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APPROVAL SHEET

Title of Dissertation: "p63 is a p53 Homologous Gene with Basal Cell Restricted Expression in the Prostate"

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Doctor of Philosophy Degree
29 July 2004

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2004		**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Novel Insights into p63 Expression and Function in Prostate	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Servicces universsity of the Health Sciences,F. Edward Herbert School of Medicine,4301 Jones Bridge Road,Bethesda,MD,20814-4799**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

Introduction: The central hypothesis of this dissertation is that p53 homologues exist and that identification of these genes will provide a better understanding of the role p53 gene family plays in biology and cancer. At the beginning of this research, no p53 related gene was known. Subsequently, p63, a p53 homolog was described by other investigators. p63 is necessary in the development of epithelial structures. One of the isoforms of p63, DNp63, is highly expressed in prostates, which led to the hypothesis that DNp63 has critical functions in the prostate. The studies reported here provide new insights into the expression and function of p63 in the prostate and prostate cancer. Results: Initially, p53 homologs were sought in human prostate epithelial derived cell cultures through degenerate RT-PCR and other techniques. When the discovery of p63 using a similar approach was reported, our effort focused on p63 evaluation in tissue microarrays of benign and malignant prostate tissues. The basal cell specific expression of DNp63 in normal prostate is very striking. DNp63 is absent in other cell types in normal prostate and is not expressed in prostate adenocarcinoma. Detectable p63 was found in immortalized and early passage cell cultures derived from benign and malignant prostate tissue, but not in senescent cultures. Experimental models using adenovirus p63 expression vectors and prostate cell culture models have been developed. Evaluation of i proteins involved in cell growth and development regulation suggested that p63 overexpression reduced serine 9 phosphorylation of GSK3β and AKT. Conclusions: The selective presence of DNp63 in basal cells of the prostate revealed it to be an excellent new bio-marker of prostate basal cells and epithelial cell cultures. DNp63 has already found translational utility in the diagnostic pathology setting for excluding prostate cancer in biopsies due to its basal cell restricted expression in only benign glands. In the prostate, DNp63 does not appear to stimulate growth, but rather functions in the maintenance of basal cell phenotype. p63 does not appear to play a role in prostate cancer. Further investigations of p63 mechanisms in basal cell biology are warranted.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**		**125**	

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Abstract:

Novel Insights into p63 Expression and Function in Prostate

Leland D. Davis, Ph.D., 2004

Thesis directed by: Dr. Shiv Srivastava, Professor, Department of Surgery, USUHS

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Novel Insights into p63 Expression and Function in Prostate

by

Leland D. Davis

Dissertation submitted to the Faculty of the
Molecular and Cell Biology Program of the
Uniformed Services University of the Health
Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 2004

Dedication

I dedicate this dissertation to my patient wife, Shauna Denise Davis, who tolerated low salaries and late nights all these years, and even helped with the final draft. I want to recognize my parents, Ted and Mary Davis, for the financial support and assistance with childcare that enabled me to attend graduate school.

Acknowledgements

I thank my advisor, Dr. Shiv Srivastava, for his great patience in training me. I should also mention the many fine people at the Center for Prostate Disease Research basic science laboratory, who often helped me to learn, and to advance my project. In particular, I thank Dr. Vasantha Srikantan, who assisted me greatly in learning RNA analytical methodologies and editing of the dissertation; and Dr. Gyorgy Petrovics, who assisted me in understanding techniques for the study of protein.

Mrs. Lakshmi Ravindranath also assisted me in protein based studies. I collaborated with Dr. Meena Augustus on spectral karyotyping. Dr. Judd Moul, as CPDR director, allowed me to study in the CPDR laboratories. Dr. Syed Shaheduzzaman assisted me in understanding the use of oligonucleotide microarrays. Dr. Zhiqiang Zou taught me to perform Northern blots and gave me additional useful advice on the graduate school process. Dr. Axel Merseburger assisted me with immunohistochemistry.

At the Armed Forces Institute of Pathology, I learned much and gained much on collaborating with Dr. Isabell Sesterhenn and Dr. Bungo Furusato on immunohistochemistry on prostate specimens.

Dr. Mary Lou Cutler, even before I asked her to serve on my dissertation committee as chair, periodically inquired about my progress as a graduate student. I thank Dr. Johng Rhim, also a member of CPDR, for serving on my dissertation committee and helping me with prostate epithelial cell cultures. Dr. Yoshitatsu Sei was gratefully willing to serve on my committee, and showed enthusiasm for my work. Dr.

Peter D'Arpa was willing to serve not just on my dissertation committee, but was also present at my qualifying examination.

Dr. Jeffrey Harmon, as program director, was willing to forgive late registration for courses, and also served on the qualifying exam committee, along with Dr. Robert Williams and Dr. Mark Adelmann.

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Table of Abbreviations

Abbreviation	Full length name
Ad-C	control adenovirus
AdDNp63	DNp63 adenovirus
Admtp53	mtp53 adenovirus
ADULT	acro-dermato-ungual-lacrimal-tooth
Adwtp53	wtp53 adenovirus
AR	androgen receptor
ATCC	American Type Culture Collection
BCA	bicinchoninic acid
BPH	benign prostatic hypertrophy
BSA	bovine serum albumin
CDK2	cyclin dependent kinase 2
cDNA	DNA made by reverse transcription of RNA
CGH	comparative genomic hybridization
ChIP	chromatin immunoprecipitation
CK19	cytokeratin 19
CWR	Case Western Reserve
DAPI	4',6-Diamidino-2-phenyindole, dilactate
dCTP	deoxycytosine-triphosphate
DNA	deoxyribonucleic acid
E6	early 6
EDTA	ethylene diamine tetraacetic acid
EEC3	ectrodactyly-ectodermal dysplasia clefting
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
FNC	fibronectin/collagen
GAPDH	glyceraldehyde phosphate dehydrogenase
GFP	green fluorescent protein
GSK3β	glycogen synthase kinase 3 beta
H&E	hemotoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HPV16	human papilloma virus 16
HRP	Horseradish peroxidas
Hsp70	heat shock protein 70
IgG	Gamma immunoglobulin
Ki-MSV	Kirstein murine sarcoma virus
LB	Luria-Bertani
LM	limb-mammary
LOH	loss of heterozygosity
MDM2	mouse double minute 2
MEM	minimum essential media

$MgCl_2$	magnesium chloride
MOI	multiplicity of infection
MOPS	3-(N-Morpholino)propane sulfonic acid
mRNA	messenger RNA
mtp53	mutant p53
NaCl	sodium chloride
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PBS-T	PBS with Tween
PCR	polymerase chain reaction
PI1	phosphatase inhibitor one
PI2	phosphatase inhibitor two
PI3 Kinase	phosphatidyl inositol 3 kinase
PIC	protease inhibitor cocktail
PIN	prostatic intraepithelial neoplasia
PrEC	prostate epithelial cell (normal)
PSA	prostate specific antigen
PSCA	prostate stem cell antigen
Rb	retinoblastoma
RNA	ribonucleic acid
RPM	rotations per minute
RT-PCR	reverse transcription - polymerase chain reaction
SDS	sodium dodecylsulphate
SKY	spectral karyotyping
SSC	sodium citrate buffer
TE	tris-EDTA
TMA	tissue microarray
TNM	tumor-node-metastasis
Tris	Tris(hydroxymethyl)aminomethane
WHO	World Health Organization
Wnt	wingless type
wtp53	wild type p53

Chapter 1
Emerging Functions of p63

Introduction

The tumor suppressor gene p53, was first reported in the literature in 1979 as a cellular protein associating with SV40 large T antigen and adenovirus E1b (DeLeo *et al*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). The past 25 years have seen unprecedented research into understanding the functions and the role of p53 in cancer. In fact, p53 is one of the most frequently altered genes in cancer cells and it is now believed that most cancer cells have some dysfunction of the p53 pathway.

The normal function of p53 is to maintain genomic integrity in response to various genomic and cellular stress signals. p53 is a transcription factor, which when activated, stimulates the transcription of specific genes. These genes can be grouped into four major pathways, apoptosis or programmed cell death, cell cycle arrest, DNA repair, and p53 autoregulation.

Whether p53 induction results in apoptosis or DNA repair depends on the cellular context of the induction. It has been reported that p53 phosphorylation at certain sites can direct p53 towards either apoptosis or DNA repair (Holmberg *et al*, 2002; Wang and Prives, 1995). It has been suggested that, in response to severe genomic damage, apoptosis results from p53 induction, while in response to less extensive damage p53 effects cell cycle arrest and DNA repair.

Autoregulation is another pathway attributed to p53. p53 is commonly regulated by MDM2, which is ubiquitin E3 and ligase mediated proteosome dependent degradation of p53. MDM2 is the transcriptional target of p53 (Vousden and Lu, 2002).

With such an important function endowed to the p53 protein, it was reasonable to hypothesize that p53 homologs exist, which would back up p53 function in case something has gone wrong with p53.

Despite the widespread study of p53 for more than two decades, it is only during the last six years that two different human homologs of p53 have been discovered. First, p73 was discovered accidentally during positional cloning on chromosome 1p36 of a candidate tumor suppressor gene in neuroblastoma (Jost *et al*, 1997). p73 has subsequently been associated with certain cancers and has been shown to function similarly to p53 (Gong *et al*, 1999; Jost *et al*, 1997; Kaghad *et al*, 1997). The overexpression of p73 can induce apoptosis. p63, which is located on 3q27, was discovered independently by multiple laboratories through systematic homology based gene discovery techniques (Osada *et al*, 1998; Parsons *et al*, 2001).

Thus, the p53 gene family currently has three members. In comparing the amino acid sequence of p53 with p73 and p63 through alignment, the conserved domains are revealed. While p63 and p73 genes show considerable divergence from p53 at their termini, the central, DNA binding, domain of p53, (amino acids 102-292), is substantially homologous by protein alignment with its counterparts in the p63 and p73 genes. Phylogenetic analysis of p53, p63, and p73 show that human p63 and p73 appear to be more closely related to the rat KET (p63) gene (Schmale and Bamberger, 1997) and to

the "p53" gene found in *Loligo forbesi* squid than to the human p53 gene. p63 and p73 are also more closely related to one another than to any vertebrate p53 (Figure 1.1).

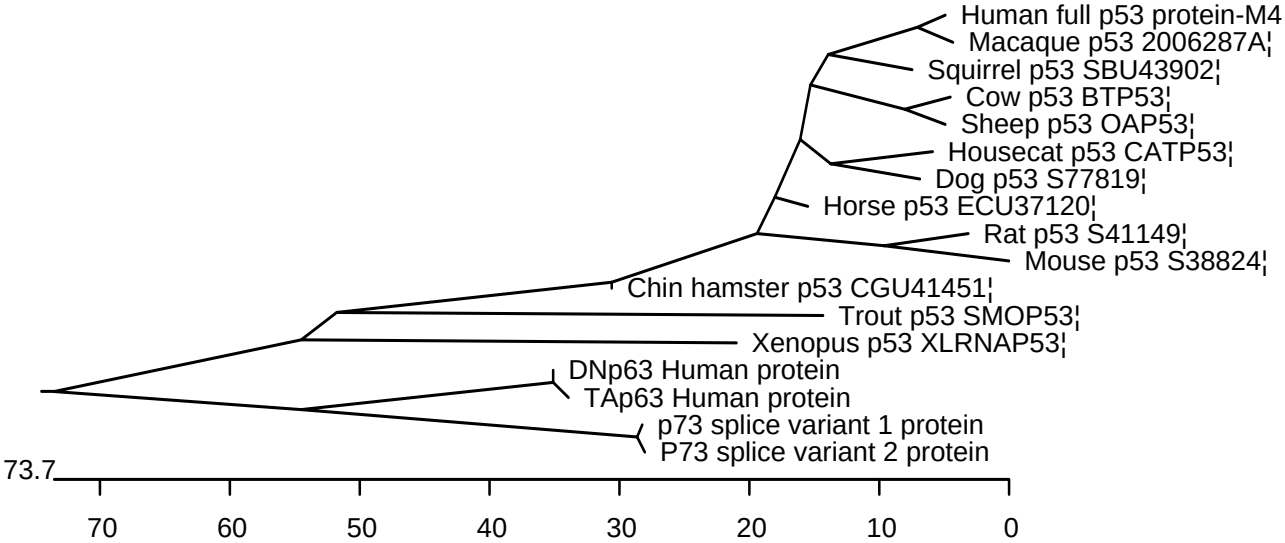


Figure 1.1: Phylogenetic tree showing ancestral position of p63 in relation to p53 and p73. Sequences of p53 from various vertebrate organisms, and human p63 and p73, were aligned by the Jotun Hein method (Bucka-Lassen *et al*, 1999) and arranged by alignment score in a phylogenetic tree.

The literature on p73 and p63 already demonstrates not only the existence of alternatively spliced gene product, but also the functional significance of these variants (Bamberger and Schmale, 2001; Kulesz-Martin *et al*, 1994; Shimada *et al*, 1999). Two and a half decades after the discovery of p53, there is still little information reported that supports biologically significant alternative splicing of p53, apart from isolated reports describing the alternatively spliced products. Due to the primary focus of this dissertation on p63, the following sections provide a comprehensive literature review on p63 (Kulesz-Martin *et al*, 1994).

p63 Structure

p63 has alternative amino termini denoted as DN and TA (Figure 1.2). These termini have been shown to have functional differences (Yang *et al*, 1998). It has been

suggested that TAp63 has transcription transactivation functions, while DNp63 acts as a dominant negative form of the protein. DNp63 is also sometimes described as Δp63, although the significance of amino acids in the N-terminus of the non-TA form of p63 remains to be better understood. For comparison, the DN terminus has 14 amino acid residues and the TA terminus has 100.

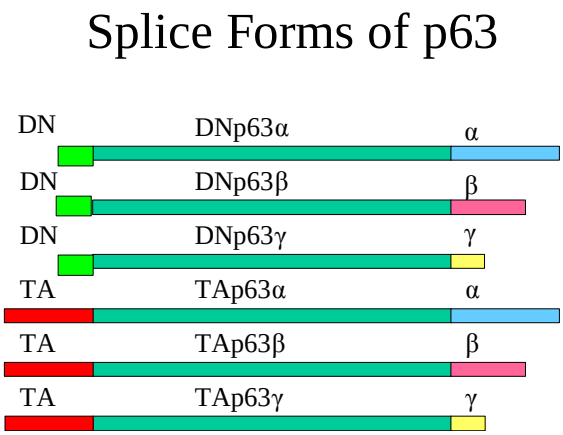


Figure 1.2: Schematic of p63 splice forms. The six known possible splice variants of p63 are compared pictorially.

There is not sufficient data to support the concept that ΔNp63 is merely an N-terminal deleted version of TAp63. We suggest that DNp63 functions as more than a dominant negative regulator of TAp63. The DN specific terminus (MLYLENNAQTQFSEP…) may be a necessary domain for DN-isoform specific p63 activity. Already, there are reports of interaction of the N-terminus of DNp63 with other cellular proteins, including oncogenes influencing cell growth (Patturajan *et al*, 2003). The N-terminal domain is readily accessible to other proteins; there are even DNp63 specific antibodies suitable for immunohistochemistry. p63 has not been shown to conclusively bind to p53 or p73. Finally, in many tissues where DNp63 is expressed, TAp63 is expressed at lower levels (Bamberger and Schmale, 2001).

There are three p63 variants based on carboxy termini known α, β, and γ (Yang *et al*, 1998). While, based on the results of predicted protein sequence analysis, there are suggested functional differences in the p63 carboxy termini; little experimental evidence has been reported beyond a suggestion that some C-termini isoforms may have different activity levels. One example of a sequence variation thought to be of significance is the sterile alpha motif, which is present in alpha C-terminus p63 proteins but not present in other forms. In other proteins where sterile alpha motif has been found, it has been reported to be involved in protein-protein interactions. Given the paucity of mechanistic data on p63 structure and function, one may expect significant further development in our understanding of the C-terminal splice variants of p63.

While the recent literature consistently names the gene in question p63, some of the earlier literature on p63 used various names, including p51, p40, AIS, and p73H (Little and Jochemsen, 2002). This may be partly explained by the multiple splice variants of the gene and its independent discovery in several laboratories. The apparent size of DNp63 which is the focus of this dissertation is about 40kD.

Biological Consequence of Germline p63 Mutations

The biological affects of p63 may be seen in intact organisms. p63 mutations give rise to genetic syndromes in humans comprised of limb and glandular epithelium abnormalities. In humans, germ line p63 mutation is associated with EEC3 syndrome (ectrodactyly-ectodermal dysplasia clefting). Individuals afflicted with this disorder exhibit claw-like or split hands and feet. They often have cleft palate, and are known to suffer from urogenital abnormalities. Sometimes, they have abnormal development of

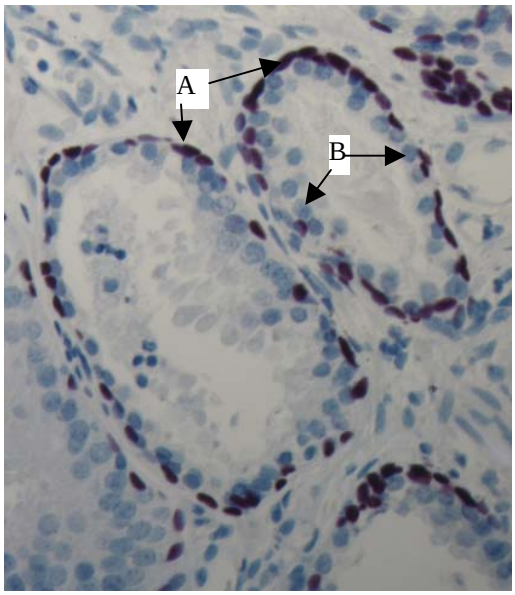
tear ducts. The mutations found to be associated with this disorder are predicted to abolish DNA binding activity of p63 (Celli *et al*, 1999). There are several other syndromes, such as Limb Mammary (LM) and Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT), with similar clinical features which have also been associated with p63 mutation (Celli *et al*, 1999).

p63 gene knockout mice have been generated to further study its biological function (Yang *et al*, 1999). Unlike the case with p53 knockout mice, the p63 knockout mice did not survive to term (Signoretti *et al*, 2000). However, these mice show a number of developmental abnormalities. The teeth and salivary glands were missing. The limbs were severely truncated. These mice lacked prostate gland. In summary these abnormalities show a disruption in the normal development of the basal layer of epithelial tissues. Even in the case of limb abnormalities, the defect appears to originate in an epithelial structure responsible for limb bud development.

The developmental abnormalities associated with p63 point to the special role played by the gene in the genesis and architecture of epithelial organs. None of the human p63 associated syndromes exhibit unusual cancer susceptibility, in marked contrast to the highly cancer prone Li-Fraumeni syndrome with germline p53 mutations (Malkin *et al*, 1990; Srivastava *et al*, 1990). This further underscores distinctions between p63 and p53.

Tissue Distribution of p63 Expression

The expression patterns and tissue distribution of p63 has provided clues about the function of p63. In various glandular organs containing epithelial tissues, we and others have shown that p63 is predominantly expressed in the basal layers of the glands (Davis *et al*, 2002; Parsons *et al*, 2001; Signoretti *et al*, 2000) (Figure 1.3). In many tissues, including the human prostate gland, DNp63 is not expressed in the luminal cells even when there is strong basal cell expression. It has been shown that in skin, p63 expression is reduced near wounds (Noszczyk and Majewski, 2001). As the wound heals, the levels of DNp63 return to normal. In cells that show DNp63 expression, a predominantly nuclear distribution of the protein is noted.



A Basal epithelial cell, p63+

B Secretory luminal epithelial cell, p63-

Figure 1.3: p63 expression in the prostate. Hematoxylin and eosin stained prostate gland, along with immunohistochemical stained with anti-DNp63. (See chapter 3 also.)

The distribution and intensity of TAp63 differs from that of DNp63. DNp63 is the predominant form of p63 in epithelial cells. In epithelial cell types, TAp63 can be coexpressed with DNp63, but as a minor component. In contrast, TAp63 is the

predominant species in muscle cells along with much lower expression of DNp63 (Bamberger and Schmale, 2001). In contrast to p63, p53 is ubiquitously expressed. Normal p53 protein is barely detectable due to its short half life. Mutations of p53 gene cause its stabilization and accumulation of the mutant p53 protein in tumor cells. Apparently, in the presence of mutant p53, the ubiquitin dependent proteolysis of p53 is inhibited, causing the levels of protein to rise, and rendering cancer cells immunohistochemically "positive" for the presence of p53 protein. The property of mtp53 is being routinely used for diagnosis of p53 defects in tumors.

Due to the homology of p63 with p53, a number of early studies have attempted to draw parallels between p53 and p63 function (Yang *et al*, 1998). It is known that p53 alterations, in the form of point mutations or deletions, are the most common gene alteration in human cancer. The data, which has emerged concerning the involvement of p63 in cancer, varies depending upon the type of cancer, and our understanding of the gene still evolving. Some cancers seem unaffected by p63, whereas there is a subset of other cancers in which either the p63 locus is amplified or the expression of p63 is increased (Hibi *et al*, 2000). Two examples of this are squamous cell carcinoma and Barrett's esophagitis. In the case of Barrett's esophagitis, the increased expression of DNp63 accompanies the transition to a columnar epithelium which is part of the etiology of Barrett's esophagitis, and precedes p53 mutation (Hall *et al*, 2001). In the case of squamous cell carcinoma, the p63 chromosomal locus is amplified in the cancer cells (Hibi *et al*, 2000). Attempts to find p63 mutations or loss of heterozygosity (LOH) associated with different cancers have not yet been successful.

In our experiments, we have shown that p63 is not expressed in prostate cancer cells at all (Davis *et al*, 2002). p63 expression is detected in the rare prostate cancer, basal cell hyperplasia, originating from the cell type where p63 is found to be expressed. Moreover, in some tissues, including the prostate, the expression of DNp63 in basal cells may be taken as evidence of the normal prostate gland because basal cells are not present in malignant prostate glands.

Just as the developmental abnormalities associated with p63 suggest its special role in epithelial tissue architecture, so do the histological observations of p63. The differing relationship of p63 to cancers depending on the origin of the cancer may indicate subtle functional differences between the roles of p63 in different cell types and tissues.

The Biochemical Functions of p63

TAp63 has been shown to induce the expression of reporter genes fused to various p53 inducible gene promoters (Shimada *et al*, 1999). However, the strength of the induction of these genes is not identical to p53. Unlike TAp63, DNp63 does not induce the transcription of p53 inducible genes. This brings up an interesting new concept where specific splice variants of p63 may inactivate wild type p53 function. Indeed, in transfection studies, DNp63 does not appear to induce apoptosis when overexpressed as does p53.

Later, by circular dichroism and calorimetry, the secondary structure of the DNA binding domain of p63 was compared to the DNA binding domain of p53 and similarities were found. However, the DNA binding domain of p63 had a higher melting temperature and a somewhat more ordered (less random coil) secondary structure (Klein *et al*, 2001).

The ability of p63 to bind to p53, MDM2, or viral proteins that bind to p53 such as HPV16 E6 has been studied in various laboratories (Calabro *et al*, 2002; Kojima *et al*, 2001; Okada *et al*, 2002). Many of the viral proteins that bind to and stimulate the degradation of p53 do not directly affect p63. There are equivocal reports on the ability of MDM2 to bind to and interact with p63. Some p53 binding proteins are being found which also bind to p63. For example, SSRP1, has been reported to interact with p53 and to TAp63 but not with DNp63 and stimulate the transcription of luciferase constructs bearing promoters from p53 responsive genes (Shelya *et al*, 2002). There have been reports on the interaction of BMP2b and p63 in zebrafish (Bakkers *et al*, 2002).

Very recent studies have revealed the possibility of p63 functioning through different pathways than p53. In glioma cell lines, several genes known to be involved in Notch signaling are induced by TAp63 adenovirus. Notch signaling has been tied to the formation of the neural tube in the embryo.

DNp63 has been contrasted with TAp63 by oligonucleotide microarray hybridization of adenovirus infected Saos2 cells (Wu *et al*, 2003). Hsp70 was found to be selectively induced by DNp63 but not by TAp63. Other genes were found to be upregulated by one isoform of p63 and down regulated by the other, including CDK2. These results were confirmed by RT-PCR.

In keratinocytes, p63 expression has been reported to be regulated by epidermal growth factor through the phosphoinositide 3-kinase (PI3 kinase) pathway (Barbieri *et al*, 2003). The promoter of p63 has been analyzed, and confirmed by chromatin immunoprecipitation (ChIP) to have binding sites for p53 and DNp63 (Harmes *et al*, 2003).

Early studies of p63 mechanisms and signaling pathways focused on similarities between p53 and p63, but more and more studies are showing the differences between p63 and p53. To date, there is more data reported on the affects of p63 on other genes rather than the affects of other genes on p63. While suitable candidates for p63 activity are being described, it is still not clear how p63, at a molecular level, allows the orderly formation of basal cell layers in epithelial tissues.

