THESIS
3
2010

LIBRARY
Michigan State
University

This is to certify that the
dissertation entitled

ANDROGEN RECEPTOR AND INTEGRIN REGULATION OF PROSTATE EPITHELIAL DIFFERENTIATION AND TUMOR CELL SURVIVAL

presented by

LAURA ELAINE LAMB

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Cell and Molecular Biology

Major Professor's Signature

2/9/10

Date

MSU is an Affirmative Action/Equal Opportunity Employer

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.
MAY BE RECALLED with earlier due date if requested.

DATE DUE	DATE DUE	DATE DUE

5/08 K:/Proj/Acc&Pres/CIRC/DateDue.indd

ANDROGEN RECEPTOR AND INTEGRIN REGULATION OF PROSTATE EPITHELIAL DIFFERENTIATION AND TUMOR CELL SURVIVAL

By

Laura Elaine Lamb

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Cell and Molecular Biology

2010

ABSTRACT

ANDROGEN RECEPTOR AND INTEGRIN REGULATION OF
PROSTATE EPITHELIAL DIFFERENTIATION AND TUMOR CELL SURVIVAL

By

Laura Elaine Lamb

Development of strategies for more effective treatment of prostate cancer is limited by an incomplete understanding of the mechanisms regulating survival of either normal prostate or prostate cancer cells. Androgen receptor (AR) signaling plays an important role in regulating differentiation and cell survival in the prostate and in prostate cancer. Prostate cancer arises from the AR expressing epithelial cells of the prostate. Adhesion to matrix through integrins is required for survival of most epithelial cells. However, AR-expressing epithelial cells of the prostate are not adherent to matrix. Paradoxically in prostate cancer, the tumor cells both express AR and are adherent to matrix, allowing for interactions between these two signaling pathways. Our hypothesis is that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while AR regulates survival of normal cells independently of integrins. We have demonstrated that PC3 and DU145 prostate tumor cell lines require PI3K signaling for cell survival. In addition, adhesion of PC3 cells to laminin 1 promotes survival via integrin-mediated activation of Src leading to increased expression of the pro-survival protein Bcl-xL. In DU145 cells, adhesion to collagen 1 drives survival through activation of EGFR and Erk. During prostate cancer progression, there is increased expression of the laminin 1-binding integrin α6β1. Previous studies have

demonstrated that AR can lead to increased expression of α6β1, suggesting that AR may drive cell survival by altering integrin expression. We have demonstrated that expression of AR in PC3 cells can rescue cells from death induced by inhibition of PI3K when adherent to laminin 1. Expression of AR in PC3 cells leads to increased expression of integrin α6β1 and Bcl-xL along with increased activation of NF-κB. Blocking each these components individually concurrent with inhibition of PI3K led to death of the AR-expressing cells, suggesting that AR regulates cell survival through enhancement of α6β1/NF-κB/Bcl-xL signaling. AR expression in PC3 tumor cells also correlates with increased filopodia formation, Src activity, and cell migration. To assess the role AR in normal cell survival, we generated an in vitro differentiation model. Confluent primary human prostate epithelial cell cultures were treated with KGF and androgen (DHT). After two weeks, a suprabasal cell layer formed in which cells no longer expressed integrins, p63, K5/14, EGFR, FGFR2IIIb, or Bcl-2, but instead expressed AR and androgen-induced differentiation markers, including K18/19, TMPRSS2, Nkx3.1, PSMA, KLK2 and secreted PSA. Differentiated prostate cell survival depended on E-cadherin and PI3K, but not KGF, DHT, AR or MAPK. Therefore, while in the prostate tumor cell line PC3, AR and integrin α6β1 cooperate to drive cell survival, neither AR nor integrins were required for survival of differentiated prostate epithelial cells.

DEDICATION

In loving memory of my mother, Joyce Elaine Gier, who succumbed to cancer but has continued to inspire me (1953-1995).

To my husband, who supports me in all my endeavors.

ACKNOWLEDGEMENTS

This dissertation is the result of the support, collaboration, and encouragement from numerous colleagues, family and friends.

I am extremely grateful for the guidance and mentorship of my advisor, Dr. Cindy Miranti. She has always had an open door, nurtured my scientific interests, encouraged my growth as a scientist, and has helped me achieve whatever goals I set for myself. I feel privileged to be her first graduate student. Thank you Dr. Miranti for your time, patience and knowledge- you have helped drive my passion for cancer research and to one day be a mentor myself. I know that I can count on your continued advice throughout my career.

I feel incredibly lucky to have Shannon Lamb as my husband, with whom I feel everything and anything is possible. He is my rock, who supports and encourages all my endeavors, and can always make me laugh. Thank you Shannon for everything that you do and continue to do to support my dreams.

I am grateful for the guidance and invaluable advice that I received from my graduate committee at MSU including Drs. Sandy Haslam, John LaPres, Bob Wiseman, and Richard Miksicek as well as investigators at VAI with whom our lab interacts including Drs. Jeff MacKeigan, Art Alberts, and Nick Duesbery as well as the members in their labs, especially Dr. Jenn White, Dr. Brendan Looyenga, and Natalie Wolters. I am also thankful for the reagents and insights of our collaborator Dr. Beatrice Knudsen (Fred Hutch Cancer Research Institute). Also, I would like to thank Dr. Melinda Frame (MSU) and Rich West (VAI) for

sharing their knowledge and expertise of microscopy and flow cytommetry with me.

I work with some of the best coworkers around in the Laboratory of Integrin Signaling and Tumorigenesis including Veronique Schulz, Dr. Elly Park, Kristen Saari, Jelani Zarif, and Jessica Trahey as well as past members Dr. Mat Edick, Lia Tesfay, Dr. Suganthi Sridhar, Susan Spotts, Eric Graf, Erica Bechtel, Gary Rajah, Fraser Holleywood, Derek Janssens, and Penny Berger. I love the culture that our lab has in that we support each other, have a sense of humor, and all work hard. I am happy that I can call you all friends and will have lifelong relationships with you all. I am especially thankful to Dr. Mat Edick who spent a lot of his time training me when I first joined the lab.

To my original VAI/MSU partners in crime, Charlie Miller, Aaron DeWard, and Sebla Kutluay, thank you for making this experience so enjoyable. I will miss our regular lunches together but I know that each one of you will go on to do incredible things. I know we will all look back and remember how spoiled we were here. I look forward to staying in touch and watching everyone's career grow.

To my MSU classmates, especially but not limited to Julie Bordowitz, Eric Marrotte, Katie Sian, Danielle Nevarez, Aaron McBride, Gabby Meyer, and Jamie Kopper, I appreciate all those times we spent together studying for classes in the fishbowl, going on our annual Uncle John's Cider trip, forwarding funny journal article titles, and summer barbeques that I will treasure as part of my graduate school experience.

I am very thankful for my family, including my parents who always encouraged my creativity and love for reading, nature, and science. Thank you for always supporting my dreams. To my sister Stephanie, for encouraging me to never settle. To my in-laws, especially my mother-in-law, for always being supportive.

Lastly, I would like to thank everyone I carpooled with for getting me to and from work safely all these years.

Thank you all from the bottom of my heart. ~LL~

TABLE OF CONTENTS

LIST OF TABLES....x

LIST OF FIGURES....xi

LIST OF SYMBOLS OR ABBREVIATIONS....xiii

CHAPTER 1
INTRODUCTION....1
- Introduction....2
- Prostate Biology and Cancer Progression....3
  - Normal Biology....3
  - Prostate Cancer Progression....6
- Androgen Receptor (AR) and Prostate Cancer....8
- Molecular Changes Associated with Prostate Cancer Progression....14
  - Phosphate and Tensin Homolog (PTEN) and PI3K/Akt Signaling.14
  - Nuclear factor-kappaB (NF-κB)....17
  - TMPRSS2-Erg....20
- Integrin Expression and Signaling in Prostate and Prostate Cancer....21
  - Integrin Signaling in Prostate and Prostate Cancer....23
- Limitations of Current Models....26
  - Canine and Murine Models....26
  - Cell Lines....30
- Framework of Dissertation....32
- References....35

CHAPTER 2
INTEGRIN-MEDIATED SIGNALING IN PROSTATE TUMOR CELLS....59
- Introduction....60
- Results....64
- Discussion....73
- Materials and Methods....79
- Acknowledgements....84
- References....86

CHAPTER 3
AR-ENHANCED α6β1 INTEGRIN AND BCL-XL EXPRSSION PROMOTES ANDROGEN-INDEPENDENT PROSTATE TUMOR CELL SURVIVAL INDEPENDENTLY OF PI3K SIGNALING....92
- Introduction....93
- Results....97
- Discussion....124
- Materials and Methods....136
- Acknowledgements....148
- References....149

CHAPTER 4
E-CADHERIN-MEDIATED SURVIVAL OF ANDROGEN RECEPTOR EXPRESSING PROSTATE CELLS....162
Introduction....163
Results....165
Discussion....187
Materials and Methods....196
Acknowledgements....204
References....205

CHAPTER 5
CONCLUSIONS AND PERSPECTIVES....214
Summary....215
Validation of AR Effects in Additional Models....219
Other Cell Lines....219
In Vivo Studies....220
Understanding Differentiation in Prostate Epithelial Cells....221
Next Generation of AR-expressing Models....221
Therapeutic Implications....223
References....225

LIST OF TABLES

Table 1. siRNA Sequences.....140

Table 2. Immunoblotting Antibodies.....142

Table 3. Flow Cytometry Antibodies.....144

Table 4. qRT-PCR Primers.....147

Table 5. Immunofluorescence Antibodies.....200

Table 6. RT-PCR Primers.....202

Table 7. Summary of AR, PTEN, and Integrin α6 Expression and Survival Pathways in Prostate and Prostate Cancer Cell Lines In Vitro.....217

LIST OF FIGURES

Images in this dissertation are presented in color.

Figure 1. Integrin and AR Expression in Prostate Cancer Progression.....4

Figure 2. Integrin-Mediated Survival Signaling in Prostate Epithelial Cells.....34

Figure 3. Integrin-mediated signaling on LM1 in PC3s.....65

Figure 4. PC3 cells depend on PI3K and Src signaling for survival.....69

Figure 5. Integrin-mediated Signaling in DU145 Prostate Tumor Cells.....71

Figure 6. Models for PC3 and DU145 integrin-mediated survival.....74

Figure 7. On LMI, AR is a survival factor that acts independently of PI3K signaling and androgen.....98

Figure 8. AR promotes survival through up-regulation of integrin α6.....105

Figure 9. AR and integrin α6 regulate Bcl-xL expression.....108

Figure 10. Bcl-xL promotes survival independent of PI3K signaling.....110

Figure 11. AR regulates Src activity.....112

Figure 12. AR promotes filopodia formation and migration.....113

Figure 13. AR regulates integrin α6 and Bcl-xL mRNA expression.....115

Figure 14. AR and integrin α6 regulate NF-κB signaling.....120

Figure 15. AR and integrin α6 regulate PAK1/2 signaling.....125

Figure 16. Model for AR signaling in PC3 cells.....127

Figure 17. AR and AR-dependent proteins are present in the differentiated cultures.....168

Figure 18. Differentiation-specific epithelial markers present in the top cells of differentiated cultures.....171

Figure 19. Prostate epithelial differentiation is accompanied by loss of integrin expression.....174

Figure 20. Prostate epithelial differentiation is accompanied by loss of laminin 5 expression.....175

Figure 21. Differentiated cells respond to androgen.....177

Figure 22. Isolation of secretory-like cells.....179

Figure 23. Signaling pathways in secretory-like cells.....181

Figure 24. Secretory-like cells are dependent on PI3K and E-cadherin, but not androgen or KGF, for survival.....183

Figure 25. AR is not required for secretory-like cell survival.....185

Figure 26. Model of Differentiation.....191

LIST OF SYMBOLS OR ABBREVIATIONS

Abbreviation/Symbol	Definition
420119	1,9-pyrazoloanthrone
3D	three-dimensional
A	adenine or alanine
α	alpha
a2	integrin α2
a3	integrin α3
a5	integrin α5
a6	integrin α6
ABI	Applied Biosystems
ABL	Abelson murine leukemia viral oncogene homolog 1
ADT	androgen deprivation therapy
AF	transactivation function domain
AG	AG1478
AG1478	Tyrphostin; N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine
Akt	protein kinases B
AP1	activator protein 1
AR	androgen receptor
AR1	PC3-AR-1
AR2	PC3-AR-2
ARE	androgen response element
ATCC	American Type Culture Collection
AZ	Arizona
β	beta
B	bottom layer cells
b1	integrin β1
b4	integrin β4
Bcl-2	B-cell leukemia/lymphoma 2
Bcl2L1	Bcl-xL
Bcl-xL	BCL2-like 1
Bclxl-1	PC3-Bclxl-1
Bclxl-5	PC3-Bclxl-5
Bclxl-7	PC3-Bclxl-7
BCR	breakpoint cluster region
Bfl-1/A1	BCL2-related protein A1
Bit-1	Bcl-2 inhibitor of transcription 1
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BPE	bovine pituitary extract

BSA	bovine serum albumin
Bub1	budding uninhibited by benzimidazoles 1 homolog
C	cytosine
CaCl	calcium chloride
CASP8	caspase 8
CCD	charge coupled device
cDNA	complementary DNA
c-FLIP	CASP8 and FADD-like apoptosis regulator
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation with on-array detection
CK	cytokeratin
CL	collagen
cm	centimeter
CMB	Cell and Molecular Biology
CO2	carbon dioxide
CRPC	castration resistant prostate cancer
CSS	charcoal stripped serum
Ct	count
d	day
D	DMSO
DAP3	death-associated protein 3
DHT	dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DR	death receptor
Ecad	blocking E-cadherin antibody
Ecad Ab	blocking E-cadherin antibody
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERE	estrogen-response element
Erg	ETS-related gene
ERK	extracellular signal-regulated kinase
ETS	E-twenty six family of transcription factors
et. al.	et alii (and others)

ETV	ets variant
FACS	Fluorescence Activated Cell Sorting
F-actin	filamentous polymers of actin
FADD	Fas-associated via death domain
FAK	focal adhesion kinase-1
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FHC	low density lipoprotein receptor
FL	full length
FN	fibronectin
FOXO	forkhead box-O transcription factors
g	gamma
G	guanine
GADD45b	growth arrest and DNA-damage-inducible, beta
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GTPase	guanosine triphosphate binding protein
h	hour
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2/ErbB	human epidermal growth factor receptor 2
HIF-1α	hypoxia-inducible factor-1α
HI-FBS	heat inactivated fetal bovine serum
HIV	human immunodeficiency virus
HO	Hoechst 33258
Hsp	heat shock protein
hygro	hygromycin
IAP	inhibitor of apoptosis
IF	immunoflourescence
i.e.	id est (that is)
IGF	insulin growth factor
IGFR	Insulin-like growth factor receptor
IgG	immunoglobulin G
IkB	inhibitor of NF-kB
IKK	IkB kinase
IL	interleukin
ILK	integrin-linked kinase
IP	immunoprecipitate
IPA-3	1,1′-Disulfanediyldinaphthalen-2-ol
IRB	institutional review board
ITG	integrin

ITGA6	integrin α6
k	kappa
K	keratin or lysine
K/D	KGF and DHT treatment
KGF	keratinocyte growth factor/FGF7
Kip1	cyclin-dependent kinase inhibitor 1B
KLK	kallikrein
LBD	ligand binding domain
LBD18	PC3-ΔLBD-AR-18
LBD28	PC3-ΔLBD-AR-28
LM	laminin
LN	LNCaP
LY	LY294002
LY294002	5-(2,2-Difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione
M	methionine
MAPK	Mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1 (BCL2-related)
MEM	Minimum Essential Medium
MI	Michigan
μL	microliter
mL	milliliter
μM	micromolor
mM	millimolar
MMP	matrix metalloproteinases
MNAR	modulator of non-genomic activity of ER
MnSOD	superoxide dismutase 2, mitochondrial
MSP	macrophage-stimulating protein
MSU	Michigan State University
mTOR	mammalian target of rapamycin
Myc	myelocytomatosis viral oncogene homolog
N	any nucleotide or the number of experimental replicates
Na3VO4	sodium orthovanadate
NaCl	sodium chloride
n.d.	not determined
NaDOC	sodium deoxycholate
NaF	sodium fluoride
NEMO	NF-kB essential modulator
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nkx3.1	NK-3 transcription factor, locus 1
NLS	nuclear localization signal

NLS30	PC3-ΔNLS-AR-30
NLS4	PC3-ΔNLS-AR-4
nM	nanomolar
NR13	anti-apoptotic protein NR13
NT	not treated
OD	optical density
P	phosphorylation or proline or PBS
P1	PD168393
P9	PD98059
PAK	p21 protein (Cdc42/Rac)-activated kinase
PBS	phosphate buffered saline
PD	PD168393
PD98059	2'-Amino-3'-methoxyflavone
PDGF	platelet-derived growth factor receptor
PEC	prostate epithelial cell
PhD	Doctor of Philosophy
phospho	phosphorylation
PI	propidium iodide
PI3K	Phosphoinositide-3 kinase
PIN	prostatic intra-epithelial neoplasia
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PL	PC3-pLKO
PMSF	phenylmethylsulphonyl fluoride
PP	PC3-puro
PP2A	serine/threonine protein phosphatase 2A
PR	progesterone receptor
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
PTEN	Phosphate and Tensin Homolog
puro	puromycin
PVDF	polyvinylidene difluoride membrane
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
R	adenine or guanine
R1881	methyltrienolone
Rac1	Ras-related C3 botulinum toxin substrate 1
Rb	retinoblastoma 1
Rel	v-rel reticuloendotheliosis viral oncogene homolog
RIPA	Radio Immunoprecipitation Assay
RLU	Relative Luminescence Units
RNA	ribonucleic acid
RNase	ribonuclease

RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
RU	RU486
SB	SB202190
SB202190	4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol
scr	scram
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis
Ser	serine
SFM	serum free media
shRNA	short hairpin RNA
siA6	siRNA against A6
siAR	siRNA against AR
siRel	siRNA against p65 (RelA)
siRNA	small interfering RNA
siSrc	siRNA against Src
si-xL	siRNA against Bcl-xL
SNP	single-nucleotide polymorphism
SP	serine protease
Sp1	Sp trans-acting transcription factor 1
Spi2A	serine (or cysteine) peptidase inhibitor, clade A, member 3G
Src	sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SRD5A2	steroid 5-alpha reductase
starv.	starvation
STR	staurosporin
SU	SU6656
SU6656	2,3-Dihydro-N,N-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]-1H-indole-5-sulfonamide
SV40	Simian virus 40
SYBR green	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
T	large T antigen, thymine, or top layer cells
t	small t antigen
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.1% Tween 20
TCL	total cell lysate
TGF	transforming growth factor
Thr	Transmembrane protease, serine 2

TMP	TMPRSS2
TMPRSS2	transmembrane protease, serine 2
TN	Tennessee
TNF	tumor necrosis factor
TRAMP	transgenic adenocarcinoma mouse prostate
Tub	tubulin
TUNEL	Terminal Deoxynucleotide Transferase dUTP Nick End Labeling
U	units
U0	U0126
U0126	1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate
uPAR	plasminogen activator, urokinase receptor
UV	ultraviolet
VAI	Van Andel Institute
VARI	Van Andel Research Institute
veh	vehicle
W	adenine or thymine
WA	Washingtom
WCL	whole cell lysate
X	any amino acid
XIAP	X-linked inhibitor of apoptosis
Y	cytosine or thymine , or tyrosine
zVAD	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

CHAPTER ONE:

INTRODUCTION

Introduction

Prostate cancer is the most diagnosed malignancy and second leading cause of cancer related death in American men. In 2009, it is predicted that in the United States alone there will be 192,280 new cases (approximately 1 in 6 men) and 27,360 prostate cancer-related deaths (approximately 1 in 35 men) (1, 2). Prostate cancer incidence strongly correlates with age and usually is detected in men sixty or older (3, 4). Autopsy studies suggest that up to 80% of 80 year old men have prostate cancer and some experts predict that all men would develop prostate cancer if they lived long enough (3, 4). However, prostate cancer is a relatively slow growing tumor and the majority of men diagnosed with prostate cancer will actually die from other causes (5). While primary prostate cancer is highly treatable by surgical resection and radiation, prostate cancer that has metastasized is not. This is reflected by a five-year survival rate of 100% for local and regional prostate cancer, and 31% for metastatic prostate cancer (6). Androgen signaling is critical in prostate cancer development and disease; androgen deprivation therapy (ADT) is the primary therapy for metastatic prostate cancer (7). Also known as androgen ablation or androgen suppressive therapy, ADT reduces levels of circulating androgen in the body in combination with blocking ligand binding to AR. While this is initially clinically successful and results in rapid tumor regression, it is not curative. Ultimately the prostate cancer will recur and will no longer respond to ADT. This is known as castration-resistant prostate cancer (CRPC), hormone-refractory, or

androgen-independent prostate cancer. Currently, there are no curative therapies for CRPC and patients ultimately succumb to the disease.

Prostate Biology and Cancer Progression

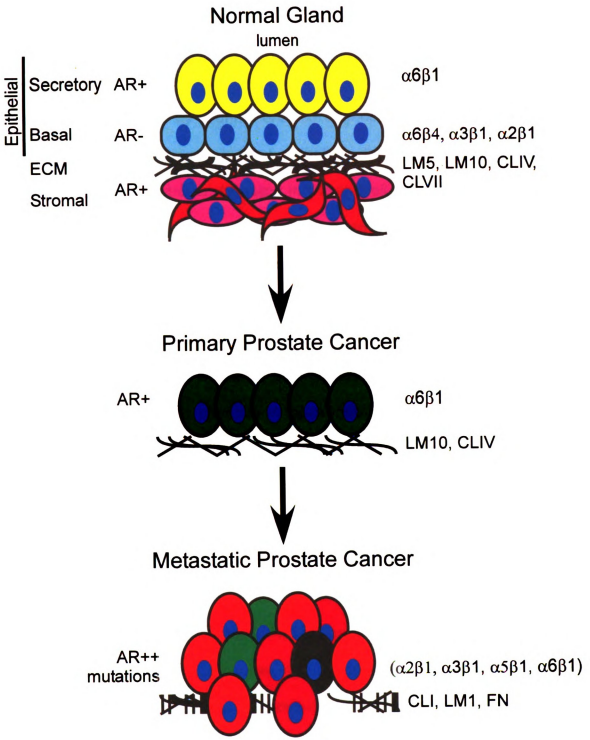
Normal Biology

The human prostate is an encapsulate glad about the size of a walnut located inferior to the bladder (5). It surrounds the urethra and functions to produce secretory alkaline proteins for the seminal fluid which nourish and protect the sperm (5). Although the prostate is thought to share common embryonic origins as well as the same blood supply as adjacent male urogenital tissues, these surrounding tissues develop neoplasia only in rare cases (5).

The prostate has a ductal-acinar histology, with stromal cells throughout and epithelial cells surrounding the acini. The epithelium of the human prostate consists of two cell layers, a basal layer and a secretory layer. The prostate basal layer is mostly made of basal epithelial cells as well as a small subpopulation of neuroendocrine cells. The basal cells are mitotic and adhere to a basement membrane containing collagens IV and VII, and laminins 5 and 10/11 (8-10) (Figure 1). Prostate basal cells give rise to terminally differentiated secretory cells (8-10). The secretory cells, also known as luminal cells, are located suprabasally and face the lumen of the prostate (Figure 1). There is also a stromal compartment of the prostate which contains myoepithelial cells,

FIGURE 1. AR, integrin, and matrix progression in prostate cancer progression. (Top panel) The prostate consists of a stromal and epithelial compartment separated by matrix composed of LM5, LM10, CLIV, and CLVII. The basal epithelial cells are adherent to this matrix by integrins α6β4, α3β1, and α2β1. The secretory epithelial cells express integrin α6β1 but are not adherent to matrix. They express AR and secrete proteins into the lumens of the prostate. (Middle panel) In primary prostate cancer, the cells are adherent to LM10 and CLIV matrix via integrin α6β1 and express AR. (Bottom panel) In metastatic prostate cancer, AR is often overexpressed or mutated. Cells are adherent to new, different matrices such as CLI, LM1, and FN as they spread to new sites in the body, primarily bone. Cell lines derived from metastatic lesions express integrins α2β1, α3β1, α5β1 and α6β1.

FIGURE 1


Normal Gland
lumen
Epithelial
Secretory
AR+
α6β1
Basal
AR-
α6β4, α3β1, α2β1
ECM
LM5, LM10, CLIV, CLVII
Stromal
AR+
Primary Prostate Cancer
AR+
α6β1
LM10, CLIV
Metastatic Prostate Cancer
AR++ mutations
(α2β1, α3β1, α5β1, α6β1)
CLI, LM1, FN

fibroblasts, and blood vessels (Figure 1). Unlike other epithelia, prostate epithelial cell differentiation is regulated by androgen signaling (11-16). AR is expressed only in the differentiated secretory cells and not in the basal cells, however AR is also expressed in the stroma (17) (Figure 1). Elegant tissue recombination studies by Cunha and colleagues demonstrated that AR is required in the mesenchyme, but not epithelium, for prostate duct development (18-20). However, androgen signaling in the epithelium is required for prostate function (18-20). Androgen signaling in the secretory cells drives AR-mediated transcription of proteins, such as prostate specific antigen (PSA/KLK3), which are secreted into the lumen of the prostate. In the normal prostate, adhesion to matrix and expression of AR are mutually exclusive. In addition to AR expression and adhesion to matrix, differentiation of basal cells into secretory cells also correlates with changes in cytokeratin, growth factor receptor, and integrin expression (discussed in more detail in Chapter Four).

Prostate Cancer Progression

Prostate cancer arises from the epithelial compartment and appears to be associated with a dysplastic lesion referred to as prostatic intra-epithelial neoplasia (PIN) (5). In low grade PIN, abnormal AR positive cells are observed in the secretory layer (21). However, as PIN progresses, there appears to be a loss of basal cells allowing the AR positive carcinoma cells to adhere to matrix (21). The loss of the basal cells is hypothesized to be due to invasion or over-proliferation of the carcinoma cells into the basal cell layer, or due to death of the

basal cells themselves (22, 23). High grade PIN precedes the appearance of prostate cancer usually by five to ten years (21). These lesions are heterogenic and multifocal; PIN can be immediately adjacent to and within the same acini structure as normal epithelium and PIN fails to permeate into the stroma (21).

Although the cell of origin for prostate cancer remains controversial, some lines of evidence strongly support that it is from a secretory or secretory precursor cell. For one, all primary prostate cancer is positive for AR, suggesting it must arise from AR positive cells, and not negative basal cells. Furthermore, secretory cells first appear during puberty when there is a dramatic and permanent increase in circulating testosterone; there are no reported cases of prostate cancer occurring before puberty and the appearance of secretory cells (4, 21).

Unlike many other solid tumors, the vast majority of primary prostate cancer grow slowly and are asymptomatic, and may take years to develop. Hence some elderly patients with other health considerations may choose "watchful waiting" rather than surgery or radiation. Prostate cancer metastasizes primarily to the bone (usually the spine and ribs) and is often the only clinically detectable site of metastasis (24). Unlike most other cancers that metastasize to bone, prostate cancer is osteoblastic (bone forming) rather than osteolytic (bone lysing), which is often painful for the patient (24). Prostate cancer also commonly metastasizes to the lymph nodes, brain, and lungs, although it can spread to other sites as well.

Androgen Receptor (AR) and Prostate Cancer

AR is critical for prostate cancer viability and growth (7, 25). Inhibiting AR expression within animal models of prostate cancer inhibits tumor development, progression, and leads to recession of established tumors (26-28). Studies in vitro demonstrate that inhibition or loss of AR using antibodies, ribozymes, or siRNA can inhibit prostate cancer proliferation and survival (26, 29-32). Thus AR is a potent mediator of cell survival and growth in prostate cancer.

AR is a nuclear transcription factor activated in response to the steroid hormone androgen (17). It consists of an N-terminal transactivation domain (AF-1) that can function in the absence of ligand, a C-terminal ligand binding domain (LBD) associated with a second transcriptional regulatory function (AF-2), and a DNA binding domain between the two. Classically, non-ligand bound AR is localized in the cytoplasm bound to molecular chaperones including Hsp90 and Hsp70, which conformationally prevent the nuclear localization signal (NLS) on AR from being exposed. This also keeps AR in a high-affinity state for ligand binding (33). Circulating androgen (testosterone) enters the prostate cells and is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (SRD5A2). DHT has a 5X higher affinity for AR compared to testosterone. This induces a conformational change in AR, allowing disassociation from Hsp70/90, homodimerization, exposure of the NLS, and subsequent translocation into the nucleus. Ligand binding of AR does not happen in cell lysates, suggesting that this is not protein autonomous, but rather requires additional cellular factors (34).

Once in nucleus, AR binds DNA at specific sequences referred to as androgen response elements (AREs) to regulate gene expression. AREs are typically two 6 base pair "half-sites" that are direct or inverted repeats separated by 3 base pairs (i.e. GGTACAnnnTGTTCT) (35). These sites are highly degenerate and divergent, making ARE prediction in gene promoters difficult (36). Microarray and chromatin immunoprecipitation (ChIP) with on-array detection (ChIP-chip) experiments suggest that as many as 20-30% of AR regulated genes do not contain predicted ARE sites (37-41). Once bound to DNA, AR can bind co-activators, co-repressors, and other transcriptional modifiers to either activate or repress transcription of target proteins (reviewed in 42, 43, 44). It has been suggested that AR is a more gradual activator of transcription after ligand stimulation compared to other steroid receptors like estrogen receptor (ER), consistent with long range interactions between enhancer elements and promoter regions (45-47). However, this has only been investigated in the regulation of a few genes and does not necessarily exclude a role of AR from having more immediate effects. Known AR targets include prostate-specific antigen (PSA/KLK3), KLK2, and TMPRSS2 (48, 49). These are often increased during prostate cancer progression; PSA detection is currently used as a marker in prostate cancer screening and for monitoring the effectiveness of ADT. However, limitations of PSA testing such as lack of specificity, elevation in benign disease, failure to detect PSA-negative tumors, and inability to distinguish slow growing tumors from aggressive ones, makes prostate cancer biomarker discovery an important area of research (50).

Furthermore, during prostate cancer progression, there is a switch in AR function. In normal prostate cells, AR functions to drive differentiation and expression of the secretory proteins of the prostate, as well as suppress cell growth (16, 51-53). In prostate cancer, AR drives proliferation and cell survival (7, 25-32). The mechanism driving this conversion is unknown but likely involves cooperation of AR with other oncogenic events.

All existing ADTs are designed to prevent AR signaling by blocking androgen binding to AR. This is done through two mechanisms. The first is to reduce systemic androgen levels in the body using chemical castration agents such as gonadotropin-releasing hormone agonists (i.e. leuprolide). The second mechanism is direct competition using competitive antagonists for AR binding (i.e. bicalutamide, flutamide). Limitations of these current generation antagonists include a 30-fold weaker affinity for AR than DHT (54) and that they do not prevent AR localization to the nucleus (55). Both of these strategies are used in combination during ADT. ADT causes remission in 80-90% of patients undergoing therapy, resulting in a median progression-free survival of 12 to 33 months (56). After remission the recurrent cancer is castration-resistant and the median survival is 23–37 months from the time of initiation of ADT (56). Currently, the next generation of AR antagonists, which are non-competitive or have a greater AR affinity, are being developed (34, 57).

AR can be post-translationally modified by phosphorylation, acetylation, sumoylation, and ubiquitination (58-66). Post-translational modification of AR can affect its stability, ligand binding affinity, protein interactions, or

transcriptional activity. These have been mostly described in vitro and have not necessarily been observed in vivo. However, in vivo detection can be difficult and transient modifications or low levels of modification may not necessarily be detected. Furthermore, mutations of some of these modification sites fail to alter AR's activity, stability, or sensitivity to ligand as expected. One of the few phosphorylation sites that has been reproducibly observed, in both in vitro and in vivo, to have an effect is Ser650, in which phosphorylation stimulates nuclear export (58, 67). Androgen-induced phosphorylation of Ser308 has also been reproducibly demonstrated to increase the transcriptional activity of AR (68).

In CRPC, AR promotes survival and proliferation, even in the absence of physiological levels of androgen. Several mechanisms have been proposed to explain this phenomenon (reviewed in 7). Other steroid receptors, including ER and progesterone receptor (PR), have been reported to be involved in non-genomic signaling, which can also have important consequences on cell behavior (69, 70). These non-genomic mechanisms are associated with rapid responses (within seconds) to ligand stimulation. This has been less well characterized for AR, but non-genomic signaling may be a mechanism by which AR can function in CRPC. AR has been reported to be in a complex with Src and ERα via its PXXP domain (71). This can lead to rapid androgen-induced mitogen-activated protein kinase (MAPK) activation and has been reported to regulate cell survival and proliferation, although the mechanism of this remains unclear (71). However, this interaction does require androgen and does not explain disease progression in CRPR. AR can also form a complex with Src and

MNAR (modulator of non-genomic activity of ER) (72). This complex is androgen dependent in LNCaP cells but is constitutively active in a castration-resistant LNCaP derivative subline. This complex activates Src and MAPK/ extracellular signal-regulated kinase (ERK) signaling (72). Hence, this may contribute to the development of CRPC.

During ADT, physiological levels of androgen are greatly reduced, but low levels of androgen (in the nanomolar range) can still be detected in tissues (73, 74). Overexpression of AR sensitizes castration-resistant prostate cancer cells to the low levels of androgens (75). Furthermore, AR is amplified and overexpressed in 20-30% of CRPC (39, 55, 76, 77). Hence, the low levels of androgens present in tissues may be sufficient for AR function. It is speculated that the source of this is the adrenal glands, which can produce testosterone and are not influenced by current ADTs. However, mice do not produce adrenal androgens, so castration results in "complete androgen ablation" (78). Furthermore, mice can still develop CRPC and AR is still found in the nucleus of these cells. This suggests that residual androgen production after castration is not playing a crucial role in driving AR function in CRPC (67, 79, 80). Alternatively, it has been proposed that prostate cancer cells can synthesize their own androgen from cholesterol (81). Currently, this has only been demonstrated in vitro and it remains to be determined if this occurs in vivo.

Another mechanism is via gain-in-function mutations in AR which allow other steroids, such as corticosteroids and anti-androgens, to bind and activate AR; similar to what has been seen in breast cancer. Mutations in AR are

generally not seen in prostate cancer until after ADT failure; AR mutations are observed in approximately 10-25% of CRPC (82, 83). Alterations in AR co-regulators can also contribute to CRPC in that these changes have been demonstrated to drive AR nuclear translocation and DNA binding (55, 84-88). Crosstalk with growth factor pathways can also lead to activation of AR in the absence of ligand (25). For example, insulin-like growth factor (IGF) signaling can lead to dephosphorylation of AR at Ser650, allowing AR to remain in the nucleus (67). It can also stimulate AR transcription; stimulation with IGF-1 can induce a 5X increased in PSA secretion in LNCaP cells (89). AR crosstalk with PI3K/AKT, NF-κB, and Src will be elaborated on below. Lastly, John Isaacs and others have suggested that CRPC develops from a subpopulation of cancer cells that never did express AR, and hence these "cancer stem cells" are not affected by ADT (90, 91). Rather the progeny of these cells undergo partial differentiation to express AR and comprise the majority of the tumor. During ADT, the AR-negative cancer stem cells have a growth and survival advantage compared to the AR-positive cancer cells, allowing the prostate cancer to reoccur after ADT.

In summary, in normal prostate cells, AR function is androgen dependent and drives differentiation, secretory protein expression, and suppresses cell growth. AR signaling is critical in prostate cancer progression where it promotes cancer growth and survival. While ADT is initially successful, patients will fail and the cancer will reoccur. AR signaling is still important despite low physiological levels of androgens. This may be due to hypersensitivity to low amounts of androgen or other non-androgen ligands, amplification, overexpression, or gain-

in-function mutations of AR or its co-regulators, and crosstalk with other pathways that are also misregulated in cancer.

Molecular Changes Associated with Prostate Cancer Progression

Prostate cancer is heterogeneous and multifocal (5, 92). The *EGFR* mutations in lung cancer, *HER2/ERBB2* amplification in breast cancer, and *BCR-ABL* gene fusion in chronic myelogenous leukemia have all been demonstrated to be important in progression of a subset of these diseases and have allowed for the development of targeted therapies; however there is not a single causative mutation or common molecular change yet identified in prostate cancer (92). Even different foci within the same patient often have different genetic alterations, suggesting that prostate cancer can develop through multiple mechanisms (5, 93). This variance may be one explanation as to why the majority of prostate cancers are slow growing and will have a low likelihood of progression while a subset will progress to an aggressive disease resulting in a slow, painful death. However, some molecular studies have identified some candidate genes that may be involved in prostate cancer development and metastasis. It is important to note that few prostate cancers have all of the molecular changes included below.

Phosphate and Tensin Homolog (PTEN) and PI3K/Akt Signaling

Phosphate and tensin homolog (PTEN) is a phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (or PIP_3) which

antagonizes of phosphatidylinositol 3-kinase (PI3K) signaling. PI3K signaling through the downstream PIP_3 target Akt (also referred to as protein kinase B) can promote cell survival, proliferation, adhesion and spreading, and thus up-regulation of PI3K/Akt signaling can provide numerous growth advantages to a cancer cell (94). PI3K activation of Akt can promote cell survival via multiple mechanisms including phosphorylation and inhibition of the pro-death proteins Bad, Bax, forkhead box-O transcription factors (FOXO), death-associated protein 3 (DAP3), and pro-caspase 9, through increased expression of the pro-survival protein survivin, and by regulating NF-κB and mTOR signaling (95, 96). Hence, PTEN acts as a tumor suppressor and inactivating mutations or loss of heterozygousity are common in many cancers including glioblastoma, breast, ovarian, colon, and endometrial cancer (97). PTEN is lost in ~30% of clinical prostate cancers and in ~60% of metastatic cancers, resulting in constitutive activation of PI3K signaling (98-100). While Akt is not amplified or overexpressed, Akt kinase activity is increased in primary prostate cancer. It is significantly increased in poorly differentiated primary prostate tumors compared to well- and moderately- differentiated tumors, is associated with advanced prostate cancer development, and is predictive of ADT failure and prostate cancer reoccurrence (101-103). *Pten*-deficient mice develop high-grade PIN, but without progression to invasive cancer (104). Therefore, PTEN loss is not sufficient for prostate cancer development. Overexpression of Akt and AR, but not Akt or AR alone, is sufficient to initiate and drive castration-resistant prostate

cancer progression in mice (105). Thus, these observations suggest a strong connection between AR and the PI3K/Akt pathway in prostate tumorigenesis.

The PI3K/Akt pathway has been reported to interact with AR by both direct and indirect mechanisms (reviewed in 106). Akt regulates AR transcription (105, 107-109), stability and expression (110, 111), and post-translational modifications (112, 113). In turn, PI3K/Akt can also be activated by AR through genomic and non-genomic mechanisms (106, 114).

Many prostate cancer cell lines are dependent on PI3K/Akt signaling for survival, and in some contexts this can be rescued by AR signaling. While the androgen sensitive prostate cancer cell line LNCaP undergoes cell death within 24 hours of inhibiting PI3K/Akt pharmacologically, addition of androgen can rescue survival (115). Long term androgen ablation of LNCaPs results in cells resistant to PI3K/Akt inhibition, and androgen-independent cell lines are less sensitive to PI3K/Akt inhibition (116). Often, Akt activity is significantly increased in androgen-independent derivates of LNCaP cells (117, 118). Also, in vivo prostate regeneration studies demonstrate that AR and Akt signaling can synergize to promote tumor formation even after androgen ablation (105). Thus AR signaling, in some contexts independent of androgen stimulation, may promote survival independent of PI3K signaling by either regulating the same downstream targets of the PI3K pathway, or through a novel mechanism. AR can also phosphorylate and inhibit FOXO proteins, thus promoting survival independent of PI3K by regulation of a common downstream target (119). The pro-survival protein Bcl-xL can also promote survival independent of PI3K

signaling in prostate cancer cells; however the mechanism by which Bcl-xL is regulated remains undetermined (120). In summary, AR along with PI3K/Akt signaling may cooperate during the progression of prostate cancer, and in some contexts, in CRPC.

Nuclear factor-kappaB (NF-κB)

Nuclear factor-kappaB (NF-κB) is a family of transcription factors (p50/p105, p52/p100, RelA, c-Rel, RelB) that classically are key regulators of pro-immune and inflammatory gene expression, but have also been demonstrated to be important regulators of cell growth, differentiation and survival (121-123). The prototypical heterodimer consists of p65 (RelA) and p50, but heterodimers and homodimers of p52, c-Rel, and RelB also exist and this diversity allows for distinct DNA-binding specificity and protein-protein interactions at promoter sites, allowing specific gene activation profiles under different physiological conditions. There is some overlapping gene regulation, but in general NF-κB dimers bind DNA sites with the general motif 5'-GGGRNWYYCC-3' (where R= A or G; N= any nucleotide; W= A or T; Y= C or T) (121).

NF-κB is held inactive in the cytoplasm bound to inhibitors of NF-κB (IκBs). Stress stimuli or other environmental clues leads to formation of the IκB kinase (IKK) complex, which consists of the catalytic kinase subunits IKKα and/or IKKβ along with two molecules of the scaffold protein NF-κB essential modulator (NEMO). The IKK complex then phosphorylates IκB at two serine residues,

leading to its ubiquitination and subsequent proteosomal degradation. The NF-κB dimers are then free to enter the nucleus and drive transcription of target genes (121).

NF-κB signaling is classically associated with driving cell survival. Mice deficient in RelA, both IKKα and IKKβ, or NEMO are embryonic lethal, probably due to extensive apoptosis of hepatocytes (124-128). Overexpression of the potent pro-survival and NF-κB-regulated protein Bcl-2 is unable to rescue liver degeneration in RelA-knockout mice, indicating that the pro-survival activity of RelA is through multiple mechanisms (129). Indeed, NF-κB signaling drives transcription of numerous pro-survival genes targeting both intrinsic and extrinsic death pathways including Bcl-2, Bcl-xL, Bfl-1/A1, NR13, c-IAP, XIAP, survivin, c-FLIP, GADD45β, and Spi2A as well as the antioxidant proteins MnSOD and FHC (reviewed in 130). NF-κB signaling can also repress transcription of the pro-death proteins BNIP3, caspase 8, DR4 (TRAIL-R1) and DR5 (TRAIL-R2) (130). However it is important to note that under certain conditions, such as radiation or treatment with chemotherapeutic drugs, NF-κB signaling can drive cell death through repression of pro-survival genes including Bcl-xL and driving expression of death receptors and their ligands by altering the NF-κB heterodimer composition, recruiting the transcriptional repressor HDAC to transcription sites, or altering NF-κB localization along with other mechanisms (130).

NF-κB signaling is important in the development, progression, and chemoresistance of many cancers, including prostate cancer (reviewed in 131). Increased NF-κB activity is associated with prostate cancer progression (132),

castration-resistance (133, 134), poor prognosis (135, 136), biochemical failure (i.e. PSA relapse) (137, 138), and has been determined to be significantly misregulated in metastatic prostate cancer based on microarray studies (139).

NF-κB can regulate AR by co-stimulating AR transcription (140-142) (133) or by regulating AR expression (140, 143, 144). In androgen-dependent cell lines, NF-κB signaling can inhibit proliferation (144) and there is generally a low level of basal NF-κB activity (145). However, in androgen-independent cell lines, NF-κB signaling is often constitutively active (145), possibly due to constitutive activation of IKKα (146). Constitutive activation of NF-κB in vivo is sufficient to maintain prostate cancer cell growth after castration in mouse models, presumably by maintaining high levels of nuclear AR (134). This suggests that there may be a functional change in NF-κB signaling in the transition to CRPC that promotes AR function independent of ligand.

PI3K/Akt signaling crosstalk with NF-κB signaling has also been described. PI3K/Akt can phosphorylate and activate IKKs, turning on NF-κB signaling (147, 148). NF-κB signaling in turn can lead to increased AR transcription independent of ligand; this has been implicated in the development of CRPC in mice models (149). Cooperative crosstalk between AR, PI3K/Akt, and NF-κB has also been described in prostate cancer cells in the regulation of interleukin-4 (IL-4) expression (150).

In summary, NF-κB signaling has strong correlations in the clinic with prostate cancer progression, can be activated by PI3K/Akt signaling, and can

stimulate AR function during castration, suggesting it plays a critical role in this disease.

TMPRSS2-Erg

TMPRSS2 is a direct transcriptional target of AR. In prostate cancer, approximately 40-60% of tumors have a TMPRSS2-Erg rearrangement, which allows for AR regulation of the ETS-related gene (Erg) and subsequent aberrant expression in prostate cancer cells (151, 152). Erg proteins are transcriptional factors that regulate cell proliferation, differentiation and tumorigenesis (153). Additionally, ETS family members *ETV1*, *ETV4* and *ETV5* have also been shown to be genetically rearranged in prostate cancer. While the biological consequence of Erg overexpression in prostate cancer has not yet been determined, it has been shown to lead to activation of the oncoprotein Myc and to repress the expression of some prostate differentiation makers (i.e. PSA, SLC45A3 (Prostein), MSMB) (151). Furthermore, there appears to be a strong correlation between TMPRSS2-Erg expression and PTEN loss. Most ERG-positive samples have reduced or absent PTEN expression (154, 155). Transgenic mice expressing Erg fail to develop PIN or develop tumors prostate tumors. However, crossing these mice with mice that were either Pten-deficient or had prostate-specific high Akt activity, resulted in offspring that developed PIN, and in the mice with a B6 background but not FVB, progression to prostate cancer was a consistent feature in offspring (154, 155). Hence, TMPRSS2-Erg

may be important in prostate cancer by allowing androgen regulation of the transcription factor Erg.

Integrin Expression and Signaling in Prostate and Prostate Cancer

Integrins are a large family of transmembrane, cell-surface receptors that bind components of the extracellular matrix (ECM) including collagen (CL), laminin (LM), fibronectin (FN), etc. (156). Upon ECM binding integrins mediate cellular adhesion, spreading, migration, proliferation, and survival by reorganizing the cytoskeleton and activating intracellular signaling pathways (157). Integrins exist as heterodimers comprising one alpha subunit and one beta subunit, the combination of which determines the ECM protein the cell can associate with. Integrins are required for survival in many cell types, and interruption of integrin binding to matrix results in a form of cell death termed anoikis (158). Integrins have no intrinsic catalytic activity, but rather interact with other structural and signaling proteins including kinases such as integrin-linked kinase (ILK) and focal adhesion kinase (FAK) via their cytoplasmic tail (159). Furthermore, integrins can activate receptor tyrosine kinases (RTKs) in the absence of exogenous ligand (160-167). Some examples of RTKs activated by integrins include epidermal growth factor (EGFR), platelet-derived growth factor receptor (PDGFR), the macrophage-stimulating protein receptor RON, and the hepatocyte growth factor receptor, c-Met (163, 168-171).

Integrin cross-talk with other signaling molecules allows coordinated cellular responses to extracellular signals such as matrix attachment and growth factors (172). Our work and that of others have demonstrated that integrin engagement is sufficient to activate receptor tyrosine kinases (160-167). For example, the macrophage-stimulating protein (MSP) receptor RON can be activated by β1 activation of c-Src independent of its ligand MSP (163). In addition, MSP enhances binding to collagen, suggesting cooperation and synergy between these two pathways (163). In another example, signaling from both integrin-mediated activation of EGFR and ligand activation of EGFR is required for cells to proliferate (162, 169). Integrin engagement of fibronectin (FN) in epithelial cells results in phosphorylation of a different subset of tyrosine residues on EGFR than ligand binding does and is enough to initiate G_1 entry and progression to mid-G_1 in the absence of growth factors (162). S phase entry requires EGF or serum, though over-expression of EGFR allows cell cycle progression in the absence of ligand, which has interesting implications in cancer where RTKs are often over-expressed (162, 169). Integrins can activate the PI3K/Akt pathway to promote cell survival (173, 174). Conversely, increased Akt activity can prevent anoikis and promote cell migration (95, 175). Integrin α6β4 adhesion to LM5 can lead to increased activation NFκB, MAPK, and Jnk (176, 177); targeted deletion of the β4 cytoplasmic tail in keratinocytes of mice inhibits their growth and migration (177). Therefore, cross-talk has important biological effects in coordinating cellular response from multiple signaling pathways and allowing different cellular responses depending on the context.

While not extensively studied, steroids have also been implicated in participating in cross-talk with integrins. Integrin α2β1- or α6β1-mediated adhesion to CLIV or LM1 respectively has been shown to regulate ERα expression and its subsequent function in mouse mammary epithelial cells, but adhesion does not regulate ERα expression in mammary fibroblasts (178). ER may also regulate α2 integrin since the gene promoter for α2 contains estrogen-response elements (EREs), which would suggest a positive feed-back loop of regulation; however this remains under investigation (178, 179). Recently, estrogen has been shown to enhance integrin α5 expression through ERα-Sp1 interaction (180). Also in MDA-MB-231 breast cancer cells expressing PR, progesterone treatment resulted in cell spreading, increased cell attachment, and increased phosphorylation of FAK and paxillin (181). Blocking β1 with antibody caused reduced spreading and significantly inhibited adhesion to ECM, suggesting that β1 plays an important role in progesterone-induced focal adhesion (181).

Integrin expression and signaling is aberrant in many cancers. For example, overexpression of LM1 can increase colon tumor growth in mouse models (182). Integrin β1 is aberrantly expressed in breast cancer and has been shown to play a central role in growth, apoptosis, invasion, and metastasis. Blocking integrin β1 with antibody has been shown to either reverse the malignant phenotype of breast cancer cells (183), or to inhibit proliferation and induce apoptosis in 3D and xenograft mice models (184). Since animals did not

appear to suffer any side effects, this suggests that blocking integrins may be a therapeutic option in patients (184).

Integrin Signaling in Prostate and Prostate Cancer

Prostate cancer arises from the prostate epithelium which comprises basal and secretory layers. The basal layer uses integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 4$ for adhesion to a basement membrane consisting of LM5 and LM10 as well as CLIV and CLVII (185). This binding of integrins to the ECM regulates survival in the basal cells (186, 187). The secretory cells do not contact a basement membrane, but rest on top of the basal cells from which they differentiate. The secretory cells only express integrin $\alpha 6\beta 1$; however they do not use this integrin to adhere to matrix (188). Rather, $\alpha 6\beta 1$ is localized to cell-cell junctions and its function there is currently unknown.

