets for therapeutics.

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List of Abbreviations

°C	degrees Celsius
ABC	ATP-binding cassette
ACTH	adrenocorticotropin
AD	androgen dependent
AF	activation function
Ago2	argonaute 2
AI	androgen independence
AR	androgen receptor
ARE	androgen response element
ASO	antisense oligonucleotides
ATRA	all-trans-retanoic acid
BARD1	BRCA1-associated RING domain 1
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
CBP	CREB-binding protein
cdk	cyclin dependent kinase
CGH	comparative genomic hybridization
ChIP	chromatin immunoprecipitation
CK	cytokeratin
cm	centimeter
CO_2	carbon dioxide
CSS	charcoal stripped serum
CZ	central zone
DBD	DNA binding domain
DBI	diazapam binding inhibitor
DEPC	diethyl pyrocarbonate
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulphate
DHT	dihydrotestosterone
dpm	decintegrations per minute
DRE	digital rectal exam
DTT	dithiothreitol
EGF	epidermal growth factor
eIF4E	eukaryotic translation initiation factor 4E
ER	estrogen receptor
ER	endoplasmic reticulum
EtOH	ethanol
FAM	fluorescein
FBS	fetal bovine serum
FMR1	fragile X mental retardation 1
GADD45G	growth arrest and DNA-damage inducible gamma
GCLM	glutamate-cysteine ligase, modifier subunit
GR	glucocorticoid receptor

GRIP	Glutamate receptor-interacting protein 1
GST	glutatione S-transferases
H&E	haematoxylin and eosin
HAT	histone acetyl transferase
HDAC	histone deacetylase
HSP	heat shock protein
IAS	intermittent androgen suppression
IGF-1	insulin-like growth-factor-1
IGFBP	IGF binding protein
JNK	c-jun kinase
KGF	keratinocyte growth factor
KLK	kallikrein
LBD	ligand binding domain
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LNA	locked nucleic acid
M	molar
MDM2	mouse double minute 2 homolog
miRNA	microRNA
mJ	milli Joules
MKNK1	MAPK-interacting kinase 1
ml	milliliter
mM	millimolar
mm	millimeter
MMS	methyl methanesulfonate
MR	mineralcorticoid receptor
MRP4	multidrug resistance-associated protein 4
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium
N_2	nitrogen
NFE2L2	nuclear factor erythroid 2-like 2
ng	nanogram
NHT	neoadjuvant hormone therapy
NLS	nuclear localization sequence
nM	nanomolar
NQO1	NAD(P)H dehydrogenase, quinone 1
NTD	N-terminal domain
PAGE	polyacrylamide gel electrophoresis
PAK6	p21-activated kinase 6
PB	processing body
PBS	phosphate buffered saline
pCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDG	6-phosphogluconate dehydrogenase
p-gp	p-glycoprotein

PIN	prostatic intraepithelial neoplasia
PKA	protein kinase A
PMS	phenazine methosulfate
poly-G	poly-glycine
poly-Q	poly-glutamine
PR	progesterone receptor
PRDX1	peroxiredoxin
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
PVDF	polyvinyl
PZ	peripheral zone
RAR	retinoic acid receptor
Rb	retinoblastoma
RISC	RNA-induced silencing complex
RNA pol II	RNA polymerase II
RSK	ribosomal protein S6 kinase
RT	room temperature
RXR	retinoic acid X receptor
SAGE	serial analysis of gene expression
SDS	sodium dodecyl sulphate
SHBG	sexual hormone binding globulin
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SRC	steroid receptor coactivator
SSC	saline sodium citrate
SUMO-1	small ubiquitin-like molecule-1
T3	3,3',5-triiodo-L-thyronine
TAMRA	tetramethyl-6-caboxyrhodamine
TFIIF	general transcription factor IIF
TGF	transforming growth factor
TMA	tissue microarray
TR	thyroid hormone receptor
TRPM-2	testosterone repressed prostate message 2
TXN	thioredoxin
TZ	transition zone
UTR	untranslated region
UV	ultraviolet
V	volts
VEGF	vascular endothelial growth factor
VSN	variance stabilizing normalization
µg	microgram
µl	microlitre

Acknowledgements and Dedication

This work could not have been completed without the help of many people. I'd like to acknowledge my supervisor, Dr. Colleen Nelson, who has been an exceptional mentor to me through the duration of my thesis. Many of my fellow graduate students have been helpful both in the lab and in keeping me sane, especially Vanessa Thomson, Lindsay Brown, Leah Prentice and Melanie Lehman. I've had the benefit of working with postdoctoral fellows who have been very nurturing, including Dr. Susan Moore and Dr. Susan Ettinger. I would like to thank Robert Shukin, John Cavanagh and Steve Hendy, whose expertise and lab skills have been invaluable. I would also like to acknowledge the help from past and present members of the array centre, especially Nadine Tomlinson and Robert Bell. I'd like to express gratitude to the staff at the Prostate Center for all the memories. And finally, I'd like to thank my family and my friends for their continual encouragement.

This thesis is dedicated to my husband, Robert Cochrane, for his support through this long process and for keeping me laughing the whole way.

Co-Authorship Statement

The work presented in this Ph.D. thesis was in part due to collaboration with other researchers. Those involved in the research are listed in the publication citations in each of the chapters. Their individual contributions to the research are outlined in a section preceding the Introduction of each chapter.

Chapter 1   Literature Review, Hypothesis and Objectives

Prostate cancer is a frequently diagnosed disease in older men and one of the leading causes of cancer related deaths in Canada. The disease is initially regulated by androgens, but following androgen ablation therapy inevitably progresses to an androgen independent form which is refractory to therapies. The androgen receptor is ligand activated transcription factor and androgen receptor activity is essential for both prostate cancer survival and the development of androgen independence. I set out to determine which genes are regulated by androgens, as these may be important for survival of the prostate cancer cells and possibly targets for new therapeutics. To accomplish this, an mRNA microarray screen was done to look for novel genes which are regulated by androgens. In this Ph.D. dissertation, I describe the androgen regulation of several genes including GADD45G and clusterin, both of which are important for cell survival. Clusterin is currently being targeted for inhibition in multi-centre phase II trials. With the data obtained in this thesis, I provide evidence to suggest that GADD45G may also be an attractive therapeutic target.

1.1   The Prostate

The prostate is a male specific glandular organ which sits below the bladder and surrounds the urethra (**Figure 1.1**). The prostate secretes proteins into the seminal fluid that are important for coating and uncoating the spermatozoa (1). One of the major secretory proteins of the prostate is the prostate specific antigen (PSA), or kallikrein 3. PSA is a protease which cleaves proteins that cause clotting of the ejaculate and the end result is increased sperm motility (2, 3). While not absolutely

necessary for fertility, prostate secretions are a major functional component of the seminal fluid and are involved in the physiological process of ejaculation.
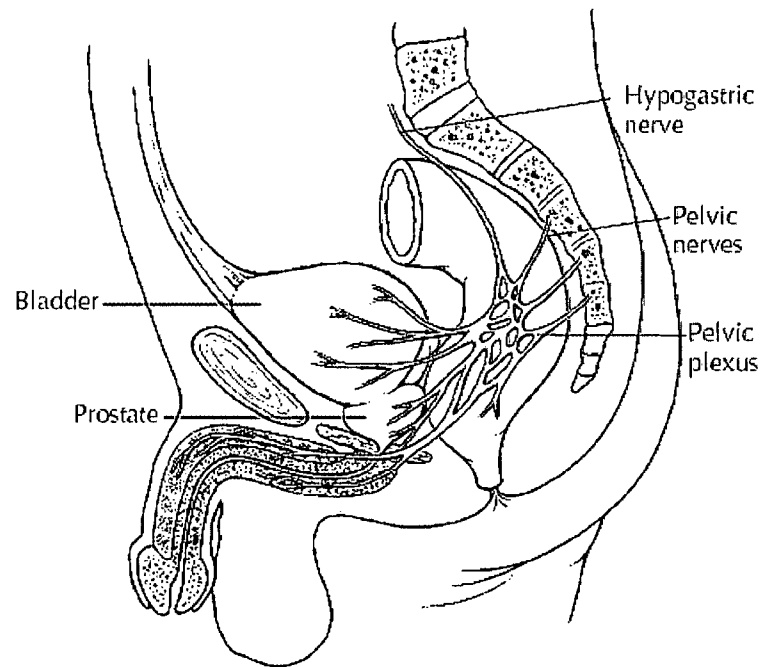
Figure 1.1 - Diagram showing the location and size of the prostate in relation to adjacent anatomical structures. Adapted from Goldenberg *et al* (4).

The prostate arises from the urogentital sinus and Wolffian ducts at 10-12 weeks of fetal human development, a process stimulated by circulating fetal androgens (5). At puberty, the maturation process is continued in response to the elevated testosterone levels. While rodent prostates develop into multi-lobed organs, the human prostate is compact and lacks distinctive lobes (6). The size of an adult human prostate is often compared to that of a walnut - 20g and 4 cm by 2.5 cm.

Although there are no distinctive lobes in the human prostate, it is divided into three zones - the central zone (CZ), the peripheral zone (PZ) and the transition zone

(TZ). The zones not only are discrete regions within the prostate, but they differ in their histology and their susceptibility to disease (7). The PZ and TZ arise from the endodermal urogenital sinus while the CZ, as well as the seminal vesicles, arise from the Wolffian ducts which are mesodermal in origin (8, 9). The majority of prostate cancers arise in the PZ, and more infrequently in the TZ, while CZ prostate cancers are very rare (7).

There are different types of epithelial cells found in the prostate - basal, luminal, intermediate, neuroendocrine and stem cells. A figure showing a cross section of prostate glands and the epithelial cells is shown in **Figure 1.2**. The basal cells are
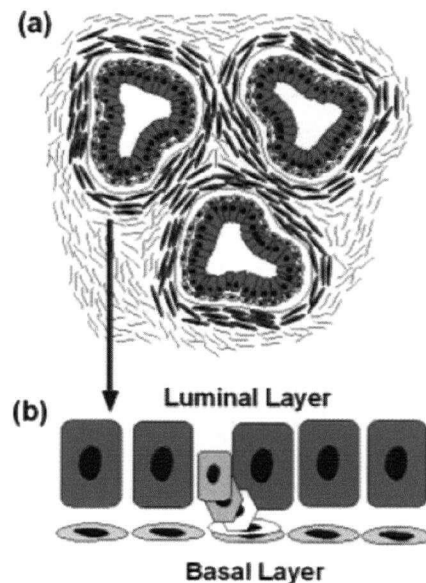
Figure 1.2 - Cells of the prostate gland. (a) A cross section of the prostate gland showing the glandular epithelial cells surrounded by the fibromuscular stroma. (b) The epithelial cells of the prostate gland showing migration of the cells from the basal layer to the luminal layer as they differentiate. Adapted from Long *et. al.* (10).

cuboidal cells with a high mitotic index, and are attached to the basement membrane (11). They have low expression levels of the androgen receptor and are androgen independent. In contrast, the prostatic, secretory luminal cells have a low mitotic index and are androgen dependent, expressing high levels of the androgen receptor (12). The basal and luminal cells each express distinctive cytokeratins, with CK 5 and 14 being indicative of basal cells and luminal cells expressing CK8 and 18 (10). The poorly differentiated basal cells migrate to become differentiated luminal cells. Between basal and luminal cells are intermediate cells (also known as transit-amplifying cells) that express a combination of basal and luminal cell markers (10). Neuroendocrine cells are terminally differentiated, androgen independent cells (13), spread out amongst the basal and luminal compartments and express the chromogranin A marker (14). Their function is not entirely clear, although it is believed that they are involved in prostate epithelial cell development and differentiation through the release of various cytokines (15). In rodents, androgen withdrawal causes involution of the prostate luminal epithelium, however the prostate can be completely regenerated once androgens are restored (16). It is likely that the human prostate responds in a similar manner following castration to induce an apoptotic regression of the secretory luminal epithelium, which can be restored upon reintroduction of androgens. This ability of the prostate to regenerate even after long periods of androgen deprivation, suggested the existence of prostatic stem cells (17). Prostatic stem cells were found to reside in the basal layer and express the Sca-1 stem cell marker (18). Underneath the epithelial layer lies the fibromuscular stroma (19).

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Stromal-epithelial interactions are important for development of the prostate as well as growth and differentiation of the epithelial cells (20).

1.2 Androgens and the Androgen Receptor

Androgens are essential for the development and maintenance of the prostate. Testosterone is the major circulating androgen and is synthesized primarily in the Leydig cells of the testis. The release of testosterone is under the negative feedback loop of the hypothalamus-pituitary-gonadal axis (**Figure 1.3**). In this axis, low levels of testosterone cause the hypothalamus to release luteinizing hormone-releasing hormone (LHRH) to the pituitary, which responds by releasing luteinizing hormone (LH). The LH induces a release of testosterone from the testes, which results in a negative regulation of LHRH release by the hypothalamus. In the blood stream, there is very little free circulating testosterone, instead it is bound by albumin or sexual hormone binding globulin (SHBG). Free testosterone can enter the cells via diffusion through the membrane and SHBG-bound testosterone enters prostate cells via receptor-mediated endocytosis (21). Testosterone is converted to the more potent androgen, dihydrotestosterone (DHT) through the actions of 5-α-reductase in target tissues such as the prostate (22, 23). The adrenal androgens androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) have some activity in the prostate, although much weaker than testosterone. The adrenal cortex synthesizes and releases adrenal androgens in response to adrenocorticotropin (ACTH) coming from the pituitary gland (24). Testosterone and its metabolite DHT exert their effects in the prostate through the actions of the androgen receptor (AR).
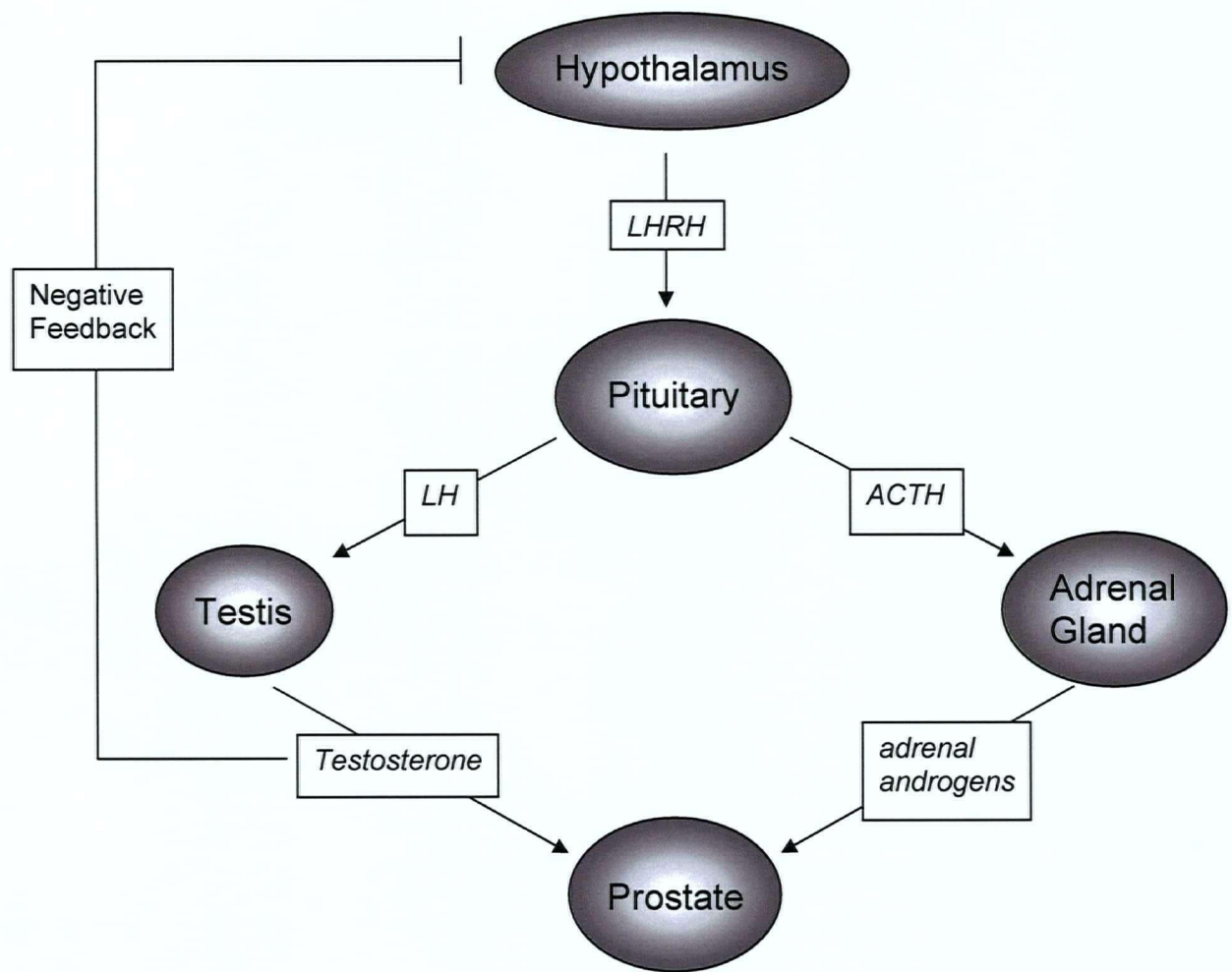
Figure 1.3 - The hypothalamus-pituitary-gonadal axis showing the steps involved in testosterone production as well as the production of adrenal androgens. The axis is controlled by a negative feedback loop.

1.2.1 Steroid Receptors

The nuclear receptor superfamily is a class of ligand activated transcription factors and members of this family can be found in all metazoans (25). The superfamily can be sub-divided into four classes, determined by the manner in which they dimerize and bind to DNA - Class I receptors are steroid receptors which bind to DNA inverted repeats as homodimers, Class II receptors form heterodimers with RXR

(retinoic acid X receptor) and tend to bind to direct repeats, Class III receptors form homodimers and bind to direct repeats and Class IV receptors bind to DNA as monomers (26).

The steroid receptor family of nuclear receptors includes the androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), mineralcorticoid receptor (MR), and estrogen receptor (ER). Steroid receptors have a common structure which relates to their common functions: a C-terminal ligand binding domain, a hinge region, a DNA binding domain and an N-terminal domain. In response to ligand binding, the receptors undergo a conformational change which allows them to bind to DNA and initiate transcription of target genes (27).

1.2.2 Androgen Receptor Gene

The androgen receptor (**Figure 1.4**) is encoded by a single copy gene of over 90 kb in length residing on the X chromosome (28). In the prostate, there are three AR transcripts of approximately 11, 8 and 5 kb (29). The gene consists of 8 exons which encodes a protein of approximately 917 amino acids and 100-110 kDa in size (30-32). There is a small variation in the size of the AR protein in individuals owing to two polymorphic regions in the gene.

1.2.3 Ligand Binding Domain

The region responsible for binding ligands resides at the C-terminal end of the AR and is approximately 250 amino acids in length forming a pocket (33). The ligand binding domain (LBD) is a well conserved region among the nuclear receptors and results in a common structure composed of 12 a-helices which assemble in a bundle with a hydrophobic center, with the 12th helix acting as a lid regulating interactions with

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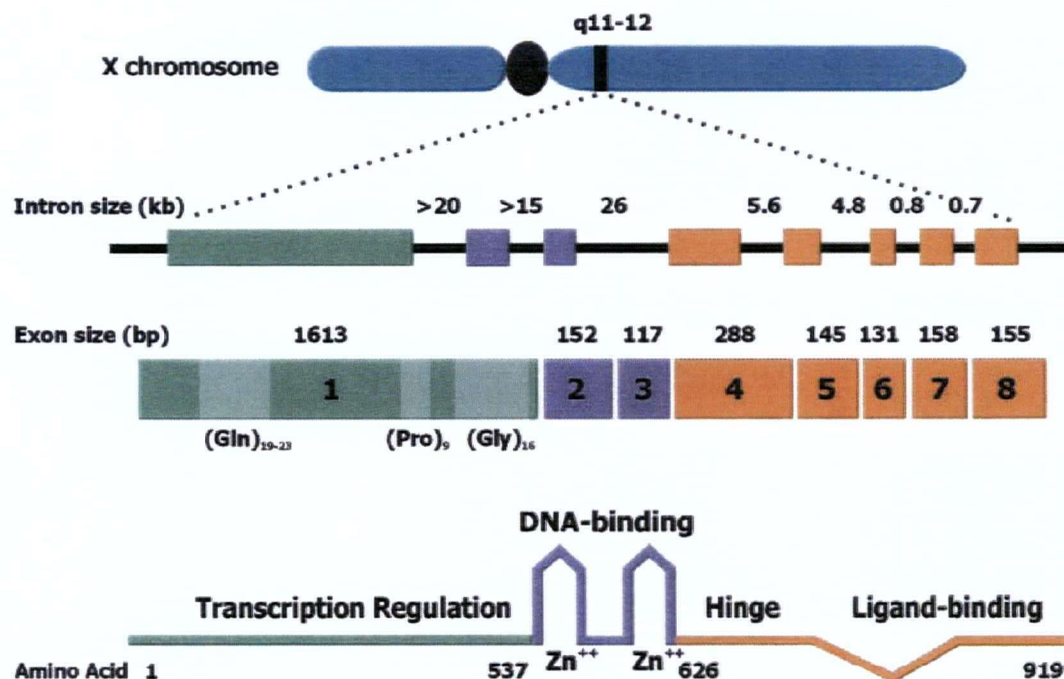
Figure 1.4 - Structure of the androgen receptor gene, mRNA and protein. The androgen receptor is encoded on chromosome Xq11-12. The mRNA is made from 8 exons and results in a protein with 4 functional domains: N-terminal domain (transcriptional regulation), DNA-binding domain, hinge region and the ligand binding domain. Adapted from Gelmann (34).

co-activators and co-repressors (35, 36). As with other steroid receptors, a ligand binding pocket is formed from 12 helices (37). The LBD has different conformations depending on whether antagonist or agonists are bound. Agonist binding results in the movement of helix 12 to a position covering the ligand binding pocket (34). Helix 12 is held away from the binding pocket when antagonists bind and in that position, it interferes with interaction of the receptor with coactivators (38). Mutations in the LBD exist that enable the antagonists to act as agonist. Crystal structure of these mutants show that their conformation while bound to an antagonist like bicalutamide mimics

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that of the conformation observed while bound to DHT (39). There are 18 key residues in the LBD which are essential for interaction with agonists and with the exception of a few ionic residues, most of these are hydrophobic (37). The LBD is necessary for trans-repression of the AR in the absence of ligand, and as such, its deletion results in a constituatively active receptor (40).

The LBD has several functions apart from allowing the receptor to bind ligand. It is also the region which interacts with heat shock proteins, contains one of the two activation function regions of the receptor and is also important for receptor dimerization. The LBD is also the location at which hsp90 and other chaperones interact with the AR (41-43). AR requires interaction with hsp90 so that it can be maintained in a conformation which has a high affinity for ligand binding (44). In the absence of hsp90, with a substantially decreased ligand binding affinity, AR has much lower activity and is degraded more rapidly (45).

Dimerization of the androgen receptor is an important factor in its ability to effectively bind to DNA and cause transactivation of target genes. Interaction of the LBD of one monomer with the DBD of the other monomer results in AR dimerization (46). The dimerization is dependant on agonist binding to the LBD, as ligand free or antagonist-occupied LBD does not induce dimerization (47, 48) and dimerized AR is more stable than monomeric AR (49). Mutations causing a disruption in the ability of the AR to dimerize decrease its transactivation activity (50-52).

There are two activation function (AF) domains in the androgen receptor and it requires both for complete transcriptional activation. The AF2 domain is the weaker of the two domains and resides in the C-terminal LBD region (53). Upon ligand binding,

there is a conformational change in the receptor which allows the AF2 region of AR to interact with coactivators, such as p160 and GRIP (54). Like other steroid receptors, AR interacts with coregulators containing the LXXLL motif (55, 56). Unlike other receptors, AR can also bind to cofactors containing FXXLF motifs (57-59). This motif is also found within in the N-terminal domain of the AR itself. The interaction between the N-terminal and C-terminal regions within AR is important for its transactivation (60).

1.2.4 DNA Binding Domain

The AR DNA binding domain (DBD) is encoded by exons 2 and 3 and consists of two zinc fingers in which 4 cysteines coordinate each zinc (61, 62). It is the most highly conserved region amongst the steroid receptors (34). The DBD is essential for DNA binding and nuclear translocation, and well as directly influences AR transactivation. Mutations in the DBD can affect the ability of the receptor to effectively interact with DNA and result in decreased or altered receptor activity (63, 64).

There are 8 conserved cysteines in the DBD that are needed to coordinate with two zinc ions to form the two zinc fingers which can then interact with the major groove of DNA (65, 66). The first (N-terminal) zinc finger is more important for determining specific binding of the receptor to DNA (67-70). The second zinc finger further contributes to the specificity of the binding to DNA, but it also important for the dimerization of the receptors while bound to DNA (71, 72). Differences in the C-terminal extensions of AR and other steroid receptors is important for their ability to recognize their appropriate DNA sequence (62, 73).

AR binds to inverted palindromic sequences separated by a 3 nucleotide spacer, called androgen response elements (AREs). The idealized consensus

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sequence for the ARE is 5'-GGA/TACANNNTGTTCT-3' (74, 75). AR is able to induce transactivation off of a single ARE, however the level of transactivation is increased substantially through cooperative binding to two or more adjacent AREs (76-78). Interestingly, GR can bind to the same consensus sequence for its response element, however the two receptors have different targets *in vivo* (79-81). The specificity of binding of AR and GR to their respective response elements may be in part due to interaction of the receptors with specific nucleotides within spacer and the flanking nucleotides (82). The cooperative nature of multiple response elements may contribute to AR being able to activate certain promoter regions, while GR is not (83). AR has also been shown to bind to response elements containing direct repeats, whereas GR cannot (73, 84, 85).

1.2.5 Hinge Region

The hinge region is located between the LBD and the DBD and is poorly conserved amongst the steroid receptors (86). The NLS (nuclear localization sequence) is located within DBD and hinge regions (87). Unliganded AR is resident in the cytoplasm and is shuttled to the nucleus upon stimulation with agonist (88). Binding of some antagonists may result in a slower rate of nuclear entry (89), but most antagonists are thought to interfere with coactivator recruitment on AREs. Mutations in the NLS results in both a decreased rate and degree of receptor import into the nucleus (90). The hinge region may also interact with some co-repressors and is therefore important for preventing activation of the unliganded AR (86, 91).

1.2.6 N-terminal Domain

The N-terminal domain (NTD) is the most divergent region of the steroid receptors. It is 541 amino acids in length in the human AR and is fully encoded by exon 1 (92, 93). It contains the two polymorphic tri-peptide repeat regions in the AR, two regions important for interaction with the LBD, as well as the AF1 region which is important for the recruitment of transcription factors.

The two polymorphic regions within the NTD are a poly-glycine (poly-G) and a poly-glutamine (poly-Q) tract. The poly-G is 10 to 31 repeats in length (94). The length of the poly-G tract does not affect the transactivation of the AR, however the length is inversely proportional to the amount of protein that gets translated (95). A poly-G tract with 14 or less repeats is more likely to be found in men with prostate cancer than in the general population (96, 97). The length of the poly-Q tract has also been associated with disease. As with the poly-G tract, an expansion of the poly-Q tract causes a decrease in AR protein expression, however there is also a decrease in transactivation (97-100). Long poly-Q repeats have been associated with infertility (101, 102) and spinal and bulbar muscular atrophy (103, 104) while shorter repeats have been associated with increased AR activity and prostate cancer (105-107).

As mentioned earlier, the NTD and the LBD interact in response to ligand binding. The two sequences in the NTD that interact with the LBD are ^{23}FQNLF^{27} and ^{433}WHTLF^{437}, with the former having a stronger interaction (108, 109). The N/C interaction is unique to the androgen receptor, which may explain some of the properties of the AR which are uncommon in other receptors. The interaction of the N and C terminal regions of the AR prevents rapid dissociation of ligand, whereas ligand

dissociates rapidly from GR where there is no N/C interaction (49). Furthermore, ligand dependent N/C interaction results in increased receptor protein stability (110). Aside from the AR and vitamin D receptor, most other nuclear receptors have decreased protein stability upon ligand binding (111-114).

The stronger of the two AF domains, the AF1, is located in the NTD. The AF1 domain has two regions which have been identified as being important for its activity, named Tau-1 (amino acids 101-360) and Tau-5 (amino acids 360-485) (115, 116). The mechanism through which AF1 is able to induce transactivation of the AR is by recruiting both coactivators as well as general transcription factors (92, 117). AF1 interacts with the general transcriptional factor, TFIIF, which has been shown to be important for assembly of the pre-initiation complex (118-120). Interaction of AF1 with coactivators such as p160 and GRIP results in enhanced transcriptional activity (121).

1.2.7 AR Coregulators

All nuclear receptors interact with proteins, termed coregulators, which regulate the receptor's ability to transactivate target genes. Coregulators positively (coactivators) or negatively (corepressors) effect gene transactivation without affecting basal transcription (122). The mechanisms through which coregulators act is through direct interaction with transcription factors or to affect chromatin structure directly or indirectly (123). AR and other steroid receptors interact with many of the same coregulators through the LXXLL motifs on the interacting proteins. One unique feature of the AR is its ability to interact with coregulators that have an FXXLF motif.

Coactivators fall into two main groups. Type I coactivators function to increase the AR occupancy of the DNA, to remodel chromatin or to recruit general transcription factors. The type II coactivators cause increased stability of the AR itself.

The best characterized group of coactivators is the steroid receptor coactivator (SRC) family and these are type 1 coactivators. Several of the SRC family members, including SRC-1 and SRC-3, have demonstrated intrinsic histone acetyl transferase (HAT) activity (124, 125). Furthermore, SRCs can recruit other coactivators to the AR transcriptional complex, such as CBP and p-CAF, which possess HAT activity (126-129). Acetylation of the histone tails by HATs causes a disruption of the nucleosomes and allows the transcriptional machinery to gain access to the location. The p300 and SRC coactivators can also recruit SWI/SNF to the AR complex (130, 131). SWI/SNF is multi-protein complex which functions in an ATP-dependent manner to remodel chromatin and allow AR to have greater access to the DNA (132, 133). Several AR coregulators such as CBP, SRC-1 and pCAF can interact directly with RNA pol II holoenzyme complex to recruit and stabilize the complex on the promoter (134-136).

As previously discussed, the interaction between the N and C termini of the AR is important for maintaining its stability, decreasing the ligand dissociation rate and for transactivation. One mechanism of coactivator action is mediation of the N/C interaction (137). The coactivators GRIP, ARA70 and CBP are able to form a bridge between the N and C termini resulting in an enhancement of AR transactivation (138-140).

There are several mechanisms through which corepressors exert their effect on the AR. Upon ligand binding, AR translocates to the nucleus where it can interact with

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DNA. Corepressors such as PAK6 and ARA67, cause the AR to be retained in then cytoplasm and therefore ablates its transactivation potential (141, 142). Like coactivators, AR corepressors can cause changes in the chromatin structure. But in the case of corepressors, it is through the recruitment of histone deacetylases (HDAC) such as TGIF (143). The HDACs have opposing actions to the HAT and cause the chromatin to become more compact and less accessible to the transcription machinery. Some corepressors bind to AR and prevent it from binding coactivators. An example of this is cyclin D inhibition of AR/pCAF interaction (144). Finally, the AR N/C interaction can be disrupted by hRad9 or filamin A, resulting in a less stable AR and decreased transactivation (145, 146).

1.2.8 AR Post Translational Modifications

The AR undergoes phosphorylation at several sites and this affects AR stability and transactivation (147-149). AKT and PKA signaling pathways, among others, have been shown to result in the phosphorylation of the AR at the specific sites. All but one of the sites on the AR are phosphorylated in response to agonist binding, with the one site constitutively phosphorylated (150, 151). AKT has been shown to directly phosphorylate AR at two sites. Paradoxically though, phosphorylation of the AR by AKT has been shown to both increase and decrease AR transactivation, depending on the context (152, 153). This is most likely due to the concentration of androgens used in the experiments; where high levels of androgens cause decreased AR activity and low levels have the reverse effect (154). PKA signaling results in an increase in AR phosphorylation and AR activity (155). Hyperphosphorylation of the AR can make it responsive to low levels or the absence of androgens (156).

The AR DBD is a target for acetylation by p300 and Tip60, which results in an increase in transcriptional activity (157, 158). Conversely deacetylation of AR by HDAC1 has the opposite effect (158). Acetylation enhances interaction with coactivators and reduces interaction with corepressors (159).

The AR can also be modified by the small ubiquitin-like molecule-1 (SUMO-1) in the hinge and NTD (160, 161). Sumoylation results in an inhibition of AR transactivation by mediating the interaction of AR with coregulators (162, 163).

Degradation of the AR occurs in the proteosome and is dependent on ubiquitination (164). Ubiquitin ligases have been identified which interact with AR including the E3 ligases MDM2 and CHIP (165-168). Interestingly, the AR also requires ubiquitination for regulation of gene expression (169). The proteosome appears to be an important factor in assembly of the AR transcriptional complex (170).

1.2.9 AR Regulated Gene Expression

Androgens, through the AR, control many important functions in the prostate including differentiation, proliferation, cell cycle and apoptosis. The AR can mediate gene expression either directly by interacting with the promoter region of the target gene, or indirectly by modulating expression of genes which can affect downstream targets. The mechanism of androgen action in prostate cells is shown in **Figure 1.5**.

Androgens are important for the differentiation of the luminal cells in the prostate. AR controls the expression of several differentiation markers such as PSA, KLK2, prostatic specific acid phosphatase and NKX3.1 (171-174). In response to androgens, the epithelial cells also express VEGF, which is an important factor in promoting angiogenesis to ensure adequate blood supply to the prostate (175, 176). In
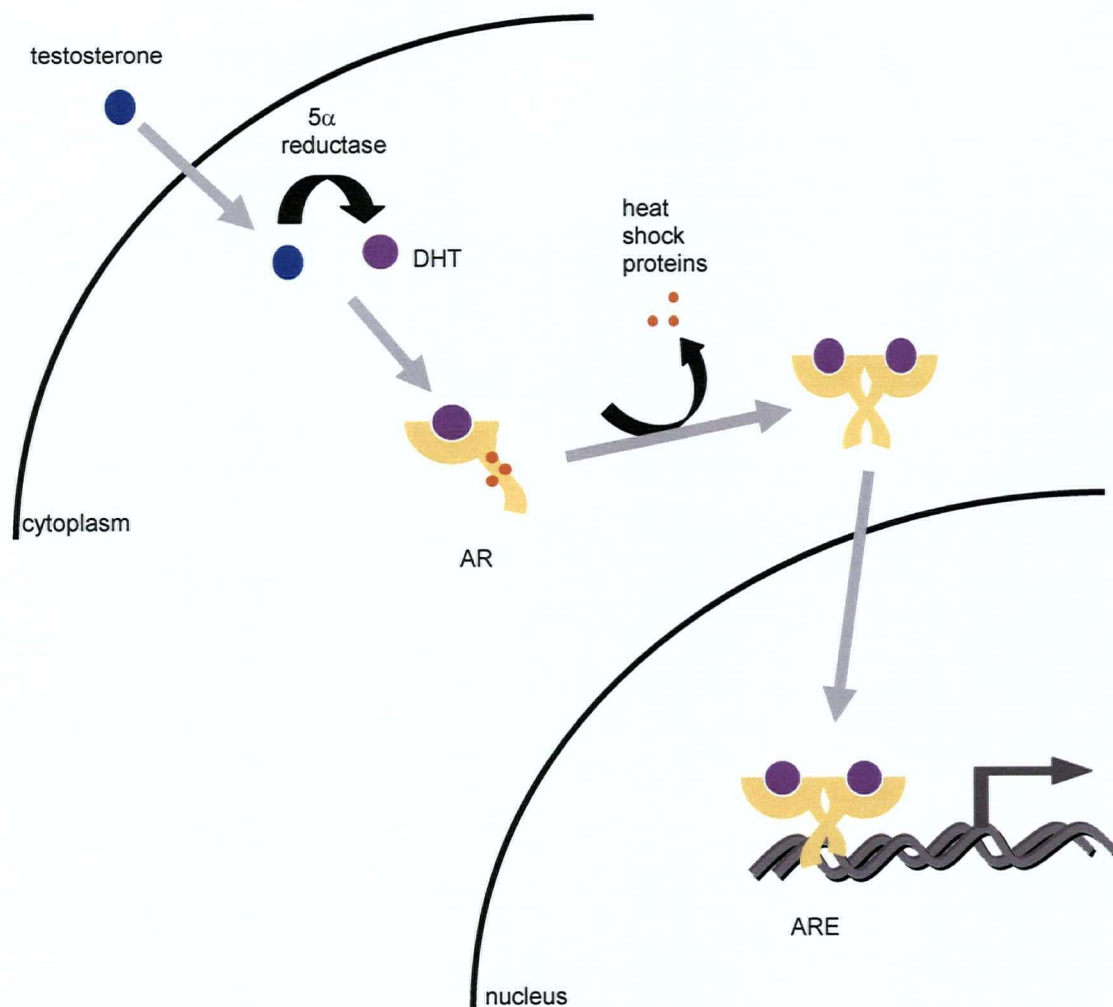
Figure 1.5 - Androgen action. Testosterone (blue circle) enters the cell and is converted to dihydrotestosterone (DHT - purple circle). The androgen receptor (AR) binds DHT and undergoes a conformational change allowing it to shed associated heat shock proteins and dimerize. The AR translocates to the nucleus and binds androgen response elements (AREs) to enhance transcription of specific genes.

the normal prostate, there is communication between the stromal cells and the epithelial cells through the excretion of androgen regulated growth factors which diffuse across the extracellular matrix. Androgens cause an induction of factors in the stromal cells such as keratinocyte growth factor (KGF), which cause an increase in cellular proliferation of the luminal cells (177, 178). Conversely, androgens inhibit TGF-B1 expression and secretion from the stromal cells, which is a negative growth factor for the epithelial cells (179).

Several large scale studies have been done to determine which genes are regulated by androgens in prostate cells. Both microarray and serial analysis of gene expression (SAGE) screens have been performed and have revealed both known and novel androgen regulated genes. The screens usually use known androgen regulated genes such as PSA as positive controls. The studies use different model systems and conditions to study androgen regulated genes and as a result, there are a surprisingly low number of genes common amongst the studies (180). What is common to the studies is that they tend to reveal certain classes of androgen regulated gene which can be functionally grouped. The genes fall into different categories involving function of the prostate as a secretory gland such as metabolism, chaperones and trafficking proteins, secretion, structural and transcriptional regulation, or into categories involving cell survival such cell cycle and regulation of apoptosis (181-183).