Summary and Comparison of p53 and p63 Features

Gene	**p63**	**p53**
Biological Effects	Allows formation of epithelial tissues. Involved in cell growth regulation and/or differentiation.	Induces apoptosis or cell cycle arrest in response to DNA damage. G1 and G2 check point protein.
Expression Characteristics	Splice variant dependent. DNp63 is found mainly in basal cells of the epithelium. TAp63 is expressed more in the muscle. Many cells do not express p63. Depending upon cancer type, overexpressed or not expressed in cancer.	Low level expression in all cells. p53 protein is stabilized in response to genomic and cellular stress. High levels of inactive p53 is present in tumor cells.
Mechanism of Action	Some splice variants transcriptionally activate genes with p53 specific response element. Evidence is accumulating of p63 involvement in cell growth regulation through protein-protein interactions.	Transcription factor with p53 specific response element. PRE are present in promoters of p53 target genes involved in the function of p53. p53 regulates cell growth by protein-protein interaction.

Table 1.1: Summary of comparisons between p53 and p63.

p63 is a recently discovered p53 homolog required for the normal development of epithelial structures. p63 expression is not ubiquitous, but widespread and highly variable. In some tissues, p63 possesses properties commonly associated with oncogenes. p53 alterations are common in a wide variety of cancers, to such an extent that p53 is thought to function primarily in the protection of the cellular genome and the prevention of cancer. p63 genomic amplification or p63 overexpression is only associated with cancer cells in some tissues, and p63 mutations have not been linked to any cancers. p63 mutation conveys a severe phenotype, EEC. It appears that, unlike p53, p63 is not central to cancer. The available reports on p63 suggest an important role in epithelial tissue organization and embryo development.

Overview

Our hypothesis that p53 homologs exist in nature led us to the search for p53 gene family members. Subsequently, early reports showing high levels of p63 in the prostate gland led us to define its expression and functions in the biologic pathways of the prostate, especially in prostate cancer. We have focused our studies on comprehensive characterization of p63 in biology of the prostate gland and prostate cancer.

Initially, our priority was to determine where, when, and how p63 is expressed in the prostate. We investigated through RT-PCR, through Northern hybridization, and through immunohistochemistry the expression of p63 in benign prostate glands, prostate tumors, and novel prostate tumor derived cultures established by us. We determined that the expression of p63 is limited to the basal cells of benign glands, and that in cell cultures p63 expression is limited to early passage primary cultures and certain categories

of immortalized prostate epithelial cell culture derived from primary tumor or benign glands (Davis *et al*, 2002).

We then moved to study the function of p63 in the prostate, using the information we had garnered in our expression study. Later, the basal cell association of p63, as well as it's loss in late passage primary cultures, focused our efforts at identifying the involvement of p63 with pathways known to be involved with cell proliferation or senescence. We found some involvement of p63 with GSK3β phosphorylation, and have subsequently been working on mechanism of DNp63 function in prostate.

Our study of the p53 homologs has brought us from the search for such genes to the expression of p63 gene to the investigation of the molecular function of a gene product. p63 appears to be necessary for the maintenance of basal cell phenotype. Current and future investigations address the very important issue of the biological functions of p63 in the prostate.

Chapter 2
The search for human p53 homologs

Introduction

The tumor suppressor gene p53 plays a central role in DNA damage response. In the presence of DNA damage caused by ionizing radiation, or by chemicals such as adriamycin, p53 accumulates in the nucleus and as a tetramer binds to promoters of genes that are transcriptional targets of p53 and mediate its functions (Friedman *et al*, 1993; Tishler *et al*, 1993). When bound to the p53 response elements in the promoter, p53 forms a transcription complex with other proteins. Transcription of genes such as GADD45 and p21/waf1/cip1 then induced by p53 can cause cells to pause in the cell cycle and stop replicating (Hofseth *et al*, 2004). Cell cycle arrest continues until DNA damage repair is effected. In cases of extensive DNA damage, the cell is guided towards apoptosis which may be triggered by the induction of genes that induce apoptosis such as BAX and caspases (Shen and White, 2001). Thus, p53 functions as a check-point protein to maintain genomic integrity.

Because p53 enables DNA repair by stopping the cell cycle, and also eliminates cells with excessive damage, the loss of p53 function results both in genomic instability and the unchecked growth of cancerous cells. p53 mutation is encountered in a variety of cancers (Vousden and Lu, 2002). p53 mutation is a hallmark of advanced stages of

colon, breast, lung, and prostate cancer (Heidenberg *et al*, 1996; Kikuchi-Yanoshita *et al*, 1992).

p53 mutation has been associated with hormone refractory prostate cancer, with a study by our laboratory indicating that in 94% of the samples analyzed, p53 protein is detected through immunohistochemistry at abnormally high levels (Heidenberg *et al*, 1995; Heidenberg *et al*, 1996). Subsequently, p53 mutation was detected in most of the samples demonstrating elevated p53 levels. Hormone refractory prostate cancer does not respond clinically to the administration of drugs that interfere with either the production of or the response to androgen. p53 mutations are also found, albeit at lower rates, in early stages of prostate cancer. p53 mutations or p53 alterations do not appear to be a necessary event in the initiation of primary prostate cancers.

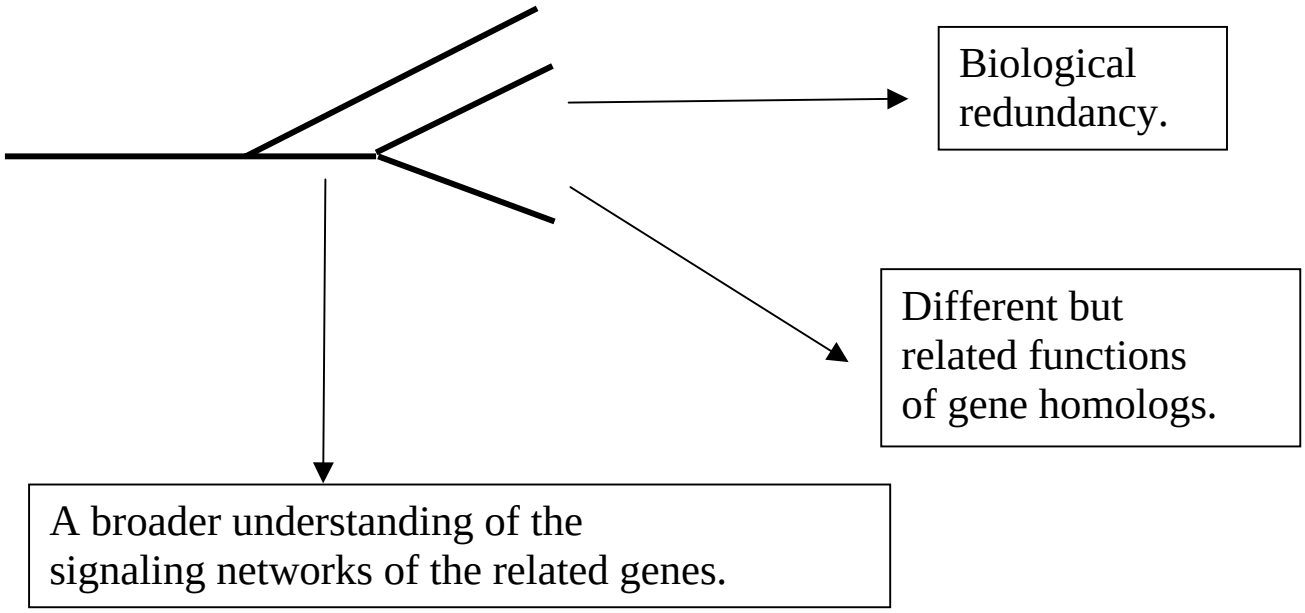


Figure 2.1 What we learn from gene families.

p53 is well conserved in evolution, it is even found in invertebrates such as squid (Winge *et al*, 1995), and in vertebrates such as mouse, dog, trout, *Xenopus*, and human. The most conserved region of p53 is its DNA binding domain. Genes with central

biological functions in diverse organisms that are found widespread among distant phylogenetic branches are generally members of gene families, which have more than one homolog in the same species.(Figure 2.1) The central role of p53 in the maintenance of genomic integrity strongly suggested the existence of additional members in the p53 gene family.

Our central hypothesis of this dissertation is that p53 homologs exist and that understanding functions of the p53 related genes may help further define the role of this "p53 net work" in biology as well as in cancer biology. We were intrigued by the fact that no p53 homologs were reported in humans. By seeking p53 homologous genes, we expected to solve the phylogenetic conundrum of p53 not having known homologs. We also expected to find alterations of other p53 related gene in those cancers that did not commonly exhibit p53 alteration such as primary prostate cancer, testicular cancers, neuroblastoma, and subsets of breast cancer.

While our hypothesis was proven correct, during the early phase of this work two p53 homologs (p63 and p73) were defined. We decided to focus our further study on p63 because of its abundant expression in prostate and our focus in defining the biology of prostate gland and prostate cancer.

Materials and Methods

Primer design and PCR methodologies

p53 protein sequences from different species were aligned to identify the most conserved sequences. The consistently identical amino acid sequences AKTCP (138-142

in human) and CACPG (275-279 in human) between p53 sequences from different species were defined and used to design degenerate primers for PCR amplification (Figure 2.2). The primer sequences were GGRCANGTYTTNGC and CCNGGRCANGCRCA. (N = A, G, C, T; R = A, G; Y = T, C). The primers were appended with T7 and M13 "DNA sequencing primer" sequences, to facilitate subsequent reamplification and sequencing. T7 and M13 (ACGACTCACTATAGGGC and ACGACTCACTATAGGGC) are phage vector derived sequences that are routinely used as sequencing primers. Due to the lower binding specificity of degenerate primers making them a poor choice for sequencing, appending the M13 or T7 primer to the 5' end of the PCR primer streamlined the sequencing of PCR products.

Poly A+ RNA obtained using the Invitrogen Fast Track kit (K1593-02) was used from LNCaP, U251, and SAOS2 cell lines. LNCaP is a well-characterized androgen responsive metastatic prostate cancer derived cell line and expressed many of the physiologically relevant genes expressed in prostate epithelium and prostate cancer, *eg* prostate specific antigen (PSA) and the androgen receptor (AR). SAOS2 is an osteosarcoma cell line which is nullizygous for p53. U251 is a glioblastoma line. Total or poly A+ RNA from prostate was also used. Later a lambda phage library of the normal human prostate (Clontech, 634122) was directly used for degenerate PCR. Reverse transcription was performed using the Superscript reverse transcriptase enzyme (Invitrogen, 18053-017).

Figure 2.2: Alignment showing conserved domain of p53. Predicted p53 protein sequence from various species was aligned using MegAlign-DNA Star software to reveal conserved sequences of p53.

PCR was performed using a variety of annealing temperatures (30-60 º) in an attempt to optimize conditions. "Touchdown PCR" was attempted where a high initial annealing temperature is used, but every few cycles the annealing temperature is reduced. PCR was performed in many cases in the presence of ^{32}P dCTP (Figure 2.3)

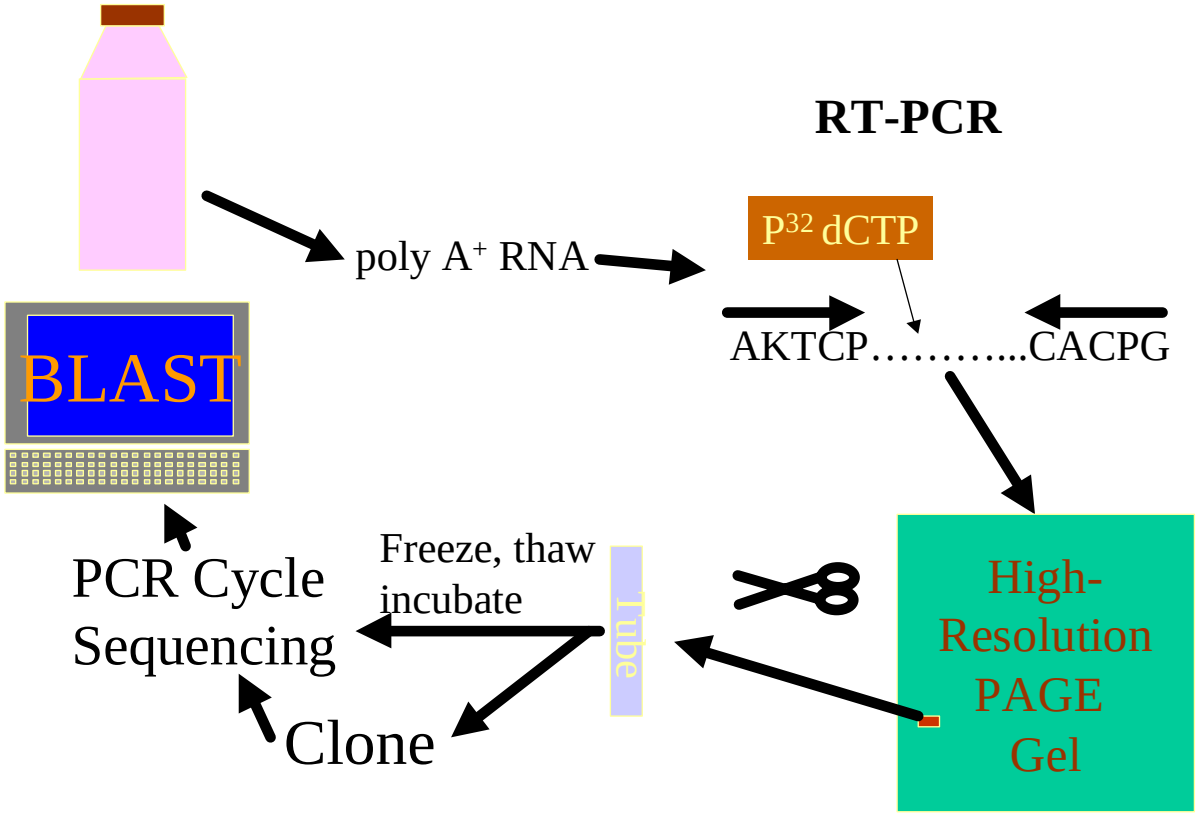


Figure 2.3: Procedure for degenerate RT-PCR approach to p53 homolog search. Cells are grown to 70% confluence, and then harvested for poly A+ RNA. Poly A+ RNA is subjected to degenerate RT-PCR. The PCR products are then run on a high resolution polyacrylamide gel, which is then used to expose X-ray film for autoradiography. Selected PCR product is excised from the gel, reamplified, and sequenced. DNA sequences were subjected to homology search.

Isolation of distinct products of degenerate RT-PCR

Radiolabelled product from degenerate PCR was run on 7% polyacrylamide sequencing gels in parallel to a known sequencing ladder derived from M13 mp8 single stranded DNA. The gel was dried on the glass plate (Genomyx System LR), and then used to expose autoradiography film. Fluorescent labels were added to the gel surface prior to film exposure to give orientation and positioning of the film relative to the gel. As a rule, two films were exposed for each gel. The first was kept as a permanent

laboratory record. The second was aligned with the fluorescent labels and taped to the gel plate. Sterile thumbtacks were pushed in multiple spots through the plastic film around bands of interest, to make a puncture pattern surrounding and defining each band. A marking pen was then applied through the pin puncture marks to color the gel surface underneath. When the bands had been so defined, the film was loosened on one side from the gel plate, and turned off of the gel surface. Prepared ahead of time, small pieces of sterile filter paper were dipped into sterile distilled water and placed, using sterile forceps, onto the bands, now outlined with ink dots. A disposable scalpel blade was used to cut the gel in the site, and the piece of paper with adherent gel was put into an Eppendorf tube containing either PCR grade distilled water or Tris-EDTA (TE pH 7.5, 10 mM Tris, 1 mM EDTA). The tube was then frozen at -80° C.

To reamplify selected bands, the frozen gel piece was thawed, incubated at a 50 ºC for 15 minutes with periodic vortexing, and 5 µl supernatant was used for PCR reamplification that was performed using T7 and M13 sequencing primers. The resulting PCR product was then run on either a 2% agarose or a Novex precast polyacrylamide gel. For PCR products, we used 10% polyacrylamide gels containing 1X tris borate EDTA (TBE) buffer (Invitrogen, catalog number EC62752BOX). Bands run on an agarose gel were cut out and purified from the agarose using Qiagens gel extraction kit (Catalog number 28704). The resulting material was then either used directly for sequencing, or cloned using the TA cloning kit from Invitrogen (Catalog number KNM2000-01).

For PCR sequencing, T7 or M13 primers were used in conjunction with Applied Biosystems (ABI) Prism's de-rhodamine cycle sequencing kit (Catalog number, 403043). For cloned PCR products, the transformed bacteria were spread on LB agar plates

containing ampicillin. Three of the colonies per clone were selected, grown in 10ml LB media, and used for making a mini preparation using Qiagen's spin miniprep kit (Catalog number, 27104). The resulting plasmid DNA was sequenced using a sequencing primer from the vector.

Detection of p53 Homologous Genes by Low Stringency Library Screening

Lambda phage library (Clontech, 634122) containing cDNA from the human prostate was spread on bacterial lawns, producing a total of 1 x 10^6 plaques on agar plates. Filters (Micron Separations Incorporated, E04HY08250) were marked for orientation and pressed onto the agar and incubated at room temperature in DNA denaturation solution which contained 0.5 M NaOH and 1.5 M NaCl (Quality Biological, 351-013-131), neutralized by soaking at room temperature in DNA neutralization solution which contained 1 M Tris at pH 7.4 and 1.5 M NaCl (Quality Biological, 351-014-130), and heated in a vacuum oven for 30 minutes at 80° C. Hybridization solution was made containing different concentrations of formamide. 40 ml low stringency hybridization solution with 30% formamide contained 4g Dextran Sulfate, 11.9 ml H_2O, 12ml Formamide, 10 ml 20X SSC, 4 ml 50X Denhardt's, 0.4 ml 1M Tris HCl pH 7.5, and 1 ml salmon sperm DNA. After a prehybridization period of 2-4 hours in the presence of this low stringency hybridization solution, ^{32}P dCTP radiolablelled p53 probe was added with hybridization solution to the filters, and the filters were incubated at 35˚C for 16-24 hours. The filters were then washed carefully under reduced stringency conditions. First, they were washed three times at room temperature for fifteen minutes

in 2XSSC with 0.5% SDS, to remove unbound probe from the filter. Then the filters were washed with more stringent conditions progressively to remove non-specifically bound probe. Each filter was washed three times at room temperature for fifteen minutes in 1X SSC with 0.5% SDS. Then, each filter was washed at 35° C three times for fifteen minutes using 1X SSC with 0.5% SDS. Between each washing, the blot was monitored by Geiger counter and/or by the exposure of X-ray film (X-Omat AR, available multiple sources, from Kodak) (Figure 2.4).

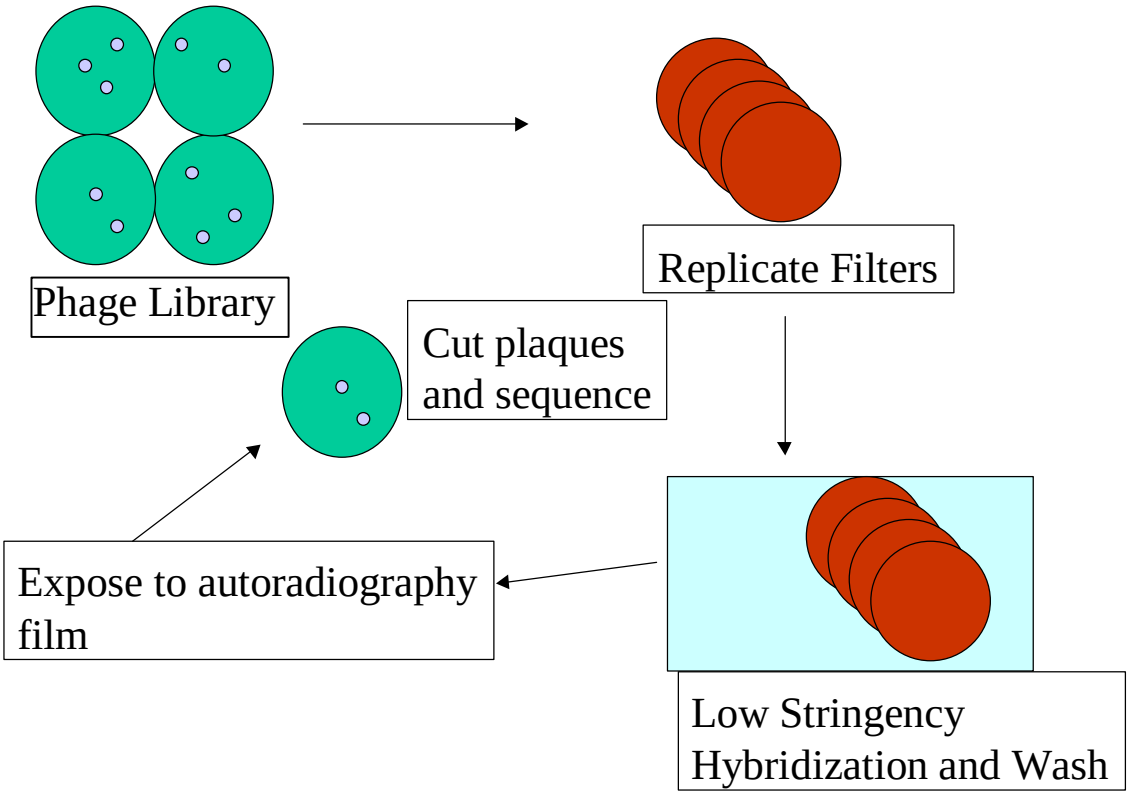


Figure 2.4: Low stringency hybridization. Replicate filters were made of library, which were subjected to low stringency hybridization with p53 cDNA, washed, and exposed to X-ray film for autoradiography.

Additional Approaches

Attempts were also made to identify p53-related genes by a combination of hybridization and degenerate PCR methodologies. Biotinylated p53 DNA was attached to diamagnetic "Dynabeads" (Dynal, 112.05/06). These beads coated with DNA were mixed with poly-A+ RNA in the presence of buffer under low stringency conditions. RNA bound to beads was captured using a magnet, washed, and then boiled to release bound RNA. Degenerate RT-PCR was then performed on the resulting nucleic acid mixture (Figure 2.5).

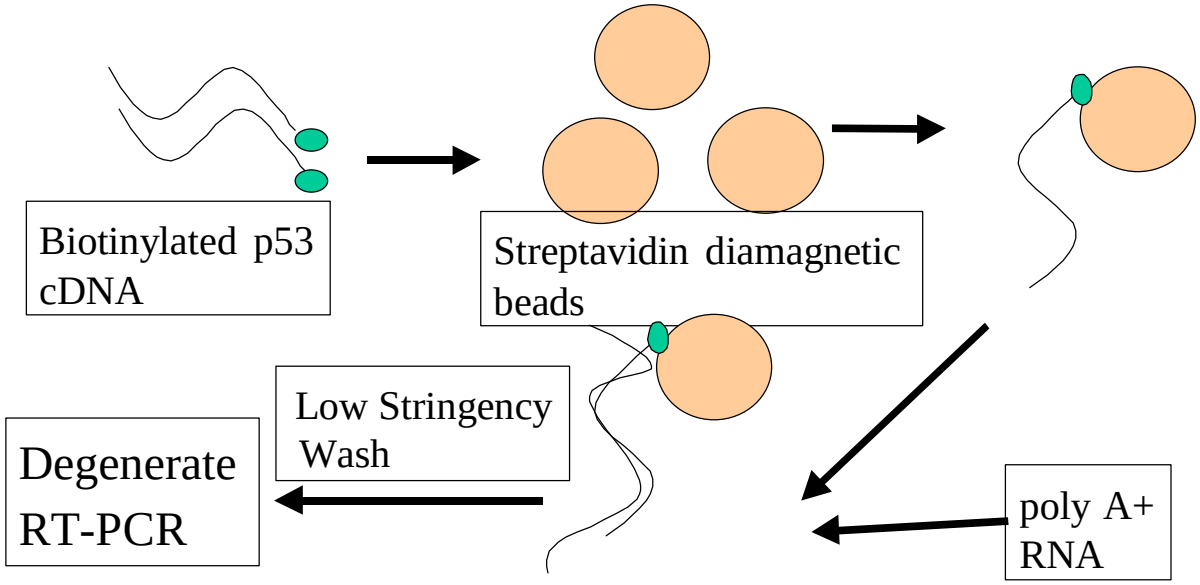


Figure 2.5: RNA enrichment. polyA+ RNA was added to streptavidin beads bound to biotinylated p53 cDNA. The beads were washed under low stringency conditions, then the RNA was removed.