In prostate cancer, there is a loss of the basal epithelial cells and prostate cancer cells are adherent to a basement membrane containing LM10 and CLIV; LM5 and CLVII are absent (185). Normal basal epithelial cells secrete and organize a LM5 matrix, so loss of these cells contributes to the altered ECM in prostate cancer. Furthermore, $\alpha 6\beta 1$ is the primary integrin expressed in both primary prostate cancer and lymph node metastases (189-195). Prostate cancer cell lines that form invasive tumors in mice have increased integrin $\alpha 6\beta 1$ expression; blocking integrin antibodies can inhibit their invasion in vitro and in vivo (196-199). One of the 22 SNPs currently associated with prostate cancer is

within the first intron of integrin α6 (200). This SNP lies within the epithelial-mesenchymal transition (EMT)-specific splice site in integrin α6; thus the integrin α6 in epithelial cells becomes altered when the cells undergo EMT and mesenchymal specific splice factors are being activated (200). In addition, a truncated form of integrin α6 (α6p) is increased during prostate cancer progression by cleavage and removal of the extracellular domain by the extracellular urokinase plasminogen activator receptor (uPAR) (201, 202). This is thought to make the cell more motile since the integrin can no longer interact with matrix, and may contribute to cancer invasion and metastasis (203). This is supported from experiments where blocking α6 cleavage with antibody prevents bone metastasis in a mouse model (188). Furthermore, two variants of integrin β1, β_{1C} and β_{1A}, have been shown to be expressed in prostatic epithelium. Interestingly, integrin β_{1C} expression is decreased in prostate cancer (204, 205), whereas integrin β_{1A} is increased in prostate cancer and in a mouse model for prostate cancer progression (206, 207). Integrin β_{1A} stimulates proliferation in vitro (207). Integrin α6pβ_{1A} may also alter integrin-mediated signaling with other molecules, contributing to tumorigenicity. Interestingly, ADT results in increased β_{1C} mRNA, suggesting a possibility of androgen regulation (208). During metastasis, additional changes in integrin and ECM expression correspond to the cells' ability to survive in distinct environments. LNCaP that were selected for increased integrin α2β1 and CLI adhesion develop tumors when injected into

tibia of nude mice, whereas parental LNCaP cells do not (209). Integrin αvβ3 or αvβ5 can drive survival, migration, and metastasis in prostate cancer cell lines and xenograft models (210-213); however these integrins have not necessarily been reported in vivo and may be an artifact from culturing cells in serum.

Expression of AR in PC3 cells has been shown to decrease α6β4, α3β1, and α2β1 expression along with a reduction in adhesion to collagen and laminin (214, 215). Expression of AR in DU145 cells decreased α6β4, but increased expression of α2β1 (216). Lastly, DHT stimulation of AR expressing immortalized non-tumorigenic rat prostate CA25 cells leads to increased integrin α6 and p21-activated kinase (PAK) activity (15). This suggests that AR can regulate integrin expression. Since integrin-mediated adhesion can regulate growth factor signaling pathways including PI3K/Akt, MAPK, and NF-κB signaling, AR and integrins can potentially crosstalk through joint regulation of these pathways. These mechanisms of cross-talk between integrins and AR are largely unexplored.

Limitations of Current Models

Canine and Murine Models

A significant challenge in understanding prostate cancer development and biology is the limitations of the models in which to study the disease. Besides humans, dogs are the only mammal in which spontaneous development of prostate cancer has been well documented (5, 217, 218). Nobel laureate

Charles Huggins used canines as a model of prostate cancer early in his career. Canine prostate cancer has many clinical and histological features in common with human prostate cancer including metastasis to bone, however there are some important differences (217). Canine prostate cancer is rare but aggressive, whereas human prostate cancer is usually slow growing (217). Most significant is that canine prostate cancer has little to no dependence on androgen and many canine prostate cancers do not even express AR (217, 219). In fact, castration is associated with increased prostate cancer risk in dogs although it can be protective if done early in life (217, 220, 221). These differences, along with the long lifespan, heterogenetic background, and considerable space and expense of housing canines make them not ideal for use as a model (217). Rodents, a common laboratory model, do not get spontaneous prostate cancer with the exception of the Dunning rat model and its derivatives (222, 223). In addition, the prostate organ itself is significantly different between rodents and humans. In humans, the organ is shaped like a walnut and is a single encapsulated body, whereas in rodents there are three distinct and separate lobes (5, 223). The prostates of rodents do not make PSA (218). Of even greater importance are differences in the organization of the epithelial compartment. In humans, as previously discussed, the basal cells form a continuous layer and, along with neuroendocrine cells, are the only epithelial cell in contact with ECM; the secretory cells are not in contact with ECM and reside on top of the basal cells facing the lumen. In contrast, rodent prostates have few basal cells and the secretory cells are in contact with ECM, which means that adhesion signaling

may occur in these cells (5). The ECM in rodents is also less tightly organized. Lastly, stromal cells are found throughout the human prostate, but in rodents the stromal cells are found associated tightly with acini (5, 223). The differences in tissue organization and matrix may also allow a more direct interaction of the secretory cells with the ECM and stromal compartment in rodents than what is found in humans.

Genetic manipulations of mice, while helpful in determining the molecular events of prostate tumorigenesis, have also failed to produce a satisfactory model of prostate cancer. Genetic knockdown or overexpression of genes known to be lost or gained during prostate cancer progression including AR, Nkx3.1, PTEN and Nkx3.1, often result in only hyperplasia or PIN (218, 223, 224). However, mice with prostate specific Myc overexpression, or PTEN loss in combination with p53 or Nkx3.1 loss, develop invasive carcinoma without metastasis (223, 225-227). Interestingly, the *Nkx3.1;Pten* mutant mice develop tumors that express AR, but are androgen-independent (225, 227). The transgenic mouse model TRAMP (transgenic adenocarcinoma mouse prostate) remains one of the few mouse models that exhibit progressive stages of androgen-dependent prostate cancer ranging from PIN to metastatic androgen-independent prostate cancer, although bone metastasis is rare (228-231). However, the TRAMP model and its derivatives rely on prostate specific transgenic expression of the SV40 T and t antigen to suppress p53 and Rb function (229, 231). While p53 and Rb are commonly lost during prostate cancer progression, it occurs only in late-stage disease, and it is not through T or

t antigen expression. There are additional T and t antigen targets that are not associated with prostate cancer; large T antigen can inactivate Bub1, a spindle assembly checkpoint protein (232), and small t antigen can modulate the activity of PP2A, which can impact a plethora of cellular signaling pathways including AR (233, 234). While some mouse models recapitulate some features of the human disease, there is some skepticism that they will to be predictive for therapeutic interventions that will work in humans (218, 223, 235, 236). For all cancers, only about 5% of therapeutics successfully tested on mice with cancer, from 1991-2000, were approved for use in human patients (237). Currently, there is not an inducible model for prostate cancer and only one report looking at the effect of altering gene expression in the stroma, although this along with more elegant compound mutational models are expected to be developed in the next few years (223, 238). Lastly, there is a need for a prostate specific promoter that is androgen independent, so that researches can better elucidate the effect of castration or ADT on prostate cancer in mice, as current prostate specific but androgen-dependent promoters would also be effected. Thus, while mouse models have been informative in characterizing the molecular events required for PIN and prostate tumorigenesis, they have to date been limited in that the rodent prostate is inherently different in its anatomy and morphology, the models rarely metastasize and when they do they rarely go to bone, there is insufficient molecular and biological diversity of models, and the models have failed to be predictive for human therapies.

Cell Lines

In vitro, there are a limited number of established cell lines due to the difficulty of successfully isolating and culturing human prostate cancer tissue despite repeated numerous attempts (239-242). This may be due to the slow growing nature of prostate cancer (5, 239, 240) and/or a lack of the proper environmental and growth factors in culture. Most in vitro work is based on three cells lines (PC3, DU145, and LNCaP) and their derivatives (for a comprehensive review of all prostate cancer cell lines see 241, 242). These were all originally isolated from metastatic lesions (bone, brain and lymph node respectively) (240); there is no human prostate cancer cell line isolated from a primary tumor. As such, most of the findings in the literature are based on a limited number of cell lines with limited genetic and biological diversity and representative of only metastatic cancer. Furthermore, their relevance in vivo has only been demonstrated in xenograft models and it remains uncertain what correlation exists in human patients. Interestingly, of these three cell lines, PC3 and DU145 cells have lost AR expression and LNCaP cells have a mutated (T877A) AR. While LNCaP cells remain responsive for androgen, the T877A mutation makes AR promiscuous to ligands other than androgen including gluccocorticoids, estrogen, progesterone, and the AR antagonist hydroxyflutamide (243). It has also been reported that all three of these cell lines have been evolving with passage in culture and thus cells from the same cell line may behave differently in different laboratories (218, 244, 245). For example, although it is well established that PC3 and DU145 cells do not express AR, there are some reports and anecdotal

stories that suggest early passages of these cells expressed low levels of AR (246-249). In LNCaP cells, IGF represses AR-mediated transcription in early passage cells, whereas IGF stimulates AR-mediated transcription in late passage cells (108). Despite this, there is no requirement for published studies using these cells to include a standardized characterization (218). Recently, some new metastatic prostate cancer cell lines have become available that express wild type AR including VCaP (250), MDA PCa 2a and MDA PCa 2b (251), and 22Rv1 (252). We are currently characterizing these cells in our lab to examine their integrin α6 expression and adhesion to LM. There is no in vitro model of androgen receptor positive primary prostate cancer cells.

Basal epithelial cells in the prostate gland express α6β4 and α3β1 integrins and adhere to a basement membrane rich in laminin 5 (253). When these cells are placed in culture they retain in vitro a majority of the properties seen in vivo, including the ability to secrete and organize their own laminin 5-rich matrix (254, 255). Hence, prostate epithelial cells (PECs) cultured from normal human prostate tissue consist primarily of AR-negative basal cells and their transient amplifying derivatives. Furthermore, although normal prostate basal cells can be isolated and cultured in vitro, AR-expressing secretory cells to date cannot. This may due to the post-mitotic status of the cells, or lack of an environmental condition that is required for their survival.

Framework of Dissertation

To develop effective and low toxicity prostate cancer therapies that are effective even in castrate resistant prostate cancer, it is imperative to understand the molecular mechanisms regulating survival of both normal and prostate cancer cells. However, these mechanisms are poorly understood, in part due to the limitations of current models. Hence the objective of this dissertation was to generate better models in which to study this disease, and to determine how survival of normal prostate and prostate cancer cells is mediated in the context of integrin and AR signaling. My overall original **hypothesis** was that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulates their survival. In normal cells, AR regulates survival. I proposed to investigate this in three specific aims.

Specific Aim 1. Identify how integrins mediate survival of PC3 and DU145 prostate cancer cells in vitro.

Specific Aim 2. Identify how integrins mediate survival in prostate cancer cells expressing AR in vitro.

Specific Aim 3. Identify how integrins mediate survival in primary prostate epithelial cells expressing AR in vitro.

Previous studies in our lab have elucidated three integrin-mediated survival pathways in PEC cells, which recapitulate the prostate basal epithelial cell population (187). Survival of PEC cells on LM5 requires $\alpha 3\beta 1$, but not $\alpha 6\beta 4$, and is dependent on integrin-mediated, ligand-independent activation of the

EGFR and the cytoplasmic tyrosine kinase Src, but not PI3K (Figure 2). Integrin-mediated EGF receptor activation supports cell survival by signaling downstream to ERK (Figure 2). Surprisingly, the death induced by inhibition of EGF receptor or Src in normal prostate cells is not mediated through or dependent on caspase activation. The presence of an autophagic pathway regulated by adhesion to matrix prevents the induction of caspases (Figure 2). Suppression of autophagy permits caspase activation and induction of classical apoptosis.

Using these pathways as a framework, I first sought to investigate the integrin-regulated survival pathways in prostate tumor cells starting with PC3 and DU145 cells, which do not express AR and are derived from a prostate metastasis, on LM1 and CLI respectively. This established which integrin-mediated signaling pathways regulate survival in the absence of AR (see Chapter 2). To generate a model of castration resistant prostate cancer, AR or AR mutants were re-expressed in PC3 cells. Using this model, I investigated the downstream targets of AR that regulate survival on LM1 independent of PI3K (see Chapter 3). Last, I generated an in vitro differentiation model in which normal human primary prostate basal cells isolated from patients are differentiated into AR-expressing cells that recapitulate many of the characteristics of secretory cells, including loss of matrix adhesion. Using this model, I investigated the requirement of AR and other signaling pathways for cell survival (see Chapter 4).

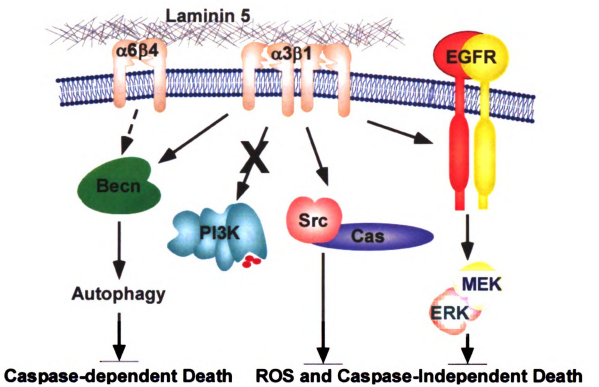


FIGURE 2. Model for LM5-mediated survival in PEC cells. Adhesion of growth factor-deprived PECs to LM5 via α3β1 and α6β4 integrin mediates cell survival by maintaining starvation-induced autophagy. Signaling via α3β1 to ERK through EGFR or through Src is also required for cell survival. The PI-3K/Akt pathway is not activated on LM5 and not required for survival. Disruption of either the EGFR/ERK or Src pathway leads to caspase-independent cell death, due to the generation of reactive oxygen species (ROS), whereas disruption of autophagy leads to caspase activation and death.

REFERENCES

1. American Cancer Society. Cancer Facts and Figures 2009. Atlanta, GA: American Cancer Society; 2009.

2. Hayat MJ, Howlader N, Reichman ME, Edwards BK. Cancer statistics, trends, and multiple primary cancer analyses from the Surveillance, Epidemiology, and End Results (SEER) Program. *Oncologist* 2007 Jan; **12** (1): 20-37.

3. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, *et al.* Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. *Hum Pathol* 2000 May; **31** (5): 578-583.

4. Sakr WA, Grignon DJ, Crissman JD, Heilbrun LK, Cassin BJ, Pontes JJ, *et al.* High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. *In Vivo* 1994 May-Jun; **8** (3): 439-443.

5. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. *Genes Dev* 2000 Oct 1; **14** (19): 2410-2434.

6. Horner M, Ries L, Krapcho M, Neyman N, Aminou R, Howlader N, *et al.* Surveillance Epidemiology and End Results (SEER) Cancer Statistics Review, 1975-2006. 2009 [cited 2009 October 2009]; Available from: http://seer.cancer.gov/csr/1975_2006/

7. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer* 2001 Oct; **1** (1): 34-45.

8. Knox JD, Cress AE, Clark V, Manriquez L, Affinito KS, Dalkin BL, *et al.* Differential expression of extracellular matrix molecules and the alpha 6-integrins in the normal and neoplastic prostate. *Am J Pathol* 1994; **145** (1): 167-174.

9. Uzgare AR, Xu Y, Isaacs JT. In vitro culturing and characteristics of transit amplifying epithelial cells from human prostate tissue. *J Cell Biochem* 2004; **91** (1): 196-205.

10. van Leenders GJLH, Schalken JA. Epithelial cell differentiation in the human prostate epithelium: Implications for the pathogenesis and therapy of prostate cancer. *Crit Rev Oncol Hematol* 2003; **46** (Supplement 1): 3-10.

11. Berger R, Febbo PG, Majumder PK, Zhao JJ, Mukherjee S, Signoretti S, *et al.* Androgen-induced differentiation and tumorigenicity of human

prostate epithelial cells. *Canc Res* 2004 December 15, 2004; **64** (24): 8867-8875.

12. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby R, Higgins SJ, *et al.* The endocrinology and developmental biology of the prostate. *Endocr Rev* 1987; **8** (3): 338-362.

13. Heer R, Robson CN, Shenton BK, Leung FY. The role of androgen in determining differentiation and regulation of androgen receptor expression in the human prostatic epithelium transient amplifying population. *Journal of cellular physiology* 2007; **212** (3): 572-578.

14. Ling MT, Chan KW, Choo CK. Androgen induces differentiation of a human papillomavirus 16 E6/E7 immortalized prostate epithelial cell line. *J Endocrinol* 2001 July 1, 2001; **170** (1): 287-296.

15. Whitacre DC, Chauhan S, Davis T, Gordon D, Cress AE, Miesfeld RL. Androgen induction of in vitro prostate cell differentiation. *Cell Growth Differ* 2002 January 1, 2002; **13** (1): 1-11.

16. Lamb LE, Knudsen BS, Miranti CK. E-cadherin-mediated survival of androgen-receptor-expressing secretory prostate epithelial cells derived from a stratified in vitro differentiation model. *J Cell Sci* 2010 Jan 15; **123** (Pt 2): 266-276.

17. Lamb DJ, Weigel VL, Marcelli M. Androgen receptors and their biology. *Vitam Horm* 2001; **62:** 199-230.

18. Cunha GR. Growth factors as mediators of androgen action during male urogenital development. *Prostate Suppl* 1996; **6:** 22-25.

19. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, *et al.* The endocrinology and developmental biology of the prostate. *Endocr Rev* 1987 Aug; **8** (3): 338-362.

20. Hayward SW, Rosen MA, Cunha GR. Stromal-epithelial interactions in the normal and neoplastic prostate. *Br J Urol* 1997 Apr; **79 Suppl 2:** 18-26.

21. Bostwick DG, Liu L, Brawer MK, Qian J. High-grade prostatic intraepithelial neoplasia. *Rev Urol* 2004 Fall; **6** (4): 171-179.

22. Bonkhoff H. Role of the basal cells in premalignant changes of the human prostate: a stem cell concept for the development of prostate cancer. *Eur Urol* 1996; **30** (2): 201-205.

23. Yu HM, Frank DE, Zhang J, You X, Carter WG, Knudsen BS. Basal prostate epithelial cells stimulate the migration of prostate cancer cells. *Mol Carcinog* 2004 Oct; **41** (2): 85-97.

24. Logothetis CJ, Lin SH. Osteoblasts in prostate cancer metastasis to bone. *Nat Rev Cancer* 2005 Jan; **5** (1): 21-28.

25. Scher HI, Sawyers CL. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J Clin Oncol* 2005 Nov 10; **23** (32): 8253-8261.

26. Cheng H, Snoek R, Ghaidi F, Cox ME, Rennie PS. Short hairpin RNA knockdown of the androgen receptor attenuates ligand-independent activation and delays tumor progression. *Cancer Res* 2006 Nov 1; **66** (21): 10613-10620.

27. Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem* 2004 Feb 15; **91** (3): 483-490.

28. Sun A, Tang J, Terranova PF, Zhang X, Thrasher JB, Li B. Adeno-associated virus-delivered short hairpin-structured RNA for androgen receptor gene silencing induces tumor eradication of prostate cancer xenografts in nude mice: a preclinical study. *Int J Cancer* 2010 Feb 1; **126** (3): 764-774.

29. Chen C, Welsbie D, Tran C, Baek S, Chen R, Vessella R, *et al.* Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004; **10** (1): 33-39.

30. Cohen MB, Rokhlin OW. Mechanisms of prostate cancer cell survival after inhibition of AR expression. *J Cell Biochem* 2009 Feb 15; **106** (3): 363-371.

31. Shanmugam I, Cheng G, Terranova PF, Thrasher JB, Thomas CP, Li B. Serum/glucocorticoid-induced protein kinase-1 facilitates androgen receptor-dependent cell survival. *Cell Death Differ* 2007 Dec; **14** (12): 2085-2094.

32. Yang Q, Fung K-M, Day W, Kropp B, Lin H-K. Androgen receptor signaling is required for androgen-sensitive human prostate cancer cell proliferation and survival. *Cancer Cell International* 2005; **5** (1): 8.

33. Georget V, Terouanne B, Nicolas JC, Sultan C. Mechanism of antiandrogen action: key role of hsp90 in conformational change and

transcriptional activity of the androgen receptor. *Biochemistry* 2002 Oct 1; **41** (39): 11824-11831.

34. Jones JO, Diamond MI. A cellular conformation-based screen for androgen receptor inhibitors. *ACS Chem Biol* 2008 Jul 18; **3** (7): 412-418.

35. Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, *et al.* Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. *J Steroid Biochem Mol Biol* 2001 Jan-Mar; **76** (1-5): 23-30.

36. Verrijdt G, Haelens A, Claessens F. Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. *Mol Genet Metab* 2003 Mar; **78** (3): 175-185.

37. Waghray A, Feroze F, Schober MS, Yao F, Wood C, Puravs E, *et al.* Identification of androgen-regulated genes in the prostate cancer cell line LNCaP by serial analysis of gene expression and proteomic analysis. *Proteomics* 2001 Oct; **1** (10): 1327-1338.

38. Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, *et al.* New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. *EMBO Rep* 2007 Sep; **8** (9): 871-878.

39. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, *et al.* Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004 Jan; **10** (1): 33-39.

40. Velasco AM, Gillis KA, Li Y, Brown EL, Sadler TM, Achilleos M, *et al.* Identification and validation of novel androgen-regulated genes in prostate cancer. *Endocrinology* 2004 Aug; **145** (8): 3913-3924.

41. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE. Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. *J Steroid Biochem Mol Biol* 2005 Aug; **96** (3-4): 251-258.

42. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 2002 Feb 22; **108** (4): 465-474.

43. Perissi V, Rosenfeld MG. Controlling nuclear receptors: the circular logic of cofactor cycles. *Nat Rev Mol Cell Biol* 2005 Jul; **6** (7): 542-554.

44. Spiegelman BM, Heinrich R. Biological control through regulated transcriptional coactivators. *Cell* 2004 Oct 15; **119** (2): 157-167.

45. Chakalova L, Debrand E, Mitchell JA, Osborne CS, Fraser P. Replication and transcription: shaping the landscape of the genome. *Nat Rev Genet* 2005 Sep; **6** (9): 669-677.

46. Wang Q, Carroll JS, Brown M. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol Cell* 2005 Sep 2; **19** (5): 631-642.

47. West AG, Fraser P. Remote control of gene transcription. *Hum Mol Genet* 2005 Apr 15; **14 Spec No 1:** R101-111.

48. Young CY, Andrews PE, Montgomery BT, Tindall DJ. Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. *Biochemistry* 1992 Jan 28; **31** (3): 818-824.

49. Murtha P, Tindall DJ, Young CY. Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. *Biochemistry* 1993 Jun 29; **32** (25): 6459-6464.

50. Bickers B, Aukim-Hastie C. New molecular biomarkers for the prognosis and management of prostate cancer--the post PSA era. *Anticancer Res* 2009 Aug; **29** (8): 3289-3298.

51. Cunha GR, Ricke W, Thomson AA, Marker PC, Risbridger G, Hayward SW, *et al.* Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development. *J Steroid Bioch Mol Biol* 2004; **92** (4): 221.

52. Wu C-T, Altuwaijri S, Ricke WA, Huang S-P, Yeh S, Zhang C, *et al.* Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. *Proc Natl Acad Sci USA* 2007 July 31, 2007; **104** (31): 12679-12684.

53. Wu CT, Altuwaijri S, Ricke WA, Huang SP, Yeh S, Zhang C, *et al.* Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. *Proc Natl Acad Sci U S A* 2007 Jul 31; **104** (31): 12679-12684.

54. Kolvenbag GJ, Furr BJ, Blackledge GR. Receptor affinity and potency of non-steroidal antiandrogens: translation of preclinical findings into clinical activity. *Prostate Cancer Prostatic Dis* 1998 Dec; **1** (6): 307-314.

55. Mohler JL, Gregory CW, Ford OH, 3rd, Kim D, Weaver CM, Petrusz P, *et al.* The androgen axis in recurrent prostate cancer. *Clin Cancer Res* 2004 Jan 15; **10** (2): 440-448.

56. Hellerstedt BA, Pienta KJ. The current state of hormonal therapy for prostate cancer. *CA Cancer J Clin* 2002 May-Jun; **52** (3): 154-179.

57. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, *et al.* Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 2009 May 8; **324** (5928): 787-790.

58. Gioeli D, Black BE, Gordon V, Spencer A, Kesler CT, Eblen ST, *et al.* Stress kinase signaling regulates androgen receptor phosphorylation, transcription, and localization. *Mol Endocrinol* 2006 Mar; **20** (3): 503-515.

59. Gioeli D. Signal transduction in prostate cancer progression. *Clin Sci (Lond)* 2005 Apr; **108** (4): 293-308.

60. Xu K, Shimelis H, Linn DE, Jiang R, Yang X, Sun F, *et al.* Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination. *Cancer Cell* 2009 Apr 7; **15** (4): 270-282.

61. Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *EMBO J* 2002 Aug 1; **21** (15): 4037-4048.

62. Faus H, Haendler B. Post-translational modifications of steroid receptors. *Biomed Pharmacother* 2006 Nov; **60** (9): 520-528.

63. Gregory CW, Fei X, Ponguta LA, He B, Bill HM, French FS, *et al.* Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. *J Biol Chem* 2004 Feb 20; **279** (8): 7119-7130.

64. Guo Z, Dai B, Jiang T, Xu K, Xie Y, Kim O, *et al.* Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell* 2006 Oct; **10** (4): 309-319.

65. Mahajan NP, Liu Y, Majumder S, Warren MR, Parker CE, Mohler JL, *et al.* Activated Cdc42-associated kinase Ack1 promotes prostate cancer progression via androgen receptor tyrosine phosphorylation. *Proc Natl Acad Sci U S A* 2007 May 15; **104** (20): 8438-8443.

66. Ueda T, Mawji NR, Bruchovsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. *J Biol Chem* 2002 Oct 11; **277** (41): 38087-38094.

67. Wu JD, Haugk K, Woodke L, Nelson P, Coleman I, Plymate SR. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. *J Cell Biochem* 2006 Oct 1; **99** (2): 392-401.

68. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, *et al.* Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *J Biol Chem* 2002 Aug 9; **277** (32): 29304-29314.

69. Cato AC, Nestl A, Mink S. Rapid actions of steroid receptors in cellular signaling pathways. *Sci STKE* 2002 Jun 25; **2002** (138): re9.

70. Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M. Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev* 2000 Dec; **52** (4): 513-556.

71. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, *et al.* Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* 2001 Mar 9; **104** (5): 719-730.

72. Unni E, Sun S, Nan B, McPhaul MJ, Cheskis B, Mancini MA, *et al.* Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res* 2004 Oct 1; **64** (19): 7156-7168.

73. Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL. Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. *Clin Cancer Res* 2005 Jul 1; **11** (13): 4653-4657.

74. Titus MA, Gregory CW, Ford OH, 3rd, Schell MJ, Maygarden SJ, Mohler JL. Steroid 5alpha-reductase isozymes I and II in recurrent prostate cancer. *Clin Cancer Res* 2005 Jun 15; **11** (12): 4365-4371.

75. Waltering KK, Helenius MA, Sahu B, Manni V, Linja MJ, Janne OA, *et al.* Increased expression of androgen receptor sensitizes prostate cancer cells to low levels of androgens. *Cancer Res* 2009 Oct 15; **69** (20): 8141-8149.

76. Gelmann EP. Molecular biology of the androgen receptor. *J Clin Oncol* 2002 Jul 1; **20** (13): 3001-3015.

77. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, *et al.* In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat Genet* 1995 Apr; **9** (4): 401-406.

78. van Weerden WM, Bierings HG, van Steenbrugge GJ, de Jong FH, Schroder FH. Adrenal glands of mouse and rat do not synthesize androgens. *Life Sci* 1992; **50** (12): 857-861.

79. Thalmann GN, Sikes RA, Wu TT, Degeorges A, Chang SM, Ozen M, *et al.* LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. *Prostate* 2000 Jul 1; **44** (2): 91-103 Jul 101;144(102).

80. Corey E, Quinn JE, Buhler KR, Nelson PS, Macoska JA, True LD, *et al.* LuCaP 35: a new model of prostate cancer progression to androgen independence. *Prostate* 2003 Jun 1; **55** (4): 239-246.

81. Dillard PR, Lin MF, Khan SA. Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. *Molecular and Cellular Endocrinology* 2008 Nov 25; **295** (1-2): 115-120.

82. Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, *et al.* Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. *Cancer Res* 1994 Jun 1; **54** (11): 2861-2864.

83. Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, *et al.* Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. *J Clin Oncol* 2003 Jul 15; **21** (14): 2673-2678.

84. Gregory CW, Hamil KG, Kim D, Hall SH, Pretlow TG, Mohler JL, *et al.* Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res* 1998 Dec 15; **58** (24): 5718-5724.

85. Fujimoto N, Yeh S, Kang HY, Inui S, Chang HC, Mizokami A, *et al.* Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. *J Biol Chem* 1999 Mar 19; **274** (12): 8316-8321.

86. Kang HY, Yeh S, Fujimoto N, Chang C. Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J Biol Chem* 1999 Mar 26; **274** (13): 8570-8576.

87. Sadar MD, Hussain M, Bruchovsky N. Prostate cancer: molecular biology of early progression to androgen independence. *Endocr Relat Cancer* 1999 Dec; **6** (4): 487-502.

88. Sadar MD, Gleave ME. Ligand-independent activation of the androgen receptor by the differentiation agent butyrate in human prostate cancer cells. *Cancer Res* 2000 Oct 15; **60** (20): 5825-5831.

89. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, *et al.* Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res* 1994 Oct 15; **54** (20): 5474-5478.

90. Isaacs JT. The biology of hormone refractory prostate cancer. Why does it develop? *Urol Clin North Am* 1999 May; **26** (2): 263-273.

91. Craft N, Chhor C, Tran C, Belldegrun A, DeKernion J, Witte ON, *et al.* Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res* 1999 Oct 1; **59** (19): 5030-5036.

92. Tomlins SA, Rubin MA, Chinnaiyan AM. Integrative biology of prostate cancer progression. *Annu Rev Pathol* 2006; **1**: 243-271.

93. Bostwick DG, Shan A, Qian J, Darson M, Maihle NJ, Jenkins RB, *et al.* Independent origin of multiple foci of prostatic intraepithelial neoplasia: comparison with matched foci of prostate carcinoma. *Cancer* 1998 Nov 1; **83** (9): 1995-2002.

94. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 2002 May; **14** (5): 381-395.

95. Reddig PJ, Juliano RL. Clinging to life: cell to matrix adhesion and cell survival. *Cancer Metastasis Rev* 2005 Sep; **24** (3): 425-439.

96. Duronio V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. *Biochem J* 2008 Nov 1; **415** (3): 333-344.

97. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A* 1999 Apr 13; **96** (8): 4240-4245.

98. Dong JT. Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Metastasis Rev* 2001; **20** (3-4): 173-193.

99. McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res* 1999 Sep 1; **59** (17): 4291-4296.

100. Schmitz M, Grignard G, Margue C, Dippel W, Capesius C, Mossong J, *et al.* Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. *Int J Cancer* 2007 Mar 15; **120** (6): 1284-1292.

101. Sun M, Wang G, Paciga JE, Feldman RI, Yuan ZQ, Ma XL, *et al.* AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am J Pathol* 2001 Aug; **159** (2): 431-437.

102. Malik SN, Brattain M, Ghosh PM, Troyer DA, Prihoda T, Bedolla R, *et al.* Immunohistochemical demonstration of phospho-Akt in high Gleason grade prostate cancer. *Clin Cancer Res* 2002 Apr; **8** (4): 1168-1171.

103. Ghosh PM, Malik SN, Bedolla RG, Wang Y, Mikhailova M, Prihoda TJ, *et al.* Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation. *Endocr Relat Cancer* 2005 Mar; **12** (1): 119-134.

104. Kwabi-Addo B, Giri D, Schmidt K, Podsypanina K, Parsons R, Greenberg N, *et al.* Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression. *Proc Natl Acad Sci U S A* 2001 Sep 25; **98** (20): 11563-11568.

105. Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON. Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. *Proceedings of the National Academy of Sciences* 2006 May 16, 2006; **103** (20): 7789-7794.

106. Wang Y, Kreisberg JI, Ghosh PM. Cross-talk between the androgen receptor and the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer. *Curr Cancer Drug Targets* 2007 Sep; **7** (6): 591-604.

107. Lin HK, Hu YC, Yang L, Altuwaijri S, Chen YT, Kang HY, *et al.* Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *J Biol Chem* 2003 Dec 19; **278** (51): 50902-50907.

108. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proc Natl Acad Sci U S A* 2001 Jun 19; **98** (13): 7200-7205.

109. Taneja SS, Ha S, Swenson NK, Huang HY, Lee P, Melamed J, *et al.* Cell-specific regulation of androgen receptor phosphorylation in vivo. *J Biol Chem* 2005 Dec 9; **280** (49): 40916-40924.

110. Manin M, Baron S, Goossens K, Beaudoin C, Jean C, Veyssiere G, *et al.* Androgen receptor expression is regulated by the phosphoinositide 3-kinase/Akt pathway in normal and tumoral epithelial cells. *Biochem J* 2002 Sep 15; **366** (Pt 3): 729-736.

111. Sharma M, Chuang WW, Sun Z. Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3beta inhibition and nuclear beta-catenin accumulation. *J Biol Chem* 2002 Aug 23; **277** (34): 30935-30941.

112. Reddy GP, Barrack ER, Dou QP, Menon M, Pelley R, Sarkar FH, *et al.* Regulatory processes affecting androgen receptor expression, stability, and function: potential targets to treat hormone-refractory prostate cancer. *J Cell Biochem* 2006 Aug 15; **98** (6): 1408-1423.

113. Fu M, Rao M, Wu K, Wang C, Zhang X, Hessien M, *et al.* The androgen receptor acetylation site regulates cAMP and AKT but not ERK-induced activity. *J Biol Chem* 2004 Jul 9; **279** (28): 29436-29449.

114. Cinar B, Mukhopadhyay NK, Meng G, Freeman MR. Phosphoinositide 3-kinase-independent non-genomic signals transit from the androgen receptor to Akt1 in membrane raft microdomains. *J Biol Chem* 2007 Oct 5; **282** (40): 29584-29593.

115. Carson JP, Kulik G, Weber MJ. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B. *Cancer Res* 1999 Apr 1; **59** (7): 1449-1453.

116. Pfeil K, Eder IE, Putz T, Ramoner R, Culig Z, Ueberall F, *et al.* Long-term androgen-ablation causes increased resistance to PI3K/Akt pathway inhibition in prostate cancer cells. *Prostate* 2004 Feb 15; **58** (3): 259-268.

117. Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, *et al.* Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J Biol Chem* 2000 Aug 11; **275** (32): 24500-24505.

118. Murillo H, Huang H, Schmidt LJ, Smith DI, Tindall DJ. Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. *Endocrinology* 2001 Nov; **142** (11): 4795-4805.

119. Li P, Lee H, Guo S, Unterman TG, Jenster G, Bai W. AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. *Mol Cell Biol* 2003 Jan; **23** (1): 104-118.

120. Yang CC, Lin HP, Chen CS, Yang YT, Tseng PH, Rangnekar VM. Bcl-xL mediates a survival mechanism independent of the phosphoinositide 3-kinase/Akt pathway in prostate cancer cells. *J Biol Chem* 2003 Jul 11; **278** (28): 25872-25878.

121. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 2006 Oct 30; **25** (51): 6680-6684.

122. Gilmore TD. The Rel/NF-kappaB signal transduction pathway: introduction. *Oncogene* 1999 Nov 22; **18** (49): 6842-6844.

123. Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* 2006 Oct 30; **25** (51): 6706-6716.

124. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* 1996 Nov 1; **274** (5288): 782-784.

125. Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, *et al.* The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med* 1999 Jun 7; **189** (11): 1839-1845.

126. Li Q, Estepa G, Memet S, Israel A, Verma IM. Complete lack of NF-kappaB activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation. *Genes Dev* 2000 Jul 15; **14** (14): 1729-1733.

127. Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL, *et al.* Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. *Immunity* 1999 Apr; **10** (4): 421-429.

128. Rudolph D, Yeh WC, Wakeham A, Rudolph B, Nallainathan D, Potter J, *et al.* Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev* 2000 Apr 1; **14** (7): 854-862.

129. Gugasyan R, Christou A, O'Reilly LA, Strasser A, Gerondakis S. Bcl-2 transgene expression fails to prevent fatal hepatocyte apoptosis induced by endogenous TNFalpha in mice lacking RelA. *Cell Death Differ* 2006 Jul; **13** (7): 1235-1237.

130. Dutta J, Fan Y, Gupta N, Fan G, Gelinas C. Current insights into the regulation of programmed cell death by NF-kappaB. *Oncogene* 2006 Oct 30; **25** (51): 6800-6816.

131. Basseres DS, Baldwin AS. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene* 2006 Oct 30; **25** (51): 6817-6830.

132. Ismail HA, Lessard L, Mes-Masson AM, Saad F. Expression of NF-kappaB in prostate cancer lymph node metastases. *Prostate* 2004 Feb 15; **58** (3): 308-313.

133. Chen CD, Sawyers CL. NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. *Mol Cell Biol* 2002 Apr; **22** (8): 2862-2870.

134. Jin RJ, Lho Y, Connelly L, Wang Y, Yu X, Saint Jean L, *et al.* The nuclear factor-kappaB pathway controls the progression of prostate cancer to androgen-independent growth. *Cancer Res* 2008 Aug 15; **68** (16): 6762-6769.

135. Lessard L, Begin LR, Gleave ME, Mes-Masson AM, Saad F. Nuclear localisation of nuclear factor-kappaB transcription factors in prostate cancer: an immunohistochemical study. *Br J Cancer* 2005 Oct 31; **93** (9): 1019-1023.

136. Lessard L, Karakiewicz PI, Bellon-Gagnon P, Alam-Fahmy M, Ismail HA, Mes-Masson AM, *et al.* Nuclear localization of nuclear factor-kappaB p65 in primary prostate tumors is highly predictive of pelvic lymph node metastases. *Clin Cancer Res* 2006 Oct 1; **12** (19): 5741-5745.

137. Domingo-Domenech J, Mellado B, Ferrer B, Truan D, Codony-Servat J, Sauleda S, *et al.* Activation of nuclear factor-kappaB in human prostate carcinogenesis and association to biochemical relapse. *Br J Cancer* 2005 Nov 28; **93** (11): 1285-1294.

138. Domingo-Domenech J, Oliva C, Rovira A, Codony-Servat J, Bosch M, Filella X, *et al.* Interleukin 6, a nuclear factor-kappaB target, predicts resistance to docetaxel in hormone-independent prostate cancer and nuclear factor-kappaB inhibition by PS-1145 enhances docetaxel antitumor activity. *Clin Cancer Res* 2006 Sep 15; **12** (18): 5578-5586.

139. Setlur SR, Royce TE, Sboner A, Mosquera JM, Demichelis F, Hofer MD, *et al.* Integrative microarray analysis of pathways dysregulated in metastatic prostate cancer. *Cancer Res* 2007 Nov 1; **67** (21): 10296-10303.

140. Delfino FJ, Boustead JN, Fix C, Walker WH. NF-kappaB and TNF-alpha stimulate androgen receptor expression in Sertoli cells. *Molecular and Cellular Endocrinology* 2003 Mar 28; **201** (1-2): 1-12.

141. Zhang L, Charron M, Wright WW, Chatterjee B, Song CS, Roy AK, *et al.* Nuclear factor-kappaB activates transcription of the androgen receptor gene in Sertoli cells isolated from testes of adult rats. *Endocrinology* 2004 Feb; **145** (2): 781-789.

142. Bhuiyan MM, Li Y, Banerjee S, Ahmed F, Wang Z, Ali S, *et al.* Down-regulation of androgen receptor by 3,3'-diindolylmethane contributes to inhibition of cell proliferation and induction of apoptosis in both hormone-sensitive LNCaP and insensitive C4-2B prostate cancer cells. *Cancer Res* 2006 Oct 15; **66** (20): 10064-10072.

143. Supakar PC, Jung MH, Song CS, Chatterjee B, Roy AK. Nuclear factor kappa B functions as a negative regulator for the rat androgen receptor gene and NF-kappa B activity increases during the age-dependent desensitization of the liver. *J Biol Chem* 1995 Jan 13; **270** (2): 837-842.

144. Nakajima Y, DelliPizzi AM, Mallouh C, Ferreri NR. TNF-mediated cytotoxicity and resistance in human prostate cancer cell lines. *Prostate* 1996 Nov; **29** (5): 296-302.

145. Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gelinas C, *et al.* Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. *Prostate* 2002 Aug 1; **52** (3): 183-200.

146. Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. *Oncogene* 1999 Dec 2; **18** (51): 7389-7394.

147. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 1999 Sep 2; **401** (6748): 86-90.

148. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 1999 Sep 2; **401** (6748): 82-85.

149. Kikuchi E, Horiguchi Y, Nakashima J, Kuroda K, Oya M, Ohigashi T, *et al.* Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. *Cancer Res* 2003 Jan 1; **63** (1): 107-110.

150. Lee SO, Lou W, Nadiminty N, Lin X, Gao AC. Requirement for NF-(kappa)B in interleukin-4-induced androgen receptor activation in prostate cancer cells. *Prostate* 2005 Jul 1; **64** (2): 160-167.

151. Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, *et al.* TMPRSS2-ERG fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. *Oncogene* 2008 Sep 11; **27** (40): 5348-5353.

152. Carver BS, Tran J, Chen Z, Carracedo-Perez A, Alimonti A, Nardella C, *et al.* ETS rearrangements and prostate cancer initiation. *Nature* 2009 Feb 12; **457** (7231): E1; discussion E2-3.

153. Seth A, Watson DK. ETS transcription factors and their emerging roles in human cancer. *Eur J Cancer* 2005 Nov; **41** (16): 2462-2478.

154. Carver BS, Tran J, Gopalan A, Chen Z, Shaikh S, Carracedo A, *et al.* Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. *Nat Genet* 2009 May; **41** (5): 619-624.

155. King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, *et al.* Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. *Nat Genet* 2009 May; **41** (5): 524-526.

156. Hynes R. Integrins: Bidirectional, Allosteric Signaling Machines. *Cell* 2002; **110** (6): 673-678.

157. Calderwood DA, Shattil SJ, Ginsberg MH. Integrins and Actin Filaments: Reciprocal Regulation of Cell Adhesion and Signaling. *J Biol Chem* 2000 July 21, 2000; **275** (30): 22607-22610.

158. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 2001 Oct; **13** (5): 555-562.

159. Schoenwaelder SM, Burridge K. Bidirectional signaling between the cytoskeleton and integrins. *Curr Opin Cell Biol* 1999 Apr; **11** (2): 274-286.

160. Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol Biol Cell* 1995; **6** (10): 1349-1365.

161. Kuwada SK, Li X. Integrin $\alpha 5/\beta 1$ mediates fibronectin-dependent epithelial cell proliferation through epidermal growth factor receptor activation. *Mol Biol Cell* 2000; **11** (7): 2485-2496.

162. Bill HM, Knudsen B, Moores SL, Muthuswamy SK, Rao VR, Brugge JS, *et al.* Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells. *Mol Cell Biol* 2004 Oct; **24** (19): 8586-8599.

163. Danilkovitch-Miagkova A, Angeloni D, Skeel A, Donley S, Lerman M, Leonard EJ. Integrin-mediated RON growth factor receptor phosphorylation requires tyrosine kinase activity of both the receptor and c-Src. *J Biol Chem* 2000; **275** (20): 14783-14786.

164. Moro L, Venturino M, Bozzo C, Silengo L, Altruda F, Beguinot L, *et al.* Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *Embo J* 1998; **17** (22): 6622-6632.

165. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol* 1996; **135** (6 Pt 1): 1633-1642.

166. Wang R, Kobayashi R, Bishop JM. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. *Proc Natl Acad Sci USA* 1996; **93** (16): 8425-8430.

167. Marcoux N, Vuori K. EGF receptor mediates adhesion-dependent activation of the Rac GTPase: a role for phosphatidylinositol 3-kinase and Vav2. *Oncogene* 2003 Sep 4; **22** (38): 6100-6106.

168. Soldi R, Mitola S, Strasly M, Defilippi P, Tarone G, Bussolino F. Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. *Embro J* 1999; **18** (4): 882-892.

169. Moro L, Venturino M, Bozzo C, Silengo L, Altruda F, Beguinot L, *et al.* Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *Embro J* 1998; **17** (22): 6622-6632.

170. Sundberg C, Rubin K. Stimulation of beta1 integrins on fibroblasts induces PDGF independent tyrosine phosphorylation of PDGF beta-receptors. *J Cell Biol* 1996 February 1, 1996; **132** (4): 741-752.

171. Wang R, Ferrell LD, Faouzi S, Maher JJ, Bishop JM. Activation of the Met Receptor by Cell Attachment Induces and Sustains Hepatocellular Carcinomas in Transgenic Mice. *J Cell Biol* 2001 May 29, 2001; **153** (5): 1023-1034.

172. Miranti C, Brugge J. Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* 2002; **4** (4): 83-90.

173. Harnois C, Demers MJ, Bouchard V, Vallee K, Gagne D, Fujita N, *et al.* Human intestinal epithelial crypt cell survival and death: Complex modulations of Bcl-2 homologs by Fak, PI3-K/Akt-1, MEK/Erk, and p38 signaling pathways. *J Cell Physiol* 2004 Feb; **198** (2): 209-222.

174. Miyazaki T, Shen M, Fujikura D, Tosa N, Kim HR, Kon S, *et al.* Functional role of death-associated protein 3 (DAP3) in anoikis. *J Biol Chem* 2004 Oct 22; **279** (43): 44667-44672.

175. Lipscomb EA, Mercurio AM. Mobilization and activation of a signaling competent alpha6beta4integrin underlies its contribution to carcinoma progression. *Cancer Metastasis Rev* 2005 Sep; **24** (3): 413-423.

176. Friedland JC, Lakins JN, Kazanietz MG, Chernoff J, Boettiger D, Weaver VM. alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini. *J Cell Sci* 2007 Oct 15; **120** (Pt 20): 3700-3712.

177. Nikolopoulos SN, Blaikie P, Yoshioka T, Guo W, Puri C, Tacchetti C, *et al.* Targeted deletion of the integrin beta4 signaling domain suppresses laminin-5-dependent nuclear entry of mitogen-activated protein kinases and NF-kappaB, causing defects in epidermal growth and migration. *Mol Cell Biol* 2005 Jul; **25** (14): 6090-6102.

178. Novaro V, Roskelley CD, Bissell MJ. Collagen-IV and laminin-1 regulate estrogen receptor alpha expression and function in mouse mammary epithelial cells. *J Cell Sci* 2003 Jul 15; **116** (Pt 14): 2975-2986.

179. Zutter MM, Santoro SA, Painter AS, Tsung YL, Gafford A. The human alpha 2 integrin gene promoter. Identification of positive and negative regulatory elements important for cell-type and developmentally restricted gene expression. *J Biol Chem* 1994 January 7, 1994; **269** (1): 463-469.

180. Sisci D, Middea E, Morelli C, Lanzino M, Aquila S, Rizza P, *et al.* 17beta-Estradiol enhances alpha(5) integrin subunit gene expression through ERalpha-Sp1 interaction and reduces cell motility and invasion of ERalpha-positive breast cancer cells. *Breast Cancer Res Treat* 2010 Jan 6.

181. Lin VCL, Ng EH, Aw SE, Tan MGK, Ng EHL, Bay BH. Progesterone Induces Focal Adhesion in Breast Cancer Cells MDA-MB-231 Transfected with Progesterone Receptor Complementary DNA. *Mol Endocrinol* 2000 March 1, 2000; **14** (3): 348-358.

182. De Arcangelis A, Lefebvre O, Mechine-Neuville A, Arnold C, Klein A, Remy L, *et al.* Overexpression of laminin alpha1 chain in colonic cancer cells induces an increase in tumor growth. *Int J Cancer* 2001 Oct 1; **94** (1): 44-53.

183. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, *et al.* Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* 1997 Apr 7; **137** (1): 231-245.

184. Park CC, Zhang H, Pallavicini M, Gray JW, Baehner F, Park CJ, *et al.* Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. *Cancer Res* 2006 Feb 1; **66** (3): 1526-1535.

185. Slack-Davis J, Parsons T. Emerging views of integrin signaling: Implications for prostate cancer. *J Cell Biol* 2004; **91** (1): 41-46.

186. Watt F. Role of integrins in regulating epidermal adhesion, growth and differentiation. *Embo J* 2002; **21** (15): 3919-3926.

187. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, Miranti CK. Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death. *Mol Biol Cell* 2007 July 1, 2007; **18** (7): 2481-2490.

188. Ports MO, Nagle RB, Pond GD, Cress AE. Extracellular engagement of alpha6 integrin inhibited urokinase-type plasminogen activator-mediated cleavage and delayed human prostate bone metastasis. *Cancer Res* 2009 Jun 15; **69** (12): 5007-5014.

189. Bonkhoff H, Stein U, Remberger K. Differential expression of alpha 6 and alpha 2 very late antigen integrins in the normal, hyperplastic, and neoplastic prostate: simultaneous demonstration of cell surface receptors and their extracellular ligands. *Hum Pathol* 1993 Mar; **24** (3): 243-248.

190. Cress AE, Rabinovitz I, Zhu W, Nagle RB. The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. *Cancer Metastasis Rev* 1995 Sep; **14** (3): 219-228.

191. Davis TL, Cress AE, Dalkin BL, Nagle RB. Unique expression pattern of the alpha6beta4 integrin and laminin-5 in human prostate carcinoma. *Prostate* 2001 Feb 15; **46** (3): 240-248.

192. Goel HL, Alam N, Johnson IN, Languino LR. Integrin signaling aberrations in prostate cancer. *Am J Transl Res* 2009; **1** (3)**:** 211-220.

193. Nagle RB, Hao J, Knox JD, Dalkin BL, Clark V, Cress AE. Expression of hemidesmosomal and extracellular matrix proteins by normal and malignant human prostate tissue. *Am J Pathol* 1995 Jun; **146** (6)**:** 1498-1507.