Androgens are important for the proliferation of prostate cells. The cell cycle is controlled by cyclins and cyclin dependent kinases (cdk), which when complexed will phosphorylate various substrates. Androgens cause an increase in several cdks, including cdk 2 and 4; as well as a decrease in cell cycle inhibitors such as p16, which

results in an increase in proliferation (184, 185). Androgens also regulate genes involved in blocking cell cycle, such as p21 (186). Therefore it is hypothesized that androgens act in a biphasic manner and under low concentrations induce proliferation and high concentrations inhibit proliferation and induce differentiation of the prostate secretory epithelium. Androgens are also important in ensuring cell survival by inhibition of apoptosis. One of the ways that androgens affect apoptosis is to regulate the expression of the Bcl-2 family, which has both apoptotic and anti-apoptotic members. Androgens induce the expression of the anti-apoptotic members, Bcl-2 and Bcl-XL, and decrease the expression levels of the pro-apoptotic members, Bik, Bax and Bak (187). Androgens also downregulate caspases 8, 3, 7, 2 and 9 (187).

Recently, a fusion protein, TMPRSS2-ERG has been discovered that is expressed in a high proportion of prostate cancer specimens (188-190). The fusion that is commonly observed in prostate cancer specimens the 5' end of the TMPRSS2 gene and the 3' end of the ERG or ETV1 gene. TMPRSS2 is expressed in the prostate and is under the control of the AR (191), therefore the fusion results in the androgen regulated expression of ERG or ETV1, which have been previously known to be overexpressed in prostate cancer (192). The ets family of genes, to which ERG and ETV1 are members, encode transcription factors that have been previously observed to be overexpressed in various types of cancers (193, 194). The TMPRSS2-ERG fusion is observed rarely in well differentiated tumors, but more often in poorly differentiated tumors (195) and is also more often observed in AI prostate cancer (196). This translocation is a novel mechanism by which AR can control the expression of a proto-oncogene.

1.3   Prostate cancer

Prostate cancer is currently the most commonly diagnosed cancer among Canadian men, with an estimated 20,700 newly diagnosed cases and approximately 4200 Canadian men will die from prostate cancer in 2006. Prostate cancer is a cancer of a glandular tissue and as such is an adenocarcinoma. The cancer generally arises in the PZ of the prostate (7). It is commonly believed that the precursor for prostate cancer is prostatic intraepithelial neoplasia (PIN) (197). In PIN, there is abnormal growth of the epithelial cells, without penetration through the basement membrane into the stroma (198). In most men, PIN will develop into prostate cancer within 10 years (198). From adenocarcinoma, approximately a third of the cases will develop into metastatic cancer, most often metastasizing to the bone, lymph and liver. Animal models, such as when PTEN is knocked out in the mouse prostate, can recapitulate this progression of prostate cancer from PIN to adenocarcinoma to metastatic tumors (199). Benign prostatic hyperplasia (BPH) can present with similar symptoms as prostate cancer, but is not thought to be a precursor for cancer (200).

The prostate luminal cells secrete PSA, some of which leaks into the bloodstream (201). The amount of PSA in the bloodstream is correlated to the number of secretory prostate cells. Furthermore, as tumors develop and begin to push through the basement membrane, there is more leakage of PSA into the bloodstream resulting in elevated serum PSA (202). Another method used to detect prostate cancer is the digital rectal exam (DRE). Smaller, tumors may not be detected by DRE and therefore it is not necessarily a good screening method for early detection. Screening blood

samples for PSA allows tumors to be detected earlier, when there is a better prognosis (203). The mortality rate for prostate cancer has been decreasing since PSA testing has become commonly used, as the cancers are detected earlier when treatment options are more effective (204, 205). One of the downfalls of PSA is the over-diagnosis of prostate cancer and this may be a problem as the treatments and their side effects can decrease the quality of life (206).

1.3.1 Risk Factors

The risk factors for prostate cancer include both genetic and environmental factors. The largest risk factor for prostate cancer is age. The incidence of prostate cancer increased after the age of 50 and is fairly uncommon in younger men (207). The early onset form of the disease is more aggressive than that later in life (208).

There is a genetic component which contributes to development of prostate cancer as there is an increase risk of prostate cancer when a first degree family member has had the disease (209, 210). Men with a relative who have early-onset prostate cancer have a greater risk of developing prostate cancer than those with relatives who developed prostate cancer later in life (211, 212). There are racial differences in the incidence of prostate cancer. In America, there is a higher incidence of prostate cancer in African Americans than in Caucasians (213). There are lower rates of prostate cancer in Europe and lower still in Asia (214). However, Asians who have migrated to America have an increased incidence of prostate cancer compared to native Asians (215), indicating that there are environmental factors impacting the development of prostate cancer.

Glutathione S-transferases (GSTs) are detoxifying enzymes which link electrophilic compounds to glutathione. GST is important for protecting DNA against oxidative stress from carcinogens. Certain polymorphisms which result in the loss of GST are associated with increased risk of prostate cancer and poor prognosis (216, 217). Loss of GST is characteristic of precancerous lesions (218). The loss of GST makes the prostate cells susceptible to DNA damage and mutations that can contribute to tumorigenesis.

There are several lines of evidence pointing to food intake affecting the risk of developing prostate cancer. Several studies have shown that obesity is a risk factor for not only developing prostate cancer, but also being associated with a more aggressive disease (219-221), however other studies have shown a decreased risk of aggressive prostate cancer in those who were obese younger in life (222, 223). There is a positive correlation between the probability of developing prostate cancer and the amount of red meat consumed (224, 225). Many dietary compounds have demonstrated some degree of protection against prostate cancer, including lycopenes, vitamin E and selenium (226-228).

It is unclear whether testosterone levels have an impact on the development of prostate cancer. Some studies have shown individuals with higher levels of circulating testosterone have an increased risk of developing prostate cancer (229, 230). Other studies, however have failed to find a correlation (231, 232). Whether or not smoking is a risk factor for the development of prostate cancer is also up for debate, as the results of epidemiological studies have been inconsistent (233, 234).

1.3.2 Gleason Grading

A grading scheme for prostate cancer was developed in the 1960s and 1970s by Dr. Donald F. Gleason, which is the gold standard of diagnosis and prognosis used today. The Gleason grade uses H&E staining of prostatic adenocarcinoma to determine the degree of differentiation of the gland and invasion into the stroma, and a grade of 1 to 5 is given (235). The drawing of the five Gleason grades is pictured in **Figure 1.6**.

Gleason grade 1 adenocarcinoma has many glands and the tumor has smooth, well defined edges, and is pushing into the stroma without invasion. A grade 2 tumor has less well defined edges than grade 1, as well as more variation in the size and shape of the glands. Gleason grade 3 tumors are moderately differentiated; they have poorly defined edges with invasion in the stroma. The glands are have a variable size and shape and are less densely packed than in the lower grade tumors. The poorly differentiated grade 4 tumors have ragged edges which infiltrate into the stroma and glands that have become fused. The highest grade, Gleason grade 5 is very poorly differentiated with ragged, infiltrating edges. Being poorly differentiated, these tumors have only a very few and often small glands.

Prostate cancer often presents as a multifocal tumor (236). In general, the two areas are scored; one is the area with the most predominant staining pattern and another with the second most prevalent pattern. The scores are combined to give a grade between 2 and 10. In general, Gleason grade 2-4 denotes a well differentiated tumor, grades 5-7, a moderately differentiated tumor and grades 8-10 denotes poor differentiation. Gleason grade is one of the best prognosticators of clinical outcome.

Gleason grading can be used to predict progression to metastatic disease, response to

therapies and survival.

PROSTATIC ADENOCARCINOMA
(Histologic Grades)
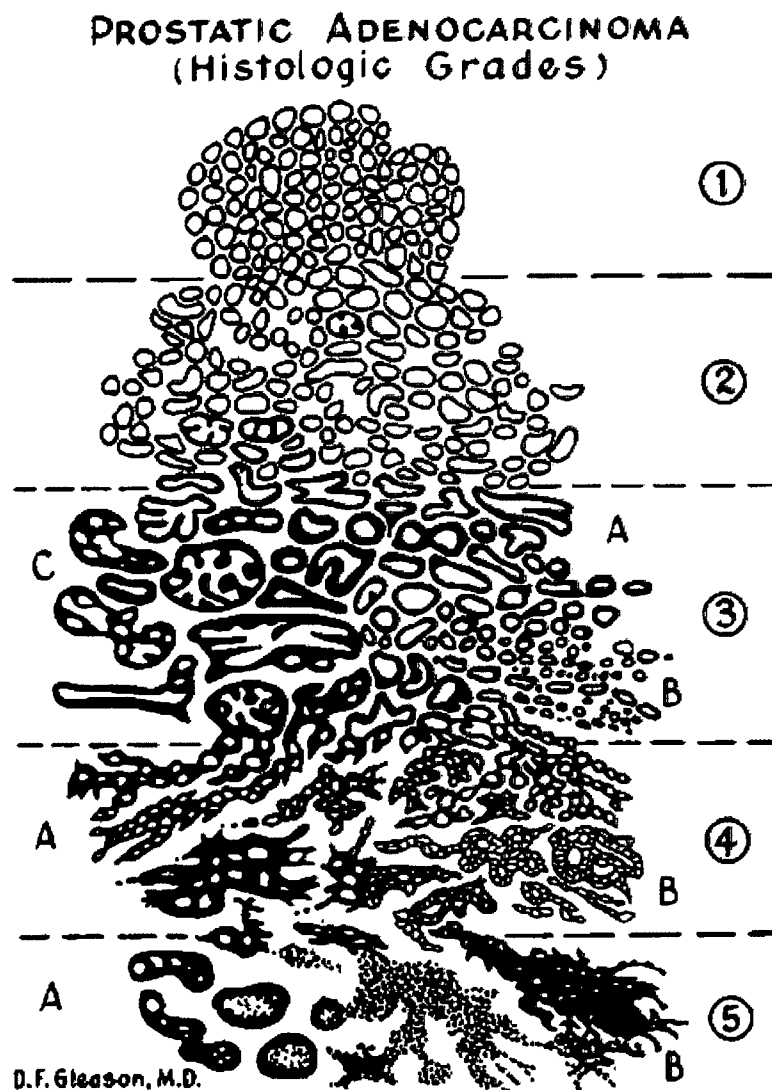
Figure 1.6 - Depiction of the histological features of Gleason grades 1 through 5, hand-drawn by Dr. Gleason. Adapted from Humphrey (235).

1.3.3 Androgen Ablation Therapy

Much like normal prostate cells, cancerous prostate cells are initially dependent on androgens for growth and survival. The first indication that prostate cancer was dependent on androgens was reported by Huggins and Hodges in 1941 (237). They reported that castration caused a reduction in prostate cancer while testosterone supplementation resulted in prostate cancer growth (238). For his pioneering work identifying the importance of androgens to prostate cancer growth, Huggins received the Nobel Prize in 1966 (239). The prostate tumors grow by taking advantage of the same androgen-regulated pathways which allow normal prostate cells to proliferate and evade apoptosis.

The dependence of prostate cancer on AR signaling has been used to develop therapies which disrupt it. The two main avenues that hormone therapy function is through physiological disruption of androgen synthesis or to directly disrupt the action of the AR. The importance of AR in both androgen dependent and independent cancers is also demonstrated *in vitro* by targeted knockdown of AR using siRNA, which causes growth inhibition and apoptosis (240, 241).

Physical and chemical castration have both been long shown to have positive results on prostate cancer. Physical castration is a simple and low risk surgery, but is not commonly used of late due to psychological side effects after the procedure (242). LHRH agonists such as leuprolide or goserelin have been used to cause castrate levels of serum testosterone. LHRH agonists will cause an increase in LH which will cause an initial surge in testosterone. However after three weeks, constant stimulation with LHRH causes a decrease in its receptors and results in levels of testosterone

similar to that achieved with physical castration (243). LHRH agonists achieve a decrease in prostate cancer mortality by approximately one third (244).

Castration reduces the levels of testicular androgens, however there are still circulating adrenal androgens which will have some activity in the prostate. Ketoconazole is an inhibitor of cytochrome P-450 and effectively suppresses the production of adrenal androgens. Ketoconazole is successful in decreasing PSA levels in patients alone or in combination with other therapies (245).

Another method of reducing androgen signaling in the prostate is to directly inhibit DHT from binding to the AR using antagonists or to inhibit 5α-reductase activity so that DHT production is limited. AR antagonists bind to the receptor, but hold it in a conformation which prevents its transactivation. Some AR antagonists such as cyprotenone acetate are steroidal hormone derivatives, while others, like bicalutamide and flutamide, are non-steroidal (246). Antiandrogens are often used in combination with castration or LHRH agonists, resulting in a higher rate of survival (247). 5α-reductase inhibitors, such as finasteride, prevent testosterone from being converted to DHT. These inhibitors cause a decrease in prostate cancer mortality when used in combination with other therapies and are potentially useful as a preventative agents (248, 249).

Other hormones or their derivatives have therapeutic use in the treatment of prostate cancer. Estrogenic compounds cause a decrease in LH release from the pituitary and also have an apoptotic effect on prostate cells (250). The synthetic estrogen, diethylstilbestrol (DES) has long been used as a therapy for prostate cancer. Despite the low cost of synthesis, cardiovascular complications are a main deterrent

for its use (24). A herbal supplement called PCSPES was initially shown to have positive effects on prostate cancer, but it was later discovered that some lots were contaminated with DES (251).

A novel course of treatment for prostate cancer was developed called intermittent androgen suppression (IAS) therapy. In IAS, there are cycles of androgen ablation therapy interspersed with periods of no treatment. The theory behind IAS is that the periods of off-treatment keep the surviving cells in a differentiated state where they remain susceptible to the androgen ablation therapy (252, 253). Studies have shown a trend of IAS therapy delaying progression of the disease (254). Other advantages of IAS over constant androgen ablation are that the off-treatment periods result in lower cost of treatment and a reprieve from the adverse side effects (255, 256).

1.4 Androgen Independence

Androgen ablation therapy, while initially successful, begins to fail after a median time of 24 months, a situation termed androgen independence (AI) (257). As the prostate tumor begins to grow at AI, the PSA levels also begin to rise. AI is resistant to traditional therapies and tends to be fatal, with a median survival time of approximately 16 months (258).

The mechanism by which an androgen dependent tumor loses its reliance on androgens is not well understood. There are two theories on how androgen independent tumors arise from androgen dependent tumors, clonal selection and adaptation, and there is evidence to support both theories (259, 260). As discussed

previously, the prostate contains several different types of cells, some androgen dependent and some androgen independent. In the clonal selection theory, androgen ablation causes the androgen dependent cells to undergo an apoptotic response, but selects for growth of the androgen independent cells that already exist within the population. In the adaptation theory, hormone therapy causes regression of the tumor, however some cells adapt to survive in the androgen depleted environment through genetic alterations. It is likely that there are aspects of both clonal selection and adaptation in the development of AI.

One of the important aspects of AI is that there is a recurrence of AR signaling. This points to the prospect that the AR is important in development of AI. There are several mechanisms through which the AR can become reactivated, as well as some non-AR related mechanisms to achieve AI.

1.4.1 AR Amplification

Several studies have looked at AR gene amplification in clinical AI samples and found the gene to be amplified in approximately 20-30% of the cases (261-264). Importantly, AR amplification found in AI tumors is not found in the same patients prior to androgen ablation treatment (263, 264). This is suggestive that AR amplification is an adaptive response to androgen ablation. Amplification of the AR gene results in higher levels of AR protein, but there was also AR overexpression found in AI tumors which had no gene amplification (265). AR overexpression may contribute to tumor growth at distal sites, as AR gene amplification was found in half the bony metastases tested (266). Increased levels of AR may allow prostate tumors to be more sensitive to

the lower levels of androgens present in androgen ablation therapy. The AR gene activation appears to result in an increase in functional AR, as there is an increase in PSA expression (267). Patients with amplified AR genes responded favorably to a combined androgen ablation therapy (antiandrogens in conjunction with LHRH agonists), showing that a functional AR continues to be important for tumor survival (268, 269).

1.4.2 Hypersensitivity to Androgens

Castrate levels of androgens are not normally sufficient to cause significant transactivation of the AR, however the AI tumors may have adapted such that they require much lower levels of androgens. *In vitro* cultures of AI prostate cells require levels of androgens that are four orders of magnitude less than androgen dependent (AD) prostate cells for proliferation (270). Furthermore, the AR was more stable and primarily nuclear in the AI cells as compared to the AD cells (270). While castration significantly decreases circulating testosterone, there are still adrenal androgens being produced. Adrenal androgens are poorer ligands for the AR compared to DHT, however AI prostate cells may adapt to the new hormonal milieu. There is an increase in the levels of enzymes converting adrenal androgens to testosterone and DHT in some hormone refractory tumors (271, 272). Prostate cells may therefore be able to increase the intracellular levels of androgens.

1.4.3 AR Mutations

There have been mutations described in the LBD of the AR which decrease its specificity, making it able to bind ligands it would not normally bind to. There is a mutational hot spot at codon 877, which resides in helix 11 of the LBD, and the

preferred mutation T877A, causes increased flexibility of the ligand binding pocket (273). These mutated ARs have demonstrated the ability to bind to progesterone, estrogens, adrenal androgens and cortisone (273-276). Furthermore, mutations in the LBD of the AR confer the ability of some ligands such as flutamide, which normally act as antagonists, to become agonists (277). These AR LBD mutations explain a phenomenon called "antiandrogen withdrawal syndrome" whereby patients undergoing combined hormone ablation therapy have a decrease in PSA when the antiandrogen is removed from their treatment regimen (278). Mutations in the LBD have also been described that have increased interaction with coactivators, while other mutations in the LBD result in nuclear accumulation of the AR (279, 280). Both these mutations result in a more transcriptionally active AR. AR mutations are rare in early stage tumors, but become more frequent in metastatic, AI tumors (281, 282). Mutations in regions other than the LBD in prostate cancer are rare, however there have also been AR mutations found in the hinge, N-terminal and DBD regions. A mutation found in the hinge region in a patient resulted in a truncated and constituatively active AR (283). Another patient was found to have a mutation in the DBD which resulted in an increase in AR transactivation at non-specific promoters regions (284).

1.4.4 AR Coregulators

Coregulators have a large impact on AR transactivation and therefore the levels of coregulators as the tumor progresses to AI may impact the ability of the AR to function in low levels of androgens. AR coactivators are able to increase AR transactivation in the presence of antiandrogens and adrenal androgens (285). Coactivators, such as cdc25B and CARM, are overexpressed in some prostate tumors

(286, 287). High levels of the AR coactivator p300 is predictive of larger tumors and progression to AI (288). Upregulation of the coactivators SRC-1, TIF-2 and GAK is observed in hormone refractory tumors (289, 290). Since corepressors have a dampening effect on AR activity, it is likely that there are cases in which a downregulation of AR corepressors can contribute to progression to AI; however none have been documented to date. The implications of overexpression of coregulators would be expected to be quite broad.

1.4.5 AR Activation by Growth Factors

Several growth factors have been shown to activate the AR in the absence of ligand, including insulin-like growth-factor-1 (IGF-1), KGF and epidermal growth factor (EGF). Whether the signaling cascades activate the AR directly or if the activation is a downstream effect is unknown. It is clear, however that a functional AR is needed, as the AR antagonist bicalutamide, blocks the stimulatory effects of these growth factors (291).

Of the growth factors discussed here, IGF-1 is the most potent stimulator of AR, with a five-fold increase in transactivation (291). IGF-1 binds to its receptor (IGF-1R) resulting in an activation of phosphorylation cascades, including PI3K (292). The ability of IGF-1 to interact with its receptor can be positively or negatively impacted by IGF binding proteins (IGFBPs). There is no significant change in the levels of IGF or its receptor in response to castration, however there is significant changes in the levels of some IGFBPs. IGFBP-2 and 5, which are activators of IGF-1 signaling, are increased post castration, while an inhibitor of IGF-1 signaling, IGFBP-3, is decreased (293-295). Increased signaling through the IGF-1 pathway may be a method through which

prostate cells adapt to a hormone depleted environment to retain AR signaling and

progress to AI. A demonstration of the importance of IGFBP-2 and 5 to the progression

of prostate cancer to AI is that their downregulation with antisense oligonucleotides

causes a delay in the progression to AI and their overexpression accelerates

progression (295-297).

Signaling through HER-2/neu, a member of the EGF-receptor family is another

route through which AR can be activated in the absence of ligand. HER-2/neu is found

to be overexpressed in AI cells lines as well as some AI tumors from patients (298,

299). Overexpression of HER-2/neu can induce ligand independent activation of AR

transcriptional activity (298). HER-2/neu signals though the MAPK pathway, and

MAPK inhibitors can abrogate its ability to activate AR. Treatment with herceptrin, an

antibody directed against HER-2/neu, in combination with taxol has antiproliferative

effects in androgen independent tumor models (300). HER-2/neu antibodies have not

been shown to be effective as a monotherapy for AI prostate cancer in clinical trials,

but may have increased efficacy when used in combination with other therapies (301,

302).

1.4.6 AKT pathway

PTEN is a phosphatase which dephosphorylates 3-phosphorylated inositol

lipids, resulting in the inactivation of the anti-apoptotic AKT pathway. PTEN is

frequently inactivated in AI tumor progression, resulting in increased AKT signaling

(154, 303). Overexpression of AKT can lead to AI progression in tumor models (304).

AKT can phosphorylate the AR and enable it to be activated in the absence of ligand,

and expression of a dominant negative AKT blocks AR signaling (153). The AKT

pathway can be activated by HER-2/neu, and therefore an increase in HER-2/neu expression may be another route through which AKT can become activated in AI tumors and for the AR to be activated in the absence of ligand (153).

1.4.7 Non-AR Related Pathways

Although the AR has been shown to be involved in prostate cancer progression to AI, it is possible that there are routes to AI that are not dependent on AR. Most notably, overexpression of anti-apoptotic pathways could allow the normally androgen dependent cells to evade death in the androgen depleted milieu. The anti-apoptotic gene Bcl-2 is often overexpressed in AI prostate cancer, while it is not expressed in normal luminal prostate cells (305). In some cases, the overexpression of Bcl-2 is a result of gene amplification (306). In xenograft tumor systems, Bcl-2 overexpressing cells were protected from castration induced apoptosis (307). Downregulation of bcl-2 causes a delay in AI progression of tumor models (308-310). Another anti-apoptotic Bcl family member, Bcl-xL is also overexpressed in AI prostate tumors (311). Targeting Bcl-xL with antisense oligonucleotides results in an elevated chemosensitivity and decreased proliferation (312). There is enhanced chemosensitivity in AI prostate cells by targeting both Bcl-2 and Bcl-xL with a bi-specific antisense oligonucleotide (313, 314).

Other anti-apoptotic proteins that are overexpressed in AI prostate cancer include HSP27 and clusterin (315-317). HSP27 is a molecular chaperone which has been shown to be cytoprotective in prostate cells (315). Targeting HSP27 for downregulation makes prostate cancer cells more sensitive to chemotherapeutics and as such, is currently being tested as a therapeutic target (318, 319). Clusterin is a

multi-functional protein which has been shown to have both pro- and anti-apoptotic functions. Overexpression of clusterin in prostate cells results in chemotherapeutic resistance (317). Conversely, targeting the apoptotic form of clusterin has shown to be an effective therapy in AI prostate cancer in mouse xenograft model systems and is currently being tested in clinical trials (320-322).

1.5 LNCaP Cell Line and Tumor Model

There are three commonly used cell lines in prostate cancer research - LNCaP, PC3 and DU145. The cell line used for the majority of the experiments herein is LNCaP. The LNCaP cell line was established from a lymph node metastasis, biopsied from a 50 year old Caucasian man (323). LNCaP cells display a high degree of aneuploidy, with the chromosome number ranging from 33 to 91 (324). LNCaP cells express AR and are androgen dependent. The AR in LNCaP cells bears the T877A mutation in its ligand binding domain making it promiscuous. With the mutated AR, LNCaPs are responsive to adrenal androgens, progesterones and estrogens (325-327). Furthermore, flutamide which is normally an AR antagonist, acts as an agonist to the mutated AR in LNCaP cells (328). Despite the mutated AR, LNCaP cells are the preferred system for studying androgen action *in vitro* as the other two commonly used cell lines do not express AR.

The PC3 prostate cancer cell line was established from the lumbar vertebra of a 62 year old Caucasian man (324). PC3 cells are androgen independent and express little or no androgen receptor. Being androgen independent, they are able to form tumors in both male and female mice.

34

The first prostate cell line to be established in culture was DU-145. The cell line is derived from brain lesion of a 69 year old, Caucasian man who suffered from both prostate cancer and lymphocytic leukemia (323). DU-145 cells are moderately differentiated cells, which express some epithelial cell markers. Importantly, DU-145 cells do not express AR, due to promoter methylation, and are thereby androgen independent (329).

LNCaP cells can form tumors subcutaneously in nude mice when co-inoculated with matrigel (330). The tumors grow and secrete PSA into the bloodstream in proportion to the tumor load. Upon castration, there is a drop in PSA levels; however there is little regression of the tumor itself. LNCaP tumors become androgen independent, there is an increase in proliferation and the serum PSA levels rise (331). LNCaP tumors grown subcutaneously do not metastasize, however intraprostatic inoculation in mice does result in metastasis to the lung and lymph nodes (332).

1.6 Scope of Thesis

1.6.1 Hypothesis

The AR is central to growth and survival of prostate cancer and to the development of AI prostate cancer. Genes regulated by the AR are likely to be involved in pathways essential for proliferation and evasion of apoptosis, and as such may prove to be potential targets at which to direct therapeutics.

1.6.2 Specific aims

The first aim of this work is to identify androgen regulated genes in LNCaP cells using an mRNA microarray screen.

The second aim is to characterize the functional significance of the genes identified in the first aim and to gauge their potential as therapeutic targets.

To complete the first aim of the project (**Chapter 2**), LNCaP cells were treated with androgens for several different time points and the resulting RNA used for microarray experiments to screen for genes both up and downregulated by androgens. Many genes were found to be dysregulated by androgen treatment including many genes previously shown to be androgen regulated. Three genes picked up from the arrays were validated, two upregulated genes (GADD45G and MRP-4), as well as one downregulated gene (BARD1). Furthermore, networks of coordinately androgen regulated genes were identified, and the NFE2L2 network of genes involved in the protection against oxidative stress was validated.

In **Chapter 3**, the importance of GADD45G expression to prostate cancer cells was established. The levels of GADD45G protein in patient samples being treated with hormone therapy were determined. GADD45G has been shown to interact with and activate MEKK4 and affect p38 signaling. To determine the signaling pathways which are dependent on GADD45G, we knocked down the protein levels of GADD45G and looked for dysregulation of signaling cascades. Many pathways which are important for cell survival were identified, including AKT, p53 and Rb. Since GADD45G seems to have an important role in controlling cell survival, we conclude that GADD45G may be an attractive target at which to direct therapeutics.

In **Chapter 4**, the regulation of GADD45G by androgens was further explored. It was discovered that the protein levels of GADD45G were downregulated by androgens, while the mRNA levels were increasing. An androgen regulated miRNA

which targets GADD45G and prevents its translation was identified. The translational inhibition could be reversed by taxol treatment. It is likely that GADD45G mRNA is being stored in the cell in the event that a stress on the cell, such as taxol, signals that it is needed.

In **Chapter 5**, the androgen regulation of clusterin and two of its transcriptional isoforms is described. The transcription of clusterin was originally thought to be downregulated by androgens, but we identified the transcript as being positively regulated by androgens. Upon further inspection we found that there are two transcripts of clusterin made from different transcriptional initiation sites and they are differentially regulated by androgens. The importance of this is that Isoform 1 has the potential to be spliced into a pro-apoptotic form and is downregulated by androgens. Isoform 2 is only capable of making an anti-apoptotic protein and is upregulated by androgens. Clusterin has already been used as a therapeutic target in prostate cancer, and here we show the importance of targeting the anti-apoptotic isoform.

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Chapter 2. Expression Profiling of Androgen Regulated Genes in LNCaP Cells Using Microarrays

2.1 Introduction

Androgens, such as testosterone, play a vital role to the development and maintenance of the prostate as well as for the development of prostate cancer (1, 2). Prostate tumors are also initially dependent on androgens for growth and survival. Removal of androgens through chemical or physical castration is initially an effective therapy against prostate cancer, however the cancer will recur in an androgen independent (AI) form, which is refractory to conventional therapies and is generally lethal (3). Prostate cancer is currently the most diagnosed cancer and has been the second leading cause of cancer deaths in Canadian men.

The androgen receptor (AR) is a ligand activated transcription factor which interacts with specific DNA sequences in response to androgen binding and promotes transcription of various genes (4). Androgen regulated genes include both those important for the normal function of the prostate, such as the prostate specific antigen (PSA), as well as genes involved in cell survival such as Bcl-2 (5, 6). AR remains important as prostate cancer progresses to AI where there is a recurrence of AR regulated gene expression (7).

Since the androgen receptor is central to prostate cancer and its progression, we sought to identify and characterize novel androgen regulated genes, which may be important for prostate cancer progression. The two most commonly used techniques for gene expression profiling are serial analysis of gene expression (SAGE) and microarrays. SAGE can allow for gene discovery as well as quantitative expression

profiling (8). However, SAGE can be cost prohibitive as well as not being as high throughput as microarrays and technically unable to detect between 10-20% of genes (9). Up to 40,000 oligonucleotide spots can be printed onto microarray slides and they are amenable to high throughput screening of mRNA expression levels in a cost and time effective manner. The microarray platform also includes many applications other than simple transcriptional profiling such ChIP on chip, SNP chips, array CGH and protein chips among others (10). Thereby microarray based expression analysis was the method of our choice for these studies,

The LNCaP prostate cell line is a moderately differentiated, androgen dependent cell line often used as a model of prostate cancer and which has also been used to study androgen regulated gene expression in prostate cells (11). We treated LNCaP cells with the synthetic androgen, R1881, for three time points and microarrays were used to determine which genes are differentially regulated by androgens in prostate cancer cells. Two approaches were undertaken - looking at single genes which are androgen regulated and looking at networks of androgen regulated genes. GADD45G, a gene involved in apoptosis and cell cycle progression, and MRP4, which is involved in chemotherapeutic drug resistance were found to be highly upregulated in response to androgens. The pro-apoptotic gene BARD1 was downregulated by androgens. Ingenuity software was used to query biological pathways which are coordinately regulated by androgens. The transcription factor NFE2L2 was upregulated with androgens as well as its downstream genes, all involved in protecting the cell against oxidative stress.

2.2 Materials and Methods

Cell Culture - LNCaP cells, passage 35-50, were grown at 37°C and 5% CO_2 in RPMI containing 5% fetal bovine serum (FBS), 50 U penicillin and 50 µg streptomycin. Charcoal stripped serum (CSS) was prepared using FBS rotated with 1% charcoal and 0.1% dextran T-70 at 4°C for 30 minutes. The solution was centrifuged at 1000 rpm for 2 minutes and the supernatant saved. The process was repeated twice and the serum filtered through a Steriflip column (0.22 µm membrane, Millipore).

Hormone Treatments - R1881 was purchased from Steraloids Inc. and stored as a stock solution in absolute ethanol. Prior to any hormone treatments, cells were grown in RPMI + 2% CSS for 48 hours. The R1881 was diluted first into 80% ethanol, then into 20% ethanol before being added to RPMI + 2% CSS and put onto the cells. The concentration of R1881 was 0.01 nM to 10 nM, as indicated.

siRNA Transfections - LNCaP cells were grown to 60% confluency before being transfected with 50 nM NFE2L3 siRNA (Ambion). Lipofectamine 2000 (Invitrogen) was added to serum free RPMI (1:50 ratio) and incubated at room temperature for 5 minutes. The lipofectamine 2000/RPMI mix was then added to an equal volume of serum free RPMI containing the negative control siRNA or NFE2L2 siRNA and allowed to incubate at room temperature for 20 minutes. The siRNAs were added to the cells to a final concentration of 50 nM. As a negative control, some cells were treated with lipofectamine 2000 only. The cells were incubated at 37°C for 4 hours before replacement of the FBS to 5%. When done in conjunction with androgen treatment, the cells were grown in RPMI + 2% CSS for 24 hours prior to siRNA treatment. After the 4 hour incubation with the siRNA, the CSS was replaced to 2%. After 24 hours, 1 nM

R1881 or an equal volume of 20 % EtOH was added. The RNA was harvested 24 hours after the androgen treatment.

Preparation of RNA - RNA was harvested from the cells using Trizol (Invitrogen), as per manufacturer's instructions. Briefly, the cell pellet was dissolved in 1 mL Trizol by pipeting and 200 µL chloroform was added and the tube shaken. The phases were separated using centrifugation (10,000 x g, 15 minutes). The aqueous (top) phase was removed to a new tube and 500 µL of isopropanol added. The RNA was precipitated with centrifugation at 10,000 x g for 10 minutes. The pellet was washed with 75% ethanol made in DEPC-H_2O, and centrifuged at 7500 x g for 5 minutes. The ethanol was aspirated off, the pellet dried and resuspended in DEPC-H_2O. The concentration of the RNA was determined using a spectrophotometer (Nano Technologies).

Microarrays - To generate labeled cDNA, 20 µg RNA was reverse transcribed in a reaction containing 200 U Superscript II reverse transcriptase (Life Technologies), 1X reaction buffer, 1 mM DTT, 3.75 µM anchor T primer, 0.5 µM dATP, dCTP and dGTP, 0.05 µM dTTP, and 2.5 µM Cy3 or Cy5 dUTP (Amersham). The reaction proceeded for 3 hours after which the RNA was hydrolysed with NaOH. The probe was purified using a PCR purification kit (Qiagen) followed by an ethanol precipitation. The probe was resuspended in hybridization buffer (50% formamide, 5 X SSC, 0.1 % SDS, 0.5 µg/µl poly dA, 0.5 µg/µl yeast tRNA and 1 µg/µl salmon testes DNA) and denatured at 65°C for 5 minutes. The arrays were printed in-house and contained 13,971 spotted 70-mer oligonucleotides (Operon). The probe was then added to a microarray and a cover slip applied. Hybridization was done in a humidified chamber at 42°C overnight. GFP PCR product was labeled with Cy3 or Cy5 using an oligolabeling kit (Amersham).

Labeled GFP was spiked into the reaction after the probe purification step. GFP cDNA was printed on the corners of all the subgrids on every microarray, and was used as an orientation marker. Once hybridized, the microarrays were scanned at a resolution of 10 μm on the Perkin Elmer ScanArray Express scanner. The resulting TIF images were quantified using Imagene software (Biodiscovery). The biological and technical replicates were averaged and the data was normalized using lowess in the GeneSpring software package. Tree diagram was generated using a standard correlation. The normalized data was loaded into the web-based Ingenuity software which generates biological networks from the differentially regulated genes.

Northern Blot Analysis - Total RNA (15 μg) was denatured in sample buffer (4 μM MOPS, I μM Na acetate, 42% formamide, 4.4% formaldehyde) and run through a denaturing 1% MOPS formaldehyde agarose gel (0.04 mM MOPS, 0.01 mM Na acetate, 1mM EDTA, 5.3% formaldehyde, 1% agarose) for 1 hour at 80 V. The RNA was transferred to a nylon membrane (Biodyne B, Pall Gelman Laboratory) in 20x SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 20 hours. The RNA was crosslinked to the membrane using a UV Stratalinker (Stratagene) according to manufacturer's instructions. Membranes were prehybridized in ExpressHyb (Clontech) containing 0.1 mg/ml denatured salmon testes DNA (Sigma) for 3 hours at 65°C. The Northern blot probes were generated by PCR amplification from LNCaP cDNA using the following primers: NFE2L2 forward 5′-CACCGCTCATCATGATGGACTTGG-3′ and reverse 5′-CTAGTTTTTCTTAACATCTGGCTT-3′; GCLM forward 5′-TCAGTCCTTGGAGTTGCACA-3′ and reverse 5′-CCCTGATACCTAGGCCAAAA-3′; NQO1 forward 5′-GCACTGATCGTACTGGCTCA-3′ and reverse 5′-

CGGAAGGGTCCTTTGTCATA-3 ; PGD forward 5 -ATGCCCTGTTTTACCACTGC-3

and reverse 5 -TTCAGCCGCATGATACAGAG-3 ; PRDX1 forward 5 -

TTGAACCCCAAGCTGATAGG-3 and reverse 5 -TCCCCATGTTTGTCAGTGAA-3 ;

TXN forward 5 -GCCAAGATGGTGAAGCAGAT-3 and reverse 5 -

GCAGATGGCAACTGGGTTTA-3 . Radioactivity was incorporated into the probe using

Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech) to a specific

activity of 1-2x10^8 dpm/μg. Probes were hybridized to the membrane overnight at 65°C

in hybridization buffer containing salmon testes DNA. The membranes were washed 4

times in Wash Buffer I (2 x SSC, 0.5% SDS) for 30 minutes each and twice in Wash

Buffer II (0.1 x SSC, 0.1% SDS) for 30 minutes each, at 65°C. The membranes were

sealed in plastic and exposed to a phosphorimager screen for 24-72 hours. The

screens were scanned on a Typhoon Scanner (GE Healthcare) at a resolution of 100

μm.

Real Time PCR - Real time PCR was used to quantify the mRNA levels of NFE2L2 in

the siRNA experiments. Before generating the cDNA, 2 ug of the RNA was treated with

DNAse1 (1 U, Invitrogen) for 15 minutes at room temperature to remove any DNA

contamination. The DNase 1 was inactivated by adding 10 mM EDTA and heating the

sample to 65°C for 10 minutes before cooling on ice. The RNA was reverse

transcribed into cDNA in a reaction containing 1X reaction buffer, 0.01 M DTT, 1 mM

dNTPs, 40 U RNAsin (Promega), 250 ng random hexamers and 200 U of MMLV

(Invitrogen). The reaction proceeded at 25°C for 10 minutes, then at 37°C for one

hour. The enzyme was heat denatured at 95°C for 5 minutes. The levels of NFE2L2

transcript were then assayed using real time PCR on the ABI 7900 HT Sequence

Detection System. The primers and probes used to amplify NFE2L2 are as follows: forward 5 –ACTCCCAGGTTGCCCACAT-3 , reverse 5 -TGCGCCAAAAGCTGCAT-3 and probe 5 -[6 FAM] TCAGATGCTTTGTACTTTGATGACTG[TAMRA-Q]-3 . For normalization purposes, rRNA control primers and probes (ABI) were used. The cycle used for the real time PCR was as follows: Step 1 – 50°C for 2 min; Step 2 – 95°C for 10 min; Step 3 – 95°C for 15 sec, 60°C for 1 min. Step 3 were repeated 39 times. The reported values are the averages and the error bars are the standard error on the mean of three biological replicates.

LNCaP xenografts – 2×10^6 cultured LNCaP cells were combined with Matrigel (Becton Dickinson Labware) at a 50:50 volume ratio of cells to matrigel and injected subcutaneously into four sites of 6-8 week old athymic nude mice (BALB/c strain; Charles River Laboratory). The mice were surgically castrated 8 weeks post injection under methoxycluorane anesthesia. To harvest the tumors, the mice were sacrificed using carbon dioxide asphyxiation, the tumors were removed and frozen immediately at -80°C. Tumors were harvested prior to castration or at 7, 14, 21, 28 or 35 days post-castration.

