MODELING HUMAN PROSTATE CANCER DEVELOPMENT USING TRANSGENIC MICE WITH HETEROGENEOUS MUTATIONS

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Pathology

December, 2009

Nashville, Tennessee

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To my parents and sister

for their endless support and encouragement

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Sarki Abdulkadir who has been my mentor for five years. He provided me with wonderful opportunity to involve in research projects using valuable transgenic mice, so I could get better understanding of carcinogenesis in *in vivo* system. In addition, he has shown me an excellent role model as an independent scientist, and been a wonderful advisor as a mentor. When we discussed about research in progress, he always led me to think the projects critically, wisely and creatively, so I could reach the goals more efficiently. Furthermore, he has suggested valuable ideas whenever I confronted difficulties.

Next, I would like to thank my dissertation committee: Dr. Richard Hoover, Dr. Harold Moses, Dr. Gregory Sephel, Dr. Stephen Hann and Dr. Neil Bhowmick. They viewed my on-going works critically and suggested priceless ideas in regular committee meetings, so guided me to minimize mistakes and finish the goals successfully. Moreover, they advised better way to prove key points and generously provided me with important reagents for my experiments whenever I requested. I am very grateful for their support and encouragement.

I am also grateful to Dr. Isam-Eldin Eltoum in the University of Alabama at Birmingham. His insightful suggestion contributed to completing my paper sooner and his independent data analysis as an unbiased pathologist helped us to be confident of the significance of our findings.

I also want to acknowledge people in Abdulkadir laboratory and the next-door labs. Dr. Meejeon Roh, a research instructor, helped me a lot to adjust to new environment when I first joined this lab. She taught and helped me many experiments when I started my projects, and she and her husband Dr. Chisoo Song sincerely advised me on living in the US. I also thank junior graduate students, Jie Wang, Sydika Banks, Erin Martinez and Monica Logan for sharing their future dreams and friendship. In addition, thanks to the lab manager Irina Doubinskaia who supported the lab to be well-organized and maintained experimental animals, I could work on my research efficiently and smoothly without wasting time. I also thank Dr. Ashish Mogal, a former graduate student for encouraging each other since we joined the lab at the same time, by which I could overcome many moments of disappointment and frustration. Moreover, I could not reach my goal without the help of former research assistants, Bernard Gary and Riet van der Meer, so I value their great support and help. I also acknowledge Nady Roodi and Dr. Chastity Bradley, a former graduate student, in Dr. Fritz Parl's lab.

In addition, I would like to thank people in Vanderbilt University Prostate Cancer Center laboratories who provided me with opportunity to present and discuss about my work in progress quarterly, and get beneficial feedbacks. Also, I thank the Human Tissue Acquisition lab that processed numerous mouse and human tissues of mine, which facilitated me to perform subsequent critical experiments.

Lastly, I would like to acknowledge my family: my parents and sister. Although they live in 7000 miles away, they supported me spiritually with enormous encouragement not to feel disappointed and depressed. Also, my parents visited US twice to stay with me and share happy moments during their vacation. Their visit and travel with them comforted and inspired me to propel my research with new energy. I am also grateful to financial support of grants CA094858 and CA123484 (S.A.A.) from the NIH.

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PZ	Peripheral zone
CZ	Central zone
TZ	Transition zone
BPH	Benign prostatic hyperplasia
UGS	Urogenital sinus
DHT	5α-dihydrotestosterone
AR	Androgen receptor
FOX	Forkhead box
Shh	Sonic hedgehog
FGF	Fibroblast growth factor
TGFb	Transforming growth factor beta isoform
ASC	The American Cancer Society
PIN	Prostatic intraepithelial neoplasia
LGPIN	Low grade PIN
HGPIN	High grade PIN
MB	MYC box
bHLH-Z	basic Helix-loop-helix leucine-zipper
NLS	Nuclear localization signal
NTD	N-terminal domain
CTD	C-terminal domain
TAD	Transactivation domain
HAT	Histone acetyl transferase
MEF	Mouse embryonic fibroblast
CDK	Cyclin-dependent kinase
GI	Gastrointestine
HDAC	Histone deacetylase
ROS	Reactive oxygen species
LDHA	Lactate dehydrogenase A
IRP2	Iron responsive element binding protein 2
PIP3	PI(3,4,5)P3 (Phosphoinositol tri-phosphate)
PI3K	Phosphoinositol 3 kinase
Jnk	c-Jun N-terminal kinase
BMP	Bone morphogenetic protein
Chk1	DNA damage checkpoint kinase 1
DSB	Double-stranded DNA break
FISH	Fluorescence *in situ* hybridization
AP	Anterior prostate
DP	Dorsal prostate
LP	Lateral prostate
VP	Ventral prostate
Pb	Probasin
RXR	Retinoid X receptor

Rb	Retinoblastoma
TRAMP	Transgenic adenocarcinoma of the mouse prostate
PRL	Prolactin
ER	Estrogen receptor
KO	Knockout
15-LO-1	15-lipoxygenase-1
TSG	Tumor suppressor gene
APC	Adenomatous polyposis coli
floxed	flanked by loxP sites
Egr-1	Early growth response gene 1
Pten (=MMAC 1)	Phosphatase and tensin homologue
MMAC 1 (=Pten)	Mutated in multiple advanced cancers 1
GEM	Genetically engineered murine
mTOR	mammalian Target of rapamycin
CTLA-4	Cytotoxic T-lymphocyte antigen 4
COX-2	Cyclooxygenase 2
α-DFMO	α-Difluoromethylornithine
ODC	Ornithine decarboxylase
ATRA	All-trans retinoic acid
GSE	Grape seed extract
PNP	Purine nucleoside phosphorylase
2-FA	2-Fluoroadenine
rAAV-6	recombinant Adeno-associated virus-6
PEV	Position-effect variegation
BLG	Beta-lactoglobulin
eGFP	enhanced Green fluorescent protein
eYFP	enhanced Yellow fluorescent protein
eCFP	enhanced Cyan fluorescent protein
NT	Nuclear transfer
REMI	Restriction enzyme mediated integration
CFAP	Glial fibrillary acidic protein
MSI	Microsatellite instability
Pms2	Postmeiotic segregation increased 2
β-geo	β-galactosidase/Neomycin resistance fusion gene
ES	Embryonic stem
S.M.	Skeletal muscle
smMHC	smooth muscle Myosin heavy chain
PCR	Polymerase chain reaction
poly A	polyadenylation signal
SD	Standard deviation
PBS	Phosphate-buffered saline
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
pHH3	phospho-Histone H3
CAA	Cell-autonomous apoptosis
NCAA	Non-cell-autonomous apoptosis

CASP3	Caspase 3
CM	Conditioned media
Dpp	Decapentaplegic
Wg	Wingless
DTT	Dithiothreitol
M-MLV	Moloney murine leukemia virus
Gapdh	Glyseraldehyde-3-phosphate dehydrogenase
TSA	Tyramide Signal Amplification
DAPI	4',6-Diamidino-2-phenylindole
ATCC	American Type Culture Collection
EGF	Epidermal growth factor
VSVG	Vesicular stomatitis virus glycoprotein
CK8	Cytokeratin 8
SMA	Smooth muscle actin
LOH	Loss of heterozygosity
HNSCC	Head and neck squamous cell carcinoma
DMSO	Dimethylsulfoxide
LCM	Laser-captured microdissection
FFPE	Formalin-fixed paraffin-embedded
rRNA	ribosomal RNA
RIN	RNA Integrity Number
MAPK	Mitogen-activated protein kinase

CHAPTER I

INTRODUCTION

The Prostate

The prostate structure

The prostate gland is a part of the mammalian male reproductive system about the size of walnut, which is located just in front of the rectum and below the bladder. The prostate consists of many small exocrine glands that produce fluid portion of semen, which contains important secretory proteins and helps the motility of the sperm. The prostate is divided into several compartments, i.e. lobes (Hutch, 1972; Lowsley, 1912) or zones (McNeal, 1968; McNeal, 1972; McNeal, 1978; McNeal, 1980; McNeal, 1981). According to McNeal, the prostate is divided into four compartments. First, the peripheral zone (PZ) occupies more than 70% of the glandular prostate and the majority of prostate cancers arise from this area. The peripheral zone is close to the rectum since it is located in the back of the prostate gland, and its ducts radiate laterally from the urethra and are lateral and distal to the verumontanum. Second, the central zone (CZ) constitutes ~25% of the glandular prostate and its ducts branch proximally to the ejaculatory duct, and the lateral branches lie parallel to the proximal branches of the peripheral zone. Third, the urethral transition zone (TZ) is located around the urethra and proximally to the base of the verumontanum. It is the primary area

where benign prostatic hyperplasia (BPH) develops. Fourth, the anterior fibromuscular stroma is entirely non-glandular and makes up about 1/3 of the prostate volume within the prostatic capsule (Figure 1).

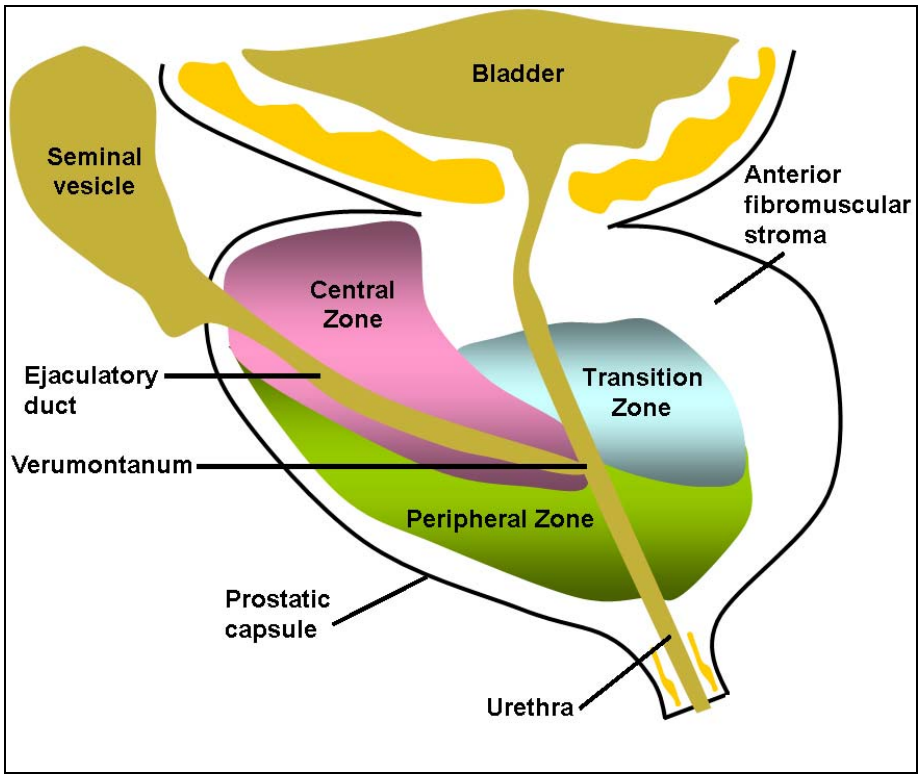


Figure 1. The zonal structure of the human prostate (Sagittal view). The prostate is mainly composed of 4 subdivisions: peripheral zone, central zone, transition zone and anterior non-glandular fibromuscular stroma.

The prostate development

Embryologically, the prostate derives from the urogenital sinus (UGS) which is endodermal, unlike the mesodermal derivation of most other male accessory sex glands (Prins, 2008). The binding of 5α-dihydrotestosterone (DHT) to the androgen receptor (AR) in mesenchymal tissue induces the prostatic buds from the UGS to initiate outgrowth (Shannon, 1983; Takeda, 1991; Takeda, 1985). The outgrowth of the buds undergoes extensive branching morphogenesis at the fetal stage. After birth, androgens affect lumen formation of the ducts and the epithelium starts to differentiate and synthesize secretory products (Timms, 2008) (Figure 2). However, full maturation of the prostate occurs at puberty.

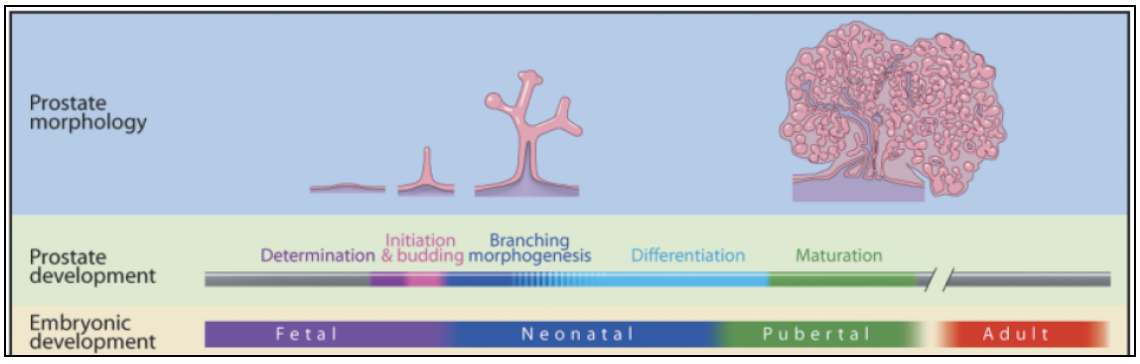


Figure 2. The stages of the prostate development. [Adapted from (Prins, 2008)]

Hormonal and genetic regulation of prostate development

During the fetal stage, androgens produced from the fetal testes regulate prostatic development. A series of studies using rodent animal models demonstrated that surgical/chemical castration during particular periods of fetal life inhibits prostate development (Cunha, 1973; Jost, 1953; Lasnitzki, 1977; Price, 1936; Price, 1961). Upon androgen ablation in *ex vivo* organ culture, however, the mouse/rat UGS retrieved at the time when fetal testes produce testosterone formed prostatic buds but did not form buds before testosterone production (Cunha, 1973; Lasnitzki, 1977), which suggests that the timing of androgen ablation is thought to be critical when the prostatic bud formation initiates. Also, DHT which more actively binds to AR induces prostatic budding more efficiently than its parental compound, testosterone, which implies that the entire dependence of prostate development is on hormonal (androgen) regulation with androgen receptor signaling at fetal stage. Hormonal action of androgen is mediated by interacting with AR and inducing transcriptional activity of AR.

In addition to the hormonal regulation with androgen receptor signaling, some candidate genes are also believed to regulate morphogenesis in prostate development either by each individual gene itself or by cross-talk among them. They are categorized into (1) nuclear transcription factors including homeobox genes and (2) secreted signaling molecules that act through paracrine/autocrine signaling between epithelial and mesenchymal cells (Hogan, 1999). As shown in Table 1, *Hox* genes are known to determine body patterning from invertebrates (fruit fly) to vertebrates (humans) (Krumlauf, 1994), and Nkx3.1 has critical roles

in prostate branching morphogenesis and differentiation (Bhatia-Gaur, 1999; Schneider, 2000; Tanaka, 2000). Transcription factors like Forkhead box genes (*Fox*) including *Fox* A1/A2 are thought to be involved in ductal morphogenesis, epithelial cell maturation and bud initiation (Kopachik, 1998; Mirosevich, 2005). Also, Notch signaling pathway determines cell fate and patterning in tissue development (Bolós, 2007), as it inhibits expansion of prostatic progenitor cells and helps differentiation of epithelial cells during prostate development (Grishina, 2005; Shou, 2001; Wang, 2006; Wang, 2004). Furthermore, paracrine or autocrine factors such as Hedgehogs, Fgfs, Bmps/Tgfβ/Activins and Wnt signaling regulators and their associated receptors mediate signals between epithelial and mesenchymal cells.

Table 1. Genes involved in the prostatic development

Gene types	Representative genes
Homeobox genes	*Hox* genes *Nkx3.1*
Transcription factors	*Fox A1/A2* *Notch1/Delta/Jagged*
Secreted signaling molecules	*Sonic hedgehog (Shh)* *Fibroblast growth factor-10* *Bmps/Tgfb/Activins* *Wnt* genes and signaling regulators

Cell types within prostatic ducts

Within human prostatic ducts, epithelial cells can be classified into at least 3 cell types. They are morphologically and functionally distinct, and are composed of secretory luminal cells, basal cells present between luminal cells and basement membrane, and neuroendocrine cells which are very rare and express various neuropeptides (Figure 3). Luminal cells are the most predominant, are androgen-dependent, and uniquely produce prostatic secretory proteins. They are distinguished by the expression of the androgen receptor (AR), cytokeratin 8 and 18 and surface marker CD57 (Brawer, 1985; Liu, 1997; Nagle, 1987; Sherwood, 1990; Verhagen, 1988). The second major epithelial cells are basal cells that express cytokeratin 5 and 14, CD44 and low levels of AR (still controversial) (Brawer, 1985; Bui, 1998; Liu, 1997; Nagle, 1987; Sherwood, 1990; Verhagen, 1988). Prostatic stem cells are believed to present as a subpopulation of basal cells which are androgen-independent (Bonkhoff, 1996; De Marzo, 1998). Third, androgen-independent neuroendocrine cells constitute a minor portion of the prostatic epithelium and are mostly distributed in the basal layer. They are thought to be involved in providing paracrine signals that facilitate the growth of luminal epithelial cells but their embryological origin is not clear. They express distinct factors like serotonin, chromogranin A and neuropeptides (Abrahamsson, 1999; di Sant'Agnese, 1992; di Sant'Agnese, 1998).

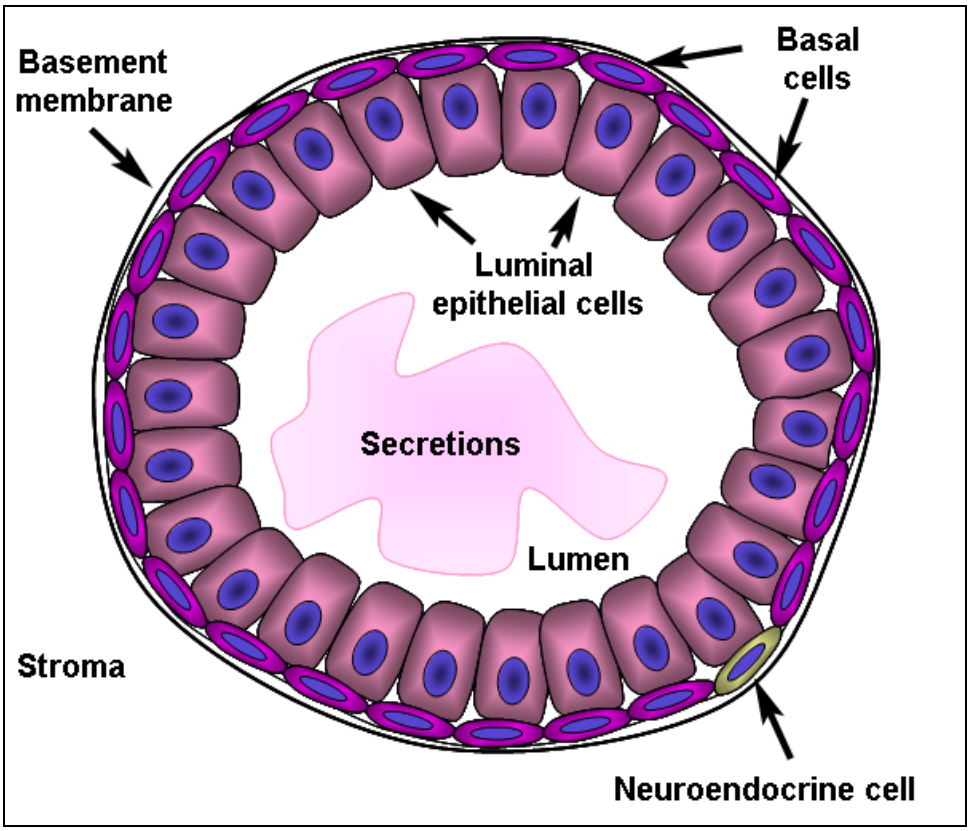


Figure 3. Cell types within a human prostatic duct. Prostatic epithelial cells are composed of luminal, basal and neuroendocrine cells which are very rarely present.

Stem cells in the prostate

The existence of prostate stem cells in the basal cell layer was hypothesized from the observation that basal cells preferentially survived upon androgen ablation (Isaacs, 1987). p63 (basal cell-specific marker) deficient mice were born without a prostate (Signoretti, 2000) and secretory prostate cells

originated from p63+ basal progenitor cells in the chimeric blastocysts consisting of p63- and p63+ cells during development (Signoretti, 2005). Recently, prostate-regenerating cells in the proximal region of mouse prostate were enriched using Sca-1, a cell surface marker, although Sca-1+ cells in the distal region or even Sca-1- cells were observed to regenerate prostatic cells (Xin, 2005). In the human prostate, stem cells were isolated using $\alpha 2\beta 1$ integrin (Collins, 2001; Hudson, 2000) and CD133 (Richardson, 2004). $\alpha 2\beta 1^{hi}$/CD133+ cells were randomly found all over the prostatic acini/ducts (Collins, 2001; Richardson, 2004) and at the base of budding areas or branching tips. Moreover, cells that were morphologically and phenotypically intermediate between basal and luminal epithelial cells were identified within normal epithelium (Brandes, 1966; Mao, 1966; Sherwood, 1990; van Leenders, 2000; Verhagen, 1992), which suggested possible hierarchical relationship between two cell types that are derived from the same origin.

In general, stem cells are quiescent and localized in a specific area called a niche. The microenvironment in the niche is known to support the stem cells to be balanced between quiescence and self-renewal (Lang, 2009). In human adult prostate, epithelial stem cells were shown to exist at the basement membrane with high expression of $\alpha 2\beta 1$ integrin (Collins, 2001). $\beta 1$ integrins are known to maintain stem cell functions and promote asymmetric division of mammary stem cells (Taddei, 2008). Inductive mesenchymal signal is reported to control epithelial cell differentiation during prostate development with the help of androgen signaling (Cunha, 1989). This is further supported by the finding that

rodent prostatic mesenchyme could regulate the generation of prostatic epithelial tissues from human embryonic stem cells (Barclay, 2008; Taylor, 2006).

Prostate cancer statistics and risk factors

Prostate cancer is the most commonly diagnosed cancer and the second largest cause of cancer deaths in American men. The American Cancer Society (ASC) estimates that nearly 192,280 new cases will be diagnosed, and 27,360 men will die of prostate cancer in the US during 2009 (www.cancer.org). Although certain links to prostate cancer are not clear, several risk factors are reported to significantly contribute to prostate cancer, including age, region, race, family history, steroid hormone, life style, and infection and inflammation of the prostate (www.cancer.org) (Fleshner, 2009; Fradet, 2009). Age is the most significant factor for prostate cancer. The disease incidence dramatically increases for men of 50 or older, and two out of every three prostate cancer cases are found in men over the age of 65. The incidence of prostate cancer in some regions like North America and northwestern Europe is known to be much higher than Asian countries. Moreover, prostate cancer is more common in African-American men than in men of other races. Although hereditary prostate cancer cases are relatively rare, certain inherited mutations are known to increase prostate cancer risk and cause prostate cancer from generation to generation in some families. Mutations in BRCA2, for example, seem to raise the risk up to 20 times (Edwards, 2003), and multiple chromosome loci on 8q24 are also reported to be important loci that could increase prostate cancer risk (Cheng,

2008). In addition to prostate development, signaling between steroid hormones and their receptors is also known to play an important role in prostate tumorigenicity, so hormonal changes, i.e. androgen/estrogen in men, can alter incidence of prostate cancer. Studies have shown that obesity increases the risk of prostate cancer, so life styles, such as diet and exercise, are also believed to be important. Furthermore, infection and inflammation of the prostate gland might affect the risk, although the link is still controversial (www.cancer.org) (Klein, 2008; Sutcliffe, 2008).

Molecular genetics of prostate cancer

Prostate cancer is thought to arise from accumulated genetic mutations that may transform benign prostatic epithelium to prostatic intraepithelial neoplasia (PIN), a precursor of prostate cancer. PIN displays four characteristic structural patterns, i.e. tufting, micropapillary, cribriform, and flat (Bostwick, 1996; Bostwick, 1999; Bostwick, 1993; Bostwick, 1987; Nagle, 1991). This stage is sub-categorized into low grade PIN (LGPIN) and high grade PIN (HGPIN), and the latter is believed to be an immediate precursor of early invasive carcinoma. Both PIN and early prostatic carcinoma commonly display reduction of E-cadherin and vimentin which are an adhesion protein and a cytoskeletal component, respectively. Invasive carcinoma exhibits discontinuous basement membrane due to microinvasion of transformed epithelial cells into stroma. PIN lesions, however, display complete glandual structure with intact and continuous basement membrane (Bostwick, 1993). With additional genetic alterations, PIN

progresses to invasive cancer, and finally to metastatic disease (Figure 4). Metastatic prostate cancer is generally accompanied by emergence of androgen-independency, marked by failure to respond to androgen ablation therapy.

It has been observed that distinct chromosomal loci including 6q, 7q, 8p, 10q, 13q, 16q, 17p and 18q are deleted in human prostate cancer specimens at different stages of prostate cancer progression, although individual deletion frequencies vary and more characterization is still needed (Figure 4) (Cooney and Wojno, 1996; Cunningham, 1996; Elo, 1997; Latil, 1994; Latil, 1997; Saric, 1999; Takahashi, 1995; Zenklusen, 1994). According to allelic loss on the chromosome locations, some tumor suppressor genes such as *NKX3.1*, *PTEN*, *RB* and *p53*, are potential candidate genes (Figure 4). In addition to candidate tumor suppressor genes, various cancer-related genes contribute to prostate cancer progression at each stage (Abate-Shen, 2000; Tomlins, 2006), i.e. initiation from normal prostate, progression to early carcinoma, and development to metastatic disease (Table 2).

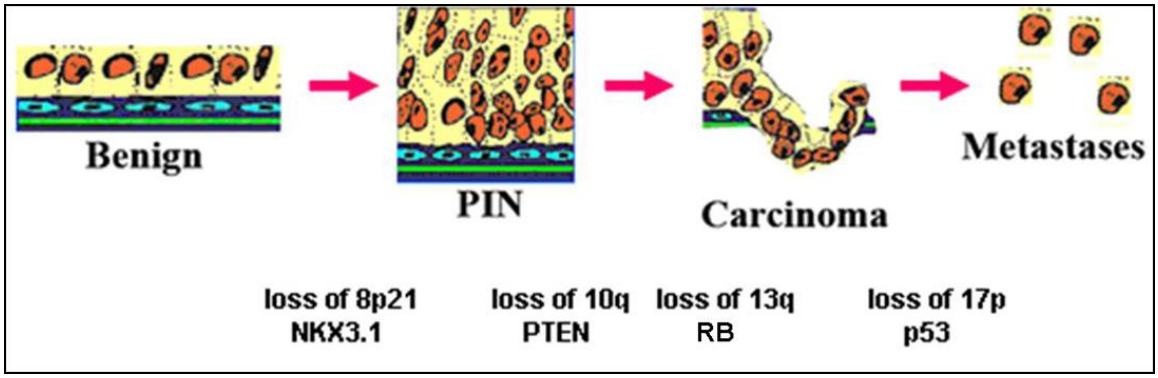


Figure 4. Multi-step model of prostate cancer progression. Distinct loci on the chromosomes are frequently deleted during human prostate cancer progression.

Table 2. Candidate genes dysregulated in prostate cancer progression

Genes	Function
Initiation (Normal to PIN)	
NKX3.1	Homeodomain transcription factor
Progression to carcinoma	
MYC, MXI1	Transcription factors
PTEN	Phosphatase
RB, p27 and p16	Cell cycle regulators
Telomerase	Reverse transcriptase
FGFs	Growth factors
E-cadherin, c-CAM, Integrins	Cell adhesion/interaction
c-MET	Tyrosine kinase receptor
Advanced carcinoma and metastatic disease	
AR	Nuclear hormone receptor
p53	Transcription factor/Apoptotic regulator
BCL2	Apoptotic regulator
IGF1, TGFα, TGFb1, EGF	Growth factors
KA1	Membrane protein

C-Myc

Gene and protein structure of c-Myc

c-Myc is an oncogenic, basic helix-loop-helix transcription factor. c-Myc was first identified as a homologue of viral oncogene *v-Myc* of avian myelocytomatosis virus MC29 (Vennstrom, 1982). *N-Myc* and *L-Myc* are also members of Myc proto-oncogene family whose sequences were found amplified in neuroblastoma and small cell lung cancer, respectively (Nau, 1985; Schwab, 1983). These three *Myc* genes have been the most intensively studied (Table 3). *c-Myc* gene is composed of three exons and it encodes a short, predominant protein product, c-Myc 2, and long form, c-Myc 1, which is translated from alternative translation initiation site, CTG on the exon 1 (Figure 5A). The expression levels of the long form c-Myc 1 are generally low, but induced to levels comparable to short form when *in vitro* cultured cells are highly confluent (Hann, 1992). The function of c-Myc 1 seems to be contradictory because it was shown to inhibit *in vitro* cell growth unlike short form (Hann, 1994) but further investigations are needed to characterize differential functions between short and long form c-Myc. c-Myc protein is basically composed of two domains. First, MYC box I and II (MBI and MBII) at the N-terminus of c-Myc are required for the transactivation of target gene expression. Second, basic helix-loop-helix leucine-zipper (bHLH-Z) domain at C-terminus is a crucial region for binding of c-Myc to a conserved DNA sequence such as E-BOX (CACGTG) for transactivation (Blackwood, 1991) (Figure 5B).

Table 3. Mammalian *Myc* family genes

Myc genes	Functions
c-Myc	Cellular transformation Apoptosis Cell cycle progression
L-Myc	Cellular transformation Cell cycle progression
N-Myc	
S-Myc	Growth suppression Apoptosis
B-Myc	Inhibition of neoplastic transformation
P-Myc/L-Myc Ψ	Pseudogenes

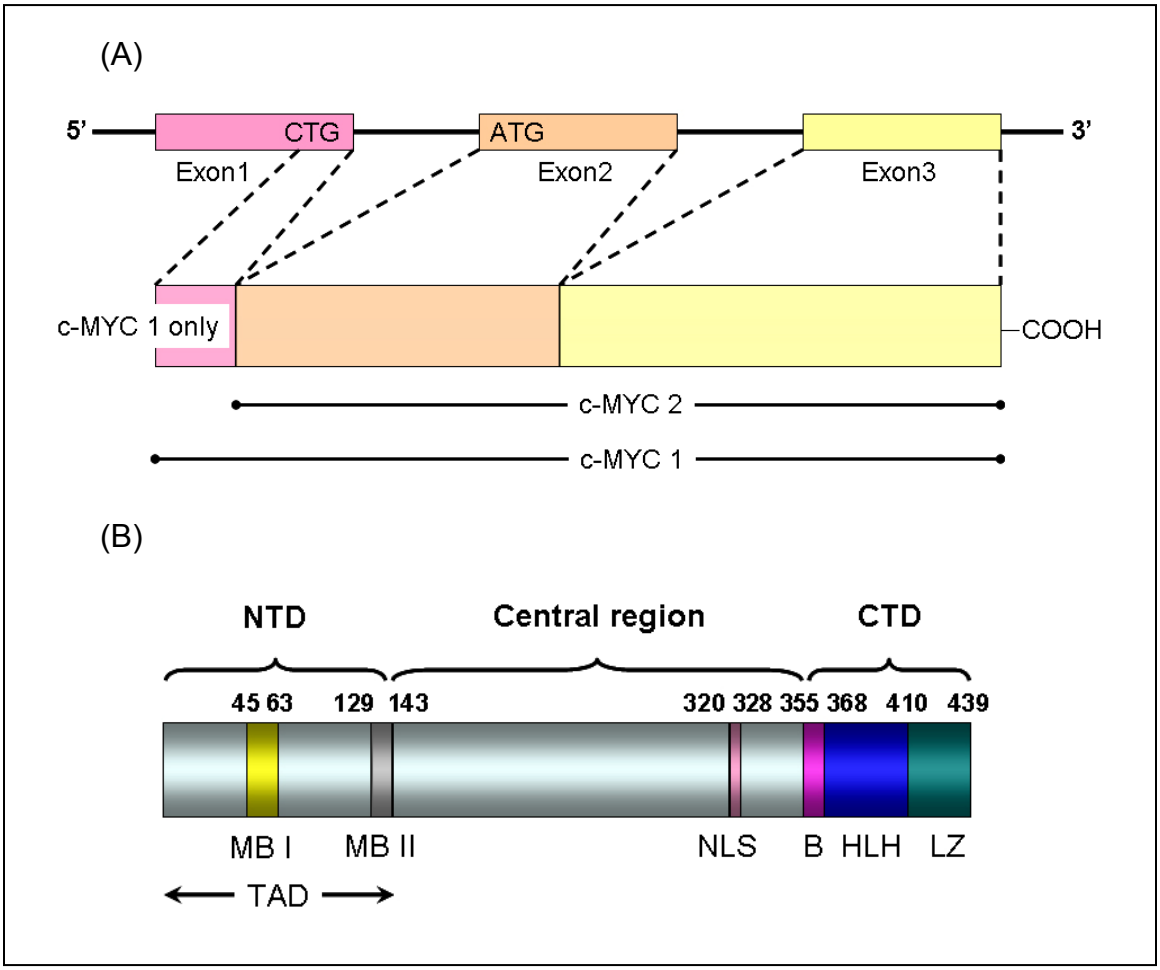


Figure 5. Schematic depiction of human *c-MYC* gene and its protein products. Long form of c-MYC protein (c-MYC 1) is initiated from alternative translation site (CTG) (A). (B) describes MYC boxes (MB I & II), nuclear localization signal (NLS), basic region (B), helix-loop-helix (HLH) and leucine-zipper (LZ) on c-MYC protein. NTD, CTD and TAD stand for N-terminal domain, C-terminal domain and transactivation domain, respectively. Numbers indicate amino acids.

Transcription regulation by c-Myc

There are multiple molecules that interact with c-Myc and they activate or repress transcription activity as transcriptional coregulators (Figure 6). To activate target gene transcription, c-Myc binds to the E-BOX sequence after forming a heterodimer with another basic helix-loop-helix leucine-zipper protein MAX (Solomon, 1993) and sometimes coactivators such as Pim-1 kinase (Zippo, 2007) and TRRAP (McMahon, 1998) with its associated HATs (histone acetyl transferases, GCN5 or Tip60) (Frank, 2003; Frank, 2001; McMahon, 2000) (Figure 6B). On the other hand, MIZ-1, a zinc-finger transcription factor, binds to c-Myc-MAX heterodimer and this interaction is believed to be important for repressing transcriptional activity of c-Myc, although the mechanism of repression is still not clear (Herold, 2002; Kime, 2002; Seoane, 2002; Seoane, 2001; Staller, 2001; van de Wetering, 2002; Wu, 2003) (Figure 6C). Table 4 shows selected target genes either induced or repressed by c-Myc, and their functions (Adhikary, 2005).