194. Nagle RB, Knox JD, Wolf C, Bowden GT, Cress AE. Adhesion molecules, extracellular matrix, and proteases in prostate carcinoma. *J Cell Biochem Suppl* 1994; **19:** 232-237.

195. Pontes-Junior J, Reis ST, Dall'oglio M, Neves de Oliveira LC, Cury J, Carvalho PA, *et al.* Evaluation of the expression of integrins and cell adhesion molecules through tissue microarray in lymph node metastases of prostate cancer. *J Carcinog* 2009; **8:** 3.

196. Rabinovitz I, Nagle RB, Cress AE. Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype in vitro and in vivo. *Clin Exp Metastasis* 1995 Nov; **13** (6)**:** 481-491.

197. Witkowski CM, Rabinovitz I, Nagle RB, Affinito KS, Cress AE. Characterization of integrin subunits, cellular adhesion and tumorgenicity of four human prostate cell lines. *J Cancer Res Clin Oncol* 1993; **119** (11)**:** 637-644.

198. Vafa A, Zhang Y, Sikes RA, Marengo SR. Overexpression of p185erbB2/neu in the NbE prostatic epithelial cell line increases cellular spreading and the expression of integrin alpha6beta1. *Int J Oncol* 1998 Dec; **13** (6)**:** 1191-1197.

199. Marengo SR, Sikes RA, Anezinis P, Chang SM, Chung LW. Metastasis induced by overexpression of p185neu-T after orthotopic injection into a prostatic epithelial cell line (NbE). *Mol Carcinog* 1997 Jul; **19** (3)**:** 165-175.

200. Eeles RA, Kote-Jarai Z, Al Olama AA, Giles GG, Guy M, Severi G, *et al.* Identification of seven new prostate cancer susceptibility loci through a genome-wide association study. *Nat Genet* 2009 Oct; **41** (10)**:** 1116-1121.

201. Fornaro M, Steger CA, Bennett AM, Wu JJ, Languino LR. Differential role of beta(1C) and beta(1A) integrin cytoplasmic variants in modulating focal adhesion kinase, protein kinase B/AKT, and Ras/Mitogen-activated protein kinase pathways. *Mol Biol Cell* 2000 Jul; **11** (7)**:** 2235-2249.

202. Demetriou MC, Pennington ME, Nagle RB, Cress AE. Extracellular alpha 6 integrin cleavage by urokinase-type plasminogen activator in human prostate cancer. *Exp Cell Res* 2004 Apr 1; **294** (2): 550-558.

203. Knudsen B, Miranti C. The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. *J Cell Biochem* 2006; **99** (2): 345-361.

204. Fornaro M, Tallini G, Bofetiado CJ, Bosari S, Languino LR. Down-regulation of beta 1C integrin, an inhibitor of cell proliferation, in prostate carcinoma. *Am J Pathol* 1996 Sep; **149** (3): 765-773.

205. Perlino E, Lovecchio M, Vacca RA, Fornaro M, Moro L, Ditonno P, *et al.* Regulation of mRNA and protein levels of beta1 integrin variants in human prostate carcinoma. *Am J Pathol* 2000 Nov; **157** (5): 1727-1734.

206. Knox JD, Cress AE, Clark V, Manriquez L, Affinito KS, Dalkin BL, *et al.* Differential expression of extracellular matrix molecules and the alpha 6-integrins in the normal and neoplastic prostate. *Am J Pathol* 1994 Jul; **145** (1): 167-174.

207. Goel HL, Breen M, Zhang J, Das I, Aznavoorian-Cheshire S, Greenberg NM, *et al.* beta1A integrin expression is required for type 1 insulin-like growth factor receptor mitogenic and transforming activities and localization to focal contacts. *Cancer Res* 2005 Aug 1; **65** (15): 6692-6700.

208. Fuzio P, Lucarelli G, Perlino E, Battaglia M, Bettocchi C, Selvaggi FP, *et al.* Androgen deprivation therapy regulation of beta1C integrin expression in prostate cancer. *Oncol Rep* 2009 Aug; **22** (2): 327-335.

209. Hall CL, Dai J, van Golen KL, Keller ET, Long MW. Type I collagen receptor (alpha 2 beta 1) signaling promotes the growth of human prostate cancer cells within the bone. *Cancer Res* 2006 Sep 1; **66** (17): 8648-8654.

210. Zheng DQ, Woodard AS, Fornaro M, Tallini G, Languino LR. Prostatic carcinoma cell migration via alpha(v)beta3 integrin is modulated by a focal adhesion kinase pathway. *Cancer Res* 1999 Apr 1; **59** (7): 1655-1664.

211. Manes T, Zheng DQ, Tognin S, Woodard AS, Marchisio PC, Languino LR. Alpha(v)beta3 integrin expression up-regulates cdc2, which modulates cell migration. *J Cell Biol* 2003 May 26; **161** (4): 817-826.

212. Chatterjee S, Brite KH, Matsumura A. Induction of apoptosis of integrin-expressing human prostate cancer cells by cyclic Arg-Gly-Asp peptides. *Clin Cancer Res* 2001 Oct; **7** (10): 3006-3011.

213. Engl T, Relja B, Marian D, Blumenberg C, Muller I, Beecken WD, *et al.* CXCR4 chemokine receptor mediates prostate tumor cell adhesion through alpha5 and beta3 integrins. *Neoplasia* 2006 Apr; **8** (4): 290-301.

214. Bonaccorsi L, Carloni V, Muratori M, Salvadori A, Giannini A, Carini M, *et al.* Androgen Receptor Expression in Prostate Carcinoma Cells Suppresses {alpha}6{beta}4 Integrin-Mediated Invasive Phenotype. *Endocrinology* 2000 September 1, 2000; **141** (9): 3172-3182.

215. Evangelou A, Letarte M, Marks A, Brown TJ. Androgen Modulation of Adhesion and Antiadhesion Molecules in PC-3 Prostate Cancer Cells Expressing Androgen Receptor. *Endocrinology* 2002 October 1, 2002; **143** (10): 3897-3904.

216. Nagakawa O AT, Hayakawa Y, Junicho A, Koizumi K, Fujiuchi Y, Furuya Y, Matsuda T, Fuse H, Saiki I. Differential expression of integrin subunits in DU-145/AR prostate cancer cells. *Oncology Reports* 2004 May 28, 2004; **12:** 837-841.

217. Leroy BE, Northrup N. Prostate cancer in dogs: comparative and clinical aspects. *Vet J* 2009 May; **180** (2): 149-162.

218. Pienta KJ, Abate-Shen C, Agus DB, Attar RM, Chung LW, Greenberg NM, *et al.* The current state of preclinical prostate cancer animal models. *Prostate* 2008 May 1; **68** (6): 629-639.

219. Khanna C, Lindblad-Toh K, Vail D, London C, Bergman P, Barber L, *et al.* The dog as a cancer model. *Nat Biotechnol* 2006 Sep; **24** (9): 1065-1066.

220. Freitag T, Jerram RM, Walker AM, Warman CG. Surgical management of common canine prostatic conditions. *Compend Contin Educ Vet* 2007 Nov; **29** (11): 656-658, 660, 662-653 passim; quiz 673.

221. Bostwick DG. Personal Communication. 2009.

222. Isaacs JT, Isaacs WB, Feitz WF, Scheres J. Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers. *Prostate* 1986; **9** (3): 261-281.

223. Ahmad I, Sansom OJ, Leung HY. Advances in mouse models of prostate cancer. *Expert Rev Mol Med* 2008; **10:** e16.

224. Abdulkadir SA, Kim J. Genetically engineered murine models of prostate cancer: insights into mechanisms of tumorigenesis and potential utility. *Future Oncol* 2005 Jun; **1** (3): 351-360.

225. Banach-Petrosky W, Jessen WJ, Ouyang X, Gao H, Rao J, Quinn J, *et al.* Prolonged exposure to reduced levels of androgen accelerates prostate cancer progression in Nkx3.1; Pten mutant mice. *Cancer Res* 2007 Oct 1; **67** (19): 9089-9096.

226. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 2005 Aug 4; **436** (7051): 725-730.

227. Gao H, Ouyang X, Banach-Petrosky WA, Shen MM, Abate-Shen C. Emergence of androgen independence at early stages of prostate cancer progression in Nkx3.1; Pten mice. *Cancer Res* 2006 Aug 15; **66** (16): 7929-7933.

228. Gingrich JR, Barrios RJ, Foster BA, Greenberg NM. Pathologic progression of autochthonous prostate cancer in the TRAMP model. *Prostate Cancer Prostatic Dis* 1999 Mar; **2** (2): 70-75.

229. Gingrich JR, Barrios RJ, Morton RA, Boyce BF, DeMayo FJ, Finegold MJ, *et al.* Metastatic prostate cancer in a transgenic mouse. *Cancer Res* 1996 Sep 15; **56** (18): 4096-4102.

230. Kaplan-Lefko PJ, Chen TM, Ittmann MM, Barrios RJ, Ayala GE, Huss WJ, *et al.* Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. *Prostate* 2003 May 15; **55** (3): 219-237.

231. Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, *et al.* Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A* 1995 Apr 11; **92** (8): 3439-3443.

232. Cotsiki M, Lock RL, Cheng Y, Williams GL, Zhao J, Perera D, *et al.* Simian virus 40 large T antigen targets the spindle assembly checkpoint protein Bub1. *Proc Natl Acad Sci U S A* 2004 Jan 27; **101** (4): 947-952.

233. Sablina AA, Hahn WC. SV40 small T antigen and PP2A phosphatase in cell transformation. *Cancer Metastasis Rev* 2008 Jun; **27** (2): 137-146.

234. Yang CS, Vitto MJ, Busby SA, Garcia BA, Kesler CT, Gioeli D, *et al.* Simian virus 40 small t antigen mediates conformation-dependent transfer of protein phosphatase 2A onto the androgen receptor. *Mol Cell Biol* 2005 Feb; **25** (4): 1298-1308.

235. Dennis C. Cancer: off by a whisker. *Nature* 2006 Aug 17; **442** (7104): 739-741.

236. Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov* 2006 Sep; **5** (9): 741-754.

237. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* 2004 Aug; **3** (8): 711-715.

238. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, *et al.* TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004 Feb 6; **303** (5659): 848-851.

239. Bright RK, Vocke CD, Emmert-Buck MR, Duray PH, Solomon D, Fetsch P, *et al.* Generation and genetic characterization of immortal human prostate epithelial cell lines derived from primary cancer specimens. *Cancer Res* 1997 Mar 1; **57** (5): 995-1002.

240. Navone NM, Logothetis CJ, Eschenbach ACv, Troncoso P. Model Systems of Prostate Cancer: Uses and Limitations. *Cancer and Metastasis Reviews* 1999; **17:** 361-371.

241. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines--part 2. *J Urol* 2005 Feb; **173** (2): 360-372.

242. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines--part 1. *J Urol* 2005 Feb; **173** (2): 342-359.

243. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, *et al.* A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun* 1990 Dec 14; **173** (2): 534-540.

244. Culig Z, Hoffmann J, Erdel M, Eder IE, Hobisch A, Hittmair A, *et al.* Switch from antagonist to agonist of the androgen receptor bicalutamide is associated with prostate tumour progression in a new model system. *Br J Cancer* 1999 Sep; **81** (2): 242-251.

245. Festuccia C, Gravina GL, Angelucci A, Millimaggi D, Bologna M. Culture conditions modulate cell phenotype and cause selection of subpopulations in PC3 prostate cancer cell line. *Anticancer Res* 2000 Nov-Dec; **20** (6B): 4367-4371.

246. Buchanan G, Craft PS, Yang M, Cheong A, Prescott J, Jia L, *et al.* PC-3 cells with enhanced androgen receptor signaling: a model for clonal selection in prostate cancer. *Prostate* 2004 Sep 1; **60** (4): 352-366.

247. Edelstein RA, Carr MC, Caesar R, Young M, Atala A, Freeman MR. Detection of human androgen receptor mRNA expression abnormalities by competitive PCR. *DNA Cell Biol* 1994 Mar; **13** (3): 265-273.

248. Tilley WD, Bentel JM, Aspinall JO, Hall RE, Horsfall DJ. Evidence for a novel mechanism of androgen resistance in the human prostate cancer cell line, PC-3. *Steroids* 1995 Jan; **60** (1): 180-186.

249. Bello-DeOcampo D. Personal Communication. 2009.

250. Korenchuk S, Lehr JE, L MC, Lee YG, Whitney S, Vessella R, *et al.* VCaP, a cell-based model system of human prostate cancer. *In Vivo* 2001 Mar-Apr; **15** (2): 163-168.

251. Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu SM, *et al.* Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin Cancer Res* 1997 Dec; **3** (12 Pt 1): 2493-2500.

252. Sramkoski RM, Pretlow TG, 2nd, Giaconia JM, Pretlow TP, Schwartz S, Sy MS, *et al.* A new human prostate carcinoma cell line, 22Rv1. *In Vitro Cell Dev Biol Anim* 1999 Jul-Aug; **35** (7): 403-409.

253. Knox JD, Cress AE, Clark V, Manriquez L, Affinito KS, Dalkin BL, *et al.* Differential expression of extracellular matrix molecules and the α6-integrins in the normal and neoplastic prostate. *Am J Pathol* 1994 Jul; **145** (1): 167-174.

254. Yu HM, Frank DE, Zhang J, You X, Carter WG, Knudsen BS. Basal prostate epithelial cells stimulate the migration of prostate cancer cells. *Mol Carcinog* 2004 Oct; **41** (2): 85.

255. Gmyrek GA, Walburg M, Webb CP, Yu HM, You X, Vaughan ED, *et al.* Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. *Am J Pathol* 2001; **159** (2): 579-590.

CHAPTER TWO:
INTEGRIN-MEDIATED SIGNALING IN PROSTATE TUMOR CELLS

Portions of this chapter were published separately as:

Edick, M.J., Tesfay, L., Lamb, L.E., Knudsen, B.S., and Miranti, C.K., *Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/ERK or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death.* Mol. Biol. Cell, 2007. **18**(7): p. 2481-2490

INTRODUCTION

In vivo, the precise regulation of epithelial cell homeostasis involves interactions between cells and their microenvironment. Cells receive signals from both the extracellular matrix in the basement membrane and soluble factors secreted by the stroma that precisely control the timing of cell division, growth arrest, differentiation, and survival. Integrins on the cell surface that interact with laminin (LM) in the extracellular matrix, such as α3β1, α6β4, and α6β1, are critically involved in mediating survival. Genetic loss of LM5, or its receptor subunits, α3, α6 or β4 integrins, in vivo results in epithelial cell detachment and induction of caspase-mediated apoptosis, even in the presence of soluble factors (1, 2). Integrin β1 null embryonic stem cells produce less LM1 (3, 4), suggesting an important relationship between integrin β1 and LM1 specifically. This detachment induced form of apoptosis has been termed anoikis (5). In vitro anoikis can be rescued by expression of an activated form of FAK, Rac, or Akt (6-8), suggesting that integrin-mediated signaling through these molecules is required to maintain cell survival. However, studies in which specific signaling pathways are inhibited while integrins are still engaged suggest alternative pathways, such as Ras/ERK or Jnk, are required for integrin-mediated survival. Whether signaling from multiple pathways is involved in mediating integrin-dependent survival and whether different pathways are unique to specific cell types has not been extensively investigated.

Anoikis has been observed to result in caspase-mediated apoptosis (9). Disruption of the mitochondria, subsequent release of cytochrome c and

activation of caspases are hallmarks of classical apoptosis (10) and caspase 3 is required for mitochondria-regulated apoptosis (11). Members of the Bcl-2 protein family are important regulators of mitochondria integrity (10). In addition to classical caspase-mediated apoptosis, several other mechanisms of cell death have been described. Other forms of cell death include caspase-independent cell death, autophagy, or cornification. The role of integrins in regulating cell survival through suppression of these other death pathways is unknown. However, some of the same integrin-induced signal transduction pathways that have been linked to survival are also important for regulating these alternative cell death pathways. For example the Ras/ERK and PI3K pathways act as positive and negative regulators, respectively, of autophagy in several cell types (12). Additionally, epithelial cells have been shown to undergo death by cornification in response to inhibition of ERK and Jnk, but not PI3K (13). Finally, death induced by over expression of Ras, or suppression of Raf in melanoma cells leads to caspase-independent cell death (14, 15). Whether integrin-induced activation of specific signaling pathways plays a role in regulating any of these cell death mechanisms, has not been determined.

While studies with various established cell lines have been extremely useful for elucidating potential signaling pathways involved in integrin-mediated survival, it is important to place the findings in the context of a defined organ system where the specific cell type, the integrins expressed, and the matrix being studied are better defined.

Our work and that of others have demonstrated that integrin engagement is sufficient to activate receptor tyrosine kinases (16-23). We demonstrated that adhesion of normal epithelial cells to matrix is sufficient to induce activation of the epidermal growth factor receptor (EGFR), independently of ligand (18). In addition, we demonstrated that integrin-mediated activation of a subset of signaling pathways, namely the Ras/ERK and PI3K/Akt pathways, are dependent on integrin-induced EGFR activation. Src kinases have been implicated in regulating integrin-mediated activation of receptor tyrosine kinases (19, 24).

Our lab has additionally demonstrated that adhesion of growth factor starved normal primary prostate epithelial cells (PECs) to LM5-rich matrix mediates survival via integrin α3β1 through two independent signaling pathways; 1) integrin mediated activation of EGFR and subsequent signaling to ERK and 2) integrin-mediated activation of Src, the former being dependent on α3β1 integrin. Interestingly, there was no activation of the PI3K/Akt signaling pathway in PECs on LM5; consequently there was no dependence on this pathway for survival in normal PECs. Inhibition of EGFR/ERK or Src is sufficient to induce cell death, but this death is mediated through a caspase-independent mechanism that is dependent on the generation of reactive oxygen species.

During the course of these studies we also discovered that adhesion of PECs to LM5-rich matrix is required to maintain autophagy (refer to Chapter 1, Figure 2) (25). Signaling through α3β1, and to a lesser extent α6β4, is required for autophagy. Disruption of autophagy, pharmacologically or by blocking α3β1, leads to caspase activation and death.

Since our studies demonstrated the importance of these pathways in regulating integrin-mediated survival, we set out to use them as a framework to investigate the integrin-mediated survival pathways in the prostate tumor cell lines. Most prostate tumor studies have been done in three metastatic cell lines, PC3, DU145, and LNCaP due to the lack of a human primary prostate tumor cell line. LNCaP and PC3 cells both lack the PI3K/Akt inhibitor Pten; however, LNCaP cells retain wild-type p53 and have a mutated androgen receptor (AR), while PC3 cells have neither (26-28). DU145 cells also do not express p53 or AR, but still retain some Pten expression (26-28). During prostate cancer progression, laminin (LM) and collagen (CL) are the predominant matrices observed (29). However, how integrin-mediated adhesion can regulate survival in these cell lines and on these matrices has not yet been determined. We chose to investigate integrin-mediated signaling in the PC3 and DU145 cell lines because they express the proper integrins to adhere and bind to laminin and collagen matrix respectively, and represent two different oncogenic profiles. Although these cell lines do not express AR and the ultimate goal of the thesis was to determine how AR and integrins regulate survival in tumor cells, we first sought to establish what integrin signaling pathways regulated survival in these cells.

We hypothesized that the prostate tumor cell line PC3, which has lost expression of PTEN, would be dependent on PI3K signaling for survival. These cells have decreased expression of EGFR, so we hypothesized that EGFR signaling would not be required for cell survival, although signaling through Src

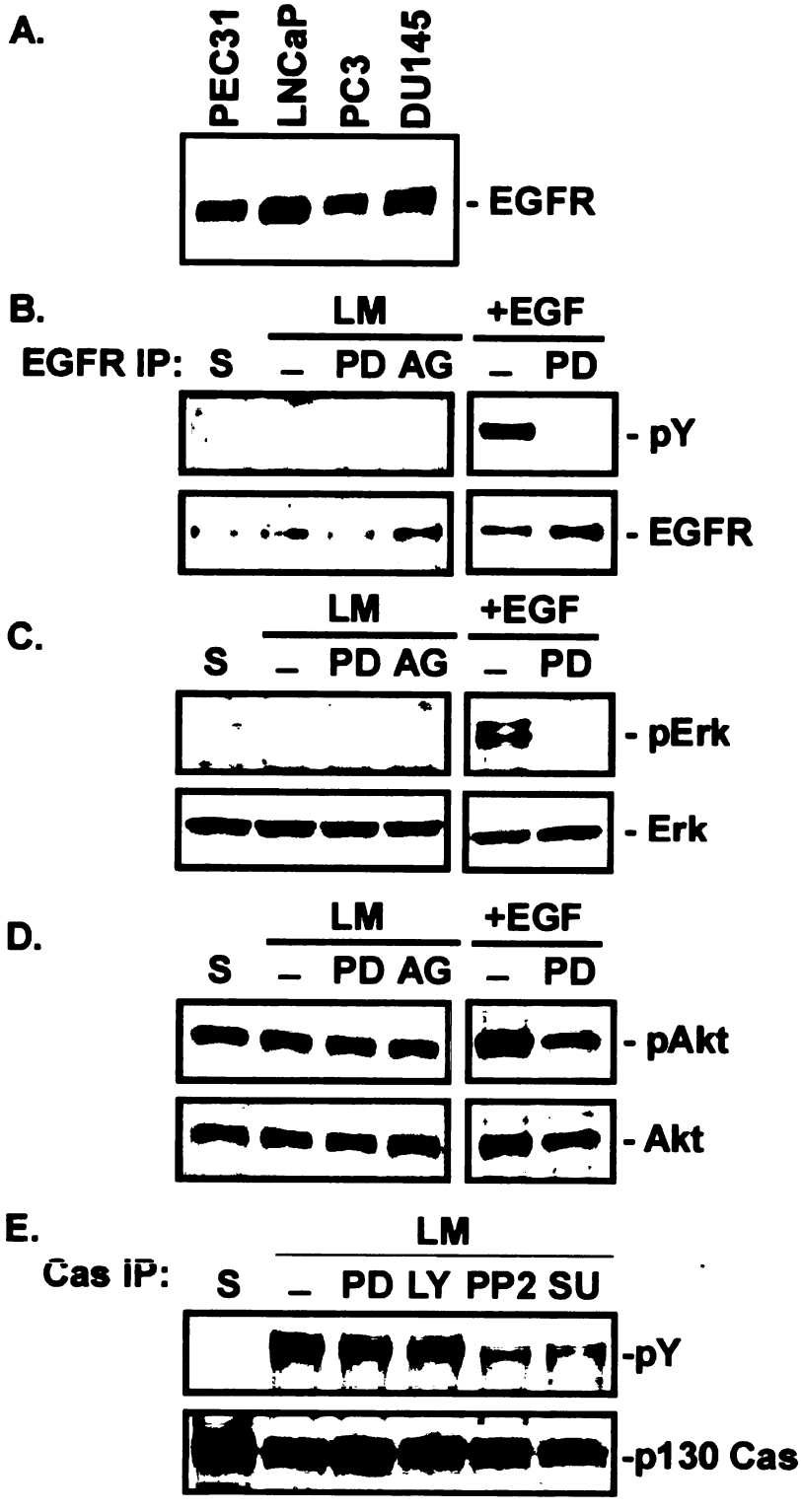
may still be important in promoting cell survival. In contrast the cell line DU145, which still express some Pten and EGFR, signaling via Ras/ERK could be mediated through integrin-dependent activation of EGFR. These cells may not be dependent on PI3K signaling since they still express Pten. To test these hypotheses, we assessed the ability of PC3 and DU145, adherent to either LM1 or CLI matrix respectively, to survive in the context of EGFR, PI3K, Src and downstream signaling inhibitors. These matrices where investigated given their importance in prostate cancer progression. Primary and lymph node metastatic prostate cancer predominately express laminin integrin α6β1 and it is hypothesized that prostate cancer escapes the gland in part by laminin-coated nerves (29-37). The primary metastatic site for prostate cancer is bone, where the microenvironment is rich in collagen, and to a lesser extent laminin (38, 39). Although PC3 cells can bind both matrices, we choose to investigate their adhesion to LM1 since this is the preferred substrate for integrin α6β1, which is critical in disease progression. Lastly, DU145 integrin-mediated adhesion was investigated on CL1, their preferential matrix.

RESULTS

In our studies we observed that the relative expression level of EGFR across prostate cancer cell lines and PECs varies (Figure 3A). Considering that EGFR expression level in PC3 cells is lower than the expression level in PECs (Figure 3A), we sought to determine if this prostate tumor cell line maintained EGFR-dependence for integrin mediated survival. We found that adhesion of

FIGURE 3. Integrin-mediated signaling on LM1 in PC3s. A) 50 μg of whole cell extracts from PEC31, LNCaP, PC3, and DU145 cells were analyzed for EGFR levels by immunoblotting. **B-E)** Growth factor-starved PC3s were placed in suspension (S) and treated with DMSO (-), 0.5 μM PD168393 (PD), 1 μM AG1478 (AG) prior to plating on LM1 (LM) for 1 hour. In some cases cells were also treated with 10 μM PP2 or 2 μM SU6656. **B)** EGFR was immunoprecipitated (IP) and levels of tyrosine phosphorylation were monitored by immunoblotting with anti-phosphotyrosine antibodies (pY). **C)** ERK and **D)** Akt activation were monitored by immunoblotting of whole cell extracts with the anti-ERK (T202/Y204) and anti- Akt (S473) phospho-specific antibodies (pERK Blot, pAkt Blot). Some cells were treated with 10 ng/ml EGF (+EGF) for 10 minutes. **E)** Src activity was monitored by immunoprecipitation (IP) of p130 Cas and levels of tyrosine phosphorylation were monitored by immunoblotting with anti-phosphotyrosine antibodies (pY). *(A-C) Dr. Cindy Miranti, (D,E) Laura Lamb.*

FIGURE 3


A.
PEC31
LNCaP
PC3
DU145
- EGFR
B.
LM
+EGF
EGFR IP: S – PD AG – PD
- pY
- EGFR
C.
LM
+EGF
S – PD AG – PD
- pErk
- Erk
D.
LM
+EGF
S – PD AG – PD
- pAkt
- Akt
E.
LM
Cas IP: S – PD LY PP2 SU
-pY
-p130 Cas

PC3 cells to laminin 5 (or laminin 1), does not induce the activation of EGFR or signal downstream to activate ERK (Figure 3 B,C; LM1 data not shown). Notably, stimulation of PC3s with EGF induces EGFR and ERK activation, both of which are inhibited by the EGFR inhibitor PD168393, demonstrating that EGFR is functional in these cells (Figure 3B,C). PC3 cells do not express the PI3K/Akt inhibitor Pten, and consequently Akt is activated independent of matrix in PC3 cells, and is not blocked when EGFR signaling is inhibited by PD168393 and AG1478 (Figure 3D). However, this pathway can be activated further by growth factor stimulation, as demonstrated by EGF stimulation (Figure 3D). Lastly, Src activity as measured by phosphorylation of the downstream effector p130 Cas, was also increased upon adhesion to LM1 (Figure 3E). Inhibition of EGFR or PI3K using pharmalogical inhibitors had no affect on p130 Cas phosphorylation, however the Src specific inhibitors PP2 and SU6656 could partially inhibit this pathway (Figure 3E).

To determine if EGFR, PI3K, and Src pathways were important in PC3 cell survival, these molecules were inhibited using the specific pharmacological inhibitors and cell death was assessed over a 124 hour time course, with maximal death occurring at 72 hours. EGFR is not activated by integrin-mediated adhesion to LM1 (Figure 3B), accordingly inhibition of EGFR does not induce significant cell death in PC3 cells as measured by Annexin V staining (Figure 4A). However, inhibition of the PI3K pathway with LY294002 induced cell death in LM-adherent PC3 cells (Figure 4A). The extent of cell death induced by inhibition of PI3K in PC3 cells was only 40% compared to 85% observed with

inhibition of EGFR in PECs suggesting that other mechanisms might be involved in regulating PC3 cell survival on matrix (25). We blocked Src signaling in PC3 cells with either SU6656 or PP2 and found that integrin-mediated survival in PC3 cells, like PECs, is also dependent on Src (Figure 4A). Inhibition of Src resulted in more extensive death than inhibition of PI3K, with >70% of the cells dying.

Loss of adhesion has been associated with a specialized form of caspase-dependent apoptosis known as anoikis. To determine if PC3s were dying via caspase-dependent apoptosis, we used an enzyme assay to directly measure caspase activity in dying PC3s. Induction of apoptosis in PC3 cells by treatment with LY294002 to inhibit PI3K activity, or SU6656 or PP2 to inhibit Src activity, induced a 2.5 fold increase in caspase activity (Figure 4B). This activity was inhibited by the caspase inhibitor zVAD. Thus, inhibition of either PI3K or Src, but not EGFR, results in caspase 3 activation and death.

Next, we wanted to determine what proteins downstream of Src and PI3K were directly regulating cell survival in the PC3 cells. Src has been reported to maintain the levels of the anti-apoptotic protein Bcl-xL (40). Inhibition of Src with SU6656 or PP2 in PC3 cells resulted in decreased expression of the anti-apoptotic protein Bcl-xL (Figure 4C). PI3K has been reported to promote survival through Akt-dependent phosphorylation and subsequent sequestration of the pro-death protein Bad on Ser136 (41), or by driving the expression of the anti-apoptotic protein Bcl-2 (42). PI3K inhibition decreased Akt-dependent phosphorylation on the pro-apoptotic protein Bad (Figure 4D). However inhibition of PI3K had no effect on Bcl-2 (data not shown) or Bcl-xL (Figure 4C). This is

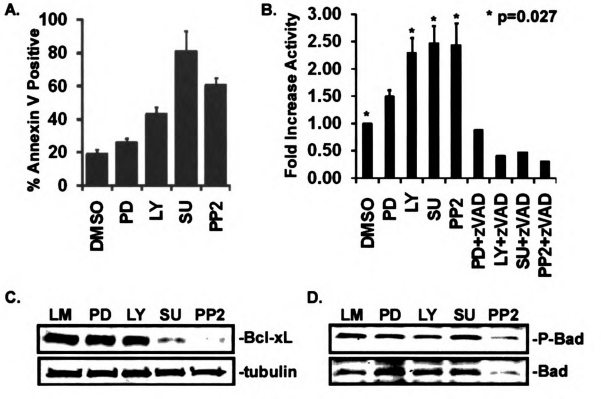


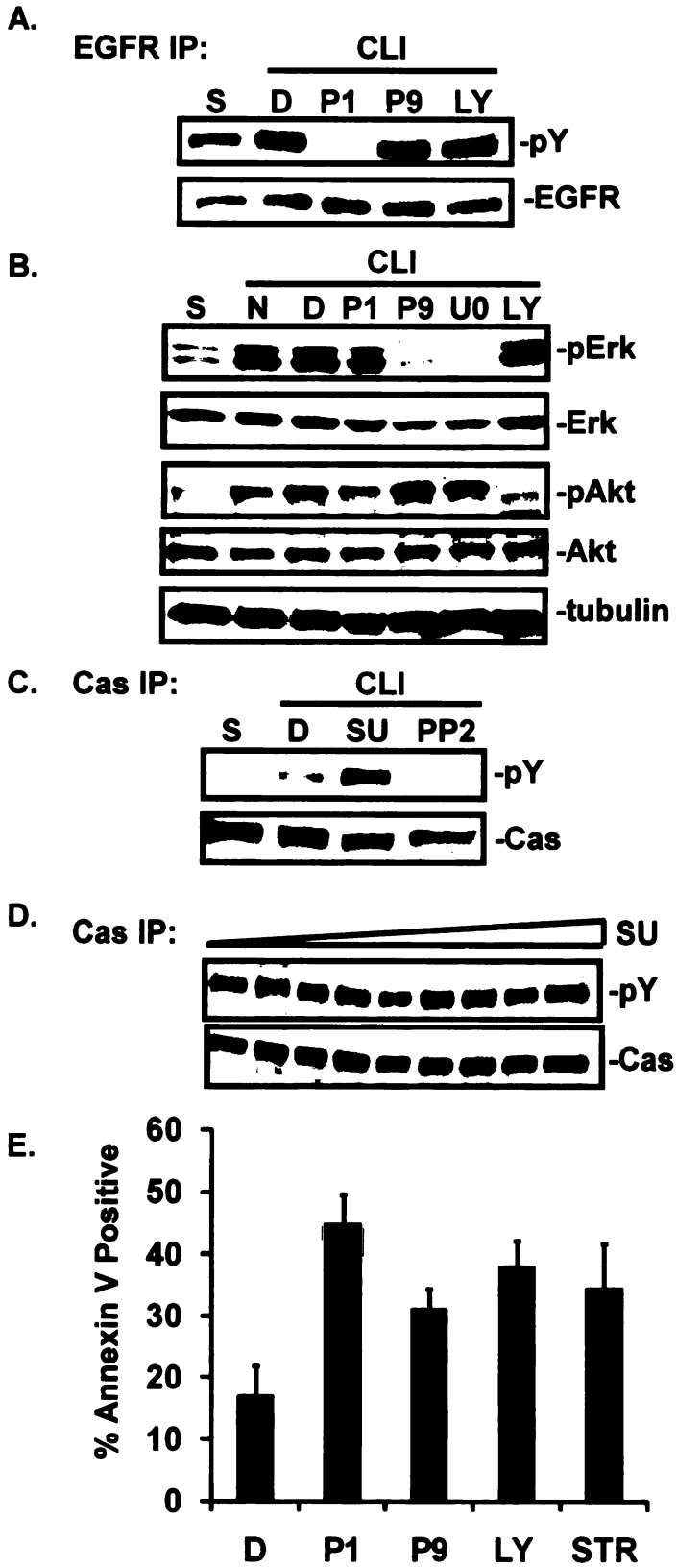
FIGURE 4. PC3 cells depend on PI3K and Src signaling for survival. PC3 cells were pretreated with DMSO (DMSO or LM), 0.5 μM PD168393 (PD), 10 μM LY294002 (LY), 2 μM SU6656 (SU), or 10 μM PP2 (PP2). In some cases 20 μM of the caspase inhibitor z-VAD was added at the time of plating as indicated (zVAD). Error bars on all graphs represent standard deviation. **A)** Cells were analyzed for percent Annexin V staining by FACS 72 hours after plating on laminin. $n = 4$. **B)** Cells were analyzed for caspase 3 activity. Caspase data are expressed as fold increase in caspase activity over that observed in DMSO-treated cells. $n = 3$. **C)** Bcl-xL expression was monitored by immunoblotting of whole cell extracts with the anti-Bcl-xL antibody. Tubulin was used as a loading control. **D)** Bad phosphorylation was monitored by immunoblotting of whole cell extracts with the anti-phospho-Ser136 (pBad Blot) Bad antibody. Total Bad expression was used as a loading control.

consistent with work done by Bondar and McConkey which also found that Bcl-2 does not regulate cell death in PC3 cells (43). Thus in PC3 cells, Src regulates survival through maintaining elevated levels of Bcl-xL and the PI3K/Akt pathway phosphorylates and inactivates Bad.

DU145 cells, another prostate tumor cell line, maintain EGFR expression comparable to PEC cells (Figure 3A). Furthermore, adhesion to CLI matrix induces activation of EGFR and treatment with the EGFR-specific inhibitors PD168393 or AG1478 (not shown) blocked integrin-induced EGFR tyrosine phosphorylation (Figure 5A). Adhesion to CLI also resulted in increased ERK activity; this appeared to be mostly independent of EGFR in that treatment of cells with EGFR inhibitor PD168393 only partially blocked ERK activation (Figure 5B). Inhibition of ERK directly with PD98059 or U0126 completely blocked ERK activation (Figure 5B). The PI3K pathway was also activated upon integrin engagement of matrix (Figure 5B). This may be partially dependent on EGFR in that the EGFR inhibitor PD198393 partially blocked Akt phosphorylation (Figure 5B). Src signaling was also activated upon integrin engagement of matrix as measured by phosphorylation of the downstream protein p130Cas, however the pharmalogical inhibitor for Src SU6656 was unable to effectively inhibit Src activity at a variety of concentrations tested (Figure 5C,D). The Src inhibitor PP2 le for Bcl-xL in prostate cells. Previous independent studies have shown thaget effects at the concentrations effective in this assay (Figure 5C). This data suggested that all four pathways, PI3K, EGFR, ERK, and Src, may be important for integrin-mediated survival since they are activated upon adhesion to matrix.

FIGURE 5. Integrin-mediated Signaling in DU145 Prostate Tumor Cells. DU145 cells were starved of growth factors for 48 hours, treated with nothing (N), DMSO vehicle (D), 0.5 μM PD168393 (PD1), 10 μM PD98059 (PD9) or U0126 (U0), 10 μM LY494002 (LY), or 2 μM SU6656 (SU) or 10 μM PP2 (PP2) and plated on CLI. Cells in suspension (S) were used as a negative control for integrin engagement. **A)** Activation of EGFR was monitored by immunoprecipitation of EGFR and immunoblotting of tyrosine phosphorylation levels (pY Blot). **B)** ERK activation was monitored by immunoblotting total cell lysates with anti-ERK phospho-specific antibody (pERK Blot). Activation of PI3K was monitored by immunoblotting total cell lysates with anti-Akt phospho-specific antibody (pAkt Blot). **C-D)** Activation of Src was monitored by immunoprecipitation of Cas and immunoblotting of phosphorylation levels (pY Blot). **D)** DU145 cells were treated with increasing concentrations of SU6656 (SU), ranging from 2 to 200 μM. **E)** After 72 hours, cells were harvested and stained with fluorescence conjugated Annexin V and P.I. and analyzed by FACS.

FIGURE 5


A.
EGFR IP:
CLI
S D P1 P9 LY
-pY
-EGFR
B.
CLI
S N D P1 P9 U0 LY
-pErk
-Erk
-pAkt
-Akt
-tubulin
C. Cas IP:
CLI
S D SU PP2
-pY
-Cas
D. Cas IP:
SU
-pY
-Cas
E.
% Annexin V Positive
60
50
40
30
20
10
0
D P1 P9 LY STR

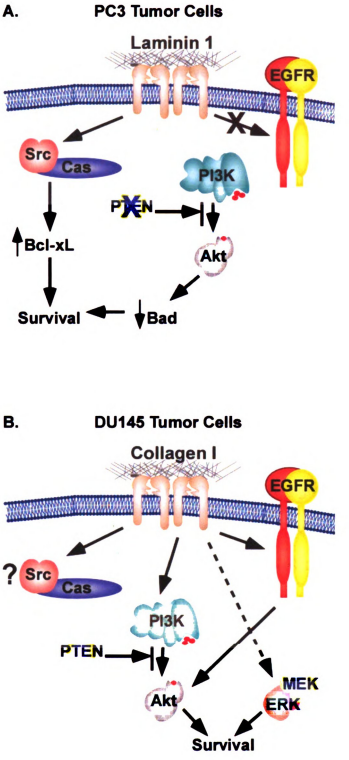
To determine if integrin regulation of the EGFR, ERK, and PI3K pathways were important for DU145 cell survival, these molecules were inhibited using specific pharmacological inhibitors and apoptosis was then measured using Annexin V and propidium iodide (P.I.) staining with FACS analysis. Staurosporin (STR) was used as a positive control for inducing cell death. Treatment of DU145 cells with PD198393, PD98059, or LY294002 for 72 hours after plating induced cell morphologies suggestive of apoptosis; notably cell condensation and membrane blebbing (data not shown). A 2.6 fold increase in Annexin V positivity was induced in the DU145 cells were treated with the EGFR inhibitor, the ERK inhibitor PD98059 induced a 1.8 fold increase, and the PI3K inhibitor induced a 2.2 fold increase in cell death (Figure 5E). Thus, the EGFR, ERK, and PI3K pathways are important in integrin-mediated survival of DU145 cells.

DISCUSSION

Using two different prostate tumor cell lines, PC3 and DU145, we have identified integrin-mediated signaling pathways whereby adhesion to matrix mediates cell survival (Figure 6). In PC3 cells, survival occurs through PI3K and integrin-mediated activation of Src, but not EGFR. Src regulates survival through maintaining elevated levels of Bcl-xL and the PI3K/Akt pathway phosphorylates and inactivates Bad. Inhibition of either pathway results in caspase-dependent cell death (summarized in Figure 6A). In contrast, in DU145 cells, cell survival occurs through integrin-mediated activation of EGFR, ERK, and PI3K

FIGURE 6. Models for PC3 and DU145 integrin-mediated survival. A) Survival of PC3 cells adherent to laminin 1 requires PI3K signaling and integrin-dependent activation of Src, but not EGFR. Src regulates survival through the pro-survival protein, Bcl-xL and the PI3K pathway phosphorylates and inactivates the pro-death protein Bad. Inhibition of either pathway results in caspase-mediated cell death. **B)** Survival of DU145 cells adherent to collagen I requires integrin-dependent activation of PI3K, ERK, and EGFR. PI3K activates Akt, while EGFR leads to partial activation of Akt. Integrin adhesion also activates Src.

FIGURE 6


A.
PC3 Tumor Cells
Laminin 1
EGFR
Src
Cas
PI3K
PTEN
Bcl-xL
Akt
Survival
Bad
B.
DU145 Tumor Cells
Collagen I
EGFR
?
Src
Cas
PI3K
PTEN
MEK
Akt
ERK
Survival

(summarized in Figure 6B). Src signaling is also increased upon adhesion to matrix, however we were unable to determine if it is important in promoting cell survival since the Src specific pharmalogical inhibitors were unable to effectively inhibit Src activity at doses that did not have off-target effects. Future studies using Src specific siRNA need to be done to determine the requirement of Src in survival of DU145 cells.

Integrin-mediated transactivation of receptor tyrosine kinases has been widely reported; however, the biological significance of this crosstalk is largely unknown. In this study we demonstrate that EGFR is a critical pathway for integrin-mediated survival in DU145s, but not PC3s. Interestingly, normal PEC cells also are dependent on EGFR for survival. DU145s are not as invasive and metastatic as PC3 cells, perhaps representing an intermediate phenotype between PEC and PC3 cells, explaining why they may have retained EGFR-dependence. While ERK signaling was also increased upon adhesion in DU145 cells similar to PECs, this was independent of EGFR signaling. However, in DU145 cells, EGFR activation may partially regulate the PI3K/Akt pathway. Similar to this, it has been reported that survival of EGFR over expressing NIH-3T3 cells on fibronectin required integrin-mediated activation of EGFR, but did not involve signaling to ERK, but rather PI3K (24). Like PECs, integrin-mediated survival of primary keratinocytes on LM5 has been shown to involve EGFR signaling to ERK (44), suggesting that in normal cells integrin activation of EGFR and subsequently ERK is important for cells survival, and that this pathway becomes aberrant during cancer progression. Thus it is interesting to speculate

that PC3 cells may have lost the requirement for EGFR for survival by becoming dependent on PI3K signaling due to Pten loss, circumventing the requirement for integrin-mediated activation. However, given that the integrin-mediated survival pathways in PC3 and DU145 cells were studied on different matrixes which engage different integrins, this could also be an explanation as to the differences in signaling observed between the two cell lines. Indeed, in short term 1 hour adhesion assays we have observed that adhesion of PECs to laminin 1, but not the laminin 5-rich matrix, is sufficient to activate Akt (data not shown), suggesting alternative signaling pathways are activated on other matrices.

Interestingly, both PC3 and DU145 cells were dependent on PI3K signaling for cell survival. LNCaP cells, which like PC3 cells also lack Pten, have also been reported to be dependent on PI3K signaling for survival (45); inhibition of PI3K signaling in LNCaPs results in caspase-dependent cell death (data not shown). However, normal PEC cells are not dependent on PI3K for survival (Figure 2) (25). Many human prostate cancers have reduced levels of the negative PI3K regulator, Pten, and the PI3K/Akt pathway is constitutively activated in those tumors (46-48). However, given that the DU145 cells still express Pten, this suggests that prostate tumor cells can still require this pathway for survival independent of Pten status. This suggests that the PI3K/Akt pathway may be favored in tumor development or progression and may provide a therapeutic target that would be non-toxic to normal prostate basal epithelial cells. However, this requires deeper investigation.

Whether integrin-mediated activation of other receptor tyrosine kinases is involved in regulating survival on matrix is not known. However, it was recently demonstrated that ligand independent activation of c-Met in PC3 cells was required for cell survival (49). The specific integrins, matrix, and signaling pathways involved in c-Met-mediated survival are currently unknown. Recent work from our lab has demonstrated that in PECs c-Met regulates survival by stabilizing α3β1 integrin via a kinase-independent mechanism (Lia Tesfay, unpublished data). α3β1 loss via inhibition of c-Met expression blocks integrin-dependent signaling, which compromises cell viability (Lia Tesfay, unpublished data). Our data indicate that PC3 cells, which express low levels of EGFR relative to PECs, do not activate EGFR or ERK upon integrin engagement and do not depend on this pathway for integrin-mediated survival. Instead survival of PC3 cells requires PI3K and Src. c-Met is known to activate these signaling pathways in response to HGF, but whether c-Met participates in integrin-mediated signaling to PI3K or Src in PC3 cells has not been determined.

Despite extensive characterization of integrin-mediated signaling molecules involved in regulating cell survival on matrix, including FAK, ILK, Src, p130Cas, Akt, ERK, Jnk, and Rac (6-8, 44, 50-54), little is known about how these molecules link up with the cell death pathways. In PC3 cells we found that loss of signaling through Src results in decreased levels of Bcl-xL and loss of PI3K signaling results in decreased Bad phosphorylation. Early anoikis studies suggested that Bcl-2 was involved in regulating cell survival, because its over expression was sufficient to rescue cell death induced by loss of adhesion (50)

and adhesion to matrix in some cells increases Bcl-2 levels (55). However, Bcl-2 expression does not correlate with sensitivity to anoikis in prostate cells (43). More recent studies have linked integrin-mediated survival to Bcl-xL (56). Furthermore, loss of androgen receptor expression in LNCaP cells leads to cell death which is accompanied by a reduction in Bcl-xL expression (57), suggesting an important role for Bcl-xL in prostate cells. Previous independent studies have shown that Bcl-xL can be regulated by signaling through EGFR, integrins, or Src (40, 56, 58). Here we demonstrate that in PC3 cells, Src but not EGFR, regulates Bcl-xL.

In summary, in the prostate tumor cell line PC3 survival on LM1 occurs through PI3K and integrin-mediated activation of Src, but not EGFR. Src regulates survival through maintaining elevated levels of the pro-survival protein Bcl-xL and the PI3K/Akt pathway phosphorylates and inactivates the pro-apoptotic protein Bad. Inhibition of either pathway results in caspase-dependent cell death. In comparison in DU145 cells on CLI, integrin-mediated activation of EGFR, ERK, and PI3K promotes cell survival.

MATERIALS AND METHODS

Antibodies

EGFR immunoprecipitating and blocking monoclonal antibodies were purified in the Monoclonal Antibody Core at VARI from hybridoma cells obtained from ATCC (HB-8508). EGFR (Ab12) immunoblotting antibodies were purchased from NeoMarkers. ERK and p130Cas antibodies were purchased from Becton-

Dickinson Transduction Labs. Phosphospecific antibodies against ERK1/2 (T202/Y204), Akt (S473), Bad (S136) and antibodies to Bcl-2, Bcl-xL, and Bad were purchased from Cell Signaling. The anti-phosphotyrosine monoclonal antibody 4G10 was obtained from Upstate Biotechnology. The Akt antibody was described previously (18). Blocking antibodies for β4 integrin (ASC-8) and α3 integrin (P1B5) were purchased from Chemicon and GoH3 α6 integrin antibody was obtained from Becton Dickinson.

Cell Culture

Primary cultures of human prostate epithelial cells (PEC) were derived from normal human prostatic tissue and cultured as described previously (59). Human samples were obtained after institutional IRB approval. PECs were maintained in Keratinocyte-SFM medium (Invitrogen) supplemented with bovine pituitary extract and EGF. All experiments were conducted on cells between passages 3 and 5. PC3 and DU145 cells were obtained from American Type Culture Collection (ATCC). PC3 cells were maintained in F12K medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U of penicillin and 50 mg of streptomycin per ml. DU145 cells were maintained in Eagle's Minimum Essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 2mM glutamine, 50U of penicillin and 50mg of streptomycin/ml.

Integrin Signaling

Preparation of cells for adhesion to extracellular matrices was carried out as described previously (25, 60). Briefly, cells were growth factor-starved for 48 hours, trypsinized, treated with soybean trypsin inhibitor (Invitrogen), washed in PBS, and placed in suspension in growth factor-free medium for 30-60 minutes. Cells were then either plated on tissue culture plates blocked with 1% BSA (Sigma) and pre-coated 10 μg/mL natural mouse laminin 1 (Invitrogen) or rat tail collagen 1 (Becton, Dickinson and Company). Similar results were obtained in PC3 cells on laminin 1 as on laminin 5. Occasionally cells were also treated with 2-10 ng/ml EGF (Upstate). A suspension control was maintained at 37°C. Two hours after plating on matrix cells were lysed either in Triton-X (50 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1% TritonX-100, 50 mM NaF, 50 mM β-glycerophosphate, 5mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM PMSF, 100 U/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) or RIPA (10 mM Tris pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NaDOC, 1% Triton-X100, 1 mM Na3VO4, 1 mM PMSF, 100U ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) buffers. Pharmacological inhibitors, PD168393, AG1478, LY294002, SU6656, or PP2, purchased from Calbiochem, were added to suspension cells 20 minutes prior to plating on matrix; except for SU6656, which was added 16 hours prior to placing cells in suspension. All working concentrations of the pharmacological inhibitors were determined by titrating to the minimum inhibitor concentration that effectively blocked the target of the pharmacological inhibitor for the duration of our experiments. Inhibitor

effectiveness was monitored by Western blotting. Specifically, PD168393 and AG1478 were tested for their ability to inhibit EGFR tyrosine phosphorylation, p130Cas tyrosine phosphorylation (a Src substrate) was used to test SU6656 and PP2, phosphorylation of Akt was used for LY294002, and U0126 was tested against phosphorylated ERK. Titrations were performed for each drug in each cell type.