2.3   Results

Many genes are regulated in prostate cells treated with androgens - To determine which genes are regulated in androgen treated cells, a set of microarrays were probed with cDNA generated from LNCaP cells treated with androgens for 24, 48 or 72 hours. The microarray results were analyzed using GeneSpring software. **Figure 2.1A** shows a line graph of the expression levels of the over 13,000 genes represented on the arrays. Green indicates downregulation and red indicates upregulation in the

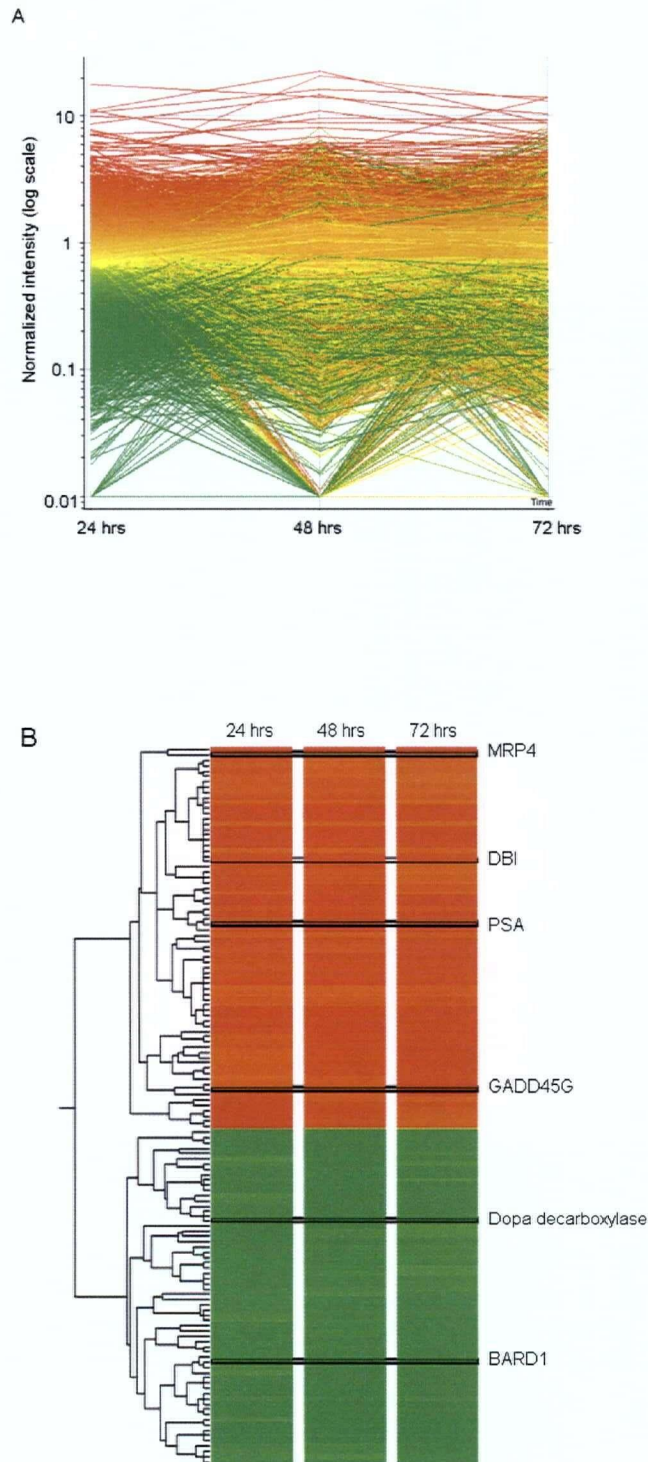**Figure 2.1 Differential gene expression in prostate cells in response to androgen treatment.** *A*, LNCaP cells were treated with 1 nM R1881 or ethanol vehicle alone for 24, 48 or 72 hours. From the treated cells, labeled cDNA was generated and used to probe microarrays containing 13,971 spotted oligonucleotides. A line graph showing the expression levels of all genes represented on the microarray for each of the three

time points. The data at each time point represents the average of two biological and two technical replicates. Green indicates upregulation, red downregulation and yellow represents no change in the androgen treated cells as compared to the ethanol treated cells. *B*, Heat map of the most differentially regulated genes in the androgen treated cells. The data was filtered to remove genes that had low or absent expression levels. Shown are 88 genes upregulated two fold or more and 64 downregulated three fold or more in the presence of androgens. Genes known to be differentially regulated by androgens are highlighted (DBI, PSA and dopa decarboxylase) as well as three genes of interest (GADD45G, MRP4 and BARD1).

R1881 treated cells versus the ethanol vehicle control. There were 88 genes found to be upregulated by androgens by at least 2 fold at all three time points and there were 64 genes downregulated by 3 fold or more. A complete list of these genes appears in **Appendix 1**. The regulated genes are shown in **Figure 2.1B**, with several important genes highlighted. Genes known to be upregulated by androgens were found to be upregulated in our arrays including PSA, diazepam binding inhibitor (DBI), ornithine decarboxylase, and fatty acid synthase, (5, 12-14) as well as several prostate specific proteins such as the prostate epithelium specific Ets transcription factor and the prostate differentiation factor. Genes downregulated by androgens are less well characterized than upregulated ones, however dopa decarboxylase has been consistently downregulated by androgens in microarrays performed in our lab and others (15); and is seen to be downregulated here. Several novel genes were found to be regulated by androgens. To validate the expression patterns observed in the microarray experiment, Northern blotting was used to confirm the mRNA expression levels. Furthermore, the expression levels of these genes in AI prostate cancer progression were determined using the LNCaP xenograft tumor model.

GADD45G and MRP4 are upregulated by androgens in LNCaP cells – Two of

the genes which were found to be upregulated by androgens in LNCaP cells, GADD45G and MRP4, were validated using northern blotting to confirm the expression patterns. Since there appears to be a recurrence of androgen regulated gene expression in AI prostate cancer, we were interested in determining whether or not the genes found to be upregulated *in vitro* were also upregulated in the LNCaP tumor progression model. LNCaP tumors are implanted subcutaneously in male nude mice. As the tumors grow, PSA is secreted into the vasculature and used to track the progression of tumor growth. Upon castration there is a decrease in androgen regulated gene expression as demonstrated by the marked decrease in serum PSA levels. The serum PSA levels begin to increase as the tumor progresses to AI, after approximately 21 days.

Of the androgen upregulated genes we were interested in was GADD45G because it has been described as a protein which is involved in stress response and has also been implicated in protection against apoptosis (16) and therefore may be important for prostate cancer progression to AI. **Figure 2.2A** shows that GADD45G mRNA levels are increased in LNCaP cells treated with androgens for 16 hours or longer. Furthermore, GADD45G acts similarly to other androgen regulated genes we have studied in the LNCaP tumor progression model, as its levels are high in both the precastrate and the AI tumors (**Figure 2.2B**).

MRP4 is a member of the ABC transporter family of proteins which are membrane transporters able to confer multidrug resistance to cells (17). Resistance to chemotherapeutics is common in androgen independent cells and therefore MRP4 was of interest. MRP-4 is strongly induced in LNCaP cells in response to androgen

Figure 2.2 GADD45G is an androgen regulated gene in LNCaP cells. LNCaP cells were treated with 1 nM R1881 or ethanol vehicle alone for 16, 24, 48 or 72 hours and the resulting mRNA run on a Northern blot and probed for GADD45G (panel A). The Northern blot of the timecourse was repeated with three independent biological replicates, with similar results. LNCaP cells were injected subcutaneously into nude mice and allowed to form tumors. The mice were castrated and tumors harvested directly prior to castration and at various time points after castration. *B*, Northern blot of the LNCaP xenograft tumor series probed for GADD45G. The two samples shown for each time point arise from tumors on different mice.

treatment from 16 hours onward (**Figure 2.3A**). Further evidence for MRP4 being androgen regulated, is that it has high levels in the LNCaP xenografts prior to castration, dropping substantially after castration, followed by an increase at AI (**Figure 2.3B**).

BARD1 is downregulated by androgens in LNCaP cells - BARD1 (BRCA1-associated ring domain protein) is thought to be a pro-apoptotic protein which interacts with BRCA1 (18). Some pro-apoptotic genes have been shown to be downregulated

A

B
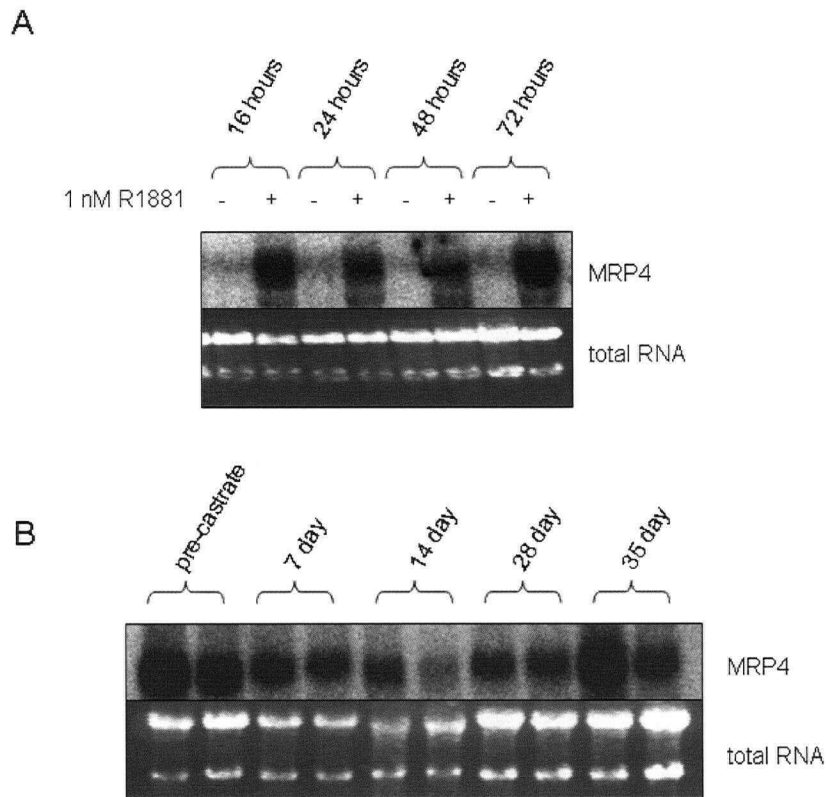
Figure 2.3 MRP4 is upregulated in response to androgens in LNCaP cells. *A*, Northern blot of LNCaP cells treated with 1 nM R1881 or ethanol vehicle alone for 16, 24, 48 or 72 hours probed for MRP4. The Northern blot of the timecourse was repeated with three independent biological replicates, with similar results. LNCaP tumor progression series was generated from subcutaneous xenograft tumors harvested at various time points following castration as well as prior to castration. *B*, Northern blot of the LNCaP xenograft tumor series probed for MRP4.

by androgens and it therefore is functionally plausible that BARD1 is also repressed by androgens. **Figure 2.4** shows a northern blot of BARD1 levels in response to androgen treatment. The levels of BARD1 in the absence of androgens is quite high, however diminishing over time, while there is little or no BARD1 mRNA in the androgen treated cells. There was no detectable BARD1 mRNA in any of the LNCaP xenograft tumors at any of the time points.
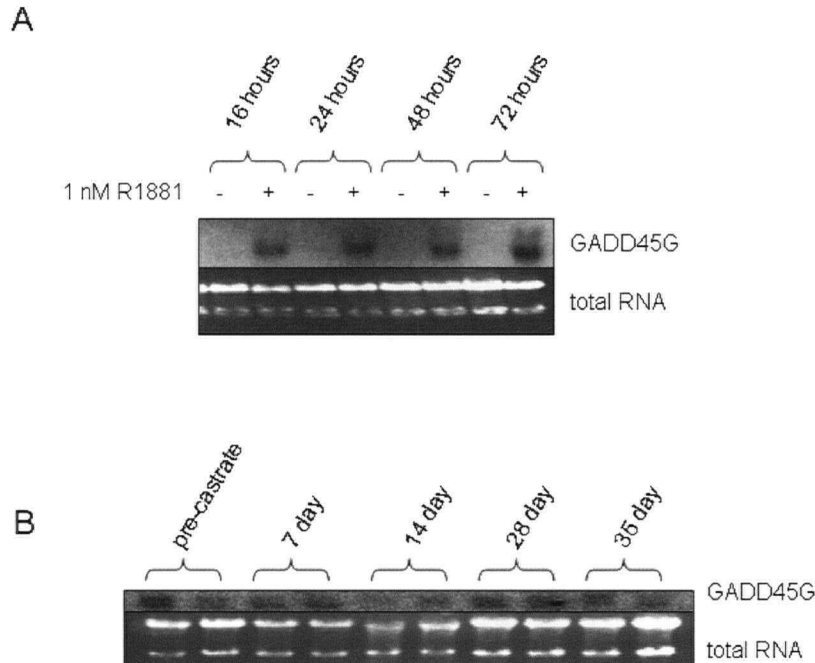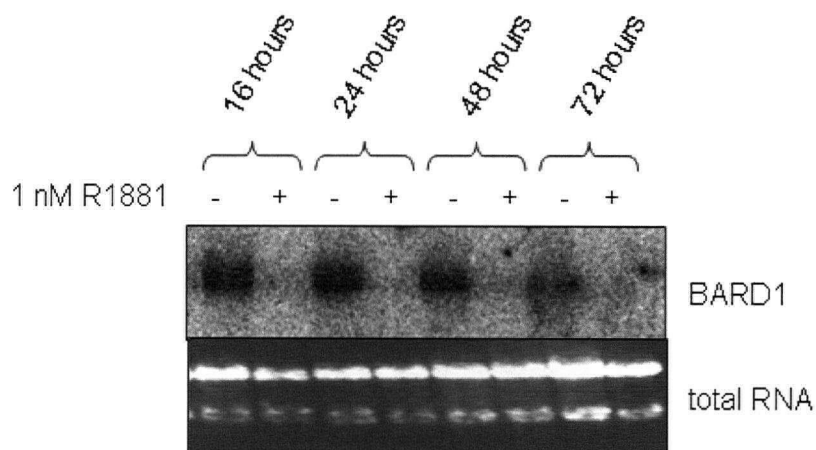
Figure 2.4 BARD1 is repressed by androgens in LNCaP cells. LNCaP cells were treated with 1 nM R1881 or ethanol vehicle alone for 16, 24, 48 or 72 hours and the resulting mRNA run on a Northern blot and probed for BARD1. Northern blotting was performed on three biological replicates with similar results.

Androgens upregulate a network of genes involved in protection against oxidative stress - Ingenuity software is a powerful tool which allows researchers to visualize array data in the context of biological pathways and interactions. The array data from the androgen treated cells was loaded into the Ingenuity software to determine networks of genes affected by androgens. One network that emerged involved the upregulation of the transcription factor NFE2L2 (nuclear factor erythroid 2-like 2). Many of the genes regulated by NFE2L2 are involved in the protection of the cell against oxidative stress (19). As NFE2L2 is upregulated by androgens, so are many of the genes it regulates (**Figure 2.5**). The array data was validated for several
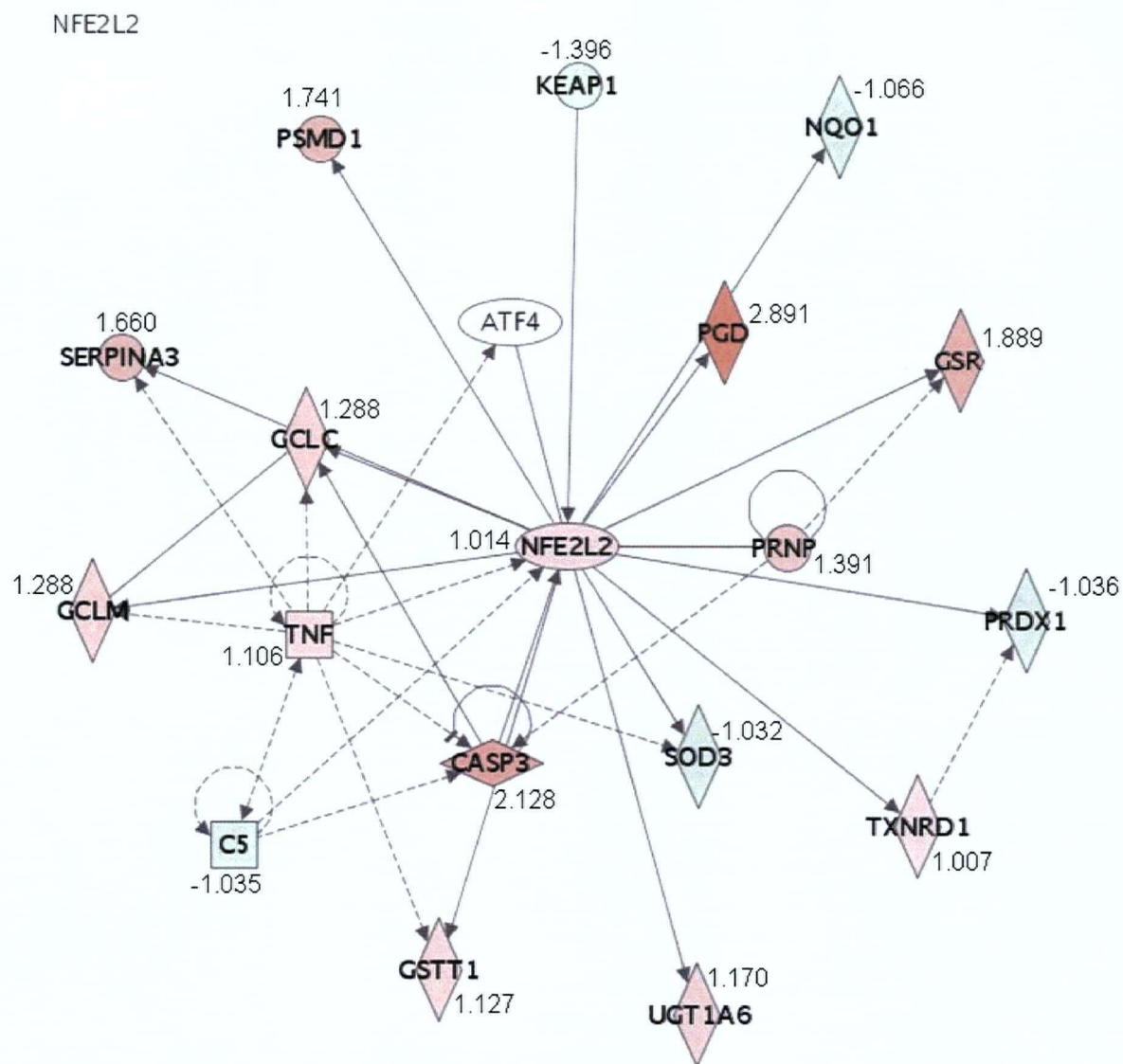
Figure 2.5 Ingenuity network showing an NFE2L2 pathway. Microarray data was analyzed with Ingenuity software, which constructs networks of biologically linked genes. Shown is the NFE2L2 pathway of antioxidant genes with expression levels overlaid from the 24 hour time point. Green indicates downregulation, red indicates upregulation in the R1881 treated cells in relation to the ethanol control and white indicates no data available.
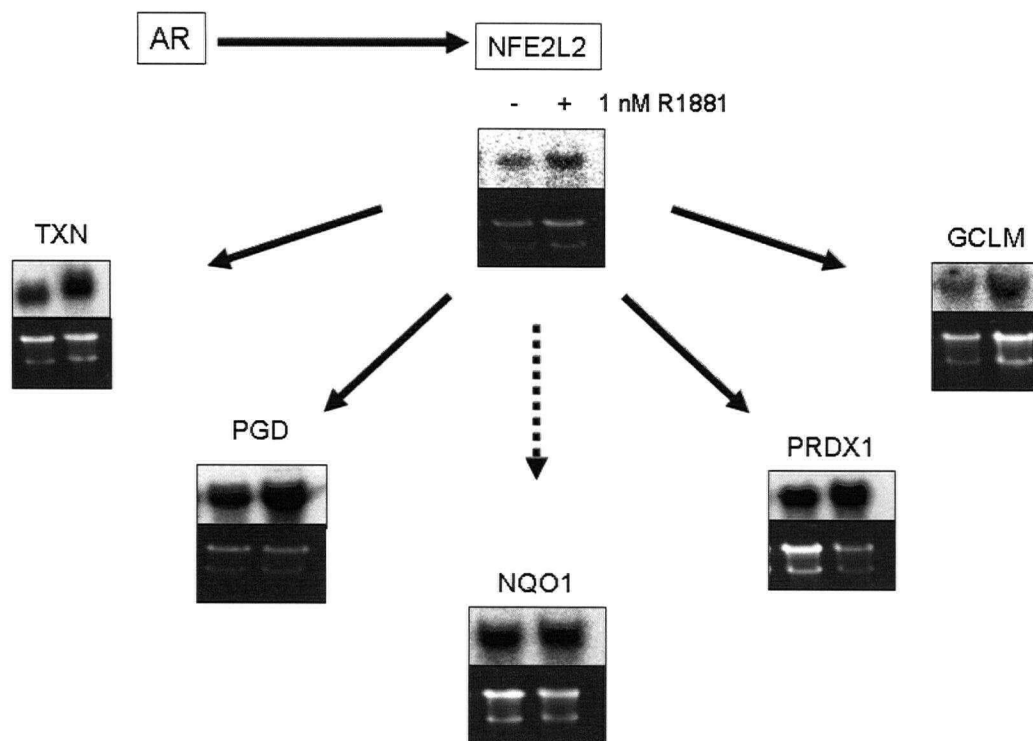
Figure 2.6 Genes in the NFE2L2 antioxidant pathway upregulated by androgens.
LNCaP cells were treated with androgens for 48 hours and the resulting RNA run on
Northern blots which were probed for NFE2L2, TXN, PRDX1, PGD GCLM and NQO1.
The northern blots were performed on three sets of independent biological replicates
and representative samples are shown.

genes in the NFE2L2 network including, thioredoxin (TXN), peroxiredoxin (PRDX1), 6-

phosphogluconate dehydrogenase (PGD), glutamate-cysteine ligase, modifier subunit

(GCLM), using northern blotting. The effects of androgens on the expression of a gene

which has been well characterized as being an NFE2L2 regulated gene, NQO1, was

also assayed even though there was no obvious upregulation observed in the

microarrays. **Figure 2.6** shows the northern blots for LNCaP cells treated with

androgens for 48 hours. NFE2L2, TXN, PRDX1, PGD and GCLM are all upregulated in the androgen treated cells. NQO1 however shows no apparent androgen regulation. This suggests that perhaps transcriptional activation of genes by NFE2L2 is different when upregulated by androgens than in other contexts.

The effect of NFE2L2 downregulation on downstream genes differs in the absence and presence of androgens - Since NFE2L2 is a transcription factor reported to increase the levels of a host of downstream genes, we wanted to confirm that the upregulation observed in response to androgens was due to an increase in NFE2L2 levels. An siRNA targeted to NFE2L2 was used and effectively downregulated NFE2L2 to approximately 10% of its endogenous levels (**Figure 2.7A**). Surprisingly, downregulation of NFE2L2 did not result in a downregulation of any of the genes picked up on the microarrays in the presence of androgens (**Figure 2.7B**). NQO1 levels, however, were decreased in the NFE2L2 siRNA treated cells. The siRNA experiment was performed in media supplemented with FBS, which will contain some testosterone. A second siRNA experiment was performed in the presence or absence of androgens. The siRNA caused NFE2L2 levels to be depressed to approximately 14% of the endogenous levels, both in the presence and absence of androgens (**Figure 2.8A**). NQO1 mRNA levels are decreased in NFE2L2 siRNA, both in the presence and absence of androgens (**Figure 2.8B**). PRDX1 is unaffected by the decrease in NFE2L2 levels, and this suggests that in LNCaP cells, PRDX1 may not be NFE2L2 regulated at all and is only regulated by androgens. Surprisingly, TXN and PGD levels are decreased only in the cells which are androgen depleted and which
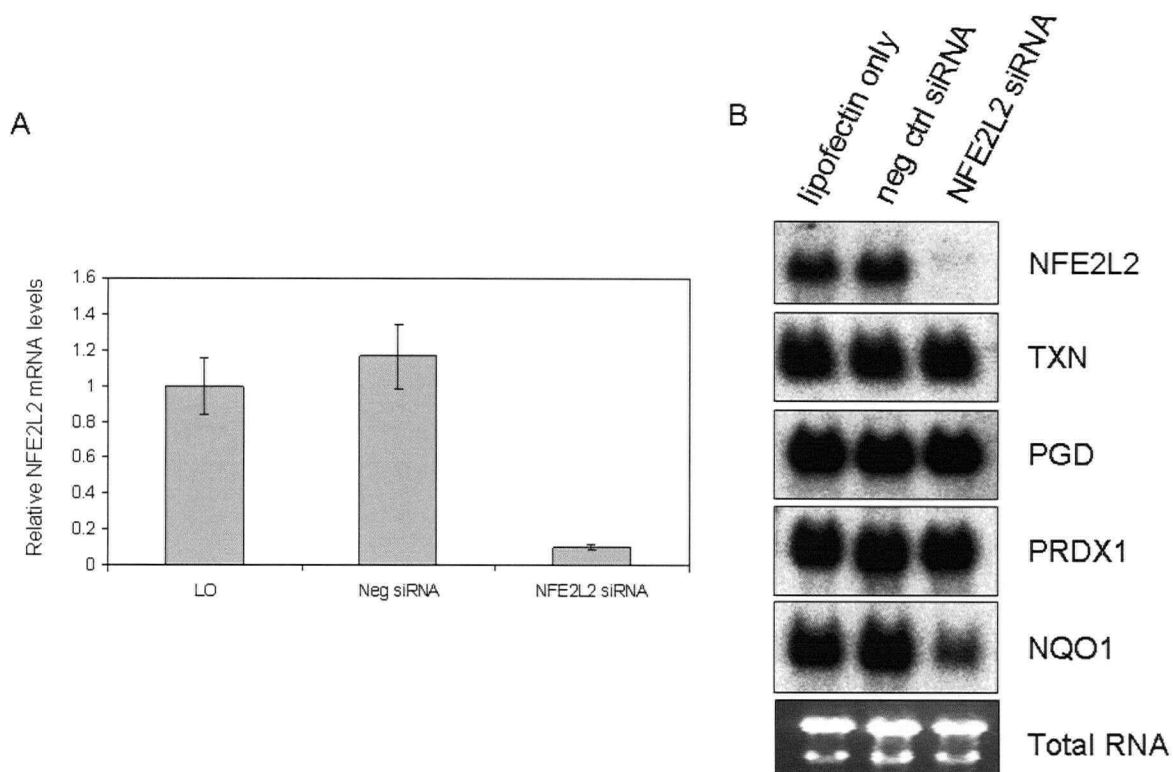
Figure 2.7 Downregulation of NFE2L2 with siRNA and effects on downstream genes. LNCaP cells were treated with 50 nM siRNA targeted to NFE2L2, a negative control siRNA or lipofectamine 2000 only. The RNA was used in real time PCR as well as Northern blotting. *A*, Quantification of three biological replicates, showing the NFE2L2 mRNA levels in response to siRNA treatment. LO indicates the lipofectamine 2000 only negative control, Neg siRNA indicates the negative control siRNA, and NFE2L2 siRNA is the siRNA specific for NFE2L2. *B*, Northern blotting of the NFE2L2 siRNA treated cells, probed for NFE2L2, TXN, PGD, PRDX1 and NQO1, with the ethidium bromide stained rRNA to show loading. The northern blots were done in triplicate with similar results.

have decreased NFE2L2 levels. This suggests that the regulation of TXN and PGD

transcription is complex and can be influenced by androgens or NFE2L2 in different

contexts.
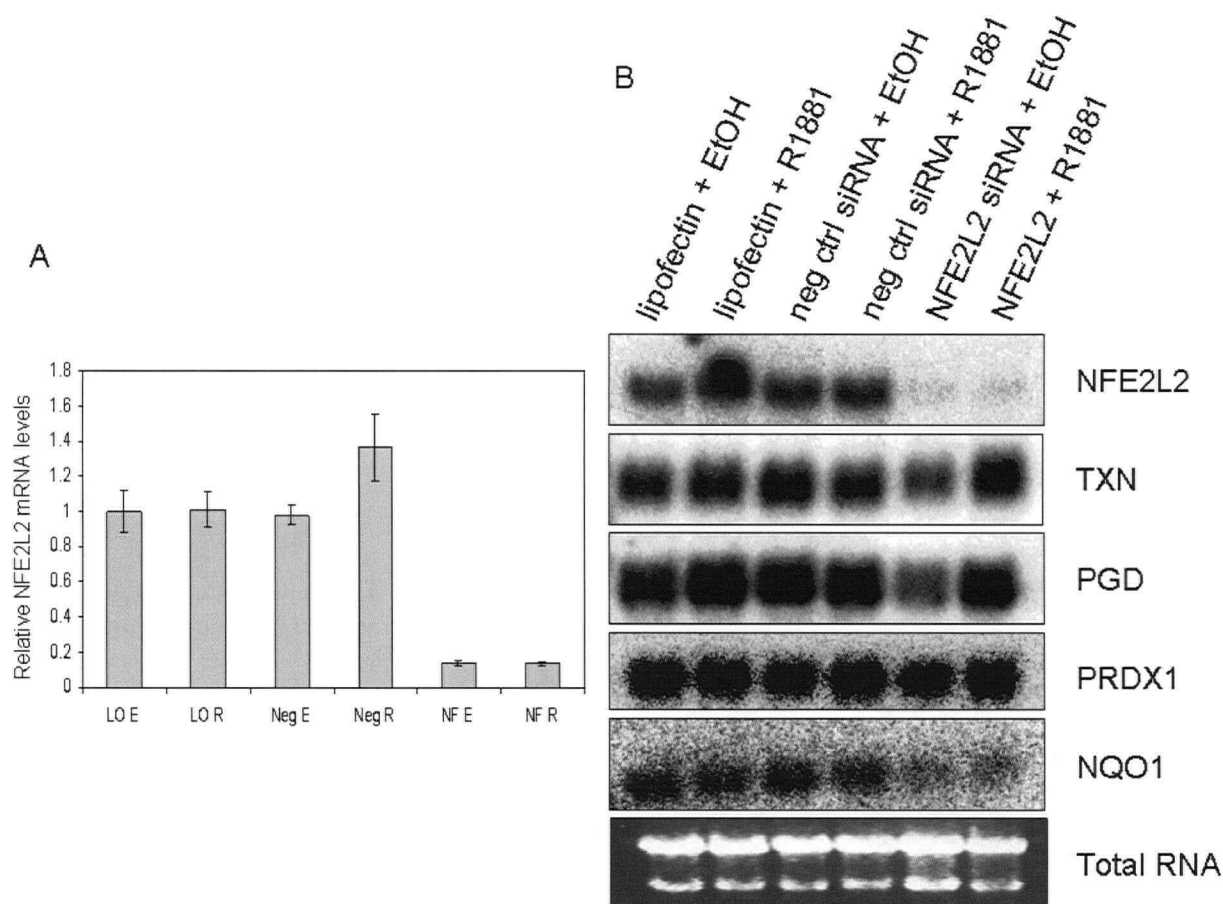
Figure 2.8 Downregulation of NFE2L2 with siRNA in the presence or absence of androgens and effects on downstream genes. LNCaP cells were treated with 50 nM siRNA targeted to NFE2L2, a negative control siRNA or lipofectamine 2000 in conjunction with 1 nM R1881 or ethanol vehicle control. The RNA was used in real time PCR as well as Northern blotting. *A*, Quantification of three biological replicates, showing the NFE2L2 mRNA levels in response to siRNA and androgen treatment using real time PCR. E - EtOH, R - R1881, LO - lipofectamine, Neg - negative control siRNA, NF - NFE2L2 siRNA. *B*, Northern blotting of the NFE2L2 siRNA and androgen treated cells, probed for NFE2L2, TXN, PGD, PRDX1 and NQO1, with the ethidium bromide stained rRNA to show loading. The northern blots were performed on triplicate samples with results similar to what is shown.

2.4   Discussion

The AR is important to the development of the prostate, prostate cancer and importantly to the progression of the disease to AI. Since the AR is a transcriptional

activator for many genes which are important for cell survival and evasion of apoptosis, we sought to characterize novel androgen regulated genes using a microarray screen. Initially, the data was filtered to determine the genes which were upregulated or downregulated by androgens. The resulting lists were mined in search of genes which may be important in prostate cancer progression. Genes known to be androgen regulated were picked up on the array providing confidence to the results.

One gene which was of interest for further characterization was GADD45G. Androgens are known to be cytoprotective for prostate cells and there is an upregulation of certain anti-apoptotic genes (6). GADD45G has been implicated in both cell cycle progression and protection against apoptosis (20). Therefore it is reasonable that GADD45G is upregulated by androgens as one of the proteins which helps protect against apoptosis. Furthermore, GADD54G mRNA levels are increased in AI prostate cancer progression. An important feature of AI progression is the evasion of apoptosis and therefore GADD45G may be involved in that. Another lab has independently demonstrated GADD45G upregulation in LNCaP cells (21). The importance of the GADD45G protein in prostate cancer is explored in **Chapter 3**. Surprisingly, I discovered that while the mRNA levels of GADD45G are increased with androgens, the protein levels are decreased. This new dimension to the androgen regulation of GADD45G is further examined in **Chapter 4**.

Another gene found to be upregulated by androgens in this initial microarray study and by northern blots was MRP4, which is one of 9 members of the ATP-binding cassette (ABC) transporter efflux pumps. MRP4 is able to confer resistance to nucleotide analogs and antiviral agents (22, 23). AI prostate cancer exhibits poor

response to chemotherapeutic agents (24, 25). A mechanism through which cells become resistant to cytotoxic agents is to increase the levels of transporters which can pump the toxins out of the cell. One of the well characterized multidrug resistance genes is p-glycoprotein (p-gp) has been shown to be overexpressed in prostate tumors and its activity is increased during progression to AI (26, 27). MRP4 may be another efflux pump which contributes to drug resistance in AI prostate cancer. In a recent report another lab has also found MRP4 to be upregulated by androgens in LNCaP cells (28). Furthermore, they demonstrated that downregulation of MRP4 sensitizes prostate cells to methotrexate (28).

One of the downregulated genes verified from the microarrays is BARD1. Studies have demonstrated that BARD1 is a tumor suppressor protein which can interact with BRCA1 (29). The interaction of BARD1 and BRCA1 is important for DNA damage repair (30). BARD1 also has a pro-apoptotic function which involves interaction with p53 resulting in its phosphorylation (31, 32). Here we demonstrate that BARD1 is repressed by androgens in LNCaP cells in vitro. Other pro-apoptotic proteins have been found to be androgen repressed (6), and BARD1 may be another such gene. There were no detectable levels of BARD1 in northern blots of the LNCaP tumor xenograft, and it is therefore difficult to speculate whether or not it has a role in prostate cancer progression. As a pro-apoptotic protein, it would be expected that there would be increased levels following androgen ablation and lower levels as the tumors progress to AI. The LNCaP tumor series does not regress however, so it may be reasonable that no BARD1 is expressed. There is an increased risk of prostate cancer in men carrying BRCA1 mutations (33, 34). It is therefore possible that

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mutations or suppression of BARD1 will result in an increased risk of prostate cancer. Certain mutations of BARD1 have been shown to result in a slightly higher risk of breast or ovarian cancer (35, 36). Whether or not BARD1 mutations are involved in the development of prostate cancer remains to be thoroughly tested, however in the sole report looking at a specific BARD1 mutation found no increased risk of prostate cancer (37).

Oxidative stress is thought to play a role in the development of prostate cancer. Evidence to this is that the loss of the detoxifying enzyme, GST, is a risk factor for the development of prostate cancer (38, 39). Also, antioxidants like lycopenes or vitamin E confer some degree of protection against prostate cancer (40, 41). One of the effects of oxidative stress is increased DNA damage which could lead to deleterious mutations. Androgens have been shown to cause an increase in oxidative stress in LNCaP cells (42).

One of the networks that became apparent in the Ingenuity pathway analysis was NFE2L2 and its downstream genes. NFE2L2 is a transcription factor which binds to antioxidant response elements and increases the expression of various genes involved in protection against oxidative stress. NFE2L2 knockout mice are susceptible to oxidative stress (43). Here we show that NFE2L2 and several of its downstream genes are regulated by androgens in LNCaP cells. While androgens increase oxidative stress in prostate cells, the upregulation of NFE2L2 and its downstream genes may provide a route to temper these effects, as an accumulation of mutations could lead to decreased cell viability.

Interestingly, the siRNA experiments revealed different regulation patterns for

the genes in the NFE2L2 network. NQO1 is solely an NFE2L2 regulated gene and PRDX1 is only regulated by androgens. PGD and TXN require either androgens or NFE2L2 for their expression. One of the GST genes, GST-P1 has been shown to have both antioxidant response elements and androgen response elements which allow for the control of the gene by both NFE2L2 and AR (44). Therefore there may be a whole group of anti-oxidant genes which are regulated by both AR and NFE2L2. Another possibility for the unexpected patterns of regulation is that the genes are being affected by off target effects of the NFE2L2 siRNA. There have been many reports of siRNAs affecting genes which share partial homology to the siRNA (45-47). Off target siRNA effects can allow for mismatches within the sequence and can result in downregulation of the RNA or inhibition of translation, similar to what is seen with miRNAs (48, 49). Therefore it is possible that the genes downstream of NFE2L2 actually share some homology to the transfected siRNA and are subject to off target effects, or these genes are downstream of a gene affected by off target siRNA effects. To control for off target effects, siRNAs that target different areas of NFE2L2 would need to be tested. If several siRNAs targeted against NFE2L2 have the same effect on the downstream genes, then it is likely that it is truly a specific result.

The data presented here demonstrate that androgens regulate a wide variety of genes in LNCaP cells. Two genes discussed are involved in apoptosis - BARD1 is a pro-apoptotic gene which is downregulated by androgens and GADD45G is an anti-apoptotic gene which is upregulated by androgens. The data suggests that androgens may also upregulate the efflux pump MRP4, which may enhance cell survival by protection against toxic agents, however it may also contribute to resistance to

chemotherapeutic agents in AI prostate cancer. Finally, we demonstrated that many genes involved in protection of prostate cells against oxidative stress are upregulated by androgens. Taken together, the data provides further evidence that the AR affects gene expression which results in increased cell survival and these genes may also be important for progression to AI.

2.5   References

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Chapter 3. GADD45G is a Key Mediator of Multiple Signaling Pathways in Prostate Cancer Cells[1]

This work was completed with the help of several people. Amy Lubik performed the MTS assay and probed the HSP27 western blot. John Cavanagh labeled proteins from cell lysates and probed the antibody arrays. Dr. Amina Zoubeidi provided expertise in the cell signaling pathways and experimental design. Dr. Ladan Fazli stained and scored the tissue microarrays.