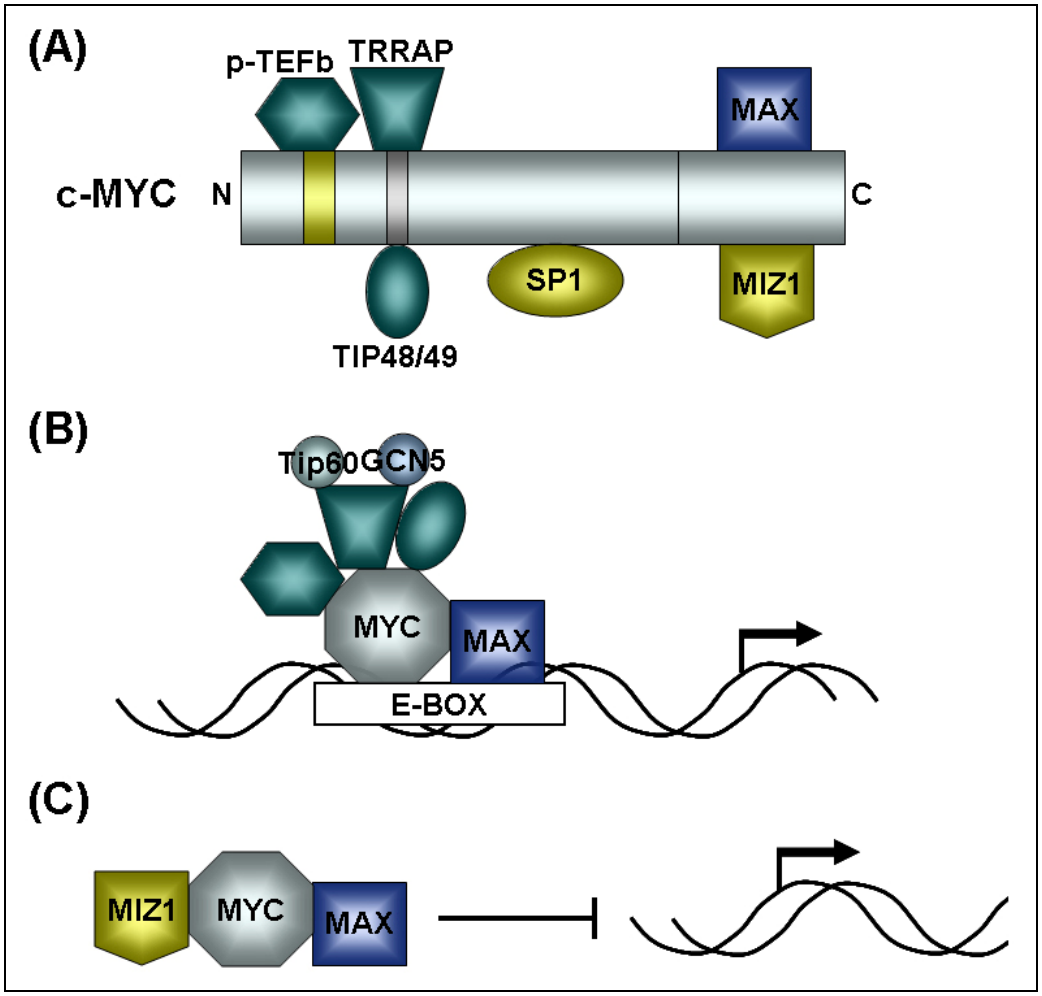


Figure 6. c-Myc binding proteins and transcription activation/repression by c-Myc with coregulators. (A) Representative c-Myc-interacting molecules and their binding sites on c-Myc. (B) and (C) describe transcription activation and repression by c-Myc and its coregulators, respectively.

Table 4. Selected c-Myc target genes and their functions

Target genes	Regulation	Functions
ODC, RCL, *HMGI/Y, PMTA*	UP	Anchorage-independent growth Transformation (rat fibroblast)
HDAC2	UP	Suppression of differentiation
CCND1, *CCND2, CDK4*	UP	G1 progression upon mitogen stimulation Proliferation, carcinogenesis
E2F2	UP	c-Myc-induced proliferation
LDHA	UP	c-Myc-induced transformation
SHMT	UP	c-Myc-induced proliferation and growth
IRP2 *H-ferritin*	UP DOWN	c-Myc-induced proliferation and transformation
$p21^{CIP1}$	DOWN	Cell cycle checkpoint Cell differentiation
$p15^{INK4B}$	DOWN	TGF-β-mediated proliferation arrest
N-cadherin, *integrins*	DOWN	Adhesion of stem cells to stem cell niche

Biological functions of c-Myc

c-Myc has multiple roles in regulating biological phenotypes including cell proliferation, apoptosis, transformation, and inhibition of differentiation (Figure 7).

Cell proliferation and cell growth

One of the most crucial functions of c-Myc is that it promotes cell proliferation by cell-cycle progression. In quiescent cells *in vitro*, c-Myc levels are not detectable, but the expression levels of c-Myc mRNA and protein are dramatically increased when cells are stimulated with mitogens or serum (Amati, 2001; Amati, 1998; Dang, 1999; Eilers, 1999). In c-Myc-deficient rat fibroblast cells, cell proliferation is dramatically suppressed and cells show cell-cycle defects in the G1 phase (Mateyak, 1997). In addition, homozygous c-Myc deletion in mice *in vivo* leads to embryonic lethality (Davis, 1993), suggesting an important role of c-Myc in regulating normal growth during embryonic development (Davis, 1993).

Cell-cycle in G1-S progression of eukaryotic cells is controlled by the complexes of cyclins and CDKs (cyclin-dependent kinases), i.e. cyclin D-CDK4 and cyclin E-CDK2, and c-Myc regulates G1-S progression by activating or repressing target genes involved in cell-cycle progression. In fact, c-Myc is known to directly activate the expression of *CCND2* (cyclin D2) and *CDK4* (Bouchard, 1999; Coller, 2000; Hermeking, 2000) or recruit HATs associated with TRRAP indirectly to change chromatin structure and enhance the accessibility of c-Myc-MAX complex to the DNA sequence (Figure 6) (Bouchard, 2001). Also, c-

Myc inhibits cell-cycle arrest by repressing the expression of cell-cycle arrest genes such as CDK inhibitors (e.g. $p15^{INK4b}$) (Staller, 2001) and $p21^{WAF1/CIP1}$ (Herold, 2002).

In addition to cell proliferation, dMyc (*Drosophila* orthologue of c-Myc) is reported to control cell size in the wing imaginal disc during fly development, although cell-cycle distribution was not affected upon diminished dMyc levels (Johnston, 1999). When c-Myc was activated in *p27*-deficient mouse embryonic fibroblasts (MEFs), cell growth was stimulated without affecting cyclin E-CDK2 activity (Beier, 2000). In P493-6 cells, a human B cell line, c-Myc induced cell growth in the absence of cell division (Schuhmacher, 1999). Moreover, Eu-Myc transgenic mice, which are engineered to overexpress c-Myc in the B lymphocytes, also displayed elevated cell growth (Iritani, 1999; Pelengaris, 2002a). Evidence demonstrates that c-Myc increased cell size without affecting proliferation through cell-cycling.

Inhibition of cell differentiation

There seems no doubt that c-Myc inhibits cell differentiation because cell-cycle progression and terminal differentiation are generally incompatible each other. However, c-Myc is reported to block cell differentiation regardless of promoting cell proliferation (La Rocca, 1994; Ryan, 1997). Some studies on the crypts of the GI (gastrointestinal) system and epithelium of the skin demonstrated that c-Myc expression was observed in proliferating cells while MAD protein expression was restricted in differentiating cells where c-Myc was undetectable

(Chin, 1995; Hurlin, 1995a; Hurlin, 1995b). It is not clear whether downregulated c-Myc caused cell differentiation or the former was just one of the consequences of the latter. However, studies demonstrated that c-Myc, MAX and MAD family transcriptional repressors, which include MAD1, MXI1, MAD3 and MAD4, were believed to be involved in regulating terminal differentiation (Foley, 1999; Grandori, 2000). Moreover, MAX dimerized with MAD and this dimer competed with c-Myc-MAX complex by binding to E-BOX. Also, MAX-MAD recruited HDACs (histone deacetylases) to E-BOX where MAX-MAD bound and repressed c-Myc target gene expression by decreasing the accessibility of transcription factors following local chromatin modification (Ayer, 1995; Schreiber-Agus, 1995). *In vivo* evidence showing that a knockout mouse model of Mad1 exhibited altered differentiation of granulocytes supported the role c-Myc in cell differentiation (Foley, 1999).

Cell apoptosis

Some oncoproteins such as c-Myc and E1A are known to enhance cell proliferation as well as apoptosis (Askew, 1991; Debbas, 1993; Evan, 1992). Although contradictory, cell proliferation index and apoptotic rate increased simultaneously when transformed cells expanded. Although cellular context such as cell types, tissue location, and presence of additional mutations affect the machinery of c-Myc-induced apoptosis, increasing evidence demonstrates a strong link between c-Myc and apoptosis. When rat fibroblasts with c-Myc overexpression were cultured under low serum, cells completely underwent

apoptosis (Evan, 1992). c-Myc is also known to sensitize cells to pro-apoptotic stimuli such as hypoxia, DNA damage through ROS (reactive oxygen species) induction, limited survival factors (Askew, 1991; Evan, 1992) and apoptotic signaling pathways (Hueber, 1997; Klefstrom, 1994; Lutz, 1998). Furthermore, c-Myc is involved more directly in apoptosis by inducing cytochrome c release from mitochondria into cytoplasm, which is an initial stage of general apoptotic pathway (Juin, 1999). c-Myc expression is important for activating BAX through a conformational change that induces cytochrome c release, and it is tightly controlled by the presence of other anti-apoptotic molecules, e.g. BCL2, BCL-X_L, or other survival factors (Brenner, 2000; Eskes, 1998; Green, 2002; Juin, 1999; Soucie, 2001). Additionally, c-Myc activates $p19^{ARF}$ expression, an inhibitor of MDM2 (E3 ligase), resulting in p53 stabilization, so is thought to contribute p53-dependent apoptosis (Zindy, 1998). It is also observed in Eu-Myc transgenic mice *in vivo* (Jacobs, 1999).

Cellular transformation

Data suggest that c-Myc induces cellular proliferation or transformation by regulating cell-cycle and metabolic pathways. First, cyclin D-CDK4 complex is believed to have an important role in c-Myc-induced transformation since cyclin D1/D2/D3-deficient fibroblasts were not transformed by c-Myc overexpression (Kozar, 2004) and CDK4-deficiency significantly repressed tumorigenic activity of c-Myc in epithelial tissues (Miliani de Marval, 2004). Second, c-Myc is known to enhance glucose uptake and glycolysis through target gene activation involved in

glucose metabolic pathways, such as lactate dehydrogenase A (LDHA) (Dang, 1999; Shim, 1997) (Table 4). When LDHA gene expression was deficient in the fibroblasts, c-Myc-induced transformation was inhibited (Shim, 1997). C-Myc also controls iron metabolism by inducing the expression of the transferrin receptor and iron responsive element binding protein 2 (IRP2), and repressing the expression of H-ferritin and NRAMP1. Both increased available iron levels in the cytosol (Bowen, 2002; Wu, 1999). Although the mechanism is not known, re-expression of H-ferritin blocked c-Myc-induced transformation by sequestering iron (Wu, 1999) (Table 4). Third, metabolic enzymes required for folate metabolism were upregulated by c-Myc and promoted cell growth and proliferation. Induction of one of these enzymes in c-Myc-null fibroblasts partially mimicked c-Myc effects in that both cyclin D and CDK4 were expressed (Nikiforov, 2002).

It is very rare that deregulated c-Myc alone transforms normal cells to tumorigenic cells, and therefore cells with enhanced c-Myc levels need additional mutation(s) to be fully transformed. Since c-Myc induces cell proliferation as well as apoptosis that limits cellular transformation, deletion of barrier genes such as $p19^{ARF}$, *p53* (Eischen, 1999) or *BIM* (Egle, 2004), or overexpression of oncogenes, e.g. *BMI-1* (Jacobs, 1999) and *TBX-2* (Jacobs, 2000) that repress $p19^{ARF}$ expression or anti-apoptotic $BCL\text{-}X_L$ (Egle, 2004; Pelengaris, 2002b), facilitated c-Myc-induced tumorigenesis.

Other functions

Numerous studies have demonstrated that high levels of c-Myc expression induce DNA damage, which is partly promoted by ROS. When c-Myc was activated, ROS was elevated in both tissue culture and *in vivo* system (Reimann, 2007; Vafa, 2002), which was probably due to disruption in mitochondrial biogenesis (Dang, 2005; Li, 2005; Morrish, 2003) or cellular metabolism (Ray, 2006; Tanaka, 2002). In addition, c-Myc was reported to promote DNA replication independently of transcriptional activity (Dominguez-Sola, 2007), which may also cause DNA damage. Moreover, c-Myc induced translation regardless of transcriptional activity by activating mRNA capping, i.e. methylation of 5' mRNA guanine, a critical step for gene translation from mRNA (Bentley, 2005; Cowling, 2007; Shatkin, 1976; Shuman, 2002).

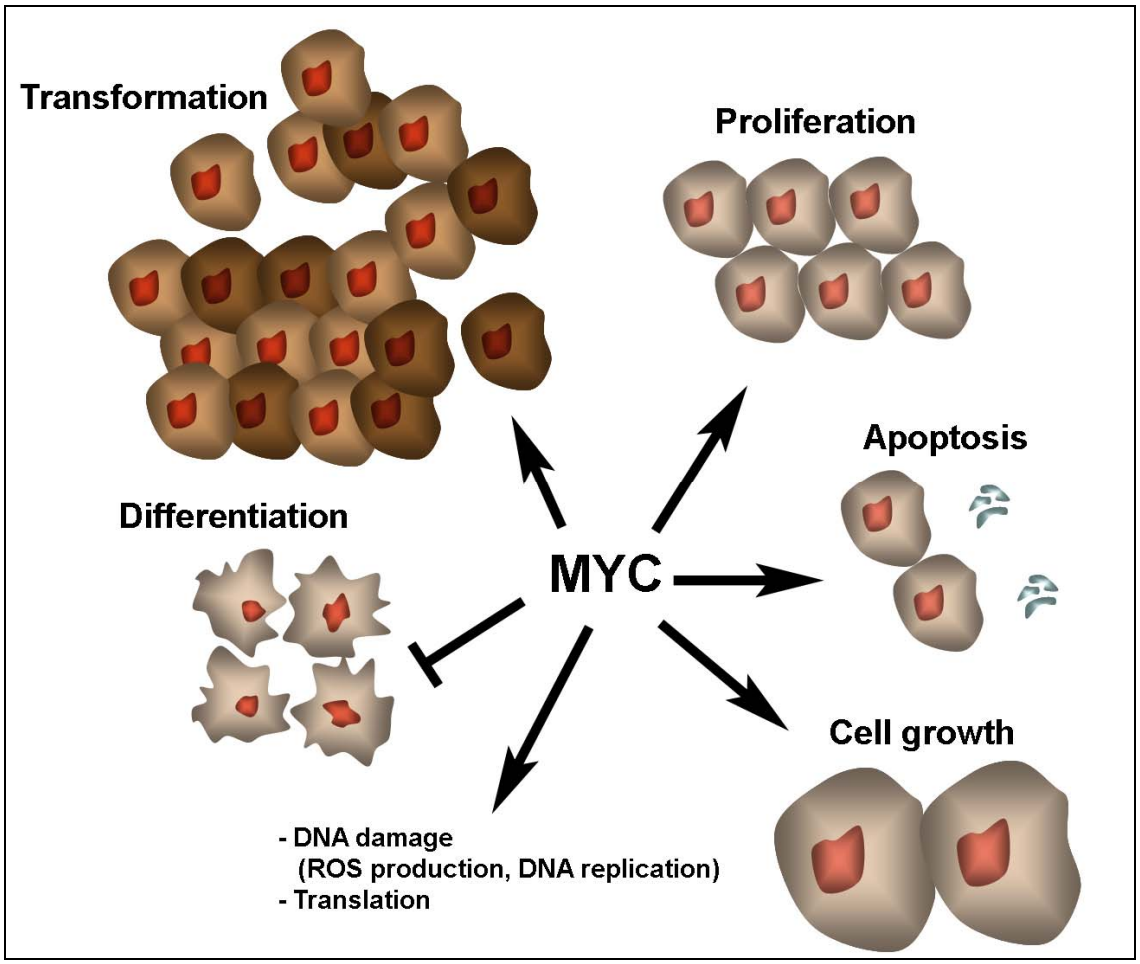


Figure 7. Biological functions of c-Myc. c-Myc induces cellular proliferation, growth, apoptosis, transformation, gene translation, and DNA damage. Also c-Myc inhibits terminal differentiation.

C-Myc and cell competition

Cell competition is the phenomenon that when there is(are) certain mutation(s) in a subset of the cells in the cell population, cells become “super-competitors” by actively eliminating neighboring “loser” cells which do not have

that mutation(s). In contrast, defective mutated cells (losers) are killed by neighboring intact cells (winners). This phenomenon was observed in wing imaginal discs during *Drosophila* development and *dMyc* (*Drosophila* homologue of *Myc*) was thought to be involved in a gene dose-dependent manner. Mutant cells with only one copy of *dMyc* gene were eliminated in the presence of wild type cells with intact two copies of *dMyc*. In addition, cells with three copies of *dMyc* became “super-competitors” by outcompeting wild type cells and were also outcompeted by cells with four copies of *dMyc* (de la Cova, 2004; Moreno, 2004). Interestingly, similar phenomenon was observed in mammals, although more investigations are needed to examine and determine cell competition. In the mouse intestine with conditional *c-Myc* deletion, the number of *c-Myc*-deficient cells per crypt was dramatically reduced compared to *c-Myc*-proficient wild type cells in the same *c-Myc*-deficient animals (Muncan V, 2006). Several pathways have been proposed to explain cell competition, but the mechanism is not still conclusive. One cell culture study recently showed that some unknown soluble factors released from high-*dMyc* cells may possibly contribute to kill low-*dMyc* cells (Senoo-Matsuda, 2007).

C-Myc and human prostate cancer

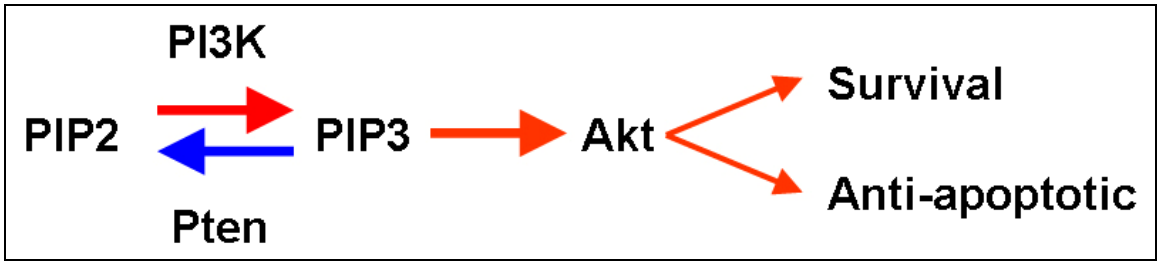
c-Myc is known to be overexpressed in many human cancers including prostate cancer. Its expression is often deregulated by copy number amplification, chromosomal translocations, proviral insertion and retroviral transduction (Marcu, 1992) but many tumors still display c-Myc activation by other unclear

mechanisms. Resuls showing that chromosomal locus (8q24) where c-MYC is present was somatically amplified in some prostate cancer patients (Jenkins, 1997; Nesbit, 1999; Qian, 1997; Sato, 1999), suggested that c-Myc may be involved in prostate cancer progression. Also c-MYC amplification showed significant correlation with higher pathological grades and a worse disease prognosis (Jenkins, 1997; Sato, 1999). Numerous studies have confirmed that mRNA levels of *c-MYC* were elevated in human prostate cancer samples, compared to benign prostate tissues (Buttyan, 1987; Dhanasekaran, 2005; Fleming, 1956; Lapointe, 2004; Meng, 2002; Tomlins, 2007; Varambally, 2005; Yu, 2004). Recently, Gurel *et al.* analyzed “functional” nuclear c-MYC protein expression levels as a transcription factor (Persson, 1984) in human prostate specimens in contrast to cytoplasmic c-MYC detection done in most previous papers, and showed that c-MYC protein was overexpressed in 76% of PIN, 81.6% of adenocarcinoma and 68% of metastatic prostate cancer (Gurel, 2008). These data suggest that c-MYC alteration is one of the early events in human prostate carcinogenesis.

Pten

Pten, Phosphatase and tensin homologue, is a phosphatase that has the function of tumor suppressor. Human *PTEN* gene is located on the chromosome 10 and this chromosomal locus is known to be frequently deleted in many human cancers, including prostate, breast, lung, and bladder cancer (Simpson, 2001). During tumorigenesis, however, Pten expression is reduced or completely deficient either by somatic gene deletion or epigenetic mechanism, including promoter methylation (Sansal, 2004). In addition to somatic mutation in PTEN, germline mutation in Pten is known to cause Bannayan-Zonana syndrome, Cowden's syndrome and Lhermitte-Duclos disease (Liaw, 1997).

Pten protein is a phosphatase that dephosphorylates phosphate group at 3-position of inositol ring and its most important substrate is phosphoinositol tri-phosphate (PI(3,4,5)P3 or PIP3) (shown below). In many tumors, Pten deficiency results in hyperactivation of Akt, which is prosurvival/anti-apoptotic because PIP3 phosphorylated by phosphoinositol 3 kinase (PI3K) activates the downstream target protein kinase B (Akt) family (Sun, 2001).



Pten plays a critical role as a tumor suppressor because Pten is a unique protein phosphatase that no other enzymes can replace (Li, 2007). Also, Pten has close a relationship with cancer-related molecules and pathways, such as p53 and Akt. Akt is known to decrease cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$ which controls G1 arrest, and leads to cell-cycle progression from G1 to S (Sherr, 1999; Slingerland, 2000). In addition, Akt inactivates Bad, a pro-apoptoic protein by phosphorylation, so cells are prevented from apoptosis (Datta, 1999; Datta, 1997).

In addition to Akt, Pten is also connected to the c-Jun N-terminal kinase (Jnk) pathway, and Jnk is thought to be activated through BMP signaling with Pten deficiency. When Pten was deleted in mice in a prostate-specific manner, BMP7 was upregulated (Yang, 2005), and BMP7 induced Jnk activity in C4-2B cells, a *PTEN*-null human prostate cancer cell line (Yang, 2006). Also, Jnk is activated in *Pten*-null cells and human prostate cancer specimens with active AKT or PTEN-deficiency (Vivanco, 2007).

Pten interacts with p53 in many ways. p53 is known to regulate Pten transcription (Stambolic, 2001) and Pten protects p53 from degradation that occurs through mdm2-mediated ubiquitinylation by forming a complex with p53 (Freeman, 2003a; Mayo, 2002; Zhou, 2003).

Pten is also thought to control genomic stability. When Pten is deficient, activated Akt induces mono-ubiquitination of DNA damage checkpoint kinase 1 (Chk1) by phosphorylating serine 280 (Puc, 2005a). This results in an impaired response to DNA damage. Additionally, Pten loss was reported to decrease

Rad51, a DNA repair protein, so that double-stranded DNA breaks (DSB) were elevated and caused genomic instability (Puc, 2005b).

Fluorescence *in situ* hybridization (FISH) studies demonstrated that *PTEN* (both heterozygous and homozygous) deletion occurs frequently in human prostate cancers. Yoshimoto *et al.* (Yoshimoto, 2006) showed that *PTEN* deletion was observed in 23% of PIN and 68% of prostate cancer without metastasis. Han *et al.* (Han, 2009) also reported that 9% of HGPIN, 17% of primary cancer, and 53% of metastatic disease displayed *PTEN* deletion. Collectively, the data indicate that *PTEN* loss is a late event in human prostate carcinogenesis compared to c-Myc overexpression.

CHAPTER II

MOUSE AS A MODEL ORGANISM

Introduction

To examine gene functions and their roles in human diseases *in vivo*, investigations have used several animal models such as nematodes (*Caenorhabditis elegans*), flies (*Drosophila*), frogs (*Xenopus*) and rodents (rats and mice). Among the frequently used model organisms mentioned above, humans and mice were the most recently diverged (evolutionarily around 75 million years ago), and there have been only 0.01 changes in gene structure per gene despite the process of evolution in each lineage (Mouse Genome Sequencing Consortium, 2002). In addition to genetic similarity, there are other multiple advantages of mice as an *in vivo* model organism for basic and cancer biology. First, mice are among the smallest mammals, so they are easy to handle and take care of. Second, their generation time is reasonably short and females have large litters (5 to 10), so they are a good model system for genetics. Third, they display high tumor incidence and relatively fast tumor growth, which additionally makes mice valuable model for cancer biology.

For prostate cancer research in particular, mice are useful because they show functional and structural similarities in the prostate to that of humans (Figure 8). The peripheral zone (PZ) in human prostate which occupies about

70% of the glandular prostate is comparable to the dorso-lateral lobe in mouse prostate. In addition, the human central zone (CZ) is comparable to the mouse anterior lobe. However, there are no homologous counterparts of the human transition zone (TZ) and mouse ventral lobe in mouse and human prostate, respectively (Ahmad, 2008). However this similarity is based on descriptive data, so molecular characterization of each compartment needs to be assessed for further comparisons. Also, mice do not spontaneously get prostate cancer but they can get cancer that is histologically similar to human prostate cancer with genetic or chemical manipulation.

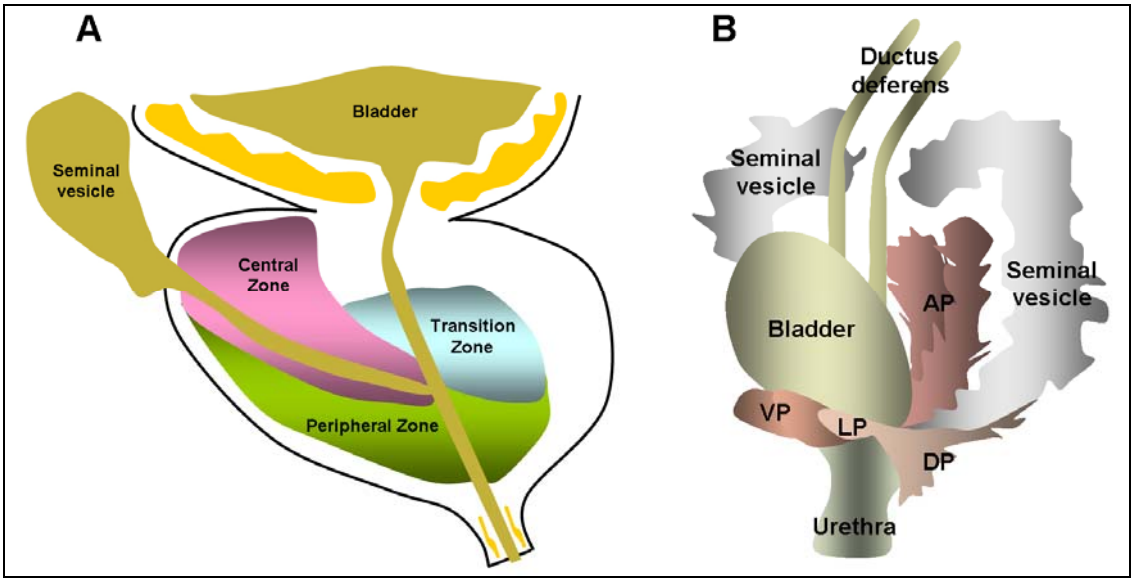


Figure 8. Comparison of human and mouse prostate structure. A and B describe human and mouse adult prostate structure, respectively. AP, DP, LP and VP in (B) stand for anterior, dorsal, lateral and ventral prostate, repectively.

Current Mouse Models of Prostate Cancer

[This part is adapted from {Abdulkadir, S.A. and Kim, J., *Future Oncology* (2005)}]

There has been substantial progress made recently in the effort to model human prostate cancer in mice. Several mutant mice have been generated which mimic various aspects of the human disease, including the development of preneoplastic lesions, invasive carcinoma, and metastases. These mouse reagents provide the research community with valuable new tools for dissecting the mechanisms of tumorigenesis, as well as for testing new targeted therapies.

Although rodents do not spontaneously develop prostate cancer, recent advances in murine modeling of human cancer have provided the scientific community with new tools for investigating the mechanisms of prostate carcinogenesis, as well as parameters in response to chemoprevention and therapy. Nonetheless, there is a need for refining the current models to address scientific questions that may not be easily examined *in vitro* or in human subjects. For the generation of transgenic murine models of prostate cancer, the choice of promoter used to target transgene expression is of paramount importance. The temporal and spatial pattern of transgene expression, which may be cell-specific, is driven by a promoter that causes dramatic effects on the resulting phenotype.

Models that develop prostatic epithelial hyperplasia/dysplasia/PIN without progression to carcinoma

Several models have been generated that develop prostatic epithelial hyperplasia/dysplasia/PIN without evidence of progression to invasive carcinoma over the course of the lifetime of the organism. These have mostly been models in which a single gene is disrupted or overexpressed in the prostatic epithelium. These results indicate that multiple pathways may have to be disrupted for progression to invasive carcinoma.

Nkx3.1 mutant mice

Nkx3.1 encodes a homeodomain transcription factor whose expression is androgen-dependent and largely restricted to the luminal epithelial cells of the prostate (Bieberich, 1996). Reduced NKX3.1 protein expression has been observed in approximately 75% of human prostate tumors and 20% of PIN lesions (Bowen, 2000). Mice lacking *Nkx3.1* develop prostatic epithelial hyperplasia that progresses to PIN over time (Abdulkadir, 2002; Bhatia-Gaur, 1999). Mice with conditional deletion of *Nkx3.1* in the mature prostate also develop hyperplasia and PIN (Abdulkadir, 2002). The latter finding is consistent with the loss of *NKX3.1* expression observed in human PIN lesions, since the PIN lesions in the conditional knockout mice arise independent of the prostate developmental defects observed in regular *Nkx3.1*-knockout animals. Epithelial hyperplasia seen in $Nkx3.1^{-/-}$ and $Nkx3.1^{+/-}$-mutant mice may be a consequence of an extended proliferative phase due to a delay in exiting the cell cycle by differentiating luminal epithelial cells (Magee, 2003). Gene expression analysis of

Nkx3.1 mutant mice revealed a class of dosage-sensitive *Nkx3.1* target genes that demonstrate a stochastic, on/off pattern of expression. Thus, a reduction in *Nkx3.1* dosage reduced the proportion of cells expressing this class of target genes. These findings are consistent with a model of haploinsufficient tumor suppression in which reduction in *Nkx3.1* dosage increases the probability of complete loss of expression of select target genes in luminal epithelial cells.

Probasin-androgen receptor transgenic mice

The androgen receptor (AR) plays an important role in the normal development of the prostate as well as in all stages of prostate carcinogenesis including androgen-independent disease. Expression of the wild-type *AR* transgene under the control of the rat *Probasin* promoter (*Pb*) resulted in increased cell growth and PIN, and provides a model system of *AR*-stimulated PIN that can be used for assessing preventative hormonal therapies and for identifying secondary transforming events relevant to human prostate cancer (Han, 2005; Stanbrough, 2001). While expression of wild-type *AR* does not lead to prostate cancer, prostate-specific expression of a mutant *AR (AR-E231G)* has recently been demonstrated to lead to the development of metastatic prostate cancer (Han, 2005).

Probasin-AKT transgenic mice

Inactivating somatic mutations of *PTEN* or loss of the PTEN protein are common in prostate cancer cell lines and tumors. Mutations in *PTEN* lead to

deregulated phosphoinositide-3 kinase (PI3K) signaling, resulting in constitutive activation of downstream targets such as the protein kinase B (Akt) kinase family (Sun, 2001). Sellers and colleagues have generated mice expressing active Akt in the prostate (murine prostate Akt kinase transgenic [MPAKT] mice) (Majumder, 2003). In these mice, Akt expression led to p70S6K activation, and PIN. Thus, the MPAKT model may be a useful model to study the roles of Akt in prostate epithelial cell transformation and to test new pathway-targeted therapies.

C(3)1-Myc and Probasin-MYC transgenic mice

Mice with low levels of *c-Myc* expression in the prostate *(C(3)1-Myc)* develop prostatic hyperplasia and PIN without progression to invasive carcinoma, although higher level expression of Myc using the *Probasin* promoter (in *Pb-Myc* and *ARR$_2$Pb-Myc* mice) leads to invasive carcinoma demonstrating the significance of the dose of an oncogene in prostate tumor progression (Ellwood-Yen, 2003; Zhang, 2000b).

Probasin-Ras transgenic mice

While the involvement of *Ras* in human prostate cancer has not been evaluated extensively, activating mutations in *H-*, *K-*, or *N-Ras* have been reported in 5–30% of human prostate cancers (Castrillon, 2001). To assess the role of *Ras* in prostate carcinogenesis, a constitutively activated form of *H-Ras* was targeted to the prostate using the minimal *Pb* promoter. The *Pb-Ras*

transgenic mice present with neoplastic changes in their prostates including low grade PIN and intestinal metaplasia (Scherl, 2004).

Retinoid X receptor mutant mice

Retinoids play an important role in development, differentiation and tissue homeostasis (Chambon, 1996). Expression of retinoid X receptor (RXR)α in the nucleus is generally decreased in prostate cancer cell lines and specimens, and prostate cancer cells in which RXRα is overexpressed demonstrates significantly increased apoptosis (Pandey, 2003; Zhong, 2003). Conditional deletion of *RXRα* in the prostate led to the development of hyperplasia and PIN which worsens with age, although invasive cancer did not develop. The heterozygous mutant mice also developed similar prostatic phenotypes but in a delayed manner, implying a role of haploinsufficiency (Huang, 2002).

Retinoblastoma mutant mice

The retinoblastoma (Rb) tumor suppressor functions in the control of cellular proliferation and differentiation (Kranenburg, 1995; Weinberg, 1995). *Rb* gene alterations can occur in all grades and stages of prostate cancer, in localized as well as metastatic disease (Brooks, 1995). In human prostate cancer, *Rb* gene mutations have been reported in approximately 16% of primary human prostatic cancers (Bookstein, 1990; Kubota, 1995). Conditional deletion of *Rb* in the prostate, however, resulted only in PIN lesions, without development of frank carcinoma (Maddison, 2004; Zhou, 2006). It is remarkable that the lesions in

these animals did not progress to carcinoma, considering the significant role Rb plays in regulating the cell cycle and the fact that it is targeted in human tumors. One possible explanation is compensation by other *Rb* family members such as *p130*, which is expressed at high levels in the mouse prostate.

Fibroblast growth factors mutant mice

The fibroblast growth factor (FGFs) family of heparin-binding growth factors plays important roles in normal prostate development and tumorigenesis (Basilico, 1992; McKeehan, 1998). The prostatic epithelial and stromal cells express various kinds of FGF ligands and their receptors (FGFR) to transduce signals which are required for biologic processes such as branching morphogenesis, differentiation and survival (Cunha, 1992). While some of the FGFs are overexpressed (Heer, 2004; Ozen, 2001; Polnaszek, 2003; Ropiquet, 2000), others are downregulated in human prostate cancer cells (Mydlo, 1988). Transgenic mice expressing FGF-2 under the control of a short rat *Probasin* promoter were established. When these infertile FGF-2-expressing transgenic founder mice were sacrificed for histologic analysis, they demonstrated a simple epithelial hyperplasia in the dorsal lobe of the prostate (Konno-Takahashi, 2004). However, FGF-2 does have a role in tumor progression as deletion of one or both copies of *FGF* in transgenic mouse model of prostate cancer *(TRAMP)* mice resulted in increased survival, decrease in metastasis, and inhibition of progression to the poorly differentiated phenotype in primary prostatic tumors

(Polnaszek, 2003). Like the situation with FGF-2, expression of FGF7, 3 or 8b in the prostate, resulted in hyperplasia and PIN (Chua, 2002; Foster, 2002).