Cell Survival Assays

Cells were serum starved for 48 hours and placed in suspension as described above and then plated on 1% BSA-blocked tissue culture plates pre-coated 10 μg/mL natural mouse laminin 1 or rat tail collagen 1. Pharmacological inhibitors, 1 μM staurosporine (Promega), 0.5 μM PD168393, 1 μM AG1478, 10 μM PD9809, 10 μM U0126, 10 μM LY294002, 0.5-2 μM SU6656, or 10 μM PP2 were then added. Cells were allowed to adhere for 4 hours and then non-adherent cells were removed and drugs were replaced. Cells were incubated for an additional 72 hours. LY294002 was replenished 48 hours after plating. To assess cell death, cells were stained with Annexin V and propidium iodide using a kit obtained from Molecular Probes (Invitrogen). Staining was carried out according the supplied protocols. For all staining procedures both attached and floating cells were collected. Attached cells were removed by trypsinization and pooled with floating cells and all cells were washed one time. For Annexin V staining, cells were resuspended in Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl, pH 7.4) containing Alexafluor488 conjugated Annexin V

and incubated in the dark for 15 minutes. Cells were then washed once with Annexin binding buffer, then incubated in 1 μg/mL propidium iodide and 100 μg/mL RNase A for 5 minutes at room temperature, and washed twice more. Samples were put on ice and immediately analyzed. Extent of staining was monitored by FACS using a FACS Caliber (Becton-Dickinson) and CellQuest version 3.1.3 acquisition and analysis software (Becton-Dickinson).

Caspase Activity Assays

Caspase 3 and 7 activity in PC3 cells was directly measured using a CaspaseGlo 3/7 kit (Promega) following the manufacturers suggested protocol. For PC3 cells, 10,000 cells/well were plated on 1% BSA blocked 96 well plates pre-coated with 10ug/ml of laminin respectively. Cells were plated in the presence of DMSO, 1 μM staurosporine, 0.5 μM PD168393, 10 μM LY294002, 2 μM SU6656, 10 μM PP2. CaspaseGlo reagent was added at various times after inhibitor treatment and incubated for 1 hour at room temperature in the dark. Relative light intensity was measured in each well using a Fluoroskan Assent FL fluorometer and software (Labsystems).

Immunoprecipitation and Immunoblotting

Immunoprecipitation mixtures containing 500-1000ug protein were incubated with the appropriate antibodies for 3 hours at 4°C with either protein A- or protein G-conjugated agarose beads (Pierce) to capture the complexes. All immunoprecipitated complexes were washed three times with their respective

lysis buffer. Immunoprecipitated samples from adhesion assays were resuspended in 2X SDS sample buffer. In some cases 50-75 μg of total cell lysates were placed directly in 2x SDS sample buffer. All resuspended samples were boiled and subjected to SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (PVDF). The PVDF membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 hours, followed by 2 hour incubation with the appropriate primary antibodies in 5% BSA/TBST. After several washes, blots were incubated with a horseradish peroxidase conjugated secondary antibody (Bio-Rad) for 1 hour in 5% BSA/TBST and visualized with a chemiluminescence reagent and captured by a CCD camera in a Bio-Rad Chemi-Doc Imaging System. Levels of activation, relative to total levels of protein, from blots captured by CCD camera were quantified using Quantity One software (Bio-Rad). Blots were stripped in low-pH 2% SDS at 65°C for 60 minutes, rinsed and reprobed for total levels of protein in the immunoprecipitates or cell lysates.

ACKNOWLEDGEMENTS

We wish to thank Mat Edick, Veronique Schulz, Matt Van Brocklin, Dr. Kate Eisenmann, and the FACS core at VARI for technical assistance. Special thanks to the laboratories of Developmental Cell Biology, Systems Biology, and Cell Structure and Signal Integration at VARI for their constructive suggestions. Dr. Miranti, Dr. Edick, Ms. Tesfay, and Ms. Lamb are supported by the American Cancer Society (RSG CSM-109378). Dr. Miranti is also supported by the

Department of Defense Prostate Cancer Research Program of the Office of Congressionally Directed Medical Research Programs (W81XWH-04-1-0044). Additional support was also provided by the generous gifts of the Van Andel Institute.

REFERENCES

1. DiPersio CM, van Der Neut R, Georges-Labouesse E, Kreidberg JA, Sonnenberg A, Hynes RO. $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrin receptors for laminin-5 are not essential for epidermal morphogenesis and homeostasis during skin development. *J Cell Sci* 2000; **113** (Pt 17): 3051-3062.

2. Ryan MC, Lee K, Miyashita Y, Carter WG. Targeted disruption of the LAMA3 gene in mice reveals abnormalities in survival and late stage differentiation of epithelial cells. *J Cell Biol* 1999 Jun 14; **145** (6): 1309-1323.

3. Sasaki T, Forsberg E, Bloch W, Addicks K, Fassler R, Timpl R. Deficiency of beta 1 integrins in teratoma interferes with basement membrane assembly and laminin-1 expression. *Exp Cell Res* 1998 Jan 10; **238** (1): 70-81.

4. Aumailley M, Pesch M, Tunggal L, Gaill F, Fassler R. Altered synthesis of laminin 1 and absence of basement membrane component deposition in (beta)1 integrin-deficient embryoid bodies. *J Cell Sci* 2000 Jan; **113 Pt 2:** 259-268.

5. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 2001 Oct; **13** (5): 555-562.

6. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 1996; **134** (3): 793-799.

7. Coniglio SJ, Jou TS, Symons M. Rac1 protects epithelial cells against anoikis. *J Biol Chem* 2001 Jul 27; **276** (30): 28113-28120.

8. Rytomaa M, Lehmann K, Downward J. Matrix detachment induces caspase-dependent cytochrome c release from mitochondria: inhibition by PKB/Akt but not Raf signalling. *Oncogene* 2000 Sep 14; **19** (39): 4461-4468.

9. Melino G, Knight RA, Nicotera P. How many ways to die? How many different models of cell death? *Cell Death Differ* 2005 Nov; **12 Suppl 2:** 1457-1462.

10. Martin S, Vuori K. Regulation of Bcl-2 proteins during anoikis and amorphosis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2004; **1692** (2-3): 145-157.

11. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999 Feb; **6** (2): 99-104.

12. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005 Sep; **5** (9): 726-734.

13. Uzgare AR, Isaacs JT. Enhanced redundancy in Akt and mitogen-activated protein kinase-induced survival of malignant versus normal prostate epithelial cells. *Cancer Res* 2004 Sep 1; **64** (17): 6190-6199.

14. Panka DJ, Wang W, Atkins MB, Mier JW. The Raf inhibitor BAY 43-9006 (Sorafenib) induces caspase-independent apoptosis in melanoma cells. *Cancer Res* 2006 Feb 1; **66** (3): 1611-1619.

15. Chi S, Kitanaka C, Noguchi K, Mochizuki T, Nagashima Y, Shirouzu M, *et al.* Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene* 1999 Apr 1; **18** (13): 2281-2290.

16. Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol Biol Cell* 1995; **6** (10): 1349-1365.

17. Kuwada SK, Li X. Integrin $\alpha 5/\beta 1$ mediates fibronectin-dependent epithelial cell proliferation through epidermal growth factor receptor activation. *Mol Biol Cell* 2000; **11** (7): 2485-2496.

18. Bill HM, Knudsen B, Moores SL, Muthuswamy SK, Rao VR, Brugge JS, *et al.* Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells. *Mol Cell Biol* 2004 Oct; **24** (19): 8586-8599.

19. Danilkovitch-Miagkova A, Angeloni D, Skeel A, Donley S, Lerman M, Leonard EJ. Integrin-mediated RON growth factor receptor phosphorylation requires tyrosine kinase activity of both the receptor and c-Src. *J Biol Chem* 2000; **275** (20): 14783-14786.

20. Moro L, Venturino M, Bozzo C, Silengo L, Altruda F, Beguinot L, *et al.* Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *Embo J* 1998; **17** (22): 6622-6632.

21. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol* 1996; **135** (6 Pt 1): 1633-1642.

22. Wang R, Kobayashi R, Bishop JM. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. *Proc Natl Acad Sci USA* 1996; **93** (16): 8425-8430.

23. Marcoux N, Vuori K. EGF receptor mediates adhesion-dependent activation of the Rac GTPase: a role for phosphatidylinositol 3-kinase and Vav2. *Oncogene* 2003 Sep 4; **22** (38): 6100-6106.

24. Moro L, Venturino M, Bozzo C, Silengo L, Altruda F, Beguinot L, *et al.* Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *Embro J* 1998; **17** (22): 6622-6632.

25. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, Miranti CK. Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death. *Mol Biol Cell* 2007 July 1, 2007; **18** (7): 2481-2490.

26. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines--part 1. *J Urol* 2005 Feb; **173** (2): 342-359.

27. Bastola DR, Pahwa GS, Lin MF, Cheng PW. Downregulation of PTEN/MMAC/TEP1 expression in human prostate cancer cell line DU145 by growth stimuli. *Mol Cell Biochem* 2002 Jul; **236** (1-2): 75-81.

28. Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. *Cancer Res* 1991 Sep 1; **51** (17): 4716-4720.

29. Slack-Davis J, Parsons T. Emerging views of integrin signaling: Implications for prostate cancer. *J Cell Biol* 2004; **91** (1): 41-46.

30. Ports MO, Nagle RB, Pond GD, Cress AE. Extracellular engagement of alpha6 integrin inhibited urokinase-type plasminogen activator-mediated cleavage and delayed human prostate bone metastasis. *Cancer Res* 2009 Jun 15; **69** (12): 5007-5014.

31. Bonkhoff H, Stein U, Remberger K. Differential expression of alpha 6 and alpha 2 very late antigen integrins in the normal, hyperplastic, and neoplastic prostate: simultaneous demonstration of cell surface receptors and their extracellular ligands. *Hum Pathol* 1993 Mar; **24** (3): 243-248.

32. Cress AE, Rabinovitz I, Zhu W, Nagle RB. The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. *Cancer Metastasis Rev* 1995 Sep; **14** (3): 219-228.

33. Davis TL, Cress AE, Dalkin BL, Nagle RB. Unique expression pattern of the alpha6beta4 integrin and laminin-5 in human prostate carcinoma. *Prostate* 2001 Feb 15; **46** (3): 240-248.

34. Goel HL, Alam N, Johnson IN, Languino LR. Integrin signaling aberrations in prostate cancer. *Am J Transl Res* 2009; **1** (3): 211-220.

35. Nagle RB, Hao J, Knox JD, Dalkin BL, Clark V, Cress AE. Expression of hemidesmosomal and extracellular matrix proteins by normal and malignant human prostate tissue. *Am J Pathol* 1995 Jun; **146** (6): 1498-1507.

36. Nagle RB, Knox JD, Wolf C, Bowden GT, Cress AE. Adhesion molecules, extracellular matrix, and proteases in prostate carcinoma. *J Cell Biochem Suppl* 1994; **19:** 232-237.

37. Pontes-Junior J, Reis ST, Dall'oglio M, Neves de Oliveira LC, Cury J, Carvalho PA, *et al.* Evaluation of the expression of integrins and cell adhesion molecules through tissue microarray in lymph node metastases of prostate cancer. *J Carcinog* 2009; **8:** 3.

38. Siler U, Seiffert M, Puch S, Richards A, Torok-Storb B, Muller CA, *et al.* Characterization and functional analysis of laminin isoforms in human bone marrow. *Blood* 2000 Dec 15; **96** (13): 4194-4203.

39. King TE, Pawar SC, Majuta L, Sroka IC, Wynn D, Demetriou MC, *et al.* The role of alpha 6 integrin in prostate cancer migration and bone pain in a novel xenograft model. *PLoS One* 2008; **3** (10): e3535.

40. Karni R, Jove R, Levitzki A. Inhibition of pp60c-Src reduces Bcl-XL expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. *Oncogene* 1999 Aug 19; **18** (33): 4654-4662.

41. Zha J, Harada H, Yang E, Jockel J, Korsmeyer S. Serine Phosphorylation of Death Agonist BAD in Response to Survival Factor Results in Binding to 14-3-3 Not BCL-XL. *Cell* 1996; **87** (4): 619.

42. Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, *et al.* Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem* 2000 Apr 14; **275** (15): 10761-10766.

43. Bondar VM, McConkey DJ. Anoikis is regulated by BCL-2-independent pathways in human prostate carcinoma cells. *Prostate* 2002 Apr 1; **51** (1): 42-49.

44. Manohar A, Shome SG, Lamar J, Stirling L, Iyer V, Pumiglia K, *et al.* Alpha 3 beta 1 integrin promotes keratinocyte cell survival through activation of a MEK/ERK signaling pathway. *J Cell Sci* 2004 Aug 15; **117** (Pt 18): 4043-4054.

45. Carson JP, Kulik G, Weber MJ. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B. *Cancer Res* 1999 Apr 1; **59** (7): 1449-1453.

46. McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res* 1999 Sep 1; **59** (17): 4291-4296.

47. Dong JT. Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Metastasis Rev* 2001; **20** (3-4): 173-193.

48. Schmitz M, Grignard G, Margue C, Dippel W, Capesius C, Mossong J, *et al.* Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. *Int J Cancer* 2007 Mar 15; **120** (6): 1284-1292.

49. Shinomiya N, Gao CF, Xie Q, Gustafson M, Waters DJ, Zhang YW, *et al.* RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. *Cancer Res* 2004 Nov 1; **64** (21): 7962-7970.

50. Frisch SM, Vuori K, Kelaita D, Sicks S. A role for Jun-N-terminal kinase in anoikis; suppression by bcl-2 and crmA. *J Cell Biol* 1996 Dec; **135** (5): 1377-1382.

51. Cheng TL, Symons M, Jou TS. Regulation of anoikis by Cdc42 and Rac1. *Exp Cell Res* 2004 May 1; **295** (2): 497-511.

52. Almeida EA, Ilic D, Han Q, Hauck CR, Jin F, Kawakatsu H, *et al.* Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. *J Cell Biol* 2000; **149** (3): 741-754.

53. Westhoff MA, Serrels B, Fincham VJ, Frame MC, Carragher NO. Src-mediated phosphorylation of focal adhesion kinase couples actin and

adhesion dynamics to survival signaling. *Mol Cell Biol* 2004 Sep; **24** (18): 8113-8133.

54. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. *Oncogene* 2000 Aug 3; **19** (33): 3811-3815.

55. Zhang Z, Vuori K, Reed JC, Ruoslahti E. The $\alpha 5\beta 1$ integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci U S A* 1995; **92** (13): 6161-6165.

56. Harnois C, Demers MJ, Bouchard V, Vallee K, Gagne D, Fujita N, *et al.* Human intestinal epithelial crypt cell survival and death: Complex modulations of Bcl-2 homologs by Fak, PI3-K/Akt-1, MEK/Erk, and p38 signaling pathways. *J Cell Physiol* 2004 Feb; **198** (2): 209-222.

57. Liao X, Tang S, Thrasher JB, Griebling TL, Li B. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol Cancer Ther* 2005 April 1, 2005; **4** (4): 505-515.

58. Stoll SW, Benedict M, Mitra R, Hiniker A, Elder JT, Nunez G. EGF receptor signaling inhibits keratinocyte apoptosis: evidence for mediation by Bcl-XL. *Oncogene* 1998 Mar; **16** (11): 1493-1499.

59. Gmyrek GA, Walburg M, Webb CP, Yu HM, You X, Vaughan ED, *et al.* Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. *Am J Pathol* 2001 Aug; **159** (2): 579-590.

60. Miranti C, Brugge J. Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* 2002; **4** (4): 83-90.

CHAPTER THREE:
AR-ENHANCED α6β1 INTEGRIN AND BCL-XL EXPRSSION PROMOTES ANDROGEN-INDEPENDENT PROSTATE TUMOR CELL SURVIVAL INDEPENDENTLY OF PI3K SIGNALING

INTRODUCTION

Prostate cancer is the second highest cancer-related death in American men, resulting in over 28,000 deaths per year (1). While primary prostate cancer can be successfully surgically removed or treated with radiation, metastatic prostate cancer cannot. Since prostate cancer is initially dependent on androgen signaling for proliferation and survival, androgen deprivation therapy, i.e. chemical castration, is the major treatment for metastatic prostate cancer. Although patients initially respond to androgen deprivation therapy, they ultimately relapse with castration-resistant tumors which no longer respond to the anti-androgen therapy, offering little hope for long-term disease-free survival. However, inhibition of androgen receptor (AR) expression or its DNA binding activity, even in castration-resistant cells, inhibits their proliferation and leads to cell death (2-4). This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen. However, the downstream targets of AR that directly regulate survival in castration-resistant cells are largely unknown.

Cell adhesion is necessary for the survival of all epithelial cells; integrins are heterodimeric cell surface receptors that mediate adhesion to extracellular matrix (5, 6). Integrin adhesion to matrix results in subsequent activation of many signaling molecules (5, 6). This integrin signaling through specific pathways is required for cell survival and detachment of cells results in rapid induction of cell death, known as anoikis (7). Integrin signaling through MAPK, PI3K/Akt, Src, Rho GTPases, ILK, and FAK signaling can promote survival

through increased expression of the pro-survival proteins Bcl-2, Bcl-xL, survivin, and Mcl-1, and through inhibition of the pro-death proteins Bad, Bak/Bax, Bid, Bit1 and Bim (7-9).

Integrin expression and signaling is aberrant in many diseases, including prostate cancer where α6β1 is the primary integrin expressed in both primary prostate cancer and lymph node metastases (10-16). In the normal gland, the basal epithelial cells express several integrins; two in particular, α6β4 and α3β1, allow the cells to adhere to laminin 5 in the matrix and are responsible for promoting basal cell survival (17). Basal cells do not express AR, but differentiate into AR-expressing secretory cells. During this process, the majority of integrins are lost as cells differentiate and detach from the matrix. The exception is α6β1, which was recently shown to be expressed in secretory cells (18). Even though the secretory cells express α6β1, they do not use this integrin to adhere to matrix. Integrin α6β1 is localized to cell-cell junctions in secretory cells and its function there is currently unknown. During prostate cancer development, the basal cells are lost and the secretory cells, expressing both AR and α6β1 integrin, now adhere to the matrix. In addition, the matrix is remodeled such that laminin 5 is no longer present and laminin 10, the preferred substrate of α6β1, predominates. Thus, the engagement of α6β1 integrin in AR-expressing cells in prostate cancer could provide a new mechanism for prostate cancer cell survival.

Furthermore, a few studies suggest that AR could, in part, be responsible for maintaining α6 integrin expressing in cancer cells. AR re-expression in

prostate cancer cell lines was reported to alter integrin expression and adhesion to matrix (19-21), and constitutive AR expression in immortalized prostate epithelial cells has been demonstrated to increase integrin α6 (22). In addition, the α6 integrin promoter contains a steroid-response element that is capable of stimulating α6 integrin expression in response to progesterone (23).

Phosphoinositide 3-kinase (PI3K) signaling, a common downstream target of both integrin and growth factor receptor signaling, is required for survival of most prostate cancers. The phosphatase and tensin homolog (PTEN), a phosphoinositide phosphatase and negative regulator of PI3K signaling, is lost in ~30% of clinical prostate cancers and in ~60% of metastatic cancers, resulting in constitutive activation of PI3K signaling (24-26). Akt is a major downstream effector of PI3K signaling and regulates survival through phosphorylation and inhibition of the pro-death proteins Bad, Bax, forkhead box-O transcription factors (FOXO), death-associated protein 3 (DAP3), and pro-caspase 9, through increased expression of the pro-survival protein survivin, and by regulating NF-κB and mTOR signaling (7, 27). While the androgen sensitive prostate cancer cell line LNCaP undergoes cells death within 24 hours of inhibiting PI3K/Akt after pharmalogical inhibition, addition of androgen can rescue survival (28). Furthermore, long term androgen ablation of LNCaPs results in cells resistant to PI3K/Akt inhibition (29). Lastly, in vivo prostate regeneration studies demonstrate that AR and Akt signaling can synergize to promote tumor formation even after androgen ablation (30). This suggests that AR signaling, and in some contexts independent of androgen stimulation, may promote survival

independent of PI3K signaling by either regulating the same downstream targets of the PI3K pathway, or through a novel mechanism. Li and colleagues demonstrated that AR can also phosphorylate and inhibit FOXO, thus promoting survival independent of PI3K by regulation of a common downstream target (31). Work by Chen's laboratory has suggested that the pro-survival protein Bcl-xL can also promote survival independent of PI3K signaling in prostate cancer cells, however the mechanism by which Bcl-xL was regulated independent of serum stimulation remained undetermined (32). Whether integrin and AR signaling crosstalk can promote survival independent of PI3K/Akt signaling remains unknown.

In this study, we tested the hypothesis that AR-dependent regulation of $\alpha 6\beta 1$ expression in prostate cancer cells promotes survival independent of PI3K signaling. AR was re-expressed in the prostate cancer cell line PC3. Re-expression of AR in PC3 cells lead to increased transcription and expression of integrin $\alpha 6$, and adhesion to laminin subsequently increased the activity of PAK1/2 and NF-κB p65, increased transcription and expression of the pro-survival protein Bcl-xL, and made the cells resistant to cell death induced by PI3K inhibition. Loss of integrin $\alpha 6$, NF-κB p65, or Bcl-xL re-sensitized AR-expressing PC3 cells to PI3K-dependent survival. Treatment of AR expressing PC3 cells with the AR inhibitor RU486 or AR-specific siRNA, or expression of AR mutants that block the ability of AR to translocate to the nucleus (ΔNLS), but not to bind ligand (ΔLBD), largely restored the parental PC3 phenotype, including

PI3K-dependent survival. These results are largely supported by siRNA knock-down of AR in LNCaP cells.

RESULTS

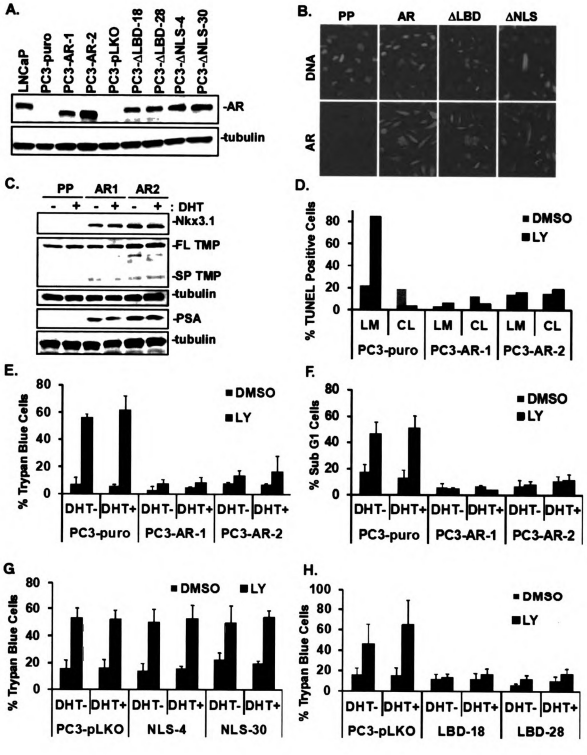
The ability to assess the role of $\alpha6\beta1$ in AR-dependent prostate cancer survival has been hampered by the lack of appropriate cell models that recapitulate the AR/$\alpha6\beta1$ profile seen in vivo. For instance, the most well characterized AR-expressing prostate cancer cell line, LNCaP, expresses $\alpha6\beta1$ (as well as others), but adheres poorly to laminin. On the other hand, the PC3 cell line expresses $\alpha6\beta1$ and adheres nicely to laminin, but does not express AR. Unfortunately, PC3 cells also express a lot of other integrins, including another laminin integrin $\alpha3\beta1$, which is not consistent with what is seen in vivo. Previous studies indicated that AR expression in PC3 cells changes integrin expression (19, 20). Furthermore, both wild type and AR mutants can be expressed in the absence of endogenous AR, which would ordinarily complicate the interpretation of the results. Lastly, any observable phenotypes can be attributed to AR by direct testing with AR siRNA.

Viruses were used to introduce empty vectors (PC3-puro or pLKO), sequence-verified wild-type AR, or two AR mutants into PC3 cells. These cell lines were selected and constantly maintained in charcoal-stripped serum to avoid selection against growth suppression, a problematic side-effect of AR expression in PC3 cells (33). AR expression was constantly monitored and only early-passage (< 20) cells were used. To understand which function of AR is

important to the observed phenotypes, two well-characterized AR mutants were selected. The ΔNLS mutant is defective in AR translocation to the nucleus and in DNA binding (34, 35). The ΔLBD mutant is unable to bind ligand (30). To ensure that AR was not too highly over expressed, stable clonal cell lines that expressed approximately the same level of AR as LNCaP cells were selected. AR expression, in the absence of androgens, was confirmed by immunoblotting and immunoflourescent (IF) staining with anti-AR antibodies (Figure 7A, B). Immunoblotting showed that the wild-type AR clone PC3-AR-1 had comparable expression to LNCaP cells, and clone PC3-AR-2 had higher AR expression (Figure 7A). All the AR mutants had approximately the same level of AR expression as the LNCaP cells (Figure 7A). AR localization was both cytoplasmic and nuclear in wild-type AR expressing clones by IF staining (Figure 7B). Surprisingly, the ΔLBD mutant was predominately nuclear, while the ΔNLS mutant was exclusively cytoplasmic as expected (Figure 7B). AR localization was not significantly altered by exogenous treatment with androgens (DHT). To access AR functionality, the expression of known AR-target genes was examined by immunoblotting. PC3-AR-1 and PC3-AR-2 cells expressed higher levels of the AR-target genes Nkx3.1 and prostate specific antigen (PSA) than the empty vector control cells (Figure 7C), indicating AR is functional. While the full-length androgen-responsive protein TMPRSS2 was expressed in the empty vector PC3-puro cells as well as the AR expressing clones, only the cleaved and activated serine protease domain could be detected in the AR expressing clones (Figure

FIGURE 7. On LMI, AR is a survival factor that acts independently of PI3K signaling and androgen. A) AR and tubulin expression levels were monitored by immunoblotting whole cell lysates of LNCaP, PC3 empty-vector (PC3-puro or PC3-pLKO) pools and AR expressing clones (PC3-AR, PC3-ΔLBD, and PC3-ΔNLS). **B)** PC3-puro (PP), PC3-AR-1 (AR), PC3-ΔLBD-28 (ΔLBD), and PC3-ΔNLS-4 (ΔNLS) cells were immunostained to detect expression of AR (green) and imaged by epifluorescence microscopy. Nuclei (blue) were visualized by Hoechst staining. Clones not shown had similar staining to the representative clones shown. **C)** Growth factor-starved and charcoal-stripped PC3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 (AR2) cells were plated on LMI and treated with vehicle or 10 nM DHT for 72 hours. Nkx3.1, the full length (FL) and cleaved (SP) forms of TMPRSS2 (TMP), and PSA expression were monitored by immunoblotting. Tubulin served as a loading control. **D)** PC3-puro, PC3-AR-1, PC3-AR-2 cells were growth factor-starved in charcoal-stripped media. Cells were then plated on CLI or LMI in the presence of DMSO or 10 μM LY294002 (LY) to inhibit PI3K signaling, for up to for 4 hours and then non-adherent cells were washed away and drugs and DHT were replaced. Cell viability was measured 72 hours later by fixing and permeabilizing cells for nicked DNA using TUNEL staining and FACS analysis. n = 2. **E-H)** PC3-puro, PC3-AR-1, PC3-AR-2, PC3-pLKO, PC3-ΔNLS (NLS), or PC3-ΔLBD (LBD) cells were growth factor-starved in charcoal-stripped media. Cells were then plated on LMI with vehicle or 10 nM DHT in the presence of DMSO or 10 μM LY294002 (LY), for up to for 4 hours and then non-adherent cells were washed away and drugs and DHT were replaced. After 72 hours cells were either counted for trypan blue staining **E, G-H)** or fixed, permeabilized, and stained with propidium iodide (PI) to detect dead cells (sub G1) by FACS analysis **F)** Error bars on all graphs represent standard deviation; n = 3-5.

FIGURE 7


A.
LNCaP
PC3-puro
PC3-AR-1
PC3-AR-2
PC3-pLKO
PC3-ΔLBD-18
PC3-ΔLBD-28
PC3-ΔNLS-4
PC3-ΔNLS-30
-AR
-tubulin
B.
PP
AR
ΔLBD
ΔNLS
DNA
AR
C.
PP
AR1
AR2
- + - + - + : DHT
-Nkx3.1
-FL TMP
-SP TMP
-tubulin
-PSA
-tubulin
D.
% TUNEL Positive Cells
DMSO
LY
LM CL LM CL LM CL
PC3-puro PC3-AR-1 PC3-AR-2
E.
% Trypan Blue Cells
DMSO
LY
DHT- DHT+ DHT- DHT+ DHT- DHT+
PC3-puro PC3-AR-1 PC3-AR-2
F.
% Sub G1 Cells
DMSO
LY
DHT- DHT+ DHT- DHT+ DHT- DHT+
PC3-puro PC3-AR-1 PC3-AR-2
G
% Trypan Blue Cells
DMSO
LY
DHT- DHT+ DHT- DHT+ DHT- DHT+
PC3-pLKO NLS-4 NLS-30
H.
% Trypan Blue Cells
DMSO
LY
DHT- DHT+ DHT- DHT+ DHT- DHT+
PC3-pLKO LBD-18 LBD-28

7C). Addition of androgen (DHT) did not significantly alter expression of Nkx3.1, PSA, or cleaved TMPRSS2, presumably because AR nuclear localization occurs independently of ligand in these cells (Figure 7B).

Inhibition of PI3K signaling in PC3 cells with the pharmalogical inhibitor LY294002 results in cell death (36). To determine if the integration of integrin signaling and AR expression could protect cells from death when PI3K signaling was inhibited, serum-starved AR-expressing clones were plated on laminin 1 (LM1), a preferred substrate for $\alpha 6\beta 1$ integrin, or the $\alpha 2\beta 1$ substrate, collagen I (CLI) in the presence or absence of LY294002, and analyzed over a 72-hour time course. Cell death was measured by Terminal Deoxynucleotide Transferase dUTP Nick End Labeling (TUNEL) staining and FACS analysis. Inhibition of PI3K signaling with LY294002 resulted in maximal death at 72 hours in the PC3-puro cells, but only when plated on LM1 (Figure 7D). In contrast, the AR-expressing clones did not experience significant cell death from LY294002 treatment on LM1 or CLI (Figure 7D). Thus, in the context of adhesion to LM1, but not CLI, AR contributes significantly to cell survival, since LY294002 was sufficient to induce death in the PC3-puro cells but not in the AR expressing cells. Treatment of PC3-AR-1 or PC3-AR-2 cells adherent to LM1 with LY294002 also failed induce cell death as measured by trypan blue staining or propidium iodide (PI) staining of sub G1 cells (Figure 7E-F). All the observed effects were independent of androgen (DHT). This difference in survival was not strictly due to cell cycle status since PC3-AR-1 cells grow at the same rate, whilePC3-AR-2 cells grow slower than PC3-puro cells (data not shown).

Furthermore, the ability of AR-expressing cells to survive on LM1 was not due to AR-mediated hyper-activation of the PI3K/Akt pathway and LY294002 was still a potent inhibitor of PI3K/Akt signaling in the AR expressing cells (data not shown). The AR nuclear localization mutant ΔNLS (Figure 7G), but not the AR ligand binding mutant ΔLBD (Figure 7H), restored sensitivity to PI3K inhibition resulting in cell death. Thus, AR can promote survival independent of PI3K signaling in PC3 cells plated on LM1. This requires AR localization to the nucleus, presumably to bind DNA.

Expression of AR in PC3 cells was previously shown to decrease α6β4, α3β1, and α2β1 expression (19, 20). On the other hand, expression of AR in DU145 cells decreased α6β4, but increased α2β1 (21). Since different matrixes engage different integrins and the dependence on PI3K signaling was only observed when the cells were cultured on LM1 but not CLI (Figure 7D), we sought to determine if AR affected integrin expression in cells plated on LM1. Integrin surface expression was analyzed by FACS in the empty vector PC3 cells and compared to the AR-expressing PC3 cells after adhesion to LM1. AR, independent of DHT, reduced expression of integrins α3 2-3 fold and α5 3-6 fold, but increased expression of integrin α6 3-5 fold (Figure 8A). AR had relatively little or no effect on integrin α2 and there was a slight decrease (<2-fold) in integrin β1 (Figure 8A). Integrin β4 levels were very low in PC3-puro cells (mean fluorescent values 18.05 -/+1.73) and did not significantly change upon AR expression (mean fluorescent value 13.82 -/+ 6.49 and 10.18 -/+ 2.79 for PC3-AR-1 and PC3-AR-2 respectively). Since integrins must be in heterodimeric

pairs to be expressed on the cell surface and there was a decrease in the α3 and α5 subunits and an increase in α6, there must be an overall decrease in integrins α3β1and α5β1 and an increase in α6β1 since integrin β4 levels did not increase to compensate for the large increase in α6 surface expression. During prostate cancer development there is a loss of most integrins and an increase in expression of integrin α6β1 (10-16), suggesting that our AR-expressing PC3 cells recapitulate some of what is seen in vivo. To verify that AR can regulate integrin α6 expression in other cell lines, endogenous AR in LNCaP cells was knocked-down by transfection with AR-specific siRNAs. After 48 hours, AR levels were greatly diminished as observed by immunoblotting (Figure 8B). This was accompanied by a ~12% decrease in integrin α6 expression as measured by FACS (Figure 8C).

Since integrin-mediated adhesion can promote cell survival and there was an increase in integrin α6 with AR expression, we sought to test the hypothesis that AR was promoting survival through up-regulation of integrin α6. To test this, integrin α6 expression in the AR expressing clones were decreased as close as possible to empty vector levels by careful titration of integrin α6 siRNA (Figure 8D). A non-specific siRNA (scram) was used as a control in these and all subsequent experiments. Loss of integrin α6 did not have a significant or consistent affect on AR expression, indicating that integrin α6 is downstream of AR (Figure 8D). Cells were treated with integrin α6 siRNA or scram for 72 hours to induce partial knock-down of integrin α6, then cells were plated on LM1 and treated with LY294002. After 72 hours, cell viability was assessed by trypan blue

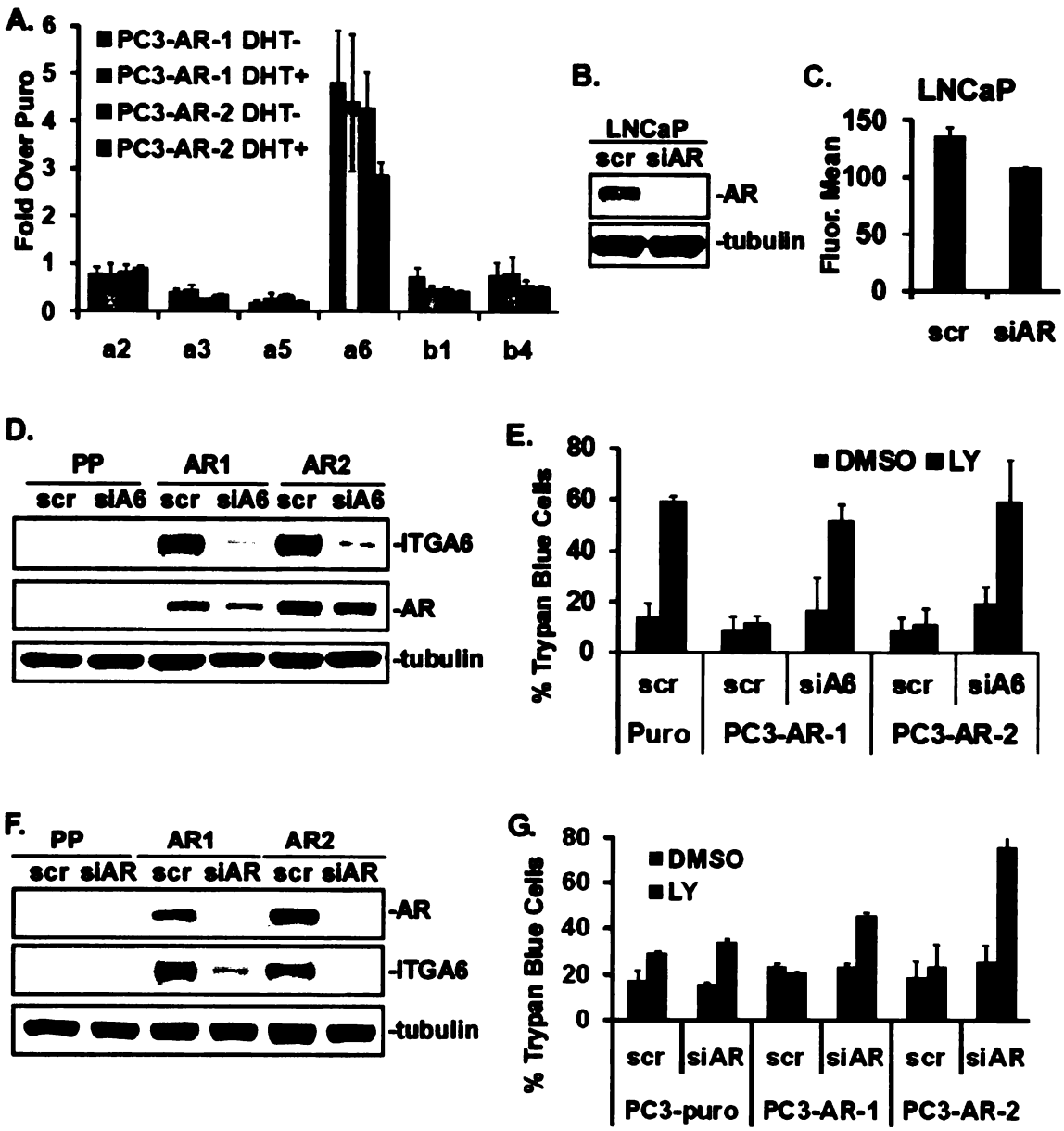
staining. A 63-73% reduction in α6 integrin expression in the presence of LY294002 is sufficient to induce cell death in AR expressing cells (Figure 8E). These data indicate that AR is promoting survival through integrin α6.

To verify that these effects were due to AR expression and not clonal selection, AR expression was knocked in the AR expressing cells using siRNA prior to treatment with the PI3K inhibitor, LY294002 (Figure 8F,G). Loss of AR in AR expressing clones lead to decreased integrin α6 expression by immunoblotting (Figure 8F) and increased cell death when the cells were treated with LY294002 (Figure 8G). Overall, these data indicate that AR is a pro-survival factor in PC3 cells that acts independently of PI3K signaling through increased integrin α6 expression.

We then wanted to determine the mechanism by which AR and integrin α6 regulate survival independent of PI3K signaling. We first tested the hypothesis that AR/integrin α6 was regulating a common downstream target of PI3K/Akt signaling. The PI3K/Akt pathway has been demonstrated to promote survival through several mechanisms, including regulation of the pro-death proteins Bad or FOXO (36-39) and the pro-survival proteins survivin and Bcl-2 (40-42). Furthermore, these proteins have also been reported to be regulated by AR (38, 40, 41, 43) or integrins (44, 45). Bcl-2 and survivin have also been associated with prostate cancer progression (46-49). However, in the AR expressing PC3 clones, there was no decrease in Bad or FOXO activity and no increase in survivin or Bcl-2 levels compared to empty vector control cells, with or without DHT or plating on LM1 or CLI matrix (data not shown). Together, these data

FIGURE 8. AR promotes survival through up-regulation of integrin α6. A) PC3-puro, PC3-AR-1, PC3-AR-2 cells were growth factor-starved in charcoal-stripped media and plated on LMI in the presence of vehicle or DHT. After 72 hours, cells were treated with flourescent-conjugated antibodies against the indicated integrins and analyzed by FACS. Mouse and Rat IgG were negative controls. IgG controls were subtracted from mean flourescent values then values for AR expressing cells were normalized to those of PC3-puro cells. Error bars represent standard error; *n* = 5-8. **B-C)** LNCaP cells were treated with siRNA against AR (siAR) or non-specific sequence (scr) for 72 hours. **B)** LNCaP lysates were immunoblotted to monitor AR expression. Tubulin was used as a loading control. **C)** LNCaP cells were treated with flourescent-conjugated antibody against integrin α6 and analyzed by FACS. Rat IgG was the negative controls. Values given are for mean flourescent values minus IgG control. Error bars represent standard error; *n* = 2. **D-G)** PC3-puro (PP, Puro), PC3-AR-1 (AR1), and PC3-AR-2 (AR2) cells were treated with siRNA against integrin α6 (siA6), AR (siAR) or non-specific sequence (scr) for 72 hours and plated on LMI. **D, F)** Integrin α6 (ITGA6) and AR expression was monitored by immunoblotting. Tubulin was used as a loading control. **E, G)** Cells were treated with DMSO or LY294002 (LY) for 72 hours. Cell viability was determined using trypan blue staining. Error bars represent standard deviation; *n* = 3. *(A,D-G) Laura Lamb, (B) Jelani Zarif, (C) Laura Lamb and Jelani Zarif.*

FIGURE 8


A.
PC3-AR-1 DHT-
PC3-AR-1 DHT+
PC3-AR-2 DHT-
PC3-AR-2 DHT+
Fold Over Puro
0
1
2
3
4
5
6
a2
a3
a5
a6
b1
b4
B.
LNCaP
scr siAR
-AR
-tubulin
C.
LNCaP
Fluor. Mean
0
50
100
150
scr
siAR
D.
PP
AR1
AR2
scr siA6 scr siA6 scr siA6
-ITGA6
-AR
-tubulin
E.
DMSO
LY
% Trypan Blue Cells
0
20
40
60
80
scr
scr
siA6
scr
siA6
Puro
PC3-AR-1
PC3-AR-2
F.
PP
AR1
AR2
scr siAR scr siAR scr siAR
-AR
-ITGA6
-tubulin
G.
DMSO
LY
% Trypan Blue Cells
0
20
40
60
80
scr
siAR
scr
siAR
scr
siAR
PC3-puro
PC3-AR-1
PC3-AR-2

suggest that in AR expressing PC3 cells Bad, FOXO, survivin, or Bcl-2 is not the downstream target of integrin- and AR-signaling.

The pro-survival protein Bcl-xL has been reported to promote survival independent of PI3K signaling in prostate cancer cells (32), and we previously demonstrated integrin-mediated adhesion to LM1 in PC3 cells led to increase Bcl-xL expression through the non-receptor tyrosine kinase Src (see Chapter 2) (36). Increased Bcl-xL expression is also associated with prostate cancer progression (46, 50, 51). Immunoblotting of total cell lysates demonstrated that Bcl-xL is up-regulated in AR expressing PC3 cells (Figure 9A), suggesting Bcl-xL could be the mechanism by which AR promotes survival independent of PI3K signaling. To demonstrate that up-regulation of Bcl-xL is due to AR expression, AR-expressing cells were treated with AR siRNA and expression of Bcl-xL was monitored. In collaboration with Jelani Zarif, we demonstrated that loss of AR in AR expressing clones resulted in down-regulation of both integrin α6 and Bcl-xL (Figure 9B). To determine if Bcl-xL expression is dependent on integrin α6, integrin α6 expression was decreased using integrin α6 siRNA. Decreased integrin α6 resulted in decreased Bcl-xL (Figure 9C). Lastly, we demonstrated that loss of AR in LNCaP cells also resulted in a modest decrease in both integrin α6 and Bcl-xL levels. Together, these data indicate that AR, acting via integrin α6, drives increased expression of the pro-survival protein Bcl-xL.

To determine if Bcl-xL is required for PI3K-independnet survival, Bcl-xL was knocked down in the AR expressing cells to the approximate levels found in

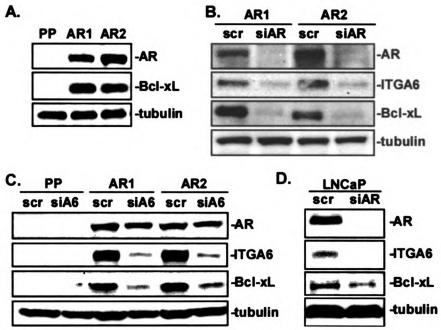


FIGURE 9. AR and integrin α6 regulate Bcl-xL expression. PC3-puro (PP), PC3-AR-1 (AR1), PC3-AR-2 (AR2) and LNCaP cells were treated with siRNA against AR (siAR), integrin α6 (siA6), or non-specific sequence (scr) for 72 hours. AR, integrin α6 (ITGA6), and Bcl-xL levels were monitored by immunoblotting of whole cell extracts using AR, integrin α6 (ITGA6), and Bcl-xL specific antibodies. Total levels of protein were monitored by immunoblotting with anti-tubulin. *(A, C) Laura Lamb, (B, D) Jelani Zarif.*

the PC3-empty control cells using Bcl-xL siRNA (Figure 10A). Partial loss of Bcl-xL in the AR expressing clones in the presence of LY294002 induced cell death to the levels found in the PC3-empty cells treated with LY294002 (Figure 10B). Complete loss of Bcl-xL resulted in complete loss of viability (data not shown). To demonstrate that Bcl-xL over-expression is sufficient to promote survival independent of PI3K signaling, retroviruses were used to infect cells with an empty vector or a vector expressing Bcl-xL and stable clonal cell lines were selected. Bcl-xL over-expression to the levels found in AR expressing clones was confirmed by immunoblotting (Figure 10C). As expected, Bcl-xL over expression did not result in AR expression or changes in integrin expression (Figure 10C, data not shown). Moreover, Bcl-xL over-expressing cells did not die when treated with LY294002 (Figure 10D). Thus, Bcl-xL is a potent pro-survival factor and can promote survival independent of PI3K signaling.

We previously demonstrated integrin-mediated adhesion to LM1 in PC3 cells led to increased Bcl-xL expression through the non-receptor tyrosine kinase Src (see Chapter 2) (36). AR has also been reported to promote Src activity though its proline-rich region, which is capable of binding the SH3 domain of Src (52). We sought to determine if increased Src activity was responsible for the increased Bcl-xL expression. Src is activated by phosphorylation at Y416 and inhibited by phosphorylation at Y527 (53). Therefore, Src expression and activity was measured by immunoprecipitation of Src and immunoblotting for phosphorylation of Y416, dephosphorylation of Y527, and total Src. There was increased Src expression and activity in the AR expressing clones (Figure 11A).

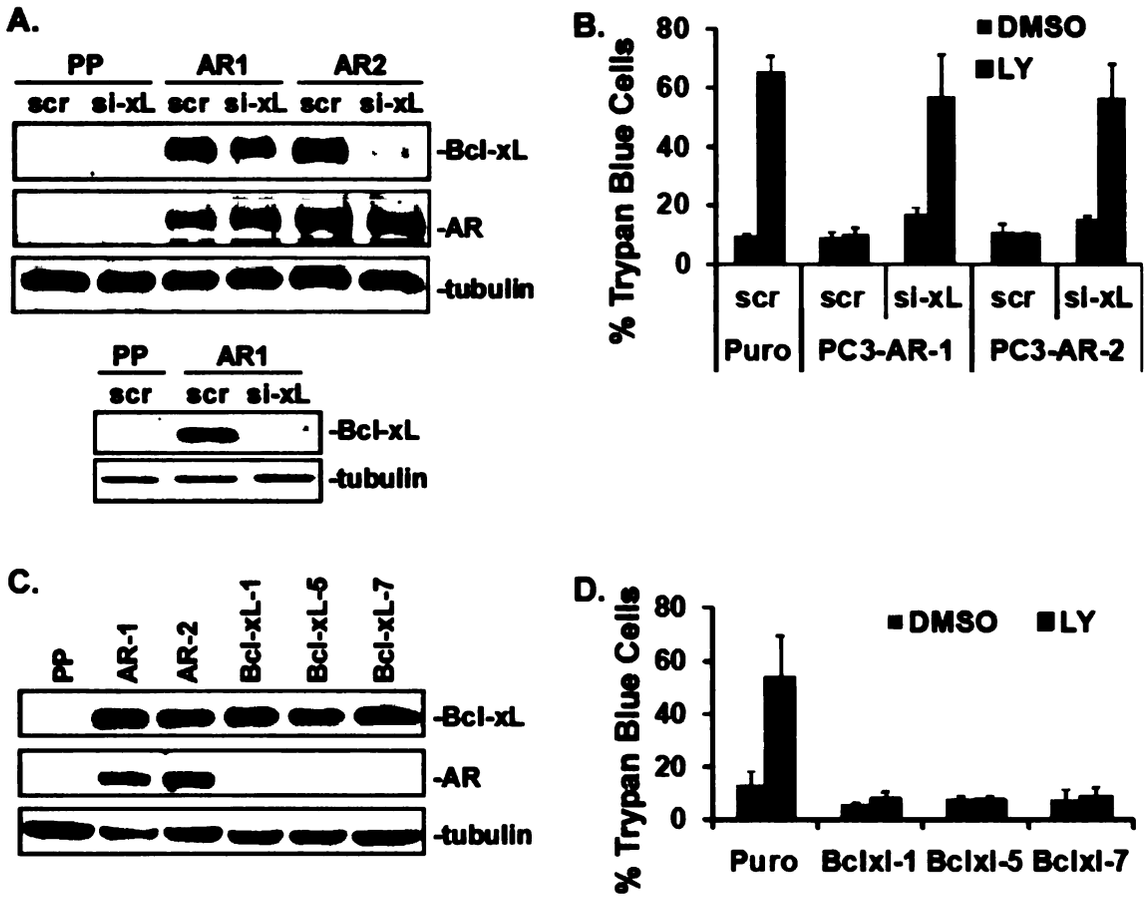


FIGURE 10. Bcl-xL promotes survival independent of PI3K signaling. A) Cells were treated with siRNA specific against Bcl-xL (si-xL) or non-specific sequence (scr) for 72 hours. Bcl-xL and AR levels were monitored by immunoblotting of whole cell extracts using Bcl-xL and AR specific antibodies. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. Treatment of PC3-AR-1 cells with siRNA for more than one experiment is shown. **B)** Cells were then plated on LMI and treated with DMSO or LY294002 (LY) to inhibit PI3K signaling. Cell viability was measured 72 hours after drug addition by trypan blue staining. Error bars represent standard deviation; *n* = 3. **C)** PC3 cells were made to over express Bcl-xL by infecting cells with a retrovirus containing an empty or Bcl-xL construct (PP and Bcl-xl respectively) and stable clones were selected in puromycin. **D)** PC3-puro (puro) and PC3-Bcl-xl (Bclxl) clones were plated on LMI and treated with DMSO or LY294002 (LY) for 72 hours. Cell viability was then measured by trypan blue staining. Error bars represent standard deviation; *n* = 3.