Results

Radiolabelled degenerate RT-PCR, followed by electrophoresis on a sequencing gel produced numerous bands of different sizes. These bands had a wide range of molecular weights. We focused on bands near the expected range of PCR product (between 200 and 800 bp) and selectively chose the darker bands for excision (Figure 2.6). Based on the following consensus p53 amino acid sequence, AKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCPHHERCSDSDGLAP PQHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNYMCNSSCMG GMNRRPILTIITLESSGNLLGRNSFEVRVCACPG, the p53 gene was expected to produce a product band at 423 bp. In the evolution of gene families, the following modifications may happen to a gene, in declining order of frequency: base substitution; deletions or additions; and gene duplication, fusion, or translocation. While less common than base substitutions, additions and deletions are not uncommon, therefore, a range of product sizes were allowed, but selections were biased towards the expected size for p53. When it became apparent that non-specific product was being obtained, the sample selection was biased towards more intense bands.

Autoradiogram of PCR products with p53 degenerate PCR primers

Sequencing ladder for molecular weight determination (A)

Band labels, for picking bands. (B, C)

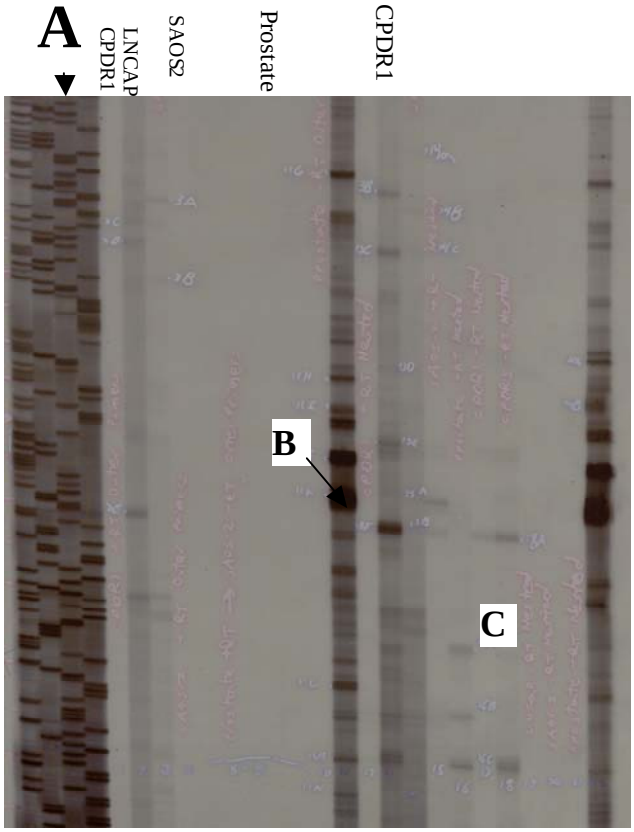


Figure 2.6: Representative sequencing gel to identify radiolabelled p53 PCR products by degenerate pcr primers. The products of degenerate RT-PCR samples were run on this sequencing gel. Also included is the M13mp8 sequencing ladder for molecular weight determination.

Resulting PCR products (more than 150 selected amplification products) were excised and subjected to reamplification. The result of PCR reamplification was to yield bands similar in size to those obtained from the sequencing gels (Figure 2.7). These bands were then subjected to DNA sequencing. Initially, direct sequencing of the PCR products using T7 and M13 primers was performed. If sufficient unambiguous sequence to perform a BLAST search was produced, the sequence was deemed adequate. Some

attempts to directly sequence the reamplified band yielded poor sequence. In such cases, the PCR product was cloned into a vector, and purified plasmid DNA from selected colonies of bacteria was sequenced. We obtained consistently good quality sequence from cloned cDNA. None of the sequences obtained from cell lines and tissues through degenerate RT-PCR showed demonstrable homology to p53. As an indicator of successful experimental strategy we were able to amplify cloned p53 using the degenerate primers. We attempted to vary the stringency of the RT-PCR reaction by performing the polymerase chain reaction with the annealing temperature reduced every three cycles. Unfortunately, we continued to amplify a wide array of different products with differing sizes.

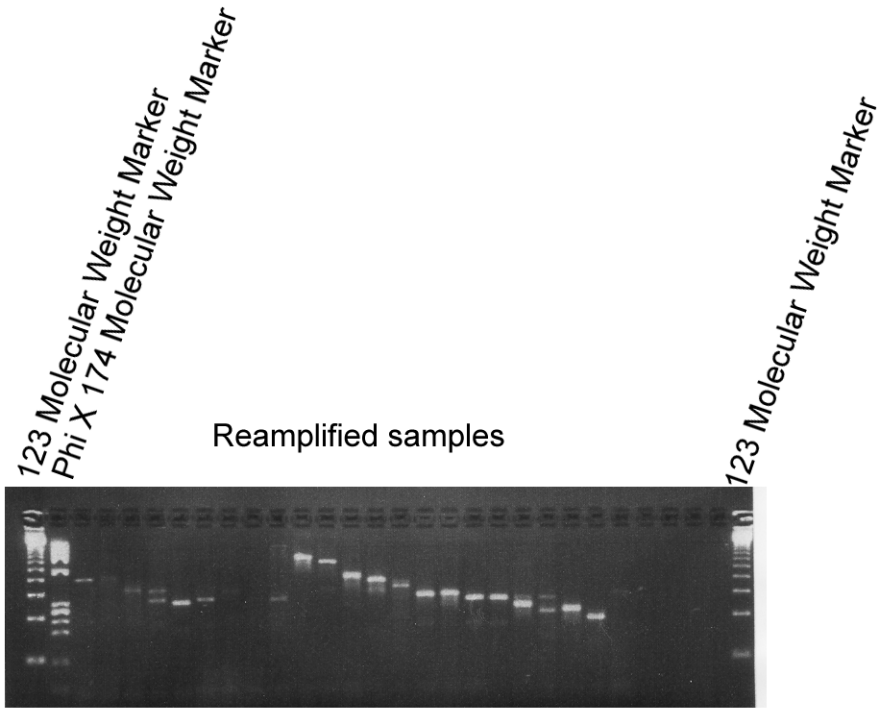


Figure 2.7: Reamplification of PCR products. PCR products excised from sequencing gel were subjected to PCR reamplification and run on an agarose gel. Samples of interest were then purified from the agarose gel for DNA sequencing.

To use diverse approaches to finding p53 homologous genes, the low stringency screening of a prostate cDNA library was pursued parallel to the degenerate RT-PCR approach. This particular methodology became more imperative as we failed to identify p53 homologous genes through degenerate RT-PCR. On our autoradiograms of filters subjected to low stringency hybridization to random primed full-length p53 cDNA, we identified several promising plaques (Figure 2.8). Upon sequencing, it was apparent that these plaques contained cloned p53, but not p53 homologous genes. p53 homologous genes were not obtained through low stringency hybridization.

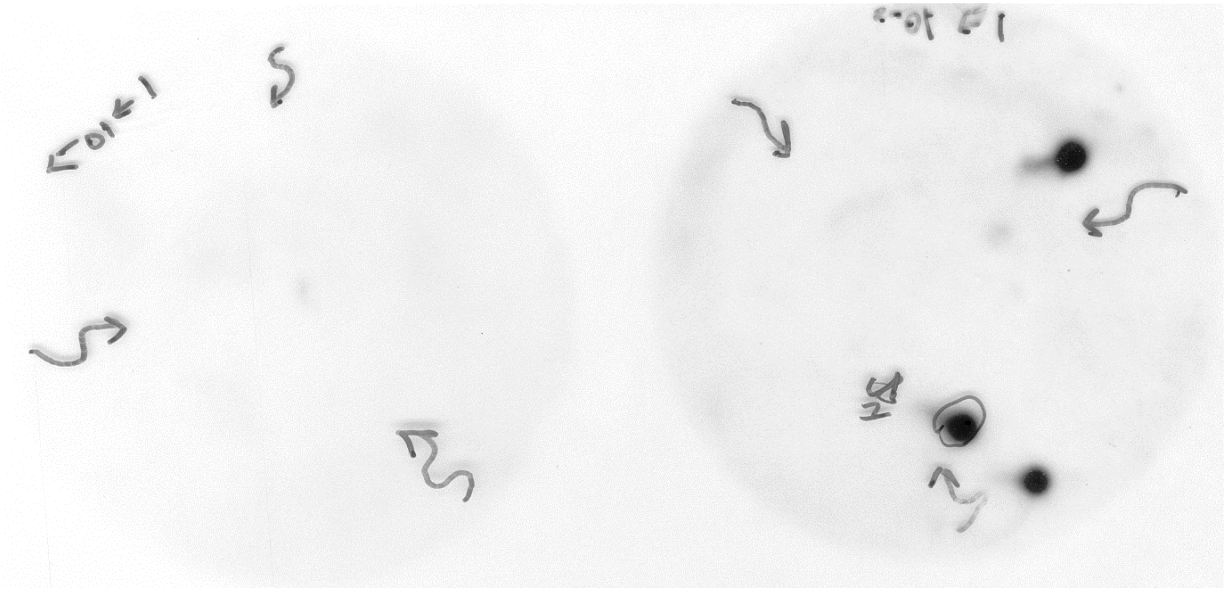


Figure 2.8: Low stringency hybridization approach to cloning p53 related genes. The arrows drawn film matched arrows drawn on the filters for orientation purposes. The circled colony was sampled from the plates for sequencing.

In an attempt to improve our odds of obtaining p53 homologous sequence, we next selected a method of partially enriching cDNA through low stringency hybridization prior to performing degenerate RT-PCR. The combination of hybridization techniques and degenerate RT-PCR was expected to bring the specificity of the hybridization and also the reduced specificity of the RT-PCR into one technique, so that novel p53

homologous genes could be identified. cDNA was annealed to “bait”, an anti-sense sequence derived from p53 cDNA that was end-labeled with biotin. The bait was attached to streptavidin beads. cDNA was bound to the bait under low stringency conditions, and then subjected to degenerate RT-PCR. By the time this phase of the project ended, two p53 homologous genes had already been reported. To verify the conditions of the experiment, some samples had added cDNA of p63 or of p53. Initially, while the pattern of amplification obtained varied from that obtained from the unenriched RNA, we were still unable to find novel p53 homologous genes.

Conclusions

We used a variety of methods in an attempt to identify p53 homologous genes. Other laboratories used similar methods, but in different tissues (Trink *et al*, 1998). The degenerate RT-PCR method we used independently was used in another laboratory, even the same primer sequences, to discover p63 in cell lines derived from squamous cell carcinoma derived from epithelial tissues of the head and neck. Subsequently, it was determined that p63 is amplified and over-expressed in these samples, and that in squamous cell carcinoma, p63 undergoes gene duplication and acts as an oncogene. In the prostate, DNp63 is highly expressed in the basal cells of benign glands though not as a result of gene duplication. However, it is not expressed in LNCaP cells. With further efforts, our degenerate RT-PCR approach conceivably could have yielded p63 from the total prostate RNA sample that we used, but our other samples could not be expected to yield p63. p73 is another p53 homologous gene which has been reported in the

literature (Jost *et al*, 1997). It was encountered as a candidate gene in a positional cloning project, and is itself not highly expressed in the prostate.

Chapter 3
p63 Expression is Restricted to the Basal Cells of the Prostate Gland

Introduction

p53 homologs p63 and p73 have been described recently (Jost *et al*, 1997). Unlike p53 expressed in a wide variety of tissues and cell types, p63 is selectively expressed in epithelial structures including prostate (Bamberger and Schmale, 2001). p73 exhibits selective expression in epithelial tissues, and like p63 is expressed predominantly in the basal layers (Puig *et al*, 2003).

As stated in chapter 1, the multiplicity of names for p63 - e.g. p40, AIS, p73H, CUSP, and p63, to which we refer as p63 - results from confusion surrounding its discovery (Osada *et al*, 1998; Trink *et al*, 1998; Yang *et al*, 1998). p63, unlike p53, has at least six different splice variant mRNAs encoding different versions of this protein, some of which were originally thought to be novel genes distinct from p63. The central portion of the protein, consisting of 330 amino acids, is the same in all the different splice forms (Klein *et al*, 2001). The differently spliced messages code for proteins with different amino and carboxy termini. There are two known potential amino termini for p63. One is named DN, which is the dominant negative form of the native p63 protein, and the other is named TA, the form of the protein that has transcription transactivating functions.

The DN form of the p63 gene (DNp63) is the major form found in epithelial cells. TA splice forms of p63 stimulate transcription via p53 gene responsive promoters (Shimada *et al*, 1999). p63 mutations predispose for the inherited disease, ectrodactyly, ectodermal dysplasia, and cleft lip (EEC syndrome), which is characterized by limb and glandular abnormalities (Celli *et al*, 1999). Finally, p63 knockout mice show abnormalities in numerous glands, including the absence of a prostate (Yang *et al*, 1999). Without p63, many epithelial structures do not form. p63 is overexpressed in certain cancers such as squamous cell carcinoma of the lung (Hibi *et al*, 2000).

Abundant expression of p63 in the prostate gland led us to hypothesize that it had important functions in the growth and differentiation of the prostate gland and that p63 alterations may be associated with prostate cancer. Our group and others have evaluated the expression of p63 in a large cohort of benign and malignant prostate tissues (Davis *et al*, 1999; Parsons *et al*, 2001; Signoretti *et al*, 2000). Here, we established p63 protein expression features in a prostate tissue microarray (TMA) containing benign and cancerous prostate tissues and primary and established prostate cancer cell lines. p63 is predominantly expressed in basal cells of benign prostate glands and only rare carcinomas derived from this cell type show p63 expression. In early passage primary cell cultures derived from primary prostate tumors, consistent expression of p63 is noted, whereas senescent primary cell cultures lose p63 expression. Thus p63 expression may serve as a potentially useful bio-marker for prostatic basal epithelium *in vivo* and *in vitro*. On the basis of our and other studies, positive p63 expression as a marker of basal cells of the prostate gland has the utility to mark benign glands in the diagnostic pathology

setting. Biologic functions of p63 remain to be defined in context of the prostate growth and differentiation.

Materials and Methods

Patient Selection and Clinical Features of Prognostic Prostate Cancer Tissue Microarray.

Prostate tissue specimens from 99 of 402 prostate cancer patients, who had radical prostatectomy at the Walter Reed Army Medical Center, were selected for developing a prognostic tissue microarray (TMA) by Dr. Judd W. Moul of Walter Reed Army Medical Center (WRAMC) and the Center for Prostate Disease Research (CPDR). The tissue microarray was constructed by Dr. Isabel Sesterhenn and her colleagues at Armed Forces Institute of Pathology (AFIP). Patient selection was based on sufficient follow-up after radical prostatectomy treatment of prostate cancer, ethnicity and pathological stage of tumors (Table 3.1). Forty one patients had cancer recurrence as evidenced by rise of serum PSA after surgery and 58 were without evidence of recurrent disease. Selected prostate specimens were used to construct a tissue microarray according to the methodology of Kononen et. al. (Kononen *et al*, 1998). From 99 patients 760 tissue samples were taken to construct the tissue microarrays.

Table 3.1. Characteristics of 99 Radical Prostatectomy Patients used for Tissue Microarray analysis.

Variable	No. Pts.	No. Recurrence (%)
Total patients	99	41 (41.4)

Table 3.1 continued

Variable	No. Pts.	No. Recurrence (%)
Age (years)		
< 55	12	7 (58.3)
55-59	24	6 (25.0)
60-64	35	14 (40.0)
65-69	19	9 (47.4)
70 or More	9	5 (55.6)
Race		
W	59	14 (35.0)
B	40	27 (45.8)
Stage (pathological)		
pT2b	3	1 (33.3)
pT2c	44	5 (11.4)
pT3a	21	8 (38.1)
pT3b	6	5 (83.3)
pT3c	19	17 (89.5)
pTx	4	3 (75.0)
pT4a	2	2 (100.0)
Stage (grouped)*		
PT2	47	6 (12.8)
PT3/4	48	32 (66.7)
Surgical margin		
Pos.	40	28 (70.0)
Neg.	58	14 (24.1)
Gleason score¥		
2-4	0	0 (0.0)
5-6	21	4 (19.0)
7	26	14 (53.9)
8-10	15	11 (73.3)

TNM staging for tumor pathology: See below. pT2 invade but do not penetrate the capsule. pT3 tumors penetrate the capsule. pT4 tumors are found in surrounding viscera. pTx are primary tumors with incomplete information.

Surgical margin: Whether tumor is visible on the edge of the surgically excised prostate after radical prostatectomy.

Gleason Score: (range 2-10) Scored on overall gland characteristics in two fields, each field scored separately (range 1-5) and the two scores summed.

Variable	No. Pts.	No. Recurrence (%)
Nuclear grade [t]		
I	32	11 (34.4)
II	51	28 (54.9)
III	10	1 (10.0)
WHO		
Well	14	3 (21.4)
Moderate	32	7 (21.9)
Poor	47	30 (63.9)
Hormonal neoadj. therapy		
Yes	8	2 (25.0)
No	91	41 (45.1)

* Excluding stage pTx specimen.
¥ Unknown for variable: Gleason score- 37
[t] Too little tumor to grade - 6.

Nuclear grade: Based on appearance of the nuclei.

WHO: Based on differentiation of the tumor.

Hormonal neoadjuvant therapy: Were given antiandrogen prior given prior to the radical prostatectomy?

Pathologic data

The capsule is a fibrous sheath surrounding the prostate. Nerve sparing radical prostatectomy does not result in the complete removal of the prostate and its capsule in the vicinity of the nerves or urethra. Prostate samples were deemed to have positive surgical margins if tumor was identified on the edge of the surgically excised portion of the prostate.

In the TNM clinical staging system, stage pT prostate tumors are primary tumors restricted to the prostate and anatomical regions near the prostate, such as the seminal vesicles. N and M refer to tumors which may be found outside the prostate, either as metastasis to local lymph nodes (N) or to distant sites (M). pT2 tumors invade but do not penetrate the prostate capsule. pT3 tumors have penetrated the prostate capsule, and may

have infiltrated the seminal vesicles. pT4 tumors may have invaded viscera near the prostate.

The Gleason score (range 2-10) is calculated as a sum, and is obtained from observing two fields in the microscope, rating each them on overall structural characteristics (range 1-5), and summing the scores. Well defined tumor areas, minimal invasion of the stroma, and an appearance of normally shaped, closely packed glands in a microscopic field provide a low score (1). Poorly defined, ragged tumor margins, severe invasion of the stroma, and lack of a visible glandular structure provide a high score (maximum 5) (Bostwick, 1997) (Table 3.2).

Table 3.2: Gleason score(Bostwick, 1997)

Score	Margins	Gland Pattern and Size	Gland Distribution	Stromal Invasion
1	Well defined	Single, separate, round, Medium	Closely packed	Minimal
2	Less definite	Single, separate, round, Medium	Spaced up to one gland diameter, average	Mild
3	Poorly defined	Single, separate, more irregular, Small medium or large	Spaced more than one gland diameter.	Moderate
	Poorly defined	Rounded masses of epithelium, Medium or large	Rounded masses with smooth, sharp edges	Expansile masses
4	Ragged infiltrating	Fused glandular masses, Small	Fused in ragged masses	Marked
5	Ragged infiltrating	Almost absent, small	Ragged anaplastic masses of epithelium	Severe
	Poorly defined	Few small lumina in rounded masses of solid epithelium, Small	Rounded masses with smooth, sharp edges	Expansile masses

Tumors frequently exhibit enlarged nuclei with a lighter appearance. While in a benign prostate gland the nuclei of the secretory cells are seen to be in a line with one another, the nuclei in tumor cells will not be in a line. Nuclear grade is a system for rating a tumor on the appearance of the nuclei.

The WHO grading system was developed by the World Health Organization (United Nations) and rates tumors on the degree of differentiation. A poorly differentiated tumor corresponds to a more aggressive tumor.

Prostate intraepithelial neoplasia (PIN) is a precancerous condition occurring in the prostate. While benign prostate hyperplasia (BPH) involves excess growth of the prostate, it is not believed to be precancerous.

The radical prostatectomy derived whole prostate glands were fixed in 10% buffered formalin, sliced at 2.25 mm intervals, processed, embedded, and sectioned as whole mounts. H&E-stained whole mounted slides from each patient were reviewed by one pathologist (I.A.S.) and the slides including the index tumor (largest and worst grade), the highest number of tumors, and the most valuable corresponding donor block with at least 1mm of remaining tissue were selected. Tissues representing tumor regions, benign glands, and prostatic intraepithelial neoplasia (PIN) were marked for tissue array core retrieval. Each tumor was recorded and graded separately according to the WHO and Gleason score and stage according to the TNM system. Tumors with extracapsular extension were coded as pT3 (Gleason, 1966; Mostofi *et al*, 1980; Ohori *et al*, 1994).

Immunohistochemistry

The sections were deparaffinized, dehydrated and immersed in 0.6% hydrogen peroxide in methanol to block endogenous peroxidase activity. Antigen retrieval was accomplished by microwaving the sections for 15 minutes in 10mM citrate buffer. To detect p63, anti-p63 antibody of clone 4A4 at 1/320 dilution was used (Santa Cruz, catalog number sc-8431). This mouse monoclonal antibody is specific to DNp63 and does not bind TAp63. To compare staining patterns, Keratin 903 (K903) antibody was used. K903 is an antibody against high molecular weight cytokeratins which is widely used to detect basal cells (Enzo Diagnostics, Farmingdale, New York, catalog number 30903) (Makin *et al*, 1984). The avidin-biotin-peroxidase system (Vectastain Elite Kit; Vector Labs) was used for visualization. The slides were then examined and graded for p63 expression by a single pathologist (I.A.S.). p63 staining was scored as positive or negative.

Metastatic Prostate Cancer Cell Lines

LNCaP, PC3, and DU145 are widely used metastatic prostate cancer cell lines available through ATCC (ATCC, Rockville, MD). DuPro is a metastatic prostate cancer cell line, and was a gift from Dr. David Paulson, Duke University Medical Center, Durham, NC. LNCaP and DuPro were grown in RPMI 1640 media, PC3 was grown in Kaign's F12, and Du145 was grown in MEM as recommended by the suppliers. The metastatic prostate cancer cell lines were grown in media from Invitrogen supplemented with 10% fetal bovine serum. The CWR human prostate cancer nude mice xenografts (CWR 693, 694, 695, 696, and 697) were kindly provided by Dr. T. Pretlow (Case Western Reserve University School of Medicine, Cleveland, Ohio). Primary epithelial

cell cultures (PrEC) obtained from benign prostate tissue was purchased from the Clonetics (division of Cambrex Corporation, East Rutherford, NJ), and were cultured according to the manufacturer's instructions.

Derivation of Prostate Tumor Derived Primary and Immortalized Cell Cultures

The primary cell cultures were from radical prostatectomy tumor derived specimens. Following the surgery, the sample was excised from the core of palpable tumor. The specimen was put in Hank's balanced salt solution (HBSS) and shipped on ice to the CPDR laboratory. Within hours of receipt, the prostate tissues were macerated with sterile scalpel blades in a cell culture dish coated with FNC coating mix (BRFF, Ijamsville, MD), or collagen, and cultured in Keratinocyte-SFM media with 5% added serum (LifeTechnologies, Carlsbad, CA). FNC coating mix is a cell attachment media and contained bovine fibronectin and collagen. Keratinocyte-SFM is a media developed for the primary culture of keratinocytes, was also used for the culture of prostate epithelial cells (Bright *et al*, 1997). The tissues which gave rise to CPDR-1 cell cultures were initially cultured in super prostate media, containing minimum essential medium (MEM) with 10% serum and a variety of enriching growth factors. After growth for 4-5 weeks, or upon reaching greater than 70% confluence, the cell cultures wer passaged using trypsin digestion and split at a ratio of 1:3 (Figure 3.1).

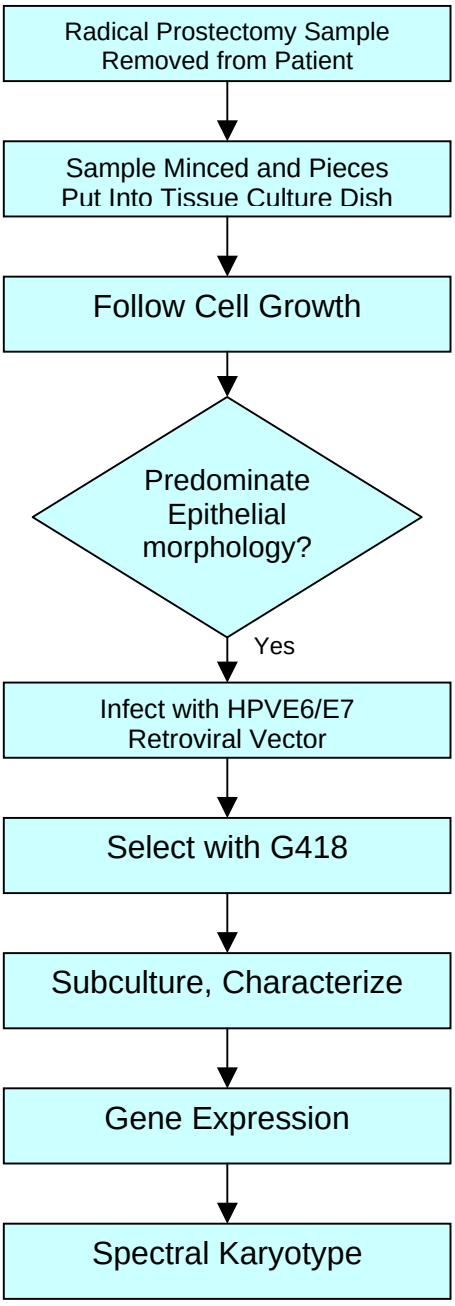


Figure 3.1: Derivation of immortalized cell cultures. Cells from radical prostatectomy specimens were cultured as primary prostate epithelial cultures, followed by immortalization by HPV16 E6/E7.