FGF receptors mutant mice

Models based on the *FGFRs* mirror the phenotypes of mice expressing FGF ligands. FGFR1 and 2 present different functions. FGFR1 activation in transgenic mice triggers hyperplasia and PIN while FGFR2 did not demonstrate observable changes in prostate (Freeman, 2003b; Jin, 2003; Roy-Burman, 2004). The expression of a truncated FGFR23b receptor also caused disorganized prostatic ducts that were not tightly associated with the basement membrane as well as disorganization of the stroma (Foster, 2002). While none of the models based on dysregulation of the FGF receptors or ligands resulted in invasive carcinoma, this does not exclude the possibility that these molecules do have a role in the later stages of tumor progression.

Skp2 transgenic mice

An ubiquitin E3 ligase, Skp2, has been implicated in ubiquitinating the CDK inhibitor $p27^{kip1}$ and the transcriptional factor E2F-1. Overexpression of Skp2 in prostate of mice decreased $p27^{kip1}$ levels, promoting proliferation, resulting in hyperplasia and high grade PIN (Shim, 2003). This phenotype is more severe than that of *p27*-deficient mice (Gary, 2004), suggesting that Skp2 impacts additional targets other than $p27^{kip1}$ in the prostate.

Probasin–prolactin transgenic mice

The pituitary hormone prolactin (PRL) has been found to have diverse effects on the prostate gland. These chiefly include actions related to proliferative activity, secretory function and regulation of specific metabolic functions (Costello, 1994; Reiter, 1999). Transgenic mice which express prostate-specific rat prolactin (rPRL) were generated to explore the role of locally produced PRL, possibly acting via an auto- or paracrine mechanism, in promoting abnormal prostate growth. The *Pb-rPRL* transgenic males developed a significant enlargement of the prostate gland, characterized primarily by hyperplasia of the stromal compartment, distended ductal structures, and focal areas of glandular dysplasia (Kindblom, 2003).

Estrogen receptor mutant mice

Estrogens have important roles in differentiation and maintenance of reproductive tissues, and also influence lipid metabolism and bone remodeling. There are two estrogen receptor (ERs) identified thus far, ERα and β. The adult male *ERα* knockout (KO) mouse prostate shows normal development and histology. The weight of seminal vesicle and ventral prostate of *ERα* KO is increased with aging (Couse, 1997), but remains histologically indistinguishable from that of wild-type littermates (Prins, 2001). Mice lacking *ERβ* were generated by inserting a neomycin resistance gene into exon 3 of the coding region by using homologous recombination in embryonic stem cells. Young, sexually mature male mice demonstrated no overt abnormalities and reproduce normally.

However, older mutant males presented with prostate and bladder hyperplasia (Krege, 1998). Furthermore, in prostates of *ERβ*-null mice, AR levels are increased and the tissue contains multiple hyperplastic foci (Weihua, 2001).

FLiMP mice

Human 15-lipoxygenase-1 (15-LO-1) metabolizes polyunsaturated fatty acids and has a physiological role in remodeling membrane. This enzyme is also known to function in atherosclerosis, inflammation and and carcinogenesis. The conditional overexpression of human 15-LO-1 using Cre-LoxP method (*PbCre* and *CAG–loxP–CAT–loxP–h15-LO-1*) led to epithelial hyperplasia and PIN lesions (Kelavkar, 2006). This model implies possible dietary prevention of prostate cancer.

Conditional p53 knockout model

p53 is a well-studied tumor suppressor gene and mutation in *p53* is associated with metastasis and hormone refractory prostate cancers (Bookstein, 1993; Heidenberg, 1995). To see the effects of altered *p53* pathway on prostate cancer development, conditional *p53* deletion model was generated (Zhou, 2006). Despite critical role of *p53* in general human carcinogenesis, these mice displayed only PIN lesions without progression to invasive carcinoma. In addition, Chen *et al.* independently showed that *p53* inactivation alone did not display histological abnormalities (Chen, 2005).

Models that develop invasive cancer without metastasis

Several models have been generated that develop invasive prostate carcinoma which, however, do not lead to the efficient progression to metastasis over the lifetime of the organism. Most of these models are obtained by enforced expression of potent oncogenes or multiple mutations in tumor suppressor genes.

Probasin–myc transgenic mice

As mentioned above, high-level expression of c-MYC in the mouse prostate resulted in the development of PIN followed by invasive adenocarcinoma, while expression of low levels results only in PIN (Ellwood-Yen, 2003; Zhang, 2000b). This dosage dependence of the cancer phenotype may be a reflection of the fact that expression of c-Myc at different levels activates distinct gene programs (Guo, 2000).

Nkx3.1$^{-/-}$ & Pten$^{+/-}$ compound mutant mice

Loss of function in *Nkx3.1* and *Pten* cooperate in prostate carcinogenesis in mice. *Nkx3.1$^{-/-}$* and *Pten$^{+/-}$* compound mutant mice display an increased incidence of high grade PIN (HGPIN)/early carcinoma lesions, resembling early stages of human prostate carcinogenesis (Kim, 2002).

LPb-Tag (LADY) transgenic mice

Several transgenic lines expressing SV40 large T-antigen under the control of the long *Probasin* promoter have been generated (Kasper, 1998;

Masumori, 2001). Some of these lines develop invasive carcinoma while only one line (12T-10) line developed metastatic disease (Masumori, 2001). Metastatic potential can be conferred on non-metastatic LADY tumors by expression of a *Probasin-Hepsin* transgene (Klezovitch, 2004). Hepsin is a cell surface serine protease that is markedly upregulated in human prostate cancer.

$Pten^{hy/-}$ mice

A hypomorphic *Pten* mouse mutant series with decreasing Pten levels and activity were generated by homologous recombination: *$Pten^{+/+}$* > *$Pten^{hy/+}$* > *$Pten^{+/-}$* > *$Pten^{hy/-}$* > *Pten* prostate-specific conditional KO mutants (two types; inactivated focally or throughout the entire prostatic epithelium). Among these mice, *$Pten^{hy/-}$* mutants displayed massive prostate hyperplasia and invasive prostate cancer. *$Pten^{+/-}$* mutants, however, developed moderate/low grade PIN (LGPIN) at incomplete penetrance but never developed invasive cancer (Trotman, 2003). This important study demonstrates the critical dependence of prostate tumorigenesis on tumor suppressor gene (TSG) dosage.

Conditional p53 and Pten knockout mice

As described above, *p53* deficiency alone did not show any abnormality in mouse prostate but it dramatically cooperated with conditional *Pten* deletion (Chen, 2005). Dual conditional inactivation of *p53* and *Pten* caused prostate cancer without distant metastasis (until 2.5 years) but all these mice died by 7 months.

Conditional APC-null mice

β-catenin is a multi-functional molecule regulating cell growth and it plays an important role in Wnt signaling pathway together with Adenomatous polyposis coli (Apc). When Apc was deleted in mouse prostate by the action of PbCre to alter Wnt signaling pathway, invasive adenocarcinoma without metastasis was developed (Bruxvoort, 2007).

Models that develop metastatic prostate cancer

Most of the models that efficiently develop metastatic prostate cancer are based on the expression of the SV40 large T- and small t (T/t)-antigens. The *PbCre* and *Pten*$^{loxp/loxp}$ conditional model is unique as a model in which deletion of a single TSG efficiently leads to the development of metastatic disease.

TRAMP mice

TRAMP mice express the T/t antigens under the control of short *Probasin* promoter and develop metastatic cancer (Gingrich, 1999; Greenberg, 1995). It has been demonstrated that the *TRAMP* mice develop adenocarcinoma that will sometimes display neuroendocrine-like features in very late stages of disease (Kaplan-Lefko, 2003). Further genetic manipulation of *TRAMP* mice has provided additional valuable insights into the mechanisms of prostate tumor initiation and progression *in vivo*. For example, *TRAMP* mice lacking the transcription factor early growth response gene *(Egr)* 1 were generated to test the role of *Egr1* in prostate tumorigenesis (Abdulkadir, 2001). Egr1 is known to be overexpressed in

human prostate tumors (Abdulkadir, 2001; Eid, 1998). *TRAMP;Egr1*$^{-/-}$ mice show a significant delay in progression from PIN to invasive carcinoma (Abdulkadir, 2001).

Other T/t antigen models

Other T/t antigen models, all of which develop metastatic prostate cancer include *C3(1)-Tag* in which the regulatory control of the rat prostatic steroid-binding protein *C3(1)* gene was used (Shibata, 1996): *PSP94-Tag* mice (Gabril, 2002), *PSP-KIMAP* mice (Duan, 2005), *gp91-phox-Tag* mice (Skalnik, 1991) and *cryptdin2-Tag* mice (Garabedian, 1998). In *cryptdin2-Tag* mice, the transgene is expressed in the neuroendocrine cells of the prostate, thus the tumors that develop in these animals are composed of neuroendocrine cells.

LADY model line 12T-10

Model line 12T-10 of *LPb-Tag* transgenic mice develops precursor lesions more analogous to human high grade PIN (HGPIN), without associated prominent stromal hypercellularity (also more similar to human prostate cancer). Furthermore, this line predictably develops invasive carcinoma with glandular differentiation (adenocarcinoma) as well as neuroendocrine prostate cancer that commonly metastasizes (Masumori, 2001).

Conditional Pten knockout model

Pten/mutated in multiple advanced cancers (MMAC) 1 is a phosphatase and a tumor suppressor gene implicated in a wide range of human cancers. Homozygous deletion and point mutations were observed in human prostate cancer samples and cell lines (Li, 1997; Steck, 1997) (CHAPTER I). *Pten* inactivation resulted in early embryonic lethality (Di Cristofano, 1998). To achieve prostate-specific deletion of *Pten*, *PbCre;Pten*$^{loxp/loxp}$ (Wang, 2003), *MMTVCre;Pten*$^{loxp/loxp}$ (Backman, 2004), *PSA-Cre;Pten*$^{loxp/loxp}$ (Ma, 2005), and *PSA-Cre-ER*^{T2}*;Pten*$^{loxp/lox}$ (Ratnacaram, 2008) have been generated although floxed exons of *Pten* gene are different each other. In all models *Pten* loss leads to a significant shortened latency of PIN formation and results in prostate cancer progression but metastasis has thus far been observed only in *PbCre* and *Pten*$^{loxp/loxp}$ mice (Wang, 2003) and *PSA-Cre;Pten*$^{loxp/loxp}$ (Ma, 2005). *PSA-Cre-ER*^{T2}*;Pten*$^{loxp/lox}$ model provides spatiotemporal control of somatic mutagenesis using *Cre-ER*^{T2} which is active upon tamoxifen treatment (Ratnacaram, 2008).

Probasin-AR (E231G) transgenic mice

Although overexpression of wild-type AR does not lead to prostate cancer, overexpression of *AR-E231G*, a mutant *AR*, leads to metastatic prostate cancer in 100% of mice examined (Han, 2005). These results established *AR* as an oncogene in prostate cancer. Furthermore, since expression of *AR-T857A*, a different mutant *AR*, does not lead to cancer phenotype, the *E231G* mutation is proved to be a specific oncogenic gain of function.

Conditional p53 and Rb double knockout mice

Although inactivation of either *p53* or *Rb* led to PIN without the phenotype of carcinoma, two mutations synergistically cooperated and induced metastatic carcinoma (Zhou, 2006). These tumors showed neuroendocrine differentiation and were resistant to androgen ablation. They shared genetic expression signatures commonly found in human prostate cancers.

Conditional Pten-heterozygous & Fgf8b-overexpressing mice

By crossing *ARR$_2$Pb-Fgf8b* (fibroblast growth factor 8 isoform b) and conditional *Pten* deletion mice *(PbCre;Pten$^{loxp/loxp}$)*, mice with *Fgf8b* overexpression and conditional *Pten* heterozygosity were generated (Zhong, 2006). As reported previously (Song, 2002), Mice either with *Fgf8b* overexpression or with Pten heterozygosity alone displayed PIN lesions, but their cooperation dramatically accelerated the disease progression by developing adenocarcinoma and metastatsis.

Therapeutic Applications of GEM Models of Prostate Cancer

Immunologically intact mice with defined genetic lesions have obvious advantages in the development of new therapeutic agents. Some of the genetically engineered murine (GEM) models described above have been used for testing new therapeutic and agents, a few of which will be described.

MPAKT model

In this model, constitutive Akt expression led to p70S6K activation and the development of PIN, a phenotype that bears many of the hallmarks of mammalian target of rapamycin (mTOR) activation. This suggests that activation of mTOR downstream of Akt may be linked to the development of PIN in these mice. To test this, MPAKT mice were treated with the mTOR inhibitor RAD001. The results of this experiment indicated that the expansion of the intraluminal cells driven by Akt expression is mTOR-dependent. mTOR inhibition induces the complete reversal of the neoplastic phenotype in the prostate of these transgenic mice through apoptosis of epithelial cells (Majumder, 2004).

TRAMP mice

TRAMP mice have been widely used in testing therapeutic and chemopreventive agents.

Immunotherapy

In one study, adoptive transfer of tumor-specific memory lymphocytes against Tag prevented tumor development and progression. This adoptive transfer did not influence on the morphology and function of tissues involved (Granziero, 1999). Anti-cytotoxic T-lymphocyte antigen (CTLA)-4, an inhibitory receptor on T cells, plays a critical role in attenuating T-cell responses (Linsley, 1991). Anti-CTLA-4 antibodies that block interactions between CTLA-4 and B7 (CD80 or CD86) (Lenschow, 1996), enhance *in vivo* T-cell responses to peptides, superantigens, and parasites (Thompson, 1997). CTLA-4 blockade in combination with irradiated tumor cell vaccines was effective at reducing tumor incidence and the severity of prostatic lesions in *TRAMP* mice (Hurwitz, 2000).

Chemoprevention

Nonsteroidal anti-inflammatory drugs such as celecoxib (Celebrex®) and E-7869 (R-flurbiprofen), have been demonstrated to significantly reduce tumor progression in *TRAMP* mice, possibly due to effects on cyclooxygenase (COX)-2 enzyme activity or mRNA levels (Gupta, 2004; Wechter, 2000). When OSU03012, a celecoxib-derived PDK1 inhibitor was used to treat *TRAMP* mice, PIN lesions were reduced but it did not affect the incidence of carcinoma or metastasis (Sargeant, 2007). Other agents demonstrated to function as chemopreventive agents in *TRAMP* mice include α-difluoromethylornithine (α-DFMO) which is an irreversible inhibitor of the enzyme ornithine decarboxylase (ODC) (Gupta, 2000), green tea polyphenols (Gupta, 2001), soy constituent genistein (Mentor-Marcel,

2001; Wang, 2007), tamoxifen derivative toremifene (Raghow, 2002), all-trans retinoic acid (ATRA) (Huss, 2004), grape seed extract (GSE) (Raina, 2007), natural polyphenolic phytochemical resveratrol (Harper, 2007), and herbal extract Nexrutine® (Kumar, 2007). The *LADY* transgenic model has also been used to demonstrate that dietary antioxidants (vitamin E, selenium, and lycopene) at a human achievable dose significantly inhibit prostate cancer development and increase the degree of survival of these mice (Venkateswaran, 2004).

Gene therapy

The *TRAMP* mice were treated with intra-prostatic injection containing OAdV220, an ovine adenovirus which expresses PNP gene under the control of the Rous sarcoma virus promoter, followed by systemic fludarabine treatment. Purine nucleoside phosphorylase (PNP) is an *Escherichia coli* enzyme that converts the prodrug fludarabine phosphate into the highly toxic metabolite 2-fluoroadenine (2-FA) can inhibit nucleic acids and protein synthesis (Martiniello-Wilks, 2002; Secrist, 1999; Voeks, 2002). The weight change of the genitourinary tract, seminal vesicles, prostate and animal survival was determined. The results indicate that PNP-gene-directed enzyme prodrug therapy delivered by ovine adenovirus vector caused significant suppression of prostate cancer progression and increased survival (Martiniello-Wilks, 2004). In addition, when *TRAMP* mice were treated with mouse endostatin and angiostatin using recombinant adeno-associated virus-6 (rAAV-6) by intramuscular injection at early stages, this therapy remarkably prolonged survival rate (Isayeva, 2007).

Conclusions

The transgenic mice described herein have greatly enhanced our knowledge of the mechanisms of prostate carcinogenesis. Among the genes with known roles in human prostate tumorigenesis, analysis of genetically engineered mice has revealed prominent roles for the tumor suppressor *Pten* and the oncogene *c-Myc* in prostate tumorigenesis. In particular, conditional deletion of *Pten* in the prostate using *PbCre* mice efficiently induces prostate carcinoma which metastasizes to distant organs. This and other models may now be used for further dissection of mechanisms of tumorigenesis and for testing new targeted therapies and chemopreventive agents.

Future Perspective

Although currently available models provide valuable tools for studying prostate carcinogenesis, several improvements can be made to generate more refined models with better utility. In general, it will be important to generate models based on genes and pathways with relevance to human prostate carcinogenesis. This will become easier as more human prostate cancer genes are discovered and their pathways defined. The manner in which the relevant genes are disrupted is also important. Like conditional *Pten* deletion model executed by tamoxifen-dependent Cre (*Cre-ER*) (Ratnacaram, 2008), temporal and spatial gene inactivation or overexpression in the adult prostate, will mimic the situation in human prostate cancer more closely. Strategies that allow regulated gene expression may provide the additional flexibility of studying the effects of mutation reversion on cancer survival. The field will clearly benefit also from the availability of models that spontaneously develop frequent osteoblastic osseous metastases similar to the situation in human prostate cancer. Another important issue is the need to develop prostate specific, yet androgen-independent promoters for transgene expression like *PbCre;Z-MYC* mice (Kim, 2009) which will be described in CHAPTER VI. This allows the effects of castration on specific oncogene-induced tumors to be investigated and separate androgen ablation from the effects of oncogene withdrawal due to transgene silencing in models generated with androgen-sensitive promoters like *Probasin*. Moreover, strategies for imaging should be incorporated when making new

models to allow easier tracking of the fate of transformed cells *in vivo*. Figure 9 outlines some strategies for generation of more refined murine models with controlled deletion or activation of cancer-related genes and/or markers to aid with *in vivo* imaging. Consideration of the influence of the tumor microenvironment is also essential. Future models should incorporate effects of alteration in the stroma as well as inflammation, free radicals, infection or aging. The study investigated by Bhowmick and colleagues who demonstrated that selective deletion of the prostatic stromal *Tgfbr2* promoted the development of PIN is a good example (Bhowmick, 2004). Finally, there is a need to use the several models of preinvasive neoplasia already generated for chemoprevention trials. This will require a standardized pathologic (Shappell, 2004) and/or a computer-aided grading system to ensure accuracy and reproducibility (Gary, 2004).

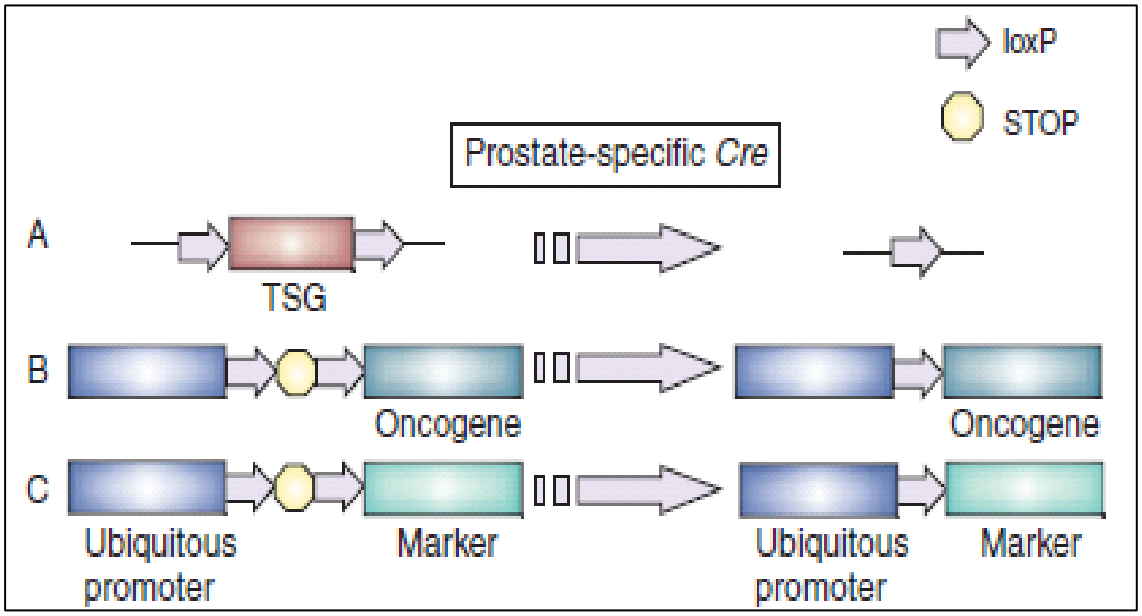


Figure 9. Cre-inducible conditional gene activation/deletion. Conditional systems such as the Cre/loxP system can be used to achieve prostate-specific deletion of tumor suppressor genes (TSGs) (A) or activation of oncogenes (B) and marker genes (C) to aid with mutant cell detection. The conditional activation of oncogenes as shown in "B" can provide models in which castration will not lead to transgene silencing if the ubiquitous promoter used (ubiquitous promoter) is not androgen dependent. Cre: Cre recombinase; STOP: Transcription termination signal; TSG: Tumor suppressor gene.

CHAPTER III

GENETIC MOSAICISM

Introduction

Genetic mosaicism describes that two cell populations with different genetic backgrounds present in one individual originated from one fertilized egg. In the area of biology, investigators who seek to characterize *in vivo* roles or functions of genes of interest in normal development or human diseases have been utilizing transgenic animals or plants by introducing foreign genes into model organisms. Especially to examine human diseases including cancers, modeling diseases using laboratory model animals is one of the crucial means to study their initiation, progression, and development and test therapeutics including drug efficacy. According to a muti-step model of carcinogenesis (Fearon, 1990), human somatic cancers are believed to initiate from a single mutation in one cell or a few cells in the tissue. The mutant cells either encounter a biological barrier, e.g. apoptosis or senescence, or expand clonally. With or without additional mutations depending on the importance of the roles of the mutated gene during the pathogenesis, the pre-cancerous cells become tumorigenic. Therefore, due to the random nature of genetic mutations during multi-step cancer development, many human cancers are thought to display heterogeneity, so genetic mosaicism is supposed to be an essential part of

human somatic carcinogenesis. In addition, during tumorigenesis, the interactions between cells with distinct mutations, i.e. between mutant cells and surrounding wild type cells or between differentially mutated cells, are believed to be important for the disease development. In this sense, it is critical to model genetic mosaicism in the same animal or in the same organ or tissue of a single animal. Current advances in developing laboratory animal models and methods for cancer research demonstrate enormous efforts to elucidate a tumorigenic mechanism by mutations in genes of interest and mimic spontaneous somatic genetic alterations during human carcinogenesis. This part of the chapter will discuss reports where genetic mosaicism or sporadic transgene expression/gene deletion in model organisms was induced either intentionally or unexpectedly. In addition, advantages and disadvantages of these models will be also addressed.

Transgene Silencing and Stochastic Pattern of Gene Expression

The expression of introduced foreign genes into living organisms is frequently silenced (Clark, 1994). This sometimes happens when too few copies of the transgene are integrated into the genome, which results in low expression or mosaic pattern of gene expression (Stam, 1997). Therefore, the copy number of integrated transgene and the integration sites are believed to be critical determinants for expression levels (Clark, 1994; Dorer, 1997; Garrick, 1998). These factors can cause complete shutdown or a mosaic pattern of transgene expression (Martin, 1996). Accordingly, understanding gene silencing mechanisms of introduced foreign genes is important to control stochastic transgene expression (Figure 10).

Integration sites of transgenes are crucial to their eventual expression. When transgenes are integrated into certain chromosome loci such as euchromatin with high accessibility of transcription factors, there is more chance of higher transgene expression and a uniform expression pattern even though low numbers of transgene copies are integrated into the genome. However, transgenes integrated close to heterochromatin or repetitive DNA sequences may show focal expression pattern/gene silencing or less chance of high expression. This has been evident in animal models showing position-effect variegation (PEV), which is also known to cause mosaic pattern gene expression by the relocation of the specific gene into heterochromatin. PEV was first shown in *Drosophila* eye color determination (Muller, 1930) where red-white mosaic eye

color is caused by inactivation of the *white* gene by mutation whose expression then results in red eye color. In mammals, BLG/7 transgenic line shows a mosaic pattern of beta-lactoglobulin (BLG) transgene expression. In BLG/7 transgenic line, a tandem array of ~25 copies of BLG transgene is thought to be integrated around the centromere of chromosome 15, which causes variegated pattern of BLG expression (Dobie, 1997; Dobie, 1996).

The number of inserted transgenes also decides expression levels. In plants, T-DNA transfer system tends to insert two or more T-DNAs at the same chromosomal region. When T-DNAs are integrated as an inverted repeat (head-to-head or tail-to-tail fashion), their expression is often low, indicating possible transgene silencing (Jones, 1987; Jorgensen, 1987). In addition, when high copies of transgene are integrated into the genome of transgenic animals or plants, this is sometimes known to cause dominant-negative or other toxic effects (Thellmann, 2003).

Strain-specific modifier genes in mice have also been reported to regulate transgenic loci (Allen, 1990; Engler, 1991; Hadchouel, 1987; McGowan, 1989). Mouse strains such as Balb/c, C57BL/6 and DDK showed very low expression of a transgene while high levels of transgene expression were observed in DBA/2 and CFLP backgrounds.

In addition, post-transcriptional gene silencing can block transgene expression by an unknown mechanism, which occurs when mRNA is not accumulated, although the gene promoter is active (Matzke, 2004; Stam, 1997).

This phenomenon is evident in virus-resistant plants when viral transgene is introduced (Smith, 1994).

DNA composition or structure of the transgene can help determine silencing. Transgene methylation (Kothary, 1992; Matzke, 1998) or DNase I hypersensitivity (Pourcel, 1990) was shown to promote gene silencing, suggesting that certain nucleotide compositions in the transgene may attract silencing methylation or be susceptible to degradation. Also, the structure of the transgene DNA is a possible cause of gene silencing. In *Xenopus* transgenesis, circular DNA is more prone to be silenced than linear DNA because the former is unable to form concatemers or undergo DNA replication, so is less likely to be integrated into genome (Marini, 1988).

In addition, transgene expression is also affected by stress effects, such as heat shock or cold shock (Kothary, 1992).

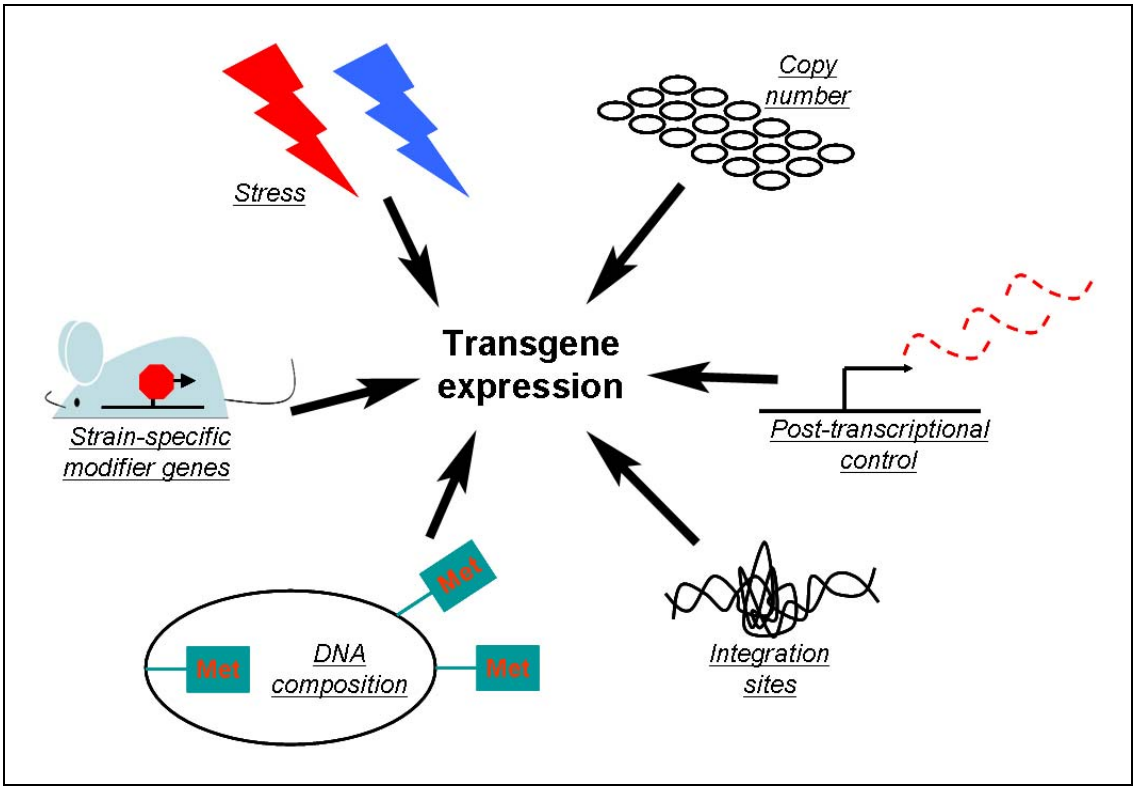


Figure 10. Factors that affect transgene expression. Multiple mechanisms are known to control transgene expression and these cause uniform, stochastic expression or even complete shutdown of transgene expression.

Methods without LoxP-Cre System for Stochastic Transgene Expression

With the understanding of transgene silencing mechanism, many efforts to generate transgenic animals with mosaic pattern of transgene expression following direct integration of transgenes have been successful despite some of the advantages and disadvantages. Enhanced green fluorescent protein (eGFP)-expressing embryos generated by a somatic cell nuclear transfer (NT) from an ear skin fibroblast cell line of a newborn pig electroporated with *eGFP* gene displayed a mosaic pattern of *eGFP* expression in contrast to uniform expression of *eGFP* in embryos generated by NT from fetal fibroblasts (Park, 2002). This was probably due to different integration sites of *eGFP* transgene in newborn cell nuclei or fetal cell nuclei. It is also possible that animal cells at different developmental stages may show different susceptibility to transgene integration or different levels of transgene expression, but more tests are needed to prove these possibilities.

Two mouse embryonic stem cell populations that overexpress either enhanced yellow fluorescent protein (eYFP) or enhanced cyan fluorescent protein (eCFP) were mixed and transplanted to get chimeric mice. The resultant chimeric mice exhibited eYFP-positive cells and eCFP-positive cells in the same organs within a single individual mouse, demonstrating possible method to control the ratio of genetic mosaicism (Hadjantonakis, 2002).

Restriction enzyme mediated integration (REMI) of DNA is often used for *Xenopus* transgenesis (Kroll, 1996). The sperm nuclei whose head membranes

are disrupted are incubated with linear transgene DNA for short periods of time (1-2min) followed by injection of the sperms into unfertilized eggs. This increased mosaic expression pattern of transgene in muscle, compared to longer periods of incubation (10-15min) (Sparrow, 2000), all of which demonstrates that mosaicism could be controlled at the stage of transgene integration by varying incubation time. In addition, direct injection of transgene DNA into fertilized eggs is also known to result in mosaicism (Christian, 1993).

All methods described above, however, utilize multi-copy integration of transgenes into the genome of target animals. This is sometimes problematic because multi-copy transgenes cause random insertional mutagenesis as well as deleterious effects on the model animals (Thellmann, 2003). Therefore, it is probably necessary to introduce a single copy transgene into the chosen chromosomal loci. Targeting single copy gene integration into chosen location in the genome also has subsequently been developed (Bronson, 1996; Misra, 2002). In this case, chromosomal sites where the foreign gene expression is favorable are selected, and however, the key is selection of integration sites where “moderate” expression of transgene can result in focal pattern expression instead of uniform expression.

Methods with LoxP-Cre System for Stochastic Transgene Expression

Since the 1980s, site-specific recombination using loxP-Cre system has allowed target genes to be deleted or expressed at the tissues of interest. Recently, several efforts using this method to introduce mosaic transgene expression or target gene deletion have been made and demonstrate promising control of stochastic gene expression or deletion in a tissue-specific manner (Figure 11). In this section, mosaic genetic alteration methods mediated by loxP-Cre recombination will be discussed.

Control of mosaicism by virus-mediated gene delivery

Virus-mediated gene delivery into target organs is reported to be ideal for mosaic transgene expression pattern (Figure 11A) (Chen, 2000; Tamamaki, 2000). To generate genetic mosaicism by activating oncogenes in a limited number of cells in the tissue of interest, Marumoto *et al*. injected lentiviral vectors that had *H-Ras* gene or activated *AKT* gene with loxP sites into Cre mice that express Cre recombinase in CFAP+ (glial fibrillary acidic protein-positive) cells in the adult brain to transduce stem/progenitor cells or terminally differentiated cells (Marumoto, 2009). Viral infection of either *H-Ras* or *AKT* did not develop a brain tumor in all the areas infected, as reported previously (Holland, 2000). However, when lentiviral vectors of *H-Ras* and *AKT* were injected together to examine cooperation of two oncogenic mutations, 50-60 cells were infected successfully demonstrating focal transgene expression - most (>98%) showed expression of

H-Ras and *AKT*. Also, these cooperative mutations resulted in tumors in some areas of infection after 4-5 months, and most of the cells in the tumor mass were positive for both oncogene expressions, suggesting expansion of highly proliferative double mutant cells (Marumoto, 2009).

Control of mosaicism by modulating *loxP* sites

Numerous transgenic mouse lines that express multiple fluorescent proteins in a mosaic pattern have been generated in neurobiology using the loxP-Cre system. Labeling neurons with XFPs, e.g. eGFP, eCFP, eRFP or eYFP, helps to examine neuronal structure dynamics and functions during brain development (Chakravarthy, 2008; Livet, 2007; Zong, 2005). Basically, two ways have been developed to affect stochastic transgene expression by modulating loxP sites (Figure 11B). First, when there are mutations in the spacer region of loxP sites, recombination efficiency became varied *in vitro* (Lee, 1998), although the efficiency was not changed *in vivo* (Livet, 2007). Second, it has been shown in an *in vivo* study that the longer the distance between two loxP sites is, the less the recombination efficiency (Zheng, 2000). Moreover, a new *in vitro* method to regulate sparseness of transgene expression has been developed based on the correlation between the loxP-loxP distance and recombination efficiency (Wang, 2009).

Control of mosaicism by modulating Cre recombinase activation

Recently, improved methods to generate transgenic mice with genetic mosaicism have developed using loxP-Cre system. Akyol *et al.* developed a method to activate *Cre* recombinase (*G22Cre*) sporadically in the caudal region of embryo, and the cecum and colon of adult mice. The mosaicism was shown by the pattern of β-galactosidase expression in *G22Cre;Rosa26* bigenic mice (Akyol, 2008; Soriano, 1999). The human *CDX2P9.5* gene promoter of this novel *Cre* construct is known to be active in caudal region of the developing mouse embryo, and in the distal small intestine, cecum and colon during late gestation and adult tissues (Hinoi, 2007). An array of 22 guanine nucleotides (22G) was added downstream of ATG initiation codon of *Cre* recombinase. Interestingly, *Cre* expression was active only if there was a frameshift reversion mutation (or microsatellite instability/MSI), i.e. conversion of 22G to 21G by frameshift mutation, which results in active *Cre* expression from the mutated construct (Figure 11C).