3.1   Introduction

Prostate cancer is the most frequently diagnosed cancer and the third leading cause of cancer death in North American men. Initially, prostate cancer is dependent on androgens for growth and survival, and most patients respond favorably to androgen ablation therapy. However, patients progress to androgen independent (AI) disease which there is currently no effective therapy. Progression to AI is a complex process by which cells acquire the ability to both survive and proliferate in the absence of androgens. The mechanism through which prostate cancer survives in an androgen depleted milieu is not entirely understood, but it is thought to involve a combination of clonal selection, adaptive upregulation of anti-apoptotic genes, and alternative growth factor pathways (1-4). It is well documented that stress genes are upregulated in prostate cancer progression to AI (5, 6) pointing out the importance to investigate their potential therapeutic for the hormone refractory prostate cancer. Understanding the

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mechanism underlying the progression from AD to AI and the implication of stress proteins in this process will help us to identify potential therapeutic targets. The GADD45 family of proteins contains three members, GADD45A, GADD45B and GADD45G, all of which respond to stresses by upregulating cell survival signaling pathways. The expression of all three family member is induced by environmental stresses such as treatment with MMS (7), however they differ in their induction to DNA damaging agents such as UV, or H_2O_2 (8). Furthermore, the three members have different tissue distributions (8). The third member of the family, GADD45G was originally identified as having an early induction in response to interleukin-2 stimulation of T lymphocytes (9). Interestingly, GADD45G has been shown previously (10) and in our studies to be upregulated by androgens in prostate cancer cells.

GADD45 proteins are potent upstream regulators of MEKK4 (MTK1/MAP3K4) activation through which they are able to initiate the key signaling pathways that allow the cell to respond to environmental stresses and DNA damage. MEKK4 responds to stresses such as UV and osmotic shock (11) and induces the activation of the p38 and JNK signaling pathways (8, 12, 13). MEKK4 contains an autoinhibitory domain within its N-terminus, and upon cell stress GADD45 proteins bind the autoinhibitory domain of MAP3K4 leading to the rapid activation of a host of downstream signaling pathways (8, 14).

Activation of the p38 kinase pathway by GADD45 likely results in the upregulation of key cell survival proteins to protect cells against stress-induced apoptosis (15). When mice deficient in GADD45A or GADD45G are stimulated with genotoxic stress, there is a marked increase in apoptosis of the hematopoietic cells

(16), demonstrating that GADD45 is important in the control of apoptosis in these cells. The GADD45G protein has also been shown to effect the cell cycle by inducing a G2/M cell cycle arrest (17, 18). It has been suggested that a decrease in GADD45 levels in cancer cells leads to a decrease in p38 kinase and JNK activity which may provide a mechanism for tumor cells to evade apoptosis (19).

GADD45G is an androgen regulated gene which is important for cell survival by controlling signaling pathways (refs). As prostate cancer progresses to AI, there is a systematic concurrent increase in androgen regulated genes as well as stress responsive genes. GADD45G could possibly be an important factor in the progression of prostate cancer to AI. In the current study, we present data showing that GADD45G levels are increased in AI tumor samples as compared to those treated with neo-adjuvant hormone therapy (NHT). We demonstrate the importance of GADD45G in controlling multiple intracellular signaling pathways including p38 kinase, HSP27, JNK, AKT and Rb, as well as its role to prostate cell survival.

3.2   Materials and Methods

Neoadjuvant Hormone Therapy (NHT) treated Tissue Microarray (TMA) - Slides (H&E) from 112 radical prostatectomy specimens (from 1989 to 2003) obtained from the Vancouver General Hospital. The treatment groups consisted of: 21 untreated patients, 21 treated with less than three months NHT, 28 treated with 3-6 months NHT, 28 treated with more than 6 months NHT and 14 AI metastatic tumors. Benign and cancer sites were identified and marked in donor paraffin blocks using matching H&E reference slides. TMA was constructed using a manual tissue micro arrayer (Beecher Instruments). Each marked block for benign and cancer was sampled 3 times with a

core diameter of 0.6 mm arrayed in a rectangular pattern with 1 mm between the centers of each core, creating a triplicate a TMA layout and ordered by treatment of the patients. The number of patients in this TMA was 112 with a total of 336 cores. The TMA paraffin block was sectioned into 0.5 micrometer sections and mounted on positively charged slides.

Immunohistochemistry - Staining was done using a Ventana autostainer with a concentration of 1:10 of GADD45G rabbit polyclonal antibody (Protein tech group. Inc).

Scoring Method - The slide was scanned with the BLISS system (Bacus Lab) and scored (0 to 3) by pathologist (L.F) based on the intensity of staining with consideration of the percentages of cells.

Cell Culture - LNCaP cells, passage 35-50, were maintained at 37°C and 5% CO_2 in RPMI containing 5% fetal bovine serum (FBS) and penicillin/streptomycin.

siRNA transfections - LNCaP cells were grown to 60% confluency before being transfected with 50 nM negative control siRNA or pre-designed GADD45G siRNA (Ambion, Austin, TX, USA). Lipofectamine 2000 (Invitrogen) was diluted in serum free RPMI and allowed to sit for 5 minutes before the siRNA was added and incubated for 20 minutes more. The transfection mixture was added to the LNCaP cells and they were incubated at 37°C for 4 hours at which point FBS was added to a concentration of 5%. Cells were harvested 48 hours post transfection and lysed in RIPA lysis buffer (1% NP40, 0.5% NaDeoxycholate, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 5 mM EDTA).

Western Blot Analysis - Cell lysate (30 μg) was separated using SDS polyacrylamide gel electrophoresis and proteins were transferred electropheretically onto PVDF

membrane (Millipore). The blots were blocked with Li-cor blocking buffer (Li-cor Biosciences). Blots were probed with the primary antibodies: phosphor-RSK1/2/4 (Ser 363) β-tubulin (Santa Cruz Biotechnology), GADD45G (Proteintech Group Inc), MEKK4 (Abnova), phospho-HSP27(Ser 78) and HSP27 (Nventa Biopharmaceuticals Corp.), phospho-p38 kinase (Thr 180/Tyr 182), p38 kinase, phospho-AKT (Ser 473), AKT, phospho-eIF4E (Ser 209), eIF4E, RSK, phospho-p53 (Ser 392), p53, phospho-JNK (Thr 183/Tyr 185), JNK, phospho-Rb (Ser 807/811) and Rb (Cell Signaling Technology Inc). Blots were probed with secondary antibodies and signal was detected on film using chemiluminescent ECL reagents (GE Healthcare) or fluorescently using the Li-cor Odyssey (Li-cor Biosciences).

MTS Assay - LNCaP cells were grown in 96-well cell culture plates. Once the cells achieved 60% confluency, the siRNAs were transfected. 24 hours post transfection, paclitaxol was added to the wells and the plate allowed to incubate for another 24 hours before addition of the MTS reagents. A 2 mg/ml MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium) (Promega Corporation) solution was combined in a 20:1 ratio with a 0.92 mg/ml PMS (phenazine methosulfate) (Sigma) solution. The mixture was added to the cell culture wells at a ratio of 1:5 and incubated at 37°C for 4 hours. The absorbance at 490 nm was read as a measure of metabolic activity and cell viability.

Antibody Arrays - Sodium carbonate (pH 8.3) at a final concentration of 0.1 M. was added to 0.1 ml (200 µg) of the lysate. Alexa Fluor 546 or 647 was added to the cell lysate and incubated for 1 hour at room temperature. 80 mM Tris pH 8.0 was added to saturate the reaction. Unbound dye was removed using an Ultrafree-0.5 Centrifugal

filter Device (Millipore). Labeled protein was finally made up to 0.2 ml with PBS. Antibody arrays (Kinexus) were washed and any remaining active sites were inactivated with 50 mM Tris for 1 hour. Slides were finally rinsed with H_2O and stored at 4°C. 50 µg of control lysate-Alexa 647 and 50 µg of treated lysate-Alexa 546 were mixed together and added to one slide. A duplicate slide with the dyes reversed was also prepared. Both slides were incubated for 1 hour and then washed with PBS-Tween 20 (0.2%) for 5 min. Sample loading, incubation and wash step were all done using an automated Protein Array Workstation (PerkinElmer). Slides were removed, washed extensively in distilled water and dried with a stream of air. The labeled slides were scanned with ScanArray Express (PerkinElmer) and the spots quantified using Imagene software (Biodiscovery Inc). Data analysis was performed using GeneSpring software (Agilent).

3.3   Results

GADD45G levels decrease with neo-adjuvant hormone therapy, but recur at androgen independence. As prostate cancer develops, it depends on androgens and androgen regulated gene expression for survival. Androgen ablation is initially an effective therapy, however as AI cancer develops the tumor begins to proliferate and there is a recurrence of androgen regulated, cell survival genes. Using microarray based expression profiling of androgen responsive genes in LNCaP cells we have found that a number of genes are regulated by androgens in a time and dose dependent manner. One gene of interest that was strongly upregulated by androgens was GADD45G, which had been previously reported to be androgen regulated in a prostate cancer cell line (10). Paradoxically, there is actually a decrease in GADD45G

protein with androgen treatment. This is due to the inhibition of translation through interaction with a miRNA, and this will be discussed in **Chapter 4**.
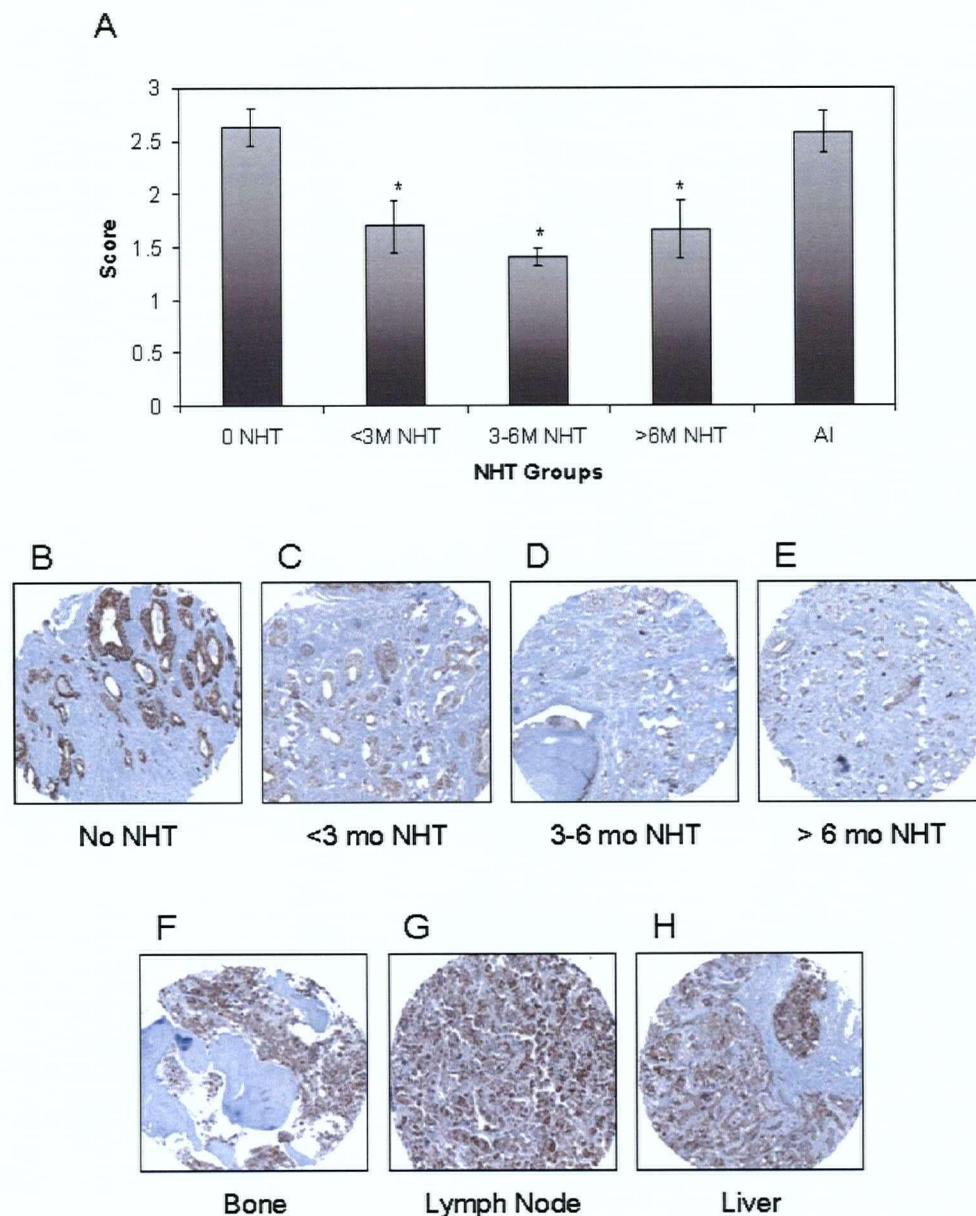
Figure 3.1 GADD45G levels are reduced with NHT treatment and increased at androgen independence. (a) Graph showing the immunohistochemical staining of the tissue tumor microarray for GADD45G. The TMA contained cores from 112 radical prostatectomy patients. The tumors were treated with varying durations of NHT from less than 3 months to more than 6 months. Staining levels of androgen independent samples are also shown. An * indicates that the value is statistically different from the non treated levels with a p-value less than 0.01 (p-value of 4.4×10^{-5} for the <3M NHT;

3.0×10^{-6} for the 1-6M NHT and 1.8×10^{-5} for >6M NHT). Representative cores are pictured for non-treated samples (b), less than 3 months NHT (c), 3 to 6 months NHT (d) and greater than 6 months NHT (e). Androgen independent samples from metastatic sites are also shown, bone (f), lymph node (g) and liver (h).

Since GADD45G is androgen responsive in cell culture, we sought to determine if it retains its androgen regulation by hormonal treatment in prostate cancer patients. A tumor tissue microarray was generated from prostate cancer specimens from patients who had been treated with various durations of neo-adjuvant hormone therapy (NHT) prior to undergoing a radical prostatectomy. The array was stained for GADD45G protein levels. **Figure 3.1** shows GADD45G staining in the granular cytoplasm of the prostatic epithelial cells. GADD45G is present in some of the cells of the benign secretory gland but almost all of the cells in the malignant glands. The levels of GADD45G are significantly decreased up to and including over 6 months of NHT. There is a high level of GADD45G staining in AI disease from metastatic sites including bone liver and lymph node. The level of GADD45G in the AI samples is comparable to the levels observed prior to NHT treatment. The expression pattern of GADD45G in these samples is consistent with previous reports and our observations that GADD45G is androgen regulated in prostate cells (10), as NHT causes a decrease in androgen regulated gene expression. As the cancer becomes hormone resistant, there is a recurrence of expression of genes that are regulated by androgens (20).

HSP27 phosphorylation is dependent on GADD45G expression. The stress responsive p38 kinase signaling pathway is thought to be important for the

development of prostate cancer as well as the progression to AI (5, 21). GADD45G is known to interact physically with MEKK4 thereby relieving its autorepression (14), and the activated MEKK4 phosphorylates p38 kinase. One of the proteins phosphorylated
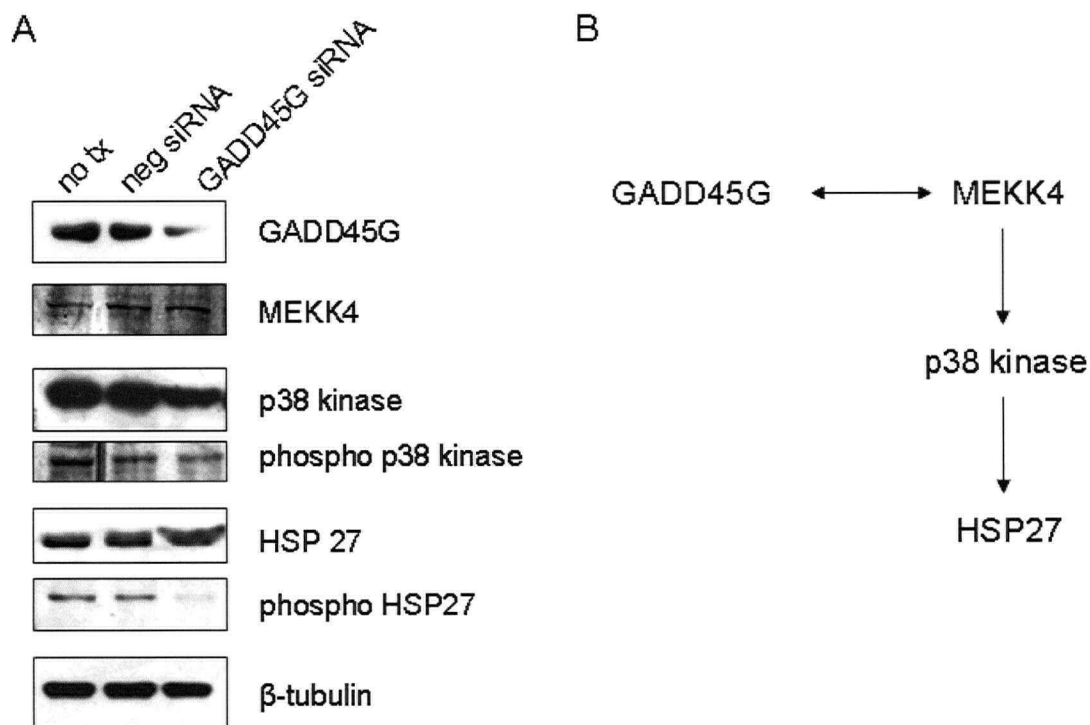
Figure 3.2 GADD45G is involved in the phosphorylation of HSP27. LNCaP cells were treated with siRNA directed at GADD45G levels. (a) Western blotting shows the protein levels of GADD45G, MEKK4, p38 kinase and HSP27. As well, the levels of phosphorylated p38 kinase and HSP27 are shown. (b) The pathway through which GADD45G effects the phosphorylation of HSP27. The Western blots were performed in triplicate with similar results each time.

by p38 kinase is HSP27 (22), a protein that has proven critically important for cell survival of prostate cancer cell lines (2, 23). To determine the importance of GADD45G in regulating the phosphorylation of these important cell survival proteins, we

downregulated GADD45G in LNCaP cells using siRNA (**Figure 3.2A**). Downregulation of GADD45G by siRNA results in a dramatic decrease in phosphorylated HSP27 in LNCaP cells. There is also a decrease in phosphorylated p38 kinase in the siRNA treated cells versus the non treated cells, however, there is also a decrease observed with the negative control siRNA, and it is therefore possible that the effects observed are the result of non-specific siRNA effects. The decrease in GADD45G levels does not affect the overall levels of MEKK4, p38 kinase or HSP27, suggesting that GADD45G is likely regulating the activity of MEKK4 as previously described. These data provide a link between GADD45G and HSP27 phosphorylation (**Figure 3.2B**).

Figure 3.3 Cell viability assay for LNCaPs with depleted GADD45G levels in response to taxol treatment. LNCaP cells were treated with siRNA targeting GADD45G in conjunction with 24 hour treatment with 0, 2 or 20 nM taxol, with 8

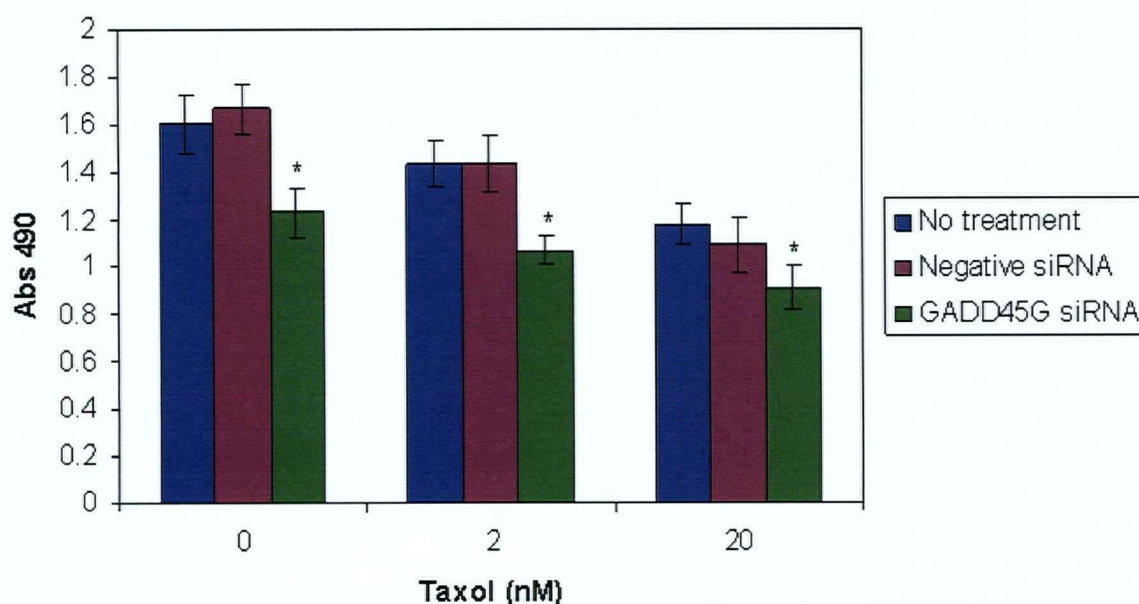biological replicates per treatment. An MTS assay was performed to determine the cell viability in response to these treatments and A490 is linear with the number of respirating cells. An asterisk indicates a significant difference between the GADD45G siRNA treated samples and the no treatment (p-value of 0.051 for 0 nM taxol; p-value of 0.010 for 2 nM taxol; p-value of 0.039 for 20 nM taxol).

The importance of GADD45G for cell viability of LNCaP cells. The implications of that GADD45G regulates critical stress activated cell survival pathways in prostate cancer cells, suggests that GADD45G knockdown may affect cell viability. The effect of GADD45G suppression was tested using the colorimetric MTS assay which provides a quantitative indicator of mitochondrial activity. The absorbance reading in the MTS assay is often used as an indicator of the amount of surviving cells. LNCaP cells were treated through transient transfection with siRNA to GADD45G in conjunction with increasing concentrations of taxol. The cells treated with GADD45G siRNA demonstrate decreased levels of survival (**Figure 3.3**) in the absence of taxol. As expected, taxol treatment results in decreased survival in a dose dependent manner. At all concentrations of taxol, there is decreased cell viability in the GADD45G treated cells in comparison to those treated with lipofectamine only or the negative control siRNA. Since there is not a 100% knockdown of GADD45G in response to the siRNA, these results are an underestimate of the effect seen in the pooled population of cells. It should be noted however that the interpretation of these results as showing that GADD45G being important for cell viability, however strictly speaking, the MTS assay measures mitochondrial activity. It is possible therefore, that the reduced levels of MTS metabolized in the assay are merely a sign of reduced respiration, as opposed to a decrease in cell survival.

GADD45G is critical for p38 kinase intracellular signaling. There are many downstream effectors of p38 kinase which are important for cell survival and prostate cancer progression. Since GADD45G is an important mediator of p38 kinase phosphorylation, we sought to determine if other downstream targets were affected by downregulation of GADD45G. p38 kinase can effect AKT phosphorylation through a variety of mechanisms (**Figure 3.4A**). The GADD45G siRNA indeed caused a decrease in AKT phosphorylation, however it is unclear whether this is a specific effect, because the negative control siRNA also has an effect on the phosohporylation of AKT. AKT has been reported to be important for prostate cancer cell survival when challenged with a number of stressors and therefore may be a key mechanism through which GADD45G exerts its cytoprotective functions. p38 kinase is also involved in a phosphorylation cascade in which MKNK1 phosphorylates the translation initiation factor eIF4E, and as such, with reduced levels of GADD45G, there is decreased phosphorylated eIF4E (**Figure 3.4B**). The phosphorylation of eIF4E can have effects on regulating protein translation and has been shown to be important for tumorigenesis (24). Another downstream effector of p38 kinase is RSK, which is a serine/theronine kinase important for cell survival (25). Studies have shown that RSK is a significant factor in the proliferation of prostate cancer cells (26). Reduction of GADD45G levels causes a decrease in the phosphorylation of RSK (**Figure 3.4C**). Each of these downstream effectors of p38 kinase has been shown individually to be important for cell survival, and we have demonstrated here that GADD45G is important for their phosphorylation.
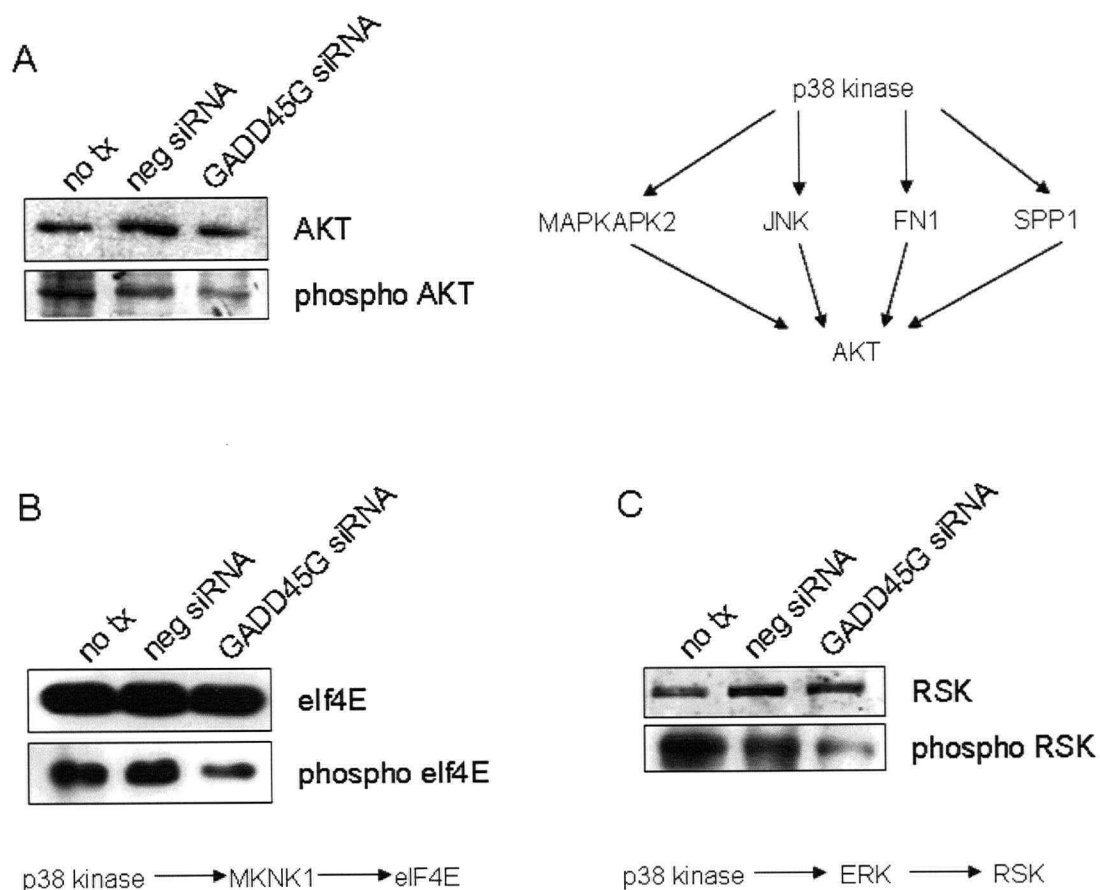
Figure 3.4 Proteins downstream of p38 kinase which are affected by GADD45G. (a) Western blots showing the levels of AKT and phosphorylated AKT and the possible pathways leading to AKT phosphorylation. Levels of total and phosphorylated eIF4E (b) and RSK (c) as visualized by western blotting are shown. The western blots were done in triplicate, with similar results each time.
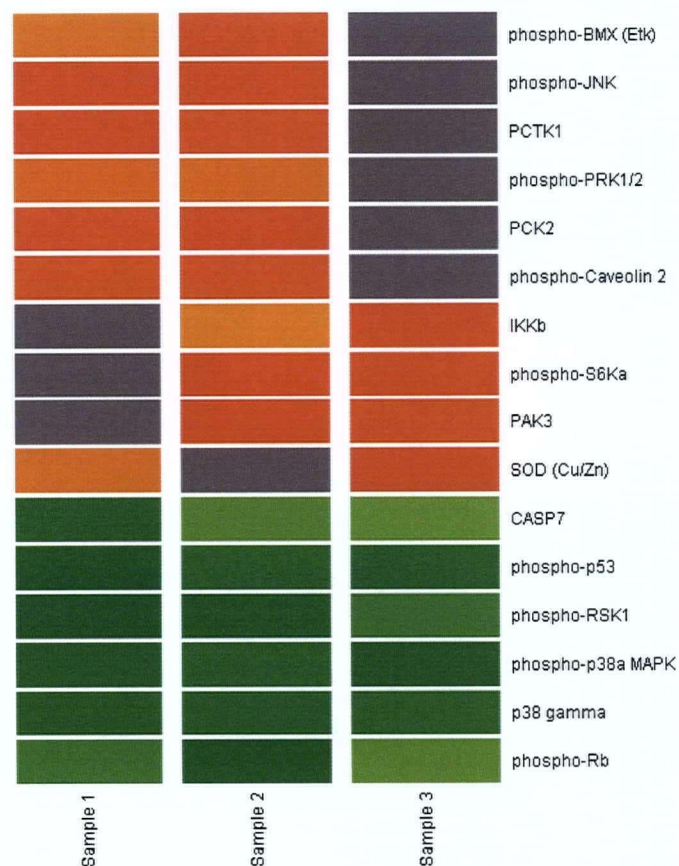
Figure 3.5 Antibody array analysis of proteins affected by reduced GADD45G levels. LNCaP cells were treated with negative control siRNA or GADD45G siRNA. The cell lysate was labeled and used to probe antibody arrays. A heat map of all of the differentially regulated (at least 1.5 fold up or down in two of the three samples) proteins is shown. Red indicates an upregulation, green indicates downregulation and grey indicates data from spots that did not pass the quality control filters and was removed from the analysis.

GADD45G is important for JNK, Rb and p53 phosphorylation. The MTS assay

revealed that GADD45G could be important to cell survival in prostate cancer cells,

and targeted evaluation of the p38 pathway are clearly downstream effectors of

GADD45G action. However, it is likely that GADD45G regulates other pathways that

have not yet been described in relation to it. Therefore we assayed LNCaP cells

treated with siRNA to GADD45G with antibody arrays containing over 600 antibodies

directed towards various signaling proteins and phospho-proteins. The arrays probed

with fluorescently labeled cell lysates isolated from GADD45G siRNA treated cells

compared to cells treated with the negative control siRNA. The data was filtered for

proteins or phosphorylated proteins which are up or downregulated in the GADD45G

siRNA treated cells versus the negative control siRNA treated cells by at least 1.5 fold

in two of the three samples. As shown in **Figure 3.5**, multiple pathways are affected by

the downregulation of GADD45G levels.

Using Western blotting, the antibody array results were validated for three key

proteins known for their role in cancer: p53, Rb and JNK. Both p53 and Rb

demonstrated decreased levels of phosphorylation following GADD45G siRNA

treatment (**Figure 3.6A** and **B**), which reproduced the effects observed in the protein

array. JNK demonstrated dramatically increased phosphorylation in response to

suppression of GADD45G (**Figure 3.6C**).

3.4   Discussion

Both GADD45G and HSP27 have been shown to be stress induced and serve

to protect cells against apoptosis. Chaperones like HSP27 are responsible for ensuring

proper folding of proteins after stress-induced misfolding (27) and are important in

protection against apoptosis (28, 29). HSP27 has been demonstrated to be an important cytoprotective factor in prostate cancer cells and its expression is linked to
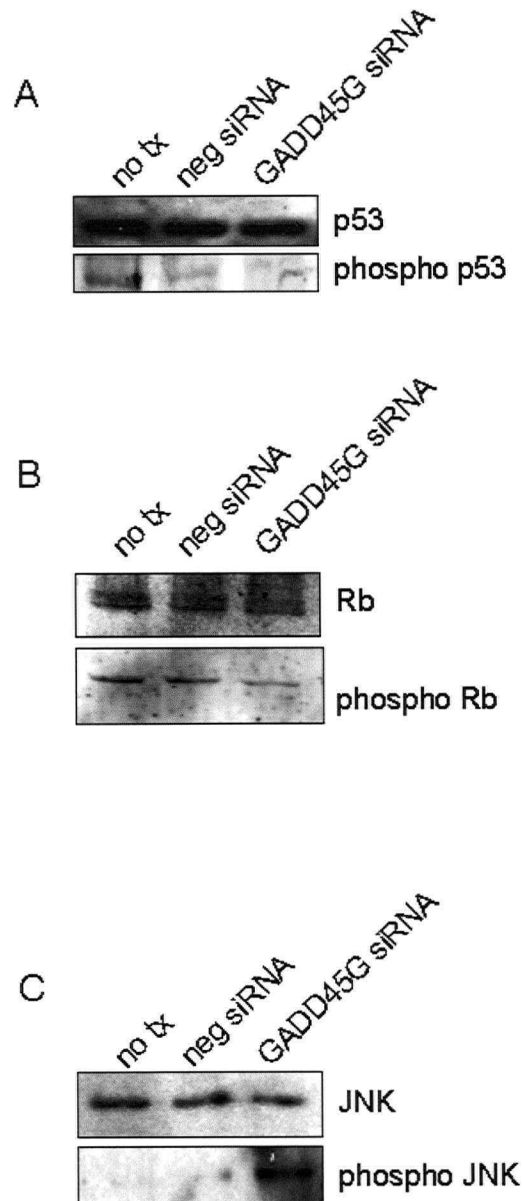
Figure 3.6 Validation of antibody arrays. Western blots of total and phosphorylated p53 (a), Rb (b) and JNK (c) in GADD45G siRNA treated cells. The western blots were done with three biological samples, with similar results each time.

progression to AI. HSP27 levels are increased in high Gleason grade prostate tumors and high HSP27 levels are linked to poor clinical outcome (30, 31). Importantly, HSP27 expression correlates with hormone refractory prostate cancer (32). Downregulation of HSP27 in prostate cancer cells causes an activation of the apoptotic program Furthermore, downregulation of HSP27 in both androgen dependent and androgen independent mouse xenograft tumors causes increased sensitivity to taxol (2, 23).

The phosphorylation status of HSP27 is linked to its activation. In unstressed cells, HSP27 largely resides in the cytoplasm in large, oligomeric units. In response to a stress such as heat shock, HSP27 becomes phosphorylated and there is a dissociation of the oligomers into tetramers (33, 34). Thermoresistance can be conferred to cells by overexpression of HSP27, however this is dependent on it being phosphorylated (34). Therefore the cytoprotective effects of HSP27 are dependent on its phosphorylation status. Phosphorylation of HSP27 occurs via the p38 kinase pathway as determined by the use of specific kinase inhibitors (35). We have demonstrated for the first time that GADD45G is a potent upstream master regulator of HSP27 phosphorylation likely acting through regulation of p38 kinase.

We have also demonstrated in our studies that GADD45G is an upstream regulator of the phosphorylation of other targets downstream of p38 kinase, such as AKT. AKT has been shown to be important in cell survival and the suppression of apoptosis in a variety of cancer model systems and is thought to be a critical element in therapeutic resistance (6, 36). Activity of AKT is pleiotropic and can suppress apoptosis directly through substrates involved in mitochondrial induced apoptosis such

as BAD (37) or caspases (38). AKT can act indirectly to provide apoptotic resistance by inhibition of FKHRL1, a forkhead transcription factor which normally induces expression of the Fas ligand gene (39, 40).

Translation is regulated by a number of elongation initiation factors. One limiting factor of translational initiation is eIF4E, which binds the 5 cap of the mRNA and as been described to be a critical factor in tumor progression and is being explored as a therapeutic target (24). eIF4E can be phosphorylated by MKNK1, which has been shown to be dependent on AKT activity (41). As well, AKT/mTOR signaling can cause phosphorylation and inactivation of eIF4E binding proteins, which normally act to inhibit eIF4E activity (42). Therefore both AKT and eIF4E are involved in the regulation of protein translation, and we show that the activity of both is dependent on GADD45G in prostate cancer cells.

Members of the GADD45 family have been shown to both upregulate and downregulate JNK signaling in different model systems. GADD45G-/- mice are deficient in their ability to activate JNK signaling (43). Conversely, GADD45A and GADD45B deficient mice demonstrated an inhibition of JNK signaling (15). The ability of GADD45 to mediate JNK activity may be dependent on the cell type or on the type of stimulus being responded to. The activity of JNK itself has been shown to be both pro-apoptotic (44) and anti-apoptotic (45) dependent on the cell context. We show that in prostate cancer cells inhibition of GADD45G by siRNA results in a profound upregulation of JNK phosphorylation.

Both p53 and Rb are important regulators of cell cycle and apoptosis and underpin many mechanisms in cancer biology. p53 is a multi functional transcription

factor that reacts to DNA damage to induce cell cycle arrest, DNA repair or apoptosis. Stress, such as DNA damage results in p53 phosphorylation, which increased the p53 protein stability by inhibiting MDM2 and subsequent ubiquitination (46). Active p53 can then cause cell cycle arrest and induction of apoptosis through both transcriptional and non-transcriptional activities (47). One of the genes reportedly being transcriptionally p53 responsive is GADD45 (48). Here we show in prostate cells that GADD45G's transcription is androgen regulated and that GADD45G acts upstream of p53. The phosphorylation of Rb is central to the progression of the cell cycle (49). GADD45G has been shown to be involved in cell cycle progression (18) and our data would suggest that one possible mechanism may be GADD45G regulating the phosphorylation status of p53 and Rb.

In several of the blots shown herein, there is a decreased level of the phosphorylation proteins in response to the negative control siRNA as well as the GADD45G siRNA. It is therefore possible that there are some non-specific effects of transfected dsRNA which results in altered levels of the phospho-proteins. It is therefore very difficult to tease apart the specific versus the non-specific results. A protein array which compares the lipofectamine treated cells versus the negative control siRNA could perhaps be used to determine the non-specific effects of the negative control siRNA, and these results compared to the GADD45G siRNA treated cells to determine the effects specific to GADD45G downregulation.

Furthermore, since there was only one siRNA targeting GADD45G used in these experiments, it is possible that this GADD45G siRNA has off-target effects (50, 51) and the results observed are not due to specific downregulation of GADD45G. The

effects observed in the western blots are at the protein phosphorylation levels, and not at the level of total protein, therefore any off target effects would not likely be directly on the proteins themselves, rather on an upstream protein which controls their phosphorylation. In future studies, to control for off target effects, several other siRNAs directed against other areas of GADD45G should be tested to determine if the effects are due to downregulation of GADD45G itself.