Immortalization of primary prostate cultures

The CPDR cell cultures (CPDR 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) were developed in our laboratory by transducing cell cultures of primary prostate cancers by HPV16-E6/E7 LXSN retroviral expression vector, kindly provided by Denise A Galloway (University of Washington, Seattle, Washington) (Leland D. Davis and Yong-Ping Su, unpublished) and by Dr. Johng Rhim's (Center for Prostate Disease Research) protocols. At 50-60% confluence, the cells were deemed ready for infection. The media was removed, and replaced with a mixture of media containing E6/E7 retrovirus derived from PA317 cells infected with E6/E7 genes contained in retrovirus. The cells were incubated in this mixture in the presence of polybrene for four to six hours, and then were changed to media containing geneticin (G418) (750 μg/ml). The neomycin resistance gene present in the retroviral vector confers resistance to G418. After 10-14 days, most of the cells died, but a few foci remained which grew to fill the dish in 2-4 weeks. These cells were

then subcultured as necessary and gave rise to the CPDR cell cultures reported here. Mock-infected cells were treated as above, but instead of filtered conditioned media from the retrovirus producing cells, regular media for use on these cells was added to the infection mixture. No mock infected cells survived in G418 selection media. The CPDR cell cultures show morphology of epithelial cells. Demonstrating their epithelial nature, all the CPDR immortalized cell cultures were positive for cytokeratin-19 by RT-PCR. Fibroblasts do not demonstrate cytokeratin-19 expression.

Other Primary Prostate Derived Cell Lines

267-B1 was developed through the transfection of neonatal primary prostate cultures with SV40 T-antigen (Kaighn *et al*, 1989). pRNS-1 was also produced from primary prostate cultures by transfection with SV40 T-antigen (Lee *et al*, 1994). Ki-267 B1 and Ki-pRNS-1 were obtained from their parent lines by transfection with Kirstein murine sarcoma virus (Ki-MSV).

Spectral Karyotyping

A T-75 flask of cells for each cell line assayed was harvested for chromosome preparation for spectral karyotyping (SKY). SKY data for the primary CPDR-1 cell-line was used to detect chromosome rearrangements and aberrations of biological significance to prostate tumorigenesis and disease progression.

The cells used for spectral karyotyping were treated with Colecemid (0.025 μg/ml), followed by fixation in Carnoy's fixative (methanol: acetic acid, 3:1) as described by Modi et. al (Modi *et al*, 1987). Prior to SKY, the slides were stored at 40° C for 1-2 weeks, pretreated with pepsin to remove excess cytoplasm, and postfixed in 1% formaldehyde in 1 X PBS/50 mM $MgCl_2$. The slides were then denatured for 1-2

minutes in 70% formamide/2 x SSC at 80° C and hybridized with SKY probes for 48-72 hours. Following hybridization, the indirect labels (biotin and digoxigenin) were detected with Avidin-Cy5, mouse antidigoxin, and sheep antimouse-Cy5.5. Subsequently, the slides were counterstained with DAPI and mounted with *para*-phenylene-diamine antifade solution.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from established cell lines or primary cultures using RNAzolB (TelTest, Friendswood, Texas), following the manufacturer's recommendations. PCR was performed on cDNAs prepared from cell line derived total RNA using the Life Technologies Superscript kit and random hexamer primers (Invitrogen, Carlsbad CA).

RT-PCR was performed using the following primers (Table 3.3):

Gene Name	Sense	Antisense	Annealing (°C)
DNp63	GCTCCCGGGCTGCAAACCTGT	GTCACCGTCACTCATGCCTCCTAAAA	57
p53	GTTGGCTCTGACTGTACCACC	GTTCCGTCCCAGTAGATTACC	60
E6	ACTTTGCTTTTCGGGATTTATGC	CAGGACACAGTGGCTTTTGACAG	50
CK19	GCGGATGTGCGAGCTGATAGTGAG	AAGGACAGCAGAAGCCCCAGAGC	59
AR	AGCCCCACTGAGGAGACAACC	ATCAGGGGCGAAGTAGAGCATC	58
p16α	CAACGCACCGAATAGTTACG	AGCACCACCAGCGTGTC	60
p16β	TACTGAGGAGCCAGCGTCTA		60
PSCA	GCAAAGCCCAGGTGAGCAACGAG	GGCCGGGTCCCCAGAGCAG	63
BRCA2(Rauh-Adelmann *et al*, 2000)	CCAAGTCATGCCACACATTC	TTGACCAGGTGCGGTAAAAT	60
GAPDH	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC	63

Table 3.3: Primers and their annealing temperatures

Note: p16 α and β used the same antisense primer. Cytokeratin 19 primers were designed specifically to not amplify cytokeratin 18 or other cytokeratins. DNp63 primers were designed to not amplify TAp63.

The cDNAs were amplified by Amplitaq Gold enzyme (Applied Biosystems, Foster City, California). To amplify DNp63, reaction mixtures were placed into a thermal cycler; which was programmed to first heat the samples to 95ºC for ten minutes; and then to perform forty cycles of 95ºC, 45 seconds; 57º C, 45 seconds; 72ºC, 45 seconds; followed by one cycle of 72ºC, 5 minutes. Other gene amplifications were

performed under the same protocol, except the annealing temperature was modified as noted in Table 3.3

Northern Blot Hybridization.

p63 cDNA construct in PCR2.1 vector was kindly provided by Dr. David Sidransky (Johns Hopkins University Medical School, Baltimore, MD). p63 cDNA from this construct was excised by Hind III and Xho I. p63 cDNA probe was labelled with ^{32}P dCTP using the Stratagene PrimeIt II kit (300385). The labeled construct was purified using a NucTrap Probe Purification Column (Stratagene, 400702) equilibrated with 1X sodium tris EDTA (STE). The sample is loaded in the top of the column, and is forced through using a syringe.

Northern blot was made by running the RNA samples on a 1% formaldehyde gel in 1X 3-(N-Morpholino)propane sulfonic acid (MOPS) buffer, and then transferring the RNA to a nylon membrane by reverse capillary transfer (Chomczynski, 2002). Northern blot was prehybridized with hybridization buffer (10mM Tris-HCl, pH7.5, 10% Dextran Sulfate, 40% formamide, 5x SSC, 5x Denhardt's solution, and 0.25 mg/ml salmon sperm DNA) for two hours at 42ºC followed by hybridization with the p63 cDNA probe in hybridization buffer at 42° C for 16 hours. Nylon membrane was washed three times at room temperature with a 2× SSC, 0.1% SDS solution followed by three washes with a 0.1× SSC, 0.1% SDS solution at 55 °C. The membrane was then exposed to X-ray film for autoradiography.

Results:

p63 Expression is Restricted to basal cells in benign glands

Selective Expression of p63 in Basal Cells of Prostate Epithelium.

Expression of DNp63 protein in benign and malignant prostate cells was evaluated because this p63 form was originally reported to be abundant in human prostate tissue (Trink *et al*, 1998). However, the pattern of expression within the prostate gland had not been determined. Tissue microarrays containing about eight samples/patient representing benign tissue, preneoplastic lesions, prostate intraepithelial neoplasia (PIN) and multiple tumor foci were stained by an anti-p63 antibody. A total of 760 tissue samples from 99 patients were analyzed (Figure 3.2). p63 staining was not detected in secretory cells or stroma of benign glands. The observed pattern of immunohistochemical staining with anti p63 antibody indicated that among normal prostatic epithelial cells, DNp63 was expressed only in the nuclei of basal cells (Figure 3.3).

Anti-p63 antibody stained basal cells with a similar intensity and tissue distribution, whereas K903 labels the cytoplasm of the basal cells, anti-p63 stained the nuclei. The nuclear-specific labelling of anti-p63 emphasizes the shape of the basal cell nuclei. p63 appears to be an excellent basal cell marker.

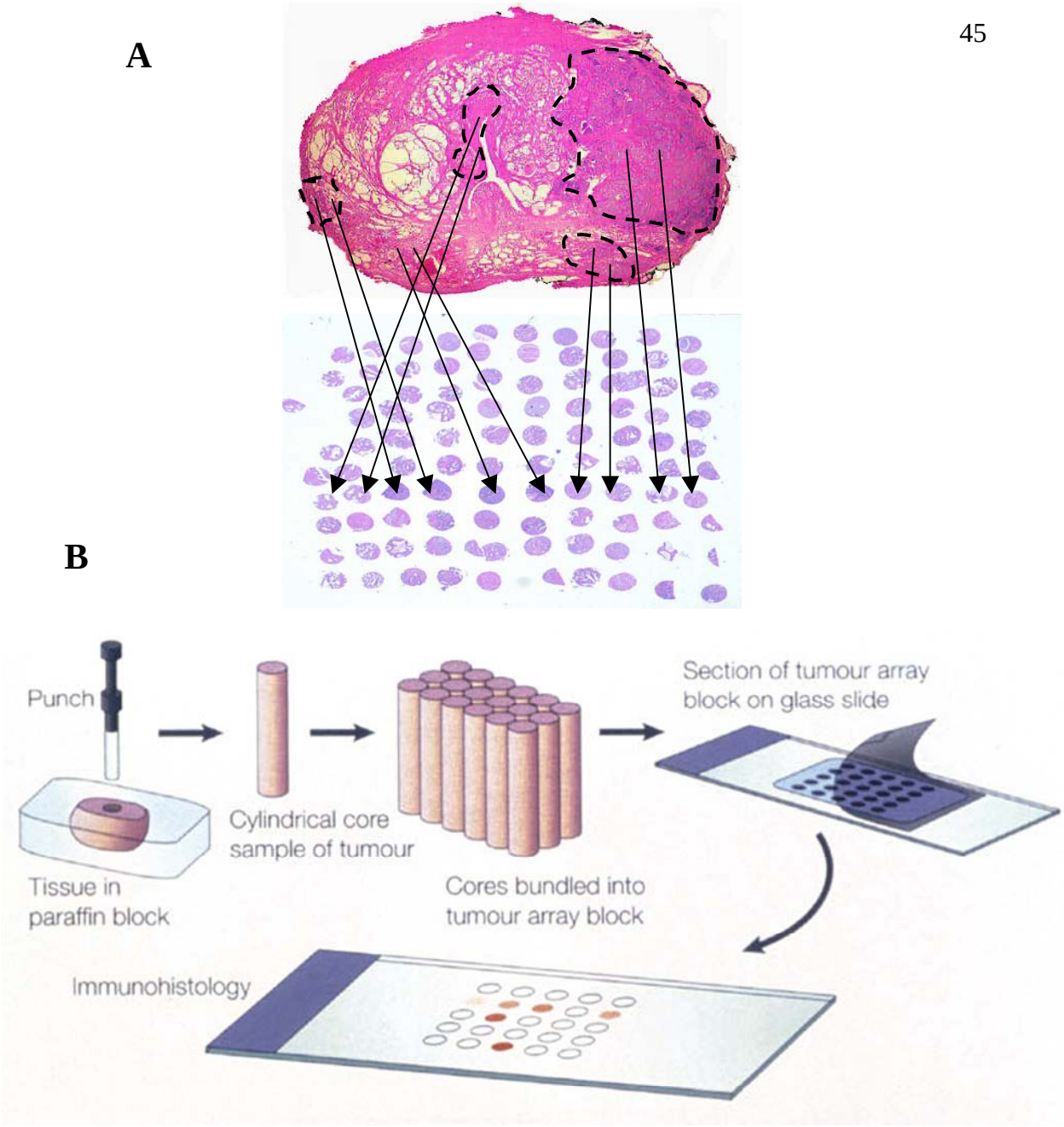


Figure 3.2: Schematic of tissue microarray construction.
A. Selected samples from tumor, benign gland and preneoplastic regions of paraffin embedded prostate whole mounts were removed and placed into receptor paraffin embedded block to create tissue microarray. B. Once constructed, the tissue microarray was sectioned to produce slides for immunohistochemistry.

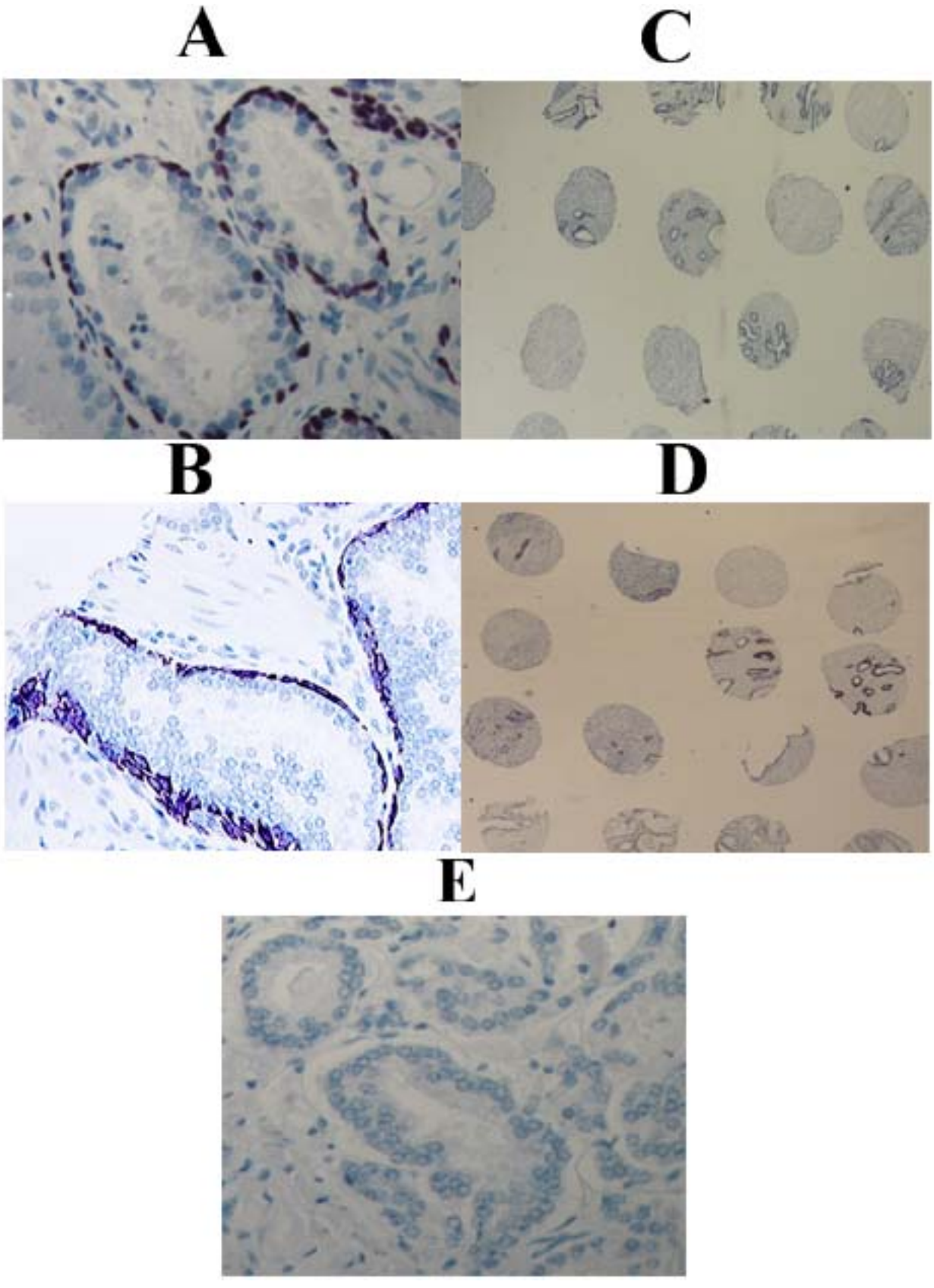

A
C
B
D
E

Figure 3.3: Sections of tissue microarray, stained through anti-DNp63 or anti-cytokeratin immunohistochemistry.
K903 and DNp63 give similar patterns of staining, however, DNp63 stains the nuclei whereas K903 stains the cytoplasm. Tumors show only rare, focal DN-p63 staining.

A. Non-malignant gland stained with anti-DNp63.
B. Non-malignant gland stained with K903.
C. A section of a prostate tissue microarray, stained with anti-DNp63.
D. A section of a prostate tissue microarray, stained with anti-cytokeratin (K903) antibody.
E. A tumor, stained with anti-DNp63.

Lack of p63 Expression in Prostate Adenocarcinoma

We have established basal cell restriction of DNp63 expression in benign prostate glands. In prostate adenocarcinoma, some of the expressed genes (*e.g.* GSTπ) are basal cell specific in benign glands, and some of the genes expressed in adenocarcinoma (*e.g.* PSA) are specific to luminal cells in the benign glands. To determine if DNp63 is expressed in prostate adenocarcinoma, the tumor sections in the tissue microarray were examined.

DNp63 is generally not expressed in primary prostate tumors or in PIN. Numerous primary tumors of different histologic characteristics, and also PIN, were stained in the tissue microarray. p63 was very rarely and focally detected under our experimental conditions. This would indicate that the absence of DNp63 expression is not an individual characteristic of specific tumor cell clones, but a consistent lack of expression in tumor cells.

In two cases of rare basal cell hyperplasia which we examined, p63 is fairly expressed in proliferating basal cells, but not in the same cells expressing K903. (Figure

3.4) Prostatic carcinomas exhibit rare focal expression of p63. These tumor cells also contained K903 possibly indicating basal cell differentiation. The identity of the basal cells was confirmed by staining with 34BR12 anti-cytokeratin, a commonly used antibody for defining basal cells of the prostate.

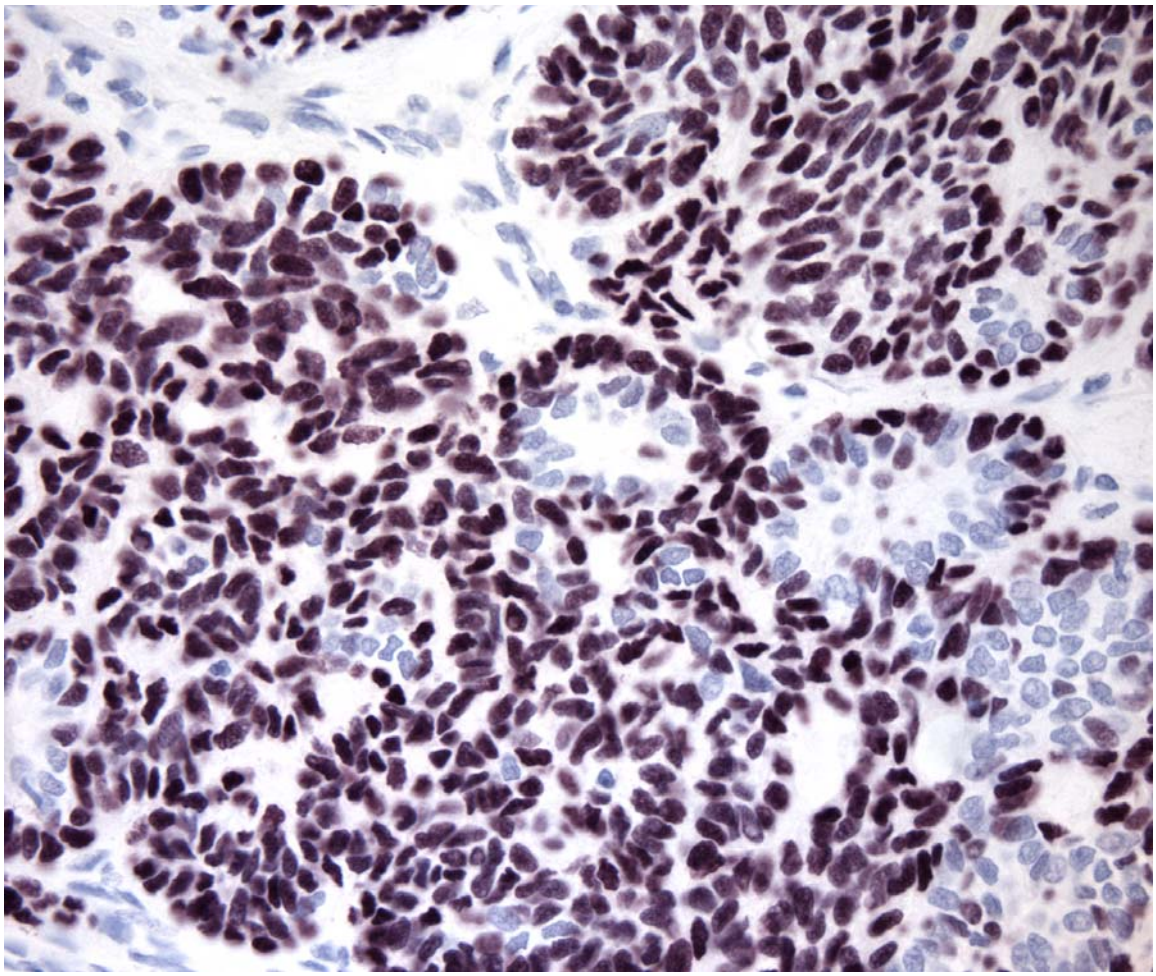


Figure 3.4: Section of prostate tissue exhibiting basal cell hyperplasia, stained with anti-DNp63.

Diagnostic Utility of p63

The novel observations of tightly restricted prostate basal cell specific expression of p63 from our and other investigators has led to the use of p63 in diagnostic pathology setting. The evaluation of prostate biopsies for prostate cancer requires the pathologist to

determine either that there is evidence of cancer in the biopsy specimen, or that there is no evidence of cancer in the biopsy specimen. DNp63 is restricted to basal cells and is not expressed in prostate adenocarcinoma. As such, a positive immunohistochemical result for DNp63 indicates a benign gland, and a negative result strongly suggests cancer. To further simplify diagnosis, p63 staining may be combined with immunihistochemistry for new discovered prostate cancer marker, alpha-methylacyl-CoA racemase (AMACR), which has been shown to be highly specific for prostate tumor cells, to produce an assay system where one color indicates cancer, the other benign glands (Luo *et al*, 2002). (Figure 3.5) The use of anti-DNp63 immunohistochemistry is expected to improve the accuracy of prostate cancer diagnosis in biopsy specimens and pathologists are already evaluating this test.

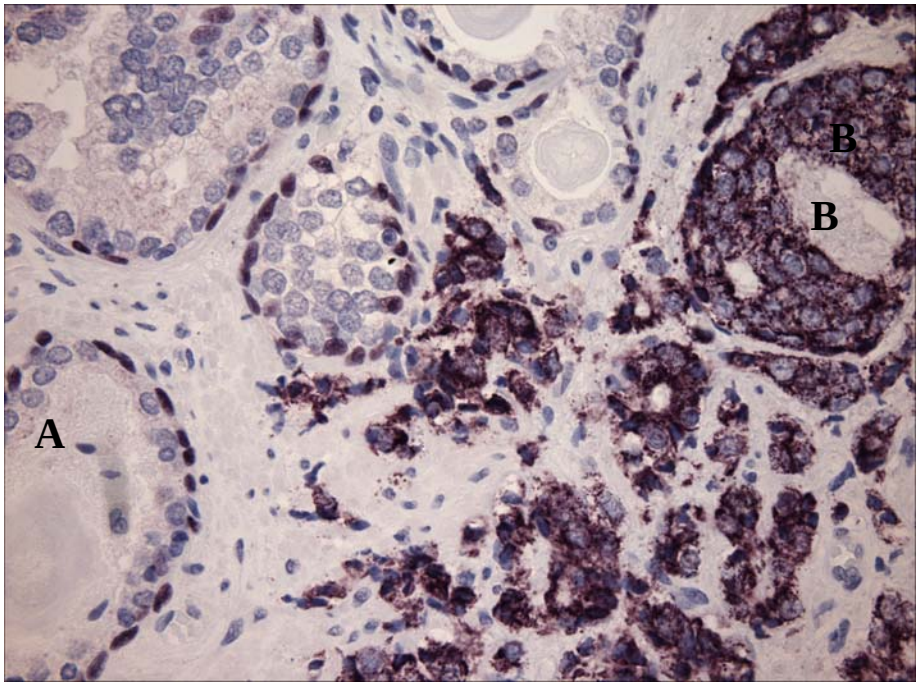


Figure 3.5: Section of prostate tissue stained by coimmunohistochemistry with anti-DNp63 and anti-AMACR.