Despite the promising nature of focal transgene expression, this model has several potential problems. According to Southern blot analysis, about ~80 copies of the transgene are integrated into the mouse genome, so it may cause unexpected insertional mutagenesis. Until an appropriate frameshift mutation occurs, unknown proteins that are generated from unmutated *Cre* constructs may affect cellular functions. In addition, although Cre activation can be transmitted through three generations, stable germline transmission of the same pattern of Cre activity was not verified due to the random nature of mutation that occurred

in the *Cre* constructs. Different copy numbers of integrated trasgene may be necessary to get a similar frameshift mutation rate in tissues other than brain cells.

Similarly, Miller *et al.* devised a novel transgenic mice that overexpressed *Cre* recombinase sporadically (Miller, 2008). *Pms2* gene is a human homologue of yeast *postmeiotic segregation increased 2* (*Pms2*) gene and is involved in DNA mismatch repair. Homozygous inactivation of *Pms2* is reported to develop lymphoma (94%) and some sarcomas, but not intestinal adenoma or adenocarcinoma (Prolla, 1998). By replacing exon 2 of *Pms2* gene with frameshifted Cre recombinase construct including a sequence of 12 adenine nucleotides (*12ACre*), either homozygous $Pms2^{cre/cre}$ or heterozygous $Pms2^{cre/+}$ is generated (Figure 11D). Since $Pms2^{cre/cre}$ results in complete loss of *Pms2* function, a frameshift mutation in the sequence of adenine nucleotides occurs frequently and the resultant *11ACre* activates *Cre* expression in the intestine, pancreas, kidney, liver and muscle in a mosaic manner, which is consistent with *Pms2* gene expression patterns (Narayanan, 1997). In contrast, Pms2 is partially functional in $Pms2^{cre/+}$, and the heterozygous $Pms2^{cre/+}$ mice displayed much less Cre activity producing a rare β-galactosidase expression in $Pms2^{cre/+}$*;Rosa26* bigenic mice.

However, this model also has several possible problems like the Akyol *et al.* model. First, a majority of *Pms2* knockout mice developed lymphoma, so the Cre line can not be used for lymphoma-related studies. Also, 50% of $Pms2^{cre/cre}$ mice died from lymphoma at 10 months, so this line is only useful for early-

developing cancer models, whose tumorigenesis study can be completed within 10 months. In addition, a defect in DNA mismatch repair may cause potential innate tumorigenesis, and this may make data interpretation difficult.

Control of mosaicism using inducible gene expression systems

In addition to conventional loxP-Cre methods, inducible temporal and spatial control of gene expression has been developed. They are ligand-inducible Cre recombinases such as *Cre-ER* (Tamoxifne-inducible) (Figure 11E) (Feil, 1996), *Cre-hPR891HBD* (RU 486-inducible) (Kellendonk, 1996), or tetracycline-controllable *tTA/rtTA* (Jaisser, 2000). In these systems, the amount or dose of ligand (e.g. Tamoxifen) given to bigenic mice (e.g. *Cre-ER;Z/EG*) to induce transgene (*eGFP*) expression was shown to alter the degree of mosicism (Shi, 2007).

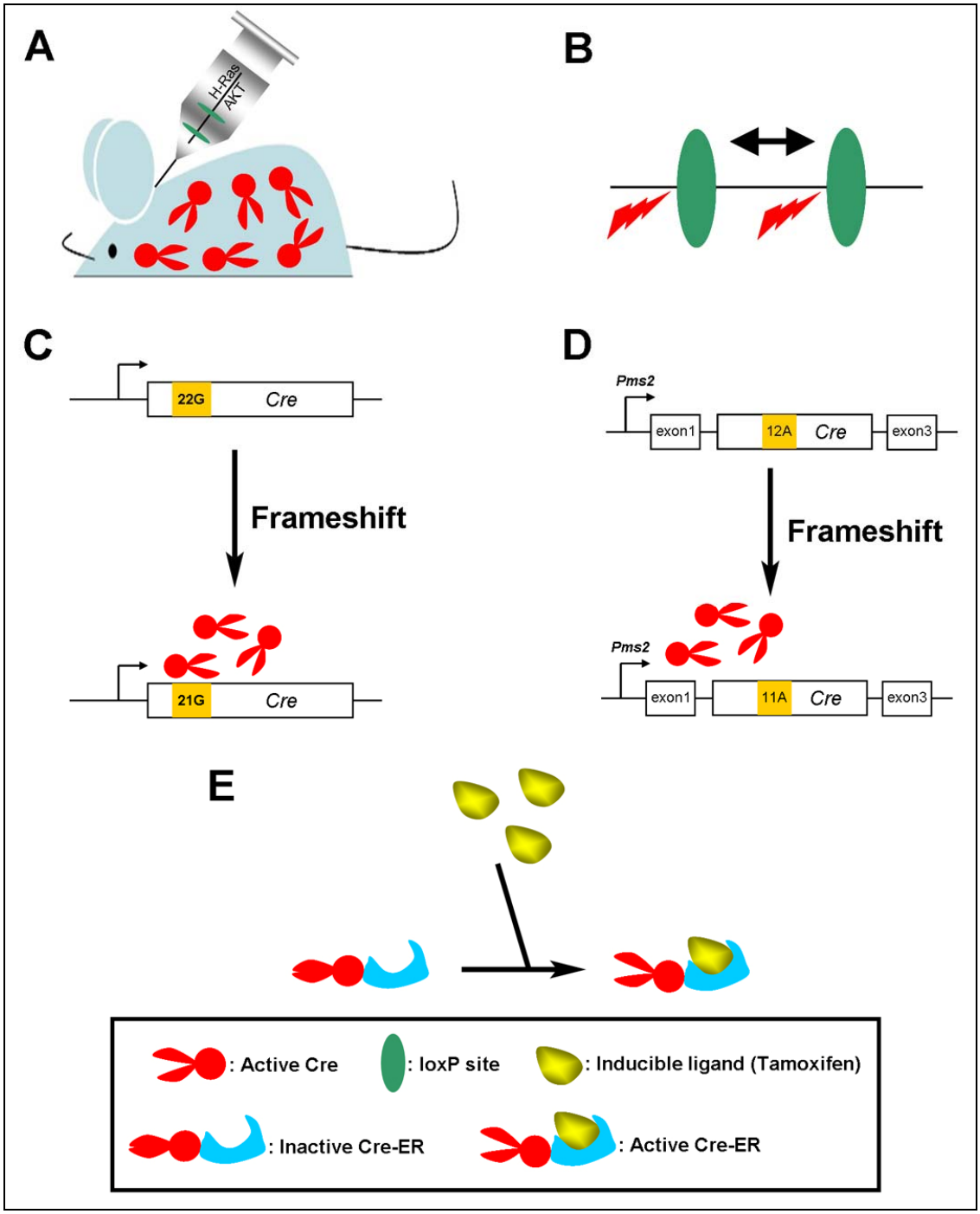


Figure 11. Methods using loxP-Cre to introduce mosaic gene alterations in model organisms. LoxP-Cre system has been widely used to induce stochastic gene alterations by virus-mediated gene delivery (A), modulating loxP sites (B) or Cre recombinase activation via frameshift mutation (C and D), and changing dose of ligands to activate Cre (E).

CHAPTER IV

GENERATION OF TRANSGENIC MICE WITH FOCAL ONCOGENE C-MYC ACTIVATION IN MULTIPLE TISSUES

[This part is adapted from {Roh, M., Kim, J., Song, C., Wills, M., Abdulkadir, S.A., *Genesis* (2006)}]

Introduction

As discussed in CHAPTER II, the analysis of the pathological role of oncogene expression will be greatly aided by the availability of suitable animal models in which specific oncogenes can be activated, in particular cell types and at particular stages of development. The c-MYC oncogenes have been implicated in tumorigenesis, including in lymphomas and prostate cancers. In human prostate cancer, for example, c-*MYC* gene amplification was reported in up to 30 % of samples (Jenkins, 1997; Nesbit, 1999; Qian, 1997; Sato, 1999) and its overexpression is thought to be an early event based on immunohistochemical analysis (Gurel, 2008) (CHAPTER I). In transgenic mice, targeted overexpression of c-Myc has been shown to be sufficient to induce cancer in various tissues (Jensen, 2003; Moroy, 1991; Nesbit, 1999; Pelengaris, 2002a; Pelengaris, 2002b; Pelengaris, 1999; Zhang, 2000b). The effects of *c*-Myc on proliferation or apoptosis differ depending on other cellular factors, for example, survival and apoptotic factors (Pelengaris, 2002a; Pelengaris, 2002b;

Pelengaris, 1999). In the prostate, targeted c-Myc expression using prostate-specific promoters such as *C3*, *Probasin (Pb)*, and modified *Probasin* promoter called *ARR_2Pb* in the prostate has been shown to induce a neoplastic phenotype (Ellwood-Yen, 2003; Zhang, 2000a). One limitation of these mouse models, however, relates to the response to androgen ablation because all these promoters are themselves regulated by androgen, as discussed in CHAPTER II.

Results

Construction of *Z-MYC* plasmid and *in vitro* validation of Cre recombination

We generated transgenic mouse lines for Cre-inducible c-MYC expression, called *Z-MYC*. The transgenic construct consists of *CMV enhancer/β-actin* promoter followed by a loxP-flanked cassette of the *β-galactosidase/Neomycin* resistance fusion gene (β-geo) and a triple polyA transcription termination signal (STOP) (Zinyk, 1998). Downstream of this cassette is human *c-MYC* (Figure 12). The presence of a "STOP" signal blocks expression of the downstream gene (*c-MYC*) until Cre-mediated recombination. The *CMV enhancer/β-actin* promoter has been shown to drive high levels of expression of downstream target genes in various cell types and tissues (Garg, 2004; Lobe, 1999; Niwa, 1991; Sawicki, 1998). The ability of the transgenic construct to serve as a Cre substrate was first evaluated *in vitro*. The *Z-MYC* construct was transfected into RWPE-1, benign human prostate epithelial cells, alone or in combination with a *Cre* expression plasmid (*EF1alpha-Cre*) (Le, 1999). Expression of *Cre* resulted in induction of expression of the transgenic c-MYC protein (Figure 12).

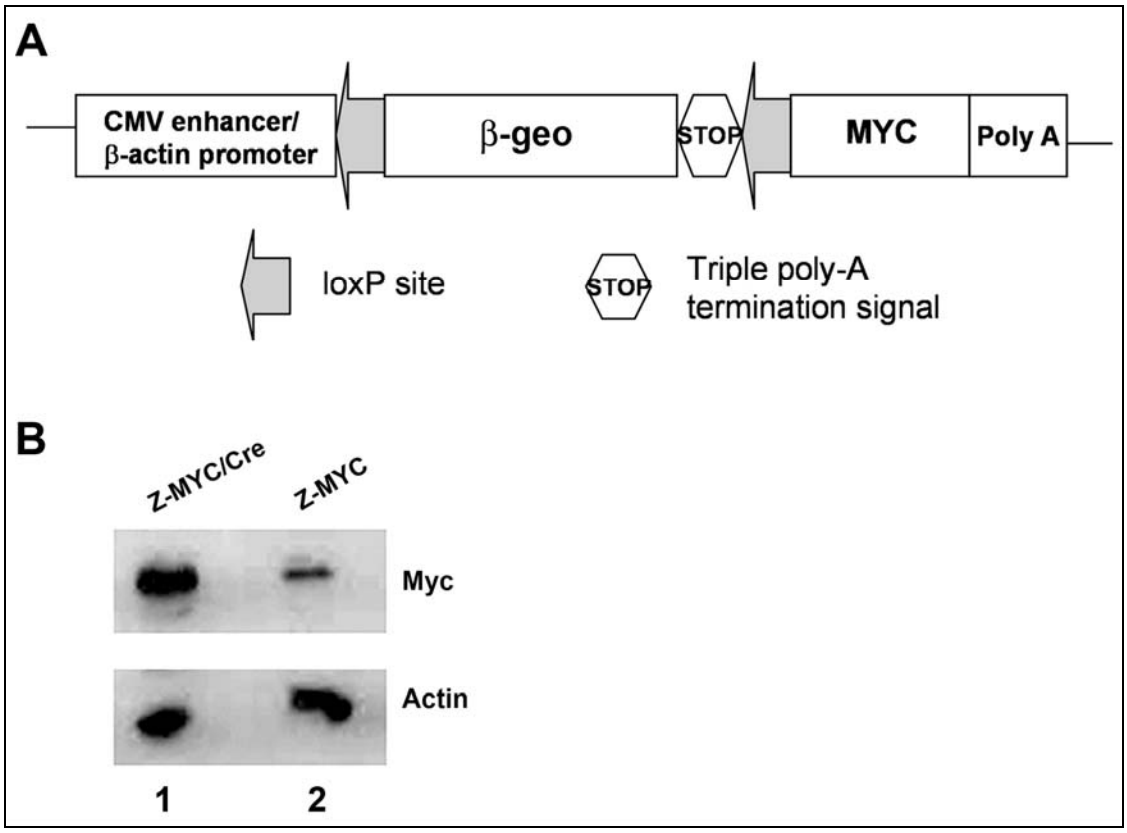


Figure 12. Schematic diagram of construct used in generating *Z-MYC* transgenic mice and *in vitro* validation of construct integrity. (A) describes schematic diagram of *Z-MYC* construct. *CMV enhancer/β-actin* promoter, *β-galactosidase/Neomycin* resistance fusion gene (*β-geo*), stop signal, human *c-MYC*, and poly-A site are depicted as boxes. (B) demonstrates *in vitro* validation of construct integrity. RWPE-1 stable cell line having *Z-MYC* plasmid was transfected with *EF1 alpha-Cre* (*Cre*), and samples were processed for c-MYC immunoblotting. The cells with both transgenes, *Z-MYC* and *Cre*, showed strong induction of *c-MYC* (lane 1), whereas single gene transfected cells did not (lane 2).

Introduction of *Z-MYC* construct into mouse ES cells and ES cell screening

Next, the *Z-MYC* construct was transfected into embryonic stem (ES) cells and the cells were subjected to G418 selection. These were then stained for lacZ (Figure 13) to identify clones displaying strong and uniform *lacZ* expression. This lacZ positive selection step is thought to improve the likelihood of generating mice with widespread transgene expression when subsequently generated from the ES clones (Lobe, 1999). For most of the clones, southern blot analysis was performed to determine the transgene copy number (Figure 13), with the goal of selecting clones with a single copy transgene for the generation of mice, to avoid possible complications that may arise due to integration of a multiple loxP containing transgene. Selected clones with a single copy transgene were further expanded, and infected with adenovirus expressing *Cre* or *GFP* control. Cell lysates were prepared and Cre-mediated recombination was confirmed by western blotting in *c-MYC* lines (data not shown). Clones displaying strong and uniform *lacZ* expression were chosen for injection into blastocysts to generate transgenic mice.

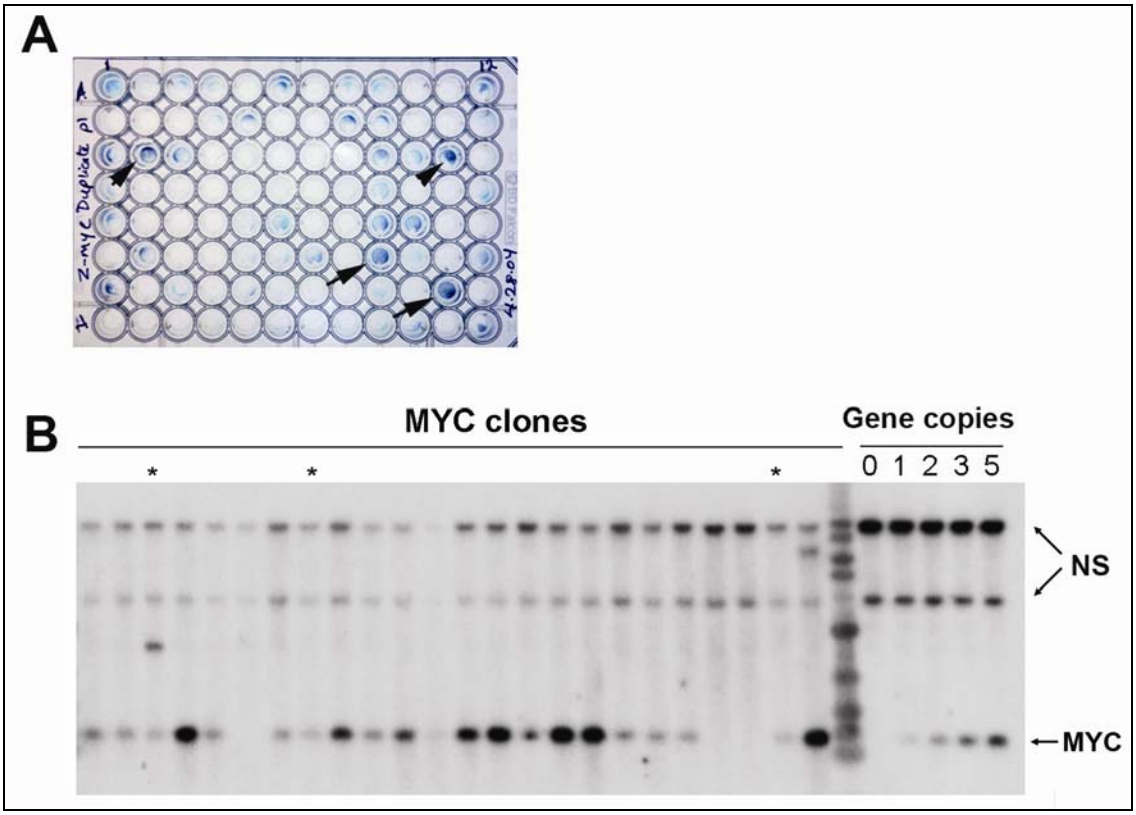


Figure 13. LacZ screening of *Z-MYC*-transfected ES cell clones and southern blot screening of ES clones for gene copy number. (A) shows lacZ screening of ES cell clones. ES cells were transfected with *Z-MYC* and stained for lacZ expression. Some lacZ positive clones are indicated (arrows). Shown in (B) is southern blot screening of ES clones. ES cell clones showing strong and uniform expression of lacZ from (A) were expanded and DNA samples were prepared. Plasmid DNA was used as a copy number control. Probes are P-32-labeled human *c-MYC* digested from *Z-MYC* plasmid. Several ES cell clones which have a single copy of transgene were indicated as *. NS: nonspecific bands.

Mosaic expression pattern of *Z-MYC* construct

The resulting *Z-MYC* male chimeric mice were bred with C57BL/6 females to establish *Z-MYC* (two lines) mouse lines. *Z-MYC* line 1 was generated from ES cells with a single-copy transgene integration event. To determine the expression pattern of transgene in various tissues, we first performed β-galactosidase staining using 8-25 week old mice. Strong β-galactosidase expression was detected in a mosaic pattern in a variety of tissues in *Z-MYC* line 1 transgenic mice, including prostatic epithelium, lung vascular and airway smooth muscle, retinal photoreceptor layer, kidney tubules, bladder smooth muscle, brain neurons, bone marrow, and spleen (Figure 14). *Z-MYC* line 2 also showed mosaic expression pattern of β-galactosidase in prostatic stroma (smooth muscle) and in the smooth muscle of various tissues including bladder, small intestine, lung (vascular and airway), skin and stomach. Cardiac muscle and skeletal muscle also showed positive staining for lacZ in this line (Figure 15). The skeletal muscle showed the most uniform expression pattern. Interestingly, all our lines showed a mosaic pattern of transgene expression, which may have distinct advantages in sporadic activation of oncogene expression in the study of tumorigenesis.

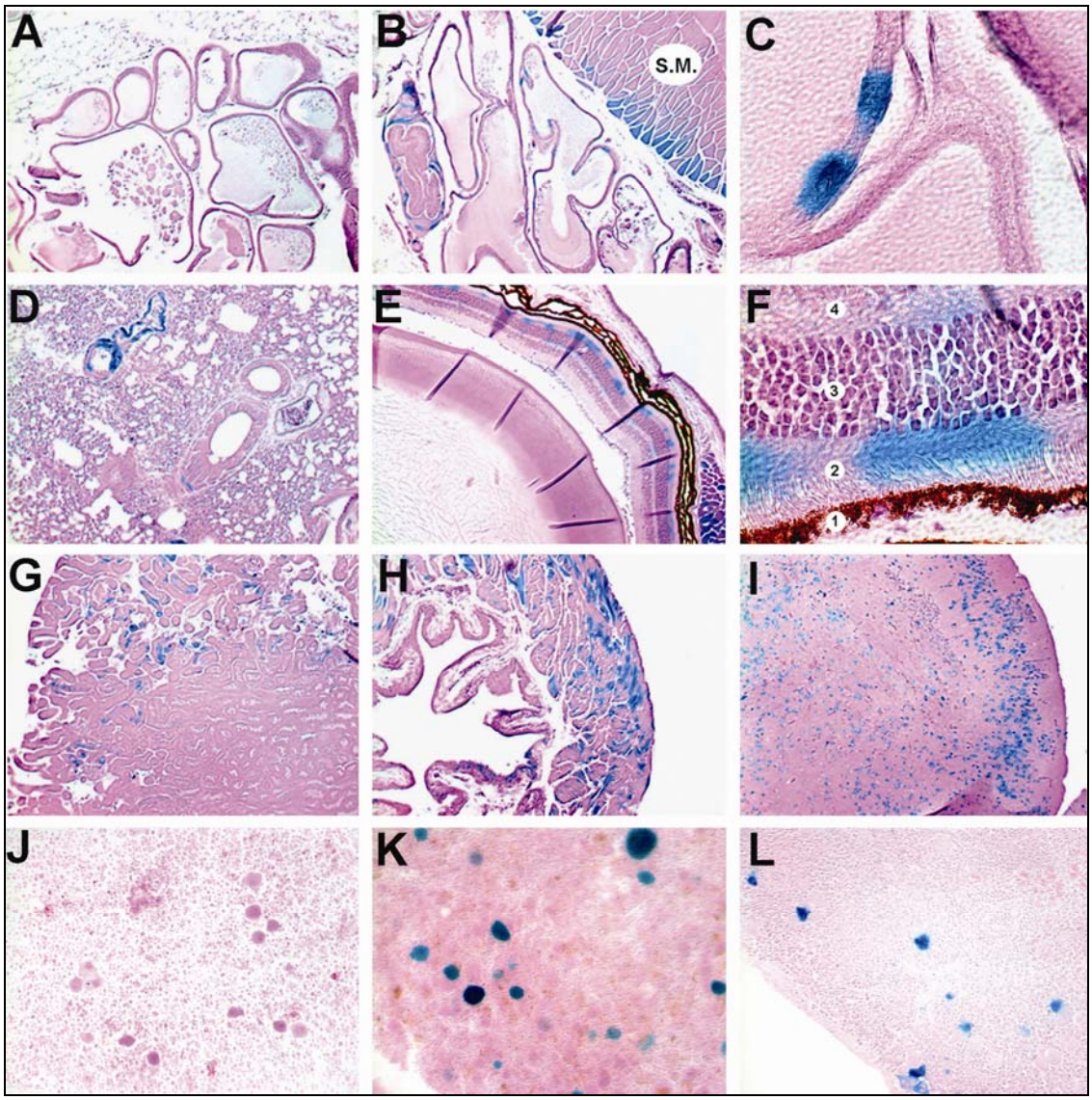

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Figure 14. LacZ expression in *Z-MYC* transgenic mouse line 1. β-galactosidase staining of tissues from *Z-MYC* line 1 (B-I, K-L), or littermate control (A, J). LacZ expression in all tissues occurs in a mosaic pattern. (A) prostate from *Z-MYC* negative control mouse. (B) Prostate and skeletal muscle (S.M.) from Z-MYC positive mouse. Note mosaic expression in prostate luminal epithelial cells. (C) High power image of prostate section in "B". (D) Lung, showing lacZ positive cells in the vascular and airway smooth muscle. (E) Transverse section through the eye with lacZ expression in part of the retina. (F) High power image of eye section from "E" showing lacZ expression in photoreceptor layer of the retina (2). 1: pigment epithelium layer; 2: photoreceptor layer; 3: outer nuclear layer; 4: outer plexiform layer. (G) Kidney, showing expression in some tubules. (H) Bladder, showing lacZ expression in smooth muscle. (I) Cerebral cortex, showing positive cells in some neurons. (J) Bone marrow from wild type animal. (K) Bone marrow from *Z-MYC* line 1. Some hematopoietic progenitor cells are positive. (L) Spleen from *Z-MYC* line 1 showing some positive cells including megakaryocytes. 10-13 week old animals were used.

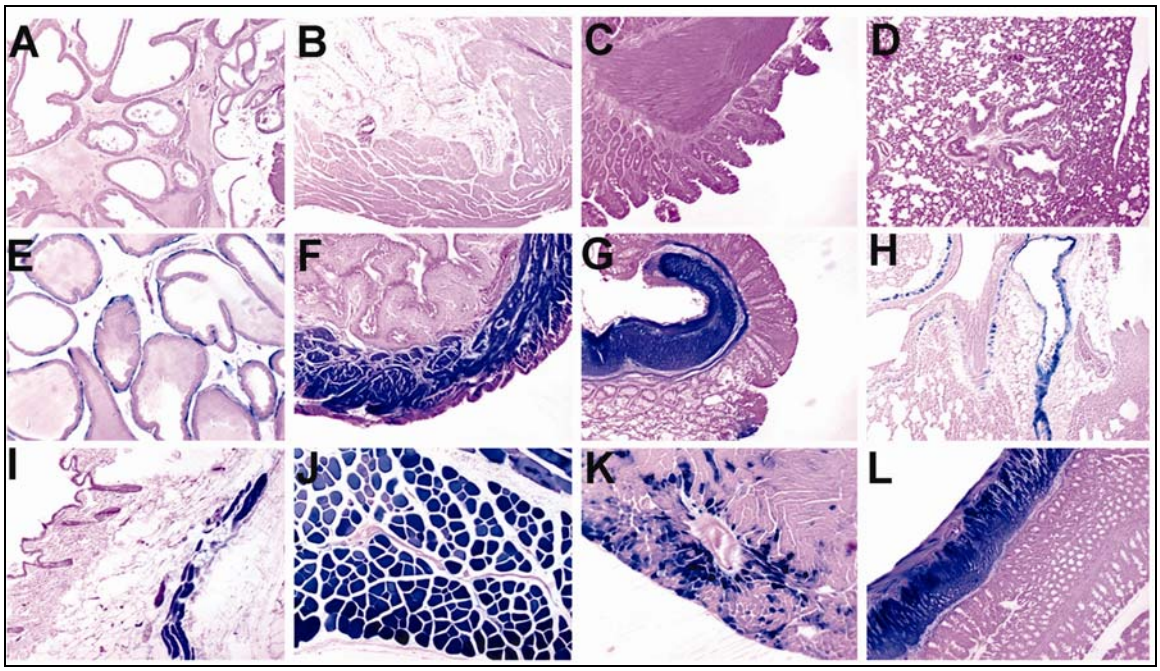

Figure 15. LacZ expression in *Z-MYC* transgenic mouse line 2. β-galactosidase staining of tissues from *Z-MYC* line 2 (E-L), or littermate control (A-D). LacZ expression in all tissues occurs in a mosaic pattern, with skeletal muscle showing the most uniform expression pattern. (A) Prostate. (B) Bladder. (C) Small intestine. (D) Lung. (E) Prostate, showing lacZ expression in stroma. (F) Bladder, showing expression in smooth muscle. (G) Small intestine, showing expression in smooth muscle. (H) Lung, showing expression in vascular and airway smooth muscle. (I) Skin, showing expression in muscle. (J) Skeletal muscle. (K) Cardiac muscle. (L) Stomach, with expression in muscle layer. 20-25 week old animals were used.

Tissue-specific overexpression of c-Myc after Cre excision

To test Cre-mediated induction of transgene expression *in vivo*, we bred *Z-MYC* positive mice with smooth muscle myosin heavy chain (*smMHC*)-*Cre* transgenic mice (Regan, 2000; Xin, 2002). The *smMHC* promoter can drive smooth muscle-specific gene expression during development as well as in the adult mouse, and has been used for the generation of mice expressing Cre recombinase in smooth muscle tissues (Regan, 2000; Xin, 2002). RT-PCR, using the *smMHC-Cre;Z-MYC* double positive mouse, confirmed smooth muscle-specific expression of human *c-MYC* transgene (Figure 16A). Western blotting, using anti-c-MYC antibody, also confirmed smooth muscle-specific expression of *c-MYC* transgene after Cre recombination (Figure 16B).

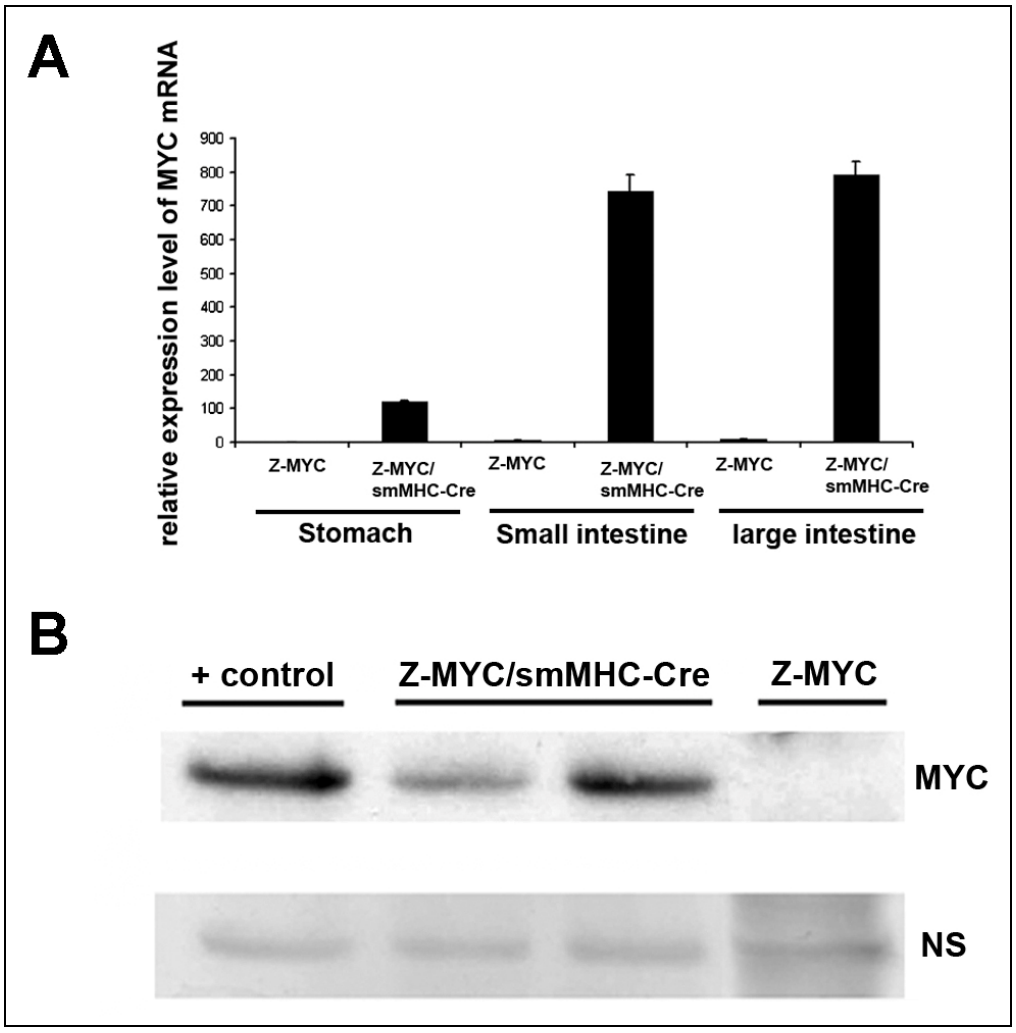

Figure 16. Evidence of Cre-mediated recombination of the *Z-MYC* construct *in vivo*. (A) RT-PCR using *smMHC-Cre;Z-MYC* double positive mouse and *Z-MYC* single positive control confirmed smooth muscle-specific expression of *c-MYC* transgene. (B) Western blotting showing c-MYC expression in *smMHC-Cre;Z-MYC* double positive mouse, but not in *Z-MYC* single positive mouse. Line 2 *Z-MYC* mouse lines were used. NS: nonspecific bands. 8-9 and 4 weeks old mice for A and B were used in these experiments, respectively. Error bars = SD

Discussion

In this study, we generated novel transgenic mice with latent c-Myc mutation in various tissues. As discussed in Genetic Mosaicism in CHAPTER III, copy number of introduced trangene and chromosomal loci where the transgene is integrated are the most critical factors to decide expression levels and expression pattern of the transgene. Based on the lacZ reporter expression and c-MYC mRNA expression after tissue-specific Cre excision, *Z-MYC* mice demonstrated quite strong expression of the transgene, although *Z-MYC* mice have only one copy of Z-MYC transagene (hemizygous). Integration of one copy of floxed transgene into the chromosomal locus is crucial because unwanted long chromosomal regions could be deleted by Cre if multiple copies are integrated into the same chromosome (Figure 17). This may cause fatal mutations and make data interpretation very difficult. Also, despite the control of the global actin promoter, transgene expression is focally activated instead of uniformly. This is probably because *Z-MYC* transgene is integrated into an intermediate area, i.e. somewhere between heterochromatin and euchromatin. Integration into this locus causes intermediate accessibility of transcription factors to activate the actin promoter and the cells display a stochastic expression pattern. Therefore, if more examination confirms that this integration site is an intermediate locus where even one copy of integrated transgene leads to focal but strong expression, it will provide an appropriate target site to introduce foreign genes

that causes stochastic gene expression in mouse models via site-directed integration of a transgene (Bronson, 1996; Misra, 2002).

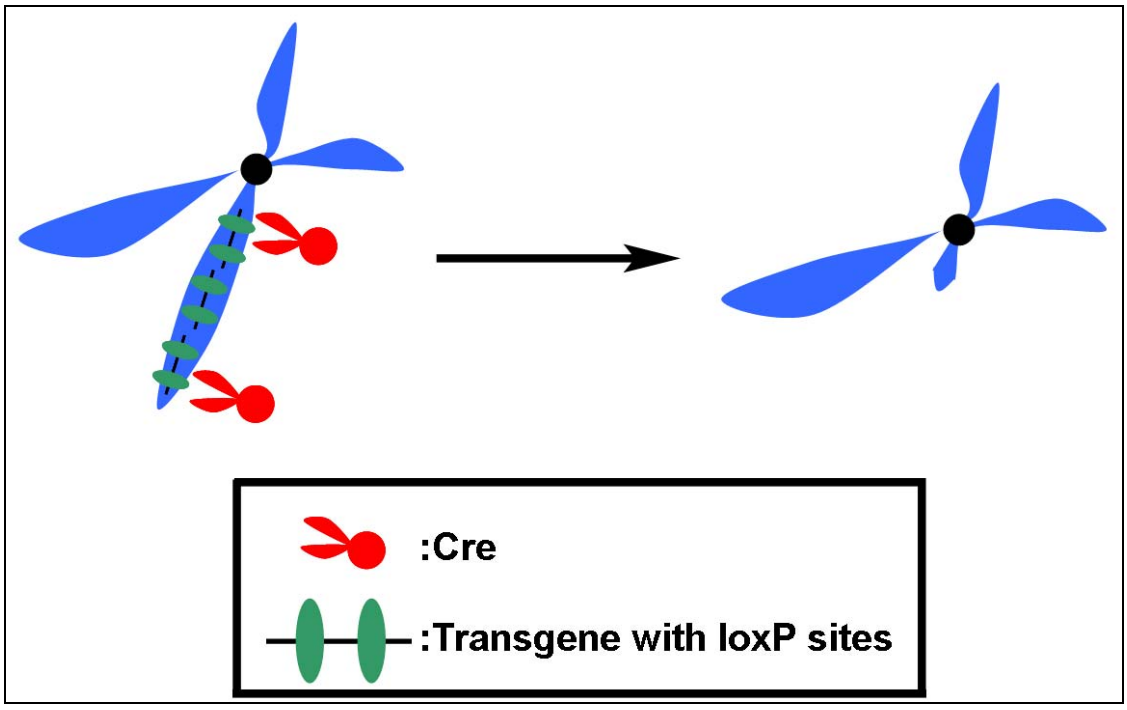

Figure 17. Possible complication due to multi-copy integration of floxed transgene. When transgenes more than one copy are integrated into the same chromosome, unwanted long chromosomal regions could be deleted and this would make the data interpretation harder.

