MYC AND PIM1 SYNERGISM IN PROSTATE CANCER

By

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Professor Sarki Abdulkadir

Dedicated to my parents, my husband and my son

for their endless support and encouragement

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TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF ABBREVIATIONS

AP	Anterior prostate
AR	Androgen Receptor
ARRs	Androgen Response Regions
ASK1	Apoptosis Signaling Kinase 1
bHLHZ	basic-Helix-Loop-Helix-Leucine Zipper
BPH	Benign Prostatic Hyperplasia
CARNs	Castration-Resistant luminal cells expressing Nkx3.1
CDK	Cyclin-Dependent Kinase
CK	Cytokeratin
CRPC	Castration-Resistant Prostate Cancer
CSCs	Cancer Stem Cells
CXCR4	CXC chemokine receptor 4
DP	Dorsal Prostate
dpc	days post-coitum
DRE	Digital Rectal Examination
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular signal-Regulated Kinase
ES	Embryonic Stem cells
FBS	Fetal Bovine Serum
GSEA	Gene Set Enrichment Analysis
GSK-3β	Glycogen Synthase Kinase-3β □
HGPIN	High Grade Prostatic Intraepithelial Neoplasia
HP	Heterochromatin Protein
I.U.	infectious units
JNK	c-Jun N-terminal Kinase
K67M	Pim1 kinase lysine to-methionine at amino acid 67
LGPIN	Low Grade Prostatic Intraepithelial Neoplasia
LP	Lateral Prostate
LTR	Long Terminal Repeat

CLL	Chronic Lymphocytic Lleukemia cells
mAbs	Monoclonal Antibodys
MMTV	Mouse Mammary Tumor Virus
MOI	Multiplicity Of Infection
MPT	MYC and Pim1 overexpressing Tumorigenic cells
MYC/Pim1	MYC and Pim1 overexpressing tumor
MYCS62D	MYC serine to aspartic acid mutant at amino acid 62
NE	Neuroendocrine
NSE	Neuron-Specific Enolase
NuMA	Nuclear Mitotic Apparatus
PIN	Prostatic Intraepithelial Neoplasia
PP2A	Protein Phosphatase 2A
Probasin	Pb
PSA	Prostate Specific Antigen
PSCs	Prostate Stem Cells
ROS	Reactive Oxygen Species
Sca-1	Stem cell antigen-1
SCID	Severe Combined Immunodeficiency
SD	Standard Deviation
SDF-1	Stromal cell-Derived Factor-1
shRNA	small hairpin RNA
SV40	Simian Virus 40
SYP, or SYN	synaptophysin
TMAs	Tissue microarrays
UGE	Urogenital epithelium
UGM	Urogenital Sinus Mesenchyme
UGS	Urogenital Sinus
VP	Ventral Prostate
YFP	Yellow Fluorescent Protein

ABSTRACT

The oncogenic PIM1 kinase has been implicated as a cofactor for the oncogene c-MYC in prostate carcinogenesis. In this study, we show that coexpression of c-MYC and PIM1 is associated with high Gleason grade in prostate cancer. Using a tissue recombination model coupled with lentiviral-mediated gene transfer we show that Pim1 is weakly oncogenic in naïve adult mouse prostatic epithelium. However, it cooperates dramatically with c-MYC to induce prostate cancer within 6 weeks. Importantly, c-MYC and Pim1 synergy is critically dependent on Pim1 kinase activity. c-MYC/Pim1-expressing tumors showed increased phosphorylation of MYC on serine 62. Expression of a phosphomimetic c-MYC S62D mutant resulted in higher rates of proliferation compared to that of wild type c-MYC. However, c-MYC S62D did not result in tumors like c-MYC/Pim1 grafts, indicating that Pim1 cooperativity with c-MYC involves additional mechanisms other than enhancement of c-MYC activity by S62 phosphorylation. In addition, c-MYC/Pim1-induced prostate carcinomas demonstrate evidence of neuroendocrine (NE) differentiation. Additional studies, including the identification of tumor cells co-expressing androgen receptor and NE cell markers synaptophysin and Ascl1 suggest that NE tumors arise from adenocarcinoma cells through transdifferentiation. These results directly demonstrate functional cooperativity between c-MYC and Pim1 in prostate tumorigenesis *in vivo* and support therapeutic strategies for targeting PIM1 in prostate cancer.