To further characterize the phosphorylation cascades and their importance in prostate cancer, future directions would include staining the NHT TMAs with the antibodies for the phospho-proteins described herein. Attempts have been made to stain the TMAs for phospho-HSP27, however this was met with limited success.

The importance of GADD45G in cancer is highlighted by the multiple signaling pathways involved in cell survival which it regulates. All the proteins discussed herein as being regulated by GADD45G have been shown to be of great significance in many types of cancers, many of which have been explored as therapeutic targets. Therefore GADD45G itself which we show is highly differentially upregulated in human prostate cancer specimens and likely to be an important player in the progression of prostate cancer. GADD45G therefore may prove to be a suitable therapeutic target as it regulates a number of key downstream pathways important for cancer cell survival.

3.5   References

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Chapter 4.   The miRNA hsa-mir-326 Targets GADD45G mRNA and Inhibits its Translation[1]

The microarray analyses were performed by Melanie Lehman, Dr. Mauricio Neira and Manuel Altamirano. The RNA labeling, array hybridizations and scanning were performed by Nadine Tomlinson.

4 1   Introduction

GADD45G (growth arrest and DNA-damage inducible, gamma) is a member of a three gene family whose proteins share a high degree of homology and functionality. These genes have been shown to have both cytoplasmic and nuclear functions. In the cytoplasm, the GADD45G proteins interact with MEKK4, reversing its auto-repression (1, 2) and activating the p38 kinase signaling cascade (3, 4). This interaction is induced by environmental stresses, such as treatment with MMS, UV or gamma irradiation, and results in decreased apoptosis (5, 6).   In the nucleus, GADD45 proteins can regulate cell cycle arrest in response to DNA damage and may contribute to DNA damage repair. The GADD45 proteins are downstream effectors of p53 and deletion of GADD45 genes leads to defects in DNA damage repair and genomic instability similar to the phenotype exhibited with a p53 deletion (7, 8), suggesting that the GADD45 proteins are important mediators of DNA damage repair. Furthermore, GADD45 proteins can stimulate DNA damage nucleotide excision repair (9, 10), while cells deficient in GADD45 have impaired DNA damage repair capabilities (11). GADD45 proteins are also involved in regulating G2/M arrest in response to various

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stresses (11-13). GADD45G interacts with both p21 and PCNA, interfering with PCNA induced growth suppression (14-16). This interaction with p21 and PCNA is important in the control of DNA damage repair (17).

Androgens are important regulators of prostate cell growth and proliferation and contribute to the progression of prostate cancer. GADD45G is highly expressed in the prostate (18). Recent studies have shown that GADD45G mRNA is upregulated by androgens in LNCaP cells, an androgen-dependent prostate cancer cell line, in a time and dose dependent manner (19). Recombinant overexpression of a GADD45G construct encoding the protein coding region in prostate cells causes growth inhibition and results in morphological changes (19).

Recently, microRNAs (miRNAs) have emerged as important mediators of gene expression. These miRNAs are short RNA molecules, approximately 22 nucleotides in length, derived from non-coding regions of the genome (20, 21). Following transcription, a hairpin in the RNA is excised by Drosha to form the 60-70 nucleotide pre-miRNAs (22, 23). The pre-miRNAs are exported from the nucleus via Exportin-5 (24-26) and further cleaved by Dicer (27-29) to yield the mature miRNA, which is then loaded into the RNA-induced silencing complex (RISC) (30).

miRNAs can affect either mRNA stability or translation of the mRNA that they target (31, 32). Degradation of mRNA is accomplished if there is a perfect match of the miRNA to the target mRNA (33), in a manner akin to siRNA, however imperfectly matched miRNAs can also cause mRNA degradation (34). Some miRNAs prevent translation of the target mRNA and cause their storage in cytoplasmic processing

bodies (PBs) (35, 36) miRNAs have also been shown to mediate a change in the poly-A tail length (37) and a removal of the 5 cap (38).

The data presented here shows that while androgen treatment causes an increase in GADD45G mRNA levels, the protein levels actually decrease. A screen for androgen-regulated miRNAs identified the miRNA, hsa-miR-326, which was further shown to target the GADD45G 3 UTR. An anti-miR directed against hsa-miR-326 prevented the androgen-mediated translational repression of GADD45G. We hypothesize that androgen stimulation causes GADD45G mRNA storage in PBs. In this study, androgen treatment caused storage of mRNA and subsequent taxol treatment caused translation of that stored mRNA and restored GADD45G protein levels.

4.2 Materials and Methods

Cell Culture and Treatments - LNCaP cells, passage 35-50, were grown at 37°C and 5% CO_2 in RPMI containing 5% fetal bovine serum (FBS) and penicillin/streptomycin. R1881, dihydrotestosterone was purchased from Steraloids Inc. and was stored as a stock solution in absolute ethanol. Prior to R1881 treatment, cells were grown in media + 2% Charcoal Stripped Serum (CSS, Hyclone) for 48 hours. For UV treatments, cells were grown to 80% confluency and washed twice with PBS before 25 mJ of UV treatment using the Stratalinker (Stratagene). After UV treatment, RPMI + 5% FBS was added back to the cells and they were incubated at 37°C for 6 hours. Cells were harvested for protein, using RIPA lysis buffer (150 mM NaCl, 1% Igepal, 0.5% Na Deoxycholate, 0.1% SDS, 50 mM Tris). RNA used in the real time PCR and northern blots was harvested using Trizol (Invitrogen). Trizol was also used to harvest miRNA

for real time PCR analysis. RNA and miRNA used for probing the microarrays were harvested using the mirVana kit (Ambion).

Anti-miR Transfections – LNCaP cells were grown to 50% confluency in RPMI + 5% FBS before being incubated with RPMI + 2% CSS for 24 hours. Lipofectamine 2000 (Invitrogen) was incubated with the control anti-miR or anti-miR-326 (Ambion) in serum free RPMI for 20 minutes. The anti-miRs were added to the cells to a final concentration of 30 nM. As a negative control, some cells were treated with lipofectamine 2000 only. The cells were incubated at 37°C for 4 hours before replacement of the CSS to 2%. After 24 hours, the cells were treated with 1 nM R1881 or the ethanol vehicle and incubated at 37°C for 48 hours prior to harvesting protein and RNA.

Taxol treatments - LNCaP cells were treated with R1881 or ethanol vehicle, as described above, for 48 hours. Following this treatment, various concentrations of Paclitaxel (Taxol, Biolyse Pharma Corp.) were added and the cells were incubated for an additional 24 hours before harvesting for protein and RNA.

Northern Blotting - Total RNA (10 μg) was denatured in sample buffer and electrophoresed through a denaturing 1% MOPS formaldehyde agarose gel for 1 hour at 80V. The RNA was transferred to a nylon membrane (Biodyne B, Pall Gelman Laboratory) in 20X SSC (3M sodium chloride, 0.3M sodium citrate, pH 7.0) for 20 hours. The RNA was crosslinked to the membrane using a UV Stratalinker (Stratagene) according to manufacturer's instructions. Membranes were prehybridized in ExpressHyb (Clontech) containing denatured salmon testes DNA (Sigma) for 3 hours at 65°C. Radioactivity was incorporated into the probe using Ready-To-Go DNA

labeling beads (Amersham Pharmacia Biotech) to a specific activity of 1-2x10^8 dpm/µg. Probes were hybridized to the membrane overnight at 65°C. High stringency washes were performed on the membranes at 65°C. The membranes were exposed to a phosphorimager screen and scanned with a Typhoon scanner (GE Healthcare).

Western Blotting - Protein sample (30 µg) was boiled at 95°C for 5 minutes in sample buffer containing β-mercaptoethanol. Samples were loaded on 10% polyacrylamide gels and subjected to electrophoresed for 1.5 hours at 110 V. Proteins were transferred electropheretically onto PVDF membrane (Millipore). Membranes were incubated with blocking buffer (Li-cor) for one hour followed by an overnight incubation at 4°C with the GADD45G antibody (Proteintech Group) or the β-tubulin antibody (Santa Cruz). The membranes were washed and incubated with Alexa-fluor 680 labeled secondary antibodies (Molecular Probes). Following a second set of washes, the bands were detected by scanning the membranes on the Odyssey scanner (Li-cor).

Real Time PCR – To quantify the mRNA and miRNA levels, real time PCR analysis was performed. Prior to generating cDNA, the mRNA was treated with DNase1 (Invitrogen) for 15 minutes at room temperature to remove any DNA contamination. The DNase reaction was stopped by adding 2.8 mM EDTA and incubating at 65°C for 10 minutes. The RNA was then reverse transcribed into cDNA in a reaction containing 1X reaction buffer, 0.01 M DTT, 1 mM dNTPs, 40 U RNAout (Invitrogen), random hexamers (250 ng) and 200 units of MMLV reverse transcriptase (Invitrogen). The reaction proceeded at 25°C for 10 minutes, then at 37°C for one hour. To detect GADD45G transcript levels, the forward primer 5 -GAGTCAGCCAAAGTCTTGAACGT-

3, reverse primer 5'-CGCAGCCAGCACACAGAA-3' and probe 5'-[6 FAM]CTGCTGCGAGAACG[MGB]-3' were used. To generate the cDNA used in the miRNA real time PCR, the TaqMan MicroRNA Reverse Transcription kit was used in conjunction with a hsa-miR-326 specific primer (ABI). The primer was designed such that only mature miRNA was detected. Specific primers and probes were used in the real time PCR reaction to detect hsa-miR-326 (ABI). For normalization purposes, a reaction using rRNA control primers and probes (ABI) was run in parallel. The real time PCR reactions were run on the ABI 7900 HT Sequence Detection System. The reported values are the means and standard errors of four biological replicates. Student's t-test was used to determine statistical significance.

Generation of miRNA Cell Line pool - Small RNAs were isolated and pooled from LNCaP, HL-60, MDA, TF-1 and PC-3 cell lines. The quality of the RNA was assessed with the 2100 Bioanalyzer (Agilent) and equal amounts of RNA were pooled from each cell line to make the cell line pool.

miRNA Labeling and Microarray Hybridization - miRNAs isolated from LNCaP cells were labeled using the NCode miRNA labeling system (Invitrogen). 500 ng miRNA were labeled following the standard procedure of 3' tagging of the miRNA with the dendrimer capture sequence. Arrays containing the NCode multi-species miRNA probe sets (Invitrogen) were pre-hybridized in 5x SSC, 0.2% SDS and 0.2% BSA for 45 minutes, followed by washing 3 times with dH$_2$0 and 1x with isopropanol. The slides were spun dry and hybridized with the 3' tagged miRNA at 52°C for 16-19 hours. Following the first hybridization, the slides were washed with a succession of 2x SSC, 0.2% SDS at 50°C, 2x SSC at RT and 0.2x SSC at room temperature for 15 minutes

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each, spun dry and hybridized with Alexa 546 (cell line pool) and Alexa 647 (test condition) dendrimer-350 (Genisphere) for 4 hours. Slides were washed similarly as after the first hybridization, except that the first wash was performed at 62°C.

mRNA Labeling and Microarray Hybridization – 10 µg of mRNA isolated from LNCaP cells were 3 end-labeled and converted to cDNA as described in the Genisphere Array 350 expression array detection kit protocol. The 3 end-labeled cDNA was co-hybridized with 3 end-labeled human reference RNA (Stratagene) to an array printed in-house with Operon Version 2 human 70-mer oligonucleotides. The expression arrays were pre-hybridized in 5x SSC, 0.2% SDS and 0.2% BSA for 45 minutes and were washed 3 times with dH$_2$0, once with isopropanol, spun dry and hybridized with the 3 tagged mRNA. The slides were hybridized at 52°C for 16-19 hours. Following the first hybridization, the slides were washed with a succession of 2x SSC, 0.2% SDS at 50°C, 2x SSC at RT and 0.2x SSC at RT for 15 minutes each, spun dry and hybridized with cy3 (human universal reference) and cy5 (test condition) dendrimer-350 for 3.5 hours. Slides were washed similarly as after the first hybridization, except that the first wash was performed at 62°C.

Scanning and Image Analysis - Each slide was scanned on the Genepix Autoloader 4200AL scanner (Axon) with laser power at 100%, PMT for Alexa 647 at 400 and Alexa 546 at 420 at a 10 µm resolution. The spot intensities were quantified with ImaGene 6.0 (Biodiscovery).

Microarray Data Analysis – To analyze the mRNA arrays, GeneSpring (Silicon Genetics) was used for a local background correction and lowess normalization. For the miRNA arrays, the Limma Bioconductor package was used for variance stabilizing

normalization (VSN) and differential expression was determined using a moderated t statistic.

4.3   Results

GADD45G mRNA levels are increased with androgens, while the protein levels are decreased – We have previously observed GADD45G upregulation by androgens in LNCaP cells in microarray experiments and similar observations have also been reported by another group (19). To confirm the microarray data, LNCaP cells were treated with 1 nM R1881 for 48 hours and the mRNA harvested and used for Northern blotting and real time PCR. Both the northern blot (**Figure 4.1A**) and the real time PCR (**Figure 4.1B**) confirm that GADD45G mRNA is upregulated with androgen treatment. Quantitatively, the real time PCR shows a statistically significant 3.17 fold increase of GADD45G mRNA levels in the R1881 treated cells versus the vehicle control. A Western blot of cell lysate from androgen treated cells was done to determine if the protein levels of GADD45G increase concomitantly with the mRNA levels. Surprisingly, GADD45G protein decreased after 48 hours of androgen treatment (**Figure 4.1C**). This disparity between RNA and protein levels was specific to androgen treatment, as UV induction of GADD45G mRNA results in a corresponding increase in GADD45G protein (**Figure 4.1D and E**). Since there is a large disconnection between the observed low protein levels and the high mRNA levels following androgen treatment, it is likely that the GADD45G mRNA is not being translated. It is unclear whether the increase in GADD45G mRNA levels is due to an increase in transcription or if there is simply an accumulation of untranslated mRNA. Since miRNAs are known to inhibit
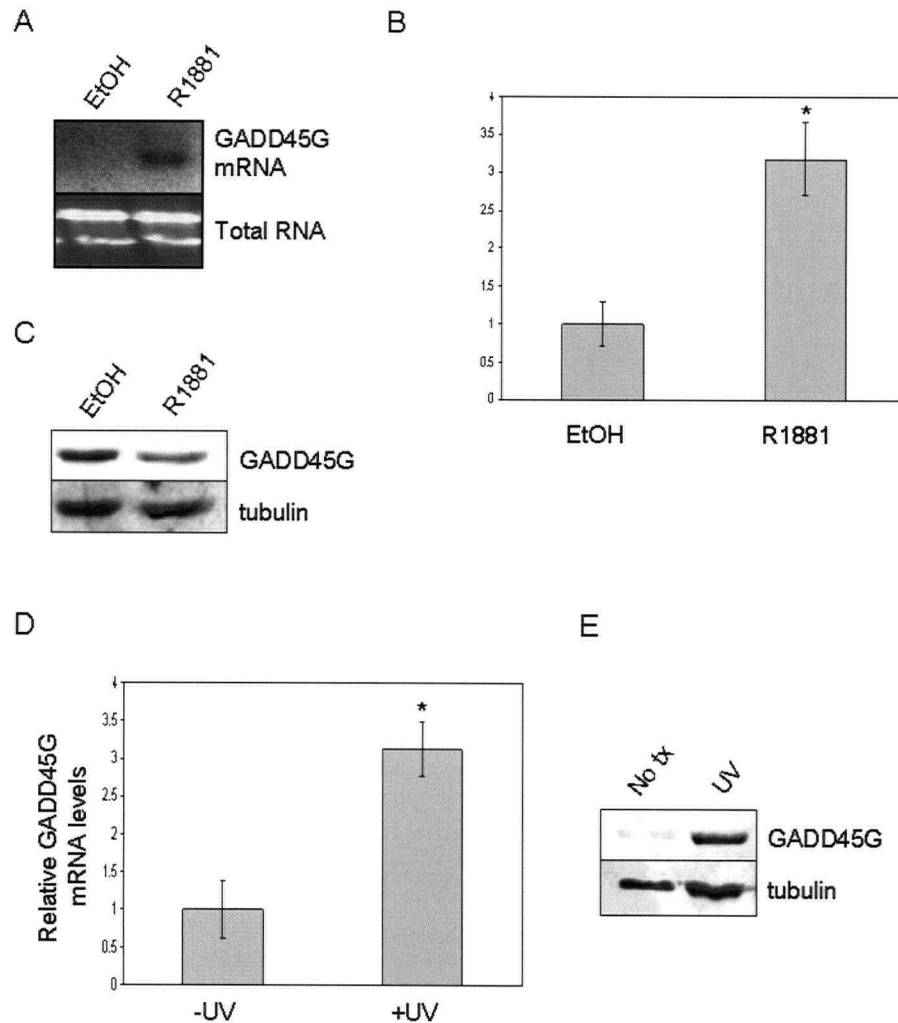
Figure 4.1 GADD45G mRNA levels increase with androgen treatment while GADD45G protein levels decrease. LNCaP cells were treated with 1 nM R1881 or ethanol vehicle alone for 48 hours before protein and RNA were harvested. *A*, Northern blot probed for GADD45G mRNA showing increasing levels with androgen treatment. Ethidium bromide stained gel shows rRNA as a loading control. The Northern blot was performed with three biological replicates, similar results achieved each time. *B*, Quantification of the GADD45G mRNA transcript levels using real time PCR. Asterisk indicates the R1881 treated levels are statistically different from the ethanol control with a p-value of 0.0016 (Student's t-test) with four replicates. *C*, Western blot of androgen treated LNCaP cell lysate probed for GADD45G and β-tubulin as a loading control, performed on three biological replicates and a representative blot shown. LNCaP cells were treated with or without 25 mJ UV and allowed 6 hours recovery before RNA and protein were harvested. Real time PCR (panel D) and Western blotting (panel E) were used to determine RNA and protein levels of GADD45G. The asterisk denotes a statistical significant difference between the UV and non treated sample in the real time PCR (p-value of 0.038, Student's t-test) for four biological replicates.

translation of targeted transcripts we reasoned that GADD45G was being targeted by a miRNA in an androgen-dependent manner.

The levels of the miRNA hsa-miR-326 are increased with androgen treatment – A microarray screen was performed to identify potential androgen-regulated miRNAs which could target GADD45G mRNA, thereby preventing translation of the GADD45G protein. Two sets of microarrays were probed. The first set was spotted with oligonucleotides specific for 21,521 mRNAs. The second contained shorter oligonucleotides specific for 883 miRNAs. Both miRNA and mRNA were extracted from LNCaP cells treated with or without androgens and these were fluorescently-labeled and used to probe the appropriate microarrays. We reasoned that it is possible that there is a miRNA which is upregulated by androgens and which targets GADD45G. The mRNA array data was filtered so that only genes which are sources of miRNAs that target GADD45G are shown (**Figure 4.2A**). One of the miRNA sources, arrestinβ1, was found to be consistently upregulated in the androgen treated samples compared the untreated samples. Arrestinβ1 is the source for the miRNA hsa-miR-326, which we found upregulated in the miRNA arrays (**Figure 4.2B**). The miRNA, hsa-miR-326 is derived from the first intron of arrestinβ1. Real time PCR using primers and probes which amplify only the mature miRNA were used to confirm the array result and this analysis showed that hsa-miR-326 was significantly upregulated by 1.60 fold with androgen treatment (**Figure 4.3A**). The Sanger miRBase Targets (39) database identified two regions in the GADD45G 3 UTR predicted to bind hsa-miR-326. The two
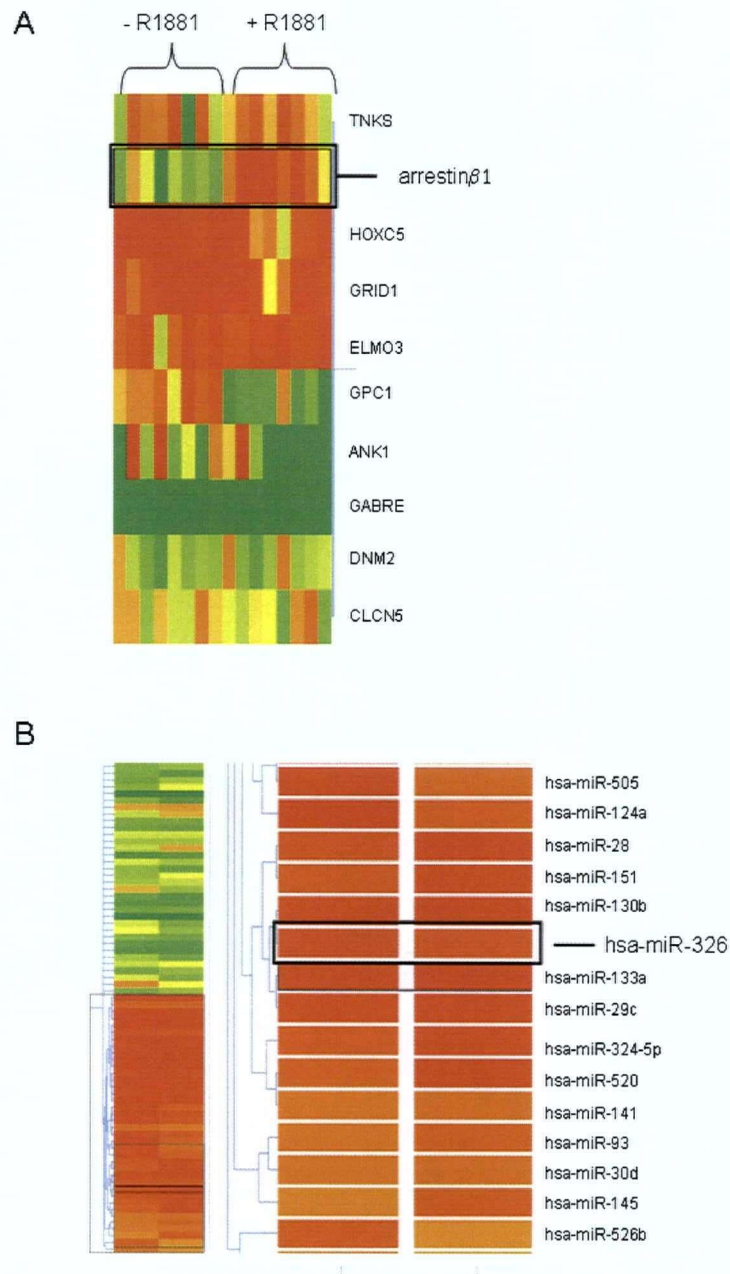
Figure 4.2 Microarray analysis of androgen treated cells reveals hsa-miR-326 and its source, arrestinβ1 are upregulated by androgens. A, LNCaP cells were treated with or without androgens and the resulting mRNA used to probe oligonucleotide arrays. The data was filtered for the sources of the miRNAs which target GADD45G. Of the sources in the microarray data, only arrestinβ1 (highlighted) is consistently upregulated with androgens. B, miRNAs were harvested from two biological replicates of androgen treated LNCaP cells and used to probe miRNA arrays. Pictured are the miRNAs which are increased or decreased with androgen treatment by 1.5 fold or more in both biological replicates. hsa-miR-326 (highlighted) is upregulated in both of the androgen treated samples.
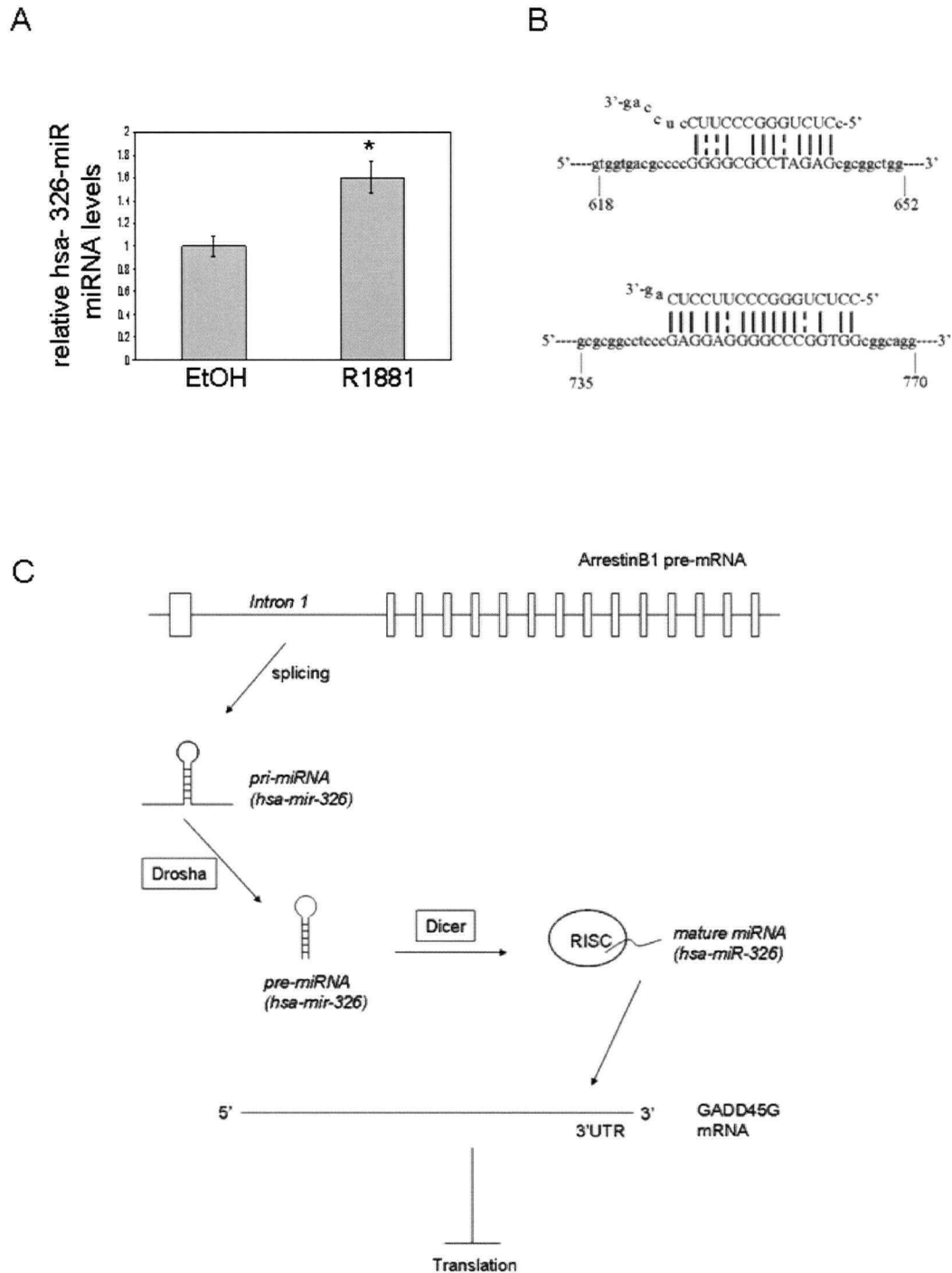
Figure 4.3 The miRNA, hsa-miR-326, is upregulated with androgens and targets the GADD45G mRNA 3 untranslated region. *A*, LNCaP cells were treated with 1nM R1881 or vehicle for 48 hour. The levels of hsa-miR-326 were assayed using real time PCR for four biological replicates. The asterisk indicates a statistically significant difference between the R1881 and vehicle-treated cells with a p-value <0.05 (Student's t-test). *B*, Two regions on the GADD45G mRNA 3'UTR are targeted by hsa-miR-326.

The top sequence shown is hsa-miR-326 and the bottom is the GADD45G mRNA. Complementary base pairing is shown by a solid line. G-U or G-T wobble base pairing is shown by a dashed line. *C*, Diagram showing the generation of hsa-miR-326 from the first intron of arrestinβ1.

predicted interaction sites are pictured in **Figure 4.3B**. **Figure 4.3C** shows a flow diagram describing the generation of hsa-miR-326 from the first intron of arrestinβ1. The first intron of the arrestinβ1 pre-mRNA, containing a hairpin loop, is spliced to generate the pri-miRNA. The pre-miRNA is produced from the pri-miRNA by digestion with drosha. Dicer further digests the pre-mir and the resulting mature, single stranded miRNA is loaded into the RISC complex. The mature hsa-miR-326, along with RISC, interacts with the 3 UTR of the GADD45G mRNA transcript and prevents its translation. It should be noted that there are hundreds of predicted targets of hsa-miR 326, however to date, none have been validated.

Inhibition of hsa-miR-326 counteracts the androgen-induced inhibition of GADD45G translation – LNCaP cells were transfected with an inhibitor (anti-miR) of hsa-miR-326, a control anti-miR or lipofectamine only, followed by androgen treatment. Protein, RNA and miRNA were harvested from the cells and the levels of hsa-miR-326, GADD45G mRNA and GADD45G protein were assayed. The levels of hsa-miR-326 were decreased to approximately 30% the levels of the lipofectamine-only controls (**Figure 4.4A**). GADD45G mRNA levels were not increased with the anti-miR treatment, and were in fact slightly decreased (**Figure 4.4B**). The GADD45G protein levels increased in the cells treated with anti-miR-326 and androgens (**Figure 4.4C**). Since there was no observable increase in mRNA levels, this increase in protein levels was likely due to increased translation in the anti-miR-326 and androgen treated cells.

There was no observable increase in protein levels in cells treated with the control anti-miR; therefore, increased translation in the anti-miR-326 and androgen treated cells is likely specific to hsa-miR-326.
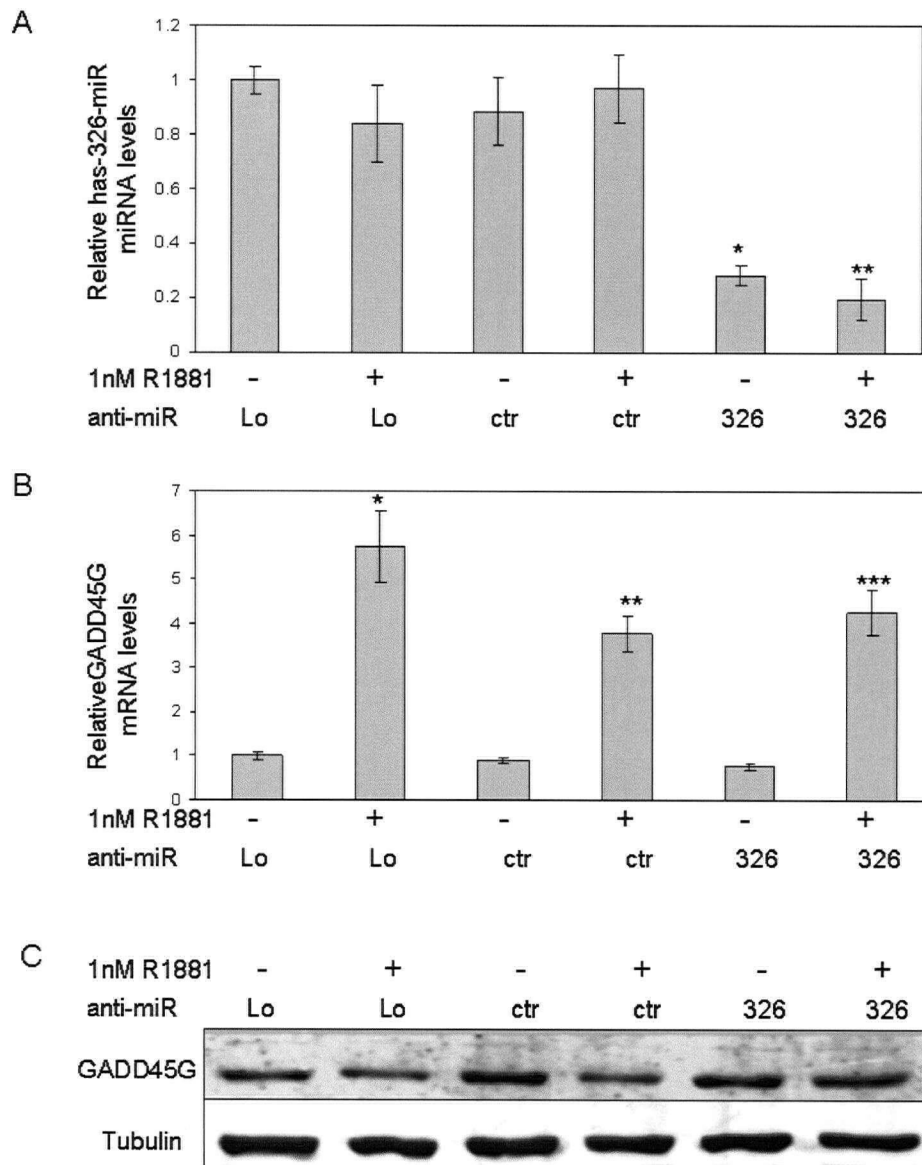
Figure 4.4 Inhibition of hsa-miR-326 relieves the translational repression of GADD45G. LNCaP cells were treated with lipofectamine 2000 only (Lo), control anti-miR (ctr) or an anti-miR that targets hsa-miR-326 (326), followed by treatment with 1nM R1881 (+) or ethanol (-). hsa-miR-326 (panel *A*) and GADD45G mRNA (panel *B*) levels were assayed using real time PCR with four biological replicates. Asterisks indicate significant differences; panel A, significant difference between Lo + EtOH and

anti-miR-326 + EtOH (* p-value 4.9×10^{-4}) and between Lo + R1881 and anti-miR-326 + R1881 (** p-value 0.027). For panel B, significance was tested between the samples treated + and - R1881 (* p-value of 0.027; ** p-value of 0.019; and *** p-value of 0.013). *C*, Western blot showing GADD45G protein levels with androgen and anti-miR treatment and β-tubulin as a loading control. Three biological replicates were performed with similar results.

Figure 4.5 Taxol treatment results in GADD45G recovery from translational inhibition. LNCaP cells were treated with 1 nM R1881 or ethanol for 48 hours followed by 24 hours of taxol (1-10 nM) treatment before protein and RNA were harvested. Real time PCR of four biological replicates was used to quantify hsa-miR-326 (panel *A*) and GADD45G mRNA (panel *B*) levels. An asterisk indicates a significant difference

between the R1881 and EtOH samples. For panel A, significant difference at 10 nM (p-value of 0.010). For panel B, significant difference between the R1881 and EtOH samples at all taxol levels (at 0 nM taxol, p-value of 0.039; at 1 nM taxol, p-value of 0.025; at 5 nM taxol, p-value of 0.0066; and at 10 nM taxol, p-value of 9.6×10^{-5}). GADD45G protein levels were assayed using Western blotting (panel C), with β-tubulin levels are shown as loading control. The Western blot was done in triplicate, with a representative blot shown.
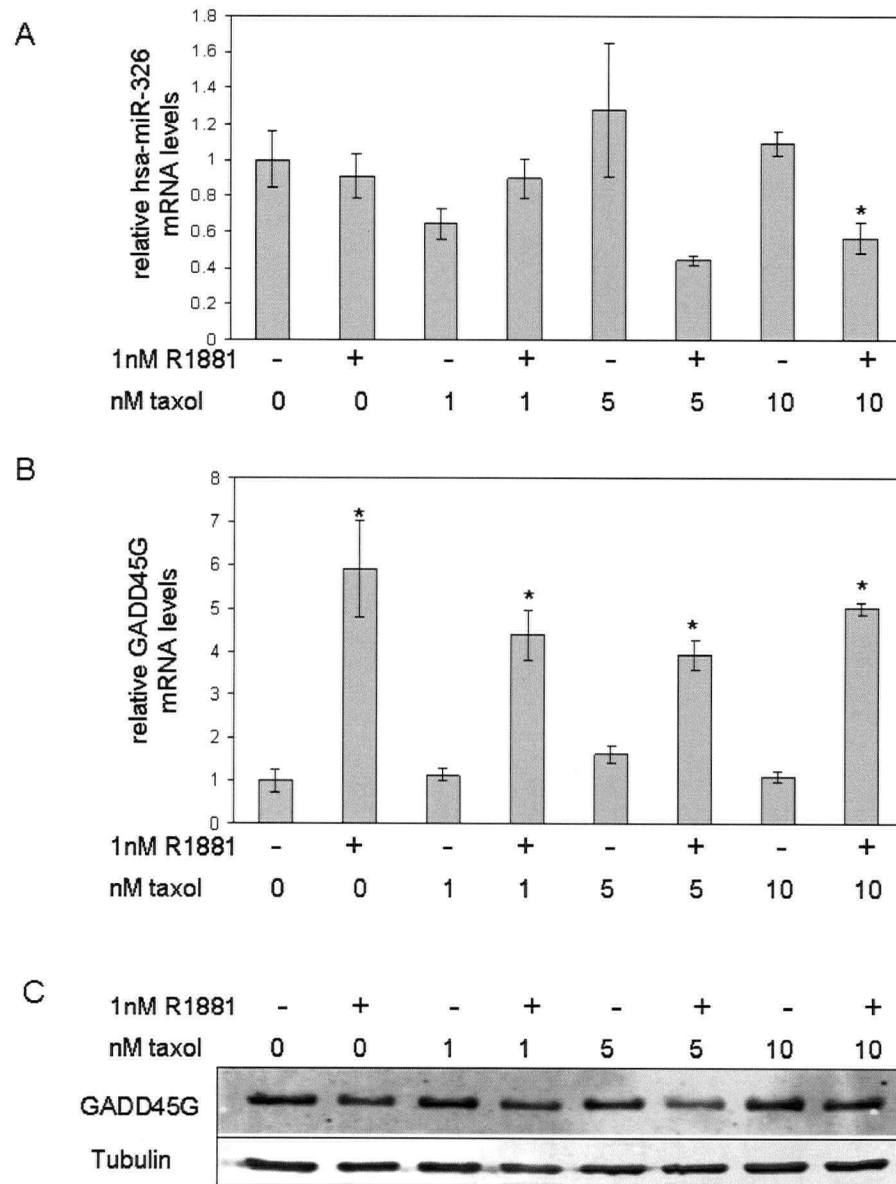
Taxol treatment relieves the GADD45G translational inhibition caused by androgen treatment – When miRNAs interact with miRNAs both become targeted to cytoplasmic processing bodies (PBs) (40). PBs contain both mRNAs being degraded as well as those which are not being translated. PBs are granular structures containing the argonaute proteins, proteins responsible for removal of the 5′ cap structure and exonucleases (41, 42). Once the appropriate trigger is sensed, the PBs can release those mRNAs that are not being translated so that the transcripts can interact with polysomes. GADD45G is inducible by cell cycle arrest and DNA damage. We reasoned that the GADD45G mRNA will commence translation when the cell senses a need for the protein and that taxol, which induces cell cycle arrest and apoptosis, may be a sufficient trigger to induce this translation. Cells were treated with or without androgens for 48 hours followed by treatment with various concentrations of taxol and assayed for hsa-miR-326 miRNA levels and GADD45G mRNA and protein levels. **Figure 4.5A** demonstrates that there is less hsa-miR-326 in the androgen treated and 5 or 10 nM taxol as compared to the ethanol controls. GADD45G transcript levels were unaffected by taxol treatment (**Figure 4.5B**); however, the protein levels increased in cells treated with androgens followed by 10 nM taxol (**Figure 4.5C**).