In collaboration with Jelani Zarif, we demonstrated that while loss of AR expression in these cells results in partial loss of Src activity it had no effect on Src expression (Figure 11B). In LNCaP cells, AR knock-down of AR also leads to a partial loss in Src activity and there is a slight decrease in total Src levels (Figure 11C). To test whether integrin α6 signaling was enhancing Src activity, integrin α6 expression was knocked down to PC3-puro levels using integrin α6 siRNA. Partial loss of integrin α6 failed to affect Src activity, suggesting that AR-enhanced Src activity was not downstream of integrin α6 (Figure 11D). In PC3-AR clones, partial knock down of Src also does not lead to decreased levels of Bcl-xL or AR (Figure 11E). Lastly, while knock down of Src in PC3-puro cells resulted in a two-fold increase in cell death, it had no significant affect on cell survival in the AR expressing cells (Figure 11F). These data indicate that AR expression alters Src activity, this dramatic change in Src activity is not involved in the integrin α6/Bcl-xL signaling axis nor is it required for survival.

In addition to promoting cell survival, Src has been well-characterized in promoting normal and cancer cell migration and invasion (54). AR expressing clones have a different morphology and display more filopodia than the PC3-puro cells as observed by immunoflourescent microscopy (Figure 12 A,B). Filopodia formation is associated with a more migratory phenotype (55). Correspondingly, the AR expressing clones are more migratory than the PC3-puro cells as determined by a Boyden chamber migration assay using LM1 as a chemoattractant (Figure 12 C, D). Work by Jelani Zarif has also demonstrated that the AR expressing clones are more invasive than PC3-puro cells, and that

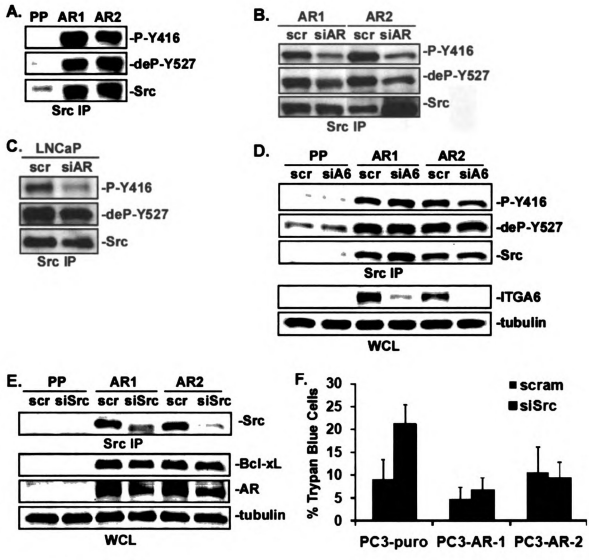


FIGURE 11. AR regulates Src activity. A-D) PC3-puro (PP), PC3-AR-1 (AR1), PC3-AR-2 (AR2), or LNCaP cells were treated with AR siRNA (siAR), integrin α6 siRNA (siA6) or a non-specific sequence (scr) for 144 **B)** or 72 **C-D)** hours. Src was then immunoprecipitated (IP) and activity was measured by immunoblotting for phosphorylation of tyrosine 416 (pY416) and loss of phosphorylation on tyrosine 527 (deP-Y527). Total Src expression was also monitored. In some cases, integrin α6 (ITGA6) and tubulin expression was measured by immunoblotting of whole cell lysates (WCL). **E)** Cells were treated with Src siRNA (siSrc) or a non-specific sequence (scr) for 72 hours, then Src activity was measured as in A-D. Whole cell lysates (WCL) were also measured for Bcl-xL and AR expression by immunoblotting. Tubulin was used as a control for loading. **F)** PC3-puro, PC3-AR-1, and PC3-AR-2 cells were plated on LMI and treated with Src siRNA (siSrc) or a non-specific sequence (scr) for 72 hours. Cell viability was measured by trypan blue staining. Error bars represent standard deviation; $n = 3$. *(A, D-F) Laura Lamb, (B-C) Jelani Zarif.*

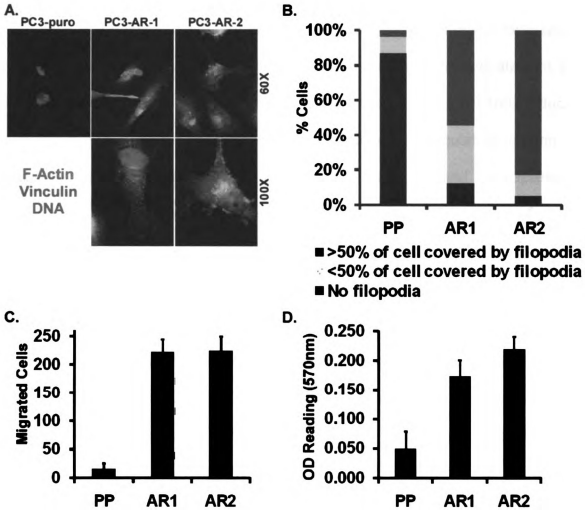


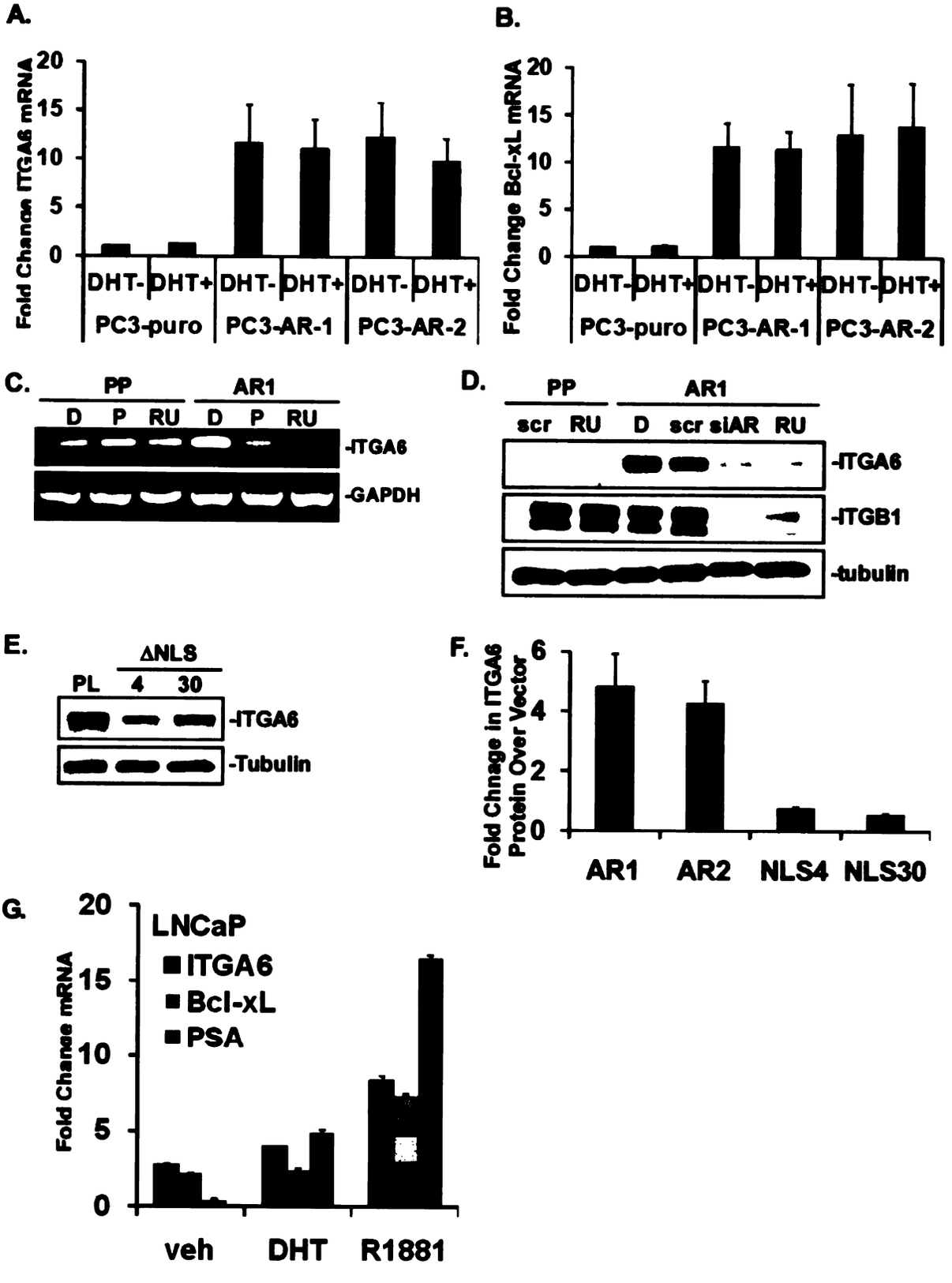
FIGURE 12. AR promotes filopodia formation and migration. A) Cells were plated on LMI for 1 hour then fixed, permeabilized, and stained for F-actin (red), vinculin to visualize focal adhesions (green specks), and nuclei (blue). **B)** Quantification of the presence of filopodia on cells. 100 cells were counted per experiment; n = 4. **C, D)** The ability of cells to migrate was tested using a Boyden-chamber migration assay with LMI gradient as the chemoattractant. Cells that migrated to the bottom chamber were then stained with crystal violet and (C) counted or (D) crystal violet staining was eluted from cells and quantified by OD reading. Error bars represent standard deviation; n = 3.

knockdown of Src or AR in the AR expressing clones significantly decreases their invasion (data not shown).

AR is a transcription factor whose activity depends on nuclear localization and expression of the AR ΔNLS mutant that is unable to translocate into the nucleus and bind DNA, was also unable to protect cells from LY294002-induced death (see Fig 7G). To determine if AR expression was regulating integrin α6 and Bcl-xL transcription, RNA was isolated from PC3-puro and AR expressing clones, reverse transcribed and quantitative RT-PCR (qRT-PCR) was preformed. There was over a 10-fold increase in integrin α6 and Bcl-xL mRNA levels compared to PC3-puro cells, independent of DHT addition (Figure 13A,B). The AR increase in Bcl-xL mRNA is in agreement with studies in LNCaP cells where treatment with androgen or AR-specific siRNA leads to a respective increase or decrease in Bcl-xL mRNA (2, 51). In collaboration with Jelani Zarif, to verify that this was an AR-dependent effect, PC3-AR-1 cells were treated with the anti-androgen RU486 or AR-specific siRNA. RU486 is reported to recruit co-repressors to AR transcriptional complexes thereby inhibiting AR-mediated transcription (56). RU486 decreased integrin α6 mRNA expression as measured by RT-PCR, (Figure 13C). RU486 treatment also resulted in a decrease in the protein levels of integrin α6 expression with approximately the same severity as AR-specific siRNA treatment (Figure 13D). There was also a decrease in integrin β1 (Figure 13D). Since integrins must be expressed as heterodimers in order to be stably expressed, loss of the integrin α6 binding partner of integrin β1 may be leading to its degradation. Since AR must be in the nucleus to act as a

FIGURE 13. AR regulates integrin α6 and Bcl-xL mRNA expression. A-B) PC3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 (AR2) cells were plated on LMI and treated with vehicle (ethanol) or DHT for 72 hours. mRNA was then isolated and reverse transcribed. Integrin α6 (ITGA6) **A)** or Bcl-xL **B)** mRNA expression was measured by qRT-PCR. Gene expression was normalized to 18s rRNA then expressed as fold change relative to vehicle-treated PC3-puro cells. Error bars represent standard deviation; *n* = 3. **C)** PC3-puro (PP) and PC3-AR-1 (AR1) were plated on LM1 then treated with DMSO (D), PBS (P), or RU486 (RU) for 72 hours. mRNA was then isolated and RT-PCR was preformed to measure integrin α6 (ITGA6) levels. GAPDH expression was used as a loading control. **D)** PC3-puro (PP) and PC3-AR-1 (AR1) were plated on LM1 then treated with DMSO (D), RU486 (RU), AR siRNA (siAR), or non-targeting siRNA (scr) for 72 hours. Cells were lysed and immunobloted to monitor integrin α6 (ITGA6) and β1 (ITGB1) expression. Total levels of protein were monitored by immunoblotting with anti-tubulin. **E)** PC3-pLKO (PL) and PC3-ΔNLS-AR (ΔNLS) clone lysates were monitored for integrin α6 (ITGA6) and Bcl-xL expression by immunoblotting. Tubulin expression was used as a loading control. **F)** PC3-puro, PC3-AR-1 (AR1), PC3-AR-2 (AR2), PC3-pLKO, PC3-ΔNLS-AR-4, and PC3-ΔNLS-AR-30 cells were growth factor-starved in charcoal-stripped media and plated on LMI. After 72 hours, cells were treated with flourescent-conjugated integrin α6 antibody and analyzed by FACS. Rat IgG controls were subtracted from mean flourescent values then values for AR expressing cells were normalized to those of the corresponding vector cells. Error bars represent standard error; *n* = 2. **G)** LNCaP cells were serum-starved in charcoal-stripped media for 48 hours, then treated with vehicle (veh), DHT, or R1881 for 24 hours. mRNA was then isolated and reverse transcribed. integrin α6 (ITGA6), Bcl-xL, and PSA mRNA expression was measured by qRT-PCR. Gene expression was normalized to 18s rRNA then expressed as fold change relative to untreated cells. Error bars represent standard deviation; *n* = 1. *(A-B, E-G) Laura Lamb, (C-D) Jelani Zarif.*

FIGURE 13


A.
Fold Change ITGA6 mRNA
20
15
10
5
0
DHT- DHT+ DHT- DHT+ DHT- DHT+
PC3-puro PC3-AR-1 PC3-AR-2
B.
Fold Change Bcl-xL mRNA
20
15
10
5
0
DHT- DHT+ DHT- DHT+ DHT- DHT+
PC3-puro PC3-AR-1 PC3-AR-2
C.
PP AR1
D P RU D P RU
-ITGA6
-GAPDH
D.
PP AR1
scr RU D scr siAR RU
-ITGA6
-ITGB1
-tubulin
E.
ΔNLS
PL 4 30
-ITGA6
-Tubulin
F.
Fold Chnage in ITGA6 Protein Over Vector
6
4
2
0
AR1 AR2 NLS4 NLS30
G.
LNCaP
ITGA6
Bcl-xL
PSA
Fold Change mRNA
20
15
10
5
0
veh DHT R1881

transcription factor, we tested the affect of expression of the AR ΔNLS mutant on integrin α6 and Bcl-xL expression. Expression of the ΔNLS AR mutant in PC3 cells did not result in increased integrin α6 expression compared to empty vector PC3-pLKO cells (Figure 13E,F). Lastly, stimulation of LNCaP cells for as little as 24 hours with DHT or the more potent synthetic androgen R1881 results in increased integrin α6 and Bcl-xL mRNA expression as determined by qRT-PCR. PSA was used as a positive control. Together, this suggests that AR transcriptionally regulates integrin α6 and Bcl-xL expression. It still needs to be determined if the effect of AR on α6 integrin is direct or indirect.

The effect of AR on Bcl-xL expression is indirect, since we demonstrated that α6 is required for Bcl-xL expression (see Figure 8D). We sought to determine how integrin α6 could drive Bcl-xL expression, since it was not through Src (Figure 11E). The transcription factor NF-κB (RelA) has been reported to directly bind the promoter of and drive transcription of Bcl-xL (57, 58). Furthermore, increased NF-κB activity is associated with prostate cancer progression (59), castration-resistance (60, 61), poor prognosis (62, 63), biochemical failure (i.e. PSA relapse) (64, 65), and has been determined to be significantly misregulated in metastatic prostate cancer based on microarray studies (66). Therefore, we determined if NF-κB signaling was increased in AR-expressing cells. NF-κB p65 activity, as determined by both NF-κB p65 phosphorylation and a reporter assay, were increased in AR-expressing cells (Figure 14 A,B). This is in agreement with previous work that expression of AR in PC3 cells can result in increased NF-κB activity (67). The increase in NF-κB

reporter activity was also independent of DHT (Figure 14B). To determine if AR and integrin α6 regulated NF-κB signaling, AR and integrin α6 were knocked down in AR expressing cells using siRNA. Knock-down of AR or integrin α6 resulted in a decrease in NF-κB p65 phosphorylation and in some cases, a modest decrease in total NF-κB protein levels (Figure 14C,D). TNFα stimulation of PC3-puro cells was used as a positive control for NF-κB p65 phosphorylation (Figure 14C,D). Thus, NF-κB p65 activity was increased in AR expressing cells in an AR- and integrin α6-dependent manner.

To determine if NF-κB p65 (RelA) was regulating Bcl-xL expression, AR expressing cells were treated with NF-κB p65 specific siRNA. Knock-down of NF-κB p65 in AR expressing cells resulted in only a partial knock-down of Bcl-xL (Figure 14E), suggesting that another pathway or another NF-κB family member may also be important in regulating Bcl-xL expression in AR expressing cells. Indeed, AR has been reported to directly regulate Bcl-xL expression (51), which could explain why NF-κB p65 knock-down only resulted in partial loss of Bcl-xL. Knock-down of NF-κB did not alter integrin α6 expression (data not shown). Nonetheless, knock-down of NF-κB p65 was sufficient to sensitize AR-expressing cells to LY294002-induced death (Figure 14F). Similar results were seen using the cell permeable small peptide inhibitor SN50, which blocks NF-κB translocation into the nucleus (data not shown). Thus, the partial knock-down of Bcl-xL by NF-κB loss may be sufficient for AR expressing cells to regain dependence on PI3K signaling. However, NF-κB signaling is known to regulate

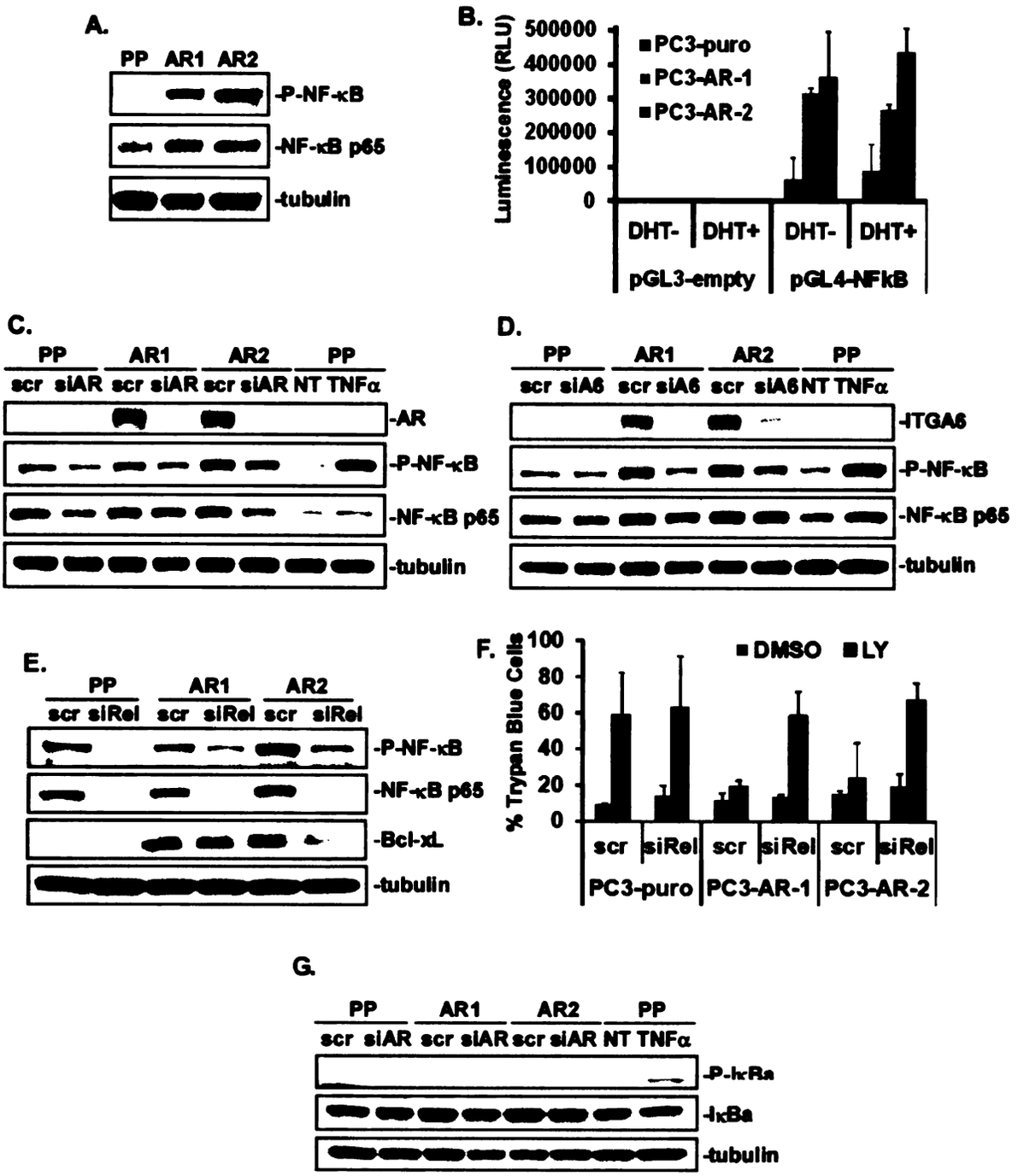
other key regulators of cell survival, including cFLIP, BFl-1/A1, c-IAP1/2, and XIAP (68), whose loss in expression may also contribute to this phenotype.

Classically NF-κB is kept in the cytoplasm bound to a family of inhibitor proteins, called IκBs (inhibitor of κB), where IκBα is the most prevalent and characterized isoform (69, 70). Activation of NF-κB classically requires phosphorylation and subsequent degradation of IκB, which allows NF-κB to translocate into the nucleus (69, 70). Surprisingly, we did not observe any detectable phosphorylation or loss of IκBα in our AR expressing cells compared to the PC3-puro cells (Figure 14G). TNFα stimulation of PC3-puro cells was used as a positive control for phosphorylation of IκBα (Figure 14G). Knock-down of AR or integrin α6 also did not have an effect on IκBα phosphorylation or degradation (Figure 14G, data not shown). IκBα is phosphorylated by IκB kinase (IKK) (69, 70). PC3 cells are reported to have constitutively active IKKα, resulting in enhanced IκBα degradation and constitutive active NF-κB activity (67, 71). IKK activity as measured by phosphorylation was also not detectable in our cells by immunoblotting, although stimulation with TNFα did not result in robust detection of phosphorylation of IKKα/β by immunoblotting either (data not shown). Therefore, we cannot yet determine if NF-κB is being activated through the canonical or a non-canonical pathway.

In MCF10A cells, integrin α6 promotes survival by p21-activated kinases 1 (PAK1) activation of NF-κB p65 (72). Others have also shown that multiple signaling pathways can activate NF-κB via PAK1 (73). Thus, we investigated if integrin α6 up-regulates NF-κB expression via activation of PAK1. PAK1 activity

FIGURE 14. AR and integrin α6 regulate NF-κB signaling. PC3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 cells were serum starved in charcoal stripped serum and plated on LMI. **A)** Cells were lysed to monitor RelA phosphorylation (P-NF-κB) and total levels of RelA (NF-κB p65). Tubulin expression was used as a loading control. **B)** Cells were transfected with empty (pGL3-empty) or NF-κB expressing Firefly reporter (pGL4-NFκB) vector and control *Renilla* reporter then treated with vehicle or DHT for 72 hours. Cells were then lysed and luminescence measured. Firefly luminescence activity was normalized to *Renilla* luciferase activity. **C-F)** Cells were treated with siRNA against AR (siAR), integrin α6 (siA6), RelA (siRel), or non-targeting sequence (scr) for 72 hours, and in some cases not treated (NT) or treated with 10 ng/mL TNFα for 1 hours. **C-E)** Cells were then lysed to monitor RelA phosphorylation (P-NF-κB) and total levels of RelA (NF-κB p65), AR, integrin α6 (ITGA6), or Bcl-xL. Tubulin expression was used as a loading control. **F)** Cells were plated on LMI and treated with DMSO or LY294002 (LY) for 72 hours. Cell viability was then measured by trypan blue staining. Error bars represent standard deviation; n = 3. **G)** Cells were treated with siRNA against AR (siAR) or non-targeting sequence (scr) for 72 hours, and in some cases not treated (NT) or treated with 10 ng/mL TNFα for 1 hours. Cells were then lysed to monitor IκBa phosphorylation (P-IκBa) and total levels of IκBa. Tubulin expression was used as a loading controlAd

FIGURE 14


A.
PP AR1 AR2
-P-NF-κB
-NF-κB p65
-tubulin
B.
Luminescence (RLU)
500000
400000
300000
200000
100000
0
PC3-puro
PC3-AR-1
PC3-AR-2
DHT- DHT+ DHT- DHT+
pGL3-empty
pGL4-NFkB
C.
PP AR1 AR2 PP
scr siAR scr siAR scr siAR NT TNFα
-AR
-P-NF-κB
-NF-κB p65
-tubulin
D.
PP AR1 AR2 PP
scr siA6 scr siA6 scr siA6 NT TNFα
-ITGA6
-P-NF-κB
-NF-κB p65
-tubulin
E.
PP AR1 AR2
scr siRel scr siRel scr siRel
-P-NF-κB
-NF-κB p65
-Bcl-xL
-tubulin
F.
% Trypan Blue Cells
100
80
60
40
20
0
DMSO
LY
scr siRel scr siRel scr siRel
PC3-puro PC3-AR-1 PC3-AR-2
G.
PP AR1 AR2 PP
scr siAR scr siAR scr siAR NT TNFα
-P-IκBα
-IκBα
-tubulin

was measured using a phospho-specific antibody that recognizes the active form of PAK1/2. PAK1/2 phosphorylation and activity was increased in both AR clones compared to PC3-puro cells (Figure 15A). Surprisingly, there was decreased expression of PAK1 in AR expressing cells compared to PC3-puro cells, although levels of PAK2 were relatively unchanged (Figure 15A). PAK3 was undetected by immunoblotting (data not shown). To determine if PAK signaling was regulated by AR and integrin α6, AR expressing cells were treated with AR and integrin α6 specific siRNA, with non-specific siRNA (scram) being used as a negative control. Knock-down of AR led to a decrease in PAK1/2 phosphorylation, (Figure 15B). Knock-down of integrin α6 in AR expressing clones to PC3-puro levels resulted in a more dramatic loss in PAK1/2 phosphorylation (Figure 15C). Thus, there is an increase in PAK1/2 activity in AR expressing cells that is both AR and integrin α6 dependent.

Since the phosphorylation-specific antibody recognizes both PAK1 and PAK2, it is difficult to determine which isoform, or if both, may be active. However, since there are low levels of PAK1 in the AR expressing cells, it is likely PAK2 is the predominant active isoform in the AR expressing cells. Immunoprecipitation of PAK1 and immunoblotting with the phospho-specific antibody of these cells suggested that PAK1 was poorly phosphorylated, further supporting that PAK2 is more active (not shown). It has also been reported that PAK2 can inhibit PAK1 activity in PC3 cells (74). If this is the case, then loss of AR or integrin α6 activation of PAK2 should lead to an increase in PAK1. Knock-down of AR in PC3-AR-1 cells, but not PC3-puro cells or PC3-AR-2 cells, results

in increased PAK1 expression (Figure 15B). Loss of integrin α6 in all cell lines resulted in increased PAK1 expression (Figure 15C). Together, this suggests that there is increased PAK activity, probably through PAK2, in AR expressing cells that is both AR- and integrin α6-dependent.

To determine if PAK signaling regulates NF-κB p65 and Bcl-xL, the cells were treated with the group I specific PAK pharmalogical inhibitor, IPA-3 (75). IPA-3 inhibits PAK1-3, with the strongest inhibition observed for PAK1 (75). Treatment of AR expressing cells with IPA-3 resulted in decreased NF-κB phosphorylation and decreased Bcl-xL expression (Figure 15D). IPA-3 treatment has no effect on AR or integrin α6 expression (data not shown). In an additional approach, AR expressing cells were treated with siRNA specific to PAK1. Knock-down of PAK1 was over 90% in AR expressing cells and did not affect PAK2 expression (Figure 15E). Knock-down of PAK1 resulted in only partial loss of PAK1/2 and NF-κB p65 phosphorylation in AR expressing cells (Figure 15E). There was a very modest decrease in total NF-κB p65 levels, and a modest decrease in Bcl-xL expression (Figure 15E). While this suggests that PAK1 may be upstream of NF-κB signaling and Bcl-xL expression, it may not be the only protein regulating this pathway since PAK1 specific knockdown did not result in a dramatic decrease in NF-κB activity or Bcl-xL expression, as was observed with AR or integrin α6 knockdown, or treatment with the group I PAK inhibitor, IPA-3 (Figure 14C,D; Figure 15D,E). PAK1 and PAK2 have some redundant functions; it may be that PAK2 is the predominant PAK as suggested earlier, or that both PAK1 and PAK2 are both required in this pathway. IKKB or p38 has also been

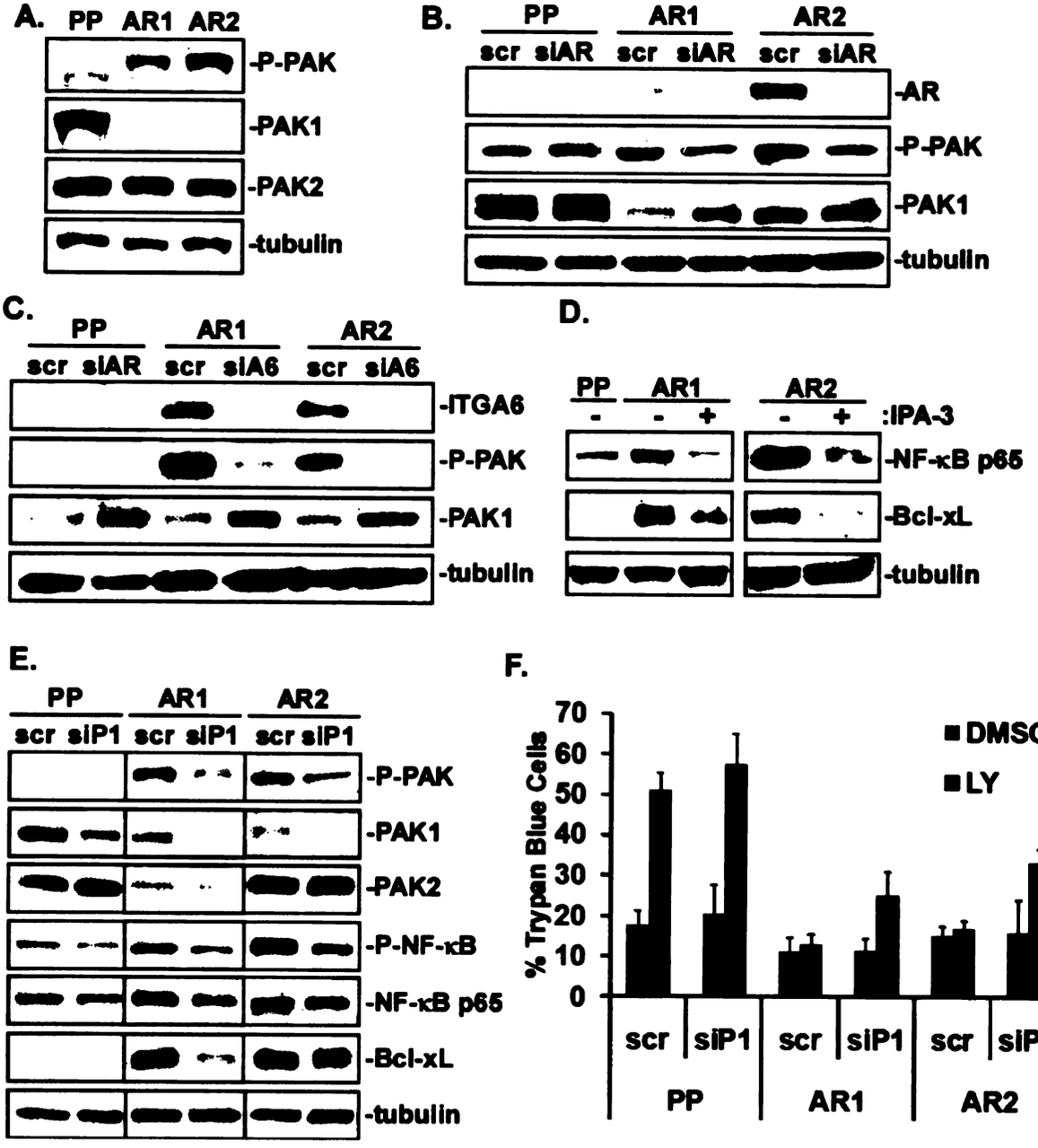
reported to phosphorylate NF-κB p65 (76-78). In agreement with this, knock-down of PAK1 alone using siRNA is unable to restore LY294002 sensitivity to death significantly in AR expressing cells (Figure 15F). PAK4 has also been demonstrated to activate NF-κB signaling (79). Therefore, PAK1 is activated by AR and integrin α6, but there may be redundancy with other PAKs. Other pathways in addition to PAK1 may also responsible for Bcl-xL regulation.

DISCUSSION

In this study, we demonstrated that re-expression of wild type AR in PC3 cells prevented the cell death normally induced upon inhibition of PI3K signaling, independent of androgen. Re-expression of AR in PC3 cells lead to increased expression of integrin α6 and subsequent activation of PAK and NF-κB and increased expression of the pro-survival protein Bcl-xL (Figure 16). Loss of AR, integrin α6, NF-κB, or Bcl-xL re-sensitized AR-expressing PC3 cells to PI3K-dependent survival. Treatment of AR expressing PC3 cells with the AR inhibitor RU486 or AR specific siRNA, or expression of AR mutants lacking the ability to translocate to the nucleus (ΔNLS), but not to bind ligand (ΔLBD), largely restored the parental PC3 phenotype, including PI3K dependent survival. These results are supported by siRNA knock-down of endogenous AR in LNCaP cells. Thus AR can support castration-resistant prostate tumor cell survival on laminin via enhanced expression of α6β1 integrin, leading to elevated Bcl-xL levels, by a mechanism that is independent of PI3K.

FIGURE 15. AR and integrin α6 regulate PAK1/2 signaling. PC3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 cells were serum starved in charcoal stripped serum and plated on LMI. **A)** Cells were lysed to monitor PAK1/2 phosphorylation (P-PAK) and PAK1 and PAK2 expression. Tubulin was used as a positive control. **B-C)** Cells were treated with siRNA against AR (siAR), integrin α6 (siA6), or non-targeting sequence (scr) for 72 hours, then lysed to monitor PAK1/2 phosphorylation (P-PAK) and expression of AR, integrin α6 (ITGA6), PAK1, and PAK2. Tubulin expression was used as a loading control. **D)** Cells were treated with vehicle or 30 □M IPA-3 for 1 hour then lysed and immunoblotted to monitor total levels of RelA (NF-κB p65) and Bcl-xL. Tubulin expression was used as a loading control. **E-F)** Cells were treated with siRNA against PAK1 (siP1) or non-targeting sequence (scr) for 72 hours. **E)** Cells were then lysed and immunoblotted to monitor phosphorylation of PAK1/2 (P-PAK) and RelA (P-NF-κB) as well as PAK1, RelA (NF-κB p65), and Bcl-xL expression. Total loading levels were monitored by probing for tubulin. **F)** Cells were plated on LMI and treated with DMSO or LY294002 (LY) for 72 hours. Cell viability was then measured by trypan blue staining. Error bars represent standard deviation; $n = 3$.

FIGURE 15


A.
PP AR1 AR2
-P-PAK
-PAK1
-PAK2
-tubulin
B.
PP AR1 AR2
scr siAR scr siAR scr siAR
-AR
-P-PAK
-PAK1
-tubulin
C.
PP AR1 AR2
scr siAR scr siA6 scr siA6
-ITGA6
-P-PAK
-PAK1
-tubulin
D.
PP AR1 AR2
- - + - +
:IPA-3
-NF-κB p65
-Bcl-xL
-tubulin
E.
PP AR1 AR2
scr siP1 scr siP1 scr siP1
-P-PAK
-PAK1
-PAK2
-P-NF-κB
-NF-κB p65
-Bcl-xL
-tubulin
F.
% Trypan Blue Cells
70
60
50
40
30
20
10
0
DMSO
LY
scr siP1 scr siP1 scr siP1
PP AR1 AR2

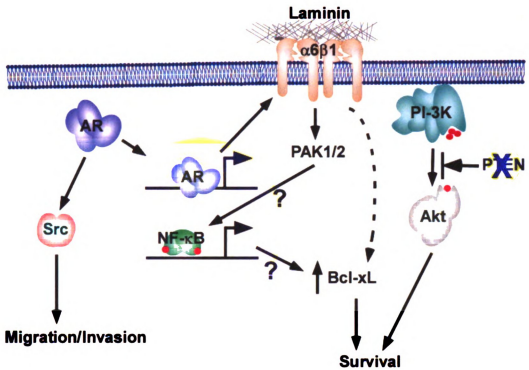


FIGURE 16. Model for AR signaling in PC3 cells. AR promotes cell survival independent of DHT or PI3K signaling. AR regulates survival via the integrin α6, which leads to phosphorylation of PAK1/2 and subsequent up-regulation of NF-κB signaling. This may lead to increased Bcl-xL expression, which regulates survival independent of the PI3K pathway. AR increases Src activity independent of integrin α6. Src activity may regulate migration and invasion.

Inhibition of PI3K signaling with LY294002 resulted in cell death of PC3-puro cells, but only when plated on LM1; PC3-puro cells plated on CLI were resistant to PI3K inhibition. Given this significant difference in survival upon different matrixes, this suggests that the different integrins responsible for adhesion to LM1 and CLI may activate different and distinct signaling pathways that regulate survival through different mechanisms. Work in our lab as well as others have demonstrated that different integrin heterodimers can activate different signaling pathways (6, 36, 45, 80-84). For example, in normal primary prostate epithelial cells (PECs), adhesion to LM1, but not the LM5-rich matrix, is sufficient to activate Akt (Dr. Mat Edick, unpublished data). In primary mammary epithelial cells, cells plated on LM1 or CLIV, but not CLI, allow insulin activation of Akt and subsequent cell survival (80, 81). Previous work from our lab has demonstrated that in PC3 cells, CLI adhesion results in greater p130Cas and FAK activation than LM1 (85). In PC3 cells, integrins $\alpha6\beta4$, $\alpha6\beta1$, and $\alpha3\beta1$ are the primary integrins that bind to laminin, with LM1 being the preferential substrate for $\alpha6\beta1$; the CLI binding integrin is $\alpha2\beta1$ (86, 87). Adhesion to CLI by $\alpha2\beta1$ can lead to activation of p38 MAPK (84), ERK (88), and Rac1 (89). While p38 MAPK is classically associated with the induction of apoptosis, p38 MAPK can also promote cell survival in specific situations, such as in response to DNA damage (90). ERK and Rac1 have also been associated with integrin-mediated survival signaling (91, 92), but ERK is not activated in PC3 cells upon LM1 adhesion (see Chapter 2) (36). However, it remains undetermined if p38, ERK, or Rac1 signaling is increased in PC3 cells adherent to CLI versus LM, and if so,

if it is sufficient to rescue survival independent of PI3K. It is interesting to note that the PC3 cell line was originally derived from a bone metastasis. The bone microenvironment, a primary site for prostate cancer metastasis, is rich in CLI suggesting that metastases to these sites may have to be treated differently therapeutically; targeting PI3K/Akt for bone metastases may not be effective.

One observation that was surprising was that the ΔLBD mutant of AR still localized to the nucleus since AR localization to the nucleus in classically thought to be driven by androgen binding to AR and this mutant cannot bind androgen. Other studies using this mutant have not looked at cellular localization directly but have verified its inability to bind DHT or other androgens (30, 34, 93). However, expression of this mutant in an in vivo prostate regeneration assay along with Akt overexpression also resulted in AR localizing predominantly in the nucleus (30). AR is kept in the cytoplasm through its interactions with Hsp90; it is interesting to speculate that the ΔLBD mutant of AR may have impaired Hsp90 binding, since Hsp90 interacts with AR within AR's ligand binding domain (94).

Akt and wild-type AR co-overexpression, but not Akt or AR overexpression alone, resulted in invasive carcinoma that was androgen insensitive and retained distinct nuclear AR localization even seven weeks after surgical castration (30). Normal prostate epithelial cells and PIN lesions within the same mice showed AR localization to the cytoplasm after castration (30). Since the PC3 cells harbor a Pten mutation resulting in constitutive Akt activity, these data suggests the possibility that high Akt activity can drive AR activity and localization to the nucleus independent of androgen. Several groups have investigated the role of

Akt on AR transcriptional activity with conflicting results, possibly due to the different cell lines, passages, and conditions used as well as the many possible and complex interactions between these two pathways (95-97). However in our cells, AR was able to promote survival independent of PI3K. This suggests that AR nuclear localization and probably transcriptional activity is independent of direct Akt phosphorylation of AR, consistent with previous in vitro and in vivo reports in which the putative Akt phosphorylation sites on AR were mutated (30, 98). This suggests that another mechanism may be driving this or that those putative Akt phosphorylation sites on AR are not required (30, 98). PI3K/Akt activation of AR may also be redundant, leading to increased integrin α6 and Bcl-xL; however this mechanism not appear to be necessary since blocking PI3K alone does not inhibit survival when AR is present.

Our observation that AR results in increased integrin α6 expression is consistent with the observation that constitutive AR expression in immortalized prostate epithelial cells leads to increase integrin α6 (22). Integrin α6 expression in prostate cancer cells is associated with increased cell migration and invasion in vitro and in vivo (83). However, previous AR re-expression studies in PC3 or DU145 cells did not report an increase in integrin α6 expression, rather these authors noticed a slight to more significant decrease in α6 (19-21).

Other studies have also reported conflicting results on AR regulation of cell survival (33, 99, 100). Possible explanations for these discrepancies include differences in the level of AR re-expression, use of FBS versus CSS, duration of growth-factor and serum starvation prior to experimental assays, and passage

number used (19-21, 33, 99, 100). Studies by Nagakawa et al. suggest that in DU145 cells, AR expression results in decreased integrin α6 expression. However, PC3 and DU145 cells may not behave the same. Indeed, AR re-expression has been demonstrated to act differently in these cells, presumably due to differences in important AR co-activator proteins (101). We are currently investigating these pathways in DU145 cells that re-express AR.

Integrin α6 and AR are both expressed in normal secretory cells (18), suggesting that in vivo AR does not necessarily inhibit integrin α6 expression. It is interesting to note that in a study by Evangelou et. al., which did not look at integrin α6, and in a study by Bonaccorsi et. al., AR alterations in integrin surface expression in PC3s were also independent of androgen, although mRNA levels were sometimes affected (19, 20). Similarly in LNCaP cells, androgen stimulation also affects mRNA expression of AR but the protein levels remain unaffected (102, 103). A significant difference was that all of the integrin expression assays in these other studies were done on plastic, while in our studies cells were adherent to LM1. Adhesion to LM1 might result in integrin α6 stabilization, explaining this observed difference. It may be that in prostate cancer, elevated integrin α6β1 expression is also dependent on engagement of the integrin by laminin. Lastly, AR localization may be important difference between our study and others. In our cells, AR localization was both nuclear and cytoplasmic; Bonaccorsi et. al. reported in subsequent studies with their cells, AR is associated with plasma membrane and specifically associates with EGFR (104). However, this localization and association with EGFR has not been

reported to date in vivo (105), and could possibly be limited by the specific antibody used.

The transcriptional mechanisms that control α6 integrin expression have not been systematically studied, much less those associated with AR. Progesterone stimulation can increase integrin α6 promoter activity six fold in MCF-7 cells, suggesting that steroid signaling can activate this promoter (23). Analysis of the cloned integrin α6 promoter did not immediately reveal any canonical AR response element binding sites (AREs) (106, 107). Also, recent expression microarray techniques and chromatin immunoprecipitation (ChIP) with on-array detection (ChIP-chip) have identified hundreds of potential AR transcriptional targets that also do not contain canonical AREs (108-112). There are some ARE half sites as well as an imperfect glucocorticoid response element (data not shown) (106, 107). The glucocorticoid element is flanked by AP1 binding sites (106, 107). Steroid receptors, including AR, have been shown to form active transcriptional complexes with AP1 (113, 114). Lastly, there is also an Ets binding site in the promoter and AR can physically interact with Ets proteins (106, 107). Furthermore, the Ets-binding sites were found ~70% of AR-rich promoters by ChIP-chip in LNCaP cells and Ets-1 is reported to be overexpressed in prostate cancer (109, 115). Recent identification of the *TMPRSS2/Erg-Etv* fusion genes in prostate cancer suggest that Ets-like transcription factors may play an important role in activating genes involved in prostate cancer development (116). The TMMPRSS2/*Erg-Etv* genes are fused to an AR-dependent promoter, therefore the Erg/Etv gene targets would be

expected to depend on AR for their expression. Thus, in prostate cancer, integrin α6 expression may be up-regulated due to the combined effect of AR and Erg/Etv transcriptional mechanisms. AR may bind to ARE-like/GRE elements within the integrin α6 promoter in conjunction with either AP1 or Ets to stimulate α6 transcription, and in AR-over expressing cells, transcription can be regulated independently of androgen. However, AR may not directly regulate the transcription of integrin α6, but rather regulate α6 through an alternative mechanism such as mRNA stability. This remains a topic of current investigation in our lab.

We observed that AR re-expression resulted in increased expression of the pro-survival protein Bcl-xL, and that knock down of AR resulted in decreased Bcl-xL. We, as well as others, have also observed that AR knockdown in LNCaP cells results in decreased Bcl-xL (2). We have proposed that this is dependent on increased integrin α6 expression, activating NF-κB signaling, which can directly regulate Bcl-xL expression. However, AR has also been reported to directly regulate Bcl-xL expression (51), which could explain why NF-κB p65 knock-down only resulted in partial loss of Bcl-xL (Figure 14E). The dramatic loss in Bcl-xL expression when integrin α6 is knocked down in our cells though suggests that in this context, AR-regulation of Bcl-xL is predominantly regulated through integrin α6. It may be that integrin α6 regulates Bcl-xL through additional mechanisms outside of NF-κB signaling.

Integrin-linked kinase (ILK) is a direct binding partner for β integrins and is activated upon adhesion (117). In prostate cancer cells, inhibition of ILK activity

results in reduced hypoxia-inducible factor-1α (HIF-1α) and overexpression of ILK results in increased activity of a HIF-1α reporter assay (118) in normoxic conditions. Recently, HIF-1α has been shown to directly drive the transcription of Bcl-xL in PC3 cells (119). Interestingly, HIF-1α can also drive LM5 expression in keratinocytes (120). HIF-1α has been reported to increase during prostate progression (121-123); expression of both HIF-1α and HIF-2α are highly correlated with AR (121) and androgen has been reported to also stimulate HIF-1α activity in LNCaP cells (124). Therefore, an alternative pathway for Bcl-xL regulation by integrin α6 may be through β1 activation of ILK, and subsequently HIF-1α. Furthermore in a tumor environment in vivo, HIF-1α could be further activated independent of ILK due to low oxygen availability within the tumor.

There are several major advantages of this PC3 model. First, the ability to test the effects of AR mutants on cell phenotype is not complicated by endogenous AR. AR was sequence verified to be wild-type and not contain any mutations. AR was not significantly "over-expressed" at levels greater than observed in LNCaP cells. Cells were isolated and grown in CSS in order to prevent continuous ligand-activation of AR. Cells were also grown in F-12K media, which has a lower concentration of phenol red, which has been demonstrated to activate steroid receptors. Multiple clones were used, verifying that any effects observed were not the result of clonal selection. We used only low passage number cells, since phenotypes can change with passage. AR expression resulted in decreased cell proliferation, consistent with previous reports (19, 20, 33). Second, any observable phenotypes can be attributed to

AR by direct testing with AR siRNA. Third, re-expression of AR generates changes in protein expression that mimic some of those seen in vivo including integrin α6β1 expression. Fourth, these cells are poorly responsive to androgens. One possible reason for this is that these cells may lack some of the AR co-activators. However, the advantage is that this allows for easier identification of castration-resistant mechanisms that can operate in the absence of classical responses, which may not be easily seen in cells that can use androgen more effectively. Last, we believe we have identified at least two distinct castration-resistant pathways that are operating in the same cell that are responsible for two different biological responses; AR regulation of survival through integrin α6/NF-κB/Bcl-xL and AR regulation of Src and migration. This model does have limitations. PC3 cells do not express AR or Pten and did not show classical androgen responses. Since no model is perfect, future analyses will examine additional cell types with several oncogenic backgrounds and express transient rather than stable expression of AR. Lastly, we are also investigating these pathways in an in vivo setting using mouse xenografts to ensure that these observations are not restricted to tissue culture.

In summary, we have developed a prostate cancer model that recapitulates some clinical aspects of advanced, hormone refractory prostate cancer. Re-expression of AR in PC3 cells and adhesion to LM1 can drive cell survival independent of PI3K signaling through up-regulation of integrin α6 expression, NF-κB signaling, and Bcl-xL expression. Loss of AR by siRNA, or a mutation inhibiting nuclear localization, restores dependence on PI3K signaling

for survival. We have also discovered a unique connection between AR and integrin α6β1 in regulating prostate cancer survival. We have verified these results in additional prostate tumor cell line, LNCaP, which expresses endogenous AR. To date, our observations have been limited to in vitro assays and we have yet to determine these mechanisms are relevant in vivo. Application of this new knowledge may lead to the development of better prostate cancer therapies, and supports the importance of targeting more than one pathway in order to effectively treat castration-resistant prostate cancer.

MATERIALS AND METHODS

Cell Culture

The prostate tumor cell lines, PC3 and LNCaP, were purchased from American Type Culture Collection. PC3 cells were grown in F-12K media supplemented with 10% charcoal-stripped and dextran-treated fetal bovine serum (CSS), 2mM glutamine, 50 U penicillin, and 50 μg/mL streptomycin. LNCaP cells were grown in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum, 2mM glutamine, 50 U penicillin, 50 μg/mL streptomycin, 0.225% glucose, 10 mM HEPES, and 1mM sodium pyruvate. For experiments, LNCaP cells were grown in phenol-red free media and 10% CSS for 48 hrs beforehand and during the experiment duration. Phoenix-ampho cells (Oribigen) and 293-FT cells (Invitrogen) were cultured in DMEM (Gibco) supplemented with 10% HI-FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate. All cells were grown at 37°C and 5% CO_2.