A. Benign gland with DNp63 positive basal cells.
B Malignant gland with AMACR tumor cells.

Development of Cell Models Used in the Study of p63

Development of Primary and Immortalized Cell Cultures from Prostate Tumor Tissues

We have produced nine different cell cultures that exhibit continued growth after infection with the E6/E7 retroviral vector (LXSN16E6E7) and selection with G418. In contrast to primary cultures, which last for only 3-4 passages, these cell cultures, CPDR 1-3, 6-10, have grown for several passages each (Table 3.4). These cell cultures have been preserved cryogenically for various types of cell biologic experiments. For example, CPDR1, 6, and 8 have been growing beyond the twentieth passage. Cells that were not infected with the E6/E7 retroviral vector uniformly died during the G418 selection process. LXSN16E6E7 is a retrovirus containing the E6 and E7 early genes from the human papilloma virus HPV16.

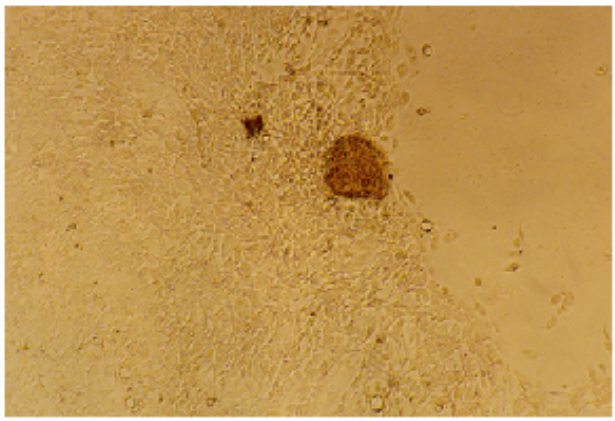
Cell Line	Number of passages currently growing
CPDR 1	22
CPDR 2	9
CPDR 3	5
CPDR 6	25
CPDR 7	14
CPDR 8	25
CPDR 9	13
CPDR 10	11

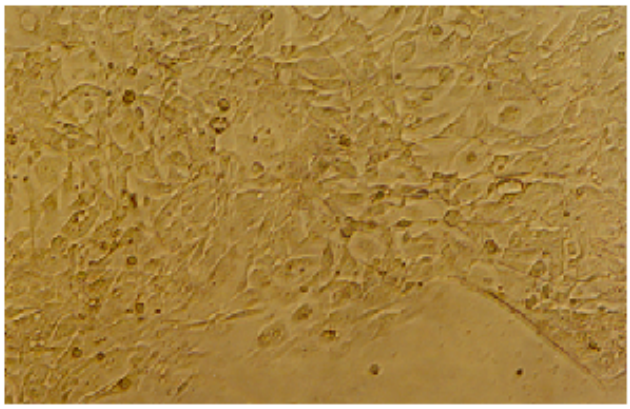
Table 3.4: Current passages of select CPDR cell lines

Fibroblasts in culture exhibit a long spindle shaped morphology. Epithelial cells are significantly shorter and closer to round in their shape, but still typically have a football shape. The morphological difference between fibroblasts and epithelial cells in culture is readily observed by phase contrast microscopy. The CPDR cultures exhibited epithelial morphology in culture (Figure 3.6).

Fibroblasts from prostate cancer derived cell culture (note the elongated shape.)

Prostate epithelial cells growing from prostate tumor explant.





Prostate epithelial cells. Note the shorter, more compact shape.

Long term culture of prostate tumor derived immortalized cell cultures of CPDR1.

Figure 3.6: Morphology of primary prostate tumor-derived cells.

Prostate tumor cell cultures that were infected with the LXSN16E6E7 retroviral construct became resistant to G418 (Geneticin). However, not all cells in the culture exhibited continued growth, for most of the cells in a culture would die after selection with G418, leaving only the cells that were successfully transformed by the vector. Our immortalized cell lines, as well as the LNCaP cells that were infected with the retroviral construct, continued to grow in G418 medium.

Gene Expression Features of the HPV16E6/E7 Transduced Prostate Tumor Derived Cell Cultures

Upon obtaining HPV16 E6/E7 transduced cell cultures of primary prostate carcinoma, we focused on the confirmation of their identity as transformed prostate epithelial cells. To confirm the expression of genes from the retroviral vector in the immortalized cell cultures, we performed RT-PCR for E6 gene in RNA from the cell cultures. All CPDR1-4 cell cultures expressed the E6 mRNA (Figure 3.7). In a representative experiment shown in figure 3.5, the parental cells from which CPDR1-4 cultures were derived did not express E6. LNCaP cells that had been infected with the viral construct and selected on geneticin also expressed E6 (data not shown) and uninfected LNCaP cells did not express E6. Since, E6 is one of the genes in the retroviral construct, its presence confirmed successful transfection with the retroviral construct.

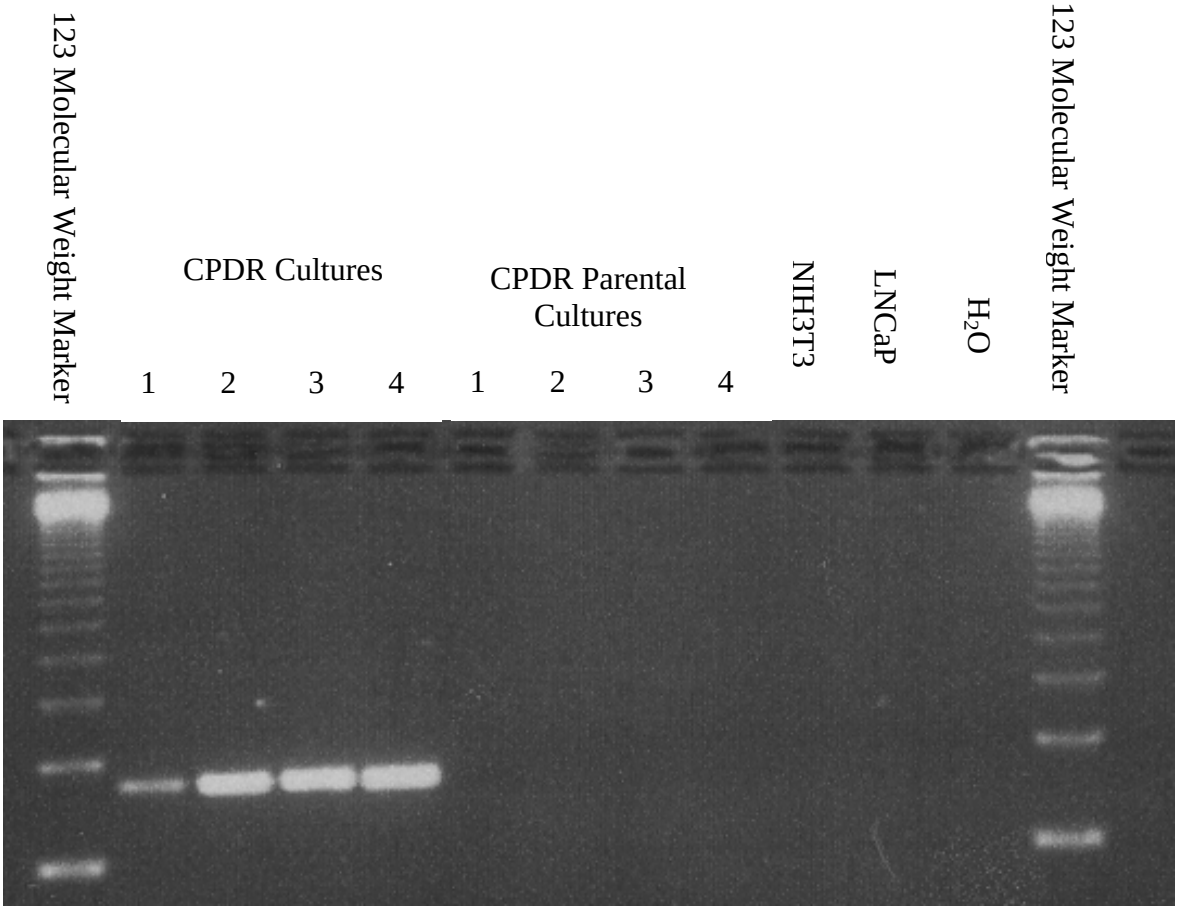


Figure 3.7: Expression of E6 gene in HPV16 E6/E7 transduced prostate tumor cells.
The HPV16 E6/E7 transduced cultures obtained from primary prostate cultures were subjected to RT-PCR to confirm expression of E6. The CPDR cultures (CPDR 1-4) were compared to samples from the parental cultures (CPDR parental 1-4). The mouse cell line, NIH3T3 and the human metastatic cell line LNCaP were also tested.

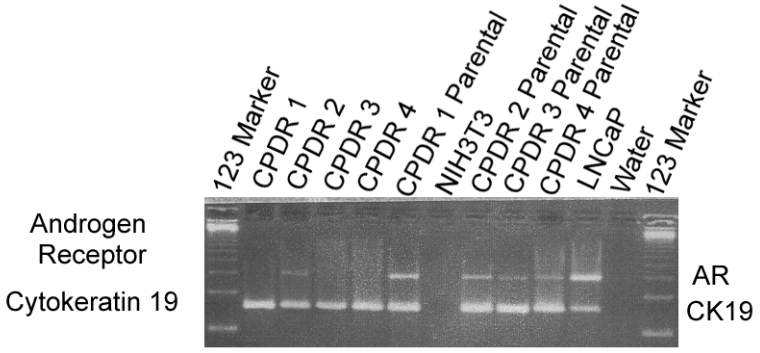


Figure 3.8: Expression of androgen receptor (AR) and cytokeratin 19 (CK19) in prostate cancer cell lines by RT-PCR.
Total RNA from the cell lines indicated was subjected to RT-PCR which used two sets of PCR primers in the same reaction mixture, and run on a 2.5% agarose gel.

The primary and CPDR cell cultures were characterized for expression of biologically relevant genes that are consistently expressed in prostate epithelial cells. Prostate epithelial cells express cytokeratin 19, but fibroblasts do not. Except for a subset of hormone refractory cancer, prostate cancer cells express androgen receptor. All of the primary cell cultures from which CPDR cultures were derived expressed androgen receptor by RT-PCR assay (Figure 3.6). Of the CPDR cell cultures, most either lost AR expression or expressed very low levels of AR that was eventually lost (Figure 3.6) expresses androgen receptor. In this regard, LNCaP cells were distinct from CPDR cell cultures and continued to express androgen receptor by RT-PCR at higher levels. Concerned that the expression of E6 or E7 may abrogate androgen receptor expression, LNCaP cells were transformed with the LXSNE6E7 vector. LNCaP cells that had been treated with the E6-E7 viral construct continued to express androgen receptor with no apparent change in expression (data not shown). As expected, the mouse line NIH3T3 did not express human androgen receptor or cytokeratin 19. By demonstrating

cytokeratin 19 expression, we confirmed the epithelial nature of the CPDR cell cultures. However, we also learned that we cannot use these cultures to model androgen response in prostate cancer.

Spectral Karyotyping

To further characterize CPDR cells and verify a pattern of cytogenetic abnormality typical of prostate cancer, they were submitted for spectral karyotyping was performed. (Augustus *et al*, 2004) (Figure 3.9) Spectral karyotypes were also performed on CPDR 1, 2, 3, 5, 6, 7, 8, 9, 10, and 11. However, we limit our discussion here to the spectral karyotyping data on CPDR1.

About 2 % of the CPDR 1 cells were hyper- triploid with a corresponding gain of entire chromosomes18 and 20. Clonal gains of chromosomes 5, 18 and 20, loss of chromosome 19, deletions at 8p21, 12p12, 17p13, 10q23, 21q21, a 9qh+ and a translocation t(Y;11)(q11;p11) were detected. The composite karyotype was interpreted as follows:

48,X,-Y,t(Y;11)(q11;p11),+5,del(8)(p21-pter),9qh+,del(10)(q23-qter),del(11)(p11-pter),del(12)(p12-pter),del(17)(p13-pter),+18,+20,-19,del(21)(q21-qter).

While CPDR1 demonstrated a variety of chromosomal abnormalities, as is common with cancer cells, it was necessary to determine that CPDR1 was not derived from the benign glands of the patient specimen. Often, cytogenetic abnormalities arise in culture. To confirm the presence of similar abnormalities in the prostate tumors from which CPDR1 was derived, fluorescence *in situ* hybridization (FISH) was performed on the prostate from which CPDR1 was derived. We confirmed that a cytogenetic

abnormality found in CPDR1 also exists in the cancer cells *in vivo*, but not the normal epithelium, of the prostate from which CPDR1 was derived.

Three copies of centromere specific signals for chromosome 18 and 20 were detected by in-situ hybridization in the tissue. Some of the prostatic epithelial cells lining a malignant large gland and practically all the cells of a neighboring small malignant gland in the frozen sections of the primary tumor biopsy, that corresponded to the cells grown in culture and used in the SKY and CGH evaluation, showed the aneuploidy.

Mono-allelic loss at 17p13 (p53) and 8p21 (NKX 3.1) was detected by FISH. Gain of chromosome 20 was confirmed using a gene probe for AIB-1 at 20q13. The modal chromosome number for CPDR-1 tumor xenografted in the mouse, was hypo-tetraploid, resembling the polyploidization of the parental CPDR-1 karyotype. The gain of 18 and 20 persisted, and 2 unbalanced, de-novo translocations, a der(3;18)t(3;18)(p10;q?), a der(12)t(13,19,12)(q?;?p,p12-ptel) and a variant der(12) t(13, 19,12) and three deleted homologs 12p-, 19p- and 13q- were observed. The deletion in 12p was interstitial, the marker chromosome retaining the 12p telomere in the rearrangement with chromosomes 19 and 13 .

As such, CPDR1 has been confirmed to be of prostate carcinoma origins, and bears cytogenetic abnormalities typical of prostate cancer cells, but also abnormalities attributable to culture.

Figure 3.9: Spectral karyotype of CPDR1 compared to data for CGH. Top panel: Comparative genomic hybridization. Bottom panel: Spectral karyotype.

Expression of p63 in Prostate Epithelial Cell Cultures

p63 Expression as a Marker for Basal Cell Features of Prostate Tissue Derived Cell Cultures.

A Northern blot of poly A+ RNA from commonly used established prostate cancer cell lines was probed with DNp63 cDNA. PC3, LNCaP, and DU145 did not show any detectable p63 expression on the Northern blot. The CWR xenografts (Pretlow *et al*, 1991), which are primary prostate tumor-derived xenografts, did not give a detectable signal either. The breast cancer cell line MCF7 also did not express p63. However, the normal prostate tissue derived primary cell culture PrEC, did give a discernable signal with the full-length p63 cDNA probe. The pattern of DNp63 expression observed by immunostaining of protein in normal tissue is supported by the presence of p63 expression in PrEC cell culture. On the basis of restricted expression of p63 in basal cell culture, we suggest that PrEC cells exhibit basal cell features (Figure 3.10).

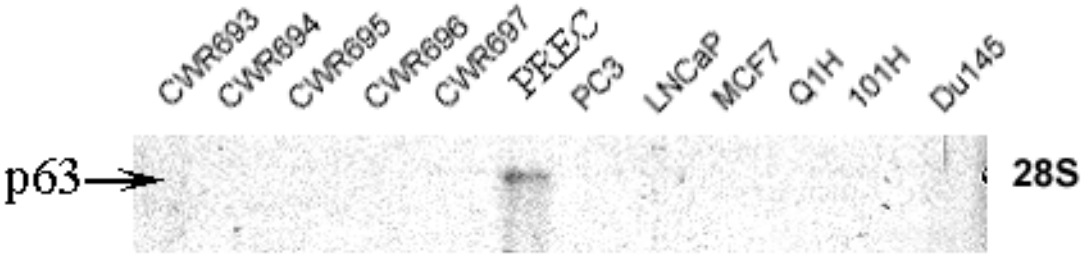


Figure 3.10: Restricted Expression of DNp63 in PrEC. A poly A+ RNA northern blot was probed with radiolabelled cDNA, showing expression of DNp63 only in PrEC.

DNp63-specific RT-PCR was performed on total RNA from a wide variety of prostate cell lines including LNCaP, PC3, Du145, DuPRO, Ki267B1, 267B1, PRNS-1, KiPRNS-1, CPDR1, CPDR3, CPDR5, CPDR6, CPDR7, CPDR8, CPDR9, and CPDR10. The PCR reaction was also performed on RNA from normal human prostate tissue. All of the CPDR cell lines showed expression of DNp63, as did Ki267B1, and to a lesser extent PRNS-1 and KiPRNS-1. As expected, RNA extracted from the human prostate gland also showed pronounced DNp63 expression (Figure 3.11).

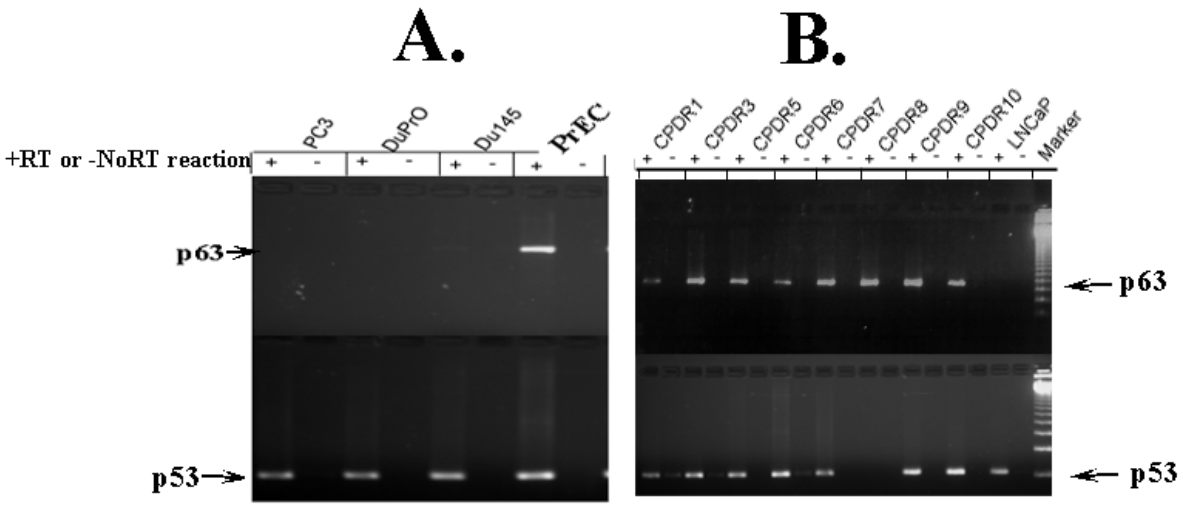


Figure 3.11: Presence of DNp63 in primary prostate cancer derived HPV16 E6/E7 transduced cell cultures, and absence of DNp63 in metastatic prostate cancer derived cell cultures.

RT-PCR for DNp63 was performed on a variety of prostate derived cell cultures. RT-PCR for p53 was performed on the same cell cultures for comparison.

A. Metastatic prostate tumor cell lines RNA (PC3, DuPro, Du145), and a normal primary prostate epithelial culture RNA (PREC).
B. CPDR primary prostate cancer derived cell lines RNA (CPDR 1, 3, 5, 6, 7, 8, 9, 10), and also the metastatic prostate tumor cell line LNCaP.

Loss of DNp63 in Late Passage Primary Prostate Cultures

The stem cells of the prostate gland are believed to be basal cells, and we have determined that DNp63 expression is limited to the basal cells. Primary CaP cultures have limited proliferative potential *in vitro* and eventually stop growing in culture. As such, late passage primary cultures may lack basal cell characteristics, including the expression of DNp63. To evaluate a possible link between the proliferative capacity of prostate cancer cells in culture and p63 expression, we analyzed p63 expression in primary prostate cancer cells at early and late passages. In primary cultures derived from two patients RC25 and RC63, p63 was expressed in the early passage (3 or 4), but not in the late passage (7), whereas the housekeeping gene, GAPDH was expressed in both late and early passage cells (Figure 3.12).

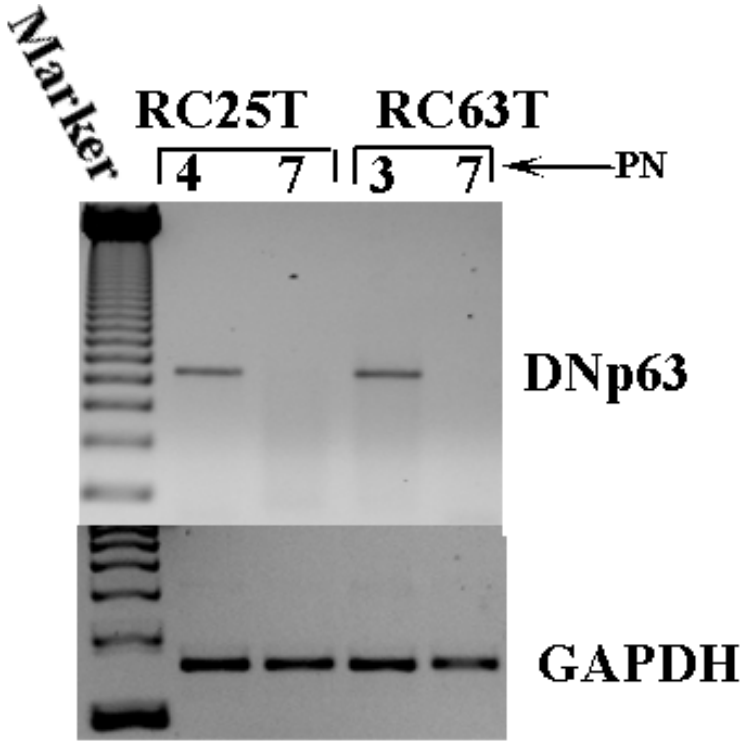


Figure 3.12: DNp63 Expression in Early and Late Passage Prostate Cancer Derived Cultures. Total RNA from passage number (PN) 4 and 7 of RC25T and 3 and 7 of RC63T were analyzed for p63 and GAPDH expression by RT-PCR as described.

To compare the results obtained from DNp63 RT-PCR with other genes associated with basal cells or senescence, PSCA and BRCA2 were amplified by RT-PCR on the same samples (RC25T passages 4 and 7, RC63T passages 3 and 7) used for DNp63 amplification. BRCA2 protein participates in the repair of double strand breaks of DNA, and it has been reported to be down regulated in quiescent cells (Vaughn *et al*, 1996). PSCA, or prostate stem cell antigen, is a gene which is restricted to basal cells in the prostate (Reiter *et al*, 1998). In both cases, late passage was associated with reduced expression of these genes (Figure 3.13).

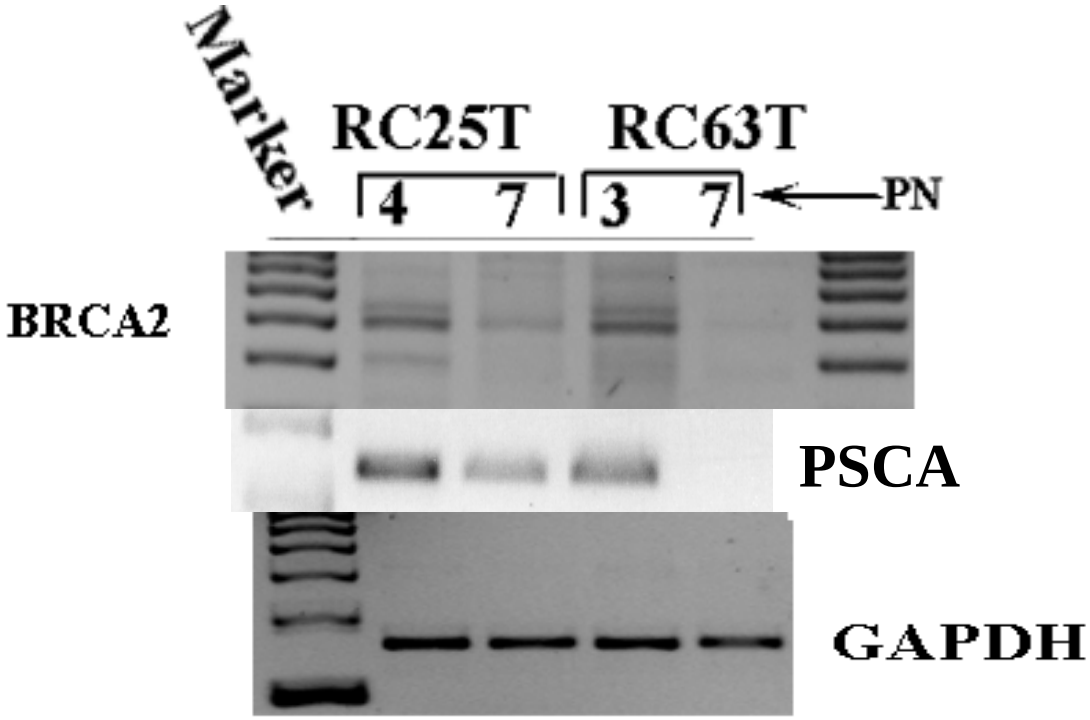


Figure 3.13: BRCA2 and PSCA Expression in Early and Late Passage Prostate Cancer Derived Cultures.
Total RNA from passage number (PN) 4 and 7 of RC25T and 3 and 7 of RC63T were analyzed for PSCA and BRCA2 expression by RT-PCR as described.

p16 or MTS1 has two isoforms, p16αINK4a and p16βARF (Stone *et al*, 1995). It has been reported that senescence is associated with increased expression of p16αINK4a

relative to p16βARF (Vogt *et al*, 1998). Therefore, we performed RT-PCR on RC25T and RC63T as before. While p16α expression intensity did not change with increasing passage number, p16β expression was reduced in later passages (Figure 3.14).