Figure 4.6 shows a model of GADD45G androgen-induced translational inhibition and subsequent recovery following taxol treatment. In androgen depleted cells, GADD45G

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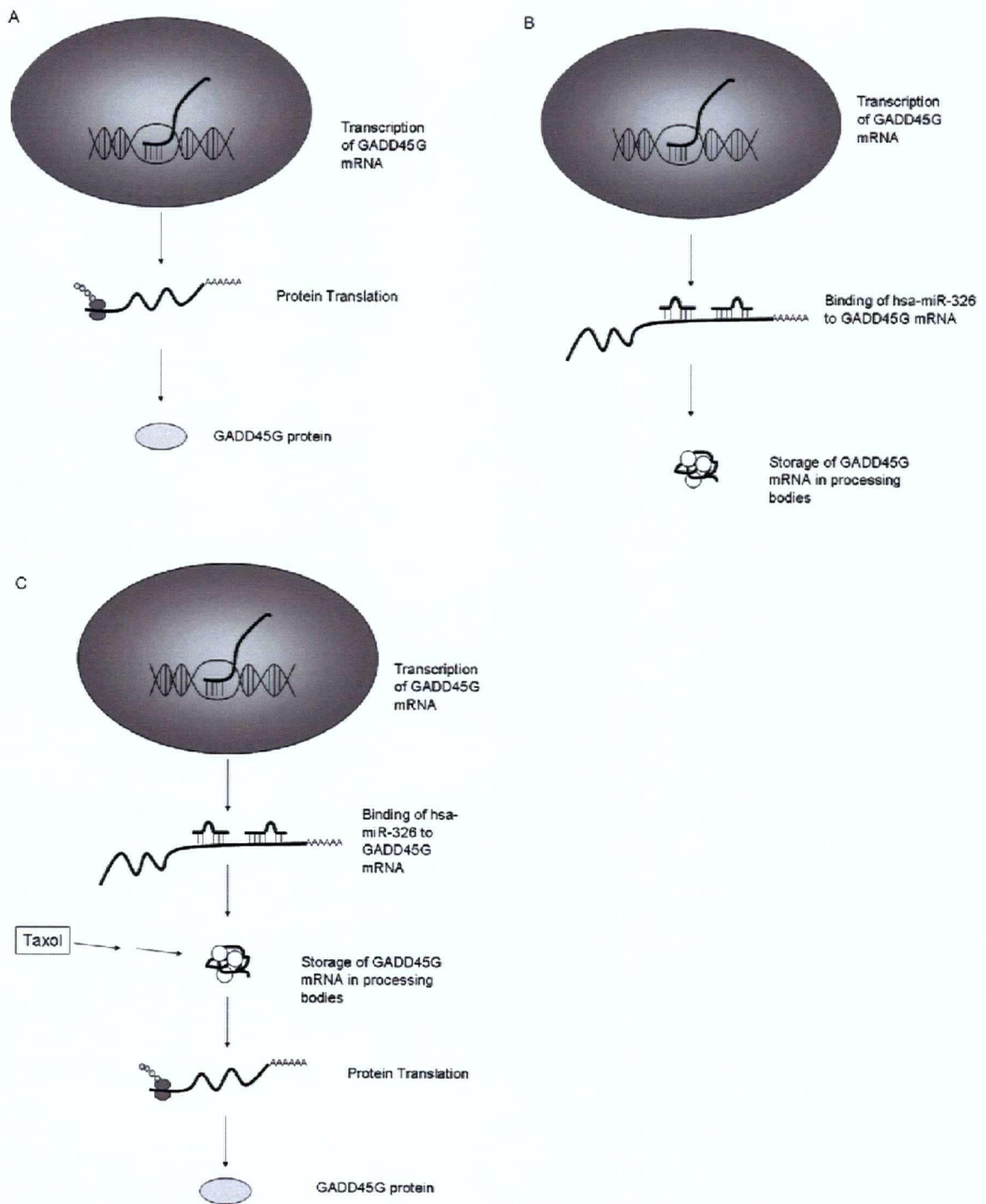
Figure 4.6 Model for androgen inhibition of GADD45G translation followed by taxol recovery. *A*, In androgen depleted cells, GADD45G mRNA is transcribed and GADD45G protein is translated. *B*, The addition of androgens to LNCaPs causes an increase in hsa-miR-326, which binds to GADD45G mRNA and causes it to be stored in cytoplasmic processing bodies. *C*, Once GADD45G mRNA is stored in processing bodies, the translational repression can be relieved through taxol treatment.

mRNA is transcribed and translated (**Figure 4.6A**). Androgen treatment induces an increase in GADD45G transcript levels, but a concurrent increase in hsa-miR-326 levels results in translational inhibition of the GADD45G protein (**Figure 4.6B**). GADD45G is important in protecting the cell against stress-induced apoptosis and androgens are cytoprotective in prostate cells. Treatment with androgens primes the cells by inducing transcription of GADD45G mRNA, but does not lead to increased translation of the GADD45G transcript, until the cells are exposed to an apoptotic stress. Cells treated with both taxol and androgens therefore show increased translation of the GADD45G protein. As shown in **Figure 4.5B**, taxol treatment itself does not cause an increase in GADD45G mRNA levels; however, when the cells are primed with androgens, GADD45G mRNA is held in stasis until a stress, such as taxol, allows the mRNA to be translated (**Figure 4.6C**).

4.4 Discussion

Several cases have been described in which miRNAs inhibit the translation of specific mRNAs. Translational repression caused by miRNAs was first described in *C.elegans*, with the inhibition of Lin-14 protein translation by the small RNA lin-4 (43, 44). Since that finding, similar miRNA activity has been described in other species, including bantam in *Drosophila melanogaster* (45) and mir-15a and miR-16-1 in humans (46). The rules for target recognition have not been fully defined; however, complementarity of pairing in the 5 region of the miRNA with the target is the most important determinant for translation inhibition (47-49). There is a requirement for the target mRNA to have a 5 cap and a poly A tail (50). The mechanism by which miRNAs

accomplish this translational silencing has not been fully elucidated. Translational inhibition requires Argonaute 2, which is a component of the RISC complex (51). The silenced mRNAs are initially targeted to polysomes (52-54). It is unclear, however, if the translation is inhibited prior to or after translation initiation (55-58). Here we show that GADD45G is targeted by a miRNA, hsa-miR-326, which prevents its translation.

Both mRNAs destined to be degraded and those whose translation is inhibited are targeted to cytoplasmic PBs. The argonaute proteins, as well as miRNAs and their target mRNAs, have been found to localize in PBs (54, 55, 59). PBs do not contain ribosomes and therefore translation is effectively blocked (60). The mRNA may not necessarily be destined to remain in the PBs indefinitely, though. PBs may simply be a storage place for mRNAs that the cell does not immediately need. There is evidence that mRNA can be released from the PBs, as seen with CAT-1 mRNA, upon stress stimuli (36). We propose that the translation of GADD45G is similarly stalled until the appropriate stress is detected by the cell, in this case taxol treatment.

GADD45G is important in mediating cell survival and protection against apoptosis in stress situations (6). Androgens also have an anti-apoptotic effect in prostate cells (61-63). It therefore makes sense that androgens would cause an increase in GADD45G levels. However, overexpression of GADD45G causes growth arrest in prostate cells and this may counteract the proliferative effects of androgens (19). Ideally, cells treated with androgens would retain high levels of proliferation while being prepared to prevent apoptosis in a stress situation.

When LNCaP cells are cultured in the absence of androgens, basal transcription of GADD45G mRNA leads to translation of the protein. For androgen-

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dependent LNCaP cells, culturing in androgen depleted media constitutes a stressful situation and the GADD45G protein would provide cytoprotection. When LNCaP cells are stimulated with androgens, GADD45G mRNA accumulates. However, in this case, the cells have an increased rate of proliferation and high levels of GADD45G protein would counteract this growth by inducing cell cycle arrest. Consequently, androgens also stimulate increased production of the miRNA, hsa-miR-326, from the arrestinβ1 intron. This miRNA binds to the 3 UTR of GADD45G leading to its storage in the PBs. We therefore observe a decrease in GADD45G protein levels following androgen treatment, presumably due to turnover of the protein that was already produced and loss of further translation due to sequestration of transcripts in the PBs. If, however, the cells are exposed to a stressor following androgen stimulation, an increase in the GADD45G protein would be expected. In this case, the cells were treated with androgens, which caused storage of GADD45G mRNA in the PBs, followed by taxol, which led to increased GADD45G protein. Taxol causes apoptosis in LNCaP cells (64, 65), while androgens are cytoprotective. We therefore propose that androgen stimulation 'primes' the cells for future apoptotic stimuli by facilitating storage of mRNA encoding anti-apoptotic genes, such as GADD45G, in PBs. It is likely that the GADD45G mRNA is not the only anti-apoptotic gene whose storage is induced following androgen exposure; however, further studies will be needed to determine which other mRNAs share the same fate.

Future studies could be done to further characterize the action of hsa-miR-326 on GADD45G mRNA. To determine if the putative binding sites on the GADD45G 3 UTR are functional, a luciferase vector can be used in which the putative hsa-miR-326

binding sites are cloned into the 3 UTR of the luciferase gene. Treatment with androgens should cause an increase in hsa-miR-326 levels and a decrease in luciferase protein, or treatment with an anti-miR will have the opposite effect. To determine if the GADD45G mRNA is being stored in PBs, in situ hybridization can be done for the GADD45G mRNA and verify that it co-localizes with components of PBs, such as the argonaute proteins. Furthermore, since hsa-miR-326 potentially targets hundreds of other mRNAs, further testing can be performed to determine if the translation or mRNA stability of any of these is impacted by androgen treatment.

Although we have shown evidence that hsa-miR-326 inhibits the translation of GADD45G, there are other possible explanations for the disconnection between the mRNA and protein levels of GADD45G in response to androgens. One such possibility is that in response to androgens, there is increased protein turnover of GADD45G. Or that the GADD45G protein become modified (cleaved or post translational modifications) which cause the protein to not be recognized by the antibody used. While other possibilities exist, the evidence provided here points to regulation of GADD45G translation by miRNAs.

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Chapter 5.   Differential Regulation of Clusterin and its Isoforms by Androgens in Prostate Cells[1]

Zhou Wang performed the rat organ culture and the Northern blot from those samples. Motosugu Muramaki aided in the design of the experiments involving the clusterin isoforms.

5.1 Introduction

Clusterin, also known as the testosterone repressed prostate message (TRPM-2), ApoJ and SPG-2, is a heterodimeric sulphated glycoprotein, first isolated in ram rete testis fluid (1, 2). Clusterin is widely expressed in many tissues including brain, ovary, testis, liver, heart, lung, breast and prostate (3) and appears to be involved in a diverse number of biological processes. There is evidence that clusterin can act as a molecular chaperone (4), and has been implicated in tissue remodeling (5), lipid transport (6, 7), cell-cell interactions (8), sperm maturation (9) and apoptosis (10). However, the exact role of clusterin in many of these processes remains unclear.

Upon castration, the prostatic epithelial cells undergo apoptosis in rat ventral prostate and clusterin mRNA levels are greatly increased (11, 12), therefore clusterin was originally thought to be repressed by testosterone. Clusterin also increases in the androgen dependent Shionogi tumor model in which castration causes a rapid apoptotic response following castration (13). However, in later studies it was shown

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that mice bearing Shionogi tumors, when treated with calcium channel blockers that prevent apoptosis, clusterin upregulation was not observed in the absence of testicular androgens (14). Similarly, if the rate of prostatic atrophy is decreased using glucocorticoid treatment, clusterin mRNA levels are reduced (15). Therefore, the current understanding is that clusterin induction is associated with apoptosis rather than being an androgen repressed gene.

Clusterin has been a somewhat enigmatic protein, being described as being both pro-apoptotic (16, 17) and anti-apoptotic (18, 19). It was later discovered that the differential translation and post-translational processing result in either a secreted or nuclear clusterin. The secreted form of clusterin has been shown to be cytoprotective whereas the nuclear form is pro-apoptotic (20, 21). An alternative splicing event generates nuclear clusterin (21-23). This splicing event removes the ER-targeting signal and allows the protein to be transported into the nucleus using a nuclear localization sequence (21).

Recent updates to GenBank have highlighted that there are two transcriptional isoforms of human clusterin (Isoform 1, NM_001831 and Isoform 2, NM_203339). These isoforms result from different transcriptional initiation sites and are only produced in human and chimpanzee. These transcriptional isoforms result in proteins that have different N-termini. Both clusterin isoforms produce proteins which are cytoprotective (24). Intriguingly, it is only Isoform 1 which is capable of producing a splice variant that results in the nuclear, apoptotic form of clusterin (21). Many studies demonstrating that clusterin has anti-apoptotic properties have been undertaken in mouse or rat and these species are only capable of transcribing the orthologue to

Isoform 1 of clusterin, however, the first exon is predicted to not be translated due to the lack of an initiating methionine in non-primate species.

Clusterin is expressed in many cancers including breast, prostate, ovarian, pancreatic and renal cancers (25-29). In prostate cancer, high levels of clusterin correlate with Gleason grade (30), which may suggest that clusterin plays a role in the aggressiveness of a tumor. The levels of clusterin increase significantly following androgen ablation therapy (31). Clusterin provides a route by which a subset of the prostate cancer cells can evade apoptosis due to androgen ablation and thereby allows the cells to grow into androgen independent (AI) cancer. In xenografts of LNCaP cells overexpressing clusterin, the tumors reach androgen independence faster than the parental cell line, suggesting that clusterin plays a role in the acquisition of the AI phenotype (11).

Prostate cells overexpressing Isoform 2 of clusterin become more resistant to Fas mediated apoptosis (32). Oligonucleotides which target Isoform 2 of clusterin endow the cells with sensitivity to radiation (33) and chemotherapeutic agents (34).

In the present study, we show that total clusterin expression is in fact upregulated by androgens. In an androgen dependent prostate cancer cell line, clusterin is upregulated at both the RNA and protein levels when treated with androgens, and this effect is reversed with anti-androgen treatment. Clusterin mRNA levels are also seen increasing in rat ventral prostate organ culture when treated with androgens. This effect is specific to androgens, as exposure to other steroid hormones does not have an effect on clusterin levels. We show that the AR directly regulates clusterin expression through interaction with an intronic enhancer region on the

clusterin gene. Furthermore, androgens have opposing effects on the two mRNA isoforms of clusterin which are generated from alternative start sites. Isoform 1 is repressed by androgens, while Isoform 2 is upregulated by androgens through direct interaction with the first intron.

5.2 Materials and Methods

Cell Culture - LNCaP cells, passage 35-50, and PC3 cells, passage 10-20, were grown at 37°C and 5% CO_2 in RPMI and DMEM, respectively, both containing 5% fetal bovine serum (FBS) and penicillin/streptomycin. Charcoal stripped serum (CSS) was prepared using FCS incubated with 1% charcoal and 0.1% dextran T-70 followed by filtration.

Hormone Treatments - R1881, dihydrotestosterone (DHT) and dexamethazone were purchased from Steraloids Inc. Progesterone, 17β-estradiol (estradiol), all-trans-retinoic acid (ATRA) and 3,3,5-triiodo-L-thyronine sodium salt (T3) were from Sigma. Casodex (bicalutamide) was from Astro Zenica, UK. All hormone stock solutions were in absolute ethanol with the exception of the T3, which was dissolved in NaOH. Prior to any hormone treatments, cells were grown in media + 2% CSS for 48 hours. Cells were harvested for protein, using RIPA lysis buffer (150 mM NaCl, 1% Igepal, 0.5% Na Deoxycholate, 0.1% SDS, 50 mM Tris) and RNA, using Trizol (Invitrogen).

Northern Blot Analysis - Total RNA (10 μg) was denatured in sample buffer and run through a denaturing 1% MOPS formaldehyde agarose gel for 1 hour at 80 V. The RNA was transferred to a nylon membrane (Biodyne B, Pall Gelman Laboratory) in 20X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 20 hours. The RNA was crosslinked to the membrane using a UV Stratalinker (Stratagene) according to

manufacturer's instructions. Membranes were prehybridized in ExpressHyb (Clontech) containing denatured salmon testes DNA (Sigma) for 3 hours at 65°C. Clusterin probes were generated using reverse transcriptase PCR from human kidney RNA using the following primers: 5-AAGGAAATTCAAAAATGCTGTCAA-3 and 5-ACAGACAAGATCTCCCGGCACTT-3. Radioactivity was incorporated into the probe using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech) to a specific activity of $1\text{-}2\times10^8$ dpm/µg. Probes were hybridized to the membrane overnight at 65°C. High stringency washes were performed on the membranes at 65°C. The membranes were exposed to Kodak MR film.

Western Blot Analysis - Protein sample (15 µg) was boiled at 95°C for 5 minutes in sample buffer containing β-mercaptoethanol. Samples were loaded on 10% polyacrylamide gels and subjected to electrophoresis for 1 hour at 150 V. Proteins were transferred electropheretically onto PVDF membrane (Millipore). Blots were probed for clusterin using a goat polyclonal clusterin-β (C-18) antibody (Santa Cruz). After incubation with a secondary antibody, the membranes were incubated with ECL reagents (Amersham) and exposed to Kodak Blue XB-1 Film. For normalization purposes, membranes were reprobed with a rabbit polyclonal β-tubulin antibody (Santa Cruz).

Real Time PCR - To quantitatively evaluate the levels of clusterin in the samples, real time PCR was undertaken. Before generating the cDNA, 2 µg of the RNA was treated with DNAse1 (Invitrogen) to remove any DNA contamination. The RNA was then reverse transcribed into cDNA in a reaction containing 1X reaction buffer, 0.01 M DTT, 1 mM dNTPs, 40 U RNAsin (Promega), random hexamers (250 ng) and 200 U of

MMLV reverse transcriptase (Invitrogen). The reaction proceeded at 25°C for 10 minutes, then at 37°C for one hour. The levels of clusterin transcript were then assayed using real time PCR on the ABI 7900 HT Sequence Detection System. To determine the levels of total clusterin, a region of Exon 3 was amplified. The primers used were; forward 5-GAGCAGCTGAACGAGCAGTTT-3, reverse primer 5-CTTCGCCTTGCGTGAGGT and probe 5-[6-FAM]ACTGGGTGTCCCGGCTGGCA[TAMRA-Q]-3. To differentiate Isoform 1 and 2 levels, primers and probes in the unique 5 untranslated regions of the respective transcripts were used. For Isoform 1, forward primer 5-CGTGAGTCATGCAGGTTTGC -3, reverse primer 5- CTGGGAGGCGCCGAAT -3 and probe 5-[6-FAM]TGTGTGCGCGAGCAGAGCGCTAT[TAMRA-Q]-3. For Isoform 2, forward primer 5-CTCTACTCTCCGAAGGGAATTGTC-3, reverse primer 5-CGGGCTGCCTGTGCAT-3 and probe 5-[6-FAM]TTCCTGGCTTCCACTACTTCCACCCC[TAMRA-Q]-3. For normalization purposes, rRNA control primers and probes (ABI) were used. The reported values are the averages and standard deviations of three biological replicates.

Organ Culture - Young adult male Harlan Sprague Dawley rats (250-300 g), from Harlan, Inc. (Indianapolis, IN), were castrated by removing testes, fat pads, and epididymis in a room dedicated for animal manipulation according to a protocol approved by the Northwestern University Animal Care and Use Committee (Chicago, IL). The castrated animals were maintained in the Northwestern University Animal Facility. The ventral prostate was dissected out from the rats 7-days after castration. Organ culture was done as described previously (35, 36). Briefly, the prostates were

minced with a scalpel to generate uniform fragments with ~1 mm in all dimensions. The prostate fragments were placed on lens paper supported at the media-air interface with a stainless steel screen in a 10 cm culture plate and incubated in 5% CO_2 incubator at 37°C. The prostate fragments were in good contact with the media but not submerged in the M-199 media consisting of Earles salts, L glutamine, and 2.2 g/L sodium bicarbonate, without phenol red, from Invitrogen, with the addition of 10% charcoal-stripped, penicillin G sodium at 200 U/ml, and streptomycin sulfate at 0.2 µg/ml. Treatment with DHT was at 1 µM final concentration for 24 hours. After treatment, the prostate fragments were harvested and frozen immediately in liquid nitrogen (N_2), and the RNA was isolated using the guanidinum/CsCl gradient method. Northern blot analysis of clusterin mRNA was carried out as described previously using a clusterin cDNA probe.

Transfections - LNCaP cells were grown to 60% confluency before being transfected with 1.5 µg of the steroid receptor expression vectors. Prior to the transfections, cells were grown in RPMI + 2% CSS for two days. Lipofectin (Invitrogen) was incubated with serum free RPMI for 30 minutes before the DNA was added and incubated for 10 minutes more. Cells were transfected with lipofectin/DNA mix for 5 hours before the media was changed to RPMI + 2% CSS. Cells were treated with 1 nM of the indicated hormone ligand for 72 hours prior to harvesting unless otherwise indicated.

Luciferase Assay - Consite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/) (37) was used to predict the location of androgen response elements (ARE's) in the clusterin gene. Luciferase reporter plasmids were created by cloning the PCR generated fragments into pTK-luc (ATCC). Both LNCaP and PC3 cells were plated into

6 well plates then using Lipofectin, the cells were transfected with 3 µg of reporter plasmid, 2 µg of rat AR in pRcCMV (Invitrogen), and 0.05 µg of the renilla expression plasmid pRLTKS (Promega) per plate. After transfection, the cells were incubated with either 2% CSS medium alone or with R1881. Cells were washed and harvested with passive lysis buffer (Promega). PC3 cells were harvested after 48 hours of hormone treatment, and LNCaP cells were harvested after 72 hours. Luciferase activity of 20 µl aliquots of lysate was determined using the Dual-Luciferase Reporter assay system (Promega) on a luminometer (Berthold, Germany). Luciferase activity was normalized for transfection efficiency using renilla activity. Experiments were done in triplicate, averaged, and expressed as fold induction.

Chromatin Immunoprecipitation - Plates of LNCaP cells were incubated with formaldehyde at a concentration of 1% for 10 minutes on a shaking platform. Glycine was then added to a final concentration of 0.125 M for 5 minutes. The cells were washed twice with ice cold PBS before being scraped off the plate with 5 mM EDTA in PBS. The cells were then pelleted and washed with ice cold PBS. The pellet was resuspended in 500 µL cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCL, 0.5% NP40) and incubated on ice for 10 minutes. The sample was centrifuged and the supernatant removed. The nuclei were then lysed by adding 1 ml nuclei lysis buffer (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1% SDS) and incubating on ice for 10 minutes. The samples were then sonicated on setting 3 (Sonic Dismembrator 550, Fisher Scientific), four pulses of 15 seconds, separated by 30 seconds. The sonicated chromatin was then precleared by rotating at 4°C for 2 hours with normal rabbit IgG (2 µg/ml), salmon sperm DNA (20 µg/ml) and 40 µL protein A/G agarose beads (Santa Cruz) that have

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been pre-washed with dialysis buffer (2mM EDTA, 50 mM Tris-Cl pH 8.0, 0.2% Sarkosyl), followed by brief centrifugation. For the immunoprecipitation, 200 µL of the precleared sample was diluted with 400 µL of dilution buffer (0.01% SDS, 1.1% Triton X 100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167 mM NaCl). The sample was then rotated overnight, at 4°C with 2 µL of normal rabbit serum or 5 µL of rabbit polyclonal AR antibody (Upstate). Protein A/G agarose beads that have been pre-washed with dialysis buffer were added and the samples were rotated at 4°C for 2 hours. The samples were centrifuged briefly and the resulting pellet was washed twice with dialysis buffer and four times with IP wash buffer (100 mM Tris-Cl pH 9.0, 500 mM LiCl, 1% NP40, 1% deoxycholic acid). Between washes, the samples were rotated at 4°C for 3 minutes. To the pellet, 150 µL of elution buffer (50 mM NaHCO3, 1% SDS) was added and the samples were shaken vigorously at room temperature for 15 minutes. The samples were centrifuged briefly, the supernatant collected and the elution process repeated. For input, 200 µL of the pre-cleared DNA is diluted into 100 µL of elution buffer. To the pooled elutions and the input sample, NaCl (final concentration of 0.3 M) was added and the samples were incubated at 67°C overnight to de-crosslink. The DNA was ethanol precipitated and the protein digested with proteinase K (Invitrogen). The DNA was purified using a PCR purification kit (Qiagen). Thirty five cycles of PCR was then performed using 1 µL of the chromatin as template. Primers for the PSA enhancer: 5 - CATGTTCACATTAGTACACCTTGCC-3 and 5 - TCTCAGATCCAGGCTTGCTTACTGTC-3 . Primers for Clu 1, which encompasses region +2094 to + 2433 of the clusterin gene were: 5 -CAGCCCTGCAGTGATTCATA- 3 and 5 -CCCGACCCCTAGTCCAGTAT-3 . Primers for Clu 2, which encompasses

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region +2443 to +2761 were: 5-CTCTACTCCCAGGGTTACCA-3 and 5-CCCCTGAAAGCAACAACTTC-3. Primes for Clu 3, encompassing +2779 and +3083 of the gene were: 5-TTCTGGCTGGCTTTGTCTCT-3 and 5-GTTCCCCTTCCTGAAATGGT-3.

LNCaP xenografts – 2 x 10^6 cultured LNCaP cells were combined with Matrigel (Becton Dickinson Labware) at a 50:50 volume ratio of cells to matrigel and injected subcutaneously into four sites of 6-8 week old athymic nude mice (BALB/c strain; Charles River Laboratory). The mice were surgically castrated 8 weeks post injection under methoxycluorane anesthesia. To harvest the tumors, the mice were sacrificed using carbon dioxide asphyxiation, the tumors were removed and frozen immediately at -80°C. Androgen dependent (AD) tumors were harvested 8-14 days post castration while AI tumors were harvested 22-35 days post castration.

Statistical analysis – Error bars on the real time PCR graphs represent standard error of the mean. The luciferase data was analyzed using a Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test to determine statistical significant differences between EtOH and R1881 treated samples using Prism (Graph Pad). For the real time PCR, statistical significance was measured using a Student's t-test (Microsoft Exel).

5.3   Results

Clusterin is Upregulated by Androgens in a Dose and Time Dependent Manner - To determine if clusterin levels are affected by androgens *in vitro*, LNCaP cells were treated with 1 nM R1881 for a time period ranging from 16 to 72 hours. Northern blot analysis of those samples reveal that when compared to the time-matched vehicle

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Figure 5.1 Clusterin mRNA levels in response to androgen treatment. *A,* Northern blot of an R1881 time course probed for clusterin in the top panel; bottom panel showing the loading control. LNCaP cells were treated with 1 nM R1881 (+) or ethanol vehicle (-) for periods of time ranging from 16 to 72 hours. Northern blots for three biological replicates were performed, all with similar results. *B,* Real time PCR quantification of clusterin mRNA levels in response to androgen treatment. The graph shows the ratio of clusterin mRNA in R1881 treated cells to vehicle control treated cells, for three biological replicates. *C,* Northern blot of R1881 titration. LNCaP cells were treated with doses of R1881 ranging from 0.01 to 10 nM R1881 for a time period of 72 hours. Northern blots for three biological replicates were performed, all with similar results. *D,* The ventral prostates from 7-day castrated adult rats were minced into small pieces and cultured in dish, as described. After overnight culture, the prostates were treated with DHT at a final concentration of 1 µM or ethanol vehicle for 24 hours. The tissues from organ culture were then harvested and isolated for Northern blot analysis. The lanes of the Northern blot shown is RNA from 50 pooled rat prostates. The lower panel is the methylene blue staining pattern, indicating the amount and quality of total RNA loaded in each lane.

control, the levels of clusterin mRNA increase with androgen treatment starting at 36 hours and continue to rise up to and including 72 hours **(Figure 5.1A).** Real time PCR was used to quantify the increase of clusterin levels in response to androgens **(Figure 5.1B)**. DNA laddering analysis of the androgen treated cells revealed no apparent increase in apoptosis over the time period when clusterin levels increase (data not shown). To verify that the response being seen is dose dependent, LNCaP cells were treated with R1881 for 72 hours in doses ranging from 0.01 to 10 nM. There is no detectable response to low levels of androgens up to 0.1 nM R1881. Clusterin mRNA levels increase in cells treated with levels of R1881 greater than 1 nM **(Figure 5.1C)**. A similar increase in clusterin mRNA levels was seen with 10 nM DHT (data not shown).

We were interested in determining if clusterin was also upregulated by androgens in the rat prostate. The rat ventral prostate is highly androgen responsive and has been used to study the affects of androgen withdrawal and as a model to study androgen regulated genes. Young male Sprague Dawley rats were castrate for 7 days and prostates were removed, microdissected and grown in organ culture with or without androgen treatment for 24 hours. RNA was harvested and analyzed for a variety of genes (38, 39). In these *ex vivo* prostate samples, the levels of clusterin RNA increased in the prostate organ culture following treatment with DHT **(Figure 5.1D)**. This data shows that clusterin is also upregulated in the normal prostate secretory epithelium in response to androgens.

To verify that levels of clusterin protein corroborate the increase detected at the mRNA level, protein from androgen treated LNCaP cells was harvested and probed by

Western blot. A time course of androgen treatment is shown in **Figure 5.2A**. In the

absence of treatment, there are low levels of clusterin expression in LNCaP cells. With

increasing duration of androgen treatment, the levels of clusterin increase starting at
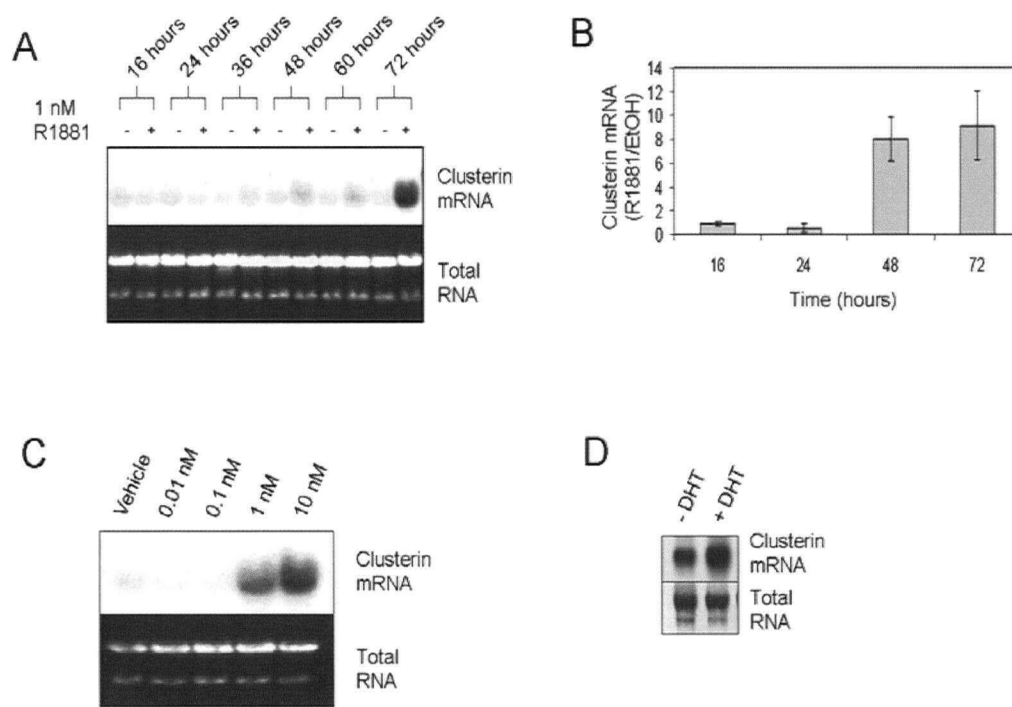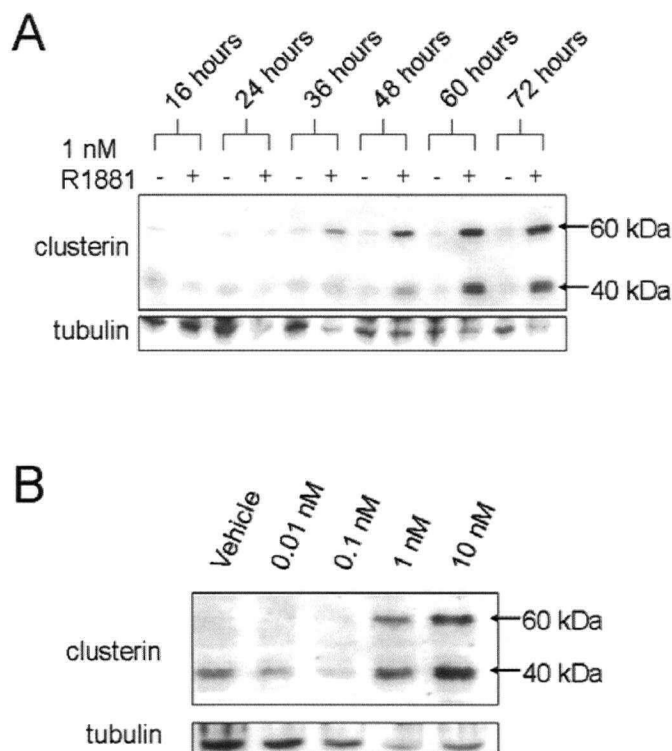

Figure 5.2 Clusterin protein levels in response to androgen treatment. *A,* Western
blot of an R1881 time course probed for clusterin in the top panel; bottom panel
showing β-tubulin levels as a loading control. LNCaP cells were treated with 1 nM
R1881 (+) or ethanol vehicle (-) for periods of time ranging from 16 to 72 hours. Shown
are both the 60 kDa (full length) and 40 kDa (processed) forms of clusterin. *B,* Western
blot of R1881 titration. LNCaP cells were treated with doses of R1881 ranging from
0.01 to 10 nM R1881 for 72 hours. The Western blots were performed in triplicate with
similar results each time.

36 hours and continuing through to 72 hours. The antibody used in the Western blots

detects the β–chain of clusterin. The two bands seen in the Western blots are the 60

kDa form and the 40 kDa cleaved form of clusterin. Both the 40 and 60 kDa bands are

seen increasing beginning at 36 hours. The dose responsiveness of the clusterin is

highly similar to that seen at the mRNA level, dramatically increasing with exposure of

R1881 at 1 nM and above (**Figure 5.2B**).

The AR is Necessary for Clusterin to be Upregulated - To prove that AR mediates the upregulation of clusterin, LNCaP cells were treated with R1881 alone or in conjunction with the AR-antagonist casodex for 48 to 72 hours. At all time points tested, casodex was able to completely block the effect of R1881 upregulation of clusterin (**Figure 5.3A and B**). The AR belongs to the steroid receptor subclass of nuclear receptors, which also includes estrogen (ER), progesterone (PR), glucocorticoid (GR) and mineralocorticoid receptors (40). Nuclear receptors are structurally similar containing a C-terminal ligand binding domain, a central DNA binding domain and an N-terminal transactivation domain (41). It is possible that the response to androgens is not specific and that other steroid receptors and their cognate hormones could also regulate clusterin expression. To test this possibility, LNCaP cells were transfected with specific hormone receptors and treated with their respective ligands to examine whether the effect being seen is confined to androgens (**Figure 5.3C**). The exogenous expression of receptors alone had no effect on clusterin levels, nor was there a noticeable effect on the mRNA levels (**Figure 5.3C**) or protein levels (data not shown) when the receptor transfected cells were ligand activated.

The First Intron of the Clusterin Gene Contains Functional ARE's - It has been reported previously that the first intron of human clusterin contained putative androgen response elements (ARE) to which the AR may bind (42, 43). To examine this further in our studies, the first intron was analyzed using Consite software, which predicts response elements within a DNA sequence and has shown the reliable prediction of validated AREs (44). Many AREs were predicted, however, a cluster of AREs was
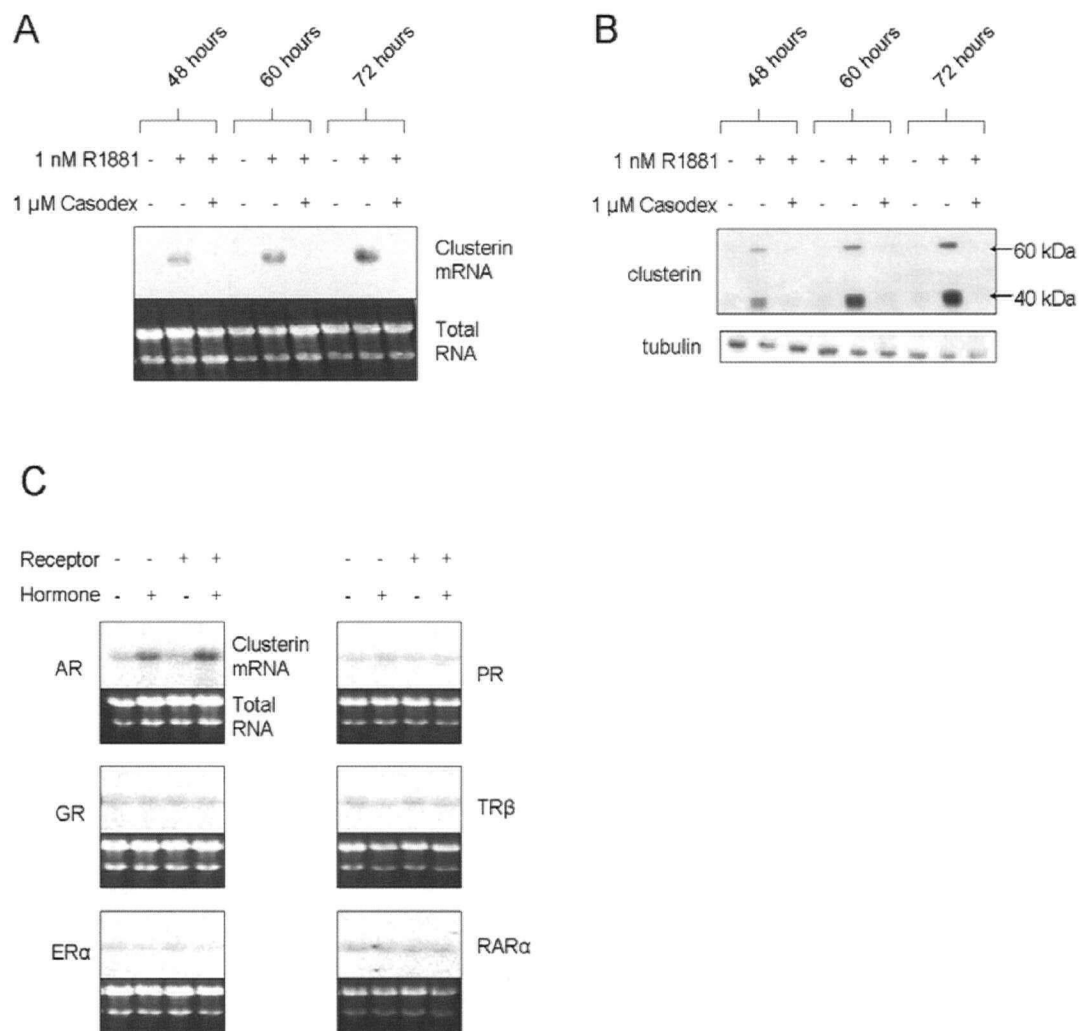
Figure 5.3 Necessity of AR in clusterin upregulation. *A,* Northern blot showing that the upregulation of clusterin is blocked by casodex, an AR antagonist. LNCaP cells were treated with 1 nM R1881 alone or in conjunction with 1μM casodex for periods of time ranging from 48 to 72 hours. *B,* Western blot showing that casodex also blocks androgen action on clusterin at the protein level. *C,* LNCaP cells were transfected with the steroid receptors AR, GR, ER, PR, TR and RAR and then treated with 1 nM of the cognate ligand (R1881, dexamethasone, estradiol, progesterone, T3 or ATRA respectively). Northern blots of these samples, probed for clusterin. The blots were performed in triplicate, with representative blots shown.

found between +2595 and +3211 of the clusterin gene (**Figure 5.4A**). It has been

These data demonstrate that an increase in clusterin levels is mediated specifically by

AR and not other steroid receptors in LNCaP cells.

shown previously that for maximal androgen responsiveness, AREs function

cooperatively (45, 46), therefore, it is likely that the androgen responsive region in the

intron lies within the cluster of AREs. Two luciferase reporter constructs were made,

both of which encompassed the region high in putative ARE's. The reporter construct

termed T21 contained the region high in ARE's in addition to the region 5 , while the

T23 construct contained the region high in putative ARE's as well as the region 3 to

this. Both constructs were tested in luciferase assays done both in LNCaP and PC3

cells. As a positive control, the same amount of a luciferase reporter construct with the

promoter region of the prostate specific antigen (PSA) was used in parallel.