DNA Plasmids

The pBabe-puro-hAR and pGL3-basic plasmids were kindly provided by Dr. Beatrice S. Knudsen (Fred Hutchinson Cancer Research Institute). The pCSCG-AR-ΔNLS plasmid, which has three point mutations (K618M, K632M and K633M) that disrupt nuclear translocation, and pCSCG-AR-N705S (ΔLBD) plasmid, which is unable to bind ligand, were generously provided by Dr. Owen N. Witte (University of California at Los Angeles) (30, 110). The pLKO.3pg was kindly provided by Dr. Jeff MacKeigan (Van Andel Institute). The pBabe-puro-Bclxl construct was a gift from Dr. Douglas R. Green (St. Jude's Children's Hospital). The Virapower packaging system was from Invitrogen. Lastly, the pGL4.32[*luc2P*/NF-κB-RE/Hygro], and phRG-TK were purchased from Promega.

All AR plasmids were sequence verified to contain wild type sequence or the respective mutations to the sequence. The following primers, designed by Dr. Aaron Putzke (Fred Hutchinson Cancer Research Institute), were used (sequences listed 5' to 3'): AAGCTCAAGGATGGAAGTGC, AGCAACCTTCACAGCCGCAG, AAGCTCAAGGATGGAAGTGC, AGCAACCTTCACAGCCGCAG, GGGCACTTCGACCATTTCTG, CTACAAGTCCGGAGCACTGG, GCGGCATGGTGAGCAGAGTG, CTTGTCGTCTTCGGAAATG, CTTGTCGTCTTCGGAAATG, CTTGTCGTCTTCGGAAATG, GTGGACGACCAGATGGCTGTC, ACATCCTGCTCAAGACGCTTC, AATGCTTCACTGGGTGTGG,

AACTCTTGAGAGAGGTGCCTC, AACTCTTGAGAGAGGTGCCTC, AACTCTTGAGAGAGGTGCCTC, AACTCTTGAGAGAGGTGCCTC, GAGGCTAGAGAGCAAGGCTG, GCAGCTTCCACATGTGAGAG, GTCCGGAGTAGCTATCCATC, TTCTCCAGCTTGATGCGAGC, CCAAAAGTGGGGCGTACATG, and GGCAGCTGAGTCATCCTCGT.

Establishment of Cell Lines

PC3-puro, PC3-AR, and PC3-Bclxl cells were made by infecting cells with pBabe-puro, pBabe-puro-hAR, or pBabe-puro-Bclxl retroviruses respectively. Retroviruses were produced in Phoenix 293 cells by transfecting 5 μg of DNA with 3 μl/μg Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) following manufacturer's directions. Cells were maintained at 32°C and 5% CO_2 and the media was changed the next day. Retroviruses in the conditioned medium were collected daily between days five and seven. Viruses were separated from cellular debris by spinning conditioned media for 5 minutes at 1,500 rpm at room temperature. The supernatant was then passed through a 0.45 μm low-binding filter (Millipore) and frozen at -80°C until use. For infection of PC3 cells, 2.5 mL of filtered virus were added per 10 cm dish of PC3 cells. After a three hour incubation, 5 μg/mL of Polybrene (Sigma) was added to cells. The media was changed the next day and infected PC3 cells were maintained at 37°C and 5% CO_2. Clones were selected for and maintained in 2 mg/mL puromycin (Sigma). Only low passage (i.e. under passage 20) cells were used for experiments.

PC3-LKO, PC3-ΔNLS, and PC3-ΔLBD cells were made by co-infecting cells with pLKO.3pgw, pCSCG-AR-ΔNLS, or pCSCG-AR-N705S lentiviruses respectively along with pLP1, pLP2, and pLP/VSVG lentiviruses. Lentiviruses were made in 293-FT cells that were pre-selected for in 1 μg/mL Diphtheria Toxin (Sigma) and 300 μg/mL of hygromycin (Invitrogen). 4 μg of each plasmid were transfected into the 293-FT cells with 2.5 μl/μg Lipofectamine 2000 in Opti-MEM following manufacturer's directions. Virus was collected from the conditioned medium four and five days after the transfection by spinning at 1,000 rpm for 10 minutes then passing through a 0.22 μm low-binding filter (Millipore). Virus was kept at -80°C until use. For infection of PC3 cells, 1.5 mL of filtered virus and 5 μg/mL polybrene were added per 10 cm dish of PC3 cells. The media was changed the next day and clones selected.

Small Interfering RNA Transfections

A pool of four small interfering RNAs (siRNA) against androgen receptor (siGENOME SMARTpool; 5 nM for PC3-AR clones and 50 nM for LNCaP cells), integrin α6 (ON-TARGETplus SMARTpool; 20 nM), Bcl-xL (siGENOME SMARTpool; 0.5 nM), RelA (ON-TARGETplus SMARTpool; 25 nM), PAK1 (siGENOME SMARTpool; 50 nM), Src (ON-TARGETplus SMARTpool; 20 nM) or a non-targeting sequence were purchased from Dharmacon. Sequences are listed in Table 1. Cells were transfected with siRNA using siLentFect lipid reagent (Bio-Rad) and Opti-MEM (Invitrogen) media following manufacturer's directions. The media was changed 16 hours after transfection.

Table 1. siRNA Sequences

Gene	NM#	Product No.	siRNA Target Sequence
AR	NM_000044	D-003400-01	GGAACUCGAUCGUAUCAUUU
		D-003400-02	CAAGGGAGGUUACACCAAAUU
		D-003400-03	UCAAGGAACUCGAUCGUAUUU
		D-003400-04	GAAAUGAUUGCACUAAUUGAUU
Bcl-xL	NM_138578	D-003458-01	CAGCUUGGAUGGCCACUUAUU
		D-003458-02	ACAAGGAGAUGCAGGUAUUUU
		D-003458-03	CCUACAAGCUUUCCCAGAAUU
		D-003458-04	GGUAGUGAAUGAACUCUUCUU
ITGA6	NM_000210	J-007214-05	GGAUCGAGUUUGAUAACGAUU
		J-007214-06	GGAUAUGCCUCCAGGUUAAUU
		J-007214-07	GAAAGGGAUUGUUCGUGUAUU
		J-007214-08	ACAGAUAGAUGAUAACAGAUU
PAK1	NM_002576	D-003521-03	CAUCAAAUAUCACUAAGUC
		D-003521-05	CAACAAAGAACAAUCACUA
		D-003521-07	AGAAAUACCAGCACUAUGA
		D-003521-21	GUGAAAAUGCUCUCGGCUAU
PAK2	NM_002577	D-003597-05	AGAAGGAACUGAUCAUUAA
		D-003597-22	GAGCAGAGCAAACGCAGUA
		D-003597-23	CGACCGGAUCAUACGAAAU
		D-003597-24	GCGACCGGAUCAUACGAAA
RELA	NM_021975	J-003533-06	GGAUUGAGGAGAAACGUAA
		J-003533-07	CCCACGAGCUUGUAGGAAA
		J-003533-08	GGCUAUAACUCGCCUAGUG
		J-003533-09	CCACACAACUGAGCCCAUG
SRC	NM_198291	J-003175-13	GCAGUUGUAUGCUGUGGUUUU
		J-003175-14	GCAGAGAACCCGAGAGGGAUU
		J-003175-15	CCAAGGGCCUCAACGUGAAUU
		J-003175-16	GGGAGAACCUCUAGGCACAUU
scram/ non-targeting	n/a	D-001210-01	UAGCGACUAAACACAUCAA
		D-001210-02	UAAGGCUAUGAAGAGAUAC
		D-001210-03	AUGUAUUGGCCUGUAUUAG
		D-001210-04	AUGAACGUGAAUUGCUCAA

Immunoblotting

Total cell lysates were prepared for immunoblotting as previously described (36, 125). Briefly, cells were lysed on ice with MAPK lysis buffer (50 mM Tris pH 7.5, 0.5 mM EDTA, 50 mM NaF, 100 mM NaCl, 50 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1% Triton-X100, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM benzamide) or RIPA (10 mM Tris pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NaDOC, 1% Triton-X100, 1 mMNa3VO4, 1 mM PMSF, 100U mL aprotinin, 10 ug/mL pepstatin, and 10 ug/mL leupeptin) buffers and 40-65 μg of total cell lysates in 2X SDS sample buffer were boiled for 10 minutes. Samples were run on SDS polyacrylamide gels following standard SDS-PAGE protocols and transferred to PVDF membranes. Membranes were blocked in 5% BSA in TBST for two hours at room temperature, and then were probed with primary antibody for two hours at room temperature. Primary antibodies were used at the indicated dilutions in Table 2. Membranes were washed three times, and incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) in 5% BSA in TBST for 1 hour at room temperature. After washing an additional three times, signals were visualized by chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

Table 2. Immunoblotting Antibodies

Protein	Clone	WB Dilution	Company
mAb AR	411	1:200	Santa Cruz
rAb Bcl-xL		1:1000	Cell Signaling
rAb Bim		1:1000	AbCam
rAb Phospho- IκBα (S32)	14D4	1:1000	Cell Signaling
mAb IκBα	44D4	1:1000	Cell Signaling
rAbITGA6	AA6A	1:10,000	Gift of A. Cress
mAb ITGB1	18/CD29	1:1000	BD Transduction
mAb ITGB4	ASC-3	1:1000	Chemicon
rAb Phopsho- NF-κB (S536)	93H1	1:1000	Cell Signaling
rAb NF-κB p65		1:1000	Cell Signaling
rAb Nkx3.1	H-50	1:500	Santa Cruz
rAb Phospho- PAK1 (S198/204)/PAK2 (S192/197)		1:1000	Cell Signaling
rAb PAK1		1:1000	Cell Signaling
rAb PAK2		1:500	Cell Signaling
gAb PAK3	N-19	1:200	Santa Cruz
goatAb PSA	C-19	1:200	Santa Cruz
rAb c-Rel		1:500	Cell Signaling
rAb Phospho- Src (Y418)		1:1000	Biosource
mAb Non-phospho- Src (Y529)		1:2000	Biosource
mAb Src	327	1:1000	VAI
mAb TMPRSS2	P5H9-A3	1:2000	Gift of P.S. Nelson
mAb α-tubulin	DM1A	1:10,000	Sigma

Immunoprecipitation

Cells were lysed in RIPA or MAPK lysis buffer as described previously and 500-1000 μg of protein was incubated with 1 μg per sample of Src clone 327 antibody (Van Andel Institute) and protein G-conjugated agarose beads (Pierce) for three hours at 4°C. The beads and immunoprecipitated complexes were then washed three times in RIPA lysis buffer. The beads and immunoprecipitated complexes were then spun down at 13,000 rpm for four minutes and resuspended in 2X

SDS sample buffer, boiled for 10 minutes, then the supernatant was immunoblotted as previously described.

Immunofluorescence

To determine AR localization, cells were fixed with 4% paraformaldehyde in PBS (Mallinckrodt Chemicals) for ten minutes and permeabilized four minutes with 0.2% TritonX-100 (EMD) at room temperature. Cells were then blocked with 10% normal goat serum (Pierce) for 2 hours at room temperature before incubation with Androgen Receptor clone 411 antibody (Santa Cruz) overnight at 4°C. Cells were incubated with goat anti-mouse Alexa Flour-488 secondary antibody (Molecular Probes, Invitrogen) for one hour at room temperature. DNA was visualized by staining with Hoechst 33258 (Sigma) for 10 minutes at room temperature. Cells were washed three times with PBS between all steps. Coverslips were mounted on the slides using Gel-Mount (Biomeda). Whole mouse IgG antibody (Pierce) was used as a control. Epifluorescent images were acquired on a Nikon Eclipse TE300 fluorescence microscope using OpenLab v5.5.0 image analysis software (Improvision).

To examine focal adhesions and actin structures, cells were fixed with 4% paraformaldehyde in PBS at 4°C for twenty minutes and permeabilized for ten minutes with TBS (10 mM Tris, pH 8.0, 150 mM NaCl) + 0.5% TritonX-100 at room temperature. Cells were then blocked with 2% BSA in TBS + 0.1% TritonX-100 for twenty minutes at room temperature before incubation with vinculin antibody (Sigma) and Alexafluor 546-Phalloidin (Molecular Probes, Invitrogen) for

one hour. Cells were incubated with goat anti-mouse Alexa Flour-488 secondary antibody for one hour at room temperature. DNA was visualized by staining with Hoechst 33258 for 10 minutes at room temperature. Cells were washed four times with TBS + 0.1% TritonX-100 over ten minutes between all steps. Slides were prepared and images acquired as previously mentioned.

Cell Surface Integrin Expression Analysis

Cells were placed in suspension by washing the cells with PBS, detached from plates using trypsin. The action of Trypsin was inhibited using soybean trypsin inhibitor (Invitrogen). Cells were then washed with wash buffer (1% sodium azide/2% FBS/PBS) and then incubated with the appropriate primary antibodies (Table 3) or control IgG molecules for 1 hour at 4°C. Cells were then washed twice and incubated with fluorescently-labeled secondary antibodies (Molecular Probes, Invitrogen) for 1 hour at 4°C in the dark. Cells were washed twice more, and fluorescence was detected by a Becton-Dickinson FACSCalibur 4-color flow cytometer with CellQUEST Pro Software v5.2.1 (Becton-Dickinson).

Table 3. Flow Cytometry Antibodies

Protein	Clone	FACS Dilution	Company
mAb ITGA2	CBL 477	1:250	Chemicon
mAb ITGA3	MAB 2056	1:250	Chemicon
mAb ITGA5	P1D6	1:250	Santa Cruz
mAb ITGB4	ASC-3	1:200	Chemicon
ratAb ITGA6	GoH3	1:200	BD Pharmingen
ratAb ITGB1	AIIB2	1:250	Iowa State Univ. Hybridoma Bank

Cell Survival Assays

Cells were serum starved for 48 hours and plated on 1% BSA blocked 24-well tissue culture plates pre-coated 10 μg/mL natural mouse laminin 1 (Invitrogen) or rat tail collagen 1 (Becton, Dickinson and Company) as described previously (36, 125). In some cases, cells had been transfected with siRNA 72 hours prior to assay. Then DMSO (vehicle; Sigma) or 20 μM of pharmacological inhibitor LY294002 (Calbiochem) was added. Cells were allowed to adhere for 4 hours and then non-adherent cells were removed and DMSO or LY294002 was replaced. Cells were incubated for an additional 72-88 hours. LY294002 was replenished 48 hours after plating. In some cases ethanol (vehicle; Decon Laboratories) or 10 nM 5α-dihydrotestosterone (DHT; Sigma or Steraloids) was also added to the cells. DHT was replenished every 24 hours.

To assess cell viability, both attached and floating cells were collected. Attached cells were removed by trypsinization, pooled with floating cells, and all cells were washed once. Cells were then spun down for 8 minutes at 800 rpm and resuspended in a small volume. Trypan Blue (Invitrogen) was mixed in an equal ratio with cells and cells that took up Trypan Blue were counted as dead. A minimum of three separate counts per well were performed using a hemocytometer, with two to three wells counted for each condition per experiment. All experiments were replicated a minimum of three times. Occasionally, DNA fragmentation was monitored using Terminal Deoxynucleotide Transferase dUTP Nick End Labeling (TUNEL) following the protocol of the APO-BrdU TUNEL Assay Kit (BD Pharmingen). On several

occasions, the sub-G1 population as determined by propidium iodide (Invitrogen) staining was also used to measure cell death. Cells were placed into suspension, washed with PBS, and fixed with 1% paraformaldehyde in PBS for 15 minutes on ice. Cells were washed twice with PBS, then incubated in 70% ethanol at 4°C overnight to permeabilize. Cells were again washed twice in PBS, incubated with 1 μg/mL propidium iodide and 100 μg/mL RNase A for 30 minutes at room temperature, and washed twice more before analyzing by flow cytommetry.

RT-PCR

Total RNA was isolated from cells using TRIzol (Gibco) and chloroform (Sigma-Aldrich). Contaminating DNA was removed using RNAse-free DNAse kit (Qiagen) following manufacturer's directions. The RNA was further purified with the RNAeasy Total RNA isolation kit (Qiagen) following manufacturer's directions. The purified RNA was eluted in 30 μl water. Concentration and purity were determined by SmartSpec3000 UV spectrophotometer (Bio-Rad).

RT-PCR was performed on 1 μg RNA with the indicated primers (Table 4) using the One-Step RT-PCR kit (Qiagen) following manufacturer's directions. The thermal cycling parameters were 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 sec, and 72 °C for 60 s, and a last step of 72°C for 10 min. RT-PCR products were analyzed on a 2% agarose/TBE gel and DNA was visualized with ethidium bromide and a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

For qRT-PCR, 0.5 μg RNA was reversed transcribed with random primers using a reverse transcription system (Promega). The synthesized cDNA was diluted 1:25 in water and amplified for qRT-PCR analysis using SYBR green master mix (Roche) with the indicated primers (Table 4) and an ABI 7500 RT-PCR system (Applied Biosystems) following manufacturer's directions. Gene expression was normalized to 18s rRNA by the $2^{-\Delta\Delta Ct}$ method (126).

Table 4. qRT-PCR Primers

Gene	Fwd Primer (5'→3')	Rev Primer (5'→3')	Ref.
Bcl2L1	CGATGGAGGAGGAAG CAAGC	GCACCACCTACATTCA AATCC	Sun et al., 2008
GAPDH	ACCACAGTCCATGCCA TCAC	TCCACCACCCTGTTGC TGTA	Sun et al., 2008
ITGA6	GCTGGTTATAATCCTT CAATATCAATTGT	TTGGGCTCAGAACCTT GGTTT	Tapia et al., 2008
ITGB1	GTGGTTGCTGGAATTG TTCTTATT	TTTTCCCTCATACTTCG GATTGAC	Tapia et al., 2008
18s rRNA	CCGCAGCTAGGAATAA TGGA	CGGTCCAAGAATTTCA CCTC	Ottosen et al., 2006

Transfection Assays

Cells were plated at 3 x 10^5 cells per well in a six-well tissue culture plate on 1% BSA blocked pre-coated 10 μg/mL laminin 1 and allowed to grow overnight. Cells were then washed twice and the media was replaced with F-12K media and 10% CSS with no antibiotics. 1.25 μg DNA of pGL3-basic or pGL4.32[*luc2P*/NF-κB-RE/Hygro], and 0.5 μg phRG-TK was incubated with Nanojuice Core Trasfection Reagent and Nanojuice Booster Reagent in Optimem following manufacturer's directions then added to cells with the following modifications: 4 μl of Core Reagent and 2 μL of Booster Reagent were used per μg of DNA. After

48 hours, cells were lysed with Dual-Luciferase Reporter Assay System (Promega) and luminescence measured using EnVision 2104 Multilabel Reader (PERKin Elmer) and Wallac EnVision Manager Software v1.11 following manufacturer's directions. Firefly luminescence activity was normalized to *Renilla* luciferase activity.

ACKNOWLEDGEMENTS

We thank Veronique Schulz, Rich West, Lia Tesfay, and Dr. Brendan Looyenga at the Van Andel Institute (VAI) for technical assistance, and we acknowledge the laboratories of Developmental Cell Biology, Systems Biology, and Cell Structure and Signal Integration at VAI for their constructive suggestions. We also thank Drs. Beatrice S. Knudsen and Peter Nelson (Fred Hutchinson Cancer Research Institute, Seattle, WA), Owen N. Witte (University of California at Los Angeles, Los Angeles, CA), Jeff MacKeigan (VAI), Douglas R. Green (St. Jude Children's Research Hospital, Memphis, TN), and Anne Cress (University of Arizona, Phoenix, AZ) for generous supply of reagents and Dr. Aaron Putzke (Fred Hutchinson Cancer Research Institute) for AR sequencing primer design. This work was supported by the Department of Defense Prostate Cancer Predoctoral Training Award (W81XWH-08-1-0058) (L.L), and the American Cancer Society (RSG-05-245-01-CSM) (J.Z. and C.K.M). Additional support was also provided by the generous gifts of the Van Andel Institute.

REFERENCES

1. American Cancer Society. Cancer Facts and Figures 2009. Atlanta, GA: American Cancer Society; 2009.

2. Liao X, Tang S, Thrasher JB, Griebling TL, Li B. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol Cancer Ther* 2005 April 1, 2005; **4** (4): 505-515.

3. Yang Q, Fung K-M, Day W, Kropp B, Lin H-K. Androgen receptor signaling is required for androgen-sensitive human prostate cancer cell proliferation and survival. *Cancer Cell International* 2005; **5** (1): 8.

4. Cohen MB, Rokhlin OW. Mechanisms of prostate cancer cell survival after inhibition of AR expression. *J Cell Biochem* 2009 Feb 15; **106** (3): 363-371.

5. Hynes R. Integrins: Bidirectional, Allosteric Signaling Machines. *Cell* 2002; **110** (6): 673-678.

6. Miranti C, Brugge J. Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* 2002; **4** (4): 83-90.

7. Reddig PJ, Juliano RL. Clinging to life: cell to matrix adhesion and cell survival. *Cancer Metastasis Rev* 2005 Sep; **24** (3): 425-439.

8. Harnois C, Demers MJ, Bouchard V, Vallee K, Gagne D, Fujita N, *et al.* Human intestinal epithelial crypt cell survival and death: Complex modulations of Bcl-2 homologs by Fak, PI3-K/Akt-1, MEK/Erk, and p38 signaling pathways. *J Cell Physiol* 2004 Feb; **198** (2): 209-222.

9. Boisvert-Adamo K, Longmate W, Abel EV, Aplin AE. Mcl-1 is required for melanoma cell resistance to anoikis. *Mol Cancer Res* 2009 Apr; **7** (4): 549-556.

10. Bonkhoff H, Stein U, Remberger K. Differential expression of alpha 6 and alpha 2 very late antigen integrins in the normal, hyperplastic, and neoplastic prostate: simultaneous demonstration of cell surface receptors and their extracellular ligands. *Hum Pathol* 1993 Mar; **24** (3): 243-248.

11. Cress AE, Rabinovitz I, Zhu W, Nagle RB. The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. *Cancer Metastasis Rev* 1995 Sep; **14** (3): 219-228.

12. Davis TL, Cress AE, Dalkin BL, Nagle RB. Unique expression pattern of the alpha6beta4 integrin and laminin-5 in human prostate carcinoma. *Prostate* 2001 Feb 15; **46** (3): 240-248.

13. Goel HL, Alam N, Johnson IN, Languino LR. Integrin signaling aberrations in prostate cancer. *Am J Transl Res* 2009; **1** (3): 211-220.

14. Nagle RB, Hao J, Knox JD, Dalkin BL, Clark V, Cress AE. Expression of hemidesmosomal and extracellular matrix proteins by normal and malignant human prostate tissue. *Am J Pathol* 1995 Jun; **146** (6): 1498-1507.

15. Nagle RB, Knox JD, Wolf C, Bowden GT, Cress AE. Adhesion molecules, extracellular matrix, and proteases in prostate carcinoma. *J Cell Biochem Suppl* 1994; **19:** 232-237.

16. Pontes-Junior J, Reis ST, Dall'oglio M, Neves de Oliveira LC, Cury J, Carvalho PA, *et al.* Evaluation of the expression of integrins and cell adhesion molecules through tissue microarray in lymph node metastases of prostate cancer. *J Carcinog* 2009; **8:** 3.

17. Knudsen BS, Miranti CK. The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. *J Cell Biochem* 2006 Oct 1; **99** (2): 345-361.

18. Ports MO, Nagle RB, Pond GD, Cress AE. Extracellular engagement of alpha6 integrin inhibited urokinase-type plasminogen activator-mediated cleavage and delayed human prostate bone metastasis. *Cancer Res* 2009 Jun 15; **69** (12): 5007-5014.

19. Bonaccorsi L, Carloni V, Muratori M, Salvadori A, Giannini A, Carini M, *et al.* Androgen Receptor Expression in Prostate Carcinoma Cells Suppresses {alpha}6{beta}4 Integrin-Mediated Invasive Phenotype. *Endocrinology* 2000 September 1, 2000; **141** (9): 3172-3182.

20. Evangelou A, Letarte M, Marks A, Brown TJ. Androgen Modulation of Adhesion and Antiadhesion Molecules in PC-3 Prostate Cancer Cells Expressing Androgen Receptor. *Endocrinology* 2002 October 1, 2002; **143** (10): 3897-3904.

21. Nagakawa O AT, Hayakawa Y, Junicho A, Koizumi K, Fujiuchi Y, Furuya Y, Matsuda T, Fuse H, Saiki I. Differential expression of integrin subunits in DU-145/AR prostate cancer cells. *Oncology Reports* 2004 May 28, 2004; **12:** 837-841.

22. Whitacre DC, Chauhan S, Davis T, Gordon D, Cress AE, Miesfeld RL. Androgen Induction of in Vitro Prostate Cell Differentiation. *Cell Growth Differ* 2002 January 1, 2002; **13** (1): 1-11.

23. Nishida K, Kitazawa R, Mizuno K, Maeda S, Kitazawa S. Identification of regulatory elements of human alpha 6 integrin subunit gene. *Biochem Biophys Res Commun* 1997 Dec 18; **241** (2): 258-263.

24. Dong JT. Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Metastasis Rev* 2001; **20** (3-4): 173-193.

25. McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res* 1999 Sep 1; **59** (17): 4291-4296.

26. Schmitz M, Grignard G, Margue C, Dippel W, Capesius C, Mossong J, *et al.* Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. *Int J Cancer* 2007 Mar 15; **120** (6): 1284-1292.

27. Duronio V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. *Biochem J* 2008 Nov 1; **415** (3): 333-344.

28. Carson JP, Kulik G, Weber MJ. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B. *Cancer Res* 1999 Apr 1; **59** (7): 1449-1453.

29. Pfeil K, Eder IE, Putz T, Ramoner R, Culig Z, Ueberall F, *et al.* Long-term androgen-ablation causes increased resistance to PI3K/Akt pathway inhibition in prostate cancer cells. *Prostate* 2004 Feb 15; **58** (3): 259-268.

30. Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON. Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. *Proceedings of the National Academy of Sciences* 2006 May 16, 2006; **103** (20): 7789-7794.

31. Li P, Lee H, Guo S, Unterman TG, Jenster G, Bai W. AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. *Mol Cell Biol* 2003 Jan; **23** (1): 104-118.

32. Yang CC, Lin HP, Chen CS, Yang YT, Tseng PH, Rangnekar VM. Bcl-xL mediates a survival mechanism independent of the phosphoinositide 3-

kinase/Akt pathway in prostate cancer cells. *J Biol Chem* 2003 Jul 11; **278** (28): 25872-25878.

33. Heisler LE, Evangelou A, Lew AM, Trachtenber J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. *Molecular and Cellular Endocrinology* 1997; **126** (1): 59.

34. Chen C, Welsbie D, Tran C, Baek S, Chen R, Vessella R, *et al.* Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004; **10** (1): 33-39.

35. Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences. *J Biol Chem* 1994 May 6; **269** (18): 13115-13123.

36. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, Miranti CK. Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death. *Mol Biol Cell* 2007 July 1, 2007; **18** (7): 2481-2490.

37. Zha J, Harada H, Yang E, Jockel J, Korsmeyer S. Serine Phosphorylation of Death Agonist BAD in Response to Survival Factor Results in Binding to 14-3-3 Not BCL-XL. *Cell* 1996; **87** (4): 619.

38. Knudsen B, Miranti C. The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. *J Cell Biochem* 2006; **99** (2): 345-361.

39. Murillo H, Huang H, Schmidt LJ, Smith DI, Tindall DJ. Role of PI3K Signaling in Survival and Progression of LNCaP Prostate Cancer Cells to the Androgen Refractory State. *Endocrinology* 2001 November 1, 2001; **142** (11): 4795-4805.

40. Zhang M, Latham DE, Delaney MA, Chakravarti A. Survivin mediates resistance to antiandrogen therapy in prostate cancer. 2005; **24** (15): 2474.

41. Huang H, Muddiman DC, Tindall DJ. Androgens negatively regulate forkhead transcription factor FKHR (FOXO1) through a proteolytic mechanism in prostate cancer cells. *J Biol Chem* 2004 Apr 2; **279** (14): 13866-13877.

42. Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, *et al.* Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem* 2000 Apr 14; **275** (15): 10761-10766.

43. Bonaccorsi L, Muratori M, Carloni V, Zecchi S, Formigli L, Forti G, *et al.* Androgen receptor and prostate cancer invasion1. *International Journal of Andrology* 2003; **26** (1): 21-25.

44. Fornaro M, Plescia J, Chheang S, Tallini G, Zhu Y-M, King M, *et al.* Fibronectin Protects Prostate Cancer Cells from Tumor Necrosis Factor-{alpha}-induced Apoptosis via the AKT/Survivin Pathway. *J Biol Chem* 2003 December 12, 2003; **278** (50): 50402-50411.

45. Zhang Z, Vuori K, Reed JC, Ruoslahti E. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci U S A* 1995 Jun 20; **92** (13): 6161-6165.

46. Krajewska M, Krajewski S, Epstein JI, Shabaik A, Sauvageot J, Song K, *et al.* Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. *Am J Pathol* 1996 May; **148** (5): 1567-1576.

47. Kishi H, Igawa M, Kikuno N, Yoshino T, Urakami S, Shiina H. Expression of the survivin gene in prostate cancer: correlation with clinicopathological characteristics, proliferative activity and apoptosis. *J Urol* 2004 May; **171** (5): 1855-1860.

48. McEleny K, Watson R, Coffey R, O'Neill A, Fitzpatrick J. Inhibitors of apoptosis proteins in prostate cancer cell lines. *The Prostate* 2002; **51** (2): 133-140.

49. Shariat S, Lotan Y, Saboorian H, Khoddami S, Roehrborn C, Slawin K, *et al.* Survivin expression is associated with features of biologically aggressive prostate carcinoma. *Cancer* 2004; **100** (4): 751-757.

50. Castilla C, Congregado B, Chinchon D, Torrubia FJ, Japon MA, Saez C. Bcl-xL is overexpressed in hormone-resistant prostate cancer and promotes survival of LNCaP cells via interaction with proapoptotic Bak. *Endocrinology* 2006 Oct; **147** (10): 4960-4967.

51. Sun A, Tang J, Hong Y, Song J, Terranova PF, Thrasher JB, *et al.* Androgen receptor-dependent regulation of Bcl-xL expression: Implication in prostate cancer progression. *Prostate* 2008 Mar 1; **68** (4): 453-461.

52. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, *et al.* Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *Embo J* 2000 Oct 16; **19** (20): 5406-5417.

53. Roskoski R, Jr. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* 2005 May 27; **331** (1): 1-14.

54. Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* 2006 Oct; **18** (5): 516-523.

55. Gupton SL, Gertler FB. Filopodia: the fingers that do the walking. *Sci STKE* 2007 Sep 8; **2007** (400): re5.

56. Hodgson MC, Astapova I, Cheng S, Lee LJ, Verhoeven MC, Choi E, *et al.* The androgen receptor recruits nuclear receptor corepressor (N-CoR) in the presence of mifepristone via Its N and C termini revealing a novel molecular mechanism for androgen receptor antagonists. *J Biol Chem* 2005 February 25, 2005; **280** (8): 6511-6519.

57. Chen C, Edelstein LC, Gelinas C. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol Cell Biol* 2000 Apr; **20** (8): 2687-2695.

58. Glasgow JN, Wood T, Perez-Polo JR. Identification and characterization of nuclear factor kappaB binding sites in the murine bcl-x promoter. *J Neurochem* 2000 Oct; **75** (4): 1377-1389.

59. Ismail HA, Lessard L, Mes-Masson AM, Saad F. Expression of NF-kappaB in prostate cancer lymph node metastases. *Prostate* 2004 Feb 15; **58** (3): 308-313.

60. Chen CD, Sawyers CL. NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. *Mol Cell Biol* 2002 Apr; **22** (8): 2862-2870.

61. Jin RJ, Lho Y, Connelly L, Wang Y, Yu X, Saint Jean L, *et al.* The nuclear factor-kappaB pathway controls the progression of prostate cancer to androgen-independent growth. *Cancer Res* 2008 Aug 15; **68** (16): 6762-6769.

62. Lessard L, Begin LR, Gleave ME, Mes-Masson AM, Saad F. Nuclear localisation of nuclear factor-kappaB transcription factors in prostate cancer: an immunohistochemical study. *Br J Cancer* 2005 Oct 31; **93** (9): 1019-1023.

63. Lessard L, Karakiewicz PI, Bellon-Gagnon P, Alam-Fahmy M, Ismail HA, Mes-Masson AM, *et al.* Nuclear localization of nuclear factor-kappaB p65 in primary prostate tumors is highly predictive of pelvic lymph node metastases. *Clin Cancer Res* 2006 Oct 1; **12** (19): 5741-5745.

64. Domingo-Domenech J, Mellado B, Ferrer B, Truan D, Codony-Servat J, Sauleda S, *et al.* Activation of nuclear factor-kappaB in human prostate carcinogenesis and association to biochemical relapse. *Br J Cancer* 2005 Nov 28; **93** (11): 1285-1294.

65. Domingo-Domenech J, Oliva C, Rovira A, Codony-Servat J, Bosch M, Filella X, *et al.* Interleukin 6, a nuclear factor-kappaB target, predicts resistance to docetaxel in hormone-independent prostate cancer and nuclear factor-kappaB inhibition by PS-1145 enhances docetaxel antitumor activity. *Clin Cancer Res* 2006 Sep 15; **12** (18): 5578-5586.

66. Setlur SR, Royce TE, Sboner A, Mosquera JM, Demichelis F, Hofer MD, *et al.* Integrative microarray analysis of pathways dysregulated in metastatic prostate cancer. *Cancer Res* 2007 Nov 1; **67** (21): 10296-10303.

67. Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gelinas C, *et al.* Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. *Prostate* 2002 Aug 1; **52** (3): 183-200.

68. Dutta J, Fan Y, Gupta N, Fan G, Gelinas C. Current insights into the regulation of programmed cell death by NF-kappaB. *Oncogene* 2006 Oct 30; **25** (51): 6800-6816.

69. Yamamoto Y, Gaynor RB. IkappaB kinases: key regulators of the NF-kappaB pathway. *Trends Biochem Sci* 2004 Feb; **29** (2): 72-79.

70. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 2006 Oct 30; **25** (51): 6680-6684.

71. Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. *Oncogene* 1999 Dec 2; **18** (51): 7389-7394.

72. Friedland JC, Lakins JN, Kazanietz MG, Chernoff J, Boettiger D, Weaver VM. alpha6beta4 integrin activates Rac-dependent p21-activated kinase 1 to drive NF-kappaB-dependent resistance to apoptosis in 3D mammary acini. *J Cell Sci* 2007 Oct 15; **120** (Pt 20): 3700-3712.

73. Frost JA, Swantek JL, Stippec S, Yin MJ, Gaynor R, Cobb MH. Stimulation of NFkappa B activity by multiple signaling pathways requires PAK1. *J Biol Chem* 2000 Jun 30; **275** (26): 19693-19699.

74. Bright MD, Garner AP, Ridley AJ. PAK1 and PAK2 have different roles in HGF-induced morphological responses. *Cell Signal* 2009 Dec; **21** (12): 1738-1747.

75. Deacon SW, Beeser A, Fukui JA, Rennefahrt UE, Myers C, Chernoff J, *et al.* An isoform-selective, small-molecule inhibitor targets the autoregulatory mechanism of p21-activated kinase. *Chem Biol* 2008 Apr; **15** (4): 322-331.

76. Sakurai H, Suzuki S, Kawasaki N, Nakano H, Okazaki T, Chino A, *et al.* Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-kappaB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *J Biol Chem* 2003 Sep 19; **278** (38): 36916-36923.

77. Yang F, Tang E, Guan K, Wang CY. IKK beta plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. *J Immunol* 2003 Jun 1; **170** (11): 5630-5635.

78. Madrid LV, Mayo MW, Reuther JY, Baldwin AS, Jr. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem* 2001 Jun 1; **276** (22): 18934-18940.

79. Cammarano MS, Minden A. Dbl and the Rho GTPases activate NF kappa B by I kappa B kinase (IKK)-dependent and IKK-independent pathways. *J Biol Chem* 2001 Jul 13; **276** (28): 25876-25882.

80. Merlo GR, Basolo F, Fiore L, Duboc L, Hynes NE. p53-dependent and p53-independent activation of apoptosis in mammary epithelial cells reveals a survival function of EGF and insulin. *J Cell Biol* 1995 Mar; **128** (6): 1185-1196.

81. Streuli CH, Edwards GM, Delcommenne M, Whitelaw CB, Burdon TG, Schindler C, *et al.* Stat5 as a target for regulation by extracellular matrix. *J Biol Chem* 1995 Sep 15; **270** (37): 21639-21644.

82. Matter ML, Ruoslahti E. A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. *J Biol Chem* 2001 Jul 27; **276** (30): 27757-27763.

83. Rabinovitz I, Nagle RB, Cress AE. Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive

phenotype in vitro and in vivo. *Clin Exp Metastasis* 1995 Nov; **13** (6): 481-491.

84. Heino J. The collagen receptor integrins have distinct ligand recognition and signaling functions. *Matrix Biol* 2000 Aug; **19** (4): 319-323.

85. Sridhar SC, Miranti CK. Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. *Oncogene* 2006 Apr 13; **25** (16): 2367-2378.

86. Kostenuik PJ, Singh G, Orr FW. Transforming growth factor beta upregulates the integrin-mediated adhesion of human prostatic carcinoma cells to type I collagen. *Clin Exp Metastasis* 1997 Jan; **15** (1): 41-52.

87. Kiefer JA, Farach-Carson MC. Type I collagen-mediated proliferation of PC3 prostate carcinoma cell line: implications for enhanced growth in the bone microenvironment. *Matrix Biol* 2001 Nov; **20** (7): 429-437.

88. Klekotka PA, Santoro SA, Wang H, Zutter MM. Specific residues within the alpha 2 integrin subunit cytoplasmic domain regulate migration and cell cycle progression via distinct MAPK pathways. *J Biol Chem* 2001 Aug 24; **276** (34): 32353-32361.

89. Arora PD, Marignani PA, McCulloch CA. Collagen phagocytosis is regulated by the guanine nucleotide exchange factor Vav2. *Am J Physiol Cell Physiol* 2008 Jul; **295** (1): C130-137.

90. Thornton TM, Rincon M. Non-classical p38 map kinase functions: cell cycle checkpoints and survival. *Int J Biol Sci* 2009; **5** (1): 44-51.

91. Coniglio SJ, Jou TS, Symons M. Rac1 protects epithelial cells against anoikis. *J Biol Chem* 2001 Jul 27; **276** (30): 28113-28120.

92. Manohar A, Shome SG, Lamar J, Stirling L, Iyer V, Pumiglia K, *et al.* Alpha 3 beta 1 integrin promotes keratinocyte cell survival through activation of a MEK/ERK signaling pathway. *J Cell Sci* 2004 Aug 15; **117** (Pt 18): 4043-4054.

93. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, *et al.* Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *J Biol Chem* 2000 Aug 25; **275** (34): 26164-26171.

94. Litvinov IV, De Marzo AM, Isaacs JT. Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling? *J Clin Endocrinol Metab* 2003 Jul; **88** (7): 2972-2982.

95. Lin HK, Hu YC, Yang L, Altuwaijri S, Chen YT, Kang HY, *et al.* Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *J Biol Chem* 2003 Dec 19; **278** (51): 50902-50907.

96. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proc Natl Acad Sci U S A* 2001 Jun 19; **98** (13): 7200-7205.

97. Taneja SS, Ha S, Swenson NK, Huang HY, Lee P, Melamed J, *et al.* Cell-specific regulation of androgen receptor phosphorylation in vivo. *J Biol Chem* 2005 Dec 9; **280** (49): 40916-40924.

98. Nan B, Snabboon T, Unni E, Yuan XJ, Whang YE, Marcelli M. The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity. *J Mol Endocrinol* 2003 Aug; **31** (1): 169-183.

99. Yuan S, Trachtenberg J, Mills GB, Brown TJ, Xu F, Keating A. Androgen-induced inhibition of cell proliferation in an androgen-insensitive prostate cancer cell line (PC-3) transfected with a human androgen receptor complementary DNA. *Cancer Res* 1993 Mar 15; **53** (6): 1304-1311.

100. Hansen N, Chang C, Chodak G, Rukstalis D. Modulation of hormone sensitivity in PC3 cells by transfectiom of a normal human androgen receptor. *Surg Forum* 1991; **43**: 745-748.

101. Litvinov IV, Antony L, Dalrymple SL, Becker R, Cheng L, Isaacs JT. PC3, but not DU145, human prostate cancer cells retain the coregulators required for tumor suppressor ability of androgen receptor. *Prostate* 2006 Sep 1; **66** (12): 1329-1338.

102. Wolf DA, Herzinger T, Hermeking H, Blaschke D, Horz W. Transcriptional and posttranscriptional regulation of human androgen receptor expression by androgen. *Mol Endocrinol* 1993 Jul; **7** (7): 924-936.

103. Yeap BB, Krueger RG, Leedman PJ. Differential posttranscriptional regulation of androgen receptor gene expression by androgen in prostate and breast cancer cells. *Endocrinology* 1999 Jul; **140** (7): 3282-3291.

104. Bonaccorsi L, Carloni V, Muratori M, Formigli L, Zecchi S, Forti G, *et al.* EGF receptor (EGFR) signaling promoting invasion is disrupted in androgen-sensitive prostate cancer cells by an interaction between EGFR and androgen receptor (AR). *Int J Cancer* 2004 Oct 20; **112** (1): 78-86.

105. Traish AM, Morgentaler A. Epidermal growth factor receptor expression escapes androgen regulation in prostate cancer: a potential molecular switch for tumour growth. *Br J Cancer* 2009 Dec 15; **101** (12): 1949-1956.

106. Nishida K, Kitazawa R, Mizuno K, Maeda S, Kitazawa S. Identification of regulatory elements of human alpha6 integrin subunit gene. *BBRC* 1997; **241** (2): 258-263.

107. Lin CS, Chen Y, Huynh T, Kramer R. Identification of the human alpha6 integrin gene promoter. *DNA Cell Biol* 1997 Aug; **16** (8): 929-937.

108. Waghray A, Feroze F, Schober MS, Yao F, Wood C, Puravs E, *et al.* Identification of androgen-regulated genes in the prostate cancer cell line LNCaP by serial analysis of gene expression and proteomic analysis. *Proteomics* 2001 Oct; **1** (10): 1327-1338.

109. Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, *et al.* New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. *EMBO Rep* 2007 Sep; **8** (9): 871-878.

110. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, *et al.* Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004 Jan; **10** (1): 33-39.

111. Velasco AM, Gillis KA, Li Y, Brown EL, Sadler TM, Achilleos M, *et al.* Identification and validation of novel androgen-regulated genes in prostate cancer. *Endocrinology* 2004 Aug; **145** (8): 3913-3924.

112. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE. Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. *J Steroid Biochem Mol Biol* 2005 Aug; **96** (3-4): 251-258.

113. Sato N, Sadar MD, Bruchovsky N, Saatcioglu F, Rennie PS, Sato S, *et al.* Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP. *J Biol Chem* 1997 Jul 11; **272** (28): 17485-17494.

114. Porter W, Saville B, Hoivik D, Safe S. Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol Endocrinol* 1997 Oct; **11** (11): 1569-1580.

115. Alipov G, Nakayama T, Ito M, Kawai K, Naito S, Nakashima M, *et al.* Overexpression of Ets-1 proto-oncogene in latent and clinical prostatic carcinomas. *Histopathology* 2005 Feb; **46** (2): 202-208.

116. Morris DS, Tomlins SA, Montie JE, Chinnaiyan AM. The discovery and application of gene fusions in prostate cancer. *BJU Intl* 2008; **102** (3): 276-282.

117. Wu C, Dedhar S. Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J Cell Biol* 2001 Nov 12; **155** (4): 505-510.

118. Tan C, Cruet-Hennequart S, Troussard A, Fazli L, Costello P, Sutton K, *et al.* Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell* 2004 Jan; **5** (1): 79-90.

119. Chen N, Chen X, Huang R, Zeng H, Gong J, Meng W, *et al.* BCL-xL is a target gene regulated by hypoxia-inducible factor-1{alpha}. *J Biol Chem* 2009 Apr 10; **284** (15): 10004-10012.

120. Fitsialos G, Bourget I, Augier S, Ginouves A, Rezzonico R, Odorisio T, *et al.* HIF1 transcription factor regulates laminin-332 expression and keratinocyte migration. *J Cell Sci* 2008 Sep 15; **121** (Pt 18): 2992-3001.

121. Boddy JL, Fox SB, Han C, Campo L, Turley H, Kanga S, *et al.* The androgen receptor is significantly associated with vascular endothelial growth factor and hypoxia sensing via hypoxia-inducible factors HIF-1a, HIF-2a, and the prolyl hydroxylases in human prostate cancer. *Clin Cancer Res* 2005 Nov 1; **11** (21): 7658-7663.

122. Du Z, Fujiyama C, Chen Y, Masaki Z. Expression of hypoxia-inducible factor 1alpha in human normal, benign, and malignant prostate tissue. *Chin Med J (Engl)* 2003 Dec; **116** (12): 1936-1939.

123. Zhong H, Semenza GL, Simons JW, De Marzo AM. Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. *Cancer Detect Prev* 2004; **28** (2): 88-93.

124. Mabjeesh NJ, Willard MT, Frederickson CE, Zhong H, Simons JW. Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3'-kinase/protein kinase B in prostate cancer cells. *Clin Cancer Res* 2003 Jul; **9** (7): 2416-2425.

125. Miranti CK. Application of cell adhesion to study signaling networks. *Methods Cell Biol* 2002; **69**: 359-383.

126. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001 Dec; **25** (4): 402-408.

CHAPTER FOUR:
E-CADHERIN-MEDIATED SURVIVAL OF ANDROGEN RECEPTOR EXPRESSING PROSTATE CELLS.

This chapter was published separately as:

Lamb, L.E., Knudsen, B.S., and Miranti, C.K., *E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model.* J. Cell Science, 2010. **123**: p. 266-276

INTRODUCTION

Epithelial cells serve several vital functions. For instance, all epithelial cells act as a barrier to protect organs from external environmental assault, as exemplified by the skin. Intestinal epithelial cells are required for the absorption of nutrients, while mammary and prostate epithelial cells are primarily secretory. Proper regulation of epithelial differentiation is crucial for the development and maintenance of barrier and organ function. Differentiation of epithelial cells has been extensively characterized in the epidermis. The basal layer of the epidermis consists of proliferating keratinocytes that adhere to a basement membrane via integrins. Loss of basal cell adhesion through integrin β1 initiates terminal differentiation, resulting in flattening of the cells, expression of differentiation proteins, and subsequent cornification, which ultimately produces several distinct stratified cell layers that make up the epidermis (1, 2).

The epithelium of the human prostate consists of two cell layers, a basal layer and a secretory layer. Similar to other stratified epithelium; prostate basal cells are mitotic and adhere to a basement membrane (3-5). Prostate basal cells give rise to terminally differentiated secretory cells (3-5). However, unlike other epithelia, prostate epithelial cell differentiation is regulated by androgen signaling (6-10). The androgen receptor (AR) is a nuclear transcription factor activated in response to the steroid hormone androgen (11). AR is expressed only in the differentiated secretory cells and not in the basal cells (11). It is unclear exactly how androgen regulates epithelial differentiation. However, tissue combination studies from AR null mice suggest that androgen stimulation of AR in the early

developing mesenchyme, and not the epithelium, is solely responsible for the induction of epithelial morphogenesis in vivo (12).

Androgen also appears to be important for secretory cell survival, in that anti-androgen therapies specifically kill the secretory cells, leaving the basal cells intact (13). Furthermore, restoration of androgens results in regeneration of the secretory cell compartment. However, tissue recombination experiments, as well as studies using conditional knockout mice that lack AR only in prostate epithelium, suggest that AR does not directly regulate epithelial survival (12, 14). Instead, androgen stimulation of the AR-positive stromal cells of the prostate may induce secreted factors that regulate secretory cell survival. Keratinocyte growth factor (KGF) and FGF10 are two factors secreted by the stromal cells, though not in an androgen-dependent manner (12, 15-17). KGF and FGF10 are both involved in murine prostate organogenesis and can induce differentiation of isolated prostate epithelial cells (15, 16, 18-22). In some cases, KGF can substitute for androgens and it is likely that KGF and AR signaling pathways interact (23). KGF has also been reported to promote differentiation and survival of the epithelium of the skin, lung, and eye (24-26). KGF acts specifically on epithelial cells and has been reported to activate p38 MAPK signaling (21).

Clarification of the roles of androgen and KGF in prostate epithelial differentiation and survival has been hampered by our inability to culture normal differentiated AR expressing secretory cells in vitro. Prostate epithelial cells (PECs) cultured from normal human prostate tissue consist primarily of AR-negative basal cells and their transient amplifying derivatives. Previous studies

in our lab have demonstrated that survival of cultured PECs is specifically mediated through α3β1 integrin-dependent adhesion (27). Similarly, basal keratinocytes are dependent on α3β1 integrin for their survival (28). During keratinocyte differentiation, basal cells lose integrin expression as well as adhesion to matrix as they are extruded to the upper layers of the skin (29). In suprabasal keratinocytes, as well as in other epithelia, cell-cell adhesion structures such as E-cadherin appear to promote survival through phosphoinositide 3-kinase (PI3K) signaling, and when PI3K signaling is lost these cells die (30-32). Whether the same survival mechanisms are operative in differentiated secretory prostate epithelial cells is unknown, and the role of KGF or androgen in prostate epithelial cell survival remains unresolved.

In this study, confluent cultured primary prostate basal epithelial cells were induced to differentiate following treatment with KGF and androgen. After two weeks, differentiated AR-expressing secretory cells appeared as a secondary cell layer above the basal cells. This model was used to identify the signaling pathways important for prostate secretory cell survival. This new model will serve as a valuable tool for understanding the biology of prostate secretory epithelial cells, a cell population previously not available for extensive analysis.