The *Z-MYC* transgenic mouse lines described herein have several advantages over other transgenic mouse models where expression of c-MYC was driven directly by tissue-specific promoters (Ellwood-Yen, 2003; Zhang, 2000a). First, the high levels of expression of c-MYC driven by *CMV enhancer/β-actin* promoter will approximate the high levels of gene expression in cancers and their pathological status. Second, it will allow examination of the effects of c-MYC overexpression on the response of specific tissue to hormone withdrawal, for example, androgen withdrawal, because once activated by Cre recombination, the transgene will be under the control of actin promoter and not affected by hormone withdrawal. Third, it will provide the wider research community with a valuable resource since it can be used in conjunction with other tissue-specific *Cre* mouse lines to activate *c-MYC* in any tissue of interest, singly or in combination with floxed allele of TSGs.

More importantly, these *Z-MYC* mice show stochastic expression of transgene although the degree of stochasticity varies depending on tissue types. As discussed in CHAPTER III, human cancers are thought to initiate from one mutation in a single cell or a few cells. In this regard, this mouse line could mimic spontaneous focal mutation in c-Myc in any tissue showing mosaic pattern of Z-MYC expression and help in better understanding of human carcinogenesis initiated c-MYC activation.

Materials and Methods

Generation of transgenic mice

Constructs were made using the pCCALL2-IRES-hAP/cg vector (gift of C. Lobe) as a backbone. This vector consists of a *CMV enhancer/chicken β̃-actin* promoter, a loxP-flanked *β-geo*, triple repeat of the SV40 polyadenylation signal (poly A), and β-globin poly-A sequence. To generate the *Z-MYC* construct, the region from the IRES to the β-globin poly-A in pCCALL2-IRES-hAP/cg was replaced with the human *c-MYC* gene from pSVMYC (gift of P. Leder), including exons 2 and 3 and poly-A site. The *Z-MYC* vector was linearized and transfected into 129SV/EV ES cells. G418 resistant ES cell clones were picked and expanded into duplicate 96 wells. The duplicate 96 wells were cryopreserved and the ES cell clones in culture expanded into 24 wells for DNA isolation and LacZ analysis. Selected clones were expanded from the cryopreserved plates and used to generate transgenic mice by injection of ES cells into blastocysts. Male chimeras were bred with female C57BL/6 mouse to establish transgenic lines. Transgene-positive mice were identified by both lacZ stain of ear biopsy and PCR of tail DNA. The PCR primers used were as follows: 5'-CGA GTC GTA GTC GAG GTC ATA-3' (forward), 5'-TAG TTC TAG CTA GTC TAG GTC GAT-3' (reverse) for *c-MYC*. *Z-MYC* positive mice were bred with *smMHC-Cre* mice that express *Cre* in smooth muscle cells (gift of M. Kotlikoff) to generate *smMHC-Cre;Z-MYC* double positive animals. All analyses, including lacZ staining and western blot analyses were done using adult mice older than 6 weeks.

Prostate cell line transfection and western blot analysis

RWPE-1 cells were transfected using Fugene 6 transfection reagent (Roche) according to manufacture's protocol. 48 hours later, cell lysates were prepared and processed for western blotting using anti-c-Myc antibody (Santa Cruz) as described previously (Roh, 2003).

Adenovirus Cre infection

ES cell clones were expanded further and infected with adenovirus expressing *Cre* (*Ad5CMVCre*) or *GFP* control (*Ad5CMVEGFP*). Briefly, adenoviruses at 100 MOI were mixed with 1 ml of ES cell medium containing 2% fatal bovine serum, added to ES cells, incubated for 12 hours, and cell lysates were prepared for western blotting.

LacZ staining

All the mouse organs were fixed in 2% paraformaldehyde containing 0.2 % glutaraldehyde for 4 hours on ice with gentle shaking. After 1-hour fixation, tissues were dissected to help penetration of fixative. To inactivate endogenous β-gal activity, the tissues were washed three times for 30 min in phosphate-buffered saline (PBS) (pH 8.6) and then incubated for 1 hour at 50C. After cooling to room temperature, tissues were incubated in prestaining solution (2mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$ and 5mM EGTA in PBS) for 1 hour at room temperature, and then stained with 0.5 mg/ml X-gal in prestaining solution for overnight at room temperature. The tissues were washed with PBS,

post-fixed in 4% paraformaldehyde in PBS, processed for paraffin embedding, and sections were counterstained with nuclear fast red.

Southern blot analysis

ES cell DNA was digested with *Bgl II* for *Z-MYC* and used for southern blot analysis according to standard protocols. Normal mouse tail DNA was included as a control after spiking with 0, 1, 2, 3, 4, or 5 copies of transgene per genome. Probes were ^{32}P-labelled transgene-specific fragments from *Z-MYC* construct.

CHAPTER V

C-MYC AND CELL COMPETITION

Introduction

As discussed in CHAPTER I, Myc is involved in cell competition by killing neighboring “loser” cells during *Drosophila* development when there are two groups of cell populations, one having relatively high levels of Myc protein and the other having low levels. According to the lacZ reporter gene expression data (Figure 14) in CHAPTER IV, our *Z-MYC* mice (line 1) display mosaic pattern of transgene expression. This suggests that there exist two cell populations in one individual animal or even in one tissue at the same time. This characteristic is very important in that there are two groups of cells that have high or low c-Myc expression at the same time. In addition, these mice are useful for investigating spontaneous human cancer development initiated from a single mutation in a few cells. Therefore, the mosaic pattern of c-Myc expression allows us to examine the idea of cell competition in mammals, which so far has been demonstrated only in invertebrates. In this chapter, evidence of c-MYC-mediated cell competition in our mouse model will be provided.

Results

Generation of *PbCre4;Z-MYC* mice with focal, prostate-specific c-MYC overexpression

To target focal c-MYC expression specifically in the mouse prostate, we used *Z-MYC* mice (line 1) that carry a single copy transgene in which the *CMV enhancer/β-actin* promoter drives expression of the *β-geo* gene and a latent *c-MYC* transgene (Figure 12, 13 and 18B) (Roh, 2006). Staining for β-galactosidase confirmed mosaic expression in the prostate epithelium (Figure 14 and 18A). To induce *c-MYC* expression focally in the prostate, we crossed *Z-MYC* mice to *PbCre4* mice (Wu, 2001) which express Cre recombinase in the prostatic epithelium (Figure 18B). As expected, human *c-MYC* transgene mRNA expression occurred only in bigenic *PbCre4;Z-MYC* mouse prostate, not in other tissues of this mouse or in *Z-MYC* mouse tissues examined, which demonstrates that *c-MYC* transgene expression is prostate-specific after Cre excision (Figure 18C). Then, to analyze protein expression of c-MYC in the prostate, immunohistochemistry for c-MYC was performed. Consistent with lacZ expression (Figure 14 and 18A), c-Myc protein expression showed a focal pattern in the prostate (Figure 18D).

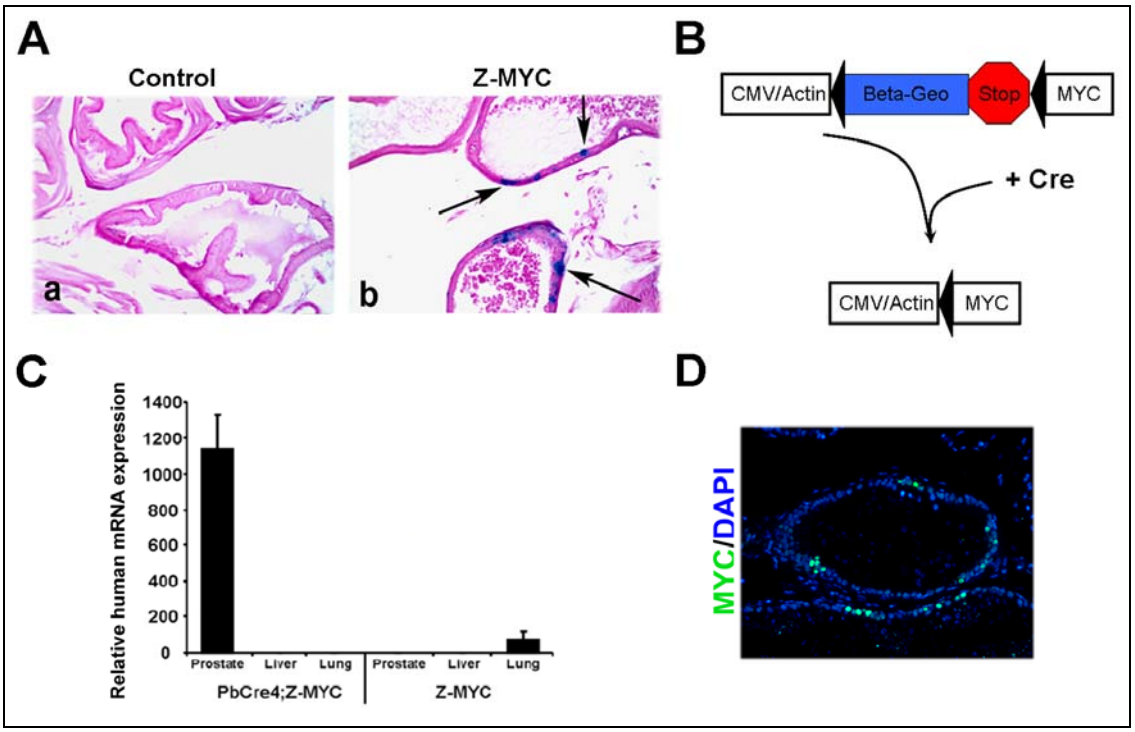

Figure 18. Mosaic transgene expression (lacZ or c-Myc) in the transgenic mouse prostate. *Z-MYC* mouse prostate shows mosaic pattern of *lacZ* reporter gene expression (A) and prostate-specific mRNA (C) and protein (D) expression of c-Myc after Cre excision. Note that c-MYC expression is directly regulated by *CMV enhancer/β-actin* promoter once Cre recombination occurs (B) and c-Myc protein expression is also focal.

c-MYC induces proliferation as well as apoptosis in the mouse prostate

First, we examined whether transgene c-MYC is functional in the mouse prostate. Since c-Myc is known to induce proliferation as well as apoptosis (see Biological functions of c-Myc in CHAPTER I), we determined its effects by quantitative RT-PCR or immunohistochemistry. To compare proliferation of mouse prostate cells between control mice (wild type, *PbCre4* or *Z-MYC*) and MYC mice (*PbCre4;Z-MYC*), we performed qRT-PCR to analyze mRNA expression levels of Ki-67, a proliferation marker. MYC mouse prostate showed more Ki-67 expression than *Z-MYC* control prostate (Figure 19A), and this was confirmed by immunohistochemistry for Ki-67 protein expression (panel "b" in Figure 19A). Increased proliferation in c-MYC-overexpressing prostates was also confirmed by double immunohistochemistry for c-Myc and phospho-histone H3, another proliferation/mitotic marker. We found that the c-Myc+ cells in the same prostatic gland displayed more phospho-histone H3 positivity (Figure 19B) and that *PbCre4;Z-MYC* prostates contained more phospho-histone H3+ cells than control prostates (Figure 19C). Next, to determine c-Myc-induced apoptosis, we stained for activated caspase 3, an apoptotic marker (Figure 19C) followed by quantitation. We also perfomed Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Figure 19D) on the control and MYC prostate sections. Both methods consistently showed increased apoptosis in the MYC prostates. Collectively, c-MYC protein in the *PbCre4;Z-MYC* mouse prostate is functional both as a proliferative agent and an apoptotic agent.

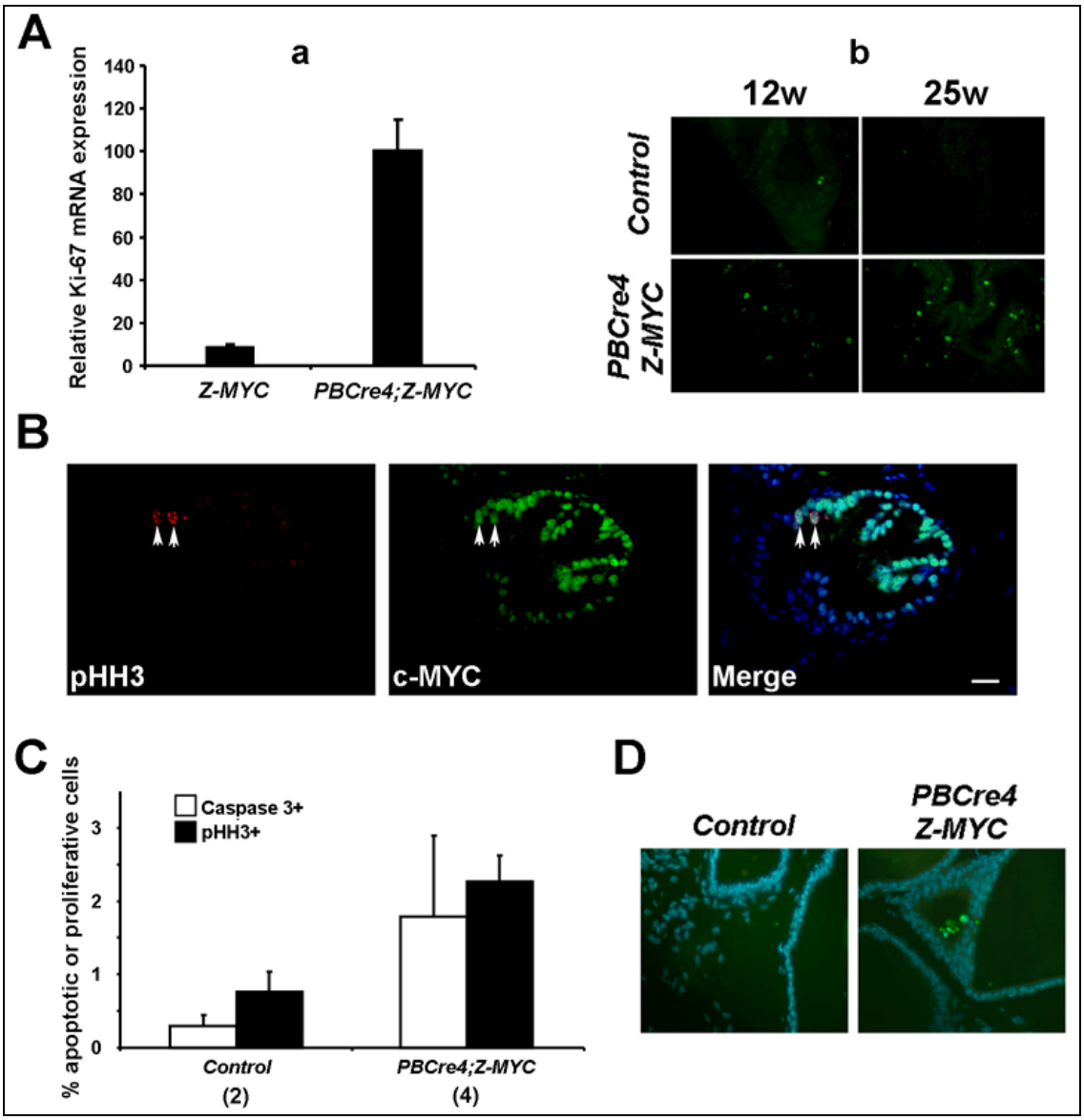

Figure 19. c-MYC expression in the *PbCre4;Z-MYC* prostates causes proliferation as well as apoptosis. Quantitative RT-PCR was performed to compare mRNA expression levels of human transgene *c-MYC* using the whole RNA isolated from *PbCre4;Z-MYC* and *Z-MYC* mouse prostates (Aa) (Error bars: standard deviation of triplicate values). This was confirmed by immunohistochemistry for Ki-67 protein (green) (Ab). Double immunohistochemistry for phospho-histone H3 (pHH3) and c-MYC (B). (C) Immunohistochemistry for activated caspase 3 and phospho-histone H3 was performed separately on the prostate sections, and the number of cells positive for each marker was counted and normalized with the number of total cells counted. Numbers in the brackets indicate the number of mice analyzed in each group (Error bars: standard deviation of the indicated number of samples). (D) TUNEL assay. TUNEL-positive cells and nuclei are stained green and blue, respectively.

Non-cell-autonomous apoptosis is dominant in mouse and human prostate tissues, but its ratio to cell-autonomous apoptosis varies depending on the type of human tissues

Although *PbCre4;Z-MYC* prostate displayed increased apoptosis, this does not necessarily mean that all the apoptoses are cell-autonomous (c-Myc+ cells are apoptotic). Non-cell-autonomous apoptosis (c-Myc- cells are apoptotic) denotes killing of neighboring “low” c-Myc cells by “high” c-Myc cells - cell death by cell competition. Therefore, we performed double immunohistochemistry for c-MYC and activated Caspase 3 to examine whether the apoptoses are cell-autonomous or non-cell-autonomous. Interestingly, the majority of apoptotic cells were c-MYC-negative (or low c-MYC) cells and the frequency of apoptosis was dramatically decreased the farther away from high c-MYC cells (Figure 20). In other words, low c-MYC cells located right next to high c-Myc cells were highly apoptotic but cells farther away were the least apoptotic. It also should be pointed out that cell-autonomous apoptosis of high c-Myc cells was relatively less frequent than non-cell-autonomous apoptosis.

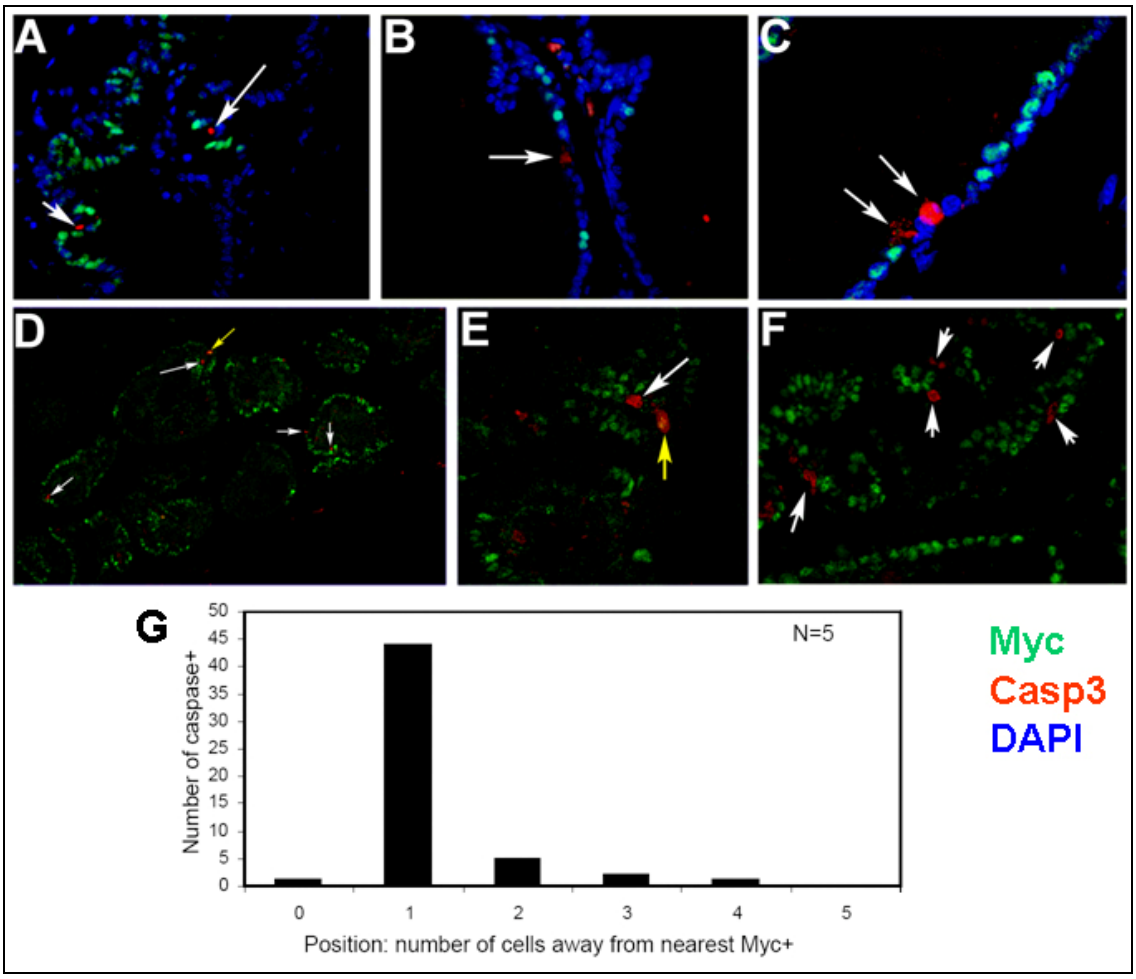

Figure 20. Non-cell-autonomous apoptoses in *PbCre4;Z-MYC* mouse prostate. Double immunohistochemistry was performed for c-MYC and activated caspase 3. White arrows and a yellow arrow indicate non-cell-autonomous apoptoses (red cells) and a cell-autonomous apoptosis (red+green cell), respectively (A-F). The number of apoptosis in either c-MYC-positive or -negative cells was counted and the apoptotic frequency depending on the distance from c-MYC-positive cells was analyzed (G). "0" denotes the position of c-MYC positive cells.

To test this phenomenon of non-cell-autonomous apoptosis in *in vitro* cell culture, we generated a c-MYC-overexpressing and vector control RWPE-1 cell line, a benign human prostate cell line using a lentiviral gene delivery system. Since the original FM-1 vector does not have an antibiotic resistance marker, we could not select cells that express infected vectors. Therefore, we regarded each pooled population with FM-1/control or FM-1/c-MYC plasmid as low c-MYC (YFP+) + low c-MYC (YFP-) (L+L) or high c-MYC (YFP+) + low c-MYC (YFP-) (H+L) population, respectively. FM-1 vector has a YFP reporter gene, so we could track high c-MYC cells in FM-1/c-MYC-infected pooled cell population (H+L) and low c-MYC cells in FM-1/control-infected pooled cell population (L+L). We cultured the cells without growth factors to induce apoptosis and analyzed cell-autonomous (CAA) and non-cell-autonomous apoptosis (NCAA) by immunocytochemistry for activated CASPASE 3. When we compared YFP+ and YFP- apoptotic cells between L+L and H+L cell population, we observed an increase in both CAA (YFP+CASP3+) and NCAA (YFP-CASP3+) in H+L population, but apoptoses of YFP+CASP3+ and YFP-CASP3+ were rarely changed in L+L population (Figure 21), even after 72-hour growth factor starvation. This suggested that c-MYC induced both CAA of c-MYC+ cells and NCAA of c-MYC- cells. Previously, it has been shown that conditioned media (CM) from the co-culture of high dMyc and low dMyc *Drosophila* cells induced apoptosis of low dMyc cells, but not that of high dMyc cells, suggesting that mixed soluble factors released from both high and low dMyc cells kill low dMyc cells *in vitro* (Senoo-Matsuda, 2007). To test this in our RWPE-1 human cell line,

we first sorted pooled cell populations to get pure c-MYC+ (high c-MYC) or control (low c-MYC) cells. 28.9% of cells in L+L (FM-1/control) population were selected for YFP+ low c-MYC cells (YFP+/c-MYC-) and 10% of cells in H+L (FM-1/c-MYC) population were selected for YFP+ high c-MYC cells (YFP+/c-MYC+) (Figure 22A). Conditioned media from two pooled populations (L+L or H+L) were then added to each sorted target cells. Interestingly, CM from high and low c-MYC cell population (H+L) dramatically elevated apoptosis in low c-MYC cells (black bars in left panel of Figure 22B) but relatively much less in high c-MYC cells (black bars in right panel of Figure 22B). CM from low c-MYC cells (L+L) caused minimal apoptosis both in low c-MYC and high c-MYC target cells (white bars in Figure 22B). Taken together, c-Myc-induced cell competition may occur in mammalian cells and soluble factors from both high and low c-Myc cells may enable killing of low c-Myc cells.

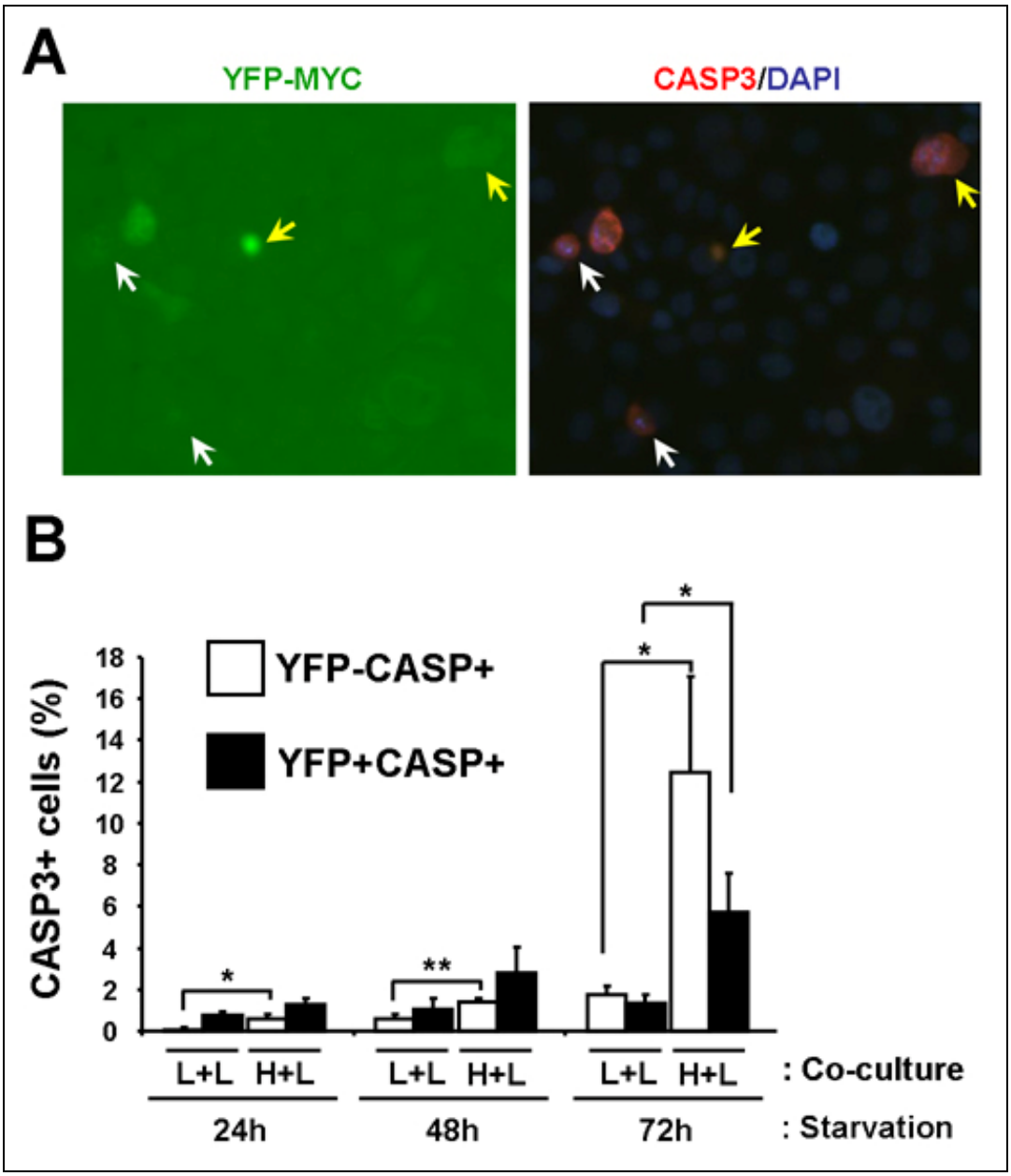

Figure 21. Analysis of *in vitro* c-MYC-induced apoptosis in co-culture condition. Pooled cell populations were starved for indicated time to induce apoptosis and immunocytochemistry was performed for activated CASPASE 3. (A) represents cell-autonomous (yellow arrows) and non-cell-autonomous apoptoses (white arrows). CASPASE 3 and nuclei were stained red and blue, respectively followed by quantitation (B). Error bars = standard deviation. **P* < 0.02, ***P* < 0.002.

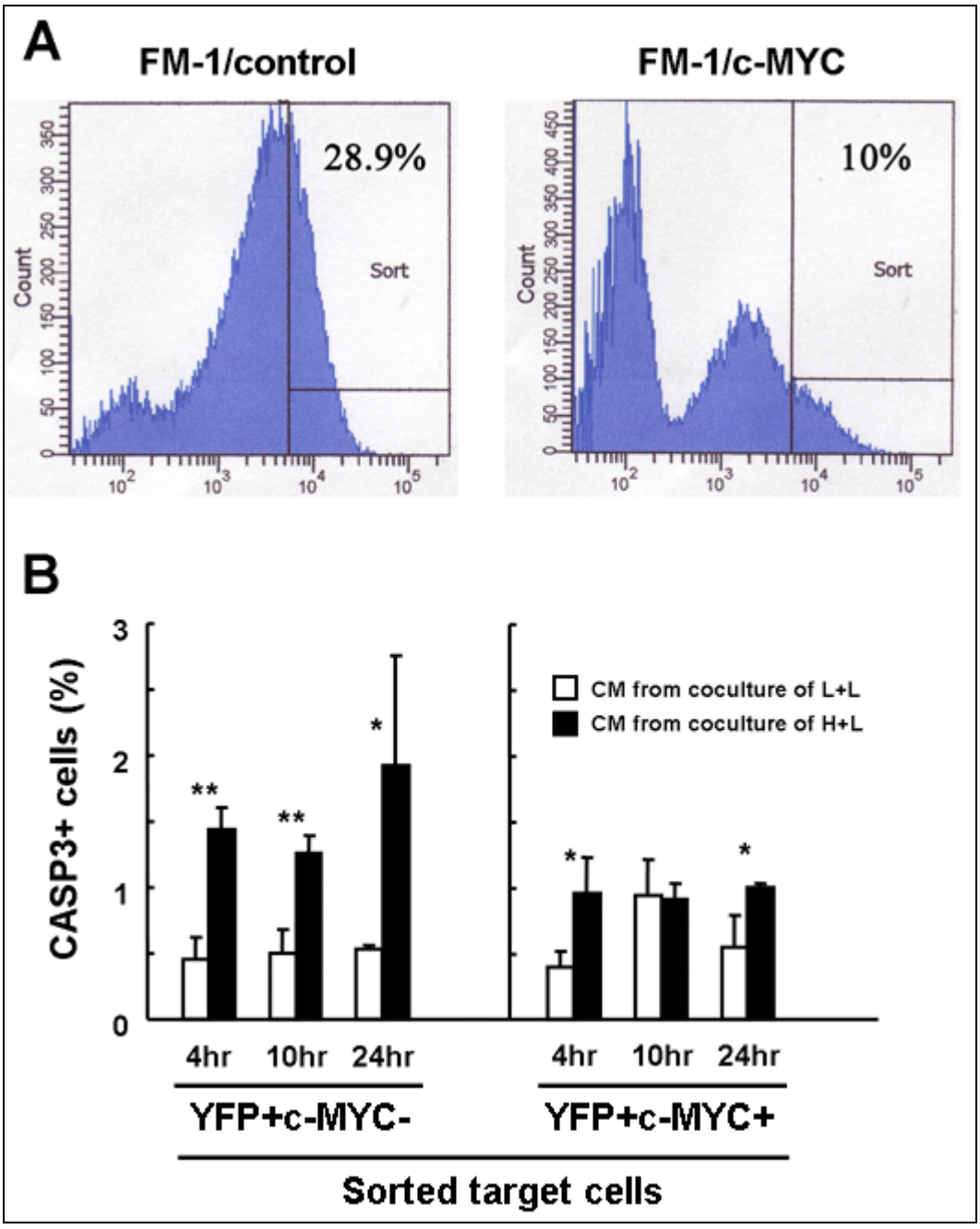

Figure 22. Analysis of *in vitro* c-MYC-induced apoptosis with conditioned media using sorted target cells. Both pooled cell populations were sorted to get pure YFP+/c-MYC- cells (28.9% in FM-1/control population) and YFP+/c-MYC+ cells (10% in FM-1/c-MYC population) (A). Conditioned media from FM-1/c-MYC-infected pooled cell population (H+L) or FM-1/control-infected pooled cell population (L+L) were added to sorted target cells (YFP+/c-MYC- cells from FM-1/control population or YFP+/c-MYC+ cells from FM-1/c-MYC population) and analyzed apoptosis following immunocytochemistry for activated CASPASE 3. Shown is the quantitated data (B). **P*<0.05, ***P*<0.005.

To confirm that there is increased NCAA in low c-MYC cells near high c-MYC cells in the human prostate tissues, we did double immunostains for c-MYC and activated CASPASE 3 in human prostate cancer specimens. Consistent with mouse and *in vitro* data, low c-MYC cells around high c-MYC cells were highly apoptotic, demonstrating a similar pattern of CAA and NCAA shown in *PbCre4;Z-MYC* mouse prostates (Figure 23). Next, we examined if c-MYC-induced NCAA is dominant over CAA in other human tissues. We stained for c-MYC and activated CASPASE 3 in other human cancer specimens. Interestingly, the ratio of NCAA over CA was clearly varied depending on tissue types (Figure 24). Prostate and rectum, for example, displayed the most frequent NCAA, as compared to other tissues. All of the prostate and rectum tissue samples analyzed and judged c-MYC+ showed more than 50% NCAA (i.e. less than 50% CAA). However, NCAA in colon and lung tissues displayed a wide range, varying between 0 to 100%. This suggests that cell types might be a critical factor to determine cell competition caused by c-MYC expression.