In LNCaP cells, the T21 construct was more responsive to androgens than the

T23 construct and slightly more active than the PSA proximal promoter construct

(**Figure 5.4B**). This suggests that there may be ARE's or other elements on the DNA

5 to the region encompassed by both T21 and T23, which can further enhance the

activity of the DNA. In PC3 cells transfected with AR, T21 has similar transcriptional

activity as PSA in response to androgens, however, T23 shows no activity at all

(**Figure 5.4C**). The difference in the activity of the T23 construct between LNCaP and

PC3 cells shows that the response is modulated by cell type and could require

additional transcription factors. To verify that AR is binding to the clusterin intron *in*

vivo, chromatin immunoprecipitation (ChIP) was performed (**Figure 5.4D**). The

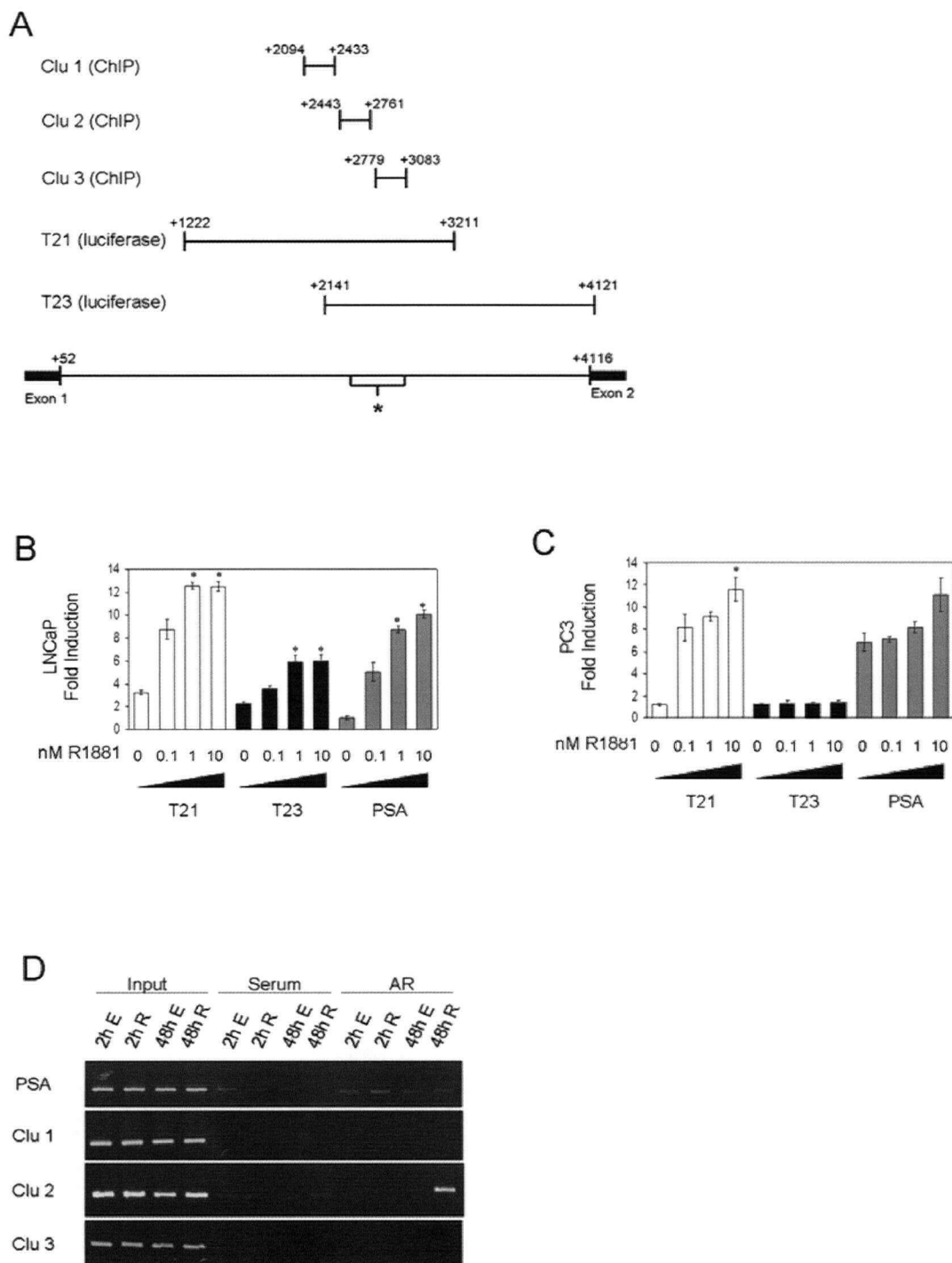**Figure 5.4 Transcriptional activation due to AR interaction with an enhancer region within the first intron of the clusterin genomic DNA.** *A,* The clusterin gene and constructs made from it for the purposes of the luciferase assay and ChIP. The asterisk denotes the region containing clusters of putative AREs. *B,* Luciferase assay in LNCaP cells. The LNCaPs were transfected with the reporter constructs, the renilla

construct and an AR expression plasmid. The cells were treated with 0, 0.1, 1 and 10 nM R1881. PSA is included as a positive control. An asterisk indicates the value is significantly different from the EtOH value (p-value <0.05) for the three biological replicates. *C*, Luciferase assay in PC3 cells. The PC3 cells were transfected with the reporter constructs, the renilla construct and an AR expression plasmid. The cells were treated with 0, 0.01, 0.05 and 0.1 nM R1881 An asterisk indicates the value is significantly different from the EtOH value (p-value <0.05). *D*, PCR products from ChIP assay. The cells were treated for 2h or 48 hours, with (R) or without (E) 1nM R1881. Immunoprecipitation using rabbit serum is a negative control showing background levels of the PCR products. PSA is a positive control for AR binding. Clu 1, Clu 2 and Clu 3 are three regions within the clusterin first intron containing putative AREs.

chromatin from LNCaP cells that had been cultured in the presence or absence of R1881 for 2 or 48 hours was immunoprecipitated with either normal rabbit serum, as a negative control, or with an AR antibody. As a positive control, PCR was performed on a region of the PSA enhancer known to be bound by AR. In the two hour time point, there is more PSA enhancer PCR product in the R1881 treated sample than the ethanol control, showing that there is more AR bound. Three regions of the clusterin intron assayed. There is no binding of AR observed in the regions of Clu1 and Clu 3. In the Clu 2 region, however, there is more PCR product in the R1881 treated samples with a 48 hour duration of treatment.

Differential regulation of clusterin isoforms - In primates, there are two transcriptional isoforms of clusterin resulting from two independent transcriptional start sites (**Figure 5.5A**). It is predicted that in human the two isoforms differ in their translational start sites, with Isoform 1 translating the first exon, whereas Isoform 2, whose transcriptional start site is in Intron 1, initiates translation in Exon 2. However, all available clusterin antibodies recognize the epitopes of the protein which are common to both isoforms, and are therefore not able to distinguish between isoforms.

Since the ARE containing region in the first intron is located between the two transcriptional start sites we were interested in determining whether androgens can regulate the transcription of one or both isoforms. Primers and probes for real time PCR were designed to the unique 5 untranslated regions of the two isoforms to measure their individual transcriptional levels. Interestingly, Isoform 2 is upregulated by androgens by 24 hours and is maintained throughout the 48 hour time point, while Isoform 1 is downregulated by androgens (**Figure 5.5B-E**). The upregulation of total clusterin mRNA levels is not observed until 48 hours, suggesting that at 24 hours, the upregulation of the Isoform 2 is masked by the downregulation of Isoform 1 and there is no observable change in total clusterin levels.

The LNCaP xenograft tumor model is often used as a model for progression from androgen dependence to androgen independence. In this model, androgen regulated genes such as PSA respond to castration by being downregulated following castration up to about day 21. However, during progression to AI, androgen regulated genes, including PSA, are reactivated by about day 28 and remain elevated in AI tumors. To determine the kinetics of clusterin expression during progression to AI, tumors from an LNCaP tumor series were harvested for RNA. A northern blot of PSA levels in the tumors prior to and following castration is shown (**Figure 5.6A**) and the corresponding serum PSA levels are shown in **Figure 5.6B**. The tumor volumes of the
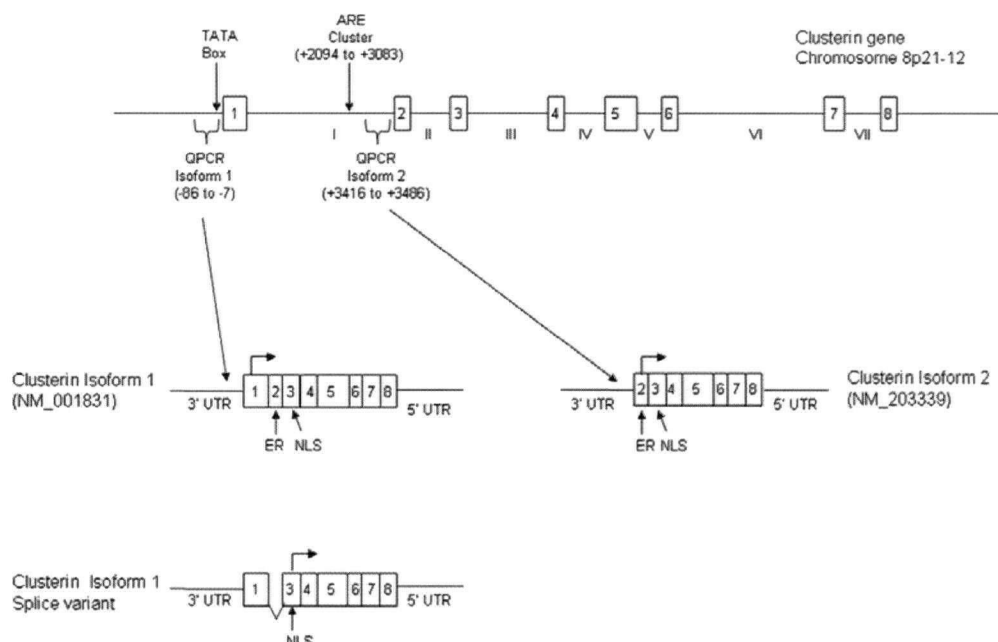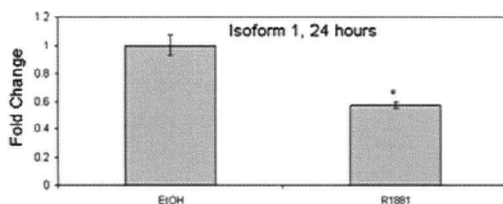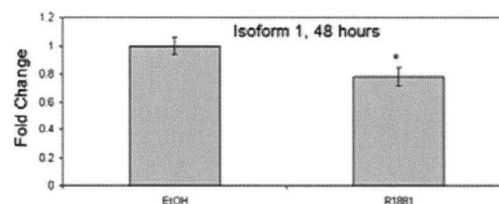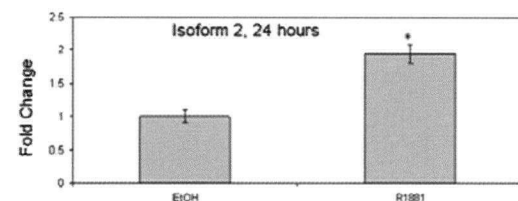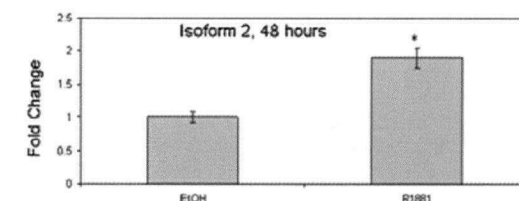
Figure 5.5 Androgenic regulation of the two clusterin isoforms. *A*, The clusterin gene and the two mRNA transcripts formed from alternate transcriptional start sites. The clusterin gene is pictured with the exons appearing as numbered boxes and the introns numbered in roman numerals. The locations of real time PCR primers used to distinguish the two isofoms is indicated on the clusterin gene as QPCR. The bent

arrows show the translational start sites on the mRNAs. Splicing out of exon 2 results in the nuclear isoform of clusterin. The ER targeting signal is found in exon2 and the nuclear localization signal (NLS) in exon 3. LNCaP cells were treated with 1 nM R1881 for 24 or 48 hours. RNA from the cells was assayed for clustering Isoform 1 and Isoform 2 levels using real time PCR. Clusterin Isoform 1 levels at 24 hours (*B*) and 48 hours (*C*) of treatment with R1881. Levels of clusterin Isoform 2 levels at 24 hours (*D*) and 48 hours (*E*). A p-value <0.05 is indicated by *. All real time PCR was done using 8 biological replicates for each time point.

tumors are shown in **Figure 5.6C**. Unlike normal prostate cells, LNCaP tumors do not undergo apoptosis in response to castration. The tumor volumes instead cease growing until AI, where proliferation continues. Real time PCR was used to determine transcript levels of clusterin and its two isoforms. PSA is shown as a positive control and its levels increase with progression (**Figure 5.6B**). Both total clusterin and Isoform 1 levels increase moderately with progression to AI (**Figure 5.6C and D**), however, the increase is not statistically significant. Isoform 2 levels do show a significant increase in levels at AI as compared to AD (**Figure 5.6E**), consistent with the androgen regulation of this isoform.

PCR has been used previously to detect the nuclear splice variant (21). Such PCR did not reveal the presence of the nuclear splice variant of clusterin (data not shown); however, neither androgen treatment *in vitro*, nor progression to androgen independence in the LNCaP tumor model is expected to have nuclear, apoptotic clusterin expressed.
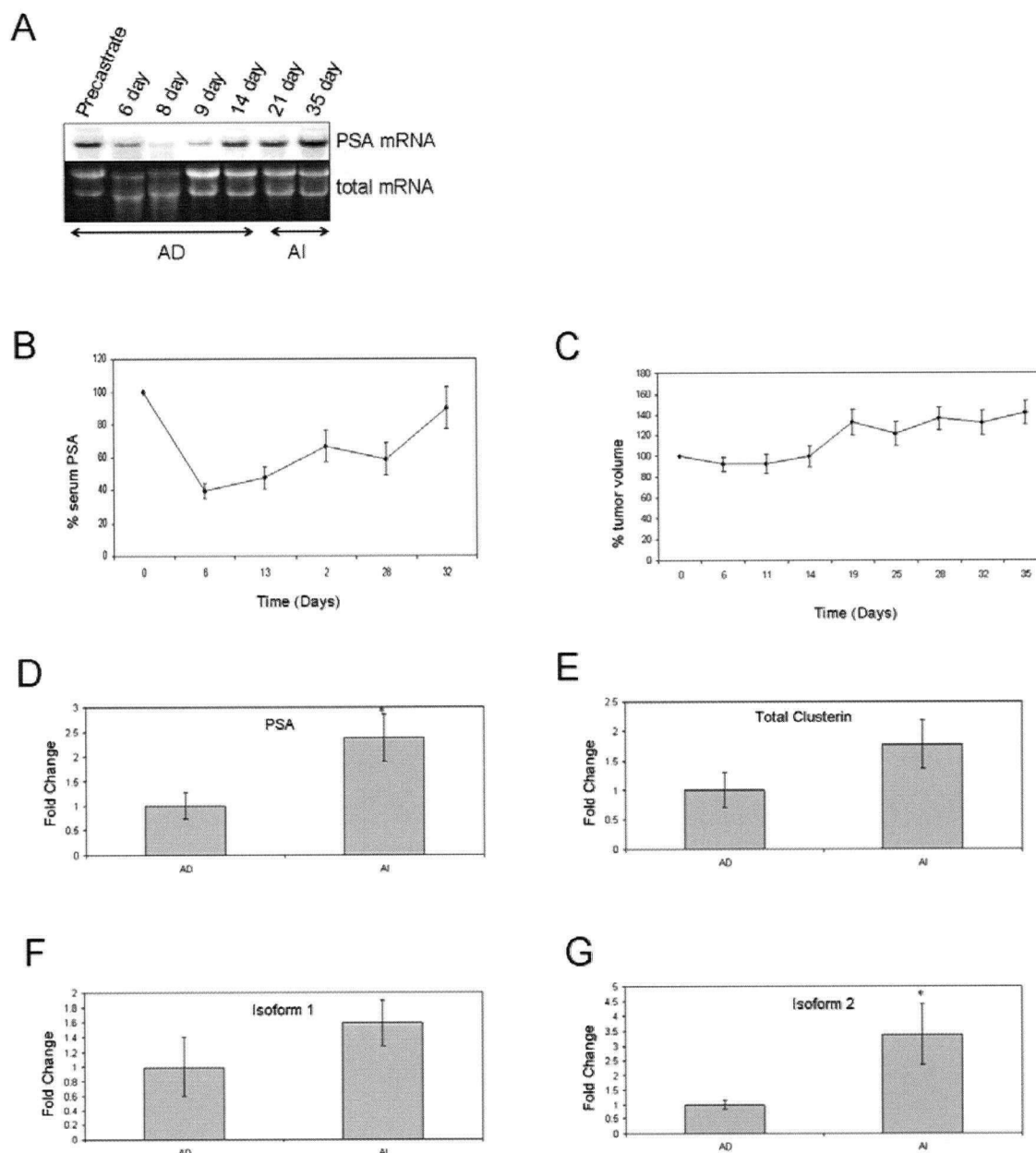
Figure 5.6 Levels of clusterin isoforms in androgen dependent and independent LNCaP xenografts. LNCaP cells were injected subcutaneously into athymic mice and tumors were harvested prior to castration and at various time points after castration. *A*, Northern blots showing the PSA levels in the LNCaP tumors as it progressed to androgen independence (AI). The serum PSA (panel *B*) and the tumor volume (panel *C*) of the tumor series is also shown. The mRNA levels of PSA (panel *D*), total clusterin (panel *E*), clusterin Isoform 1 (panel *F*) and Isoform 2 (panel *G*) were determined using real time PCR for androgen dependent (AD, using day 8 samples) or AI (using day 35 samples). A p-value <0.05 is indicated by *.

5.4   Discussion

Levels of clusterin increase dramatically in the rat ventral prostate upon castration, leading to its designation as a testosterone repressed message. Later studies demonstrated that the increase in clusterin was most likely a stress response brought about by castration-induced apoptosis of the prostatic epithelial cells and is perhaps regulated in concert with the heat shock proteins (16, 47). The androgenic regulation of clusterin has been primarily studied *in vivo* by removal of androgens by physical or hormonal castration of rats or on mice bearing xenograft tumors (48-50). However, in this context, it is difficult to dissociate the androgenic and the apoptotic stimuli. In the Shionogi tumor model, apoptosis is blocked by calcium channel blockers, clusterin levels do not increase upon castration (14). Therefore, studying the effects of androgens on prostate cells in cell culture and in organ culture avoids the confounding influence of the multiple signals present in the *in vivo* system.

In the present study we have demonstrated that in both human prostate cancer cells and in the rat ventral prostate, total clusterin is in fact upregulated by androgens. Androgen treatment causes an increase in the levels of both the total clusterin mRNA as well as the resulting protein, clusterin. The effect seen is both time and dose dependent, while independent of apoptosis.

Androgens are necessary for the growth and differentiation of normal prostatic epithelium. It has been shown that clusterin plays a role in many different types of cancer as a cell survival factor and helps cancerous cells to evade stress induced apoptosis. We propose herein, that clusterin may also play a cytoprotective role in normal prostate cells under the regulation of androgens. Androgens themselves have

long been known to promote survival of prostatic epithelium and they appear to increase the cell's ability to withstand apoptotic stimuli (47). Androgens have been shown to play a cytoprotective role by decreasing the levels of pro-apoptotic proteins such as the caspases and members of the Bcl-2 family of proteins (51, 52) as well as preventing the release of cytochrome c from the mitochondria (53). It is therefore not surprising that androgens could increase the levels of anti-apoptotic proteins such as clusterin, which would act to protect prostate cells against apoptosis.

The transcriptional regulation of the clusterin gene is likely quite complex. When a cell undergoes a stress response to stimuli such as heat shock, clusterin is induced. It was shown that in this scenario, the heat shock transcription factor, HSF-1, binds to a heat shock element in the 5 promoter region of the murine clusterin gene and induces its expression (54). In addition to stress induction, there are various growth factors that can induce clusterin expression, including transforming growth factor β, nerve growth factor and epidermal growth factor (55-57). These growth factors have been shown to require an AP-1 site found in the 5 proximal promoter region of the clusterin gene to induce its expression.

Other steroid hormones have been shown to have effects on clusterin expression in other cell types. A number of studies have reported that estradiol treatment can cause an increase or decrease of clusterin levels in uterus and endometrium (58-60). As well, vitamin D has been shown to increase clusterin levels in human breast cancer MCF-7 cells (61, 62). Although these steroids have been implicated in clusterin regulation, there has not been shown to be direct interaction of the cognate steroid receptor with the clusterin gene and they are possibly downstream

effects. In contrast, we have shown that the AR does interact with a regulatory region found in the first intron of the clusterin gene and increases the transcription of clusterin in prostate epithelial cells.

It has been postulated that there are AREs within the first intron of both the rat and human clusterin genes and that the AR could bind to these response elements and repress the expression of clusterin (42, 43, 63). We have shown that the AR does bind specifically to the clusterin intronic DNA, resulting in increased transactivation in a reporter assay. The ChIP assay demonstrated that although AR does bind a region of the clusterin first intron *in vivo*, binding does not occur at 2 hours post androgen treatment as it does for the PSA enhancer region. Rather, AR binding occurs later, within 48 hours after androgen treatment. We therefore hypothesize that there may be repressor proteins at play which prevent AR from binding or other positive acting factors or chromatin remodeling needed for AR to bind and initiate assembly of a transcription complex.

AR is known to induce transcriptional activity through interaction with AREs. However, there is little known about its ability to directly cause transcriptional repression. Therefore, it is unclear as to whether the direct interaction of AR with the intronic region of clusterin is the cause of the downregulation of Isoform 1 or if this is an effect of transcriptional interference.

The differential regulation of two clusterin isoforms may be critical to regulation of the opposing apoptotic and anti-apoptotic functions of the two isoforms. Both isoforms are cytoprotective (24), however, Isoform 1 has the capacity to produce the nuclear, apoptotic form of clusterin generated by alternative splicing (21). In the

context of androgens, which are cytoprotective in prostate cells, we show that androgens specifically upregulate Isoform 2 of clusterin while downregulating Isoform 1. Studies performed in other species demonstrate the anti-apoptotic nature of clusterin, and interestingly these other species only produce an orthologue equivalent to Isoform 2, as they lack an initiating methionine in the first exon.

Isoform 2 is shown to increase during progression to AI in LNCaP xenograft tumors and this correlates with the induction of PSA at AI. This is consistent with Isoform 2 being an androgen regulated gene as there is a reactivation of AR signaling at AI, demonstrated by the recurrence of PSA and other androgen regulated genes (64, 65). AR remains important as tumors progress to AI, and as such these tumors retain high expression levels of AR (66) and downregulation of AR using siRNA causes an increase in apoptosis (67). Therefore as prostate tumors progress to AI, the reactivation in AR signaling is functionally important for maintenance of cell viability. Clusterin is also important for evasion of apoptosis during progression to AI (31, 68). In that context, AI prostate cells will seek to upregulate the anti-apoptotic form of clusterin and as such, will upregulate Isoform 2, which cannot be spliced into the apoptotic form.

The data presented herein suggests that androgens may be cytoprotective in secretory prostatic epithelial cells in human in part through upregulation of clusterin Isoform 2 and downregulation of clusterin Isoform 1. This is not only important in the maintenance of normal prostate, but also during the progression to androgen independent prostate cancer.

5.5 References

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Chapter 6 Discussion

In this Ph.D. dissertation, I have described a microarray screen for androgen regulated genes in LNCaP cells and I have further characterized two important genes, GADD45G and clusterin. The following discussion will focus on the significance of the results, the technologies used and how they have helped achieve the results, the strengths and weaknesses of the research and future directions.

6. 1 Microarray Technologies

The microarray platform allows for a single experiment to test a large number of data points. In the work presented here I relied on several different types of microarrays, used either as screens or to validate previous studies. Having the ability to perform a single experiment that results in hundreds or thousands of data points is very powerful. One of the advantages to working at the Prostate Centre is that it houses an array printing facility which can generate the oligonucleotide, miRNA and antibody arrays, as well as a pathology lab which can generate the tissue microarrays. Having the arrays being produced in house makes them readily available for use in experiments.

Since AR signaling is important to the development of prostate cancer and its progression to AI, it was of interest to determine which genes it regulates. Of particular interest were genes predicted to be involved in cell survival of prostate cancer cells as these may be potential therapeutic targets. Microarrays were chosen as the method to screen for androgen regulated genes. The advantages of using a microarray screen are the large number of genes that can be queried, the high throughput nature of the

technology and in-house printing of the slides makes the experiments relatively inexpensive (1).

Since the microarray experiment presented here was performed, there have been several improvements to the technology. The oligonucleotide set used for this screen contained 13,971 individual oligos, while the newer set contains 21,000 individual oligos. As time continues, there will be larger oligo sets and theoretically, every gene in the human genome can be represented on a microarray. When one considers that many genes have multiple isoforms resulting from differential transcriptional start sites or differential splicing - one oligo per gene is not sufficient for a complete screen of mRNA expression. Using clusterin as an example, the two isoforms arising from different transcriptional start sites can have vastly different biological consequences. An interesting footnote here is that the oligo for clusterin recognizes both isoforms and we have not observed any changes in its expression in response to androgens in our array experiments, possibly because one isoform is repressed by androgens while the other is activated, cancelling out the measured response.

One of the issues with the cDNA labeling is that there may be dye bias when the labeled nucleotides are incorporated by the reverse transcriptase into the cDNA strand (2). To normalize against dye bias, dye swap experiments were performed. To avoid the dye bias altogether, now we use a dendrimer kit from Genisphere in which the oligo used in the generation of the cDNA contains a capture sequence to which the dyes anneal to. In this way, there is no differential incorporation of the two dyes.

In the years since performing the microarray experiment, there have been numerous other array experiments performed in our lab with samples in the presence or absence of androgens. These experiments have confirmed the androgen regulation of the genes (GADD45G, MRP4, BARD1, NQO1, etc) that I had found in my array experiment, which lends additional confidence in the results found here.

Although mRNA microarray screen formed the basis of the research presented here, there were several other types of microarrays used. In **Chapter 3**, a tissue microarray (TMA) was used to show the expression of GADD45G in clinical samples. Although we use cell lines as models for prostate cancer, they are not necessarily representative of how a cancer acts *in vivo*. Biological diversity in the population can affect the genetic profile of a tumor, which in turn affects the biology of a particular tumor, such as its aggressiveness or how it responds to treatment. TMAs therefore offer a route to observe the expression of many patient samples with diverse genetic backgrounds. The TMAs can be stained for specific proteins, mRNAs, miRNAs or genes. In the TMA used in this study, the samples were taken from patients that had been treated with various durations of anti-hormone therapy and therefore a method of observing androgen regulated gene expression in tumors from a variety of patients.

An antibody array was also used in **Chapter 3** to determine which proteins are affected by GADD45G siRNA. While an mRNA microarray can give information about gene expression, the antibody array can give information about signaling pathways and protein expression in a given sample. The antibody array contained antibodies that recognize either a specific protein or phospho-protein and therefore can be used to look at phosphorylation cascades. This is important since these signaling cascades

often result in a change in the activity of a given protein through its phosphorylation status, with no change in total protein levels. Furthermore, there is often no change in mRNA levels of these signaling proteins and so there would be no change in expression observed in the mRNA arrays.

The technology of antibody arrays is newer than the mRNA arrays and therefore is still undergoing the process optimization for the technical aspects. There is a host of different problems involved in the printing and hybridizing of the antibody slides in comparison to the oligonucleotide arrays. Antibodies are stored in a variety of different solutions and have different viscosities which cause them to have different binding properties to the slides. There is therefore more of a variation in spot morphology than is seen in oligonucleotide spots, which must be taken into consideration when analyzing the images. When working with antibodies in western blotting, there is usually optimization of the conditions used for the most effective binding of the antibody and the protein, and often different antibodies require different conditions. With antibody arrays, however, one set of hybridization conditions has to be used for all the antibodies on the array and therefore some antibodies will not be at their optimal conditions. The proteins are labeled directly with the dyes and we have observed a strong dye labeling bias, so it is very important that dye swaps are performed for each pair of samples probed.

In **Chapter 4**, we found that although GADD45G mRNA was upregulated by androgens, the protein was actually downregulated. The lesson here was not to assume that the results seen in an mRNA array is automatically translated to protein expression. In many cases miRNAs affect protein translation without affecting mRNA

levels (3) and those effects would not be picked up if only an mRNA array was performed. Ideally mRNA arrays and antibody arrays would be probed in parallel to confirm both the RNA and protein changing. Unfortunately there is only a fraction of antibodies on the array in comparison to the mRNA arrays (approximately 600 versus 21,000). The synthesis and validation of oligonucleotides is much more rapid and consistent than for antibodies, and as a result, the antibody array is much smaller. For antibodies to be used for the array, they must first be validated to show that they do not bind any other proteins non-specifically and there is only one band seen on a western blot. Furthermore, there are many proteins for which no suitable antibody is commercially available. The antibody array is continually expanding as more antibodies become available and are validated.

The final type of array performed for this work is a miRNA array. The miRNA arrays are short oligos which are complementary to miRNAs. Since miRNAs are often derived from mRNAs, you must be sure that only miRNAs are labeled and hybridized to the slides. Kits are commercially available which will separate small RNAs (< 200 nt) from the larger ones. This will contain some small RNAs, such as 5S RNA, pre-miRNAs and miRNAs. A fraction containing only the smaller, mature miRNAs (<40 nt) can be separated from the larger ones using SDS PAGE. The miRNAs are labeled by adding a polyA tail with modified nucleotides, to which dyes can bind. Since this is the newest of the array technologies used here, there are many issues that need to be addressed. There is not much known about what the normal expression profile of miRNAs is, and importantly, there are not any validated "housekeeping" miRNAs, whose expression is not expected to change under many conditions. Furthermore,

since there is a smaller number of oligos on the miRNA slides as compared to the mRNA slides, assumptions made when normalizing the data, such as the expression of most of the genes does not change, may not be necessarily be true.

6.2   siRNA Technology

In **Chapter 3**, siRNA was used to decrease the levels of GADD45G in order to determine downstream effects on various cell signaling pathways and in **Chapter 2**, siRNA was used to downregulate NFE2L2 to look at downstream gene expression. The advantages to siRNA are that they are commercially available, inexpensive and efficient. Many companies offer siRNAs targeting to a wide range of genes, with guaranteed results. There are however disadvantages to siRNAs. One is that although transfection of dsRNA can be readily performed in cell culture, administration of dsRNA *in vivo* elicits a strong cellular based immune response (4). Another disadvantage is that there are off-target effects of siRNAs, and since only a single siRNA was used for each target gene, there is a possibility that the effects observed are not due to specific siRNA effects. Off target effects occur when there is sufficient homology of the siRNA to genes other than the target genes and this can cause them to be downregulated (5, 6). Homology in the 5' end of the siRNA is the most important for effecting downregulation of off target transcripts (7), which is also the region which is important for miRNA binding to target transcripts. To control for off-target effects, several siRNAs targeted to different regions on the target genes should be tested. If all siRNAs have the same effect, it is likely that the observed effect is specific. If only one or some of the siRNAs show the desired effect, then the observed effect is an off-target effect.

Negative control siRNA is used to correct for non-specific effects due to transfection of dsRNA into the cell. Our lab as well as others, has noted that certain genes are affected by the negative control siRNA. An example of this non-specific downregulation can be seen in **Figure 3.6A** for p53. This is not a global effect, as it is only observed for certain genes and it appears to be irregardless of the sequence of the negative control siRNA used. Since Dicer recognizes dsRNA, we hypothesized that the non-specific siRNA effects are due to an activation of Dicer which causes an upregulation of miRNAs, with resulting effects on their targets. We have shown that dsRNA, and not ssRNA, ssDNA or dsDNA causes an induction of Dicer RNA and protein in a dose dependent manner (see **Appendix 2**). To determine which genes are affected by the negative control siRNA, we have performed mRNA and miRNA and plan to also run protein arrays. When the arrays are analyzed, we will try to determine if there is a global effect on miRNA generation and activity. These results provide a possible explanation for some of the non-specific effects observed in the siRNA experiments performed here, however many of the effects will only be seen at the protein level as miRNAs often inhibit translation. One important implication of this is that an observation that the negative control siRNA has a strong effect on a particular gene is suggestive that the gene either possesses a level of homology or is a target for miRNAs.

6.3 miRNA Technologies

The existence of miRNAs was only recently described and as such, there is currently much interest in this field of study. Originally described in 1993 in *C.elegans*, it is now known that miRNAs are likely expressed in all eukaryotic cells (8). The

expression of miRNAs is important for post transcriptional gene regulation and also for cell survival (9). There are still many aspects of miRNA regulation and activity which are unknown.

In the work presented here, I have shown a specific miRNA, hsa-miR-326, which acts on the GADD45G transcript to prevent its translation. Furthermore, I showed that this translational repression could be relieved in a stress situation, such as higher levels of taxol treatments. Since the field of miRNAs is relatively new, there are still many technologies which are in development.

To show that hsa-miR-326 is upregulated with androgens, I used real time PCR. There are several different types of real time PCR that can be used to detect miRNAs, however the one used in this thesis from ABI is able to specifically detect the mature, active miRNA. This is important as other technologies, such as sybr green real time PCR, will detect the mature miRNA, as well as all of its inactive precursors. One of the difficulties with real time PCR for miRNAs mentioned earlier is that there are no known housekeeping miRNAs which can be used to normalize the real time PCR results. Since the ABI real time PCR only detects mature miRNAs, there is no need to purify miRNA from the total RNA. Therefore when running the miRNA reverse transcription reaction, a parallel reaction is run using random hexamers which I can then use to detect rRNA, which is the approach I took to normalize our real time PCRs for miRNA. In the future, we hope to identify a small RNA which can be used to normalize the real time PCRs.

Another miRNA technology that was used in this work was to downregulate the miRNA using anti-miRs from Ambion. These are used to show that the effect observed

is a result of the specific miRNA. In our scenario, we used the anti-326-miR to show that hsa-miR-326 targets the GADD45G transcript. One of the issues in working with miRNAs is that they bind to the target mRNA transcripts with inexact matches, and there are therefore many predicted targets for each miRNA. The prediction algorithms are constantly being updated and refined and the databases of miRNA targets are still under development. The number of transcripts that are predicted to be bound by a single miRNA can number in the hundreds. At times, one miRNA has several predicted hits on a single mRNA transcript. We assume that the more predicted hits on a transcript, the more likely it is that the prediction is true and that the effect may be stronger, however this may not necessarily be true. Altering the expression of a miRNA, such as I have done in the anti-miR experiments should have a larger effect on gene expression than just the one gene that we were interested in. There will be a downregulation of many of the target mRNA as well as an inhibition of translation of others. Since the expression of mRNA is associated with the production of miRNAs, then we would expect to see far reaching results in which networks of mRNAs and miRNAs are affected. Currently we are probing microarrays (mRNA and miRNA arrays) with the RNA from cells treated with the anti-326-miR to determine if there is a network of genes and miRNAs that are affected by aberrant hsa-miR-326 expression. Furthermore, this will allow us to validate which of the miRNA databases is best at predicting miRNA targets, which will prove to be valuable in future miRNA experiment.

There is a similar technology called pre-miRs, in which specific pre-miRs are transfected into cells to observe the effects of overexpressing a specific miRNA. This technology has been previously used to change the genetic profile of a cell. For

example, transfection of miR-124, which is preferentially expressed in brain, into other cell types will shift their mRNA expression profiles to be more like brain (10). To date, I am continuing the optimization process for the use of pre-miR technology and would expect that overexpression of hsa-miR-326 will result in decreased GADD45G expression, even in the absence of androgens.

We have used anti-miRs to show that hsa-miR-326 affects GADD45G translation. There are techniques which can confirm binding of the miRNA to the transcript and affecting translation. One such technique is to use a luciferase reporter plasmid to which the putative binding region of the RNA can be cloned into the 3' UTR of the luciferase gene. A cotransfection of anti-miR or pre-miRs along with the luciferase vector is performed and the luciferase activity tested. If the cloned sequence is the target sequence for the miRNA, then the specific pre-miR or anti-miR will cause a decrease or increase respectively in the luciferase activity. We are currently testing such a reporter system for confirmation that hsa-miR-326 binds to the two regions within the GADD45G transcript, as well as for other miRNAs and their targets which are of interest.