RESULTS

Differentiation of Confluent PECs by KGF and DHT

Previous studies have demonstrated that KGF may be an important epithelium differentiation factor in many tissues, including prostate epithelium

(15, 16, 19, 21, 33). Androgen, acting via the androgen receptor, also plays an important role in prostate epithelial cell differentiation (6-10). To determine if the combination of KGF and androgen is sufficient to induce differentiation of prostate cells grown in culture, human primary basal prostate epithelial cells (PECs) grown to confluency in monolayer cell cultures were treated with 10 ng/ml KGF and 5-10 nM androgen (DHT). Culturing the cells for 10-15 days with KGF/DHT resulted in the formation of stratified cell patches consisting of at least two cell layers, resembling the bilayer of basal and secretory cells observed in the prostate epithelium in vivo (Figure 17A,B,C).

To determine if the stratified cells expressed differentiation markers specific to prostate secretory cells, expression of AR and the AR-target protein prostate-specific antigen (PSA) were examined by fluorescence confocal microscopy. Cells in a higher z-plane than the bottom cells, stained positive for AR and PSA (Figure 17B). AR expression was both nuclear and cytoplasmic, whereas the secreted protein PSA had the expected cytoplasmic localization (Figure 17B). While AR expression was uniform throughout the top cells, PSA expression was often concentrated at the upper membrane of the top most cells, consistent with that of a secreted protein (not shown). Neither AR nor PSA was found in the bottom cells (Figure 17B). Additionally, the AR-regulated proteins Nkx3.1 and TMPRSS2 (34-37) were expressed in top cells and not in bottom cells (Figure 17C).

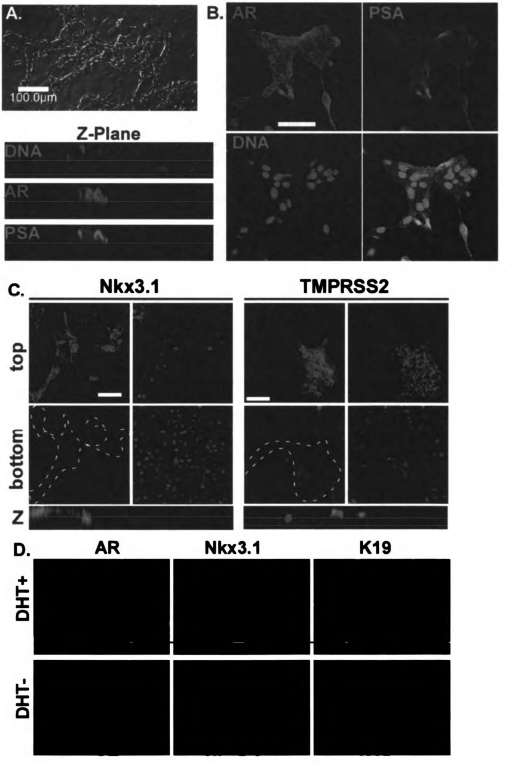
To determine the extent to which androgen stimulation contributes to PEC differentiation, PECs were treated for 10-14 days with KGF in the presence or

absence of DHT, and the expression of AR, AR target proteins, and differentiation cell markers was monitored. PSA, Nkx3.1, and TMPRSS2 were only expressed when DHT was present (Figure 17D, PSA and TMPRSS2 not shown). Intriguingly, cytokeratin markers, K18 and K19, were also expressed only in the presence of androgen (Figure 17D, K18 data not shown). Furthermore, there was a dramatic increase in AR expression itself when DHT was present.

KGF, in the absence of DHT, was sufficient to induce formation of stratified cells, with maximal formation occurring between ten to fifteen days. PECs treated with KGF in the presence of KGF-blocking antibody did not stratify. Confluency of the cultures was essential. Subconfluent cells treated with KGF and DHT did not form stratified clusters. KGF-induced stratification occurred equally efficiently, with or without the supplementary bovine pituitary extract (BPE) and EGF in the culture medium. Occasionally, a few small stratified clusters appeared in BPE-containing medium without KGF treatment, suggesting the presence of low levels of KGF and/or an additional unknown factor(s) in BPE that can promote differentiation at a low efficiency. KGF-blocking antibodies prevented the appearance of these occasional clusters. The optimal concentration of KGF was 10 ng/ml. Lower doses (1-5 ng/ml) resulted in fewer clusters and higher doses (20-50 ng/ml) did not generate more clusters. DHT alone was not sufficient to induce stratification. DHT plus KGF treatment dramatically increased the number of top cells seen after fifteen days. DHT was

FIGURE 17. AR and AR-dependent proteins are present in the differentiated cultures. Confluent primary prostate epithelial cells (PECs) were induced to differentiate with 10 ng/ml KGF and 5nM DHT for 10-14 days. Scale bars: 100 μm. **A**) DIC image of a differentiated culture shows an upper layer of cells (outlined with dashed white line) on top of a confluent bottom cell layer. **B**) 14-day differentiated culture was immunostained for AR (*green*) and PSA (*red*). Nuclei (*blue*) were visualized by Hoechst 33258 staining. (Left panel) A z-section image was compiled from 30 confocal x-y sections representing a thickness of 38.0μm. Horizontal lines demarcate top and bottom cell layers. (Right panels) Confocal images of top cells in 14-day differentiated cultures. Scale bar: 50.0 μm. **C)** Differentiated PEC cultures were immunostained for Nkx3.1 and TMPRSS2 (*green*) and imaged by confocal microscopy. Nuclei were stained with Hoechst 33258 (*blue*). Representative top and bottom cells and z-plane images are shown (Z). Scale bar: 100 μm. **D)** PECs were induced to differentiate for 14 days with KGF in the presence or absence of 10 nM DHT. Cells were immunostained with AR, Nkx3.1, and K19 and imaged by epifluorescence microscopy.

FIGURE 17


A.
100.0µm
Z-Plane
DNA
AR
PSA
B.
AR
PSA
DNA
C.
Nkx3.1
TMPRSS2
top
bottom
Z
D.
AR
Nkx3.1
K19
DHT+
DHT-

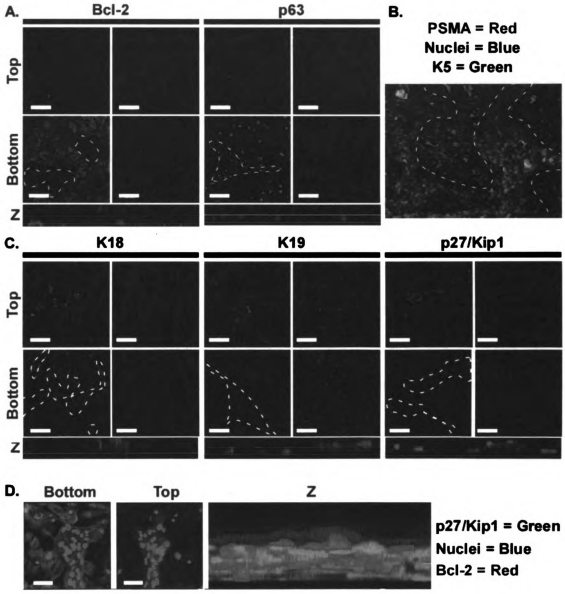
required for expression of androgen-dependent markers in the top cells. FGF10, a functionally related FGF family member shown to be important for prostate development in vivo (22, 38), could also induce PEC differentiation in the presence of DHT. Differentiation was reproducibly observed in cells derived from two different patients at three different passage numbers (passages 2, 3, and 4). It was observed however, that once cells reached passage 5, the efficiency of differentiation was dramatically reduced. Furthermore, we were able to induce differentiation in an immortalized cell line derived from a third patient. We observed that these more proliferative immortalized cultures took a few days longer to reach maximal differentiation.

Stratified Cells Express Additional Differentiation Markers

Markers specific to basal and differentiated epithelial cells populations were examined in the stratified cultures. The basal markers Bcl-2, K5, and K14 (39, 40) were expressed predominantly in the bottom cells; occasionally a few K5- and K14- positive cells were seen in the top cells (Figure 18A,B, K14 not shown). Basal marker p63 (41, 42) was associated only with bottom cells (Figure 2A). EGFR, which is predominately expressed in basal cells (43), was associated primarily with bottom cells (not shown). Epithelial cell markers K19 and PMSA were expressed only in the top cells and not in the bottom cells (Figure 18B,C). K18, as well as the cell cycle inhibitor p27 (Kip1) (44-47), was expressed predominately in the top cells (Figure 18C). Z-plane analysis of cells co-stained for Bcl-2 and p27 (Kip1) demonstrate the co-distribution of a basal cell

FIGURE 18. Differentiation-specific epithelial markers present in the top cells of differentiated cultures. 10- to 14-day differentiated cultures were immunostained for **A**) Bcl-2, p63 (*green*), **B**) K5 (*green*), PSMA (*red*), **C**) K18, K19 (*red*), and p27/Kip1 (*green*) expression and images were captured by **A,C**) confocal microscopy or **B**) epifluorescence. Nuclei were stained with Hoechst 33258 (*blue*). Representative top and bottom cells are shown. Representative z-section images (*Z*) were compiled from 10-15 confocal x-y sections representing a thickness of 17.04 (+/- 3.27) μm. Horizonal lines demarcate top and bottom cells. Scale bars: 100.0 μm. **D**) 11-day differentiated cultures were immunostained for basal marker Bcl-2 (*red*) and differentiated marker p27/Kip1 (*green*). Nuclei were stained with Hoechst 33258 (*blue*). Z-section image was compiled from 20 confocal x-y sections representing a thickness of 28.64 μm. Scale bar: 50 μm.

FIGURE 18


A.
Bcl-2
p63
Top
Bottom
Z
B.
PSMA = Red
Nuclei = Blue
K5 = Green
C.
K18
K19
p27/Kip1
Top
Bottom
Z
D.
Bottom
Top
Z
p27/Kip1 = Green
Nuclei = Blue
Bcl-2 = Red

marker and a differentiation marker, respectively, in the stratified cultures (Figure 18D).

Differentiation Induces Integrin Loss

Consistent with previous observations of differentiating epithelium in vitro and in vivo (1, 21, 48, 49), epifluorescence and confocal imaging revealed that the subpopulation of the cells undergoing differentiation lost expression of many integrins, including α2, α3, α6, β1, and β4 (Figure 19A,B). Basal cells also expressed αv, but not β3 or β5 integrin subunits. None of these integrins were present in the differentiated cells (not shown). Cultured PECs secrete and organize a laminin 5 (LM5)-rich matrix (50); the differentiating cell population that lost integrin expression also no longer produced LM5 (Figure 19A,B). Although it appears by confocal imaging that the cells directly below the top cells do not express integrin or LM5, it is possible that there is incomplete antibody penetrance into the lower cells. To address this, a time course study was performed. We observed a decrease in LM5 expression as early as three days after KGF and DHT treatment and a complete loss after eight days. At eight days decreased β1 integrin expression was observed in LM5-negative cells prior to formation of the second cell layer (Figure 20A,B). Therefore, cells directly underneath the top layer also lose LM5 and integrin expression. LM5 loss may be the trigger that initiates differentiation.

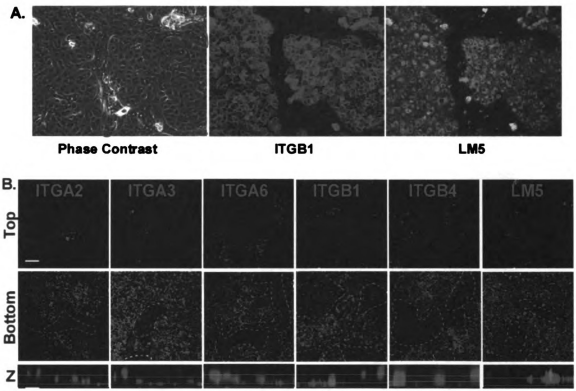


FIGURE 19. Prostate epithelial differentiation is accompanied by loss of integrin expression. **A**) Integrin β1 (ITGB1, *green*) and laminin 5 (LM5, *red*) expression in 10 day differentiated cultures were monitored by DIC (left panel) and epifluorescence microscopy following immunostaining. **B**) 14-day differentiated cultures were immunostained to detect expression (*green*) of integrins α2 (ITGA2), α3 (ITGA3), α6 (ITGA6), β1 (ITGB1), β4 (ITGB4), and laminin 5 (LM5) and imaged by confocal microscopy. Nuclei (*blue*) were visualized by Hoescht 33258 staining. Representative confocal x-y sections of the top and bottom cells are shown. The area located directly beneath the top layer of differentiated cells is indicated with dashed white lines in the bottom image. Representative z-section images (*Z*) were compiled from 10-15 confocal x-y sections representing a thickness of 17.04 (+/- 3.27) μm. Horizontal lines demarcate top and bottom cells.

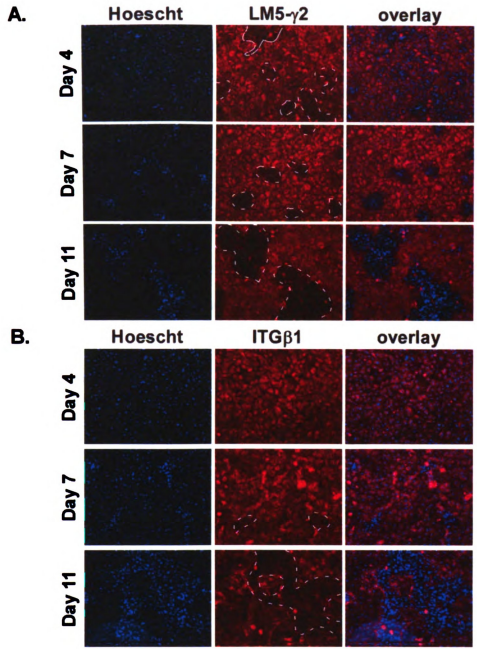


FIGURE 20. Prostate epithelial differentiation is accompanied by loss of laminin 5 expression. Confluent PECs were induced to differentiate in the presence of KGF and DHT for 4, 7 or 10 days. Cells were immunostained (*red*) for **A**) laminin 5 (LM5-γ2) or **B**) β1 integrin (ITGB1) and Hoechst 33258 (*blue*) and visualized by epifluorescent microscopy. Areas of decreased LM5 and β1 integrin expression are outlined with dashed white lines.

Differentiated Cells Respond to Androgen

AR expression could be detected by immunoblotting of cell lysates from whole cultures treated with KGF and DHT (Figure 21A). Expression of the androgen- dependent secreted proteins, KLK2 and PSA, was monitored in differentiated cultures by RT-PCR. KLK2 and PSA mRNAs were present only when DHT was present in the culture (Figure 21B). Furthermore, secreted PSA, up to 0.8ng/ml, could be detected by ELISA (Figure 21C). PSA secretion required androgen and increased with increasing DHT concentration. The expression and secretion of an androgen-regulated protein in an androgen-dependent manner indicates the presence of differentiated prostate secretory cells in the culture, and that AR is functional and regulates expression of differentiation markers.

Overall, this KGF and DHT-induced in vitro differentiation model recapitulates many aspects of in vivo differentiation as assessed by the specific markers (Fig 21D). In addition to the induction of markers common to most differentiating epithelial cells, the presence of DHT markedly stimulates the expression of markers unique to prostate secretory epithelial cells. Hereafter when referring to this model, the AR-expressing top cells will be referred to as secretory-like cells and the AR-negative bottom cells as basal cells.

Isolation of Secretory-like Cells

Treatment of differentiated cultures with dissociation buffer preferentially dislodges the secretory-like cells. FACS analysis indicates that 96.6% (+/- 0.8%)

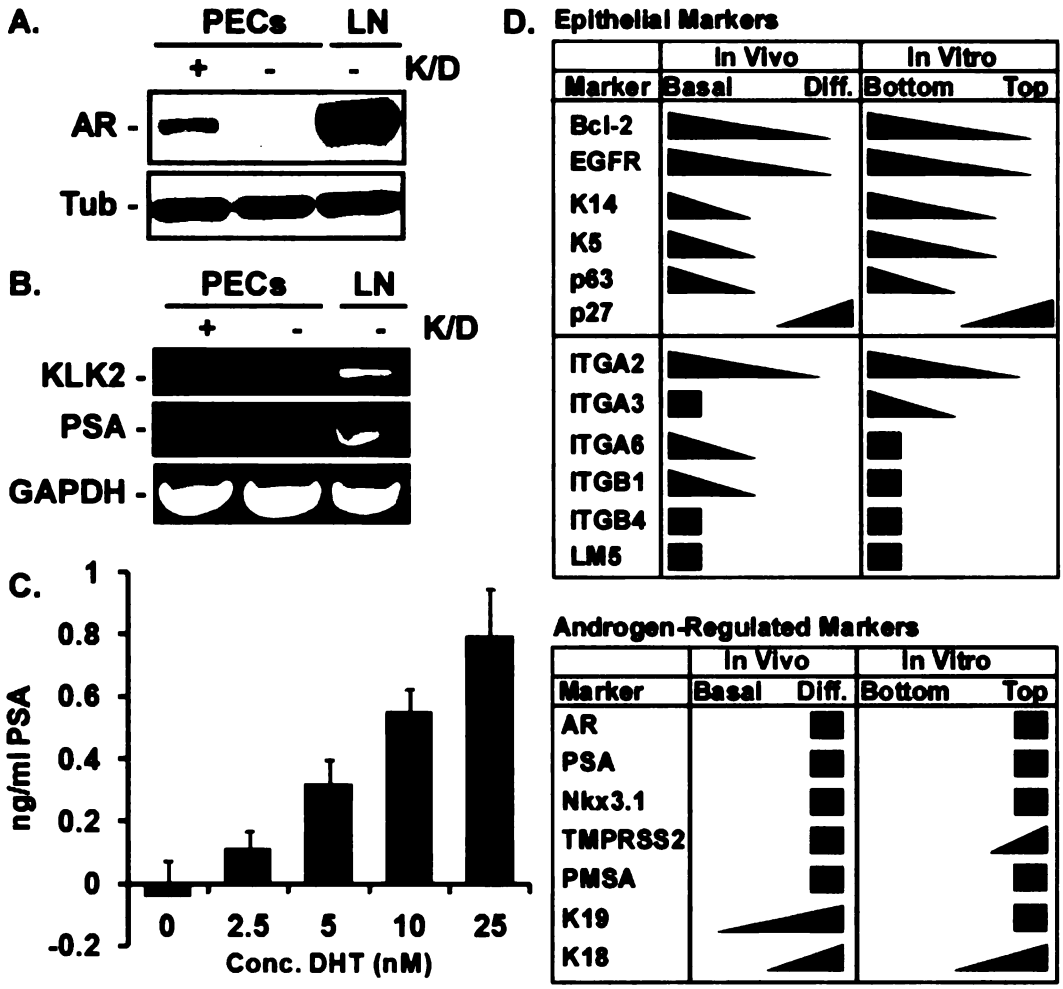


FIGURE 21. Differentiated cells respond to androgen. **A**) Immunoblot for AR expression in cultures of PECs treated with or without KGF and DHT (K/D) for 16 days. LNCaP cells (LN) were used as a positive control for AR expression. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. **B**) Levels of KLK2 and PSA mRNA isolated from 14-day differentiated (K/D) cultures were analyzed by PCR and compared to LNCaP (LN) cells. GAPDH served as a control. **C**) Secreted levels of PSA from 14-day differentiated cultures treated with KGF and increasing amounts of DHT were determined by ELISA. **D**) Summary of epithelial and androgen-dependent markers observed and their relative expression in the lower (Bottom) and upper (Top) cells. Expression observed in vitro is compared to that reported in vivo.

of the isolated dislodged population is negative for cell surface α6 integrin, while 97.19% (+/- 1.70%) of the cells not dislodged are positive for α6 integrin (Figure 22A). Further FACS sorting based on surface staining of α6 integrin and TMPRSS2 revealed that on average 87.92% (+/- 3.71%) of the α6-integrin-negative cells were positive for TMPRSS2. A representative example is provided in Figure 22B. Immunoblotting of separated cells indicated that some remaining basal cells expressed AR as well as full-length TMPRSS2 protein; however, only the secretory-like cells expressed the cleaved and activated form of TMPRSS2 (Figure 22C) (51). Conversely, only the basal cells expressed Bcl-2 and EGFR, while K5 was predominately found in the basal cells (Figure 22D).

Secretory Cell Survival is Dependent on PI3K and E-cadherin, but not KGF or Androgen

In previous studies we demonstrated that integrin-mediated activation of EGFR and downstream signaling to ERK, but not PI3K signaling, is required for the survival of basal PECs (27). However, the differentiated secretory-like PECs have lost integrin expression, no longer adhere to the LM5 matrix, and have significantly lower levels of EGFR, suggesting that other survival pathways must be important for secretory cell survival. It has been suggested that secretory cell survival may be dependent on stromal derived growth factors, including KGF (52). One possibility is that the KGF used to induce differentiation, may also be necessary for survival. To test this, the KGF receptor FGFR2IIIb (53) mRNA levels were analyzed in the isolated secretory-like cells and basal cells by RT-

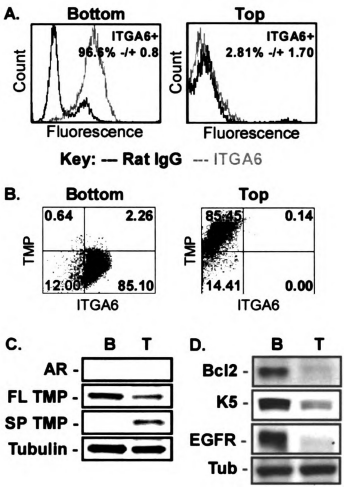


FIGURE 22. Isolation of secretory-like cells. A) Following treatment of 14-day cultures with dissociation buffer the separated upper (*Top*) and lower cells (*Bottom*) were subjected to FACS to measure cell surface α6-integrin expression (*gray line*) versus control antibody (*black line*). Dead cells were excluded using PI staining. Values are percentage α6-integrin-positive cells. **B**) Cells isolated and sorted for α6 integrin as in A were further sorted based on TMPRSS2 (TMP) surface expression. Values are percentage of positive cells in each quadrant. Data is from one typical experiment. **C,D**) Bottom (B) and top (T) cells, obtained after treatment with dissociation buffer, were analyzed by immunoblotting for **C**) AR, TMPRSS2, **D**) Bcl-2, K5, and EGFR expression. Tubulin (Tub) immunoblotting served as a loading control.

PCR. Only the basal cells expressed FGFR2IIIb mRNA (Figure 23A). Furthermore, removal of KGF after 15 days of differentiation did not induce cell death (not shown). Thus it is unlikely that KGF is regulating cell survival in the secretory-like cells.

Dissociated secretory-like cells and the remaining basal cells were screened for ERK and Akt activation by immunoblotting. Active ERK was present only in the basal cells, but not in the secretory-like cells (Figure 23B). Activated Akt was present in both types of cells (Figure 23C). Thus, ERK signaling probably does not regulate survival in differentiated cells, whereas the PI3K pathway could. Since the differentiated cells remain adherent to the bottom basal cells, we also investigated whether there is an increase in expression of the cell-cell adhesion molecule E-cadherin in the secretory-like cells. Compared to the basal cells, E-cadherin levels were elevated in the secretory cell population that also does not express $\alpha 6\beta 1$ integrin (Figure 23D). E-cadherin can lead to activation of PI3K signaling in skin and colonic epithelium as well as in some tumor cell lines (30, 54, 55). Blocking antibodies to E-cadherin suppressed Akt activity in both the secretory-like (Figure 23E) and the basal cells (not shown).

The relative importance of the different signaling pathways on secretory-like cell survival was investigated. Fourteen-day KGF and DHT-differentiated cultures were placed in KGF- and DHT-free basal medium without any pituitary extract or EGF supplement for 72 hours to reduce any signaling induced by the growth medium (Figure 24A). Visually, the starved cell cultures appeared viable,

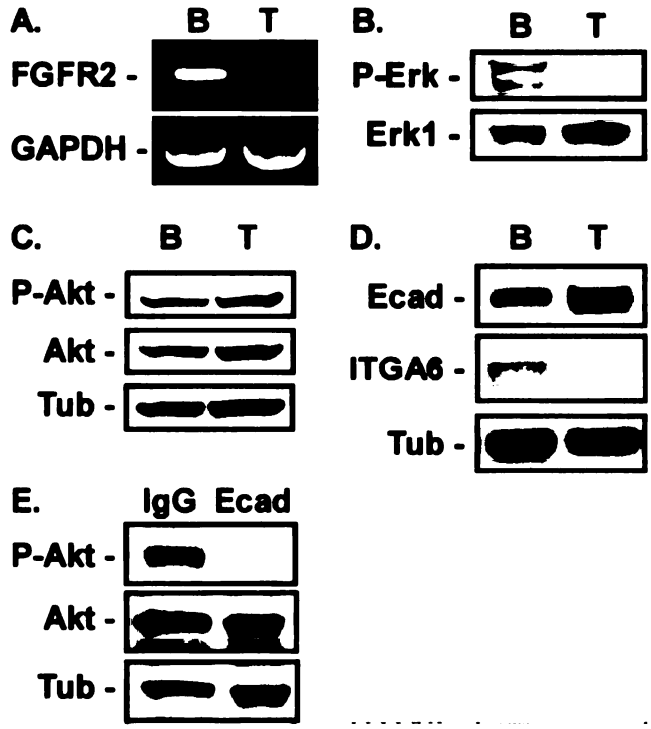
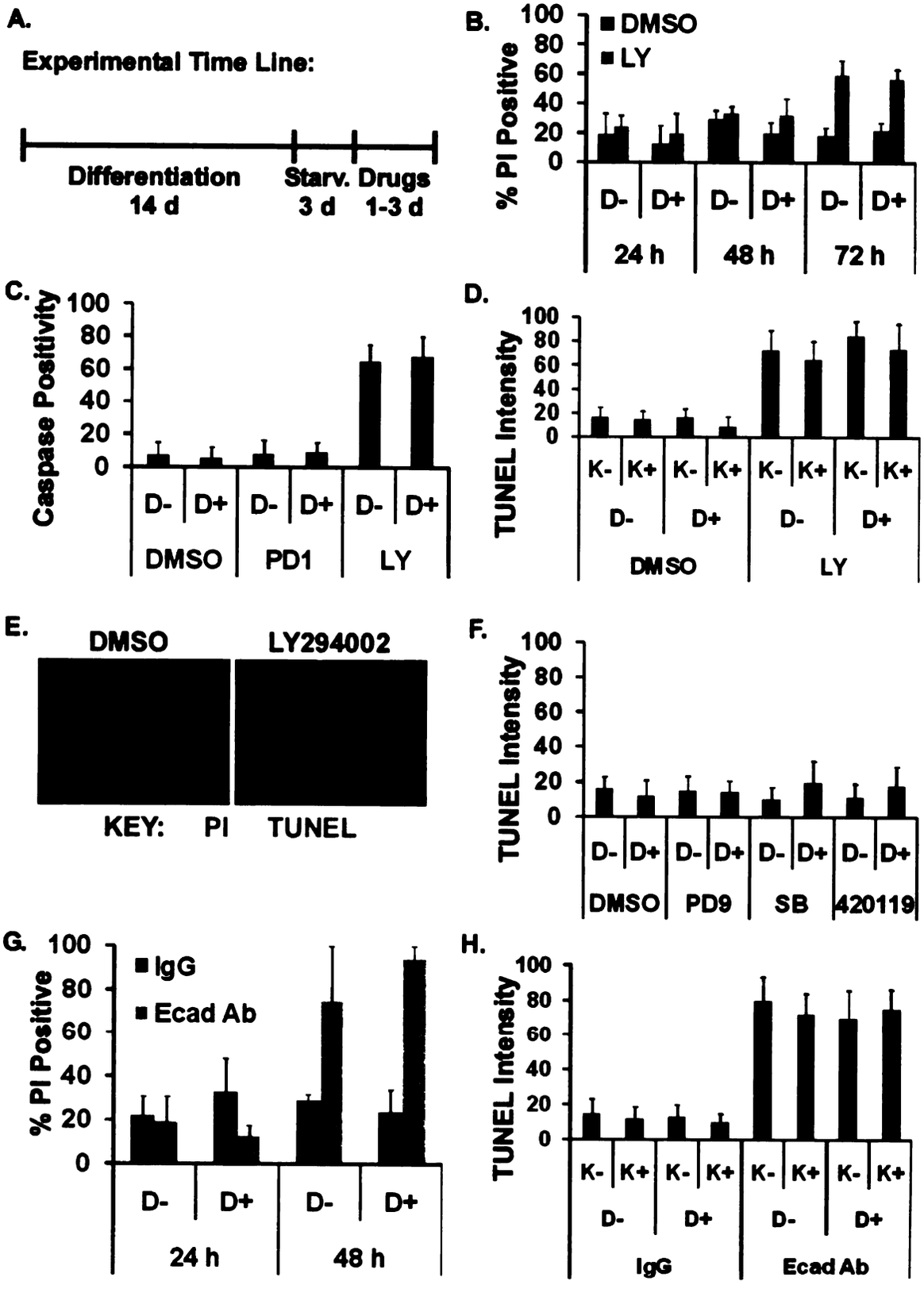


FIGURE 23: Signaling pathways in secretory-like cells. Following treatment of 14-day cultures with dissociation buffer, mRNA or protein was isolated from the lower (B) and upper cells (T). **A**) Levels of FGFR2IIIb mRNA (FGFR2) were assessed by PCR. GAPDH served as a control. **B**) Levels of ERK activation (P-ERK) in the lower (B) and upper cells (T) were monitored by immunoblotting of cell lysates with phosphospecific ERK antibodies. Total levels of ERK and tubulin in the lysates were measured by immunoblotting. **C**) Levels of Akt activation (P-Akt) in the lower (B) and upper cells (T) were monitored by immunoblotting of cell lysates with phosphospecific Akt antibodies. Total levels of Akt and tubulin in the lysates were measured by immunoblotting. **D**) Levels of E-cadherin (Ecad) and α6 integrin (ITGA6) in the lower (B) and upper cells (T) were analyzed by immunoblotting. Tubulin immunoblots served as loading controls. **E**) 14-day cultures were treated with control IgG or E-cadherin-blocking antibody (Ecad) for three hours. Levels of Akt activation (P-Akt) in the isolated upper cells were monitored by immunoblotting of cell lysates with phosphospecific Akt antibodies. Total levels of Akt and tubulin in the lysates were measured by immunoblotting.

and the upper secretory-like cell layer remained intact (data not shown). Then the starved differentiated cultures were treated with specific inhibitors in the presence or absence of freshly added DHT or KGF and analyzed over a 72 hour time course. Cell death was measured in the upper secretory-like cell layer by immunostaining for active caspase 3/7, TUNEL staining, or propidium iodide (PI) uptake. Staining was quantified as described in Materials and Methods. Inhibition of PI3K signaling with LY294002 resulted in maximal secretory-like cell death at 72 hours, where 60% of the cells stained positive for PI (Fig 24B). Furthermore, inhibition of PI3K, but not EGFR, induced a 7.0- to 7.5-fold increase in secretory cell caspase 3 activity (Fig 24C), and a 5.5- to 5.7-fold increase in TUNEL staining (Figure 24D,E). Maximal Annexin V staining was observed 66 hours after LY294002 treatment (not shown). Secretory-like cell survival was not dependent on DHT or KGF, and addition of DHT or KGF was unable to promote cell survival in the absence of PI3K signaling (Figure 24B-D). Although KGF should not be present in the media and prostate epithelial cells have been reported not to produce KGF, KGF-blocking antibodies were used to prevent any endogenous or remaining KGF from promoting cell survival. KGF-blocking antibodies had no effect on cell survival (data not shown). KGF has been reported to activate p38, and Jnk can promote survival during stress (21, 56, 57). Inhibiting p38 with SB202190, Jnk with 420119, or ERK with PD98059 did not result in cell death, suggesting these pathways are not critical for secretory cell survival (Figure 24F). The lack of effect of the inhibitors on cell survival was not

FIGURE 24. Secretory-like cells are dependent on PI3K and E-cadherin, but not androgen or KGF, for survival. A) PECs were treated with KGF and DHT for 14 days (Differentiation), then starved of growth factors and DHT for 3 days (Starv), and then treated with pharmacological inhibitors (Drugs) for 1-3 days. In some cases DHT or KGF were also added back with the inhibitors. **B)** Differentiated cultures were treated with vehicle (DMSO) or PI3K inhibitor LY492004 (LY) in the presence or absence of DHT (D) for 24, 48 or 72 hours. Cell viability in the top cells was measured by quantifying the number of cells with high PI staining and expressed as percentage PI-positive cells. **C, D, E)** Differentiated cultures were treated with vehicle (DMSO), EGFR inhibitor PD168393 (PD1), or PI3K inhibitor LY492004 (LY) in the presence or absence of DHT (D) or KGF (K). After 72 hours cell viability in the top cells was assessed using **C)** cleaved caspase-3 or **D)** TUNEL staining. Total DNA was stained with PI. Six fields per experiment and condition were examined and positive pixels counted using the software program Imagine as outlined in Material and Methods. TUNEL- or cleaved caspase-3-positive pixels were normalized to the total number of stained DNA pixels in the region of interest and expressed as relative intensity of caspase-3 or TUNEL staining. Error bars are standard deviation. *n*=3. **E)** Cell viability in the upper cells was accessed using TUNEL (*green*) staining and confocal microscopy. Nuclei were stained with propidium iodide (PI, *colorized blue*). **F)** 14 day differentiated cultures starved of growth factors for 3 days were treated with vehicle (DMSO), MEK1/2 inhibitor PD98059 (PD9), p38 inhibitor SB202190 (SB) or Jnk inhibitor 420119 in the presence of KGF and the presence or absence of DHT. Cell viability was measured by TUNEL staining 3 days later. Error bars are standard deviation. *n*=3. **G)** Differentiated cultures were treated with non-specific mouse IgG (IgG) or with E-cadherin blocking antibody (Ecad Ab; lot 2) in the presence or absence of DHT (D) for 24 or 48 hours. Cell viability was measured by PI staining. Error bars are standard deviation. *n*=3. **H)** Cell viability of differentiated cultures treated with non-specific mouse IgG (IgG) or with E-cadherin blocking antibody (Ecad Ab; lot 1) in the presence or absence of DHT (D) or KGF (K) for 72 hours was measured by TUNEL staining. Error bars are standard deviation. *n*=3.

FIGURE 24


A.
Experimental Time Line:
Differentiation 14 d
Starv. 3 d
Drugs 1-3 d
B.
DMSO
LY
% PI Positive
100
80
60
40
20
0
D-
D+
24 h
48 h
72 h
C.
Caspase Positivity
DMSO
PD1
LY
D.
TUNEL Intensity
K-
K+
DMSO
LY
E.
DMSO
LY294002
KEY: PI TUNEL
F.
TUNEL Intensity
DMSO
PD9
SB 420119
G.
IgG
Ecad Ab
% PI Positive
H.
TUNEL Intensity
IgG
Ecad Ab

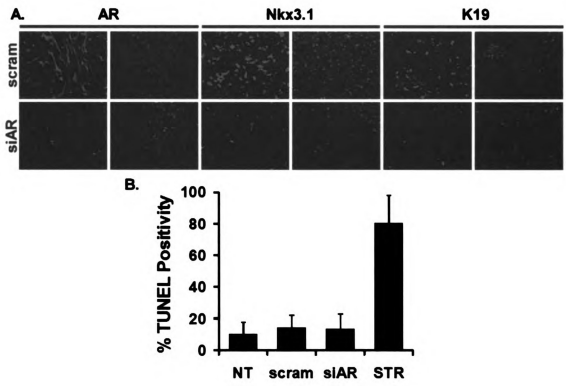


FIGURE 25: AR is not required for secretory-like cell survival. PECs were treated with KGF and DHT for 14 days and then transfected with scrambled siRNA (scram) or AR-specific siRNA (siAR) to block AR expression. **A**) Cells were immunostained with AR, Nkx3.1, and K19 (*green*) and imaged by epifluorescence microscopy. Nuclei were stained with Hoechst 33258 (*blue*). **B**) Differentiated cultures left untreated (NT) or treated with scrambled siRNA (scram), AR siRNA (siAR), or staurosporine (STR) were measured for cell viability by TUNEL staining.

due to a failure to inhibit signaling, as the concentrations of drugs used here did effectively block signaling to their specific targets in basal cells.

Inhibition of cell-cell adhesion with one application of E-cadherin blocking antibody resulted in maximal cell death at 48 hours with over 80% of the cells staining positive for PI (Figure 24G). By 66 hours, no secretory-like cells remained in the cultures. A second application of E-cadherin antibody resulted in a 7- to 8-fold increase TUNEL staining 72 hours after treatment (Figure 24H). The presence of DHT or KGF could not protect cells from death due to loss of E-cadherin function. No cell death was observed in the lower basal cells. Furthermore, blocking E-cadherin lead to a decrease in Akt activation (see Figure 23E), indicating that cell-cell adhesion mediated by E-cadherin promotes secretory-like cell survival through PI3K signaling.

Although DHT was not important for survival of the differentiated secretory-like cells, it is theoretically possible that AR, acting via an androgen-independent mechanism might still be important for cell survival. To address this, 14-day KGF and DHT-differentiated cultures were transfected with an AR-specific siRNA pool or a scrambled siRNA sequence. Confocal imaging of the transfected cells 72 hours later demonstrated the absence of AR expression in the upper cells (Figure 25A). Absence of AR expression also resulted in loss of androgen-dependent cell markers such as Nkx3.1 and K19 (Figure 25A). Cell viability of the AR siRNA-treated cells was assessed by TUNEL staining. Loss of AR had no effect on secretory-like cell viability (Figure 25B). Thus, AR and

androgen signaling are not required to maintain the viability of differentiated secretory-like cells derived from our in vitro culture system.

DISCUSSION

By treating cultured primary prostate basal epithelial cells with androgen and KGF, we have established an in vitro differentiation model of the prostate epithelium. The differentiated cells in our culture system possess the important features of terminally differentiated secretory prostate epithelial cells in vivo: they do not proliferate, they adhere to a basal cell layer and not to the basement membrane, they express AR protein, and they respond to DHT by inducing AR-dependent genes. Specifically, the cells express androgen-sensitive proteins, such as KLK2, PSA, Nkx3.1, PMSA, and TMPRSS2. In addition, cleaved TMPRSS2 is present in the upper, but not the lower cells and PSA is secreted into the culture medium. Furthermore, cytokeratin K18 and K19 expression was found to be dependent on androgen. K18 expression has previously been reported to be regulated by androgen (8, 9), and K19 has been suggested to be responsive to estrogen (58); however, both K18 and K19 promoters lack classical androgen response elements, making the mechanism of regulation unclear.

Further evidence for terminal differentiation is that the cells did not revert to basal cells when isolated and re-plated, and they failed to reattach, likely due to continued loss of integrin and/or matrix expression. Furthermore, after 21-25 days in culture the upper cells sloughed off and a few activated caspase 3 positive cells were seen in the aging cultures (data not shown), similar to what is

observed in vivo. Oddly, no more differentiated cells reappeared. Only about 20% of the cells appeared to be capable of undergoing differentiation, suggesting that the differentiated cells are derived from a distinct subpopulation of basal cells. The lack of continued differentiation after 25 days may indicate depletion of these special cells and a lack of ability to renew. The population of differentiation-competent cells is not likely to be stem cells, since 20% of the cells are capable of undergoing differentiation. However, we can't rule out the possibility that these cells arose from some stem cell-like progenitor within the culture. Further analysis would be required to determine if the progenitors are analogous to the Nkx3.1 positive luminal stem cell recently described (59). However, whatever the progenitor, it apparently can't renew in the context of our culture conditions.

Although many aspects of the differentiated cells recapitulate what is observed in vivo, there still remain some differences. For instance, the distribution of AR demonstrates a significant amount of cytoplasmic expression in the in vitro culture system, while in vivo AR is primarily nuclear. Another difference is the absence of columnar cells. In addition, a few K5- and/or K14-positive cells were sometimes seen in the upper layer, which has also been reported in another differentiation model (60). Hence, we cannot unequivocally say whether our secretory-like cells represent completely terminally differentiated prostate cells and there are still some distinctive morphological differences between our cultures and what is seen in the prostate gland in vivo.

Other studies have reported on prostate epithelial differentiation in vitro. While these studies were informative, they were limited since AR and AR-regulated proteins were not expressed (48, 61-65). A few studies have reported seeing stratified layering similar to ours after treating prostate epithelial cells in vitro with retinoic acid, FGF, and/or insulin (44, 48, 60, 66); however, in these models the top layer of cells either failed to express AR or still expressed basal markers. In our model, the top secretory-like cells expressed AR and lost basal marker expression. In one case, gland-like buds and extensions were observed to form from confluent cell cultures, reminiscent of acini structures in overall shape but without lumens (60). We have also observed cases where cells appear to form mounds. By confocal imaging, some of them appear to have formed a hollow mound (data not shown). A recent study demonstrated that co-treatment of prostate basal cells with the monoamine oxidase A inhibitor clorgyline, 1,25- dihydroxyvitamin D_3, all-trans retinoic acid, and TGF-β1 induced AR expression and loss of basal marker K14 (67), suggesting that there may be alternative mechanisms to inducing prostate epithelial cell differentiation.

In contrast to other published systems, we have demonstrated that our model can be utilized for biochemical and genetic manipulation. It is amenable to treatment with pharmacological inhibitors or siRNA to study signaling and biological pathways. Furthermore, exploitation of differential cell surface markers and adhesion properties can be used to separate basal from secretory-like cells to separately analyze RNA and protein expression.

It is unknown if AR represses integrin expression or if loss of integrin expression must precede expression of AR. Unpublished data from our laboratory and others demonstrates that re-expression of AR in prostate cancer cell lines results in decreased integrin expression (68, 69). However, in our model we observed that not all integrin-negative cells were AR positive, suggesting that integrin loss may precede AR expression. Furthermore, LM5 matrix loss preceded integrin loss, which preceded stratification and robust AR expression in our time course studies. Heer et al. have demonstrated that blocking integrin β1 is sufficient to induce partial differentiation; however, cells do not reach terminal differentiation since the cells do not express AR-regulated genes (21). This suggests that loss of adhesion can initiate early differentiation and may even be required, but that integrin loss alone is not sufficient for terminal differentiation. In contrast, unbound integrin β1 is sufficient to initiate terminal differentiation in keratinocytes (1, 29). On the other hand, in mammary epithelium loss of integrin β1 suppresses differentiation (70).

Interestingly, in most of the reported prostate differentiation models (including ours), confluent cultures were necessary for stratification. In addition, previous studies suggest that cell cycle inhibition is a prerequisite for expression of secretory cell markers K18, K19, and AR (48, 62, 65, 71). We similarly saw a loss in cell proliferation in the differentiating cell population (data not shown). This led us to develop the following model for prostate differentiation (Figure 26). Basal cells are proliferative and a subset begins to undergo growth arrest once the cells are confluent. Treatment with KGF causes a select population of cells,

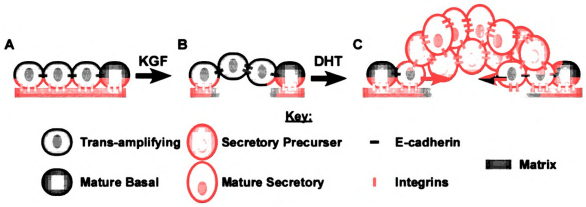


FIGURE 26: Model of Differentiation. A) Confluent primary prostate basal cells secret and adhere to a matrix rich in LM5 via integrins, which physically separates the epithelial cells from the stromal cells. **B)** After treatment with KGF, a sub-population of transient amplifying cells loses expression of LM5 and subsequently integrins, resulting in loss of adhesion. Concurrently, there is increased cell-cell adhesion via E-cadherin. **C)** Secretory-like precursor cells arise in concert with androgen treatment, which induces their differentiation into mature secretory cells. Transient amplifying cells at the edge continue to proliferate to fill in the space generated by detachment of cells and movement into the top layer. After 10-14 days, cells become stratified as more transient amplifying cells are committed to terminal differentiation.

perhaps those that express higher levels of the KGF receptor FGFR2IIIb (53), to lose LM5 and then integrin expression, causing the cells to detach. Integrin loss and detachment may then trigger low AR expression. AR expression was not detectable by immunostaining in cultures treated with only KGF, where integrin expression was lost; however, some AR expression was detectable in the basal cells from the differentiated cultures by immunoblotting. The presence of androgen in the culture appears to be necessary to allow the integrin-deficient cells to express AR at a higher level, which then turns on AR-dependent differentiation-specific genes.

Work by Heer et al. suggests that AR may be expressed at low levels in primary prostate epithelial cells and is rapidly degraded by the proteosome (8); hence androgen treatment may stabilize and/or help drive production of AR protein. In fact AR mRNA has been detected in some cultured prostate epithelial cells (71). However, in our studies and those of others, androgen alone is poorly effective in inducing AR expression (71). Thus, additional events are required to induce stable AR expression even in the presence of androgen. Reduced cell proliferation caused by strong growth suppression or loss of cell adhesion, which is also growth suppressive, may be necessary. Significant increases in AR expression can be detected in isolated suspended cells in the presence of androgen (8), thus supporting cell detachment as a potential mechanism required for stabilizing AR.

Previous work from our laboratory has demonstrated that integrin-mediated survival of primary prostate basal cells requires integrin-induced EGFR

signaling to ERK, but not PI3K signaling (27). In this study we have expanded our analysis of survival mechanisms to secretory-like prostate epithelial cells and demonstrated that secretory-like cells depend on a non-integrin-dependent mechanism for cell survival that involves cell-cell interactions through E-cadherin. Interestingly, there is switch from ERK-dependent survival in the basal cells to PI3K-dependent survival in the secretory-like cells. In the secretory-like cells EGFR levels dropped dramatically and EGFR-dependent signaling to PI3K was not required for survival (blocking EGFR had no effect on secretory cell survival). Interestingly, in prostate cancer, there appears to be a strong dependence on PI3K signaling for survival, as these cells tend to acquire mutations in Pten, a negative regulator of PI3K signaling (27, 72-74). This suggests that prostate cancer may arise from a more differentiated cell that has already acquired dependence on PI3K for its survival.

In our studies, secretory cell survival was not dependent on the presence of androgen, and knock-down of AR with siRNA in differentiated cells did not induce their death. The lack of dependence on androgen or AR for secretory cell survival in our human culture system is in agreement with genetic and tissue recombination studies in mice. Conditional knock-out of AR in mature mouse prostates results in decreased numbers of secretory cells without inducing cell death, suggesting that AR functions to increase secretory cell numbers by promoting differentiation rather than cell survival in mature glands (14). Tissue recombination experiments using mesenchyme and epithelium from AR-negative or wild-type mice demonstrate that AR expression in the epithelium is not

required for early prostate development, indirectly ruling out a role for AR in epithelial cell survival in newly formed glands (12). Thus, in both models, as well as ours, androgen is responsible for the synthesis of secretory proteins and the secretory function of the prostate.

If androgen and AR do not act cell autonomously to control epithelial cell survival, then why do only the AR-expressing epithelial cells die upon castration-induced androgen deprivation (75, 76)? One possibility is that AR signaling in the stromal cells promotes survival by paracrine factors that act on the epithelial cells (77). In our model the paracrine function of KGF, known to be expressed by stromal cells in vivo, was required for differentiation; however, it was dispensable for cell survival in committed differentiated cells. Thus, the nature of the paracrine survival factor(s) remains undetermined. In our in vitro model, survival was highly dependent on E-cadherin-based cell-cell adhesion and signaling to PI3K. Whether paracrine factors in vivo are responsible for maintaining survival via E-cadherin or whether they act on other pathways remains to be determined.

Our study supports a simpler concept that the role of stromal-derived paracrine factors is to act primarily on the stem and/or basal cells, whose proliferation and regenerative capacity is driven by these factors. As terminally differentiated cells are sloughed into lumen, basal cells are triggered to proliferate and differentiate to replace the lost cells. Under androgen-ablative conditions, the loss of paracrine factors in the stroma prevents stem and/or basal cell renewal and the terminally differentiated cells eventually slough off and are

differentiation, and subsequent restoration of secretory cells. This model would preclude the need for stromal factors acting directly on the secretory cells.

An alternative model to explain castration-induced loss of prostate secretory cells involves the observation that castration reduces blood flow and microvasculature collapse in the gland, inducing a state of hypoxia (78). It would appear that secretory cells are much more sensitive to such stress than the basal or stromal cells. This may be related to a lack of extracellular matrix support that provides additional survival signaling cues to the basal and stromal cells. Alternatively, hypoxia may affect the production of the paracrine factors required for maintenance of epithelial differentiation or survival.

In summary, we have established an in vitro differentiation model of human prostate epithelium composed of stratified cells that recapitulates many in vivo characteristics of basal and secretory cells, including AR-dependent differentiation and function. This model can be treated with pharmacological inhibitors and siRNA to study biochemical and genetic effects and the differentiated secretory-like cells can be isolated for further analysis. We have further established that while KGF, AR, and androgen are important for initiating the differentiation process and AR is important to maintain the androgen-dependent phenotype of secretory-like cells, these factors are not required for survival of the committed differentiated cells. The primary critical mechanism driving cell survival is E-cadherin-based cell-cell adhesion and subsequent activation of the PI3K signaling pathway.

MATERIALS AND METHODS

Cell Culture

Human primary prostate epithelial cells (PECs) derived from prostectomy specimens were isolated, cultured, and verified to be free of stromal contamination as described previously (27, 79). Specific patient samples used in this study were again verified to be negative for the stromal cell marker smooth muscle actin by immunostaining. PECs were grown in Keratinocyte-SFM medium (Invitrogen) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF). Experiments were reproducibly performed in cells derived from two different patients at three different passage numbers (passage 2, 3, and 4). In addition, at least three separate primary cultures from each patient were used. Experiments were verified at least three times for each of the two patients. We were also able to induce differentiation in an immortalized cell line derived from a third patient. The AR-positive prostate cancer cell line LNCaP was purchased from ATCC. LNCaP cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 2mM glutamine, 50 IU penicillin, 50 μg/ml streptomycin, 0.225% glucose, 10 mM HEPES, and 1mM sodium pyruvate.