The primary cultures analyzed exhibited a number of alterations typically associated with senescence. Among these changes detected, DNp63 expression was sharply reduced, indicating that loss of DNp63 is associated with senescence.

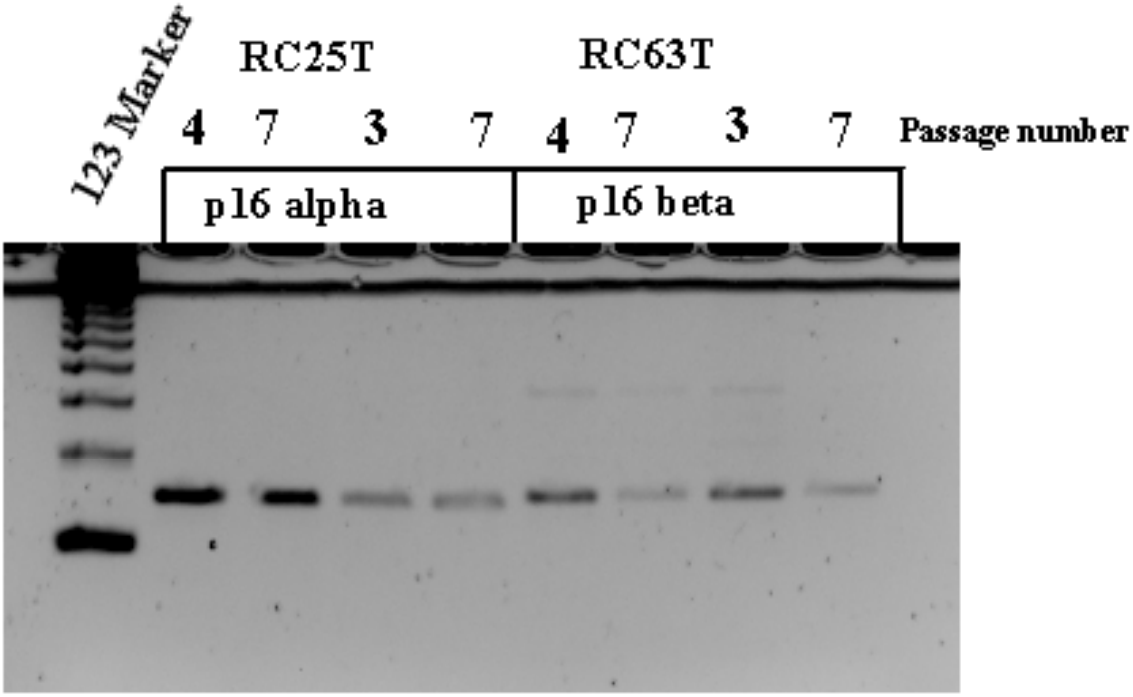


Figure 3.14: p16α/β comparative RT-PCR on senescence samples. RT-PCR was performed on early and late passage primary prostate cultures.

Discussion:

Prostate glands consist of a lumen surrounded by columnar epithelial cells, as well as secretory cells, which actively secrete components of semen. Between the secretory cells and the basement membrane line the compact appearing basal cells (Coffey, 1986). Basal cells reportedly function as stem cells replenishing the luminal secretory epithelial cells, or neuroendocrine cells (Cohen *et al*, 1993; Robinson *et al*, 1998).

Among prostate epithelial cells, DNp63 is expressed exclusively in basal cells and not in secretory cells. The almost exclusive pattern of DNp63 expression suggests that the function of DNp63 is restricted to specific cell types. On the contrary, p53 is expressed universally and has a function in all cell types. The nuclear distribution of DNp63 staining suggests a nuclear role for the protein, however, it is not clear how DNp63 may function in basal cells as DNp63 is reported to lack transcription transactivation function and exhibit inhibitory functions towards TAp63 and p53 (Yang *et al*, 1998).

DNp63 is rarely expressed in prostate tumors or established metastatic CaP cell lines. The absence of DNp63 expression, either as RNA or as protein, in CaP tumors suggests that either its expression is lost in the tumors, or that tumors are derived from cells which do not express the p63 gene.

Our data is in general agreement with recent reports (Parsons *et al*, 2001; Signoretti *et al*, 2000) as Partsons et. al (Parsons *et al*, 2001) found some weak p63 expression in a small (six percent of radical prostatectomy specimens, out of 70) subset

of tumors, and Signoretti et al (Signoretti *et al*, 2000) found consistent expression in basal cells of nonmalignant prostate tissue. We found focal and rare p63 expression in tumors. Further, new findings of p63 expression in cell cultures reported by us herein point to its utility with characterization of prostate tissue primary cultures (Signoretti *et al*, 2000).

The presence of p63 expression in numerous immortalized primary prostate cancer cell cultures derived from prostate cancer tissues suggests some basal cell features in cultures grown from primary tumor explants. p63 expression in cell cultures provides its new utility in typing the *in vitro* cultures of normal and malignant prostate tissue and it appears that prostate tumor derived primary or immortalized cell cultures either have some characteristics of basal cells or are admixed with basal cells.

On the basis of accumulating evidence from evaluation of prostate associated gene expression markers *in vitro* and *in vivo*, it is apparent that prostate tumor cells appear to express genes characteristic of both basal cells and secretory cells. These observations suggest adaptive changes in gene expression programs that favor cell proliferation in a given setting For example, NKX3.1, which is predominantly expressed in secretory epithelial cells *in vivo* (Bowen *et al*, 2000), is consistently expressed in immortalized primary prostate cancer cell cultures. On the other hand, basal cell gene expression markers DNp63 and PSCA are also expressed in a majority of immortalized prostate cell cultures.

How does DNp63 relate to cell proliferation? Basal cell phenotype is thought to be associated with proliferative phenotype. Several observations suggest that DN-p63 not only associates with the basal cell phenotype but also may regulate proliferation, especially in the prostate. It has been suggested that DNp63 may act as a dominant

negative repressor of p53 function, which would be expected to favor greater cell proliferation. Association of DNp63 with basal cells would itself suggest a role in proliferation. Finally, loss of p63 in senescent prostate epithelial cultures, noted here, as well as fibroblasts (Djelloul *et al*, 2002), support the role of p63 in either cell proliferation or survival of given cell type.

Most importantly, expression of p63 points to its critical biologic function in basal cells that remains to be understood. On the basis of our experience, DNp63 has great potential as a marker of basal cells of the prostate, possibly tumors of basal origin, and *in vitro* typing of prostate epithelial cell cultures.

Chapter 4
Probing the Biochemical Functions of p63 in the Prostate: Modulation of Glycogen Synthase Kinase 3β Phosphorylation by DNp63

Introduction

The elucidation of the basal cell specific expression of p63, a novel p53 homolog in the human prostate gland, provided an extremely important clue with respect to biological functions in the context of prostate growth and development. Our current hypothesis is that DNp63 plays critical roles in the cell growth or maintenance of prostate basal cell phenotype. Here, we report the results of a substudy undertaken to explain the mechanism of function of p63 in the prostate through phosphoprotein read-out that play important roles in cell growth and differentiation.

The epithelial cells in the prostate gland are frequently involved in both prostate cancer, and BPH (Coffey, 1986). Prostate cancer is the most commonly diagnosed non-skin cancer in American males. Although stromal/epithelial interactions play a very important role in the disease process, the cancer cells are of epithelial origin. Whether the cancer cells are from basal cells or from luminal secretory cells is still debated. Conventional cancer biology principles suggest that basal cells are the origin of cancer cells, however, gene expression markers associated with luminal (PSA or AR) or basal

(telomerase and Bcl2) cells suggest either luminal or basal epithelial origin of prostate cancer cells. It is likely that some trans-differentiation processes may also take place in epithelial cell carcinogenesis or the prostate.

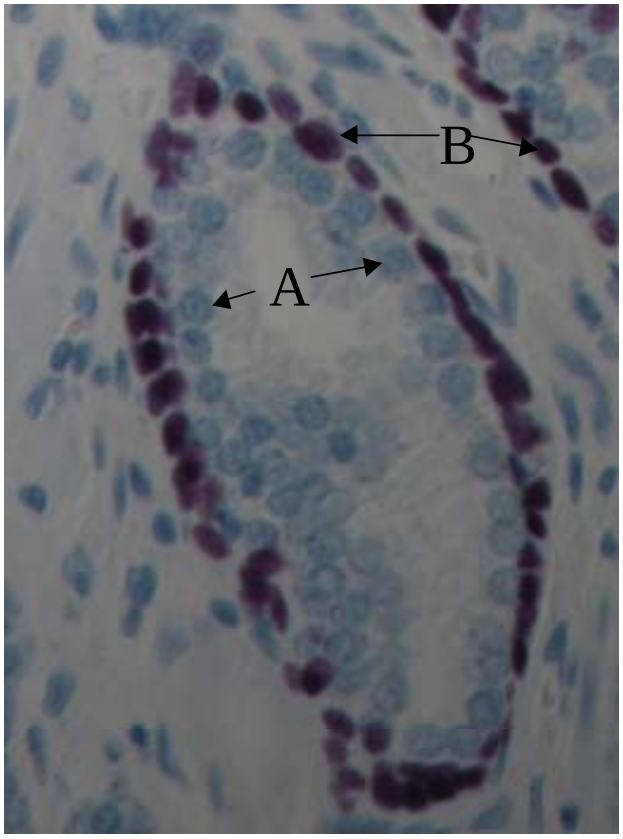


Figure 4.1: Basal cell specific expression of p63 in the prostate.
A section of benign prostate gland stained with H & E and anti-DNp63 antibody. Basal cells in purple represent p63 staining (B). Secretory cells stained blue do not express p63 (A).

As noted earlier, DNp63 is basal cell specific and is not expressed in luminal cells of normal gland or in carcinoma. p63 knockout mice experiments have shown p63 to be necessary for the formation of epithelial structures including the prostate glandular structure (Signoretti *et al*, 2000).

We have shown detectable p63 expression in a series of E6/E7 immortalized cell cultures, derived from prostate tumor tissues. The E6 and E7 genes of virus inactivate

p53 and retinoblastoma (Rb) genes respectively (Dyson *et al*, 1989; Scheffner *et al*, 1990). Primary cultures of prostate without immortalization express p63; however, their expression of p63 is significantly reduced or lost in late passages.

Previously, we had shown that p63 expression is lost in late passage primary prostate epithelial culture. p16 (MTS1) has been associated with senescence. RT-PCR results indicated that p16 may also be modified in late passage cells. The data suggested that p63 expression inversely correlated with senescence and further suggested the link of p63 in cell proliferation or maintenance of specific cell type of basal cells in the prostate gland. Therefore, we hypothesized that p63 functions may interfere with common biochemical pathways involved in cell growth and differentiation. In order to further understand the biochemical functions of p63, we have analyzed the RB, p53, and Wnt signaling pathways which include proteins/phosphoproteins commonly involved in cell growth and differentiation (Table 4.1).

Rb	**Wnt**	**p53**
Rb	β-catenin	p53
Rb (P-807/811)	Pi- β-catenin	p53 (P-6)
Rb (P-795)	Cyclin D1	p53 (P-9)
Rb (P-780)	GSK3β	p53 (P-15)
	GSK3β (P-9)	p53 (P-20)
	Akt	p53 (P-37)
	Akt (P-308)	p53 (P-46)
	Akt (P-473)	p53 (P-392)

Table 4.1: Antibodies by pathway involved.

Therefore, to experimentally address the biochemical functions of p63 in the prostate, we have used the most widely used prostate cancer epithelial cell model, LNCaP, with exogenous expression of DNp63. LNCaP cells are androgen responsive and closely resemble the gene expression patterns of the prostatic epithelial cells *in vivo*.

The experimental strategy to study p63 gene functions was to employ exogenous expression of DNp63 in prostate epithelial cell culture model. Adenoviral vectors have several advantages over other systems used for the expression of exogenous genes in cell culture lines. In excess of 95% of cells in a culture may be transfected through adenovirus with a desired time kinetics. Transient expression of p63 in this cell culture model provides a flexible experimental system to evaluate down stream targets of p63.

p16 (MTS1), as we have described earlier (Chapter 3), has been implicated in senescence (Vogt *et al*, 1998), as has the retinoblastoma tumor suppressor protein (Rb) (Alcorta *et al*, 1996). p53 activation has been associated with senescence. These proteins have been shown under some circumstances to be affected by GSK3β. When it is active, GSK3β has been reported to promote the serine 33 phosphorylation of p53 (Alcorta *et al*, 1996). GSK3β has been reported to phosphorylate β-catenin, which phosphorylation results in the proteolytic degradation of β-catenin (Hart *et al*, 1998). High levels of β-catenin have been reported to increase the levels of p16INK4a relative to p16ARF, which in turn has been reported to stimulate Rb activity (Damales *et al*, 2001). All of these proteins have biologically relevant phosphorylated forms, and are implicated in the regulation of cell growth/ cell cycle (Figure 4.2).

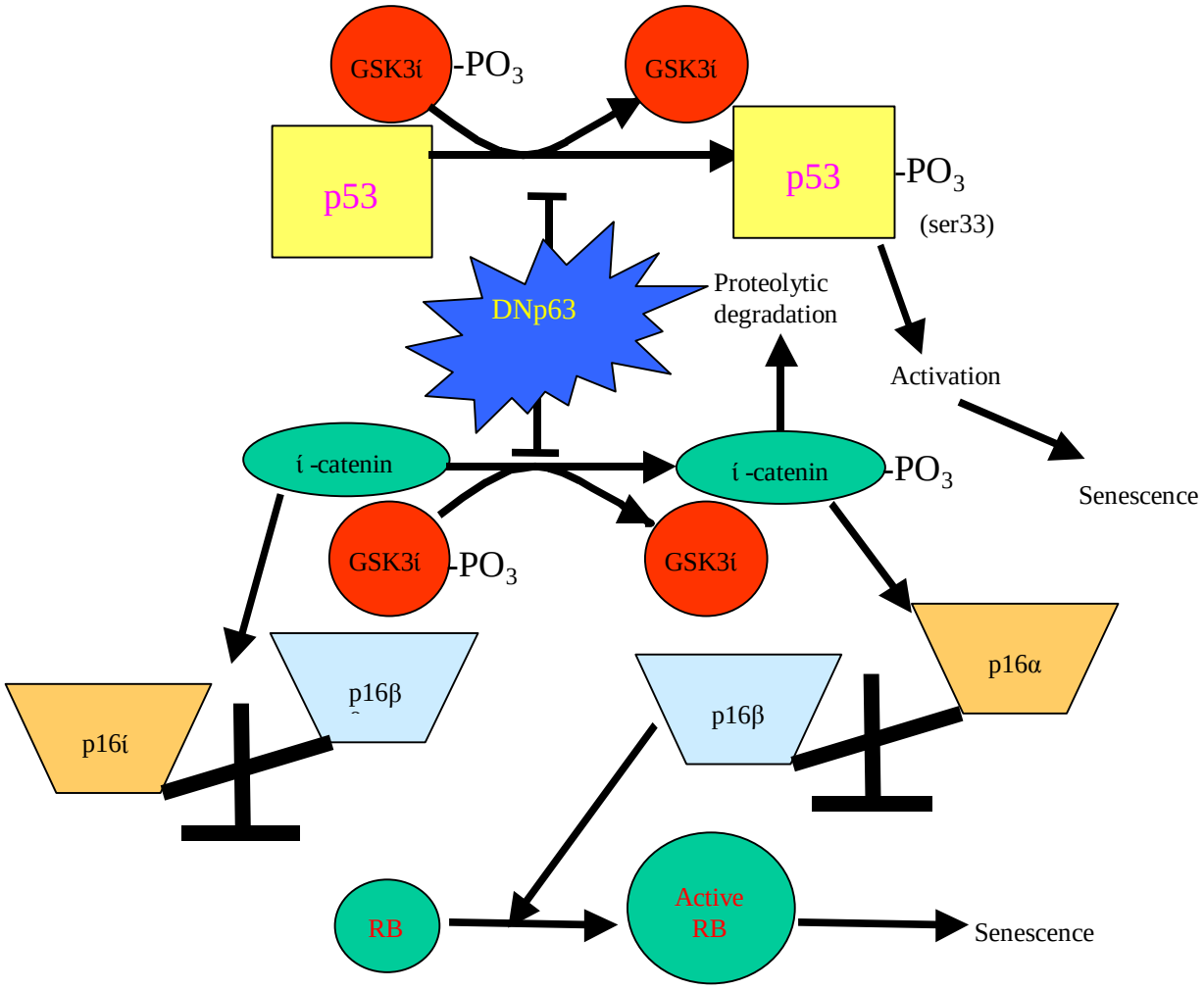


Figure 4.2: Evaluation of the Potential Interface of p63 with Critical Cell Signaling Pathways

DNp63 has been shown to increase β-catenin concentration in squamous cell carcinoma, with a cell growth stimulatory affect. DNp63 was found to bind to protein phosphatase 2A (PP2A) and glycogen synthase kinase 3β (GSK3β), which form part of the Wnt signaling pathway. Interestingly, this pathway may be involved in contact inhibition, as β-catenin is associated with transmembrane proteins including N-cadherin that make adherens junctions. When cells are unable to bind to other cells, the β-catenin may localize to the nucleus and stimulate cell proliferation, unless it is targeted for

degradation by phosphorylation by GSK3β. Apparently, the presence of DNp63 in squamous cells leads to increased cell proliferation and increased levels of β-catenin. The N-terminal domain of DNp63 interacts with PP2A and GSK3β which may lead to inhibition of GSK3β and decreased phosphorylation stabilizing β-catenin so that it translocates to the nucleus (Patturajan *et al*, 2003).

Glycogen synthase kinase 3β (GSK3β), which has been implicated in multiple signaling pathways, exhibits reduced serine 9 phosphorylation in LNCaP cells in the presence of exogenous p63. Serine 9 phosphorylation of GSK3β has been reported to inhibit the kinase activities of GSK3β, which include phosphorylation of β-catenin, and its translocation into the nucleus for β-catenin to function as a transcription factor. Therefore, increased GSK3β serine 9 phosphorylation is a signal for β-catenin translocation to the nucleus and cell proliferation.

The highlight of our study is that DNp63 overexpression causes a significant reduction of serine 9 phosphorylation in GSK3β in LNCaP cells. However, β-catenin expression or phosphorylation is not modified by p63. Also, other genes such as cyclin D1 which are associated with Wnt pathway are not modified by DNp63, indicating a distinct regulatory function of GSK3β in response to DNp63 expression in LNCaP. Further studies may be needed to fully clarify this issue.

Materials and methods

Preparation of adenovirus wtp53 and DNp63 expression vectors

DNp63 cDNA clone was obtained from Dr. David Sidransky's (Johns Hopkins Medical Institutions, Baltimore) (Invitrogen, KNM2000-01). DNp63 cDNA was released from the vector using XhoI (New England Biological, R0146S) and HindIII (New England Biological, R0104S) restriction enzymes. The DNp63 insert was purified from the vector by agarose gel electrophoresis followed by purification with the Qiagen gel purification kit. pAdEasy vectors and BJ5183 bacteria were obtained from Drs. Bert Vogelstein and Kenneth Kinzler's laboratory (Johns Hopkins Medical Institutions, Baltimore) (Figure 3). pAdTrackCMV, which uses a cytomegalovirus (CMV) promoter and expresses green fluorescent protein (GFP) via a separate expression, was digested with XhoI and HindIII. The restriction enzyme digested pAdTrackCMV DNA was then treated with calf intestinal phosphatase (New England Biological, M0290S), and subjected to proteinase K digestion (New England Biological, P8102S) to remove the phosphatase. The HindIII and XhoI digested DNp63 and vector DNA were subjected to phenol chloroform extraction, and ethanol precipitation. The precipitated DNAs were dissolved in molecular biology grade water. Both DNp63 and vector DNAs were quantitated by ultraviolet spectroscopy, then they were ligated together by incubation with T4 DNA ligase (New England Biological, M0202S).

The resulting reaction mixtures were then electroporated into Invitrogen's DH10B Electromax cells (Invitrogen, 18290-015). The DH10B cells marketed as Electromax

were suspended in glycerol rich buffer supplied by the manufacturer. The DH10B cells were mixed with the ligated DNp63 and vector DNA, put into an electroporation cuvette, and subjected to a short duration high electrical field, generated with the use of a capacitor bank. The transformed bacterial cells were then incubated in SOC (Quality Biological, 340-031-100) growth media for one hour at 37ºC, and spread on LB agar plates containing 25 µg/ml kanamycin.

Five colonies derived from ligated DNA and one from the unligated DNA were picked. The colonies were then put in LB media containing 25 µg/ml kanamycin and cultured for six hours, before refrigeration at 4º C. 5 µl aliquots from each bacterial culture were drawn and added directly to the PCR reaction using DNp63 PCR primers. Two pAdTrackCMV-DNp63 colonies were further cultured and used to make plasmid minipreps using the Qiagen spin mini-prep kit (Qiagen, 27104). One of the colonies was linearized with PmeI restriction enzyme, phenol/chloroform extracted, and resuspended in molecular biology grade water. It was then mixed with the pAdEasy-1 vector, and this mixture was then electroporated into BJ5183 cells which had been rendered electrocompetent by replacing the LB growth media with a solution of 10% glycerol in water. As recA participates in homologous recombination, which often can result in unwanted modification of cloned genes, most laboratory strains of *E. coli* used for cloning are recA-. In the AdEasy system, homologous recombination in bacteria is necessary to combine the cloning vector with the full-length adenovirus genome. The full-length genome is too large to use for cloning with restriction enzymes. Therefore BJ5183 are recA+ to allow for the necessary homologous recombination.

After initial incubation, the cells were plated on 25 μg/ml kanamycin Luria-Bertani (LB) agar plates. It is critical to avoid picking abnormally small colonies. One of the properties of these large insert vectors is that they are slightly growth inhibiting to bacteria, so one gets a bimodal distribution of bacterial colony size. The larger colonies are from pAdTrackCMV that has been able to recircularize, and do not contain the needed recombinant DNA. The slightly smaller colonies often contain the recombinant DNA. Ten colonies were picked to make minipreps, and the resulting plasmid DNA was run on a 1% agarose gel for size determination. Recombinant plasmids (pAdDNp63) were expected to exhibit dramatically higher molecular weight than pAdTrackCMV.