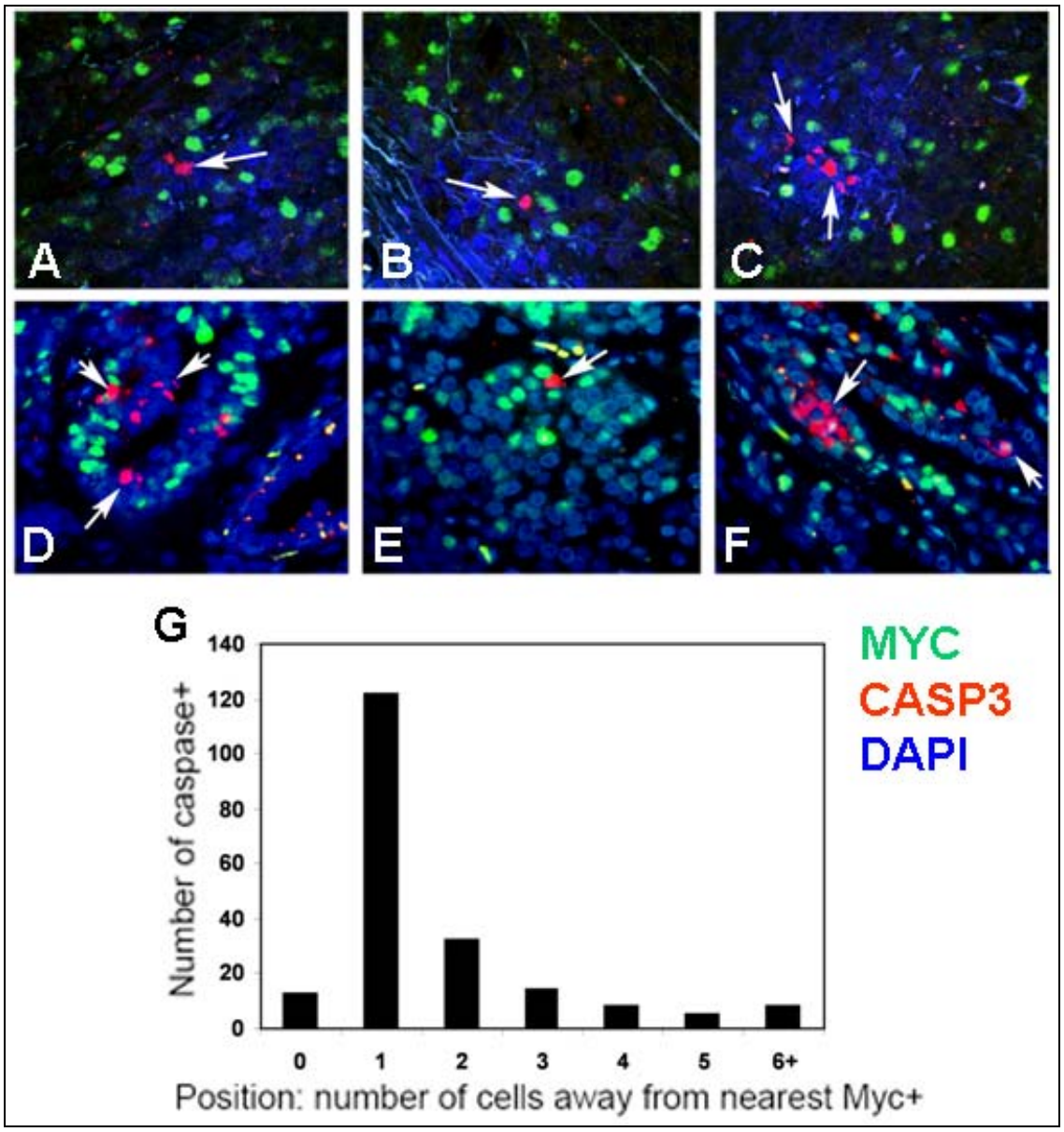

Figure 23. Non-cell-autonomous apoptoses in human prostate cancer specimens. Double immunohistochemistry was performed for c-MYC and activated CASPASE 3. Arrows indicate non-cell-autonomous apoptoses (A-F). The number of apoptosis in either c-MYC-positive or -negative cells was counted and the apoptotic frequency depending on the distance from c-MYC-positive cells was analyzed (G). "0" denotes the position of c-MYC positive cells. N=8 human prostate cancer specimens were analyzed.

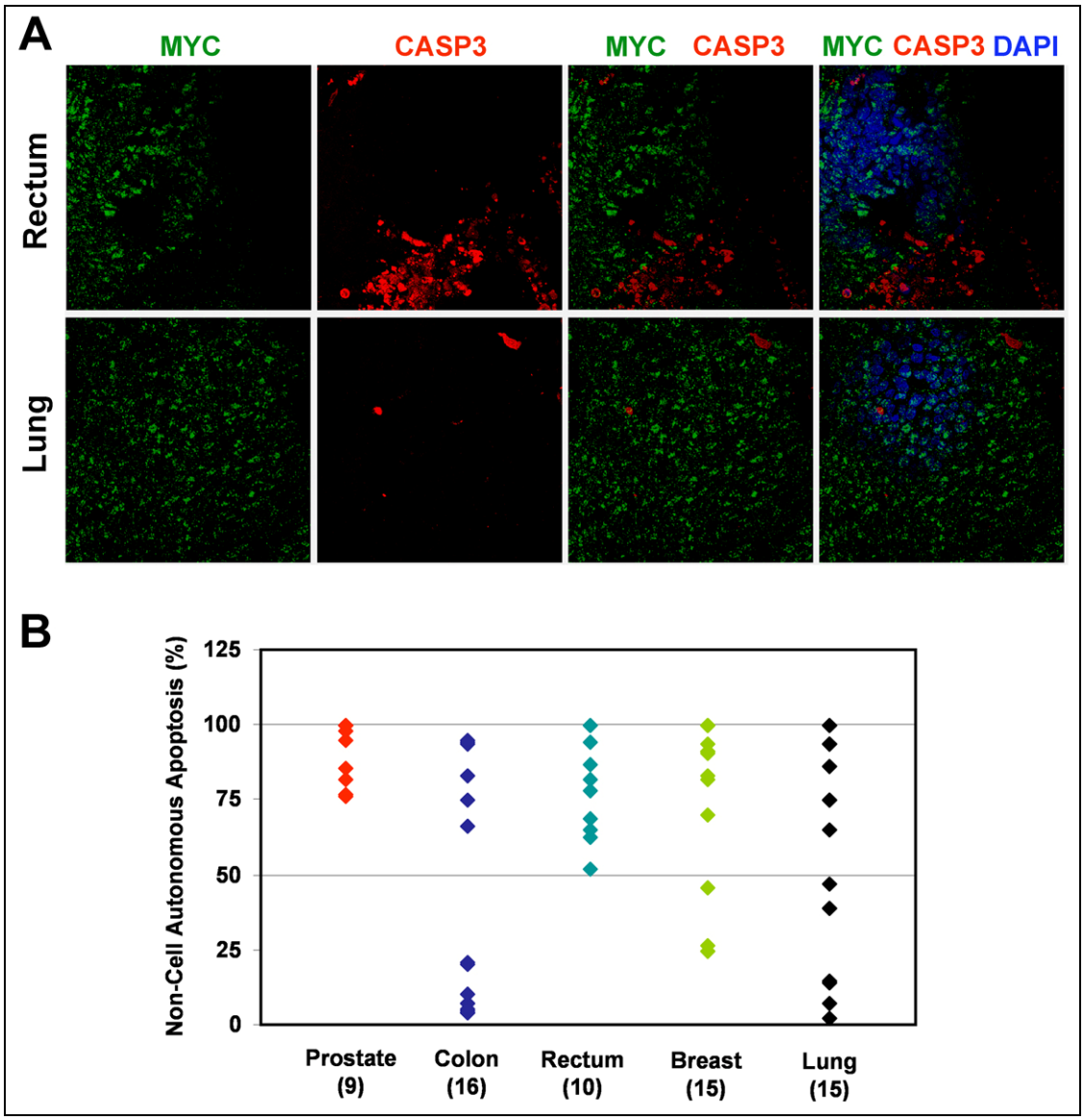

Figure 24. Comparison of NCAA and CAA in various human tissues. Human tissue microarray sample containing human prostate, colon, rectum, breast and lung cancer specimens was subjected to immunostain for c-MYC and activated CASPASE 3. Both NCA and CA apoptosis were counted and % NCAA of each sample was quantitated (B). Note that prostate and rectum tissues show the most frequent % NCAA, and colon and lung tissues show various frequency of NCAA (0-100%). Numbers in the brackets indicate the number of tissue samples analyzed. (A) represents examples of NCAA in rectum (most frequent NCAA) and lung (rare NCAA) tissues.

Discussion and Future Directions

Cell competition was first shown in *Drosophila* studies and our data suggest that active cell death of “losers” with low c-Myc caused by “winner” cells with high c-Myc may also happen in mammalian tissues. Although the data seem promising, critical questions still remain to be answered. c-Myc is a labile protein known to have a relatively rapid turn-over rate (half-life is about 20-40 mintues (Hann, 1984)), so it is important to show that the c-Myc protein is not degraded when cells are apoptotic in the prostate. To overcome this problem, other transcription factors, e.g. androgen receptor, alternatively can be labeled with an apoptotic marker to evaluate the integrity of nuclear proteins when cells undergo apoptosis. If not degraded dramatically, it will be more conclusive that non-cell-autonomous apoptosis occurs in low c-Myc cells, not in high c-Myc cells whose c-Myc proteins were degraded due to cell death. This problem can also be overcome with *in vitro* cell culture experiments. Senoo-Matsuda *et al.* showed that low *dMyc* cells become apoptotic by culturing two types of cells (high and low *dMyc* cells) separately in transwell plates when soluble factors released from each group of cells mix, excluding any possibility that cell-autonomous apoptosis was due to c-Myc degradation. Other data to consider are that the human tissues used here were all cancer samples, so it is possible that genetic alterations other than c-MYC may have already occurred. Therefore, additional genetic mutations could possibly have caused non-cell-autonomous apoptosis requiring more examination in the future to exclude this possibility.

A model has been proposed that is involved in *dMyc*-induced cell competition in *Drosophila* development. Myc is known to increase ribosomal activity by elevating transcription of ribosomal components (Arabi, 2005; Eisenman, 2001; Gomez-Roman, 2003; Grandori, 2005; Grewal, 2005; Hulf, 2005; Levens, 2003; Orian, 2003). When there is a loss of function mutation in *dMyc* (low Myc), the mutant cells lose ribosomal activity and have impaired ability to transduce the morphogen *Decapentaplegic* (Dpp) signals. Due to reduced Dpp signaling, the mutant cells are either eliminated through apoptotic program (activation of Jnk pathway or *Hid* gene) or release growth factors like Dpp or *Wingless* (Wg) to stimulate compensatory proliferation of neighboring high Myc cells (Gallant, 2005). Therefore, it would be interesting to search for the link between non-cell-autonomous apoptosis and activated Jnk pathway through Tgf-β signaling to demonstrate c-Myc-induced cell competition in mammals because Dpp is Tgf-β family member in *Drosophila*. In addition to Myc, the Warts-Hippo pathway was recently shown to cause cell competition in *Drosophila* (Tyler, 2007). This pathway is also known as a tumor suppressor pathway and deletion of its mouse homologue (Lats 1) induces cancers in mice (Pan, 2007). *Drosophila* cells with mutation in this pathway have been shown to become “super-competitors” by killing neighboring wild type cells (Tyler, 2007).

The complete pathway analyses have not been elucidated yet, but accumulating data and ideas lead to a correlation between cell competition and carcinogenesis (Baker, 2008; Moreno, 2008). Since many tumors are found genetically heterogeneous, interactions between mutant cells and wild type cells

may cause cell competition and the surviving “winner” cells outgrow the other cells to be tumorigenic. In this sense, our c-Myc transgenic mice with focal c-MYC expression should be an important model to correlate cell competition and prostate carcinogenesis.

Materials and Methods

Animals

Z-MYC and *PbCre4* mice have been described (Roh, 2006; Wu, 2001). Female *Z-MYC* mice (B6/129) were bred to male *PbCre4* mice (B6) obtained from MMHCC, Frederick, to generate *PbCre4;Z-MYC* offspring and littermate controls. Animal care and experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committees at Vanderbilt University.

LacZ stain

β-galactosidase staining followed by counterstaining with nuclear fast red was performed as described (Roh, 2006).

RNA isolation and quantitative RT-PCR

Total RNA was extracted from mouse tissues according to routine protocol with TriZol (Invitrogen). 1μg of RNA was used to synthesize cDNA from reverse transcription. Reagents used for reverse transcription were 5X First Strand Buffer, dithiothreitol (DTT), M-MLV reverse transcriptase (Invitrogen), RNase inhibitor, oligo dT, random hexamer and dNTPs mixture (Promega).

Real time quantitative PCR reaction was run in Taqman gene amplifying system (Applied Biosystems), using SYBR Green 2X master mix (Applied Biosystems) after reverse transcription reaction. Primers used were: ACCACCAGCAGCGACTCTGA for forward primer of human *c-MYC*,

TCCAGCAGAAGGTGATCCAGACT for reverse primer of human *c-MYC*, CAAAAGGCGAAGTGGAGCTT for forward primer of murine *Ki-67*, GGAGGGACATGTTTCGCAACT for reverse primer of murine *Ki-67*, CATGGCCTTCCGTGTTCCTA for forward primer of murine *Glyseraldehyde-3-phosphate dehydrogenase* (*Gapdh*), and GCGGCACGTCAGATCCA for reverse primer of murine *Gapdh*. The quantity of *c-MYC* and *Ki-67* in triplicate samples was normalized with that of *Gapdh*.

Histology and immunohistochemistry

Tissues were prepared for histopathological analyses as described (Abdulkadir, 2001). Human tissue microarrays were purchased from US Biomax, Inc. Immunohistochemical analyses were performed as described (Abdulkadir, 2001). The following antibodies were used, in some cases with Tyramide Signal Amplification (TSA; Perkin Elmer): anti-activated Caspase 3 (rabbit, 1:500, Cell Signaling), anti-phospho-histone H3 (rabbit, 1:500, Upstate), anti-c-MYC (rabbit, 1:15,000 with TSA, Santa Cruz) and anti-Ki-67 (rabbit, 1:50, abcam). For double immunofluorescenct stains, c-MYC detected by 1st primary antibody was amplified by TSA system (green, Fluorescein). Alexa Fluor 594 (red)-labeled 2nd secondary antibody (Molecular Probes) was used to detect 2nd primary antibodies (anti-phospho-histone H3 and anti-activated Caspase 3). Nuclear stain (DAPI) and sample mounting were performed using Vectashield mounting medium (Vector Laboratories).

TUNEL assay

In situ cell death detection kit was purchased from Roche Applied Science and TUNEL assay was performed to label apoptotic cells, referring to manufacturer's instruction manual.

Cell lines

RWPE-1, benign human prostate epithelial cell line (ATCC) was cultured in keratinocyte serum-free media supplemented with bovine pituitary extract and EGF (Invitrogen). We used lentiviral-mediated gene transfer to generate c-MYC overexpressing cells. 293FT packaging cells were plated on 10cm culture dishes and transfected with the FM-1/control or FM-1/c-MYC plasmid along with vesicular stomatitis virus glycoprotein (VSVG) envelope plasmid and delta 8.9 packaging plasmid to produce lentivirus. The FM-1 vector was obtained from J. Milbrandt (Araki, 2004) and was used to clone in human c-MYC cDNA. Three days after transfection, medium containing viral particles was collected and added to RWPE-1 for infection with polybrene (8μg/ml). 24 hours post infection, medium was changed. YFP+/c-MYC+ or YFP+/c-MYC- control cells were sorted by flow cytometry.

In vitro cell competition analysis

Coverslips were placed on the 24-well plates and 2-300,000 cells were plated on the coverslips and incubated under normal condition. For co-culture of pooled cell populations, media were changed to supplement-free media to induce

apoptosis. After 24, 48 or 72 hours, media were discarded and immunocytochemistry was performed for activated CASPASE 3. For conditioned media experiments, each pooled cell population was cultured in 10cm dishes under supplement-free media for 72 hours and the media were collected. Conditioned media were then added back to either sorted YFP+/c-MYC+ cells or YFP+/c-MYC- cells plated on the coverslips in the 24-well plates and incubated for 4, 10 or 24 hours. Immunocytochemistry was performed for activated CASPASE 3 after media were removed. Apoptosis was quantitated from triplicate data per group.

Statistical analyses

We compared groups by using *t*-test. Values were considered statistically significant at $P < 0.05$. Quantitative variables were expressed as means $\pm$ SD while categorical variables were expressed as numbers (%).

CHAPTER VI

MODELING PROSTATE CANCER USING TRANSGENIC MICE WITH PROSTATE CELLS WITH DISTINCT MUTATIONS IN C-MYC AND PTEN

[This part is adapted from {Kim, J., Eltoum, I.E., Roh, M., Wang, J., Abdulkadir, S.A., *PLoS Genetics* (2009)}]

Introduction

Prevailing models of multi-step carcinogenesis posit that oncogenic mutations arise in isolated cells *in situ* followed by clonal expansion. This implies that important competitive interactions occur between mutant and normal cells as well as between cells with distinct oncogenic mutations during tumorigenesis. A detailed understanding of these interactions will further efforts aimed at therapeutic targeting of neoplastic and preneoplastic lesions. However, these interactions have not been well studied *in vivo* due to a paucity of appropriate models. We report here our attempt to model these interactions in a new transgenic model of prostate cancer, focusing on the oncogene *c-MYC* and the tumor suppressor *Pten* (Phosphatase and tensin homologue), both of which are implicated in human prostate tumorigenesis (Tomlins, 2006). *c-MYC* overexpression is a common early event in prostate cancer (Ellwood-Yen, 2003; Gurel, 2008) while *PTEN* is deleted/mutated in ~30% of primary human prostate cancers (Dahia, 2000; Li, 1997; McMenamin, 1999; Sellers, 2002; Steck, 1997).

As discussed in CHAPTER II, previous attempts at modeling c-MYC overexpression in the mouse prostate have used prostate-specific promoters that target transgene expression to a majority of the cells in the prostatic epithelium. Depending on the strength of the promoter used, this resulted in various grades of mouse prostatic intraepithelial neoplasia (mPIN) or adenocarcinoma (Ellwood-Yen, 2003; Zhang, 2000b). Similarly, *Pten*-mutant mice develop mPIN and prostate cancer (Chen, 2005; Ma, 2005; Ratnacaram, 2008; Wang, 2003) and *Pten* inactivation can cooperate with mutations in oncogenes and tumor suppressors in prostate tumorigenesis, including $p27^{Kip1}$ (Di Cristofano, 2001; Kim, 2002), *Trp53* (Chen, 2005) and *Fgf8b* (Zhong, 2006).

To model the sporadic genetic alterations that are thought to occur during human somatic tumorigenesis (Fearon, 1990), we previously generated a transgenic mouse in which a latent *c-MYC* transgene can be focally activated in the prostatic epithelium by Cre expression (CHAPTER IV). We have also deleted one or both copies of *Pten* in the prostate concurrently with focal c-MYC overexpression, in order to examine the interactions of cell populations with distinct mutations within the same gland.

Results

PbCre4;Z-MYC mice with focal, prostate-specific and androgen-independent c-MYC overexpression

We previously generated *Z-MYC* transgenic mice (CHAPTER IV) and let them overexpress c-MYC in a focal, prostate-specific manner by crossing to *PbCre4* mice (CHAPTER V), in contrast to past models showing uniform c-Myc expression in the mouse prostate epithelium (Figure 25). To determine what type of cells overexpresses c-MYC in the prostate, the bigenic *PbCre4;Z-MYC* mouse prostate samples were subjected to immunostain for c-MYC in combination with either cytokeratin 8 (CK8, a prostate luminal epithelial cell marker) or p63 (a basal cell marker). Our results show that c-MYC is focally expressed in CK8+ prostate luminal epithelial cells but not p63+ basal cells (Figure 26C). Furthermore, c-MYC expression is not abrogated in castrated animals indicating that the use of the *CMV enhancer/β-actin* promoter in our model has uncoupled prostate-specific expression from androgen-dependent gene regulation (Figure 26B).

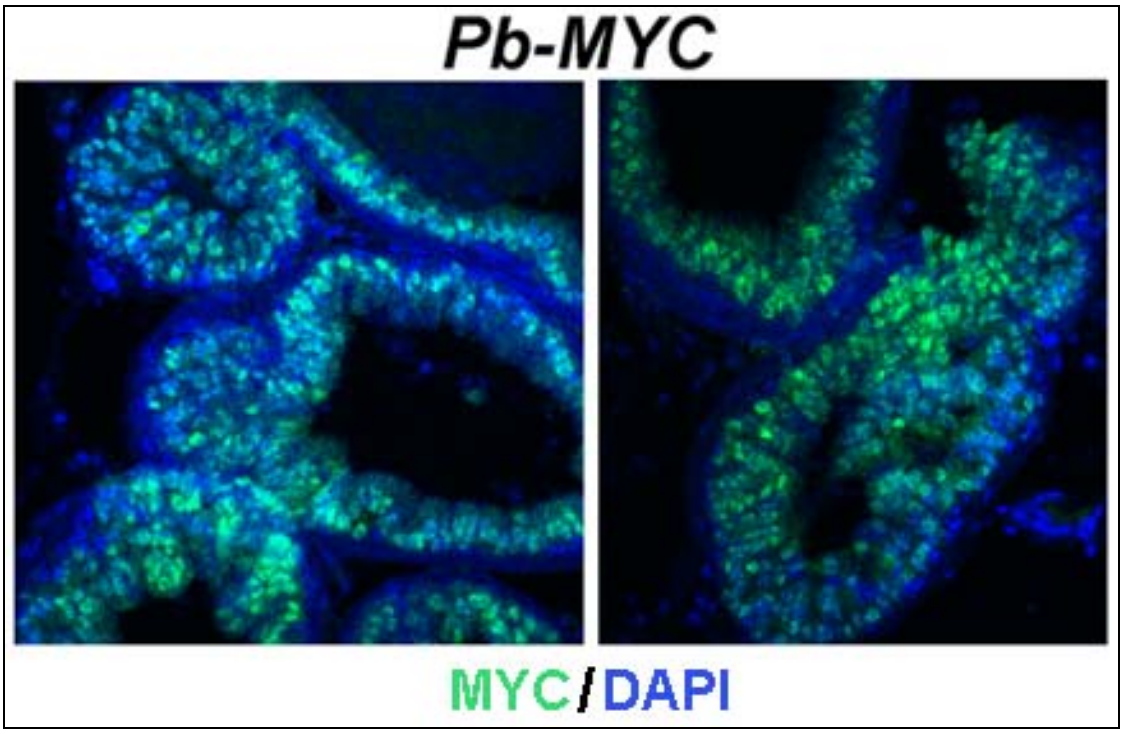

Figure 25. Uniform expression of c-MYC in the *Probasin-c-MYC* mouse prostates. *Probasin-c-MYC* prostatic intraepithelial neoplasia sample (Ellwood-Yen, 2003) was stained for c-MYC. Nuclei were stained blue. In contrast to our *PbCre4;Z-MYC* mouse prostates, this line shows uniform expression of *c-MYC* transgene.

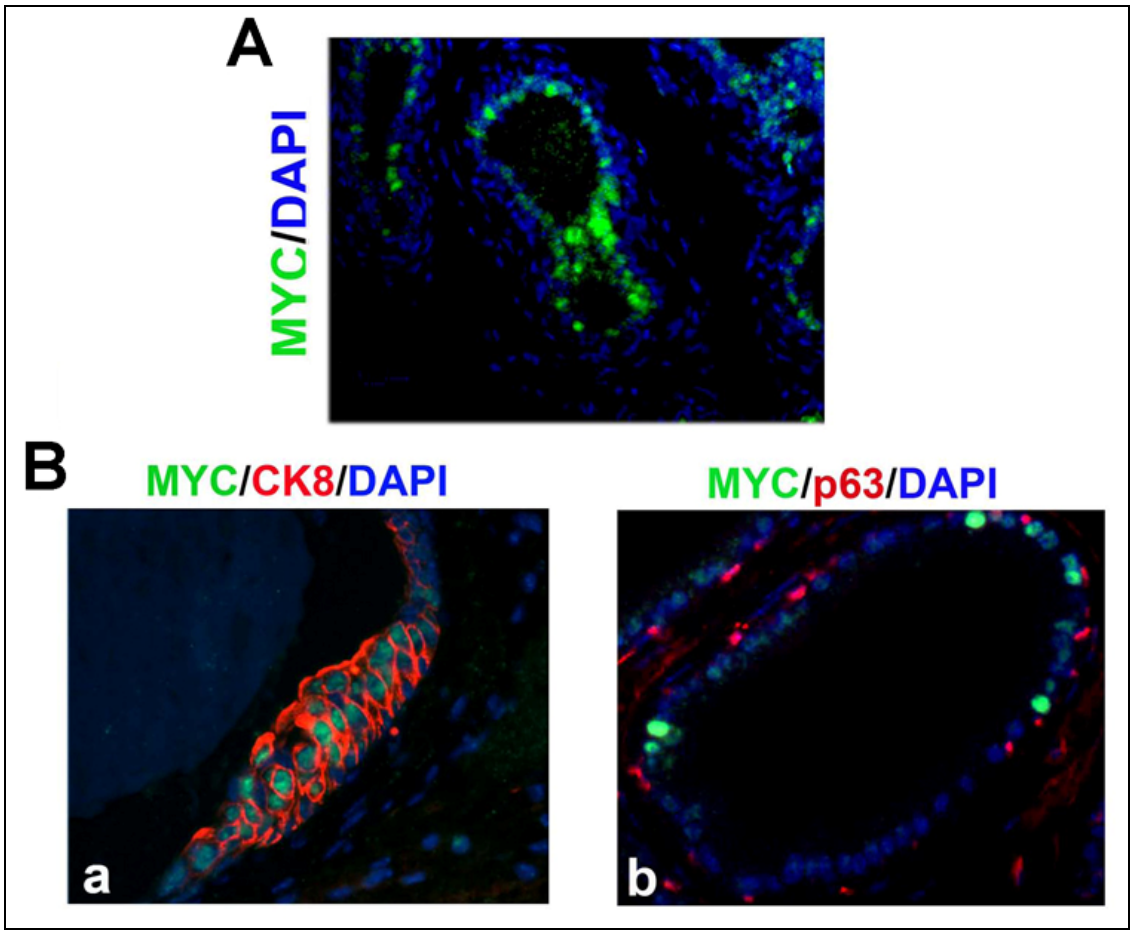

Figure 26. Focal and androgen-independent c-MYC expression in *PbCre4;Z-MYC* mouse prostate epithelial cells. (A) Immunostaining displays sporadic c-MYC expression in *PbCre4;Z-MYC* prostate epithelium 17 weeks after castration. (B) c-MYC colocalizes with cytokeratin 8 (CK8) (a) but not with p63 (b) in *PbCre4;Z-MYC* mouse prostates.

Mild pathology due to focal c-MYC overexpression

Focal c-MYC activation resulted in mild pathology, with most prostates showing normal histology or low grade mPIN (LGPIN) lesions up to 2 years of age (Figure 27A and 27B). This is unlikely due to low level c-MYC expression as the *CMV enhancer/β-actin promoter* is known to drive high level transgene expression. A closer examination of the c-MYC expression pattern in the prostates of *PbCre4;Z-MYC* mice with no pathology showed that in young mice, the frequency of c-MYC-positive cells was ~18% of the epithelial cells in c-MYC-positive glands (Figure 28G), evocative of the frequency of lacZ-positive cells (~17%) in *Z-MYC* prostates (Figure 26A). By 1 year, the frequency of c-MYC positive cells has increased to ~43% (Figure 28G). The lack of discernible histological abnormality in the prostates of a large fraction of older *PbCre4;Z-MYC* mice in the face of abundant c-MYC expression is reminiscent of the phenomenon of "field cancerization" in human tumorigenesis where incipient mutant cells occupy tissue fields without any apparent pathology (Figure 28A-F) (Slaughter, 1953). These histologically normal but mutant cells may serve as targets for transformation with additional genetic mutations.

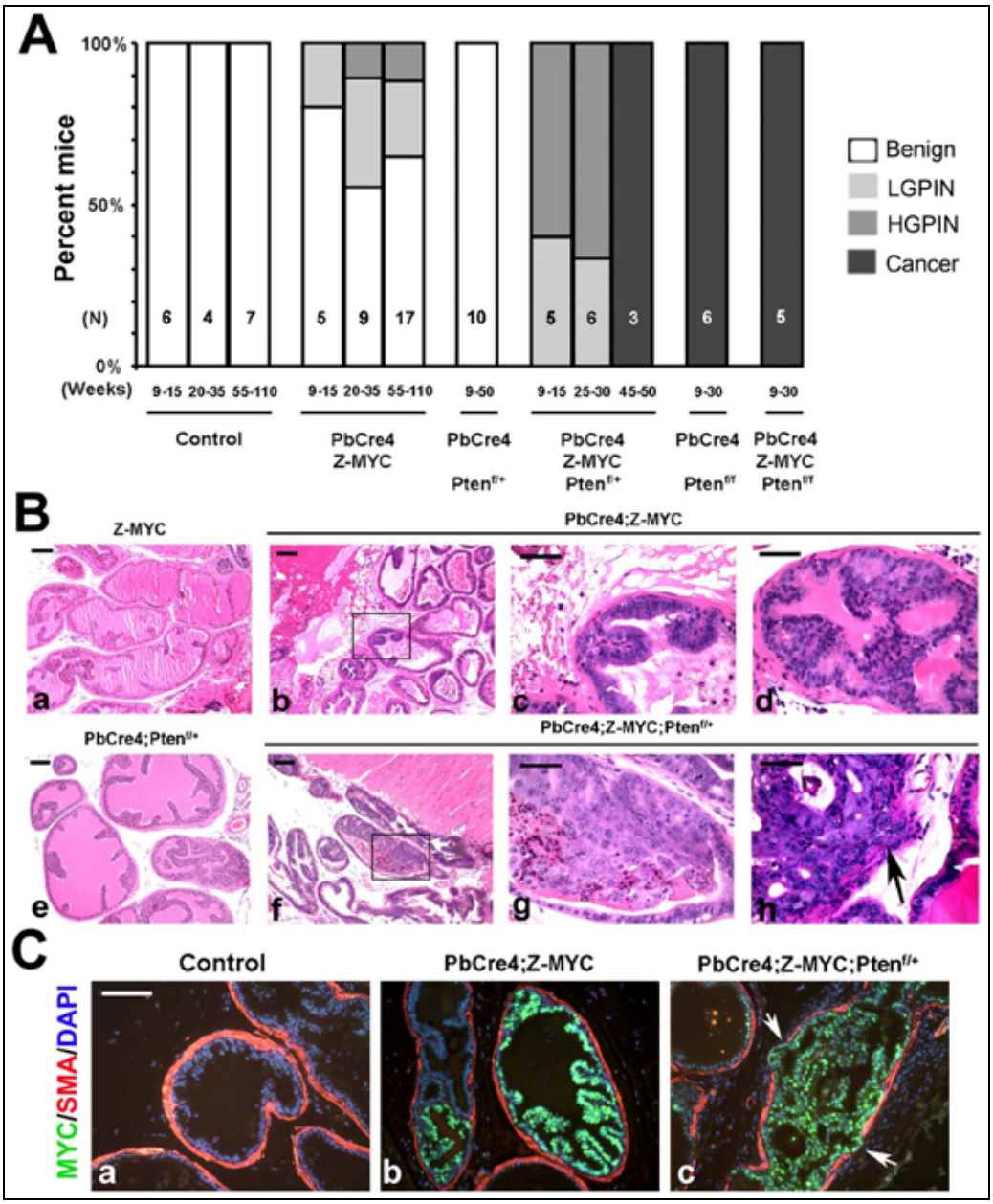

Figure 27. Pathology of *PbCre4;Z-MYC* and compound *c-MYC/Pten* mutant mice. (A) H&E-stained prostate sections were analyzed and the summary of the pathological grading is shown. (N, number of mice analyzed). (B) Representative images of H&E-stained sections show benign glands in control (a), focal LGPIN in *PbCre4;Z-MYC* (boxed region in b and higher magnification in c), focal HGPIN lesion in *PbCre4;Z-MYC* (d), benign glands in *PbCre4;Pten*$^{f/+}$ (e), focal HGPIN lesions in *PbCre4;Z-MYC;Pten*$^{f/+}$ (boxed region in f and higher magnification in g) and focal micro-invasive cancer lesion (arrow) in *PbCre4;Z-MYC;Pten*$^{f/+}$ (h). Scale bars: 100μm in a, b, e and f, and 50μm in c, d, g and h. (C) c-MYC and smooth muscle actin (SMA) staining. Arrows in "c" indicate focal areas of disruption of SMA (micro-invasion) in *PbCre4;Z-MYC;Pten*$^{f/+}$ prostate. Scale bar: 100μm.

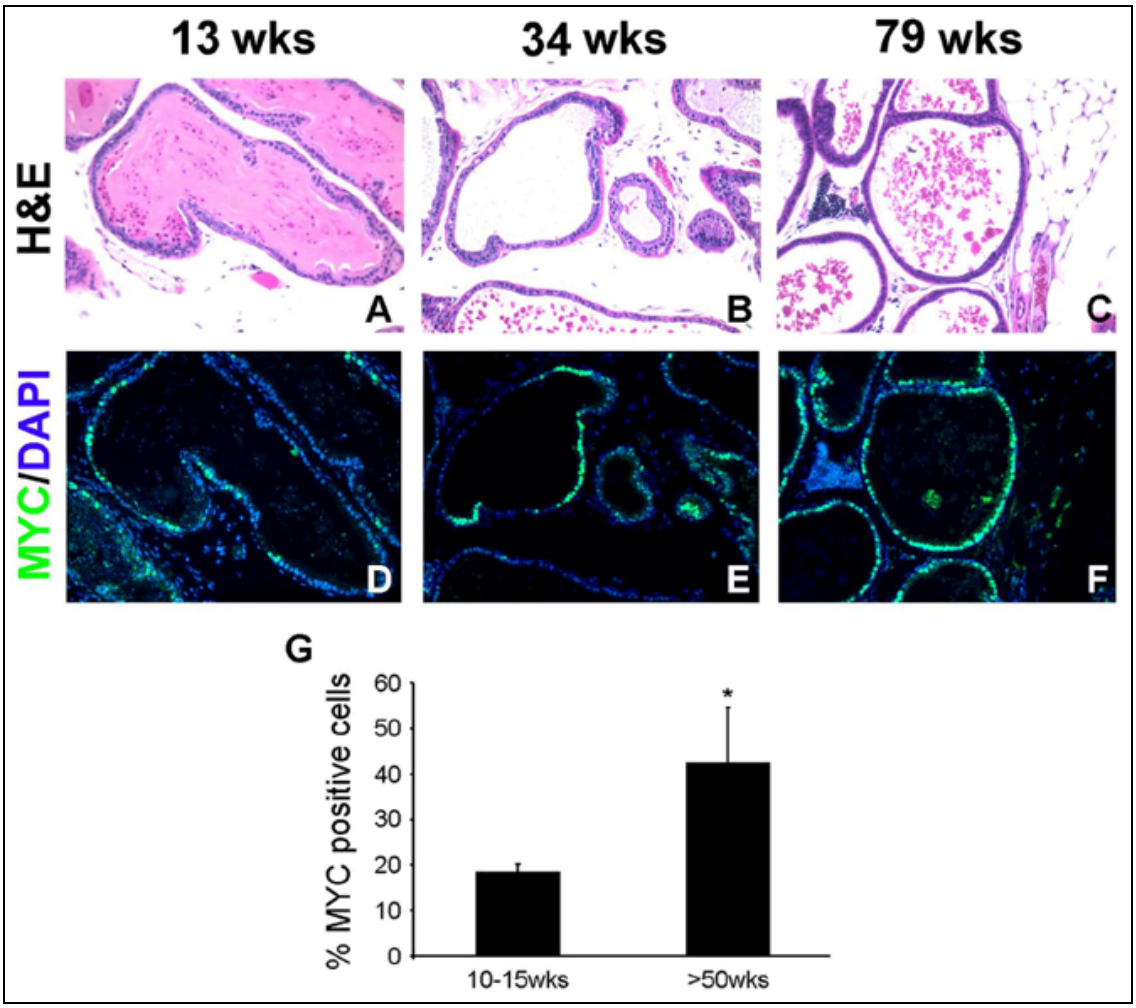

Figure 28. Time-dependent increase of c-MYC-expressing cells without discernible histopathology in a subset of *PbCre4;Z-MYC* mice. (A-C) H&E images demonstrate absence of histopathological abnormalities in *PbCre4;Z-MYC* prostates at various ages. (D-F) Adjacent sections subject to immunofluorescent staining for c-MYC demonstrate the numbers of c-MYC-overexpressing cells increase in a time-dependent manner. (G) The number of c-MYC-positive cells in any c-MYC-positive gland was quantitated based on the immunostaining shown in D-F. N = 3-4 prostate samples per group. *P<0.05.