Differentiation Assay

To induce differentiation, a confluent 10-cm culture dish of PECs was divided equally between three 8-chambered slides (Lab-Tek). Cells were grown in Keratinocyte-SFM supplemented with BPE, EGF, 10 ng/ml keratinocyte growth

factor (KGF) (Calbiochem), and 5-10 nM dihydrotestosterone (DHT) (Sigma) for 10-18 days. KGF and DHT were replenished three and five times a week respectively. For larger-scale experiments, three 10-cm plates of confluent PECs were combined onto one 10-cm dish and treated with KGF and DHT for 21-30 days.

KGF-blocking Experiments

KGF/FGF7 blocking antibody (clone 29522) was purchased from R&D Systems. 2 μg/ml KGF-blocking antibody or IgG control was added immediately prior to KGF addition. Differentiation of PECs was then assessed by immunofluorescent staining for differentiation markers.

Cell Surface Integrin and TMPRSS2 Expression Analysis

Whole cultures of differentiated PEC cultures were placed in suspension by washing the cells twice with PBS, treating with cell dissociation buffer (Gibco, Invitrogen) for 5 minutes, then adding TrypLE Express trypsin (Gibco, Invitrogen). Cells were then washed with wash buffer (1% sodium azide/2% FBS-PBS) and incubated with primary antibodies or control IgG molecules for 1 hour at 4°C. Cells were washed twice and incubated with fluorescently-labeled secondary antibodies for 1 hour at 4°C in the dark. Cells were washed twice more, and fluorescence was detected by a Becton-Dickinson FACSCalibur 4-color flow cytometer with CellQUEST Pro® Software v5.2.1 (Becton-Dickinson).

Isolation of Differentiated Cells

Differentiated PEC cultures were washed with 1mM EDTA in PBS without calcium or magnesium, and then incubated for 5 minutes with 1mM EDTA/PBS. Cells were then incubated with cell dissociation buffer (Gibco, Invitrogen) for 6-8 minutes. The top layer of cells could then be removed by pipetting the cell dissociation buffer over the cells; the bottom confluent cell layer remains attached to the culture vessel. The isolated cells were used directly or undifferentiated α6-integrin-expressing cells were separated from the differentiated cells using α6 integrin antibodies and FACS as described above using fluorescently-conjugated integrin α6 antibody (BD Pharmingen). Cells were sorted on a Becton-Dickinson FACSAria special order system 12-color flow cytometer using FACSDiVa® software v5.2 (Becton-Dickinson).

Immunoblotting

Total cell lysates were prepared for immunoblotting as previously described (27, 80). Briefly, cells were lysed with Triton-X 100 lysis buffer, and 45-75 μg of total cell lysates in 2X SDS sample buffer were boiled for 10 minutes. Samples were run on SDS polyacrylamide gels following standard SDS-PAGE protocols and transferred to PVDF membrane. Membranes were blocked in 5% BSA in TBST for two hours at room temperature, then were probed with primary antibody overnight at 4°C. Membranes were washed three times, and incubated with horseradish peroxide-conjugated secondary antibodies (Bio-Rad) in 5% BSA in TBST for 1 hour at room temperature. After washing an additional three times,

signals were visualized by a chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

Immunoblotting Antibodies

Antibodies for phospho-specific Akt (S473) or phospho-specific ERK 1/2 (T202/Y204) were purchased from Cell Signaling. Antibodies for total ERK were from Becton-Dickinson Transduction Labs and total Akt antibodies have been described previously (81). Integrin α6 and TMPRSS2 antibody were gifts from Dr. Anne Cress (University of Arizona, Phoenix, AZ) and Dr. Peter Nelson (Fred Hutchinson Cancer Research Institute, Seattle, WA) (82) respectively. Androgen receptor antibody (441) was purchased from Santa Cruz. E-cadherin antibody (clone HECD1) was purchased from Zymed. Tubulin antibody (clone DM1A) was purchased from Sigma.

Immunofluorescence

Differentiated PEC cultures were fixed with 4% paraformaldehyde (Mallinckrodt Chemicals) for ten minutes and permeabilized four minutes with 0.2% Triton-X 100 (EMD) at room temperature. Cells were then blocked with 10% normal goat serum (Pierce) for 2 hours at room temperature before incubation with primary antibodies overnight at 4°C. Cells were incubated with appropriate secondary antibodies for one hour at room temperature. DNA was visualized by staining with Hoechst 33258 (Sigma) for 10 minutes at room temperature. Cells were

washed three times with PBS between all steps. Coverslips were mounted on the slides using Gel-Mount (Biomeda).

Specific antibodies against proteins of interest were obtained as indicated in Table 5 and used for immunofluorescent (IF) staining at the stated dilutions. Whole IgG antibodies for controls were purchased from Pierce. Species appropriate Alexa Fluor 488 or 546 antibodies (Molecular Probes, Invitrogen) were used as secondary antibodies for indirect fluorescence.

Table 5. Immunofluorescence Antibodies

Protein	Clone	IF Dilution	Company
goatAb PSA	C-19	1:100	Santa Cruz
mAb AR	411	1:500	Santa Cruz
mAb Bcl-2	100	1:50	Santa Cruz
mAb E-cadherin	HECD-1	1:100	Zymed/Invitrogen
mAb EGFR	Ab12	1:200	Neomarkers
mAb ITGA2	P1H5-E9	1:10	Gift from W.G. Carter
mAb ITGA2	PIE6-1-1	1:10	Gift from W.G. Carter
mAb ITGA3	P1F2-1-1	1:10	Gift from W.G. Carter
mAbITGAV	272-17E6	1:250	AbCam
mAb K18	CY-90	1:100	Sigma
mAb K19	A53-BIA2	1:50	Sigma
mAb LM5 (γ2 chain)	D4B5	1:100	Chemicon
mAb p63	4A4	1:100	Santa Cruz
mAb PSA	18127	1:100	R&D Systems
mAb PSMA	YPSMA-1	1:250	AbCam
mAb SMA	1A4	1:100	Zymed/Invitrogen
mAbTMPRSS2	P5H9-A3	1:250	Gift of P.S. Nelson
rAb K5	AF138	1:500	Convance
rAb Ki67		1:200	Zymed/Invitrogen
rAb Kip1/p27	G173-324	1:100	Pharmigen
rAb Nkx3.1	H-50	1:500	Santa Cruz
ratAb ITGA6	GoH3	1:100	BD Pharmingen
ratAb ITGB1	AIIB2	1:100	Iowa State Univ. Hybridoma Bank
ratAb ITGB4	P4GH-1	1:10	Gift from W.G. Carter

Microscopy

Epifluorescence images were acquired using a Nikon Eclipse TE300 fluorescence microscope using OpenLab v5.5.0 image analysis software (Improvision). Confocal images were acquired by sequential detection using a Zeiss 510 Meta NLO v4.2, or Olympus FluoView 1000 LSM using FluoView software v5.0.

PSA Quantification

Differentiated PEC cultures in 8-chambered slides were grown in the presence or absence of DHT for 72 hours in 200 μL per well of growth medium. To quantify PSA concentrations in conditioned medium, a human PSA ELISA kit (Abzyme) was used according to manufacturer's directions with the following modifications: The entire 200 μl samples were incubated 50 μl at a time per well for 1 hour each. PSA standards were added to coated wells during the final 50 μl of sample incubation.

Reverse Transcription PCR (RT-PCR) for Differentiation Markers

Human KLK2, human KLK3 (PSA), FGFR2IIIb, and GAPDH mRNA levels were quantified in differentiated cells by RT-PCR. Total RNA was isolated from upper and lower cell populations of dissociated cells from differentiated cultures or from LNCaP cells using TRIzol (Gibco) and chloroform (Sigma-Aldrich). Contaminating DNA was then removed using RNAse-free DNAse kit (Qiagen) following manufacturer's directions. RT-PCR was performed on 1-2 μg RNA with

the primers listed in Table 6 using the One-Step RT-PCR kit (Qiagen) following manufacturer's directions. RT-PCR products were analyzed on a 2% agarose/TBE gel and DNA was visualized with ethidium bromide and a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

TABLE 6: RT-PCR Primers

Target	Fwd Primer (5'→3')	Rev Primer (5'→3')	Ref
ITGA6	GCTGGTTATAATCCTTCAATATCAATTGT	TTGGGCTCAGAACCTTGGTTT	(83)
ITGB1	GTGGTTGCTGGAATTGTTCTTATT	TTTTCCCTCATACTTCGGATTGAC	(83)
AR	TTTTCAATGAGTACCGCATGC	TCTCGCAATAGGCTGCACG	(84)
FGFR2IIIb	ATTGTTCTCCTGTGTCTG	CTTTTCAGCTTCTATATCC	(85)
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	(86)
KLK2	GGCAGGTGGCTGTGTACAGTC	CAACATGAACTCTGTCACCTTCTC	(87)
KLK3 (PSA)	CCCACTGCATCAGGAACAAAAGCG	GGTGCTCAGGGGTTGGCCAC	(87)

Small Interfering RNA Transfections

A pool of four small interfering RNAs (siRNA) against androgen receptor (siGENOME SMARTpool) or a non-targeting sequence were purchased from Dharmacon. Differentiated cultures were transfected with 20 nM siRNA in keratinocyte-SFM medium using siLentFect lipid reagent (Bio-Rad) and Opti-MEM (Invitrogen) medium following manufacturer's directions. The medium was changed 16 hours after transfection.

Cell Survival Assays

Differentiated PECs were growth factor-starved in keratinocyte-SFM medium containing no supplements, KGF, or DHT for 72 hours. Then DMSO (control; Sigma), pharmacological inhibitors 0.5 μM PD168393, 2 μM LY294002, 20 μM PD90859, 10 μM SB209102, 10 μM 420119 (all purchased from Calbiochem), 1 μM staurosporine (Promega), or 1 μg/ml E-cadherin blocking antibody (SHE78-7, Calbiochem) or non-specific mouse IgG (Sigma) was added; in some experiments, siRNAs were used to knock down AR expression (Dharmacon). Cells were incubated for 24, 48, 66, or 72 hours after drug, antibody, or siRNA addition. LY294002 was replenished 48 hours after its initial addition. To assess cell viability, cells were fixed and DNA fragmentation was monitored using Terminal Deoxynucleotide Transferase dUTP Nick End Labeling (TUNEL) following the protocol of the APO-BrdU TUNEL Assay Kit (BD Pharmingen). On several occasions, cleaved caspase 3 (Asp175) staining with antibody clone 5A1 from Cell Signaling was also used to measure cell viability of fixed cells. TUNEL and caspase activity were quantified using Imagine software (88). Total TUNEL- or caspase-positive pixels were normalized to total propidium iodide-stained DNA pixels in fixed cells and expressed as relative intensity of TUNEL staining. This quantification is based on pixel counts and does not necessarily reflect the percentage of positive cells, but rather the relative intensity of TUNEL or caspase 3 staining between treated and untreated cultures. As an alternative method for measuring cell viability, unfixed cells were treated with propidium iodide (PI).

High intensity PI staining of dead, i.e. permeabilized cells, was quantified on a per cell basis and expressed as % PI positive cells.

ACKNOWLEDGEMENTS

We thank Veronique Schulz, Rich West, and Dr. Jim Resau at the Van Andel Research Institute (VARI) and Dr. Melinda Frame at Michigan State University for technical assistance, and we acknowledge members of the laboratories of Developmental Cell Biology, Systems Biology, and Cell Structure and Signal Integration at VARI for their constructive suggestions. We also thank Dr. Doug Green (St. Jude Children's Research Hospital, Memphis, TN) for his constructive suggestions on the cell survival analyses. Integrin α2, α3 and β4 antibodies where a kind gift of Dr. William Carter (Fred Hutchinson Cancer Research Institute, Seattle, WA); the TMPRSS2 antibody was a generous gift of Dr. Peter Nelson (Fred Hutchinson Cancer Research Institute, Seattle, WA) and the α6 integrin blotting antibody was a gift from Dr. Anne Cress (University of Arizona, Phoenix, AZ). This work was supported by the Department of Defense Prostate Cancer Predoctoral Training Award (W81XWH-08-1-0058) to L.L., and the American Cancer Society (RSG-05-245-01-CSM) to C.K.M. Additional support was also provided by the generous gifts of the Van Andel Institute.

REFERENCES

1. Levy L, Broad S, Diekmann D, Evans RD, Watt FM. Beta 1 integrins regulate keratinocyte adhesion and differentiation by distinct mechanisms. *Mol Biol Cell* 2000 February 1, 2000; **11** (2): 453-466.

2. Lippens S, Denecker G, Ovaere P, Vandenabeele P, Declercq W. Death penalty for keratinocytes: apoptosis versus cornification. *Cell Death Differ* 2005; **12** (S2): 1497-1508.

3. Knox JD, Cress AE, Clark V, Manriquez L, Affinito KS, Dalkin BL, *et al.* Differential expression of extracellular matrix molecules and the alpha 6-integrins in the normal and neoplastic prostate. *Am J Pathol* 1994; **145** (1): 167-174.

4. Uzgare AR, Xu Y, Isaacs JT. In vitro culturing and characteristics of transit amplifying epithelial cells from human prostate tissue. *J Cell Biochem* 2004; **91** (1): 196-205.

5. van Leenders GJLH, Schalken JA. Epithelial cell differentiation in the human prostate epithelium: Implications for the pathogenesis and therapy of prostate cancer. *Crit Rev Oncol Hematol* 2003; **46** (Supplement 1): 3-10.

6. Berger R, Febbo PG, Majumder PK, Zhao JJ, Mukherjee S, Signoretti S, *et al.* Androgen-induced differentiation and tumorigenicity of human prostate epithelial cells. *Canc Res* 2004 December 15, 2004; **64** (24): 8867-8875.

7. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby R, Higgins SJ, *et al.* The endocrinology and developmental biology of the prostate. *Endocr Rev* 1987; **8** (3): 338-362.

8. Heer R, Robson CN, Shenton BK, Leung FY. The role of androgen in determining differentiation and regulation of androgen receptor expression in the human prostatic epithelium transient amplifying population. *Journal of cellular physiology* 2007; **212** (3): 572-578.

9. Ling MT, Chan KW, Choo CK. Androgen induces differentiation of a human papillomavirus 16 E6/E7 immortalized prostate epithelial cell line. *J Endocrinol* 2001 July 1, 2001; **170** (1): 287-296.

10. Whitacre DC, Chauhan S, Davis T, Gordon D, Cress AE, Miesfeld RL. Androgen induction of in vitro prostate cell differentiation. *Cell Growth Differ* 2002 January 1, 2002; **13** (1): 1-11.

11. Lamb DJ, Weigel VL, Marcelli M. Androgen receptors and their biology. *Vitam Horm* 2001; **62:** 199-230.

12. Cunha GR, Ricke W, Thomson AA, Marker PC, Risbridger G, Hayward SW, *et al.* Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development. *J Steroid Bioch Mol Biol* 2004; **92** (4)**:** 221.

13. Denis LJ, Griffiths K. Endocrine treatment in prostate cancer. *Sem Surg Oncol* 2000; **18** (1)**:** 52-74.

14. Wu C-T, Altuwaijri S, Ricke WA, Huang S-P, Yeh S, Zhang C, *et al.* Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. *Proc Natl Acad Sci USA* 2007 July 31, 2007; **104** (31)**:** 12679-12684.

15. Alarid ET, Rubin JS, Young P, Chedid M, Ron D, Aaronson SA, *et al.* Keratinocyte growth factor functions in epithelial induction during seminal vesicle development. *Proc Natl Acad Sci USA* 1994 February 1, 1994; **91** (3)**:** 1074-1078.

16. Sugimura Y, Foster BA, Hom YK, Lipschutz JH, Rubin JS, Finch PW, *et al.* Keratinocyte growth factor (KGF) can replace testosterone in the ductal branching morphogenesis of the rat ventral prostate. *Int J Dev Biol* 1996; **40** (5)**:** 941-951.

17. Thomson AA. Role of androgens and fibroblast growth factors in prostatic development. *Reproduction* 2001 Feb; **121** (2)**:** 187-195.

18. Cooke PS, Young P, Cunha GR. Androgen receptor expression in developing male reproductive organs. *Endocrinol* 1991; **128** (6)**:** 2867-2873.

19. Cunha GR. Growth factors as mediators of androgen action during male urogenital development. *Prostate Suppl* 1996; **6:** 22-25.

20. McKeenhan WL. Growth factor receptors and prostate cell growth. *Cancer Surv* 1991; **11:** 165-175.

21. Heer R, Collins AT, Robson CN, Shenton BK, Leung HY. KGF suppresses alpha2beta1 integrin function and promotes differentiation of the transient amplifying population in human prostatic epithelium. *J Cell Sci* 2006 April 1, 2006; **119** (7)**:** 1416-1424.

22. Donjacour AA, Thomson AA, Cunha GR. FGF-10 plays an essential role in the growth of the fetal prostate. *Developmental biology* 2003 Sep 1; **261** (1): 39-54.

23. Thomson AA, Foster BA, Cunha GR. Analysis of growth factor and receptor mRNA levels during development of the rat seminal vesicle and prostate. *Development (Cambridge, England)* 1997 Jun; **124** (12): 2431-2439.

24. Geiger RC, Waters CM, Kamp DW, Glucksberg MR. KGF prevents oxygen-mediated damage in ARPE-19 cells. *Invest Ophthalmol Vis Sci* 2005; **46** (9): 3435-3442.

25. Marchese C, Sorice M, De Stefano C, Frati L, Torrisi MR. Modulation of keratinocyte growth factor receptor expression in human cultured keratinocytes. *Cell Growth Differ* 1997 September 1, 1997; **8** (9): 989-997.

26. Ray P, Devaux Y, Stolz DB, Yarlagadda M, Watkins SC, Lu Y, *et al.* Inducible expression of keratinocyte growth factor (KGF) in mice inhibits lung epithelial cell death induced by hyperoxia. *Proc Natl Acad Sci USA* 2003 May 13, 2003; **100** (10): 6098-6103.

27. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, Miranti CK. Inhibition of integrin-mediated crosstalk with epidermal growth factor receptor/Erk or Src signaling pathways in autophagic prostate epithelial cells induces caspase-independent death. *Mol Biol Cell* 2007 July 1, 2007; **18** (7): 2481-2490.

28. Manohar A, Shome SG, Lamar J, Stirling L, Iyer V, Pumiglia K, *et al.* $\alpha 3\beta 1$ integrin promotes keratinocyte cell survival through activation of a MEK/ERK signaling pathway. *J Cell Sci* 2004 Aug 15; **117** (Pt 18): 4043-4054.

29. Watt FM. Role of integrins in regulating epidermal adhesion, growth and differentiation. *The EMBO journal* 2002 Aug 1; **21** (15): 3919-3926.

30. Calautti E, Li J, Saoncella S, Brissette JL, Goetinck PF. Phosphoinositide 3-kinase signaling to Akt promotes keratinocyte differentiation versus death. *J Biol Chem* 2005 September 23, 2005; **280** (38): 32856-32865.

31. Espada J, Galaz S, Sanz-Rodriguez F, Blazquez-Castro A, Stockert JC, Bagazgoitia L, *et al.* Oncogenic H-Ras and PI3K signaling can inhibit E-cadherin-dependent apoptosis and promote cell survival after photodynamic therapy in mouse keratinocytes. *Journal of cellular physiology* 2009 Apr; **219** (1): 84-93.

32. Rivard N. Phosphatidylinositol 3-kinase: a key regulator in adherens junction formation and function. *Front Biosci* 2009; **14:** 510-522.

33. Peehl D, Wong S, Rubin J. KGF and EGF differentially regulate the phenotype of prostatic epithelial cells. *Growth Regul* 1996; **6** (1): 22-31.

34. Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, *et al.* Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. *Cancer Res* 2000 November 1, 2000; **60** (21): 6111-6115.

35. Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, *et al.* Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. *Cancer Res* 1999 September 1, 1999; **59** (17): 4180-4184.

36. Murtha P, Tindall DJ, Young CY. Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. *Biochemistry* 1993; **32** (25): 6459-6464.

37. Young CY, Andrews PE, Montgomery BT, Tindall DJ. Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. *Biochemistry* 1992; **31** (3): 818-824.

38. Igarashi M, Finch PW, Aaronson SA. Characterization of recombinant human fibroblast growth factor FGF-10 reveals functional similarities with keratinocyte growth factor (FGF-7). *The Journal of biological chemistry* 1998 May 22; **273** (21): 13230-13235.

39. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LWK, Hsieh J-T, *et al.* Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res* 1992 December 15, 1992; **52** (24): 6940-6944.

40. Wang Y, Hayward SW, Cao M, Thayer KA, Cunha GR. Cell differentiation lineage in the prostate. *Differentiation* 2001; **68** (4-5): 270-279.

41. Parsons JK, Gage WR, Nelson WG, De Marzo AM. p63 protein expression is rare in prostate adenocarcinoma: implications for cancer diagnosis and carcinogenesis. *Urology* 2001; **58** (4): 619-624.

42. Signoretti S, Waltregny D, Dilks J, Isaac B, Lin D, Garraway L, *et al.* p63 Is a prostate basal cell marker and Is required for prostate development. *Am J Pathol* 2000 December 1, 2000; **157** (6): 1769-1775.

43. Sherwood ER, Lee C. Epidermal growth factor-related peptides and the epidermal growth factor receptor in normal and malignant prostate. *World J Urol* 1995; **13** (5): 290-296.

44. Peehl DM, Leung GK, Wong ST. Keratin expression: a measure of phenotypic modulation of human prostatic epithelial cells by growth inhibitory factors. *Cell Tissue Res* 1994; **277** (1): 8-11.

45. Tsihlias J, Kapusta LR, DeBoer G, Morava-Protzner I, Zbieranowski I, Bhattacharya N, *et al.* Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma. *Cancer Res* 1998 February 1, 1998; **58** (3): 542-548.

46. Wernert N, Seitz G, Achtstätter T. Immunohistochemical investigation of different cytokeratins and vimentin in the prostate from the fetal period up to adulthood and in prostate carcinoma. *Pathol Res Pract* 1987; **182** (5): 617-626.

47. Yang RM, Naitoh J, Murphy M, Wang H-J, Phillipson J, Dekernion JB, *et al.* Low p27 expression predicts poor disease-free survival in patients with prostate cancer. *J Urology* 1998; **159** (3): 941-945.

48. Gustafson MP, Xu C, Grim JE, Clurman BE, Knudsen BS. Regulation of cell proliferation in a stratified culture system of epithelial cells from prostate tissue. *Cell Tissue Res* 2006; **325** (2): 263-276.

49. Li H, Zhou JJ, Miki J, Furusato B, Gu Y, Srivastava S, *et al.* Telomerase-immortalized non-malignant human prostate epithelial cells retain the properties of multipotent stem cells. *Exp Cell Res* 2008; **314** (1): 92-102.

50. Yu H-M, Frank DE, Zhang J, You X, Carter WG, Knudsen BS. Basal prostate epithelial cells stimulate the migration of prostate cancer cells. *Mol Carcinog* 2004; **41** (2): 85-97.

51. Wilson S, Greer B, Hooper J, Zijlstras A, Walker B, Quigley J, *et al.* The membrane-anchored serine protease, TMPRSS2, activates PAR-2 in prostate cancer cells. *Biochem J* 2005; **388**: 967-972.

52. Kurita T, Wang YZ, Donjacour AA, Zhao C, Lydon JP, O'Malley BW, *et al.* Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. *Cell Death Differ* 2001; **8** (2): 192-200.

53. Giri D, Ropiquet F, Ittmann M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. *Clin Cancer Res* 1999 May 1, 1999; **5** (5): 1063-1071.

54. Hofmann C, Obermeier F, Artinger M, Hausmann M, Falk W, Schoelmerich J, *et al.* Cell-cell contacts prevent anoikis in primary human colonic epithelial cells. *Gastroenterol* 2007; **132** (2): 587-600.

55. Pang J-H, Kraemer A, Stehbens SJ, Frame MC, Yap AS. Recruitment of phosphoinositide 3-kinase defines a positive contribution of tyrosine kinase signaling to E-cadherin function. *The Journal of biological chemistry* 2005 January 28, 2005; **280** (4): 3043-3050.

56. Leppä S, Bohmann D. Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* 1999; **18** (45): 6158-6162.

57. Mehta P, Robson CN, Neal DE, Leung HY. Keratinocyte growth factor activates p38 MAPK to induce stress fibre formation in human prostate DU145 cells. *Oncogene* 2001; **20** (38): 5359-5365.

58. Choi I, Gudas LJ, Katzenellenbogen BS. Regulation of keratin 19 gene expression by estrogen in human breast cancer cells and identification of the estrogen responsive gene region. *Molecular and cellular endocrinology* 2000; **164** (1-2): 225-237.

59. Wang X, Kruithof-de Julio M, Economides KD, Walker D, Yu H, Halili MV, *et al.* A luminal epithelial stem cell that is a cell of origin for prostate cancer. *Nature* 2009 Sep 24; **461** (7263): 495-500.

60. van Leenders G, Dijkman H, Hulsbergen-van de Kaa C, Ruiter D, Schalken J. Demonstration of intermediate cells during human prostate epithelial differentiation in situ and in vitro using triple-staining confocal scanning microscopy. *Lab Invest* 2000; **80** (8): 1251-1258.

61. Dalrymple S, Antony L, Xu Y, Uzgare AR, Arnold JT, Savaugeot J, *et al.* Role of Notch-1 and E-cadherin in the differential response to calcium in culturing normal versus malignant prostate cells. *Canc Res* 2005 October 15, 2005; **65** (20): 9269-9279.

62. Garraway LA, Lin D, Signoretti S, Waltregny D, Dilks J, Bhattacharya N, *et al.* Intermediate basal cells of the prostate: In vitro and in vivo characterization. *Prostate* 2003; **55** (3): 206-218.

63. Gu Y, Li H, Miki J, Kim K-H, Furusato B, Sesterhenn IA, *et al.* Phenotypic characterization of telomerase-immortalized primary non-malignant and malignant tumor-derived human prostate epithelial cell lines. *Exp Cell Res* 2006; **312** (6): 831-843.

64. Yasunaga Y, Nakamura K, Ewing CM, Isaacs WB, Hukku B, Rhim JS. A novel human cell culture model for the study of familial prostate cancer. *Cancer Res* 2001 August 1, 2001; **61** (16): 5969-5973.

65. Danielpour D. Transdifferentiation of NRP-152 rat prostatic basal epithelial cells toward a luminal phenotype: regulation by glucocorticoid, insulin-like growth factor-I and transforming growth factor-beta. *J Cell Sci* 1999; **112:** 169-179.

66. Robinson EJ, Neal DE, Collins AT. Basal cells are progenitors of luminal cells in primary cultures of differentiating human prostatic epithelium. *Prostate* 1998; **37** (3): 149-160.

67. Zhao H, Nolley R, Chen Z, Reese SW, Peehl DM. Inhibition of monoamine oxidase A promotes secretory differentiation in basal prostatic epithelial cells. *Differentiation* 2008; **76** (7): 820-830.

68. Bonaccorsi L, Carloni V, Muratori M, Salvadori A, Giannini A, Carini M, *et al.* Androgen receptor expression in prostate carcinoma cells suppresses alpha6beta4 integrin-mediated invasive phenotype. *Endocrinology* 2000; **141** (9): 3172-3182.

69. Nagakawa O AT, Hayakawa Y, Junicho A, Koizumi K, Fujiuchi Y, Furuya Y, Matsuda T, Fuse H, Saiki I. Differential expression of integrin subunits in DU-145/AR prostate cancer cells. *Oncol Rep* 2004 May 28, 2004; **12:** 837-841.

70. Naylor MJ, Li N, Cheung J, Lowe ET, Lambert E, Marlow R, *et al.* Ablation of beta1 integrin in mammary epithelium reveals a key role for integrin in glandular morphogenesis and differentiation. *J Cell Biol* 2005; **171** (4): 717-728.

71. Litvinov IV, Vander Griend DJ, Xu Y, Antony L, Dalrymple SL, Isaacs JT. Low-calcium serum-free defined medium selects for growth of normal prostatic epithelial stem cells. *Cancer Res* 2006 September 1, 2006; **66** (17): 8598-8607.

72. Bertram J, Peacock JW, Tan C, Mui ALF, Chung SW, Gleave ME, *et al.* Inhibition of the phosphatidylinositol 3'-Kinase pathway promotes autocrine Fas-induced death of phosphatase and tensin homologue-deficient prostate cancer cells. *Canc Res* 2006 May 1, 2006; **66** (9): 4781-4788.

73. Lin J, Adam RM, Santiestevan E, Freeman MR. The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival

pathway in LNCaP human prostate carcinoma cells. *Cancer Res* 1999 June 1, 1999; **59** (12): 2891-2897.

74. Wen Y, Hu MCT, Makino K, Spohn B, Bartholomeusz G, Yan D-H, *et al.* HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res* 2000 December 1, 2000; **60** (24): 6841-6845.

75. Evans GS, Chandler JA. Cell proliferation studies in the rat prostate: II. The effects of castration and androgen-induced regeneration upon basal and secretory cell proliferation. *Prostate* 1987; **11:** 339-351.

76. Mirosevich J, Bentel JM, Zeps N, Redmond SL, D'Antuono MF, Dawkins HJ. Androgen receptor expression of proliferating basal and luminal cells in adult murine ventral prostate. *J Endocrinol* 1999 September 1, 1999; **162** (3): 341-350.

77. Verhoeven G, Swinnen JV. Indirect mechanisms and cascades of androgen action. *Molecular and cellular endocrinology* 1999 May 25; **151** (1-2): 205-212.

78. Buttyan R, Ghafar MA, Shabsigh A. The effects of androgen deprivation on the prostate gland: cell death mediated by vascular regression. *Current opinion in urology* 2000 Sep; **10** (5): 415-420.

79. Gmyrek GA, Walburg M, Webb CP, Yu H-M, You X, Vaughan ED, *et al.* Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. *Am J Pathol* 2001 August 1, 2001; **159** (2): 579-590.

80. Miranti CK. Application of cell adhesion to study signaling networks. *Methods Cell Biol* 2002; **69:** 359-383.

81. Bill HM, Knudsen BS, Moores SL, Muthuswamy SK, Rao VR, Brugge JS, *et al.* Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells. *Mol Cell Biol* 2004 October 1, 2004; **24** (19): 8586-8599.

82. Lucas JM, True L, Hawley S, Matsumura M, Morrissey C, Vessella R, *et al.* The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. *The Journal of pathology* 2008 Jun; **215** (2): 118-125.

83. Tapia A, Salamonsen LA, Manuelpillai U, Dimitriadis E. Leukemia inhibitory factor promotes human first trimester extravillous trophoblast

adhesion to extracellular matrix and secretion of tissue inhibitor of metalloproteinases-1 and -2. *Hum Reprod* 2008; **23** (8): 1724-1732.

84. Heer R, Robson C, Shenton B, Leung F. The role of androgen in determining differentiation and regulation of androgen receptor expression in the human prostatic epithelium transient amplifying population. *Journal of Cellular Physiology* 2007; **212** (3): 572-578.

85. Mehta P, Robson CN, Neal DE, Leung HY. Fibroblast growth factor receptor-2 mutation analysis in human prostate cancer. *BJU International* 2000; **86** (6): 681-685.

86. Sun A, Tang J, Hong Y, Song J, Terranova PF, Thrasher JB, *et al.* Androgen receptor-dependent regulation of Bcl-xL expression: Implication in prostate cancer progression. *The Prostate* 2008; **68** (4): 453-461.

87. Shaw JLV, Diamandis EP. Distribution of 15 Human Kallikreins in Tissues and Biological Fluids. *Clin Chem* 2007 August 1, 2007; **53** (8): 1423-1432.

88. Qian C-N, Berghuis B, Tsarfaty G, Bruch M, Kort EJ, Ditlev J, *et al.* Preparing the "soil": the primary tumor Induces vasculature reorganization in the sentinel lymph node before the arrival of metastatic cancer cells. *Cancer Res* 2006 November 1, 2006; **66** (21): 10365-10376.

CHAPTER FIVE:
CONCLUSIONS AND PERPECTIVES

SUMMARY

In conclusion, we have generated two models for understanding the role of AR and integrins in regulating cell survival in both prostate and prostate cancer. In the first model, wild-type or mutant AR was re-expressed into the metastatic prostate tumor cell line PC3. Similar to primary prostate cancer, there was an increase in integrin α6β1 with a simultaneous decrease in the other integrins. Like metastatic castrate-resistant prostate cancer, these cells were not dependent on androgen for AR-mediated phenotypes. While this model has several limitations inherent from its parental line and the caveat that AR is being re-expressed in these cells, it is currently one of the better models to use in the context of understanding the relationship between AR and integrin α6 in CRPC. AR-dependent phenotypes can be elucidated without the complication of endogenous AR, siRNA against AR can be used to validate and directly test AR-dependent effects, AR mutants can be expressed to refine mechanisms, and the cells express the laminin integrin α6β1, which is the predominant integrin in primary prostate cancer. We also developed a novel in vitro differentiation model in which normal human primary prostate basal epithelial cells isolated from patients can be induced to differentiate into AR-expressing and androgen responsive cells that recapitulate many of the in vivo characteristics of secretory cells (1). To our knowledge, this is the first in vitro differentiation model in which sufficient numbers of AR-expressing prostate cells can be generated for experimental investigation. These cells can be treated with pharmacological

inhibitors and siRNA to study biochemical and genetic effects. Furthermore, the differentiated AR-expressing cells can be isolated for further analysis.

Using these and other models, we have identified some of the pathways required for survival of prostate and prostate cancer cells (summarized in Table 7). Previous studies from our lab have demonstrated that in normal prostate basal epithelial cells, adhesion to laminin 5 via $\alpha 3\beta 1$ to ERK through EGFR or through Src is required for cell survival (2). The PI3K/Akt pathway is not activated on LM5 and not required for survival. Disruption of either the EGFR/ERK or Src pathway leads to caspase-independent cell death, due to the generation of reactive oxygen species (2). We have demonstrated that in differentiated, AR-expressing prostate epithelial cells, while KGF, AR, and androgen are important for initiating the differentiation process and AR is important to maintain the androgen-dependent phenotype of secretory-like cells, these factors are not required for survival of the committed differentiated cells (1). The primary critical mechanism driving differentiated cell survival is E-cadherin-based cell-cell adhesion and subsequent activation of the PI3K signaling pathway (1).

We have further demonstrated that in two prostate tumor cell lines, PC3 and DU145, PI3K signaling is required for cell survival. We and others have also shown PI3K signaling to be required for survival in LNCaP cells. In addition, adhesion of PC3 cells to laminin 1 promotes survival via integrin-mediated activation of Src leading to increased expression of the pro-survival protein Bcl-xL (2). Inhibition of PI3K or Src results in caspase-dependent cell death (2). In

DU145 cells, adhesion to collagen 1 drives survival through activation of EGFR and ERK. Re-expression of AR in PC3 cells can rescue cells from death induced by inhibition of PI3K when adherent to laminin 1. Expression of AR in PC3 cells leads to increased expression of integrin α6β1 and Bcl-xL along with increased activation of NF-κB. Blocking each of these components individually concurrent with inhibition of PI3K led to death of the AR-expressing cells, suggesting that AR regulates cell survival through enhancement of α6β1/NF-κB/Bcl-xL signaling.

Keeping in mind the caveat that different cell lines were used and that the survival pathways of these cells were studied on different matrices, several broad conclusions can be drawn from this data. First, all of the prostate cancer cell

TABLE 7. Summary of AR, PTEN, and Integrin α6 Expression and Survival Pathways in Prostate and Prostate Cancer Cell Lines In Vitro

		Normal		Tumor			
		Basal (PEC)	Differentiated	PC3	DU145	LNCaP	PC3-AR
Expressed	AR	No	Yes	No	No	Yes	Yes
	PTEN	Yes	n.d.	No	Yes	No	No
	ITGA6	Moderate	No	High	Moderate	Low	Highest
Survival	ECM-Cell Adhesion (Integrin)	Yes	No	Yes	Yes	Yes	Yes
	Cell-Cell Adhesion (E-cadherin)	n.d.	Yes	No	n.d.	n.d.	No
	EGFR	Yes	No	No	Yes	n.d.	n.d.
	ERK	Yes	No	No	Yes	n.d.	n.d.
	PI3K	No	Yes	Yes	Yes	Yes	Yes*
	Src	Yes	n.d.	Yes	n.d.	n.d.	No
	AR	No	No	No	No	Yes	Yes*
Chapter (Reference)		Ch. 1 (2)	Ch. 4 (1)	Ch. 2 (2)	Ch. 2	Ch. 2 (3)	Ch. 3

* Inhibition of both pathways required to induce cell death

lines tested were dependent on PI3K signaling. While the PC3 and LNCaP cell lines do not express Pten (a negative regulator of PI3K signaling) and therefore their dependence on PI3K for survival might be expected; the DU145 cells do express Pten, but still depend on PI3K for survival. Integrin-mediated activation of PI3K/Akt suggests that Akt activity can still be elevated in prostate cancer in the absence of Pten loss. Furthermore, targeting PI3K may still be an effective therapy in tumors that retain Pten expression. Second, PEC cells are not dependent on PI3K signaling, but differentiated AR-expressing cells are. This suggests that prostate cancer may arise from a more differentiated cell that has acquired dependence on PI3K for its survival and expresses AR. Third, while PEC cells are dependent on integrin-mediated adhesion to matrix and subsequent activation of EGFR for survival, differentiated AR-expressing cells do not express integrins, are not adherent to LM or CL matrix, and have reduced levels of EGFR. Subsequently, the cells are not dependent on EGFR or matrix adhesion for survival and instead are dependent on cell-cell adhesion and PI3K to promote cell survival. Fourth, PC3 cells adherent to collagen rather than laminin were not dependent on PI3K signaling for survival. Since the primary metastatic site for prostate cancer in humans is to bone which consists mostly of collagen, this has interesting implications for designing therapies for advanced disease (i.e. targeting PI3K signaling may not be effective in bone metastasis).

DU145s are not as invasive and metastatic as PC3 cells, perhaps representing an intermediate phenotype between PEC and PC3 cells. In support of this, DU145 cells, like PEC cells, were dependent on EGFR for survival. PC3

cells were not dependent on this pathway for cell survival. Interestingly, both PEC and PC3 cells were dependent on Src for survival, suggesting that maintenance of this pathway is critical during disease progression. However, expression of AR in the PC3 cells significantly altered the survival pathways in PC3 cells. Loss of Src or PI3K signaling alone was no longer sufficient to induce cell death. Rather, loss of AR expression or one of its downstream pro-survival targets (i.e. integrin α6, NF-κB, or Bcl-xL) was also required to restore dependence on PI3K signaling for survival. Thus, AR-expressing cells have a distinct survival advantage over AR-negative cells. Furthermore, this supports the need for combinational therapies in order to target and kill prostate cancer cells.

Validation of AR Effects in Additional Models

Other Cell Lines

Although we have established a relationship between AR, integrin α6, NF-κB, and Bcl-xL in PC3-AR cells, the PC3-AR model is not perfect, in that it did not originally express AR, has lost Pten expression, and only represents one oncogenic profile that could be important in this disease. Therefore, it is necessary to extend these studies to additional cells lines in order to generalize whether or not these relationships exist in other cell lines and determine, if oncogenic status (i.e. expression of Pten) influences this relationship, if endogenous AR has the same effects as exogenously expressed AR, and if there is an effect due to androgen sensitivity. We have already demonstrated

some of these relationships in LNCaP cells, which express endogenous but a more promiscuous and mutated AR. We have recently acquired C4-2B cells, a derivative of LNCaP cells that have lost their androgen sensitivity but still retain AR expression. Furthermore, we have re-expressed AR in DU145. Fortunately, some additional new cell lines that express endogenous AR and have different oncogenic backgrounds have also become available, including VCaP, LAPC4, MDA PCa 2b, and 22Rv1 cells. We are currently characterizing relative AR, integrin α6, and Bcl-xL expression levels as well as NF-κB activity in these cells. Lastly, the ability to differentiate primary human prostate epithelial cells needs to be tested in cells derived from other patients as well as from other sources.

In Vivo Studies

In addition to investigating AR and integrin survival pathways in other cell lines, we also need to establish whether they are important in vivo. We are currently investigating existing clinical microarray data sets to determine if a correlation exists between integrin α6 and prostate cancer progression to CRPC. Furthermore Jelani Zarif, a fellow graduate student in the lab, has developed lentiviral constructs that express tetracycline-inducible shRNAs against AR and has demonstrated their effectiveness in vitro. These will be used to inhibit components of the AR survival pathway in PC3 cells and their ability to form tumors when injected orthotopically into nude mice. Castration and PI3K inhibitor studies individually or in combination will be used to test the importance of the pathways in tumor survival. The tumors will also be analyzed for expression of

AR, integrin α6, and Bcl-xL as well as NF-κB or caspase activity by either immunohistochemistry or immunoblotting to validate activation of the pathways in vivo.

Understanding the Process of Differentiation in Prostate Epithelial Cells

Using the in vitro differentiation model we have generated, we can now begin to interrogate the processes that are required and sufficient to drive differentiation. Preliminary data from our lab suggests that inhibition of p38 signaling, but not EGFR or ERK, inhibits the bilayer formation, suggesting that p38 plays an important role in early differentiation. One of the most dramatic observations was loss of the γ2 chain of LM5. This appeared to occur prior to integrin β1 loss. We still need to determine if this is observed for other LM chains and other matrices as well. Is loss of LM5 driving loss of integrin expression, or is integrin lost through another mechanism? Furthermore, what is the mechanism driving LM5 loss? Is it by regulation of the LM5 chains directly, or indirectly through secretion of matrix metalloproteinases (MMPs)? Is loss of LM5 sufficient to drive early stages of differentiation? These questions can be tested in part by using LM5 specific siRNA, qRT-PCR, and zymography to test for the presence of MMPs.

Next Generation of AR-expressing Models

The differentiation model we have described can now be further optimized for generating AR-expressing cells. It may be that pre-selecting cells with "stem-

cell" characteristics before treating with KGF and DHT may enhance this process (4-6). Since KGF is a stromal-derived growth factor, it is exciting to postulate that culturing with additional stromal factors or by performing co-cultures with stromal cells may help further differentiate these cells to appear more columnar in appearance and have increased nuclear AR localization and function. Also, using the culture conditions we have described, it would be interesting to see if this would allow for culturing of human AR-expressing primary prostate cells, since an in vitro model for these cells do not exist.

Another approach to generate a model of primary prostate cancer may be to genetically alter the differentiating cells to become tumorigeneic. In the lab, we have already generated immortalized PEC cells from two patients by expressing the viral oncogenes *E6/E7* and *hTERT*. Using the method described, we can induce differentiation in these cells as measured by AR, PSA, and K18/19 positivity. Interestingly, subsets of these differentiated cells retain integrin β1 expression. The co-expression of AR and integrins in the same cells suggests that genetic manipulation of this model is possible. Since patients do not develop prostate cancer from *E6/E7* and *hTERT* overexpression, we would also want to immortalize or transform PEC cells using oncogenic mutations associated with prostate cancer progression. This includes c-Myc and TMPRSS2/Erg overexpression, or loss of the tumor suppressor proteins Pten and p27/Kip1.

Development of this differentiation model may allow us to test potential prostate cancer therapeutics to see if they will be toxic in normal prostate

epithelial cells, develop a model for early prostate cancer tumorigenesis, and once a number of different patient samples are tested and characterized, may be able to develop a model for slow growing, benign cancer versus faster growing aggressive cancer.

Therapeutic Implications

This data supports the concept that more than one pathway may need to be targeted in cancer in order to effectively target and kill the cells. This has been effective in the treatment of other diseases, such as HIV where a combination of three or four anti-retroviral drugs can provide years of extended life. This has been demonstrated in mouse models of prostate cancer where targeting AR and mTOR, a downstream target of PI3K/Akt signaling, has additive effects in reducing tumor size (7). Based on our studies, we propose that targeting AR, integrin α6, NF-κB, and/or PI3K/Akt may be an effective combination for advance metastatic prostate cancer. Drugs targeting NF-κB and PI3K/Akt are currently available or are in phase I/II clinical trials (reviewed in 8, 9). Integrin αvβ3 blocking antibody has been tested in clinical trials (10-12) and intratumoral administration of liposome-encapsulated integrin αv-siRNA has been shown to inhibit prostate tumor growth in bone xenografts (13). Systemic administration of integrin αv has had some side effects, perhaps due to its role in angiogensis (10-12, 14). However, we would predict that blocking integrin α6 would not have toxic side effects, at least in the prostate and in skin since both these cell types do not depend on integrin α6 for survival and adhesion to laminin

can also be mediated by integrin α3β1 (2, 15). In addition to ADT drugs, AR may be targeted directly. Recently, pre-clinical studies have demonstrated that adeno-associated virus (AAV)-delivery of shRNA specific for AR can reduce tumor burden of human prostate cancer xenografts in nude mice (16). Although AAV has been successfully utilized for gene therapy in some phase I/II trials, there are some concerns that this can lead to insertional mutagenesis and immunogenesis (17-19). This makes nanoparticles an attractive alternative, where multiple drugs or RNAi's targeting AR, integrin α6, NF-κB, and/or PI3k/Akt could be attached (20). Nanoparticles have already shown to be well tolerated in animal models and effective at targeting prostate cancer cells in xenograft models (21). It may be that by targeting several pathways, lower levels of drugs could be used. Using an integrin α6 recognizing antibody would have the additional advantage of targeting the nanoparticles to prostate cancer cells. Lastly, attaching a luminescent or fluorescent marker would allow imaging of the cancer, and could be used to monitor the effectiveness of the therapy.

REFERENCES

1. Lamb LE, Knudsen BS, Miranti CK. E-cadherin-mediated survival of androgen-receptor-expressing secretory prostate epithelial cells derived from a stratified in vitro differentiation model. *J Cell Sci* 2010 Jan 15; **123** (Pt 2): 266-276.

2. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, Miranti CK. Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/ERK or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death. *Mol Biol Cell* 2007 July 1, 2007; **18** (7): 2481-2490.

3. Carson JP, Kulik G, Weber MJ. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B. *Cancer Res* 1999 Apr 1; **59** (7): 1449-1453.

4. Heer R, Collins AT, Robson CN, Shenton BK, Leung HY. KGF suppresses {alpha}2{beta}1 integrin function and promotes differentiation of the transient amplifying population in human prostatic epithelium. *J Cell Sci* 2006 April 1, 2006; **119** (7): 1416-1424.

5. Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ, Collins AT. CD133, a novel marker for human prostatic epithelial stem cells. *J Cell Sci* 2004 July 15, 2004; **117** (16): 3539-3545.

6. Leong KG, Wang BE, Johnson L, Gao WQ. Generation of a prostate from a single adult stem cell. *Nature* 2008 Dec 11; **456** (7223): 804-808.

7. Zhang W, Zhu J, Efferson CL, Ware C, Tammam J, Angagaw M, *et al.* Inhibition of tumor growth progression by antiandrogens and mTOR inhibitor in a Pten-deficient mouse model of prostate cancer. *Cancer Res* 2009 Sep 15; **69** (18): 7466-7472.

8. Karin M, Yamamoto Y, Wang QM. The IKK NF-[kappa]B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004; **3** (1): 17-26.

9. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov* 2009 Aug; **8** (8): 627-644.

10. Posey JA, Khazaeli MB, DelGrosso A, Saleh MN, Lin CY, Huse W, *et al.* A pilot trial of Vitaxin, a humanized anti-vitronectin receptor (anti alpha v

beta 3) antibody in patients with metastatic cancer. *Cancer Biother Radiopharm* 2001 Apr; **16** (2): 125-132.

11. McNeel DG, Eickhoff J, Lee FT, King DM, Alberti D, Thomas JP, *et al.* Phase I trial of a monoclonal antibody specific for alphavbeta3 integrin (MEDI-522) in patients with advanced malignancies, including an assessment of effect on tumor perfusion. *Clin Cancer Res* 2005 Nov 1; **11** (21): 7851-7860.

12. Burke PA, DeNardo SJ, Miers LA, Lamborn KR, Matzku S, DeNardo GL. Cilengitide targeting of alpha(v)beta(3) integrin receptor synergizes with radioimmunotherapy to increase efficacy and apoptosis in breast cancer xenografts. *Cancer Res* 2002 Aug 1; **62** (15): 4263-4272.

13. Bisanz K, Yu J, Edlund M, Spohn B, Hung MC, Chung LW, *et al.* Targeting ECM-integrin interaction with liposome-encapsulated small interfering RNAs inhibits the growth of human prostate cancer in a bone xenograft imaging model. *Mol Ther* 2005 Oct; **12** (4): 634-643.

14. Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, *et al.* Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994 Dec 30; **79** (7): 1157-1164.

15. Manohar A, Shome SG, Lamar J, Stirling L, Iyer V, Pumiglia K, *et al.* Alpha 3 beta 1 integrin promotes keratinocyte cell survival through activation of a MEK/ERK signaling pathway. *J Cell Sci* 2004 Aug 15; **117** (Pt 18): 4043-4054.

16. Sun A, Tang J, Terranova PF, Zhang X, Thrasher JB, Li B. Adeno-associated virus-delivered short hairpin-structured RNA for androgen receptor gene silencing induces tumor eradication of prostate cancer xenografts in nude mice: a preclinical study. *Int J Cancer* 2010 Feb 1; **126** (3): 764-774.

17. Park K, Kim WJ, Cho YH, Lee YI, Lee H, Jeong S, *et al.* Cancer gene therapy using adeno-associated virus vectors. *Front Biosci* 2008; **13:** 2653-2659.

18. Stedman H, Wilson JM, Finke R, Kleckner AL, Mendell J. Phase I clinical trial utilizing gene therapy for limb girdle muscular dystrophy: alpha-, beta-, gamma-, or delta-sarcoglycan gene delivered with intramuscular instillations of adeno-associated vectors. *Hum Gene Ther* 2000 Mar 20; **11** (5): 777-790.

19. Moss RB, Milla C, Colombo J, Accurso F, Zeitlin PL, Clancy JP, *et al.* Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. *Hum Gene Ther* 2007 Aug; **18** (8): 726-732.

20. Pirollo KF, Chang EH. Targeted delivery of small interfering RNA: approaching effective cancer therapies. *Cancer Res* 2008 Mar 1; **68** (5): 1247-1250.

21. Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, *et al.* Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy in vivo. *Proc Natl Acad Sci U S A* 2006 Apr 18; **103** (16): 6315-6320.

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03063 5415