One of the exciting, new techniques being used in miRNA research is in situ hybridization in tissue samples. Since miRNAs are so small, an RNA probe itself will not have strong enough binding to be useful for in situ hybridization. Instead, an oligonucleotide containing a modified RNA molecule is used. The modification of choice is the locked nucleic acid (LNA). LNAs have a high affinity binding to RNA due to their higher melting temperature (11, 12). In the future we plan to probe patient tissues with LNAs specific to specific miRNAs. Initially the optimization will be done in

paraffin embedded LNCaP cell pellets which have been treated with or without androgens. Since we have confirmed that hsa-miR-326 is an androgen regulated miRNA, it will be used initially for the optimization. We will also use other miRNAs that have come up in our screen which have high levels in LNCaP cells and which are androgen regulated such as hsa-miR-198 and 337. The power of this technology is that patient samples, such at the NHT TMA, can be probed for miRNAs which may prove to be important in the progression of prostate cancer. If a particular miRNA is found to be upregulated in AI, then it may also be a potential target for therapeutics.

In **Chapter 4**, I demonstrated that relief of the miRNA mediated translational inhibition could be achieved with taxol treatment. We do not currently understand through which mechanism taxol releases the mRNA to be translated. It is unlikely however that the GADD45G mRNA is uniquely released and it is possible that there are other transcripts with a similar function to GADD45G which undergo the same type of stress-induced regulation. To determine which transcripts are in the processing bodies in response to androgens, and which ones are released upon taxol treatment, we plan to immunoprecipitate proteins from the processing bodies and then perform microarrays in a procedure analogous to ChIP on chip.

6.4   GADD45G as a Therapeutic Target

One of the genes of interest from the microarray screen was GADD45G, a protein involved in apoptosis and cell cycle progression. We showed the importance of GADD45G in cell viability and as a controller of important signaling pathways. Several pathways that are controlled by GADD45G have been shown to be important in prostate cancer progression to androgen independence and as such make good

therapeutic targets. One example is HSP27 - its levels are increased during progression to AI, and downregulation using antisense oligonucleotides (ASO) renders the cells more sensitive to chemotherapeutics (13). Therefore GADD45G may be a superior therapeutic agent as its downregulation will affect many important signaling pathways.

ASO technology is currently being used as a therapeutic in clinical trials for cancer. Unmodified oligonucleotides have a very short half life *in vivo*, and therefore for clinical applications, ASOs must be chemically modified to increase their serum and tissue half lives (14). Several groups have found that targeting anti-apoptotic genes with modified ASOs in combination with chemotherapeutics delays progression of the cancer over chemotherapeutics alone (15). For AI prostate cancer, some of the targets currently being evaluated include clusterin, ribonucleotide reductase, HSP27, AR and an ASO that targets both IGFBP2 and IGFPB5 (15). We believe that GADD45G transcript will be a good therapeutic target and we will be testing ASOs directed at GADD45G for their ability to effect androgen dependent and androgen independent prostate cancer growth.

Several different ASOs will be developed which target sequences along the length of the GADD45G transcript and tested for their efficacy in knocking down GADD45G protein. Previously, we have found that the ASOs that span the initiating ATG are the most successful at suppressing transcript levels. We have shown here that siRNA targeted to GADD45G can knockdown its levels and affect downstream pathways, however siRNA is not amenable to *in vivo* work and therefore currently has limited capacity for therapeutics. The most effective ASO will be tested *in vitro* to

determine if it has similar effect on the signaling pathways as observed with the siRNA, as well as its effect on cell viability. These experiments will be performed in both the androgen dependent cell line, LNCaP, and the androgen independent cell line, PC3 and they will be done in conjunction with paclitaxel to determine if there is an additive effect of the two therapeutics.

The most efficacious ASO will be tested *in vivo* in LNCaP and PC3 tumor xenografts. For the LNCaP tumor series, two sets of experiments will be performed. In the first, ASO will be administered with or without paclitaxel, in intact mice. The PSA and tumor volume will be followed to determine if GADD45G ASO is able to slow the androgen dependent growth of the tumors, and if there is increased efficacy in conjunction with taxol. In the second experiment, the tumors will be allowed to grow before the mice are castrated. The GADD45G ASO with or without taxol treatment, will be administered to the castrated mice. The PSA and tumor volumes will be tracked to determine if the GADD45G ASO is able to delay progression to AI. Finally, the GADD45G and taxol will be given to mice bearing PC3 tumors, which are not dependent on androgens for their growth, to determine if there is an effect on growth of AI tumors.

6.5 Clusterin as a Therapeutic Target

Clusterin was originally thought to be downregulated by androgens due to its upregulation in rat prostate following castration (16). It was later shown that clusterin expression is linked to the apoptotic regression which occurs upon castration rather than due to direct action of the androgen receptor (17). It has recently been demonstrated that a splice variant in which exon 2 is removed results in the nuclear,

apoptotic form of clusterin protein, whereas in the absence of splicing, the anti-apoptotic form is expressed. For the first time, I have described the regulation of the two transcriptional forms of clusterin. The importance of the two isoforms is that Isoform 2 is incapable of generating the apoptotic splice variant and therefore is always anti-apoptotic. Full length Isoform 1 is anti-apoptotic, however it has the potential to be spliced into the apoptotic form. Much of the research that has been done with clusterin's involvement in prostate cancer has been purely with the Isoform 2.

Clusterin has already proven an effective target for therapeutics using an ASO called OGX-011. Initial studies in mouse xenografts have shown that OGX-011 in combination with paclitaxel can delay progression to AI (18). Following the success of the xenograft experiments, OGX-011 was tested in Phase 1 clinical trials, in which the appropriate dosage was determined (19). The clinical trial also revealed that there was a decrease in clusterin expression in the tumor and only minor toxicity. OGX-011 is currently being tested in multi-centre phase II clinical trials across North America (19). OGX-011 is also being tested as a treatment for breast cancer and non-small cell lung cancer in addition to prostate cancer (20, 21).

One important factor is that OGX-011 targets the start codon in exon 2. Studies in our lab have now demonstrated that in LNCaP cells, treatment with OGX-011 causes a decrease in clusterin protein, but it causes an induction of the splicing event in which exon 2 is removed (unpublished data). This type of ASO-mediated "exon skipping" has been previously described with the dystrophin gene (22). In the case of clusterin, treatment with OGX-011 therefore has two effects on the same gene - it

downregulates the anti-apoptotic form while inducing a splicing event to generate the apoptotic form, an ideal situation for therapeutic uses.

6.6   Androgen Regulated miRNAs

In **Chapter 4**, hsa-miR-326 was originally found to be upregulated in response to androgens. There were many other miRNAs found to be both up and down regulated by androgens in the microarray. The question arises as to the mechanism by which these miRNAs are regulated by androgens. Undoubtedly, since some miRNAs are generated from mRNAs, androgen regulation of various genes will affect the levels of the miRNAs derived from those genes. We have however found an additional explanation as to how miRNAs are androgen regulated. In some mRNA arrays we have found that Dicer and two components of the RISC complex (Ago2 and FMR1) are upregulated with androgens, and this has been confirmed using real time PCR (**Appendix 3**). Since many of the components of the machinery which generates and aids in miRNA function are upregulated with androgens, there may be a global increase in miRNA processing from the genes being expressed that harbor miRNAs. This presents a potential problem when attempting to normalize the miRNA arrays in experiments in the presence or absence of androgens, as normalization often assumes that there are no global changes being made.

While other nuclear receptors have been shown to directly repress target genes, the AR has not. It is therefore possible that many of the genes repressed by androgens are a result of androgen upregulation of miRNAs. Androgen regulated genes have been shown to be important for progression to AI, and therefore it is possible that the miRNA machinery and miRNAs themselves play a role in progression. A recent report

has indicated that Dicer is increased in clinical prostate cancer specimens and correlates with Gleason grade (23). It would therefore be of interest to determine if Dicer levels are increased in AI clinical samples. And perhaps Dicer itself could be a potential therapeutic target. Currently, we are using an siRNA to downregulate Dicer in LNCaP cells and then to screen for its effects using mRNA, miRNA and protein array microarrays. These results will be compared to the arrays of cells treated with or without androgens to find genes common to both experiments as these will be the genes whose androgen regulation is mediated through Dicer.

There are several androgen regulated genes which are of interest to our lab which are possibly regulated by miRNAs. In **Chapter 5**, I demonstrated that the two isoforms of clusterin are differentially regulated by androgens - Isoform 1 is decreased and Isoform 2 is increased with androgens. The upregulation of Isoform 2 is a result of AR binding to AREs within the intron which is upstream of the transcriptional start site of Isoform 2. It is possible that the regulation of Isoform 1 repression is also mediated directly by the AR through this region. However another explanation is that there is a miRNA upregulated by androgens which targets the Isoform 1 transcript for degradation. Generally, miRNAs target the 3' UTRs of transcripts and the databases indicate that there are two possible 3' UTRs in the clusterin gene. There is however a lack of agreement between the databases as to which 3' UTR is associated with which isoform. We have found that the miRNA which are both androgen regulated and which target a 3'UTR of clusterin and efforts are being made to determine if these affect expression of either isoform.

It has long been known that there is a disconnection between the mRNA and protein levels of the AR gene in response to androgens. Androgen binding to the AR induces a stabilization of the protein and therefore with androgen treatment, the AR protein levels increase, however the AR mRNA levels decrease in response to androgens (24). We therefore hypothesize that there are miRNAs upregulated by androgens which can target the AR mRNA for degradation. Two androgen induced miRNAs, hsa-miR-337 and 29a, are predicted to target the AR 3'UTR.

Finally, one of the miRNAs which we have shown to be repressed by androgens is hsa-miR-1, and it is predicted to target Dicer itself. It is possible that the androgen upregulation of Dicer is not direct, rather through the downregulation of a repressive miRNA.

6.7   Significance of the Research

The hypothesis of this research was that AR regulated genes are important for the progression of prostate cancer to AI. In a microarray screen I have shown that there are androgen regulated genes which are involved in protection against oxidative stress, chemotherapeutic resistance and apoptosis. Further studies on GADD45G have demonstrated that it is a key regulator of intracellular signaling cascades which are important for progression to AI. We believe that GADD45G is an attractive target for therapeutics such as ASO to induce downregulation, and furthermore, it will be a superior therapeutic to some of the current targets as it will affect many important pathways that have been characterized to have a profound role in cancer. I have also described GADD45G as a target for translational inhibition by the miRNA hsa-miR-326. And importantly, the release of the translational inhibition by a stress such as taxol

treatment is suggesting of a novel mechanism of translational regulation in response to cellular stress. Clusterin has already been shown to be an effective therapeutic target, and here we demonstrate the differential regulation of its transcriptional isoforms by androgens. While Isoform 2 is anti-apoptotic, Isoform 1 has the capacity to produce an apoptotic splice variant. The differential regulation of two transcriptional isoforms of the same gene by androgens is a unique method of gene expression regulation and potentially has significant biological effects.

6.8 References

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Appendix 1 Up and Down Regulated Genes in LNCaP cells

Table 1 Genes upregulated 2 fold or more in all three timepoints in LNCaP cells treated with 1 nM R1881

Systematic name	fold change		
	24 h	48 h	72 h
D87953^N-myc downstream regulated	7.8157	3.0278	2.3363
L10333^reticulon 1	3.0000	2.4015	5.2750
L29008^sorbitol dehydrogenase	5.8932	9.5433	9.3026
D25215^hect domain and RLD 3	3.3018	5.5719	4.6763
D29954^KIAA0056 protein	3.4392	3.5657	4.4549
D49490^for protein disulfide isomerase-related	2.3810	2.3621	2.7670
NM_001963^epidermal growth factor (beta-urogastrone)	3.6884	2.8831	2.1813
NM_004024^activating transcription factor 3	2.2020	2.1966	2.4475
NM_004917^kallikrein 4 (prostase_ enamel matrix_ prostate)	2.4035	2.9920	2.2891
M14200^diazepam binding inhibitor (GABA receptor modulator_ acyl-Coenzyme A binding protein)	7.1542	8.0397	6.5073
M26326^keratin 18	4.8661	5.0702	3.7424
AL117666^DKFZP586O1624 protein	6.1944	2.8524	2.0481
AL122098^Homo sapiens mRNA; cDNA DKFZp434B1517 (from clone DKFZp434B1517); partial cds	8.6332	14.7893	6.9326
AL133645^Homo sapiens mRNA; cDNA DKFZp434C107 (from clone DKFZp434C107)	9.9519	8.7515	8.7053
AL050290^spermidine/spermine N1-acetyltransferase	4.6435	6.9221	5.3311
AL080081^DKFZP564F1862 protein	2.8750	2.4420	3.4736
AL117441^DKFZP434N126 protein	6.2318	5.4747	4.2391
D00726^ferrochelatase (protoporphyria)	2.0161	2.4827	3.2846
D13643^KIAA0018 gene product	2.6577	6.1148	6.2507
NM_001634^S-adenosylmethionine decarboxylase 1	2.5043	3.1151	3.6689
AL137316^Homo sapiens mRNA; cDNA DKFZp727K101 (from clone DKFZp727K101); partial cds	2.4469	2.1219	2.0200
AL137506^Homo sapiens mRNA; cDNA DKFZp761O031 (from clone DKFZp761O031); partial cds	4.7801	5.6018	6.2182
X16277^ornithine decarboxylase 1	14.9929	14.1679	8.8503
X57985^H2B histone family_ member Q	2.5449	2.0725	2.1415
X59766^alpha-2-glycoprotein 1_ zinc	3.2161	4.2948	4.7022
U96876^insulin induced gene 1	2.2391	4.6356	4.7087
X07730^kallikrein 3_ (prostate specific antigen)	5.3092	10.9480	6.5156
X12433^putative transmembrane protein	2.3120	2.2656	2.0008
X74929^keratin 8	7.2090	9.9197	9.0647
Z11793^selenoprotein P_ plasma_ 1	2.5720	3.7181	2.9641
Z83821^Human DNA sequence from PAC 296K21 on chromosome X contains cytokeratin exon_ delta-aminolevulinate	10.4102	6.3946	5.6510
X62250^glycine N-methyltransferase	3.7640	11.2933	15.8443
X66276^myosin-binding protein C_ slow-type	3.2209	3.3705	2.1704
NM_013451^fer-1 (C. elegans)-like 3	2.1002	2.1264	2.0402
NM_014182^HSPC160 protein^(PRHU04-C1-0145-BG013-001~F~3)	2.2617	2.4051	2.0547
NM_016336^Ubc6p homolog	2.9129	2.6988	3.9459

AL137663^Homo sapiens mRNA; cDNA DKFZp434G227 (from clone DKFZp434G227)	2.0615	2.1278	2.3906
NM_006117^peroxisomal D3_D2-enoyl-CoA isomerase	4.4613	6.4352	5.5698
NM_006705^growth arrest and DNA-damage-inducible_ gamma	2.0955	2.1238	4.1831
U75329^transmembrane protease_ serine 2	4.3932	4.4143	3.9383
U95006^Human D9 splice variant B mRNA_ complete cds	2.5058	2.8302	2.0416
X69141^farnesyl-diphosphate farnesyltransferase 1	2.9577	3.9782	5.3289
U07919^aldehyde dehydrogenase 6	3.0105	3.2433	3.5272
U29344^fatty acid synthase	3.7979	5.2795	4.9704
AF052107^Homo sapiens clone 23620 mRNA sequence	2.1428	4.9861	7.8038
AF054184^Sec61 gamma	2.3664	2.7279	2.6111
AF055001^KIAA0025 gene product; MMS-inducible gene	2.3334	2.2161	2.1205
AC004839^Homo sapiens PAC clone DJ0593H12 from 7p31	2.7503	3.1736	3.9210
AD001528^spermine synthase	10.6894	16.2068	14.1001
AF014403^Phosphatidic acid phosphatase type 2a	4.7198	3.9685	4.4351
AF099989^Ste-20 related kinase	3.6608	20.8987	13.0956
AF126799^delta-6 fatty acid desaturase	4.0233	7.6111	8.8959
AJ000512^serum/glucocorticoid regulated kinase	7.1648	3.7618	2.2913
AF055376^v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog	2.2479	2.4565	4.8151
AF071202^ATP-binding cassette_ sub-family C (CFTR/MRP)_ member 4	4.9248	2.1632	4.0440
AB015019^BAI1-associated protein 2	2.3793	3.5545	2.3388
AB020637^KIAA0830 protein	3.3244	3.9864	4.5000
AB020676^KIAA0869 protein	3.0711	3.0836	2.4950
AB000584^prostate differentiation factor	2.1257	3.2913	4.2328
AB011004^UDP-N-acteylglucosamine pyrophosphorylase 1; Sperm associated antigen 2	2.2021	4.6392	4.4409
AB013818^peroxisome biogenesis factor 10	2.0424	3.4418	4.9182
AB033091^Homo sapiens mRNA for KIAA1265 protein_ partial cds	2.2961	3.2413	3.6998
AC004770^hypothetical protein of unknown function	7.0639	3.8507	3.3037
AF071538^prostate epithelium-specific Ets transcription factor	4.0731	4.4714	6.8104
AB022427^stress-associated endoplasmic reticulum protein 1; ribosome associated membrane protein 4	2.4164	2.6428	2.6271
AB029020^KIAA1097 protein	2.4427	2.5575	2.0160
AL022718^odz (odd Oz/ten-m_ Drosophila) homolog 1	2.9898	4.8391	2.6735
AL031903^Human DNA sequence from clone 1032F13 on chromosome Xq25-26.3. Contains a pseudogene similar to Kera	5.6769	5.7791	5.1300
AL034348^Human DNA sequence from clone 84D21 on chromosome 6q15-16.3 Contains keratin pseudogene_ STSs_ GSSs	2.7164	3.2369	2.7366
AK002126^hypothetical protein FLJ11264	5.7388	5.0442	4.6329
AL022068^Human DNA sequence from clone 167F1 on chromosome 6p22.1-22.3. Contains a KRT18 (Keratin_ type 1 Cyt	4.6585	6.0953	6.0164
AL022333^Human DNA sequence from clone 474I12 on chromosome 22q13.1-13.2 Contains cytokeratin 18 pseudogene_	5.3007	5.0477	4.7294
AL035541^Human DNA sequence from clone 718J7 on chromosome 20q13.31-13.33. Contains part of a gene for a nove	3.2018	2.5451	2.6759
AL049977^Homo sapiens mRNA; cDNA DKFZp564C122 (from clone DKFZp564C122)	2.6391	3.8932	7.4466
U42031^FK506-binding protein 5	11.0591	22.9359	8.7910

AL034374^Homo sapiens clone 23904 mRNA sequence	3.1607	3.5734	3.0928
AL034417^Gene 33/Mig-6	7.5504	3.4729	2.3963
AK000631^hypothetical protein FLJ20624	11.0973	5.0587	13.8855
AK000759^hypothetical protein	3.0388	2.4025	2.2417
AK000860^Homo sapiens cDNA FLJ20853 fis_ clone ADKA01365_ highly similar to CORO_BOVIN CORONIN-LIKE PROTEIN P	3.1407	2.6732	2.0382
AB033062^DKFZP434N178 protein	2.7455	3.0881	3.4528
AJ001017^TBP-associated factor 172	2.8317	2.3756	2.0267
AJ002744^UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc-T7)	2.2809	2.0775	2.4402
AK001700^amino acid transporter 2	2.5112	2.6650	2.2155
AK002107^Homo sapiens cDNA FLJ11245 fis_ clone PLACE1008629	2.4268	3.5065	4.3835
AL035304^DKFZP564B167 protein	3.4655	5.2943	5.4234
AK001212^hypothetical protein FLJ10350	4.4439	2.4538	2.2774
AK001471^fatty-acid-Coenzyme A ligase_ long-chain 3	6.8060	6.5100	5.0990
AK001478^Homo sapiens cDNA FLJ10616 fis_ clone NT2RP2005453	17.8659	14.0492	10.3717

Table 2 Genes downregulated 3 fold or more in all three timepoints in LNCaP cells treated with 1 nM R1881

	fold change		
Systematic name	24 h	48 h	72 h
M62840^acyloxyacyl hydrolase (neutrophil)	0.2845	0.2666	0.1615
M76180^dopa decarboxylase (aromatic L-amino acid decarboxylase)	0.1135	0.0257	0.0692
L48728^Homo sapiens T cell receptor beta (TCRBV10S1) gene_ complete cds	0.2147	0.0442	0.1090
M36821^GRO3 oncogene	0.3067	0.1023	0.1510
NM_012133^coatomer protein complex_ subunit gamma 2	0.1697	0.3074	0.3083
NM_012275^interleukin-1 receptor antagonist homolog 1	0.2197	0.2412	0.2505
NM_006063^sarcomeric muscle protein	0.1589	0.1376	0.1533
NM_006144^granzyme A (granzyme 1_ cytotoxic T-lymphocyte-associated serine esterase 3)	0.3145	0.2050	0.2005
AL137688^copine VI (neuronal)	0.1201	0.1549	0.0783
AL161979^Homo sapiens mRNA; cDNA DKFZp761G1823 (from clone DKFZp761G1823)	0.1673	0.1089	0.3080
AL137507^Homo sapiens mRNA; cDNA DKFZp761P211 (from clone DKFZp761P211)	0.1315	0.2734	0.2779
AL137654^Homo sapiens mRNA; cDNA DKFZp434C0814 (from clone DKFZp434C0814)	0.0965	0.2427	0.0480
D86962^growth factor receptor-bound protein 10	0.2759	0.1934	0.2112
L25664^Human interferon mRNA_ complete cds	0.2076	0.2639	0.2942
D70830^double C2-like domains_ beta	0.1428	0.0535	0.2684
D86407^apolipoprotein E receptor 2	0.1987	0.1304	0.0808
U67784^Human orphan G protein-coupled receptor (RDC1) mRNA_ partial cds	0.1290	0.0224	0.1132

U76638^BRCA1 associated RING domain 1	0.2314	0.1716	0.1839
U43843^Neuro-d4 (rat) homolog	0.1297	0.2880	0.0724
U59209^UDP glycosyltransferase 2 family_ polypeptide B17	0.1789	0.1471	0.1283
X02761^fibronectin 1	0.3202	0.1550	0.2089
Y00636^CD58 antigen_ (lymphocyte function-associated antigen 3)	0.3016	0.1283	0.3219
U79248^Human clone 23826 mRNA sequence	0.3172	0.1980	0.1470
X02330^calcitonin/calcitonin-related polypeptide_ alpha	0.3297	0.3037	0.1067
S68805^glycine amidinotransferase (L-arginine:glycine amidinotransferase)	0.0794	0.0329	0.0952
S73591^upregulated by 1_25-dihydroxyvitamin D-3	0.2243	0.2292	0.2452
NM_013261^peroxisome proliferative activated receptor_ gamma_ coactivator 1	0.0199	0.1097	0.2196
NM_015980^HMP19 protein	0.3117	0.2813	0.1985
U38545^phospholipase D1_ phophatidylcholine-specific	0.2036	0.2454	0.1693
U38805^a disintegrin and metalloproteinase domain 2 (fertilin beta)	0.3001	0.2836	0.3080
U03877^EGF-containing fibulin-like extracellular matrix protein 1	0.2260	0.0100	0.1857
U24578^complement component 4B	0.2431	0.2375	0.2146
AF039555^visinin-like 1	0.1098	0.2116	0.0621
AF090099^Homo sapiens clone IMAGE 21721	0.0479	0.0638	0.3202
AF035315^Homo sapiens clone 23664 and 23905 mRNA sequence	0.1561	0.1219	0.1503
AF038963^RNA helicase	0.2295	0.1265	0.1618
AF134160^claudin 1	0.3128	0.3144	0.2025
AF134891^nuclear factor (erythroid-derived 2)-like 3	0.1573	0.0815	0.1691
AF131740^Homo sapiens mRNA; cDNA DKFZp434L1021 (from clone DKFZp434L1021); partial cds	0.1768	0.2785	0.0650
AF131838^Homo sapiens clone 25107 mRNA sequence	0.1449	0.0869	0.0649
AB011538^slit (Drosophila) homolog 3	0.1664	0.2013	0.2546
AB013924^similar to lysosome-associated membrane glycoprotein	0.1347	0.2749	0.2485
AB007865^fibronectin leucine rich transmembrane protein 2	0.1920	0.2854	0.2876
AB007872^zinc finger protein 264	0.1205	0.0439	0.1300
AB023227^KIAA1010 protein	0.1213	0.2418	0.3208
AB026187^protocadherin 11	0.2950	0.2060	0.3145
AB020631^KIAA0824 protein	0.0702	0.3095	0.3290
AB023165^KIAA0948 protein	0.2913	0.1208	0.2389
AL050281^DKFZP586G1219 protein	0.1054	0.2997	0.3279
AL117435^DKFZP434I216 protein	0.2289	0.1516	0.0473
AL050020^DKFZP564C196 protein	0.2592	0.0100	0.2108
AL050078^Homo sapiens cDNA FLJ10784 fis_ clone NT2RP4000448_ highly similar to Homo sapiens mRNA; cDNA DKFZp5	0.3272	0.0738	0.2284
AL133415^DNA (cytosine-5-)-methyltransferase 2	0.2807	0.3045	0.1614
AL137291^hypothetical protein FLJ10357	0.0882	0.2918	0.1044
AL117557^DKFZP564D177 protein	0.2146	0.0697	0.1590
AL133013^chimerin (chimaerin) 2	0.2320	0.1525	0.0100
AJ389983^Homo sapiens partial TCR beta gene for T cell receptor beta chain variable region_ germinal center 3	0.1061	0.1525	0.0886
AK000885^Homo sapiens cDNA FLJ10023 fis_ clone HEMBA1000608_ moderately similar to HYPOTHETICAL PROTEIN KIAA0	0.3109	0.2564	0.0910
AJ000730^solute carrier family 7 (cationic amino acid transporter_	0.0541	0.1154	0.1893

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y+ system)_ member 4			
AJ251847^Homo sapiens partial CHI3L1 gene for cartilage glycoprotein-39_ exons 10-13	0.1929	0.0100	0.2454
AL035122^soluble adenylyl cyclase	0.1855	0.3300	0.0224
AL049270^Homo sapiens mRNA; cDNA DKFZp564G223 (from clone DKFZp564G223)	0.1304	0.2658	0.1508
AK002137^hypothetical protein FLJ11275	0.1635	0.1343	0.1278
AK002160^Homo sapiens cDNA FLJ11298 fis_ clone PLACE1009794	0.2536	0.2236	0.0747
Y10601^ankyrin-like with transmembrane domains 1	0.1855	0.3046	0.3209

Appendix 2 Effect of dsRNA on Dicer Levels
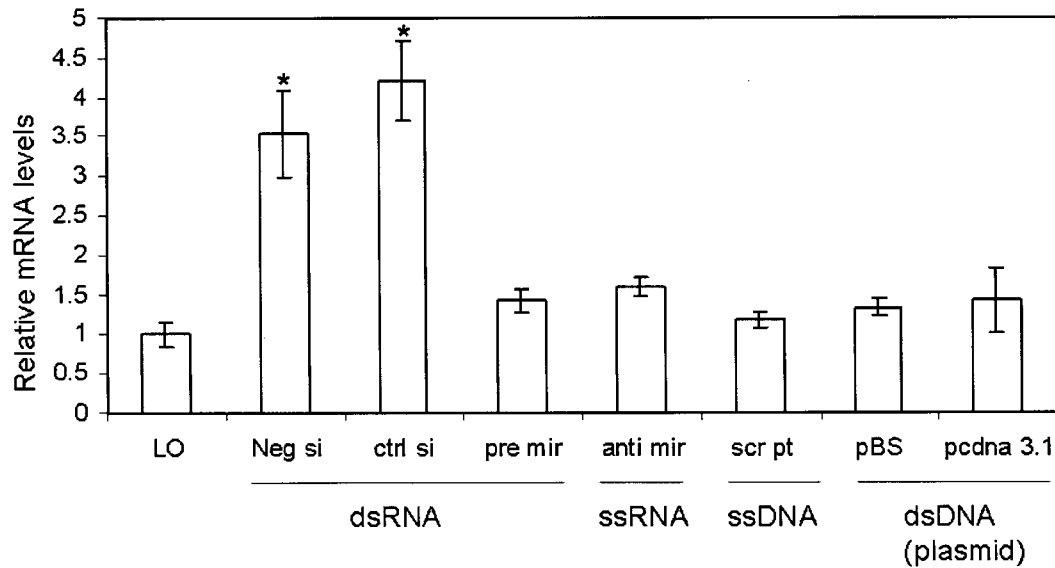
Figure AP 2.1 Dicer mRNA levels are increased with transfection of dsRNA. LNCaP cells were transfected with 50 nM of various negative control dsRNA, ssRNA or ssDNA, or 0.2ug plasmid DNA. The resulting RNA was used for real time PCR to detect Dicer levels. An asterix denotes p-value < 0.05 (Student's t-test). LO = lipofectamine 200; Neg si = Ambion negative control siRNA; ctrl = qiagen negative control siRNA; pre-mir = negative pre-mir RNA; anti-mir = negative control anti-mir; scr pt = scramble phosphothioate antisense oligonucleotide; pBS = pBlueScript empty vector; pcdna 3.1 = pcDNA 3.1 empty vector
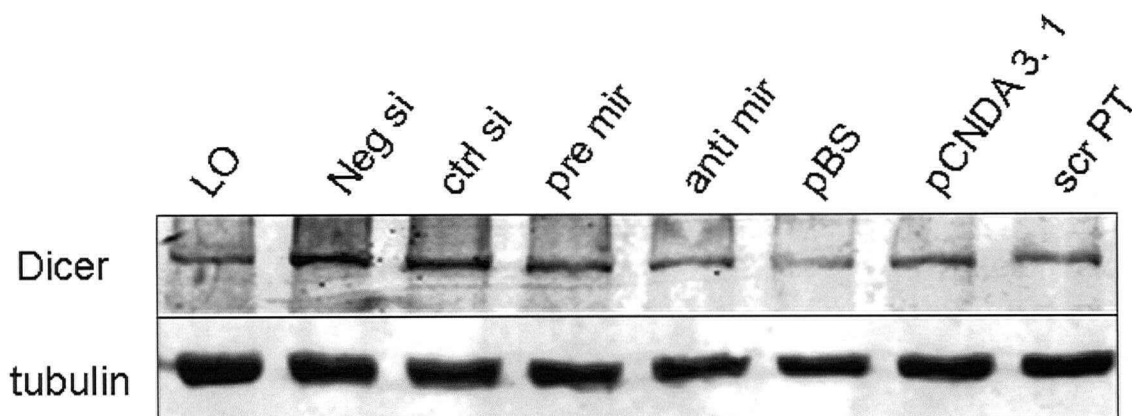
Figure AP 2.2 Dicer protein levels are increased with transfection of dsRNA. LNCaP cells were transfected with 50 nM of various negative control dsRNA, ssRNA or ssDNA, or 0.2ug plasmid DNA. The resulting protein was used in Western blotting. β-tubulin is shown as a loading control. LO = lipofectamine 200; Neg si = Ambion negative control siRNA; ctrl = qiagen negative control siRNA; pre-mir = negative pre-mir RNA; anti-mir = negative control anti-mir; scr pt = scramble phosphothioate antisense oligonucleotide; pBS = pBlueScript empty vector; pcdna 3.1 = pcDNA 3.1 empty vector
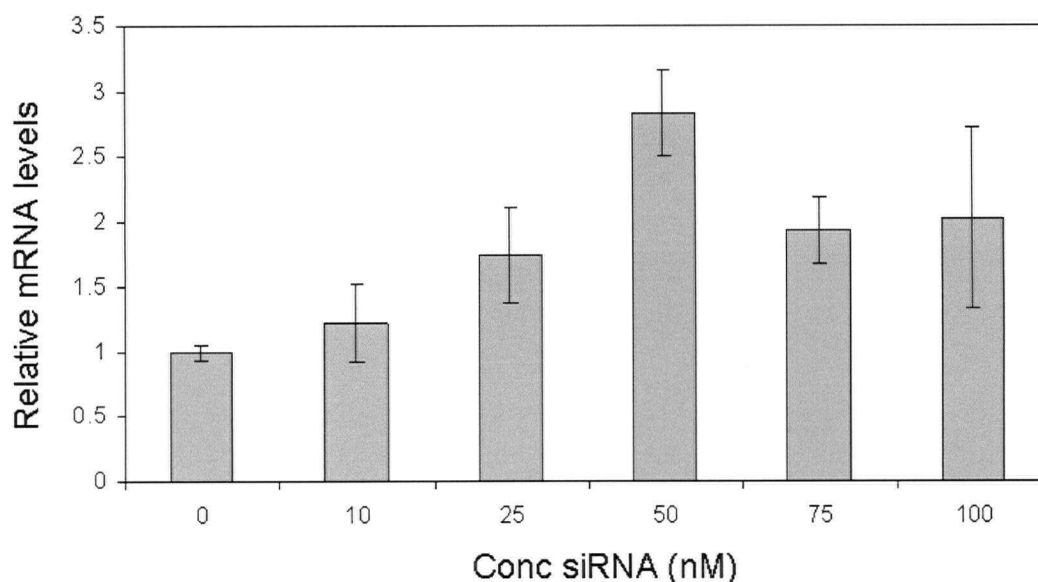
Figure AP 2.3 Dicer mRNA levels increase in a dose responsive manner with transfection of dsRNA. LNCaP cells were transfected with various concentrations of Ambion negative control siRNA. The RNA was used in real time PCR for Dicer.

Appendix 3. Androgen Regulation of miRNA Processing Machinery
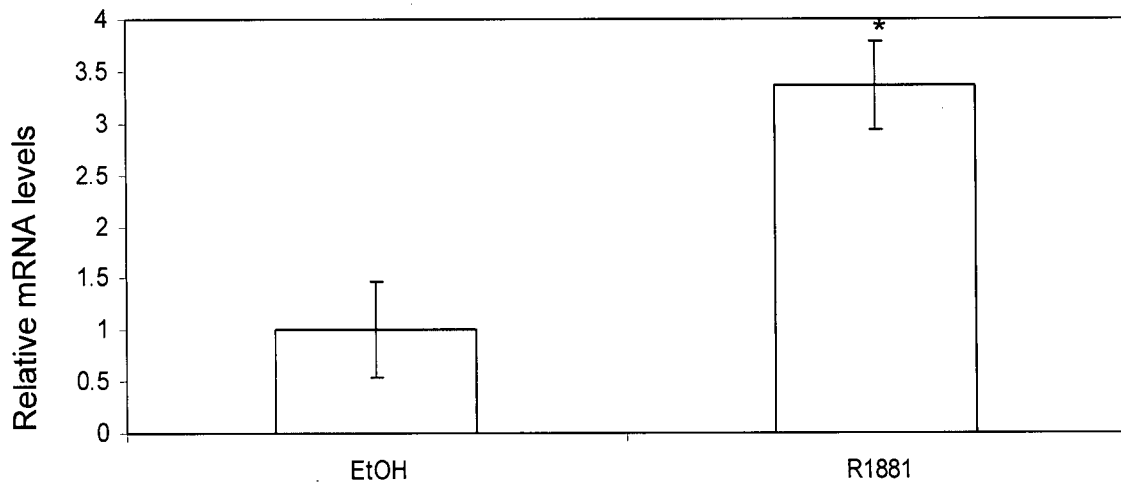

Figure AP 3.1 Dicer mRNA levels increase with androgen treatment. LNCaP cells were treated with 1 nM R1881 for 48 hours. RNA was harvested and used for real time PCR to detect Dicer mRNA. Asterisk indicates p-value < 0.01 (Student's t-test).
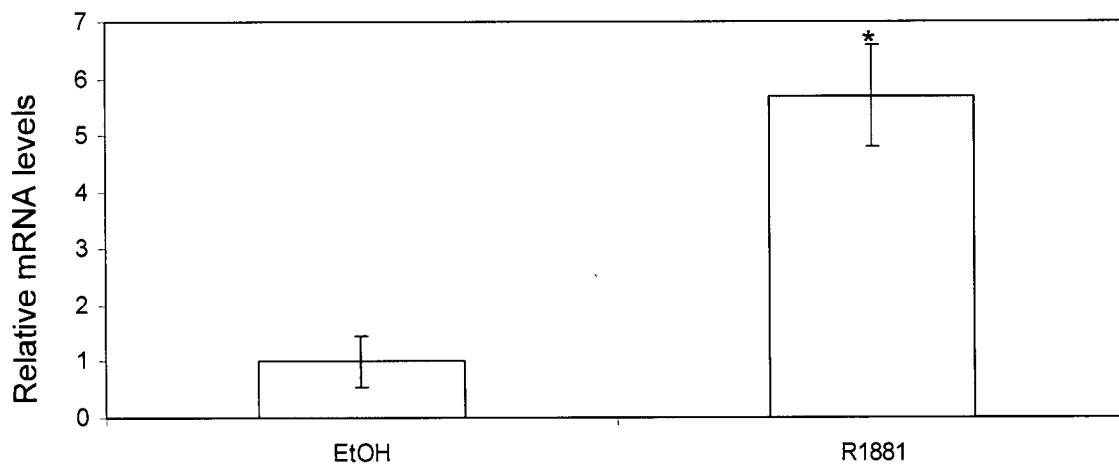

Figure AP 3.2 Ago2 mRNA levels increase with androgen treatment. LNCaP cells were treated with 1 nM R1881 for 48 hours. RNA was harvested and used for real time PCR to detect Ago2 mRNA. Asterisk indicates p-value < 0.01 (Student's t-test).
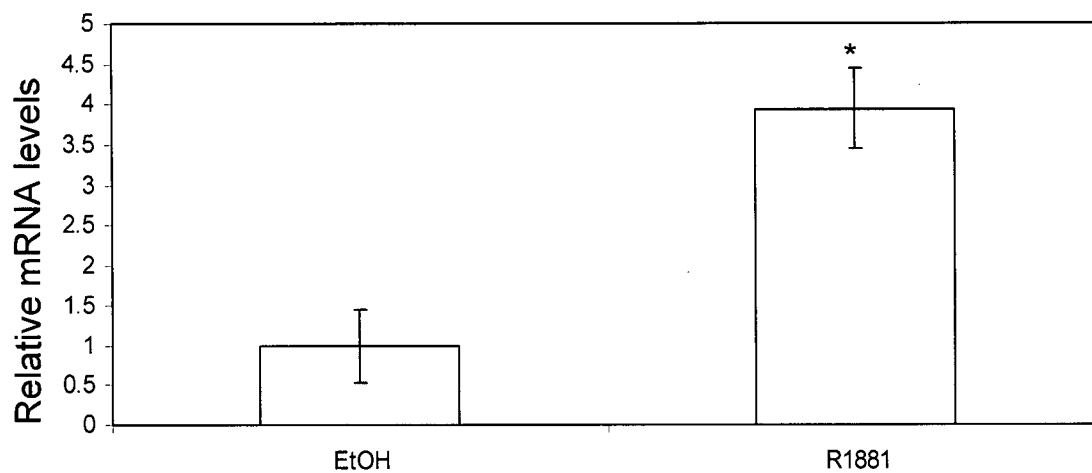
Figure AP 3.3 FMR1 mRNA levels increase with androgen treatment. LNCaP cells were treated with 1 nM R1881 for 48 hours. RNA was harvested and used for real time PCR to detect FMR1 mRNA. Asterisk indicates p-value < 0.01 (Student's t-test).