Focal c-MYC expression cooperates with *Pten* heterozygosity

Next, we generated compound mutant mice with prostate-specific deletion of one or both alleles of *Pten*, concurrently with focal activation of c-MYC. Examination of *PbCre4;Z-MYC;Pten*$^{f/+}$ prostates revealed clear cooperation between c-MYC overexpression and *Pten* heterozygosity (Figure 27). As reported previously (Ma, 2005; Wang, 2003) and confirmed by us here, conditional deletion of a single *Pten* allele had minimal effects on the prostate, with mice up to 50 weeks of age showing no abnormalities (Figure 27A and 27B). By 10 weeks of age, however, *PbCre4;Z-MYC;Pten*$^{f/+}$ mice already have evidence of focal HGPIN lesions. Over time, these animals develop micro-invasive cancer as confirmed by the presence of areas with disruption in smooth muscle actin (SMA) immunoreactivity (Figure 27B and 27C). We used immunohistochemistry to examine the status of the wild type *Pten* allele in the HGPIN/cancer lesions in *PbCre4;Z-MYC;Pten*$^{f/+}$ mice. Consistently, all lesions examined (N=8 mice) showed loss of Pten protein expression and phosphorylation of its downstream signaling components Akt and Foxo1 (Huang, 2007) (Figure 29).

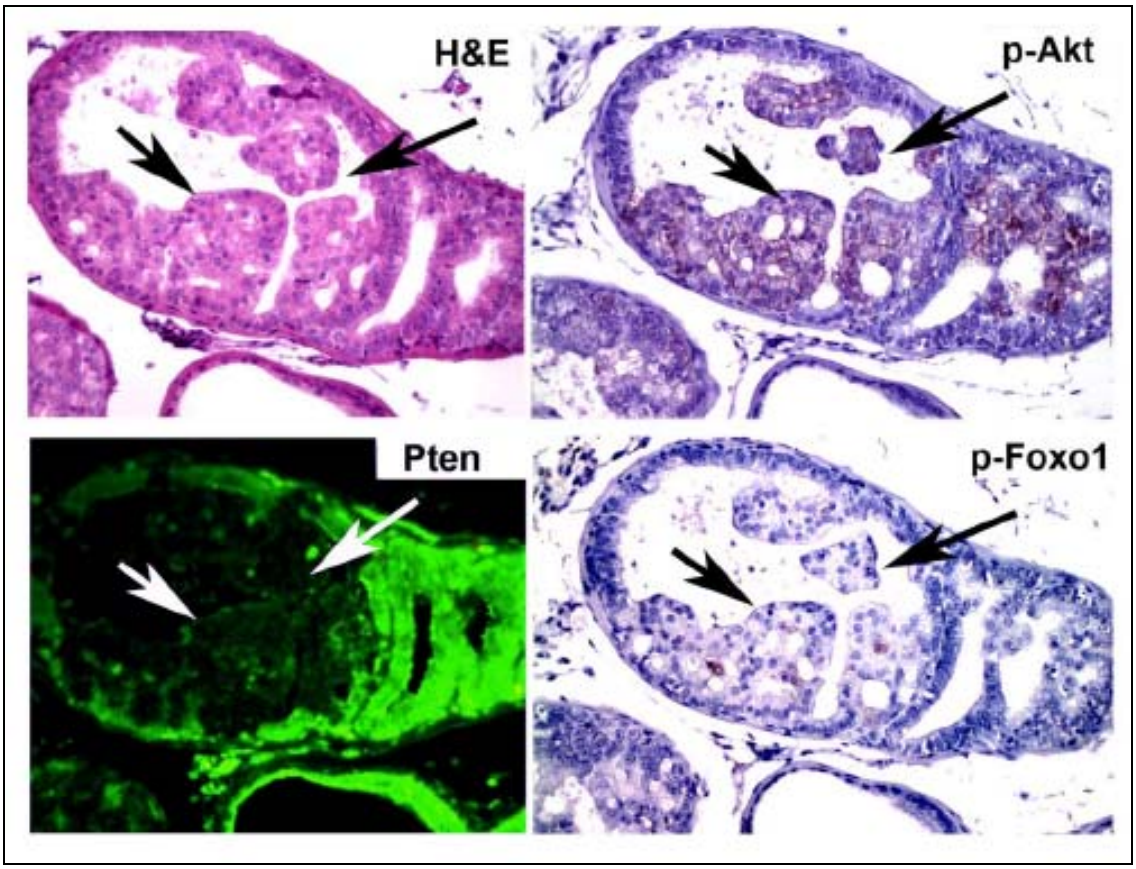

Figure 29. Focal loss of Pten expression in PIN lesions of *PbCre4;Z-MYC;Pten*$^{f/+}$ mouse prostate. Immunohistochemistry for Pten, phospho-Akt, and phospho-Foxo1 was performed in the adjacent serial sections of *PbCre4;Z-MYC;Pten*$^{f/+}$ mouse prostate. In focal PIN lesion (arrows), Pten protein expression is lost and phosphorylation of Akt and Foxo1 increased, both of which are known to be phosphorylated when Pten is lost.

c-MYC+;Pten-null cells outcompete Pten-*null* cells in the same glands

We analyzed proliferation by staining for phospho-histone H3, a mitotic marker. Proliferation was increased significantly in *PbCre4;Z-MYC* prostates relative to controls, and *Pten* heterozygosity synergistically increased it further (Figure 30A). The proliferation rates in *PbCre4;Pten*$^{f/f}$ and *PbCre4;Z-MYC;Pten*$^{f/f}$ were similarly elevated. However, the focal nature of c-MYC expression in our model means that analysis of total proliferation in the *PbCre4;Z-MYC;Pten*$^{f/f}$ prostates may not be an accurate measure of the proliferation in foci of c-*MYC+;Pten*-null cells. To overcome this, we performed double staining for c-MYC and phospho-histone H3. As shown in Figure 30B, double mutant (*c-MYC+;Pten*-null) cells were more proliferative than single mutant (*Pten*-null) cells within the same prostate glands. Furthermore, double mutant cells were histologically distinct from adjacent single mutant cells. The double mutant cells were of higher pathological grade with larger nuclei, high nuclear:cytoplasmic ratios, hyperchromatic nuclei with prominent chromocenters, focal chromatin clearing and prominent single or sometimes multiple nucleoli (Figure 30C and Figure 31). In addition, apoptotic and mitotic figures were prominent. Single mutant (*Pten*-null) cells on the other hand showed low nuclear grade with comparatively small and uniform nuclei, abundant pale cytoplasm and low nuclear:cytoplasmic ratios. These cells also have inconspicuous nucleoli and the chromatin is comparatively fine (Figure 30C). These observations suggest that *c-MYC+;Pten*-null cells may out-compete *Pten*-null cells within the same prostate gland over time. Indeed, analysis of *PbCre4;Z-MYC;Pten*$^{f/f}$ animals showed that

at early ages, c-MYC expression was focal within glands, but in older mice, lesions showed uniform c-MYC expression, suggesting clonal expansion of c-MYC-positive cells in a time-dependent manner (Figure 30D).

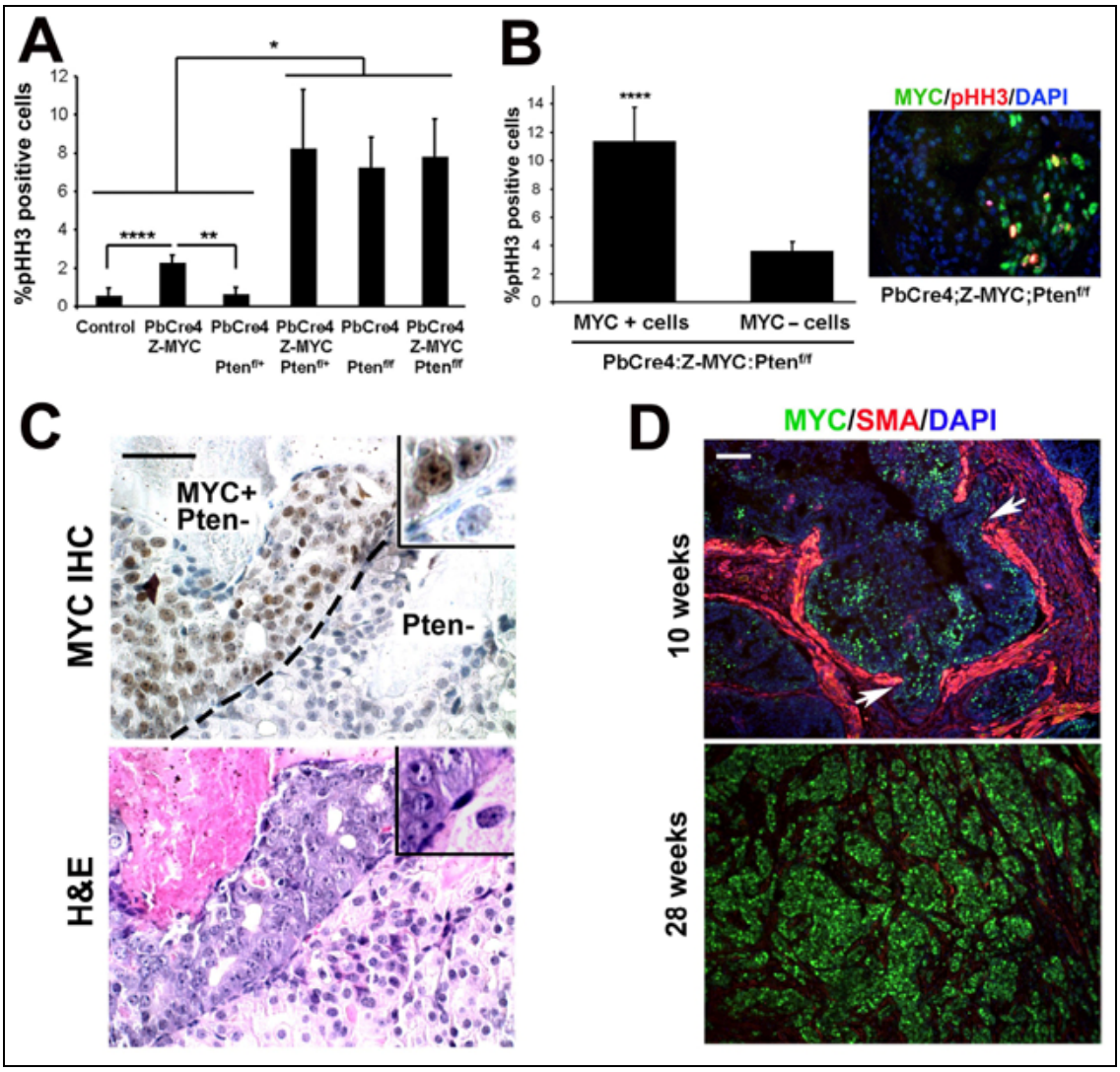

Figure 30. c-MYC expression increases the proliferation and tumorigenicity of *Pten*-deficient cells. (A) Proliferation was determined by analysis of phospho-histone H3 staining. (B) Phospho-histone H3 index in c-MYC-positive or c-MYC-negative cells in *PbCre4;Z-MYC;Pten*$^{f/f}$ prostates. Inset: double staining shows colocalization of phospho-histone H3 with c-MYC in *PbCre4;Z-MYC;Pten*$^{f/f}$ prostate. N=3-4 mice per group. *p<0.05, **p<0.005, ****p<0.01. (C) c-MYC staining identifies MYC-expressing cells next to MYC-negative cells in the same gland of *PbCre4;Z-MYC;Pten*$^{f/f}$ mouse prostate. An adjacent H&E-stained section is also shown. Note distinct, higher grade pathology of c-MYC+ cells. Scale bar: 50μm. (D) *c-MYC+;Pten*-null cells outcompete *Pten*-null cells. Prostates from a 10 week and 28 week old *PbCre4;Z-MYC;Pten*$^{f/f}$ mice were stained for c-MYC and smooth muscle actin. At 10 weeks c-MYC expression is focal; at 28 weeks it is uniform. Arrows indicate discontinuity of smooth muscle actin (focal micro-invasion). Scale bar: 100μm.

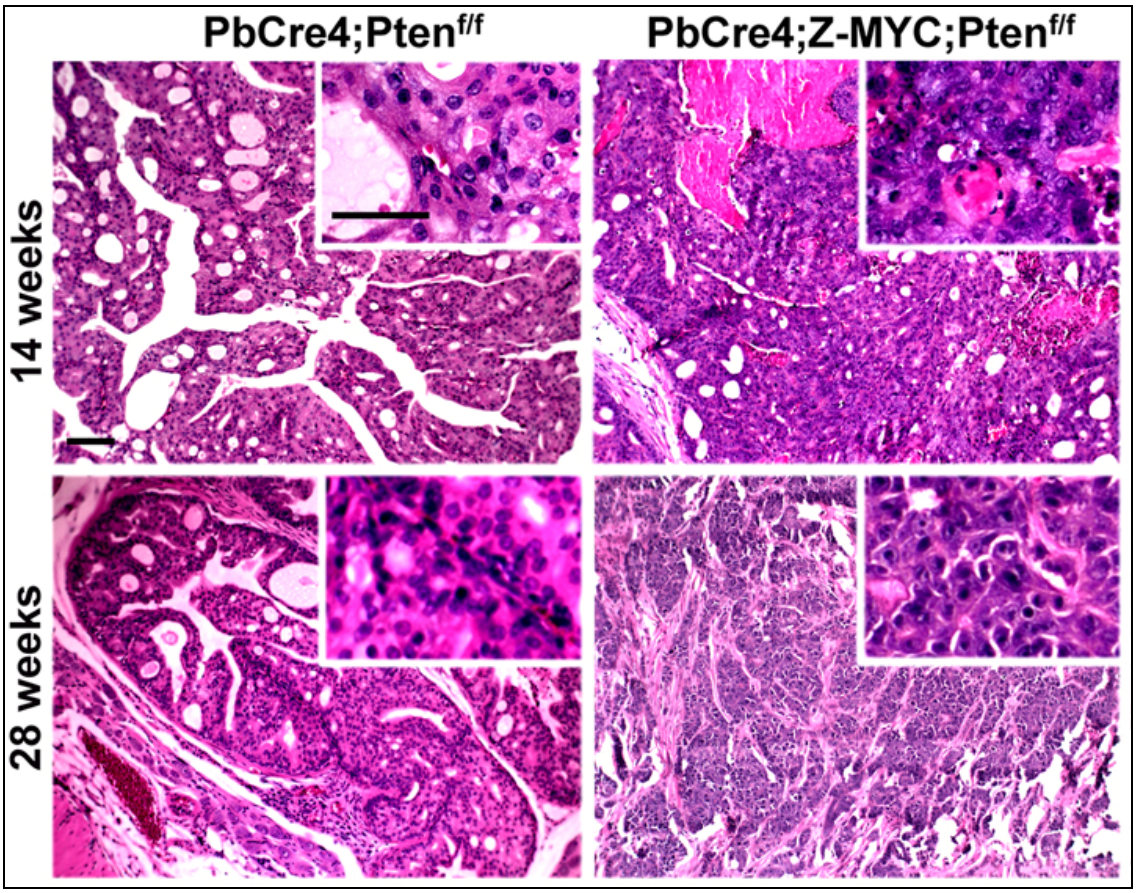

Figure 31. Histopathology of *PbCre4;Pten$^{f/f}$* and *PbCre4;Z-MYC;Pten$^{f/f}$* tumors at different ages. *PbCre4;Z-MYC;Pten$^{f/f}$* mice show higher grade lesions. Scale bars: 100μm (50μm in insets: higher magnifications).

Pten loss does not protect c-MYC overexpressing prostate cells from apoptosis

Analysis of apoptosis by staining for activated Caspase 3 showed that control and *PbCre4;Pten*$^{f/+}$ prostates had low levels of apoptosis, consistent with their normal histology, while focal expression of c-MYC in *PbCre4;Z-MYC* prostates modestly increased apoptosis (Figure 32A). Although c-MYC overexpression is known to induce apoptosis in several tissues, this depends on many variables including the level of c-MYC overexpression and the "tissue context" (Murphy, 2008; Pelengaris, 2002a). The levels of apoptosis seen in *PbCre4;Z-MYC* prostates were consistent with increased cell turnover due to enhanced proliferation. *Pten*-null prostates also had increased rates of apoptosis, and c-MYC overexpression further enhanced this effect (Figure 32A). These results were surprising as Pten loss is known to activate pro-survival pathways. Therefore, we sought to determine if apoptosis was increased in HGPIN/cancer cells that had lost Pten expression in our *PbCre4;Z-MYC;Pten*$^{f/+}$ mice. Double staining for Pten and activated Caspase-3 and quantitative analysis indicated higher rates of apoptosis in Pten-negative cells compared to Pten-positive cells (Figure 32B). Thus, Pten loss does not protect prostate cells from apoptosis due to c-MYC overexpression.

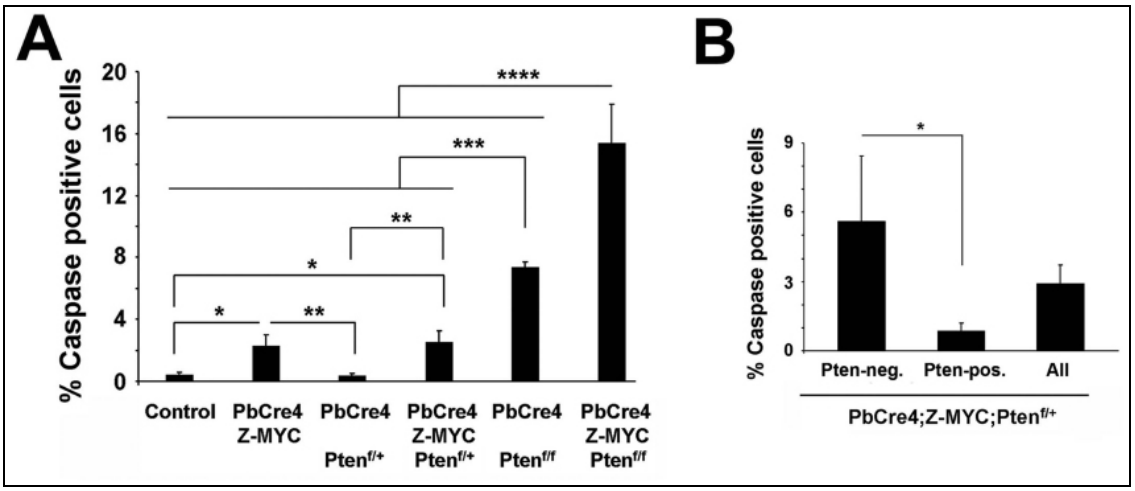

Figure 32. Analysis of apoptosis in *c-MYC/Pten* compound mutant mice. (A) Apoptosis was analyzed by staining for activated Caspase 3. (B) Sections from *PbCre4;Z-MYC;Pten*$^{f/+}$ prostates were doubly stained for activated Caspase 3 and Pten, and the number of apoptotic cells was quantitated in Pten-positive and Pten-negative epithelial cells. N=3-4 mice per group. **P*<0.05, ***P*<0.005, ****P*<0.001 and *****P*<0.01.

In addition to Akt, the c-Jun N-terminal kinase (Jnk) pathway is known to be activated in *Pten*-deficient cells and tumors (Vivanco, 2007; Yang, 2006). We confirmed that the Jnk pathway is activated in both *Pten*-null and c-MYC-overexpressing/*Pten*-null prostates by immunohistochemistry for phospho-Jnk (Figure 33A). Since Jnk is well known to have the ability to activate apoptosis, cell survival and proliferation, depending on cellular signal stimuli and cellular contexts (Bode, 2007), we asked if increased Jnk activity sensitizes *Pten*-deficient cells to apoptosis. We used small hairpin RNA to stably downregulate PTEN in the benign human prostatic cell line RWPE-1. However, treatment with the Jnk inhibitor (SP600125) led to an increase in apoptosis in PTEN knockdown cells in a dose-dependent manner, suggesting that PTEN loss-induced Jnk activity is anti-apoptotic, rather than pro-apoptotic (Figure 33).

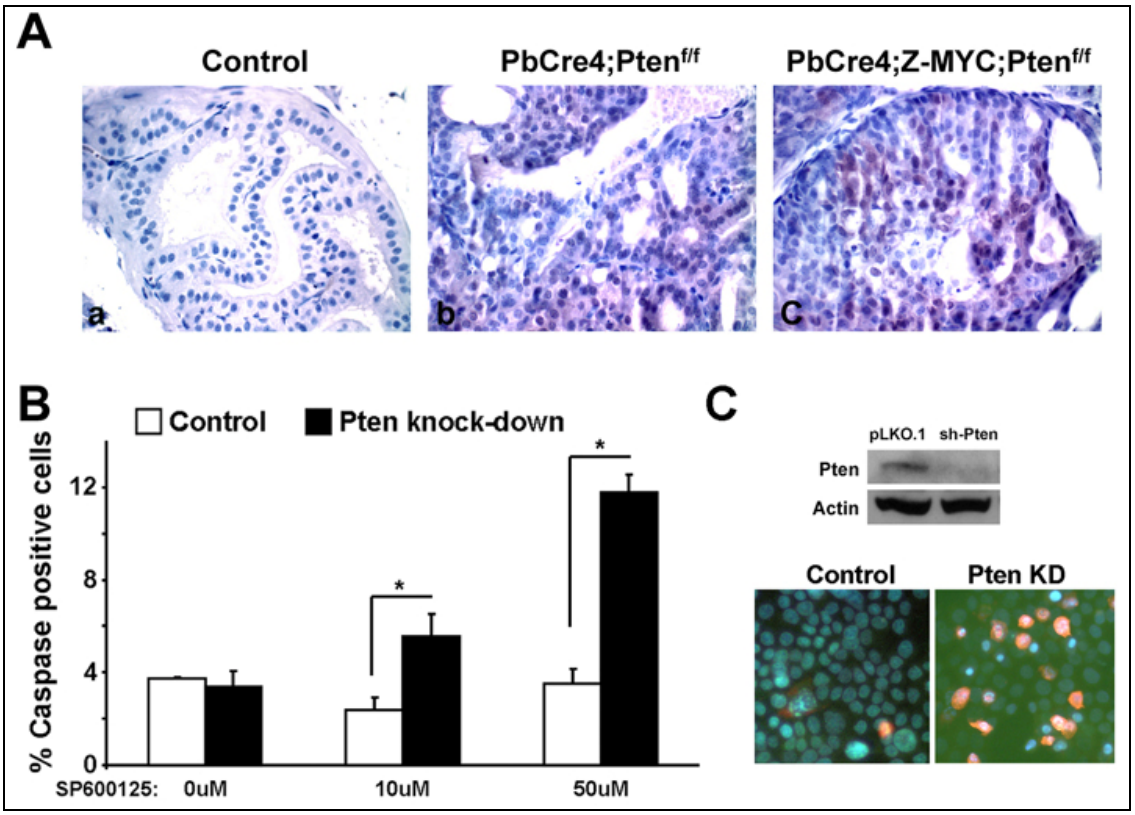

Figure 33. Jnk is activated in Pten-deficient cells and is anti-apoptotic. (A) Immunohistochemistry for phospho-Jnk demonstrates apparent Jnk activation in *PbCre4;Pten*$^{f/f}$ and *PbCre4;Z-MYC;Pten*$^{f/f}$ prostates (brown). (B) Apoptosis increases in Pten-knockdown RWPE-1 cells when treated with Jnk inhibitor (SP600125). (C) Western blots show that Pten knockdown is efficient in PTEN-shRNA-infected RWPE-1 cells. Immunofluorescence images represent increased apoptosis in PTEN-knockdown cells (red, activated Caspase 3). Nuclei were stained blue. *P<0.01.

c-MYC shifts the p53 response in *Pten*-deficient prostate cells from senescence to apoptosis

Pten loss has been reported to activate the p53 pathway, leading to senescence (Chen, 2005; Kim, 2007; Nogueira, 2008). Activation of p53 may lead to cell cycle arrest or apoptosis depending on the downstream target genes induced (i.e. cell cycle arrest genes such as $p21^{cip1}$ versus apoptotic genes such as *PUMA*). We therefore asked if activation of the p53 pathway occurs in our *Pten*-null model and whether this sensitizes the cells to apoptosis based on the target genes induced. We observed induction of p53, its targets $p21^{cip1}$ and PUMA in *Pten*-null prostates (Figure 34A). However, while p53 and PUMA were induced in c-MYC-overexpressing *Pten*-null prostates, $p21^{cip1}$ expression was not (Figure 34A), consistent with the notion that c-MYC represses $p21^{cip1}$ expression (Seoane, 2002). Similar results were obtained in RWPE-1 cells (Figure 34B). While p53 and $p21^{cip1}$ were induced with PTEN knockdown, c-MYC overexpression repressed $p21^{cip1}$ expression (Figure 34B). We hypothesized that in *Pten*-deficient cells with activation of the p53 pathway, repression of $p21^{cip1}$ by c-MYC may switch the senescent response to apoptosis. Indeed, using immunofluorescence, we found that in *PbCre4;Z-MYC;Pten*$^{f/f}$ prostates, $p16^{ink4a}$ expression (a marker of senescence) was mainly localized to c-MYC-negative cells, while apoptosis (activated Caspase 3) was found predominantly among c-MYC-positive cells (Figure 34C). Thus, *Pten*-deficiency activates the p53/$p21^{cip1}$ pathway, but concurrent c-MYC overexpression shifts the output of the pathway from senescence to apoptosis, at least partly by repressing $p21^{cip1}$.

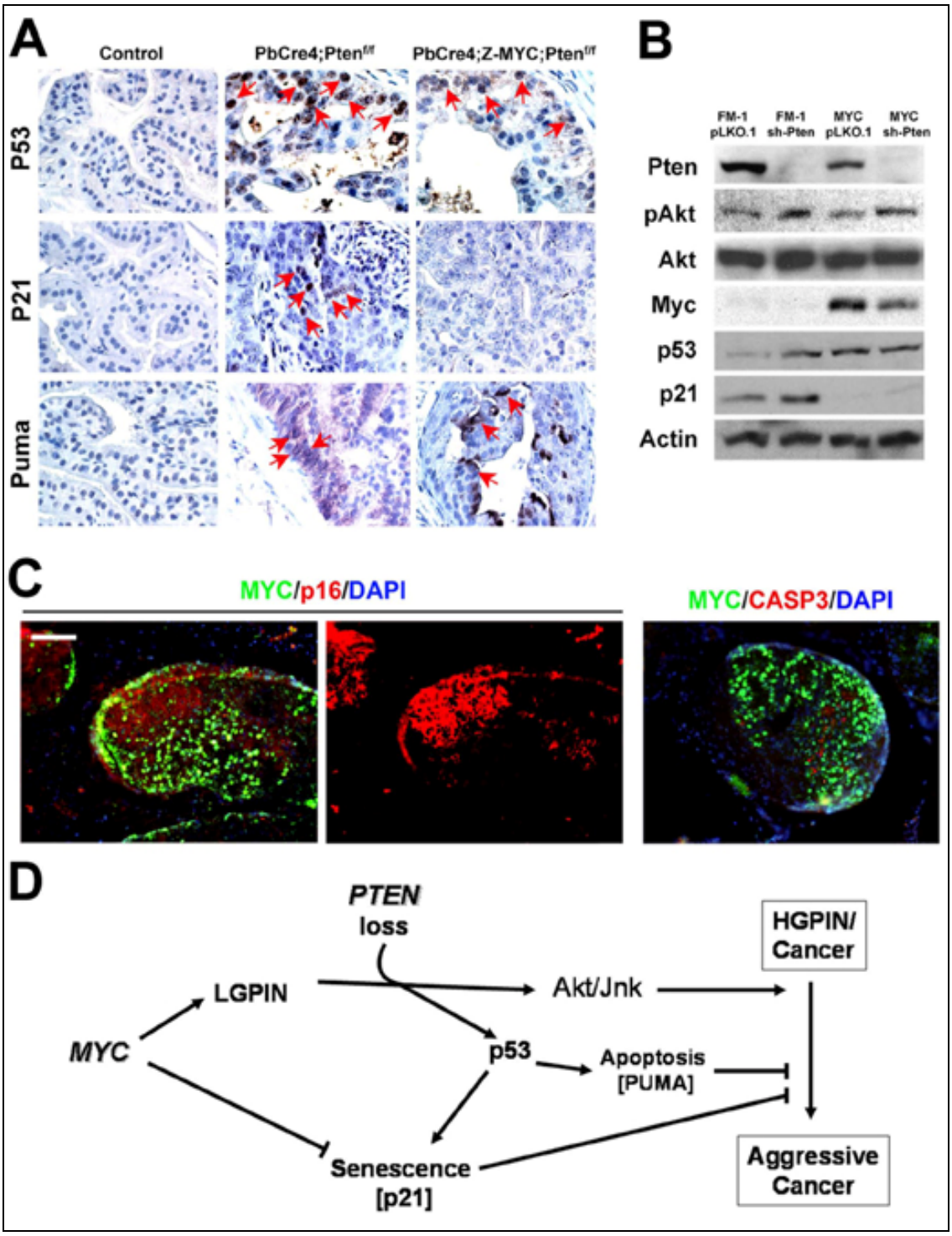
A
Control
PbCre4;Pten f/f
PbCre4;Z-MYC;Pten f/f
P53
P21
Puma
B
FM-1 pLKO.1
FM-1 sh-Pten
MYC pLKO.1
MYC sh-Pten
Pten
pAkt
Akt
Myc
p53
p21
Actin
C
MYC/p16/DAPI
MYC/CASP3/DAPI
D
PTEN loss
HGPIN/ Cancer
LGPIN
Akt/Jnk
MYC
p53
Apoptosis [PUMA]
Senescence [p21]
Aggressive Cancer

Figure 34. c-MYC and p53 pathway status in *c-MYC/Pten* mutant prostate cells. (A) Immunohistochemistry for p53, p21 and Puma. Prostate sections from mice of the indicated genotypes were stained with the indicated antibodies and nuclei were counterstained with hematoxylin. Positive cells (brown) are indicated by red arrows. (B) Western blots show expression of the indicated proteins in RWPE-1 cells expressing c-MYC, PTEN knockdown, or control vectors (FM-1, pLKO.1). PTEN and phospho-Akt (pAkt) blots confirm efficiency of PTEN knockdown. (C) Double immunofluorescent stains for c-MYC/p16 and c-MYC/activated Caspase 3 in *PbCre4;Z-MYC;Pten*$^{f/f}$ prostates. Scale bar: 100μm. (D) Model of interactions between c-MYC and Pten with the p53 pathway in c-MYC-initiated prostate cancer. Focal c-MYC overexpression leads to LGPIN and facilitates loss of Pten leading to HGPIN/invasive cancer. Activation of the p53 pathway due to Pten loss could lead to senescence or apoptosis. c-MYC expression is proposed to shift this response towards apoptosis by repressing p21 expression.

Concurrent c-MYC overexpression and loss of PTEN expression are correlated with higher Gleason grades in human prostate cancer

We used immunostaining to look for cooperativity between c-MYC and PTEN in human prostate carcinogenesis. As depicted in Figure 35, 22.4% of the 58 human prostate cancer samples examined exhibited concurrent c-MYC overexpression and reduced PTEN expression, and these samples had significantly higher Gleason grades. We also noted some focal PIN lesions that display concurrent c-MYC overexpression and loss of PTEN expression (panel c, c' and c" in Figure 35A), reminiscent of the HGPIN lesions in our *PbCre4;Z-MYC;Pten*$^{f/+}$ mice.

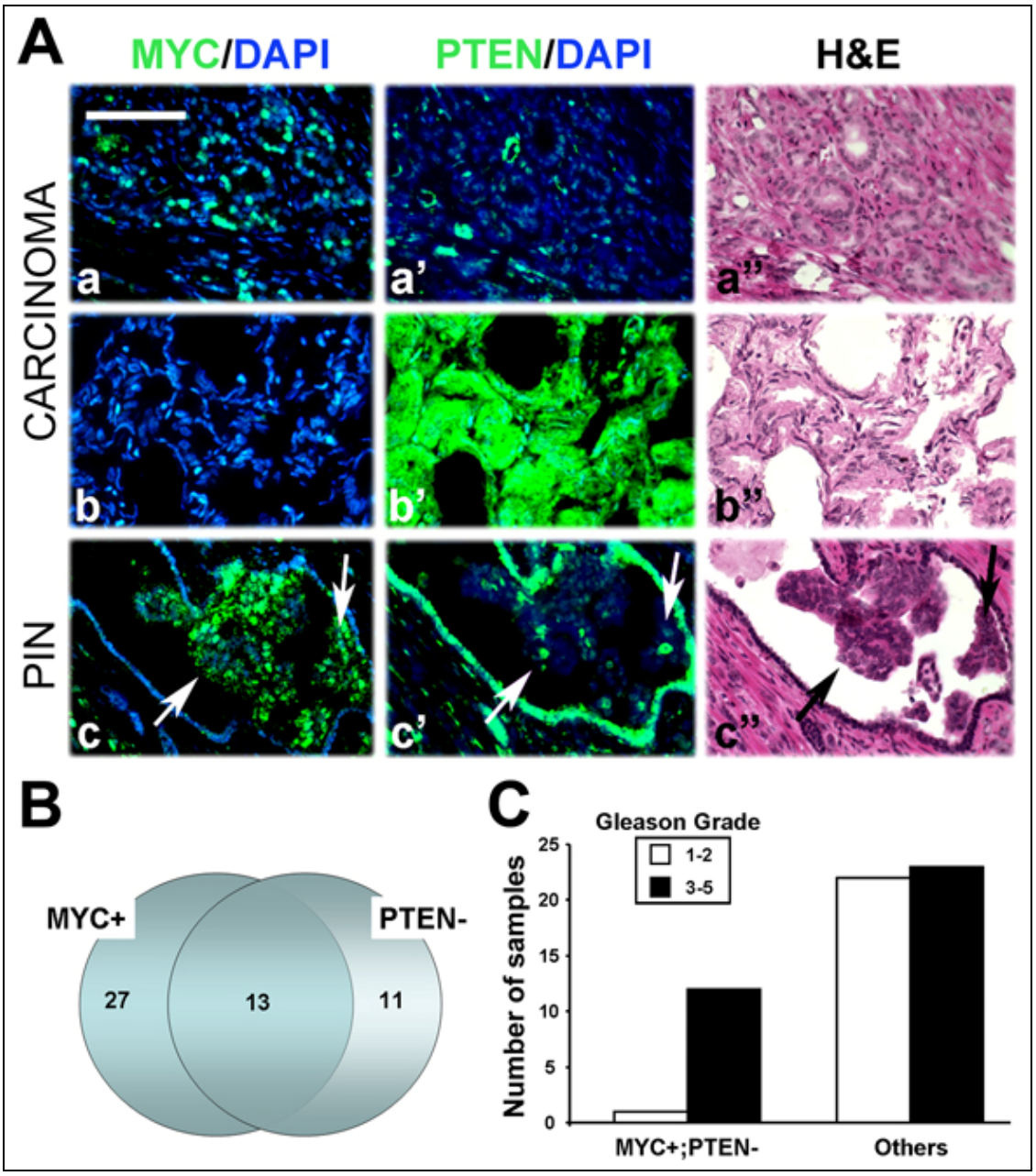

Figure 35. Immunohistochemical analysis of c-MYC and PTEN in human prostate cancer specimens. (A) Human prostate cancer with (a, a' and a") or without (b, b' and b") c-MYC overexpression and PTEN loss. Isolated PTEN (green) expression seen in a' is due to stromal PTEN expression. Panels c, c' and c'' show focal PIN lesions with concurrent c-MYC overexpression and PTEN loss (arrows). Scale bars: 100μm (B) Venn diagram showing incidence of c-MYC expression and PTEN loss in 58 human tumors (C) Correlation between c-MYC/PTEN status and Gleason grade. c-MYC+;PTEN- status is significantly associated with higher Gleason grades (P<0.01).