All of the picked colonies gave plasmids of a large size, indicating that they were recombinants. After verification of DNp63 gene by PCR amplification with DNp63 specific primers (see chapter 3), one colony was selected for further work. (Figure 4.3)

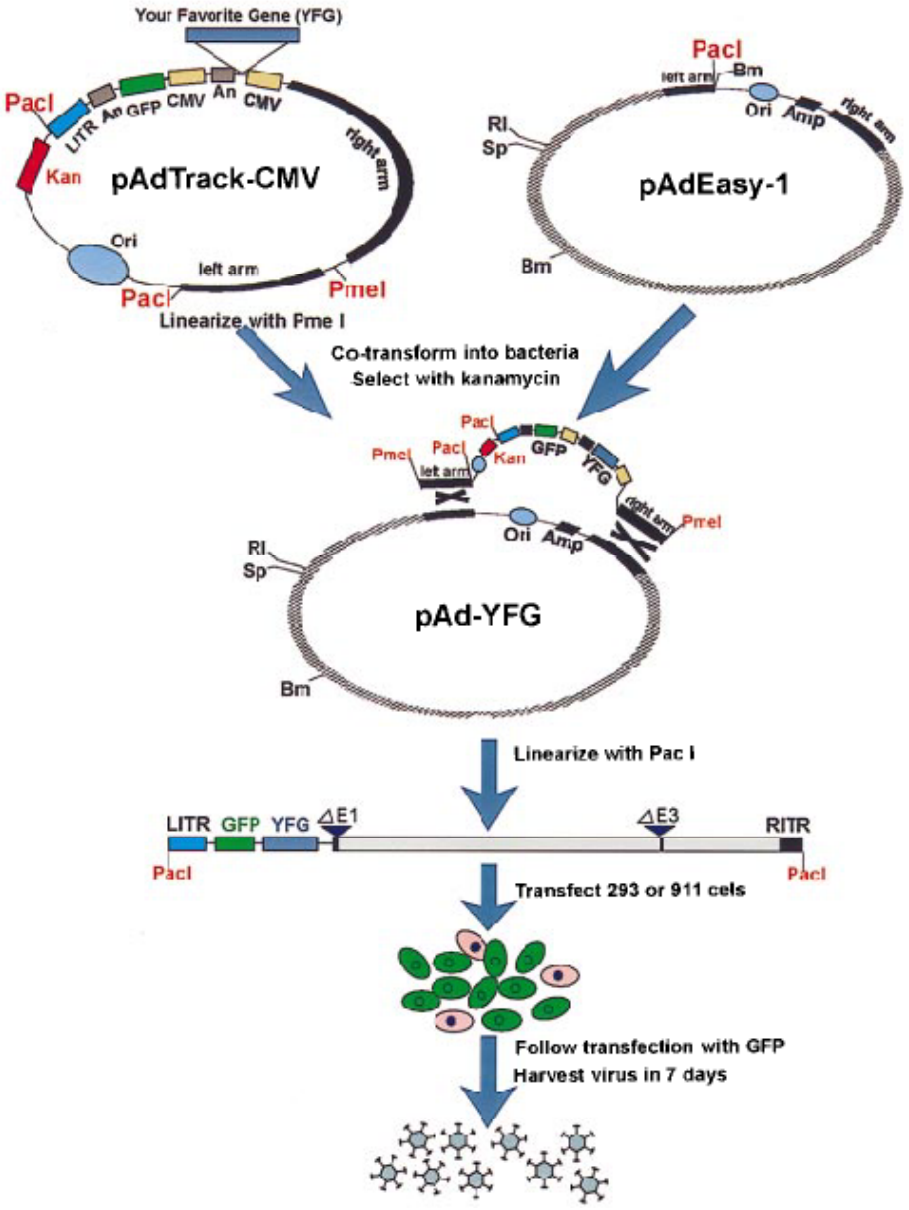


Figure 4.3: Procedure for producing recombinant adenovirus in AdEasy system.(He *et al*, 1998)

Production of DNp63 adenovirus (pAdDNp63) in Eukaryotic Cells

The recombinant plasmid (pAdDNp63) to be used for transfection into mammalian cells was made in greater quantities using the Qiagen midi-prep plasmid purification protocol (Qiagen, 12143), cut with PacI, and then transfected the plasmid into 293 cells using lipofectamine (Invitrogen, 18324-111). The adenoviruses produced in the AdEasy system are replication deficient due to the lack of the adenovirus gene E1. This improves laboratory safety, but requires the use of mammalian cells, such as 293 or 911, which express the missing E1 from their chromosomes. 293 cells are E1-transformed human embryonic kidney cells, and 911 cells are E1-transformed human embryonic retinal cells. pAdTrackCMV and the adenoviruses made with it contain a gene expression cassette entirely separate from the multiple cloning site for the expression of green fluorescent protein (GFP). As a result, cells infected with these recombinant adenoviruses express GFP, which is readily visible by fluorescent microscopy. After two weeks, green fluorescent cells were identified, and the cells were scraped into Hank's Balanced Salt Solution (HBSS). The 293 cells were subjected to several cycles of freezing in a dry ice/methanol bath and thawing in a 37º C water bath, and the cell debris was removed by low speed centrifugation at 3000 x g. The supernatant containing adenovirus was used to infect new batches of 293 cells. This was done three times, until 293 cells were consistently becoming green and rounding after two days. Because the adenovirus can multiply in 293 cells, 293 cells can show cytopathic effects from adenovirus growth even if the trans-gene does not itself have any cytopathic affects. Cell lines such as LNCaP which do not contain adenovirus E1 gene

do not show cytopathic effect from adenovirus alone except at very high multiplicities of infection (MOI).

For the production of high titer virus, the adenovirus containing cell supernatant was used to infect 293 cells. After most of the cells detached (in two days), the viral supernatant was prepared as stated above, by multiple cycles of freezing and thawing followed by centrifugation at 3000 g to remove cellular debris. 4.4 g cesium chloride (CsCl) was added to the supernatant, and the resulting fluid was spun18 to 24 hours at 32,000 RPM (126,400 g average) in an SW41 rotor, resulting in a turbid band approximately midway between the top and the bottom of the tube. This turbid band was withdrawn using a needle, mixed with 2X storage/dialysis buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 0.1% BSA, and 50% glycerol, filter sterilized), and then the sample was dialyzed in Slide-A-Lyzer dialysis cassettes (Pierce, 66405) against dialysis buffer (100 mM Tris, pH 7.4, 20% glycerol by volume) in order to remove CsCl in the adenovirus preparation. The adenovirus preparation was then aliquoted and frozen at -70˚C until needed for further use.

Titering was accomplished on the high titer stocks by serially diluting the virus, and seeding onto wells in six well dishes containing 80% confluent 293 cells. After allowing for 16 hours or more of infection, the number of green cells present were counted under a fluorescent microscope.

The recombinant p53 adenovirus was made following a similar protocol as for the production of pAdDNp63. Full-length wtp53 cDNA was provided by Dr. Shiv Srivastava which was flanked with XbaI sites. pAdTrackCMV was digested with XbaI, prior to ligation of p53. As it was possible for p53 to be oriented in either direction,

orientation was checked by sequencing and by PCR. Control adenovirus (N2) was produced by not inserting a gene into the multiple cloning site.

A protocol for producing recombinant adenovirus is described in a detailed protocol, “A Simplified System for Rapid Generation of Recombinant Adenoviruses”, at http://www.coloncancer.org/adeasy/protocol.htm.(He *et al*, 2004)

Adenovirus infection of LNCaP Cells:

Prostate cancer or primary prostate cell cultures were infected with adenovirus vectors at 70% confluence. LNCaP was the main cell line used in this study. LNCaP cells were obtained from ATCC (CRL-1740) and grown according to suppliers recommendation. LNCaP is cultured in RPMI 1640 media (Invitrogen, 21870-076) containing 10% heat inactivated fetal bovine serum. For adenovirus infection of LNCaP cells on T75 flasks, the cell culture media was replaced with 2 ml of RPMI 1640 containing the adenovirus and only 2% fetal bovine serum (FBS). Other cell cultures were infected in a similar manner.

After two hours incubation at 37 °C in 5% CO_2 incubator, a 8 ml of 10% FBS media was added to the cells. For time course experiments, a simultaneous initial infection of all samples was followed by staggered harvesting of samples.

Preparation of Cellular Proteins for Western Blot Analysis:

To harvest the adenovirus infected cells, cells were scraped in the cell culture media, and transferred into 15 ml tubes. The cells were centrifuged at 700 x g for five minutes at room temperature. After aspirating the media (10 ml for cells grown on 75 cm^2), cold 1X PBS (Invitrogen, 14040-133) was added and the cells were suspended by inversion. Cells were spun down as before and the PBS was aspirated.

To 10 ml T-Per lysis buffer (Pierce, 78510), was added 100 µl each of the three protease and phosphatase inhibitor cocktails which included Protease Inhibitor Cocktail (PIC) (Sigma, P-8340); Phosphatase Inhibitor 1 (PI1)(Sigma, P2850); and Phosphatase Inhibitor 2 (PI2)(Sigma, P-5726)

Cells were lysed in freshly prepared T-Per cell lysis buffer. Typically, 1 ml was used for a cell pack from a 75 cm^2 flask, and 200 µl was used for cells from a well of a six well dish. Cells were incubated on ice for 15 minutes, transferred to Eppendorf tubes, then spun for 5 minutes in a microcentrifuge at 5000 x g at room temperature, before collecting the supernatant.

Protein quantitation was performed by BCA assay, (Pierce, 2325). Assay was performed per supplier's recommendation. A known quantity of standard protein (bovine serum albumin) was used for determining the total protein concentration in cell lysate.

Western blot analysis

Polyacrylamide gels (4-20%, Tris-glycine) were used (Invitrogen, EC6025 Box). The gel apparatus (Invitrogen, EI0001), buffers (Gel running buffer, Invitrogen, LC2675, Transfer buffer, LC3675), and nitrocellulose (Invitrogen, LC2000) were used per supplier's recommendation. Twenty micrograms of protein sample was mixed with 2X loading buffer (Invitrogen, LC2676) loaded in each lane of the gel. A dye tagged molecular weight marker mixture was run in parallel in one lane of the gel. The gels were run at 100 V until the lowest molecular weight marker was near the bottom (approximately 90 minutes.) When protein entities were to be compared in the same sample, multiple gels containing the same samples were run simultaneously.

The protein from the gels was then electrotransfered to the membrane, using the transfer apparatus (Invitrogen, E19051) and transfer buffer at 25 V for two hours.

Immunodetection of Proteins on Western Blots:

Once the Western blots had been prepared, these were put into 1 X PBS (diluted from 10X, Quality Biological, 119-069-101) containing 0.1 % Tween (BioRad, 170-6531) and 5% powdered milk (Richfood Quality Menu), and incubated in trays with rocking at room temperature for one hour. The blots were then washed with 1X PBS containing 0.1% Tween 20 (hereafter, PBS-T) once for thirty minutes, followed by two additional washes of fifteen minutes each. The primary antibody was added in PBS-T at the optimal dilution determined in our laboratory (Table 4.2) and blots were then incubated at room temperature for two hours, or at 4°C for sixteen hours. The blots were then washed as before, prior to the addition of horse radish peroxidase (HRP) conjugated

secondary antibody at 1/5000 dilution in PBS-T containing 2% milk. The blots were incubated in secondary antibody for thirty minutes at room temperature, and then washed again in PBS-T as before.

The blots were then treated with chemiluminescence reagent for use with HRP and exposed to X-ray film ("Western Lightning" chemiluminescence kit from Perkin Elmer, and X-Omat K film from Kodak). Exposure time varied according to band intensity and background, and the correct time for a blot was determined by serial exposure of films.

Table 4.2: Antibody characteristics.

Target of antibody	Company	Catalog Number	Molecular Weight of Target	Species of antibody source	Dilution
GSK3-beta Phospho-Ser 9	Cell Signaling Technology	9336	46 kD	Rabbit	1/1000
GSK3-beta	Cell Signaling Technology	9332	46 kD	Rabbit	1/1000
beta-catenin Phospho-(Ser 33/37/Thr 41)	Cell Signaling Technology	9562	92 kD	Rabbit	1/1000
beta-catenin	Cell Signaling Technology	9561	92 kD	Rabbit	1/1000
Cyclin D1	Cell Signaling Technology	2926	35 kD	Mouse	1/1000
AKT phospho-Thr 308	Cell Signaling Technology	9275	60 kD	Rabbit	1/1000
AKT phospho-Ser 473	Cell Signaling Technology	9271	60 kD	Rabbit	1/1000
AKT	Cell Signaling Technology	9272	60 kD	Rabbit	1/1000
p53	Santa Cruz Biotechnology	126	53 kD	Mouse	1/1000
alpha-tubulin	Santa Cruz Biotechnology	5286	58 kD	Mouse	1/5000
p63	Santa Cruz Biotechnology	8431	40 kD	Mouse	1/1000
Rabbit IgG, HRP linked	Cell Signaling Technology	7074		Goat	1/5000

Target of antibody	Company	Catalog Number	Molecular Weight of Target	Species of antibody source	Dilution
Mouse IgG HRP-linked	Cell Signaling Technology	7076		Horse	1/5000

Cytotoxicity Assay to Determine Effect of the wtp53 and DNp63 on the Growth of Prostate Cancer Cells

PC3 cells were trypsinized and plated on six well plates at a density of 50,000 cells per well. After the cells were well attached to the plates (48 hours), the cells were infected with either pAdp53 or pAdDNp63 adenovirus as described above at various MOI. After 48 hours, the cell culture media was aspirated, and the cells were washed with 1XPBS (phosphate buffered saline), followed by 15 minutes washing in 1XPBS containing 2% formaldehyde. Then, the cells were incubated 15 minutes in 1XPBS containing 0.5% crystal violet. The cells were washed with distilled water and allowed to dry. Purple stained colonies of cells were analyzed visually.

Results

Characterization of Adenovirus Expression Vectors of DNp63 and wtp53 and Optimization of Infection Conditions

Adenovirus DNp63 (AdDNp63), adenovirus wtp53 (Adp53wt), and the control adenovirus (Ad-C) were from our laboratory. Adenovirus mtp53 (AdmtR175Hp53) was a gift from Dr. Bert Vogelstein. (Johns Hopkins Medical Institutions, Baltimore) Adenovirus mediated expression of DNp63 (Figure 4.4) and other genes (data not shown) was confirmed by Western blot.

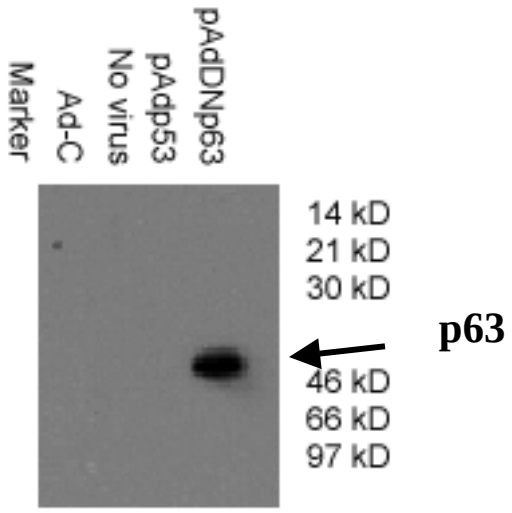


Figure 4.4: Characterization of adenovirus by Western blot.
Western blot of pAdDNp63 adenovirus infected LNCaP cells in comparison to LNCaP cells infected with other adenoviruses. Anti-DNp63 antibody was used for immunodetection.

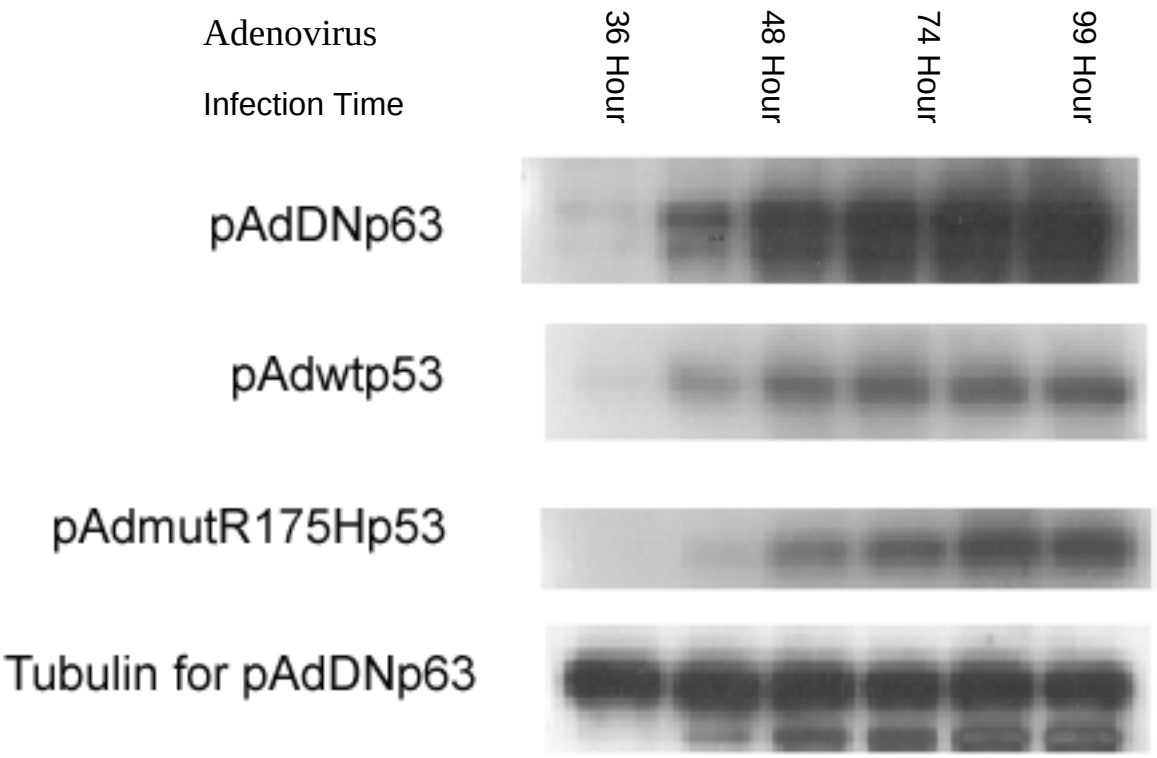


Figure 4.5: Optimization of adenovirus vectors dose and time of infection in LNCaP cells.
Timecourse to establish kinetics of exogenous gene expression resulting from adenovirus infection protocol. 5 pfu/cell virus was used to infect LNCaP cells in 6 well plates, and the cells were harvested at the times indicated. Detection was accomplished by Western immunodetection.

We have optimized protocols for the infection of prostate cancer cells with adenovirus vectors expressing wtp53, mtp53, and DNp63. The high efficiency (greater than 90%) and robustness of adenovirus transfections have made recombinant adenovirus expression vectors of choice for infection of prostate epithelial cells. We determined the dose reponse and time course of infection in LNCaP cells (Figure 4.5) by Adwtp53, Admtp53, AdDNp63, and Ad-C. (Figure 3). We chose 5 and 25 pfu/cell of adenovirus particles as optimal multiplicity of infection (MOI) and 12 and 24 hours as time of infection for subsequent experiments.(Figure 4.5)

DNp63 Modulation of Proteins Involved in p53, RB, and Wnt Signaling Pathways

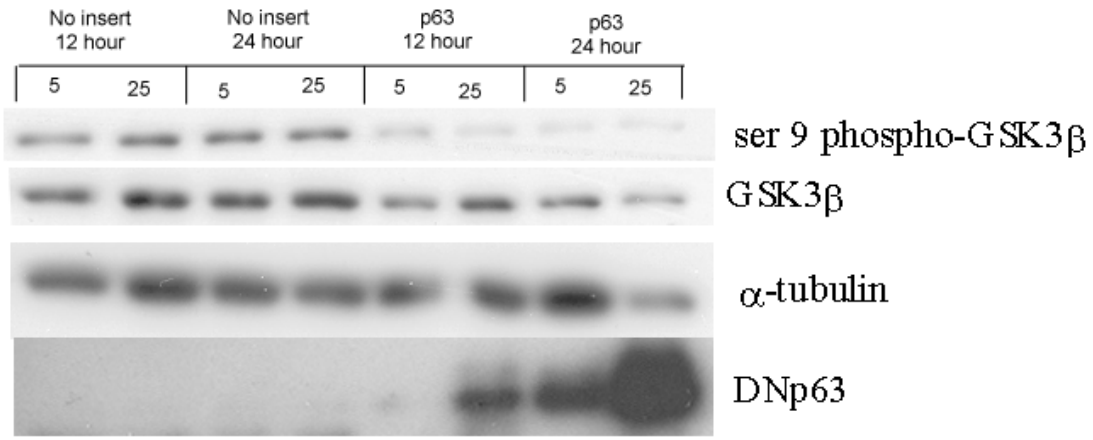
p63 is believed to play a critical role in maintenance and growth of basal epithelial cells in the prostate. Because of our observation that DNp63 expression is lost in late passage primary prostate epithelial cultures, and DNp63 expression is retained in immortalized prostate tumor-cell cultures, we chose to study the effects of p63 on the phosphorylation status of proteins involved in cell growth/ cell cycle regulation. Many of these proteins are known to be regulated by phosphorylation. Biochemical pathways interfacing with p63 are not known in prostatic epithelial cells.

We reasoned that using the LNCaP cell culture model transiently overexpressing DNp63 in the null background of prostatic epithelial LNCaP cells, may be able to ientify those protein phosphorylation signals which interface with its biochemical functions. In order to observe early regulatory changes induced by p63, we sampled at a time shortly after detectable p63 expression in the LNCaP cells. Early sampling times was also expected to reduce the non-specific effects of p63. Since we were interested in assessing DNp63 specific effects, adenovirus control infected cells were processed in parallel.

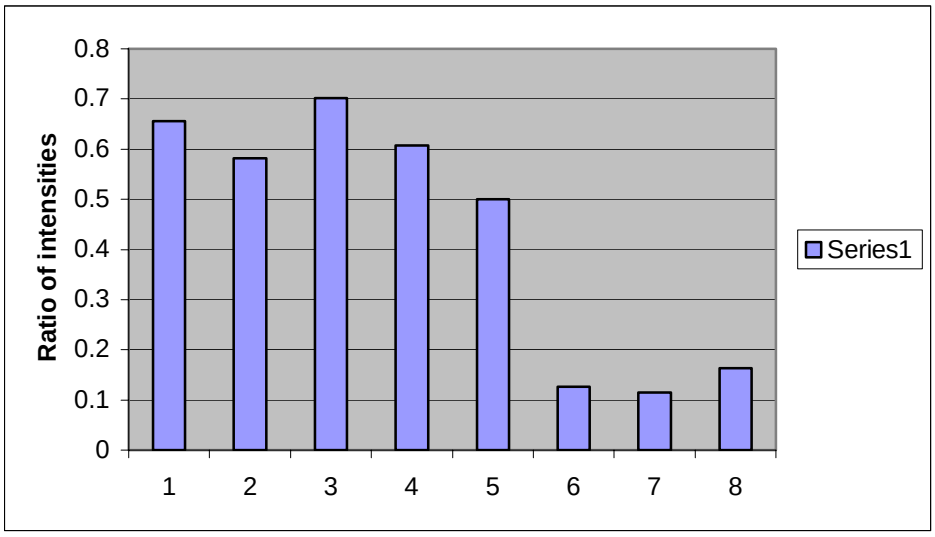
Having established the protocol to be used for adenovirus infection, we infected LNCaP cells and harvested their protein for Western blots. No consistent change in the various phosphorylation sites of p53 or Rb proteins was detected. However, reduced phosphorylation levels of GSK3β in pAdDNp63 infected cells was noted. Relative to total levels of GSK3β, there was significantly less serine 9 phosphorylation of GSK3β in DNp63 adenovirus treated LNCaP than in control adenovirus treated LNCaP cells.

(Figure 4.6). This observation has been confirmed in two independent experiments. (Data not shown)

A Loss of serine 9 phosphorylation in the Presence of DNp63 expression



B Loss of Serine 9 Phosphorylation in GSK3β in p63 Overexpressing Cells, by Ratio of phospho-GSK3β to GSK3β Band Intensities



1: No insert adenovirus, 5 pfu/cell, 12 hours.
2: No insert adenovirus, 25 pfu/cell, 12 hours.
3: No insert adenovirus, 5 pfu/cell, 24 hours.
4: No insert adenovirus, 25 pfu/cell, 24 hours.
5: p63 adenovirus, 5 pfu/cell, 12 hours.
6: p63 adenovirus, 25 pfu/cell, 12 hours.
7: p63 adenovirus, 5 pfu/cell, 24 hours.
8: p63 adenovirus, 25 pfu/cell, 24 hours.

Figure 4.6: Reduction of GSK3β serine 9 phosphorylation in the presence of DNp63 expression.

A. Western blots analysis. Immunodetection was performed by antibodies indicated. Note the loss of GSK3β with increasing p63 expression.
B. Quantitation of GSK3β phosphorylation in relation to total GSK3β. Corresponding GSK3β and ser 9 GSK3β bands were selected from scanned images of the X-ray films and analyzed for mean density by Scion Image software.

The phosphorylation of serine 9 on GSK3β has been reported to reduce the kinase activities of GSK3β. This inhibition of GSK3β is reported to prevent the phosphorylation mediated degradation of β-catenin. Therefore in Wnt signaling a critical biochemical pathway involved in development, cell growth, and differentiation, increased GSK3β ser 9 phosphorylation leads to reduced β-catenin phosphorylation and increased nuclear β-catenin. Elevated nuclear β-catenin functions as a transcription factor to promote cell growth. However in our experiments, we observed reduced GSK3β ser 9 phosphorylation in response to DNp63 overexpression. These observations are also distinct from increased GSK3β phosphorylation in squamous cell head and neck carcinoma by DNp63 expression. DNp63 overexpression in lung and head and neck squamous cell carcinoma is believed to promote cell proliferation. In the context of prostate cancer, however, DNp63 is rarely expressed in cancer cells and its expression is restricted to basal epithelial cells of normal glands. Therefore, it is reasonable to postulate that reduced GSK3β-ser 9 phosphorylation in response to DNp63 signal suggests distinct functions of p63 in prostate basal epithelial cells.

DNp63 Modulation of Other Wnt Pathway Proteins

GSK3β is involved in many aspects of cell physiology, from glucose metabolism, to growth regulation, to cell-cell interactions. The establishment of GSK3β modification by DNp63 led us to question targets of interest in relation to GSK3β. AKT, otherwise known as protein kinase B, is one of the major downstream effectors of PI3 (phosphatidyl-inositol) kinase. It has been reported in the literature that GSK3β is modified by AKT. It has also been reported that GSK3β modifies β-catenin. β-catenin levels are increased in the face of cell growth stimulation in the Wnt pathway. Cyclin D1 is an eventual transcriptional target in this pathway.

We did not consistently observe changes in levels of β-catenin or cyclin D1 in response to pAdDNp63. In squamous cells, it was reported that increased expression of DNp63 increased levels of β-catenin.(Patturajan *et al*, 2003) Cyclin D1 is one of many downstream effectors of β-catenin induced proliferation, but we did not see consistent changes in cyclin D1 by Western blot either. (Figure 4.7)

Wnt pathway effectors cyclin D1 and β-catenin are not altered by DNp63.

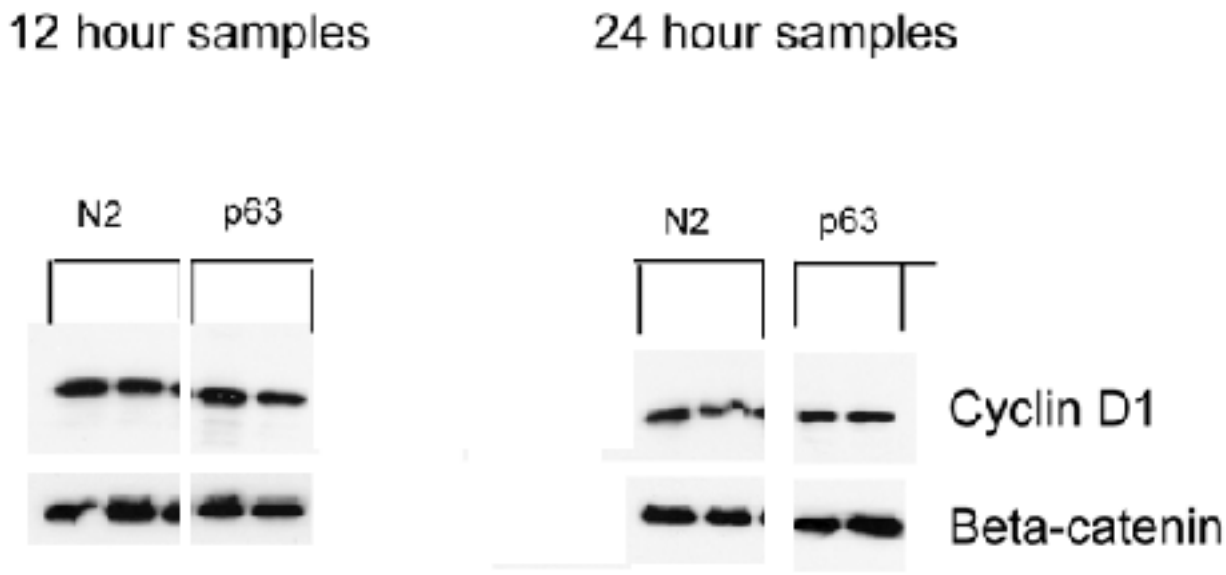


Figure 4.7: p63 overexpression does not affect cyclin D1 and β-catenin protein levels in LNCaP cells.

DNp63 modulation of AKT

AKT has been reported to inactivate GSK3β by serine 9 phosphorylation. AKT has been reported to become more active when it is phosphorylated either on serine 473 or threonine 308. Having seen an effect on GSK3β caused by DNp63 expression in LNCaP, we asked if this change could be mediated through AKT. AKT threonine 308 phosphorylation was not detectably modified in the presence of p63 as observed by phospho-specific Western immunodetection. It is possible that AKT could play a contributing upstream role to GSK3β phosphorylation in p63 expressing LNCaP cells. However, we need further confirmation of AKT phosphorylation data.

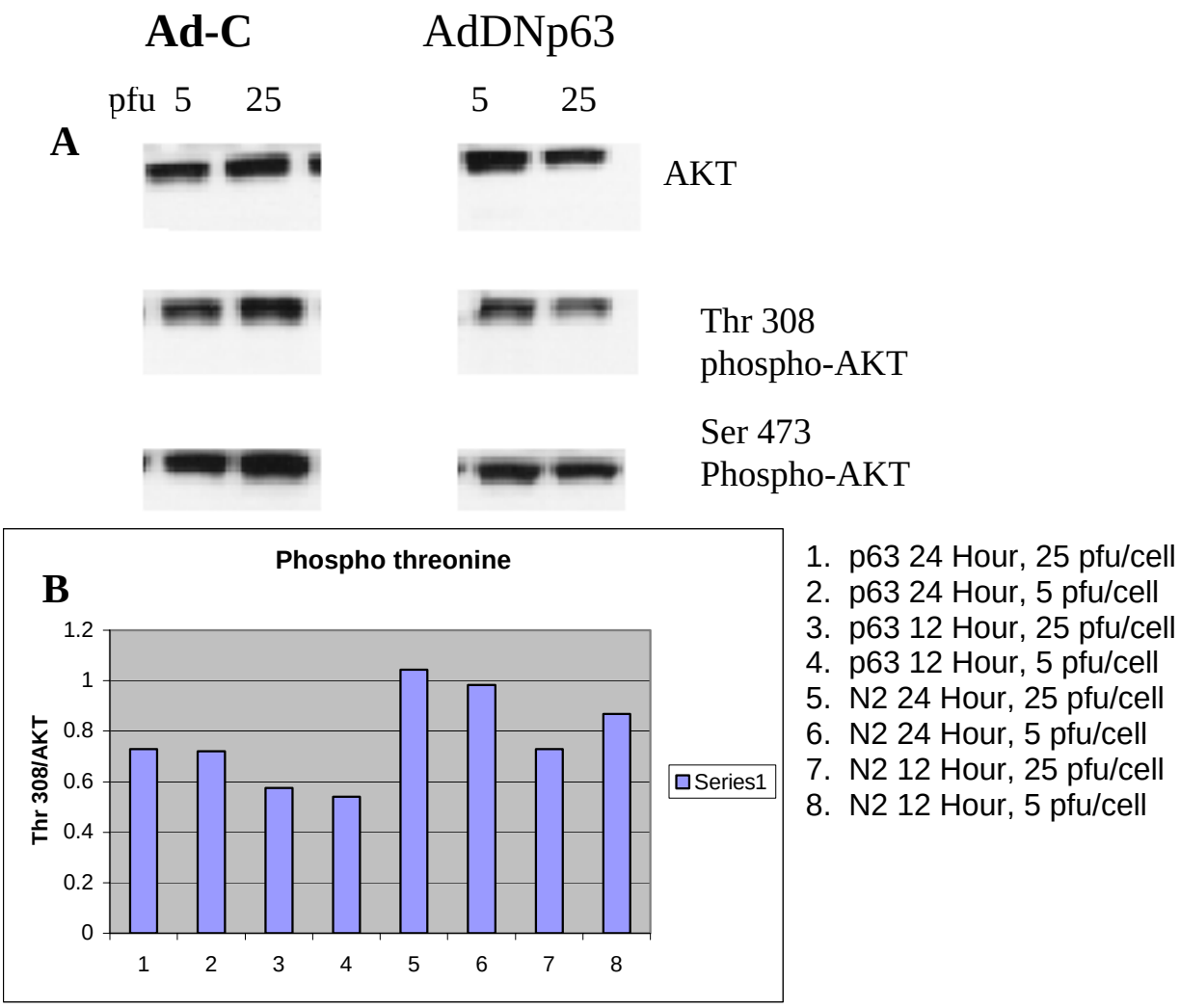


Figure 4.8. AKT response to p63.
The signal from two different phospho-specific antibodies was compared with the signal obtained with an antibody to total AKT.

A. Western blots analysis. Immunodetection was performed by antibodies indicated. Note the loss of decrease of phosphothreonine AKT with increasing p63 expression.
B. Quantitation of phosphothreonine AKT phosphorylation in relation to total AKT. Corresponding AKT and threonine 308 AKT bands were selected from scanned images of the X-ray films and analyzed for mean density by Scion Image software.

Effect of DNp63 on Growth of PC3 Cells

Modulation of DNp63 expression under various cell growth conditions such as immortalization and senescence and regulation of GSK3β serine phosphorylation suggested that DNp63 might regulate cell growth characteristics of LNCaP cells. To evaluate the effect of p63 on cell growth, PC3 cells were infected with either DNp63, or wtp53 adenovirus and growth of these cells was evaluated after two weeks by staining with crystal violet. DNp63 did not show any appreciable affect on cell growth whereas wtp53 exhibited potent cell growth inhibitory effects. This experiment, along with decreased GSK3β phosphorylation and the distinct cell type association of DNp63 in the prostate suggest novel and yet undiscovered biological targets of DNp63 in prostatic epithelial cells. (Figure 4.9).

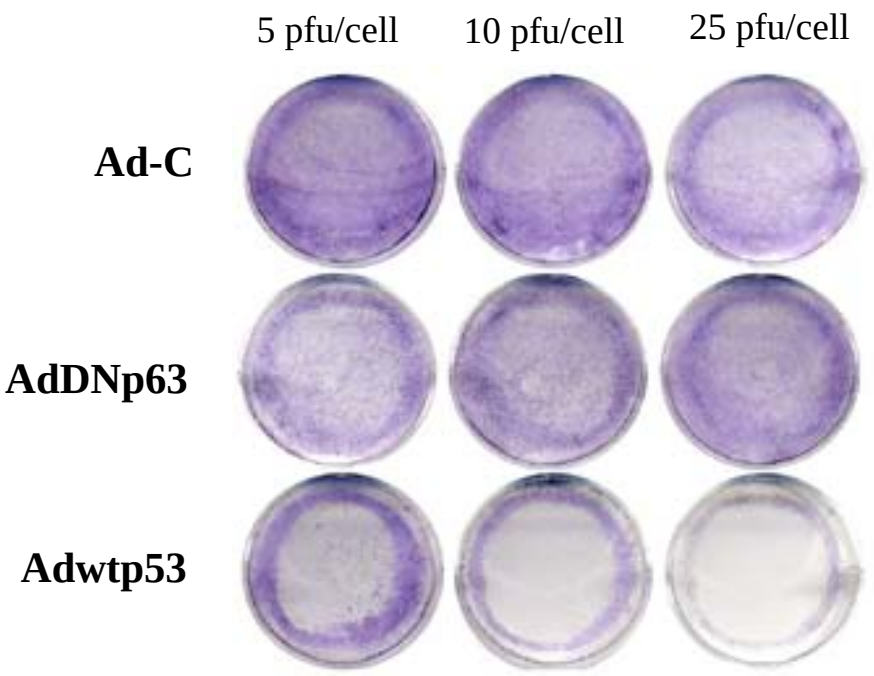


Figure 4.9: Cytotoxicity assay comparing p63, p53, and control adenovirus. PC3 cells were infected with the multiplicity of infection indicated, and 48 hours afterwards the cells were fixed with formalin and stained with crystal violet.

Discussion:

We have demonstrated that DNp63 reduces the levels of serine 9 phosphorylation of GSK3β when introduced into LNCaP cells, a null background. This can be expected to lower β-catenin levels, resulting in less proliferation or differentiation, the opposite of what has been reported for squamous cell carcinoma (Patturajan *et al*, 2003). Despite preliminary findings of changes in phosphorylation of β-catenin, we did not see any changes in the levels of total β-catenin in the LNCaP cells.

Others, working with squamous cells as their model system, reported that serine 9 phosphorylation was increased in the presence of exogenous p63 (Patturajan *et al*, 2003). A critical difference between squamous cell carcinoma and prostate adenocarcinoma is that in squamous cell carcinoma, p63 functions as an oncogene, whereas in the case of prostate carcinoma, as we have shown, there is the complete lack of expression (Davis *et al*, 2002; Hibi *et al*, 2000).

Some work published recently suggests that active GSK3β is necessary to maintain androgen responsiveness in prostate epithelial cells (Liao *et al*, 2004). For many prostate epithelial cells, androgen response is necessary to maintain proliferation. In this case, GSK3β activity may correlate positively with cell growth and proliferation, rather than negatively as has been found in other tissues.

Does reduced GSK3β phosphorylation in response to DNp63 suggest a more active GSK3β whose targets are not yet defined in basal cells of the prostate? Our study underscores that DNp63 may have a different role in the biology of prostate

adenocarcinoma than what has been reported for lung or other types of cancer. Previously, we and others reported in the literature that p63 is not expressed in prostate adenocarcinoma, in contrast to what has been reported for lung and squamous cell carcinomas.

For benign tissues, both in the prostate and in other tissues, DNp63 is found predominantly in the basal epithelial cells. This gives the impression that p63 should perform a similar function among the varying epithelial tissues. It is not uncommon for genes to have differing affects in different tissues. Steroid receptors, for example, can cause differential activation of transcription by interacting with different protein coactivators. We do not believe that the function of p63 differs completely in different tissues, rather it is possible that differing aspects of the p63 gene are emphasized by different cellular environments. The fundamental and shared function of DNp63 in various epithelial tissues appears to be in the maintenance and formation of basal cells. An expansion of our efforts to identify p63 effectors in the prostate, if successful, could result in an enhanced explanation of mechanism of p63 function, not just for the prostate, but for other tissues.

Chapter 5
Summary, Conclusions and Future Directions

We initiated our studies with the hypothesis that p53 has structural and functional homologs. Despite our unsuccessful attempt in identifying novel p53 homologs, we learned from other studies that these homologs do exist. Identification of only two additional members of p53 gene family p63 and p73 has underscored the technical difficulties in identifying these genes due to tissue restricted expression. In fact, the p73 gene was identified accidentally. We further hypothesized that DNp63 has important functions in the prostate, and our studies thus far have supported this concept. First, by studying where DNp63 is expressed in the prostate and how its expression is modulated during immortalization and senescence. These observations have also led to the development of diagnostic pathology assays now routinely used evaluating p63 expression in biopsies as an indicator of non-cancerous benign glands. Finally, we have begun studying mechanism of p63 function by developing new experimental models to identify p63 targets, which will enhance the understanding of p63 function.

Our first goal was to find p53 homologous genes. While both our research and other reports offer differing functions for the p53 homologous genes, when we commenced the investigation, it was with the concept of finding biological redundancy for p53. Biological redundancy guards against the calamity of mutation, providing

stability to cellular functions. To find p53 homologous genes, we used a variety of techniques based on sequence conservation.

When the discovery of p63 by degenerate RT-PCR was reported in the literature, we refocused our efforts on studying this gene in the prostate (Osada *et al*, 1998; Schmale and Bamberger, 1997; Trink *et al*, 1998). We determined that p63 expression is limited to the basal cells of normal prostate, and not expressed in tumors or in the secretory cells of benign glands (Davis *et al*, 2002). We demonstrated the utility of anti-DNp63 immunohistochemical staining to identify benign glands of the prostate. In the context of prostate epithelial cell culture, we determined that p63 expression is limited to cultures derived from the prostate, and not metastatic tumors. We also determined that in primary, unimmortalized cultures, p63 expression is lost in late passages.

The novel observations of tightly restricted prostate basal cell specific expression of p63 from our and other investigators has led to the use of p63 in diagnostic pathology setting. The evaluation of prostate biopsies for prostate cancer requires the pathologist to determine either that there is evidence of cancer in the biopsy specimen, or that there is no evidence of cancer in the biopsy specimen. To further simplify diagnosis, p63 staining may be combined with immunihistochemistry for new discovered prostate cancer marker, alpha-methylacyl-CoA racemase (AMACR), which has been shown to be highly specific for prostate tumor cells, to produce an assay system where one color indicates cancer, the other benign glands (Luo *et al*, 2002). The use of anti-DNp63 immunohistochemistry is expected to improve the accuracy of prostate cancer diagnosis in biopsy specimens and pathologists are already evaluating this test.

Finally, we developed experimental models to investigate p63, by inducing the expression of p63 in various prostate epithelial cell cultures, mainly in LNCaP cells. While others have reported that DNp63 has a proproliferative affect in other tissues, we did not find evidence for this in prostatic cell cultures. In our experimental model, p63 did not stimulate growth in the prostate by inactivating GSK3β, as shown by our preliminary phosphoprotein read-out data. In fact, DNp63 may even be responsible for activating GSK3β in the prostate by reducing serine 9 phosphorylation.

Our findings show us that DNp63 plays an important role in prostate biology, but is not, functioning as an oncogene or a tumor suppressor gene. DNp63 is not found in prostate cancer, nor does it stimulate the prostate cell growth and proliferation as found for p63 cancer cell types. While p63 may be a p53 homologous gene, in the prostate it has different functions, most likely in the maintenance of basal cell phenotype.

Model of p63

Competing theories on the function of p63 have been advanced, informed by p53 homology and the experimental evidence reported to date on p63. Based on what has been reported on p63 knockout mice, p63 function is critical to the proper development of mammals. Since the knockout mice lack prostates, basal cell specific expression of p63 appears critical to the development of the prostate (Signoretti *et al*, 2000; Yang *et al*, 1999).

p63 function has been proposed to mirror p53 mechanism of function, by binding to p53 response elements and stimulating transcription. As evidence, the results of reporter assays using TAp63 and luciferase constructs containing the promoters of p53 inducible genes are cited (Yang *et al*, 1998). However, the TAp63 isoform, which is

primarily expressed in muscle, is not the most important isoform present in epithelial tissues, where the most severe phenotype of knockout mice is evident (Bamberger and Schmale, 2001). The activities of TAp63 in inducing the expression of p53 inducible genes may only be one aspect of the function of p63.

DNp63 has been presumed to act as a dominant negative regulator of TAp63 or p53. There is scant evidence that DNp63 binds to p53, and it is unlikely that a dominant negative regulator of TAp63 would need to be more strongly expressed than the supposed target gene, as is the case with DNp63 in epithelial tissues.

While evidence of DNp63 activity as a transcription factor has not been reported, increasingly, there is evidence to indicate a role in direct protein-protein interactions.

Most of the pathways implicated in p63 function to date (WNT, Notch) are also known to have involvement in developmental processes (Patturajan *et al*, 2003). Some of these relationships which have been uncovered are not found with p53.

In some tissues, DNp63 has been shown to behave as an oncogene, exhibiting increased levels of expression in tumors and inducing pathways known to stimulate cell proliferation and growth. In head and neck squamous cell carcinoma, not only is DNp63 found to be expressed at increased levels, the gene locus to p63 is specifically amplified. In head and neck carcinoma, p63 expression is thought to stimulate the Wnt signaling pathway, causing increased phosphorylation of the inhibitory site serine 9 of GSK3β (Patturajan *et al*, 2003). However, the evidence to date in the prostate indicates a very different behavior for p63 in the prostate, not compatible with the concept of oncogenes. While p63 expression may be found in benign glands of the prostate, p63 is not expressed in prostate adenocarcinoma (Davis *et al*, 2002; Parsons *et al*, 2001; Signoretti *et al*,

2000). We may ask, if p63 is necessary for the formation of the prostate, how can it not be responsible for directly stimulating cell proliferation?

Why does GSK3β respond differently to the expression of DNp63 in prostate epithelial cells than in head and neck squamous cells, by not inactivating GSK3β? Is it possible that GSK3β has a different relationship to proliferation in the prostate than in other tissues?

The secretory epithelial cells of benign prostate glands have limited proliferative capacity. In the ordinary course of events, it is the basal cells which replace other epithelial cells in the prostate (Coffey, 1986), so lack of a basal cell layer would be detrimental to normal growth of prostate and need for basal layer is bypassed in malignant prostate.

We showed basal cell specificity of p63 in the prostate, and have also shown that the overexpression of exogenous DNp63 in LNCaP cells does not cause increased cell division. If p63 were necessary for the maintenance of basal cell phenotype, this would make p63 necessary for the ability of prostate epithelial cells to be proliferatively replenished, even if DNp63 does not directly stimulate cell proliferation. In keratinocytes, there is a report that p63 overexpression can block maturation (King *et al*, 2003). Keratinocytes mature from live basal keratinocytes into the physiologically dead skin cells which populate the outer layers of human skin.

A property of late passage primary cultures derived from benign or malignant cells is that they lose proliferative capacity, and if these properties are artificially stimulated, as by the expression of exogenous protein component of telomerase, the usual senescence is lost.(Yasunaga *et al*, 2001; Yasunaga *et al*, 2001) p63 may or may not

itself prevent senescence, but with its association with basal cells, and its loss of expression in cell cultures of benign and malignant prostate, it suggests some role of p63 in the maintenance of proliferative phenotype cells.

We propose that DNp63 functions in the prostate to maintain the basal layer of the prostate by preventing the differentiation of basal cells into secretory cells. The lack of a signal to prevent differentiation may be expected to result in the inappropriate, premature differentiation of the basal cells, leading to the lack of a basal layer, and a concomitant lack of stem cells. The lack of stem cells may be expected to result in involution of the organ (Figure 5.1).

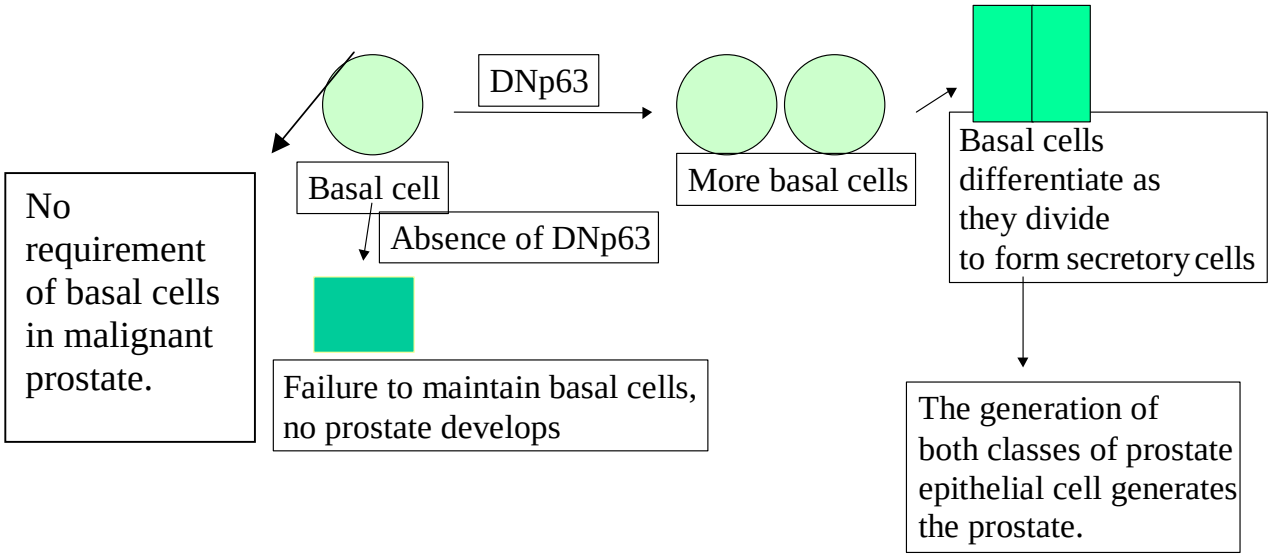


Figure 5.1: DNp63 allows the maintenance of basal cell phenotype.

Future Directions

In the prostate, we need to uncover pathways through which p63 may function. There is much that remains undiscovered and unknown about p63 mechanisms of function in general and in the context of prostate. We need to understand first, which

properties of basal cells are influenced by p63. Second, we need to understand how p63 on a molecular level is responsible for these actions. Towards this end, we have outlined here several approaches to future p63 research likely to be useful.

p63, as we have shown, is predominantly expressed in basal cells, many of which appear to be stem cells. There are studies suggesting that the prostate epithelial cells that remain in a castrated animal are stem cells, many with active telomerase, which would suggest to us a high percentage of p63 expression (Meeker *et al*, 1996; Ravindranath *et al*, 2001). What is the expression pattern of p63 in the involuted prostates of castrated animals? This question is best answered by performing immunohistochemistry on the prostate glands of castrated animals. We should expect the remaining prostate epithelial cells to be p63+.

A property of prostate epithelial basal cells is that they are less responsive to androgen than prostate secretory cells. Prostate basal cells respond more to estrogen than secretory cells. If p63 maintains basal cell phenotype, one affect of p63 expression may be to shift the pattern of hormone responsiveness towards the basal cell pattern.

We actually attempted to evaluate the effects of p63 on androgen receptor mediated signaling using AdDNp63 vector and LNCaP cells and luciferase reporter constructs driven by androgen responsive elements. However, these experiments need to be further developed.

We suggest the development of a new basal cell culture model to fully evaluate the benign prostate gland and study the role of p63 by siRNA strategy.

One important avenue of study is the further investigation of GSK3β and p63 connection in prostate epithelial cells to p63 in prostate cell cultures, by using our western blot system.

To further elucidate the molecular pathways of p63, the hybridization of p63 adenovirus treated RNA to a cDNA microarray, with no insert adenovirus as a control, would be useful. These experiments are currently in progress.

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