Discussion

Human prostate carcinogenesis is focal, random, and incremental, but current mouse models do not faithfully recapitulate this. Consequently, the competitive/cooperative interactions that may occur between mutant and normal cells during the early stages of tumorigenesis have not been well studied. The model described here exploits the stochastic expression of a “Cre-activatable” c-MYC transgene (*Z-MYC*) to induce c-MYC expression in isolated cells surrounded by normal cells. As illustrated by our studies when the *Z-MYC* mouse is crossed with prostate-specific *Pten* deletion, the focal nature of c-MYC expression allows analysis of cell populations with different genetic alterations within the same prostate gland.

Our studies have yielded several insights. First, focal expression of c-MYC in prostate luminal epithelial cells, even though driven by the *CMV enhancer/β-actin* promoter, results in remarkably mild pathology with many mice showing histologically normal prostates and a subset of mice demonstrating LGPIN lesions. These results imply a remarkable tolerance of luminal epithelial cells to c-MYC expression. We showed that the acquisition of additional genetic mutations is essential for the appearance of discernable pathology by the fact that introduction of *Pten* heterozygosity into these animals resulted in cooperativity, with the development of HGPIN/cancer lesions which in all cases were associated with loss of Pten protein expression from the wild type allele. These observations highlight an important point about c-MYC-expressing cells in

histologically “normal” glands, as may occur in tumors and tissues with “field cancerization” (Almadori, 2004; Braakhuis, 2003; Chandran, 2005; Grizzi, 2006; Slaughter, 1953) in that the overexpression of c-MYC in histologically “normal” cells may facilitate the acquisition of secondary mutations. Although it remains to be established whether loss of Pten expression is due to genetic, epigenetic or post-transcriptional control, c-MYC expression may facilitate acquisition of secondary mutations by increasing cell turnover and/or genomic instability (Felsher, 1999; Neiman, 2008).

Our *PbCre4;Z-MYC;Pten*$^{f/f}$ mice allowed us to examine the behavior of prostate cells with distinct mutations in the same prostate. c-MYC expression clearly confers an additional proliferative advantage to *Pten*-null prostate cells, allowing *c-MYC+;Pten*-null cells to outcompete *Pten*-null cells. However, *Pten* deficiency did not alleviate apoptosis in *c-MYC+;Pten*-null cells. This may appear surprising in light of the well-known, pro-survival effect of Pten loss (Suzuki, 2003) and a report that Pten loss decreased the apoptosis engendered by the inactivation of retinoblastoma (pRb) family proteins by a truncated SV40 T large antigen in the mouse prostate (Hill, 2005). Nevertheless, previous studies of mice with conditional deletion of *Pten* in the prostate and testicular germline cells have noted an increased rate of apoptosis upon *Pten* deletion (Kimura, 2003; Ma, 2005; Wang, 2003) and Radziszewska *et al* recently showed that deleting *Pten* concurrently with c-MYC activation in pancreatic beta cells led to increased apoptosis (Radziszewska, 2009). Furthermore, *Pten* deficiency has been reported to activate the p53 pathway leading to senescence (Chen, 2005; Kim,

2007; Miyauchi, 2004) as well as to sensitize cells to ROS-induced apoptosis (Nogueira, 2008).

Based on our studies and published reports, we propose the following model of cooperativity between c-MYC and Pten in prostate cancer (Figure 34D): Overexpression of c-MYC initiates tumorigenesis by facilitating loss of *Pten.* The latter leads to the activation of the p53 pathway, which can result in either senescence or apoptosis depending on the predominant *Trp53* target genes induced (i.e. cell cycle arrest genes e.g. $p21^{cip1}$ versus pro-apoptotic genes e.g. *PUMA, Bax* etc.). The expression of c-MYC drives cells down the apoptotic pathway as it selectively represses the cell cycle arrest-inducing target gene $p21^{cip1}$.

To summarize, we report a new Cre-dependent prostate cancer mouse model that reflects the focal, random and incremental nature of human prostate carcinogenesis. We show that focal c-MYC expression cooperates with *Pten* heterozygosity to promote tumor progression due to the selection of cells with loss of Pten expression. In addition, cells mutant for both *c-MYC* and *Pten* outcompete single *Pten*-mutant cells within the same prostates although *Pten*-deficiency sensitizes cells to apoptosis that is associated with activation of the p53 pathway and exacerbated by c-MYC expression. Our results highlight the utility of modeling focal oncogene activation to study the interactions between cell populations with different genetic alterations in tumorigenesis.

Implications

As discussed earlier in CHAPTER III, introduction of genetic mosaicism in model animals is very important and one of the great concerns in modeling human somatic genetic diseases. In addition to the methods to induce focal genetic mutations discussed in CHAPTER III, our mice can be utilized as a new cancer model initiated by focal c-MYC activation. In contrast to previous *Pb*- and *ARR$_2$Pb-MYC* mouse models which display uniform expression c-MYC (Figure 25), *PbCre4;Z-MYC* mice show entire stages of multi-step prostate cancer development mimicking human cancer development (Figure 4). They first show cancer initiation by focal c-MYC mutation in a few cells, i.e. ~18% of epithelial cells in any c-MYC+ prostate gland. Then, the c-MYC mutant cells expand to form uniform genetic mutation in prostate glands with time (Figure 28). To avoid any possibility that the *Z-MYC* construct itself caused cell proliferation by displaying expansion of mutant cells shown in Figure 28, it needs to be proven that the number of cells with β-galactosidase activity in *Z-MYC* prostates is not significantly changed with time, although it is highly unlikely. c-MYC mutation alone was not enough to cause invasive cancer, but it led to an “expanding field” which did not exhibit histologically visible abnormalities in a subset of *PbCre4;Z-MYC* mice. However, with a second mutation in *Pten*, the compound mutant mice developed invasive carcinoma, showing dramatic cooperation between *c-Myc* and either heterozygous or homozygous *Pten* deletion.

The theory of “field cancerization” was first proposed by Slaughter *et al.* in 1953 to explain initiation and development of neoplastic and premalignant lesions

in the oral cavity (Slaughter, 1953). With the recent hypotheses of monoclonal origin of cancer and multi-step carcinogenesis (Fearon, 1990), a new model for field cancerization has arisen. A genetically altered cell has growth advantages, proliferating and displacing normal cells through clonal expansion. This "expanding field" is macroscopically normal tissue but has sub-sufficient (some but not enough) genetic alterations to develop cancers (Almadori, 2004; Braakhuis, 2005; Braakhuis, 2003), (Grizzi, 2006). Interestingly, when macroscopically normal tissues adjacent to tumors and actual cancer biopsies were analyzed in head and neck squamous cell carcinoma (HNSCC) and breast cancer, 36% (10/28) and 26.7% (8/30) of the patient samples have at least one loss of heterozygosity (LOH) in putative tumor suppressor loci which are lost frequently in HNSCC (Figure 36) and breast cancer, respectively (Deng, 1996; Tabor, 2001). In addition, Chandran *et al.* (Chandran, 2005) confirmed this in prostate tissues by comparing genetic expression changes in normal tissues from tumor-free donors, normal-looking tissues adjacent to tumors, and tumor samples using microarray analysis. Thus, data suggest that there exist normal-looking "fields" with genetic abnormalities. In this regard, our c-MYC transgenic model *(PbCre4;Z-MYC)* displayed gradual displacement of normal cells with c-Myc mutant cells, but the "expanding field" included pathologically normal areas, which is reminiscent of field cancerization (Figure 28 and 36). Moreover, in terms of tumor recurrence, this theory of field cancerization has a crucial implication. "Expanding field" in the surgically un-excised tissues which are pathologically

normal still has genetic alterations, and the potential to develop primary tumor when additional hits of mutations occur (Tabor, 2001).

Thus, based on basic concepts of multi-step carcinogenesis and field cancerization, our murine prostate cancer model could be a valuable source to help understand basic human cancer biology in terms of initiation, progression, maintenance and even tumor recurrence in particular by breeding it to other mouse models with genetic alterations which confer additional mutations.

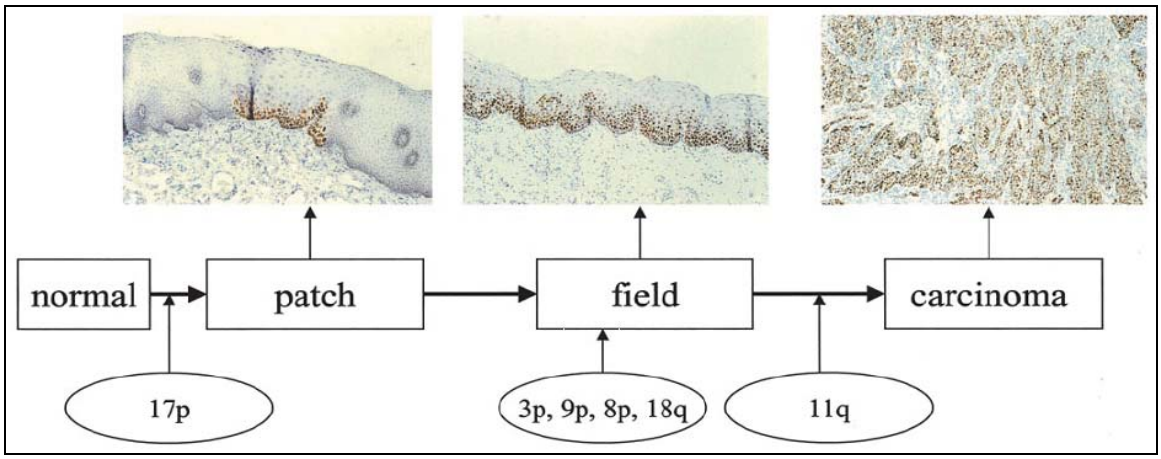

Figure 36. Model of field cancerization in head and neck squamous cell carcinoma (HNSCC). In normal tissues, cells form a "patch" (clonal unit) when there is focal mutation (loss of chromosomal locus 17p). With additional mutation(s), a "patch" becomes a "field" showing uniform genetic mutations without histologically discernable pathology. However, this normal-looking "field" gets cancerous with next oncogenic mutation(s). Chromosomal loci in the ovals indicate deletion mutation occurred at each stage. [Adapted from (Braakhuis, 2003)]

Materials and Methods

Animals

Z-MYC, PBCre4 and *Pten*$^{f/f}$ mice have been described (Groszer, 2001; Roh, 2006; Wu, 2001). The generation of *PbCre4;Z-MYC* bigenic mice have been described (CHAPTER V). To generate compound mutant mice, we generated *PbCre4;Pten*$^{f/+}$ males and *Z-MYC;Pten*$^{f/+}$ females which were further bred to obtain *PbCre4;Z-MYC, PbCre4;Pten*$^{f/f}$*, PbCre4;Z-MYC;Pten*$^{f/+}$ and *PbCre4;Z-MYC;Pten*$^{f/f}$ offspring for experiments as well as their littermate controls. Animal care and experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committees at Vanderbilt University.

Histology and immunohistochemistry

Tissues were prepared for histopathological analysis as described (Abdulkadir, 2001), and slides were reviewed by IEA based on published criteria (Mentor-Marcel, 2001). Human prostate cancer tissue microarrays were purchased from US Biomax, Inc. Immunohistochemical analyses were performed as described (Abdulkadir, 2001). The following antibodies were used, in some cases with Tyramide Signal Amplification (TSA, Perkin Elmer): anti-activated Caspase 3 (rabbit, 1:500, Cell Signaling), anti-phospho-histone H3 (rabbit, 1:500, Upstate), anti-phospho-Akt (rabbit, 1:100, Cell Signaling), anti-phospho-Foxo1 (rabbit, 1:50, Santa Cruz), anti-c-MYC (rabbit, 1:15,000 with TSA, Santa Cruz), anti-Pten (rabbit, 1:200 with TSA, Cell Signaling), anti-cytokeratin 8 (mouse, 1:2000,

Sigma), anti-p63 (PIN Cocktail, Biocare Medical), anti-p53 (rabbit, 1:5000 with TSA, Santa Cruz), anti-p21 (mouse, 1:50, Santa Cruz), anti-smooth muscle actin (mouse, 1:2000, Sigma), anti-p16 (rabbit, 1:1000, Santa Cruz), anti-phospho-Jnk (mouse, 1:100, Cell Signaling) and anti-Puma (rabbit, 1:200, Cell Signaling). For double immunofluorescenct stains, c-MYC or Pten detected by 1st primary antibodies were amplified by TSA system (green, Fluorescein). Alexa Fluor 594 (red)-labeled 2nd secondary antibodies (Molecular Probes) were used to detect 2nd primary antibodies (anti-cytokeratin 8, anti-p63, anti-smooth muscle actin, anti-phospho-histone H3, anti-p16 and anti-activated Caspase 3). Nuclear stain (DAPI) and sample mounting were performed using Vectashield mounting medium (Vector Laboratories).

Proliferation and apoptosis assay

At least 500 cells per sample were counted and quantitated after immunohistochemistry for phospho-Histone H3 and activated Caspase 3. N = 3-4 prostate samples from 10-15 week-old mice per group.

Cell lines

RWPE-1 cell culture and c-MYC-overexpressing RWPE-1 cells have been described in CHAPTER V. To generate PTEN knockdown cells, we used lentiviral-mediated gene delivery system which has been summarized in CHAPTER V. PTEN shRNA construct and pLKO.1 vector control were obtained

from Sigma. 24 hours post infection, medium was changed and another 24 hours later puromycin (1μg/ml) was added for selection of sh-Pten/pLKO.1 cells.

Western blot analyses

These were performed as described (Roh, 2005) using the following antibodies: anti-Pten (mouse, 1:1000, Cell signaling), anti-phospho-Akt (rabbit, 1:2000, Cell signaling), anti-total Akt (rabbit, 1:2000, Cell signaling), anti-c-MYC (mouse, 1:500, Santa Cruz), anti-p53 (mouse, 1:1000, Santa Cruz), anti-p21(mouse, 1:1000, Santa Cruz) and anti-β-actin antibody (goat, 1:1000, Santa Cruz).

Jnk inhibitor treatment and immunocytochemistry for activated Caspase 3

Coverslips were placed in the 24-well plates and 300,000 control or Pten knockdown RWPE-1 cells were plated on the coverslips. Next day, cells were treated with the Jnk inhibitor (SP600125) or vehicle (DMSO) at 0, 10 or 50μM for one hour. Then cells were washed with phosphate-buffered saline (PBS) and supplement-free medium was added to induce apoptosis. After 48 hours, immunocytochemistry for activated Caspase 3 was performed and apoptosis was quantitated from triplicate data per group.

Statistical analyses

We compared groups by using *t*-test or *Chi*-square test (Preacher, 2001) (http://www.quantpsy.org). Values were considered statistically significant at $P <$

0.05. Quantitative variables were expressed as means ± SD while categorical variables were expressed as numbers (%).

CHAPTER VII

FUTURE DIRECTIONS AND OVERALL CONCLUSIONS

Future Directions

We have examined prostate cancer progression using transgenic mice with distinct mutations in *c-Myc* and *Pten* and observed interactions between single mutant cells (*Pten*-null) and double mutant cells (*c-Myc+;Pten*-null) followed by expansion of the latter in the same prostatic glands. Next question is what would be the next favorable mutation to facilitate prostate tumor progression, since mutations in c-Myc and Pten encounter p53-dependent biological barriers to overcome (Figure 34). In addition, c-Myc mutation in Pten-deficient cells switched the p53 response from cellular senescence to apoptosis, with an increase of apoptosis in double mutant cells (*c-Myc+;Pten*-null) in comparison to single mutant cells (*Pten*-null). Interestingly, we noticed poorly differentiated cancer development with a dramatic decrease of apoptosis in the prostates of old *PbCre4;Z-MYC;Pten*$^{f/f}$ mice (~30 weeks) (data not shown) which showed well differentiated cancer with elevated apoptosis at younger ages (10-15 weeks). This is probably an indication of suppression in p53 activity as a third mutation to progress from micro-invasive cancer to advanced cancer due to either *p53* deletion or inactivation of p53 expression. We will be examining p53 expression in these old mouse (*PbCre4;Z-MYC;Pten*$^{f/f}$) prostates and possible

loss of *p53* gene locus in the focal areas showing poorly differentiated carcinoma by laser-captured microdissection (LCM).

Previously, it has been shown that p53 deficiency alone does not cause histological abnormalities in mouse prostates (Chen, 2005). To elucidate further the cooperation between mutations in c-Myc and p53, we generated *PbCre4;Z-MYC;p53*$^{f/+}$ and *PbCre4;Z-MYC;p53*$^{f/f}$ mice by breeding *PbCre4;Z-MYC* mice to *p53*-floxed mice (Jonkers, 2001) and observed focal HGPIN/prostate cancer development in both lines (Figure 37). This reflects obvious cooperation between mutations in *c-Myc* and *p53* based on mild phenotype of *PbCre4;Z-MYC* prostates and lack of pathological phenotype in *p53*-deficient prostates.

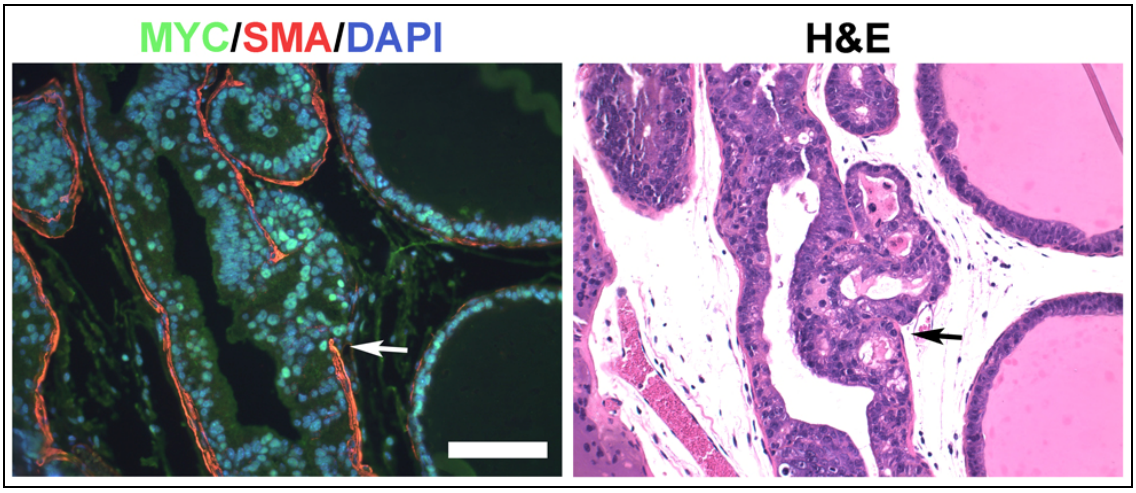

Figure 37. Focal microinvasive cancer in *PbCre4;Z-MYC;p53*$^{f/f}$ mouse prostate. Shown is 34-week old mouse prostate displaying microinvasion (arrows) at focal area. Scale bars: 100μm.

As discussed in CHAPTER I, one of c-Myc-dependent apoptosis pathways is through p53 activity. Since c-Myc activates *p19*^{ARF} expression which inhibits MDM2 E3 ligase, p53 protein is stabilized and induces p53-dependent apoptosis (Zindy, 1998). We have seen that c-Myc-overexpressing mouse prostate cells showed increased apoptosis (Figure 32) and this is probably due to p53 activation. We monitored p53 activation with c-MYC overexpression in human prostate cell line (3rd lane in Figure 34B), and in the future we will test this by immunostaining p53 protein in the prostate sections of *PbCre4;Z-MYC*, *PbCre4;Z-MYC;p53*$^{f/f}$ and *PbCre4;Z-MYC;p53*$^{f/+}$ mice.

In addition, utilizing the focal nature of our mouse prostate will provide information which will compare genetic profiles between cell populations with distinct mutations in the same prostate (Figure 38). We should perform laser-captured microdissection (LCM) from formalin-fixed paraffin-embedded (FFPE) tissues and isolate RNA followed by RNA amplification. This protocol is very challenging because RNA extracted from FFPE tissues is susceptible to degradation and being cross-linked to protein (RNA-protein) or to RNA (RNA-RNA) due to formalin fixation. RNA isolation yield is generally low and cross-linked RNA does not serve as a good enzymatic substrate for traditional microarray platforms (Abramovitz, 2008; Bibikova, 2004; Cronin, 2004; Godfrey, 2000; Masuda, 1999). Since the purpose of this is to compare genetic profiles between focal areas (Figure 38), expected isolated RNA amount will be small, which contributes to the difficulty. We initially tried to optimize the protocol and analyzed RNA quality isolated from the FFPE tissues (Figure 39). Fortunately,

we got promising results for RNA quality and amount that could be used for microarray analysis (sample #3 in lane 4 in Figure 39A and 39B). After more optimization followed by genetic profile analysis, we expect to see signaling differences between two cell populations, such as p53 target gene expression (either senescence- or apoptosis-inducers).

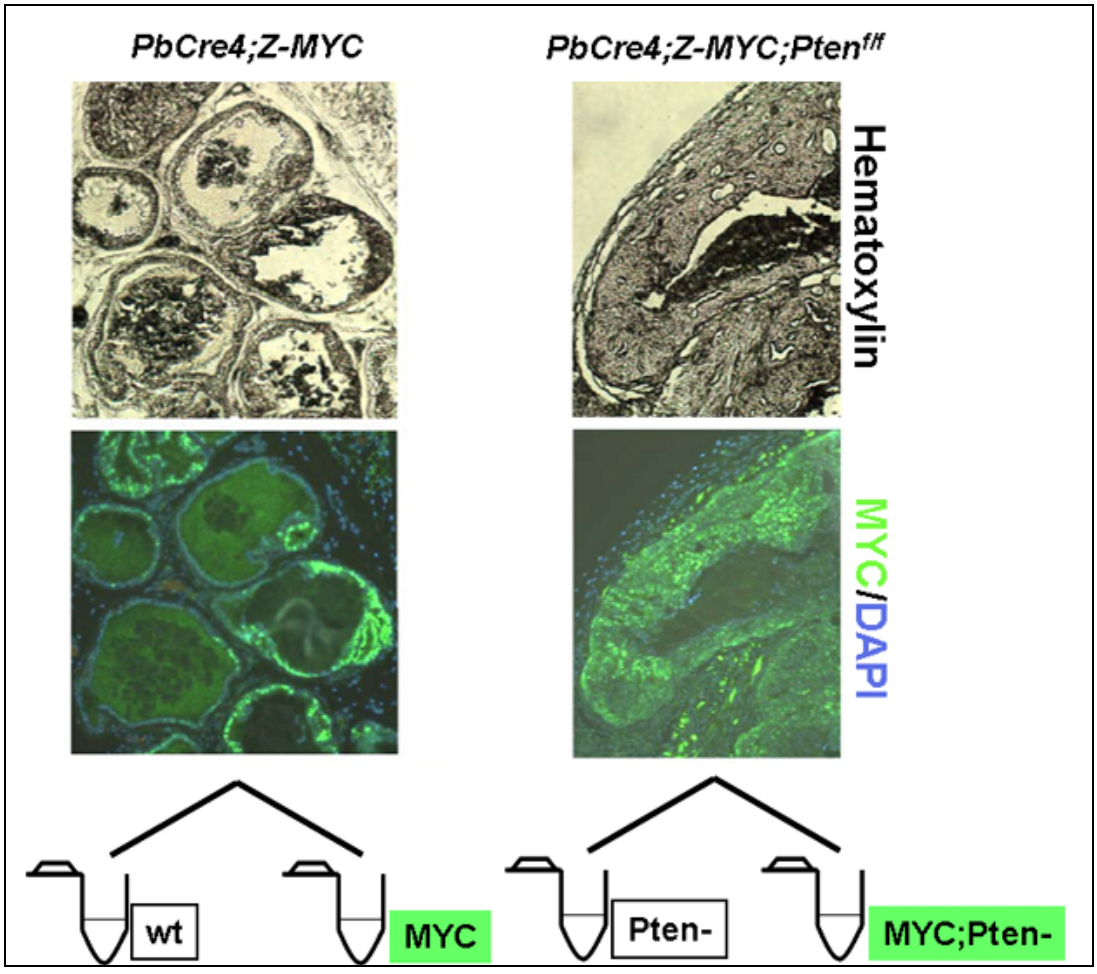

Figure 38. Tissue microdissection from *PbCre4;Z-MYC* and *PbCre4;Z-MYC;Pten*$^{f/f}$ mouse prostate sections. Laser-captured microdissection will be performed on weakly hematoxylin-stained sections (top) to take two distinct cell populations (wild type/c-Myc+ OR Pten-/c-Myc+;Pten-) based on c-Myc immunostaining (bottom).

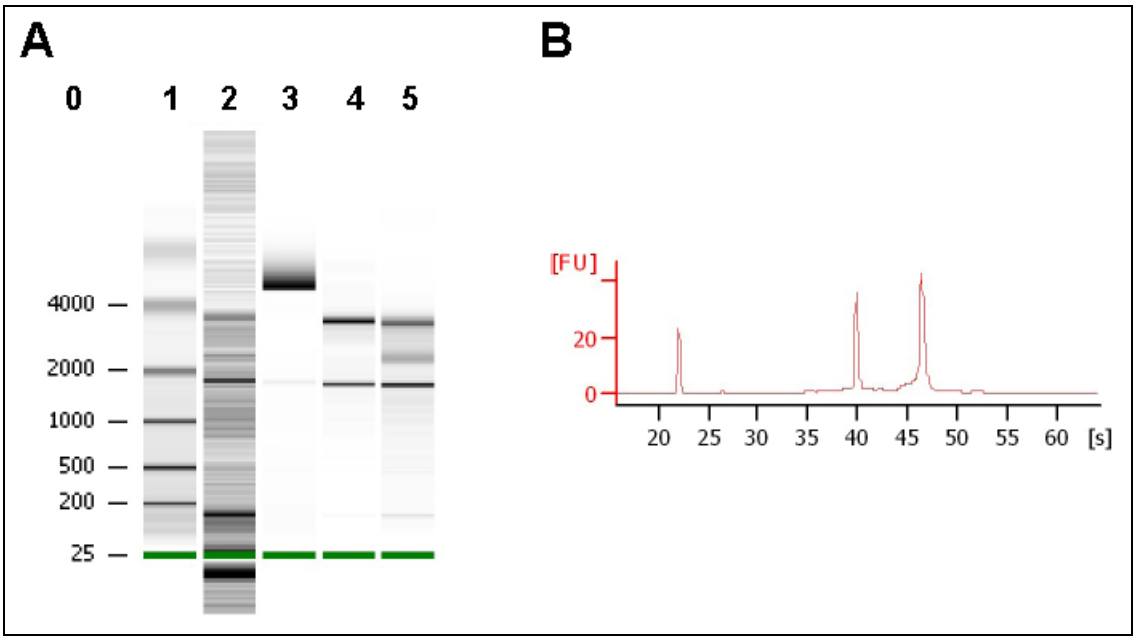

Figure 39. RNA isolation from FFPE tissues and quality analysis. RNA samples extracted from FFPE tissues were subjected to electrophoresis on bioanalyzer to analyze RNA quality. (A) lane 0 and 1 indicate size marker (size in nucleotides), lane 2-4 are isolated RNA samples (sample #1-#3, respectively) and lane 5 is an 18S/28S ribosomal RNA (rRNA) standard. (B) demonstrates an electropherogram of sample #3 in lane 4 in (A). Sample #3 shows enough amount and quality for microarray analysis (concentration: 9.8ng/ul; absorbance ratio (A260/A280): 1.35; rRNA ratio (28S:18S): 1.7; RNA Integrity Number (RIN): 9.3)

Lastly, the transgenic mice described herein are self-controlled mouse models, meaning that we can analyze the effect of one mutation by comparing two cell populations consisting of wild type/c-Myc+ cells or Pten-/c-Myc;Pten- cells in one animal. Once we get gene expression profile data, we may be able to identify some differences in specific signaling pathways or gene expression profiles between two groups of cells with distinct mutations. We can then compare cell fate changes depending on drug susceptibility between control groups (wild type or Pten- cells) and positive groups (c-Myc+ or c-Myc+;Pten- cells, respectively) in the same prostate of one mouse after the mouse has been treated with drugs that inhibit specific signaling pathways (e.g. mitogen-activated protein kinase (MAPK) or PI3 kinase (PI3K) inhibitors) or suppress general cancer effects, like increased reactive oxygen species (ROS) (Nyska, 2003; Tam, 2006).

Overall Conclusions

Based on the theories of multi-step carcinogenesis and field cancerization (Fearon, 1990; Slaughter, 1953), human somatic cancers are thought to initiate from an oncogenic mutation either in an oncogene or in a tumor suppressor gene in a single or a few cells (Figure 40A and 40B). This single mutation is normally not enough to lead to cancer development because the mutated cells often encounter barriers, such as cell-cycle arrest (senescence) or apoptosis (Figure 40B'). Depending on the strength of the mutation, the mutant cells stop proliferating until there is an additional mutation or they expand clonally without displaying histological abnormalities ("field") (Figure 40C). At this stage, the mutant cells are sub-sufficient to be cancerous, but have more of a potential to be malignant than completely normal un-mutated cells. When that next hit that suppresses the barriers comes, the cells start to develop lesions with histological abnormalities (Figure 40D), which ultimately progress to carcinoma (Figure 40E).

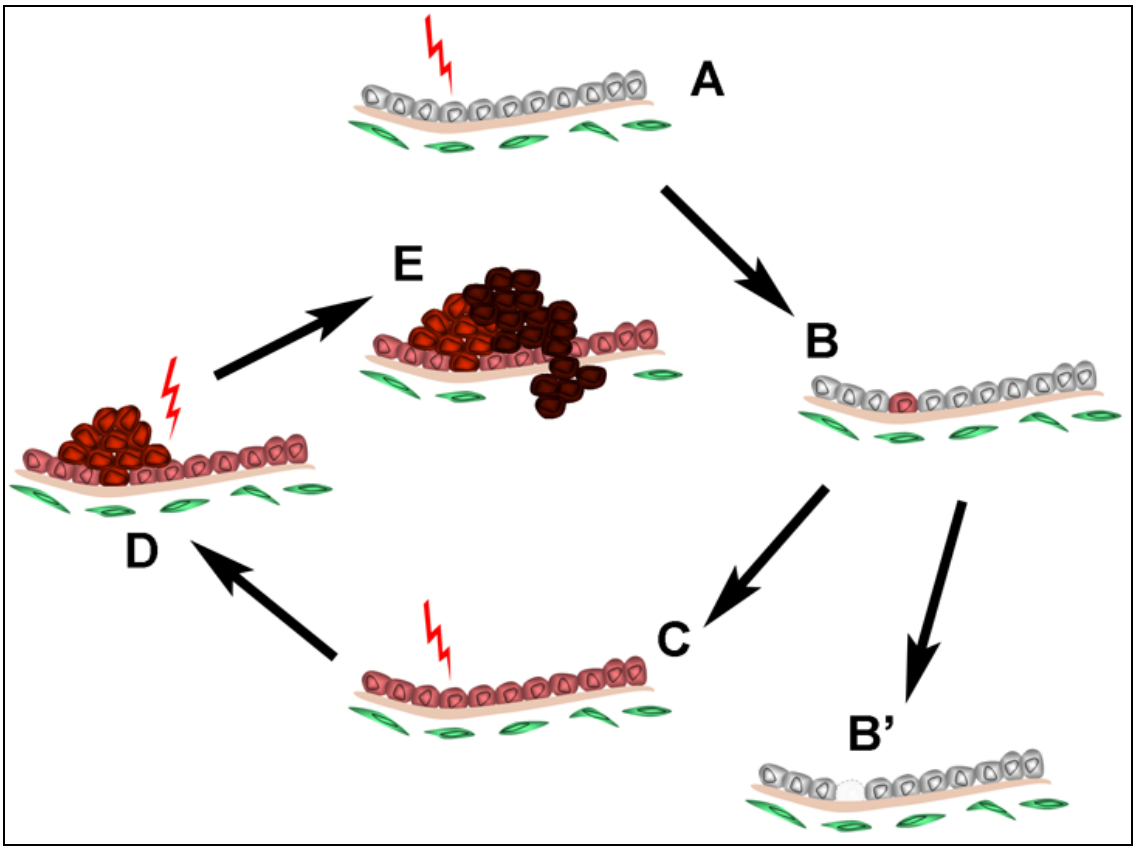

Figure 40. Model of multi-step carcinogenesis and field cancerization. When a normal cell is mutated (A and B), it is either eliminated (B') or clonally expanded to form a "field" (C) which is histologically normal. An additional mutation induces abnormal phenotypes (D) and sometimes leads invasive cancer development (E) which is more likely pushed by a third oncogenic mutation.

Numerous mouse models of human prostate cancer have been generated (CHAPTER II) and exhibit common characteristics of human prostate cancer. However, most of them may not mimic spontaneous human carcinogenesis because of uniform activation of oncogene(s) or inactivation of tumor suppressor gene(s) in the majority of prostate cells. Consequently, a lot of efforts have been made to model focal the nature of spontaneous cancer initiation using various methods (CHAPTER III). Here, we first report on transgenic mice *(Z-MYC)* that can express c-Myc in multiple tissues in a stochastic manner even though the rate of mosaicism is varied depending on tissue types (CHAPTER IV). When we activated c-Myc specifically in the prostate *(PbCre4;Z-MYC* mice*)*, we found that ~18% of prostate epithelial cells in any c-Myc+ gland showed c-Myc overexpression at young age. With time, the number of c-Myc mutant cells increased and displayed an “expanding field” without abnormal pathology in a subset of *PbCre4;Z-MYC* mice, probably due to a suppressive mechanism. When we introduced Pten deletion as a second genetic alteration, however, most of the compound mutant mice developed a precursor stage (HGPIN) of prostate cancer or invasive carcinoma. Biological barriers were still present in these compound mutant mice, but they (*PbCre4;Z-MYC;Pten*$^{f/f}$ mice) developed advanced, poorly differentiated cancer with time, which was probably due to loss of p53 activity as a third mutation.

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