Since Pim1 deficiency is well tolerated in vivo, it has been proposed that Pim1 inhibition may offer an attractive option to impede prostate cancer progression. In the current study, we used small hairpin interfering RNA (shRNA) directed against Pim1 to

determine the anti-tumor effects in prostate cancer cell lines. In mouse c-MYC/Pim1 prostate tumor-derived cells and human prostate cancer cells, Pim1 knockdown markedly decreased cellular proliferation, survival, and tumorigenicity. Further studies indicate that in prostate cancer cells, Pim1 is required to maintain ERK/MAPK signaling pathway activation. Thus, Pim1 is necessary to maintain tumorigenicity, and may represent an efficient target for prostate cancer therapy.

(Note: Human MYC and mouse Pim1 are cloned in lentiviral constructs and used for this study)

CHAPTER I

INTRODUCTION

Overview

Prostate cancer is the most common cause of cancer in men and is a leading cause of cancer-related death among men. More than 186,000 American men are diagnosed with prostate cancer each year. One out of every 6 men will get prostate cancer and one out of every 36 men will die of it (American Cancer Society, 2009). It has been shown that many features of prostate organogenesis are paralleled in the initiation and progression of prostate cancer. Therefore, understanding the development of the normal prostate should provide insights into tumor formation and progression, which will help in the identification of novel biomarkers and the development of efficient therapies to treat prostate cancer (Figure 1). Using in vivo tissue recombination models, we have examined the role of two oncogenes, Myc and Pim1 kinase and their synergism in prostate cancer development. We have also analyzed the functional role of Pim1 kinase in prostate tumorigenesis and its potential role as a therapeutic target.

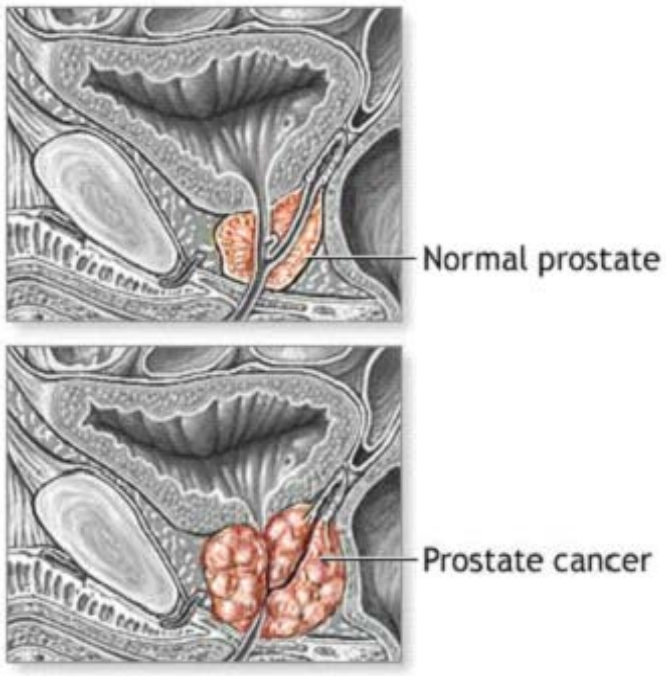

Figure 1. The diagram of normal prostate and prostate cancer (Adapted from http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/18038.jpg).

Prostate gland anatomy

The prostate is a male accessory sex gland located in front of the rectum and below the urinary bladder, encircling the urethra (Aumuller, 1989) (Figure 2). The main function of the prostate is to produce components of the seminal fluid during ejaculation. The fluid secreted by the prostate gland is rich in proteins and ionic components including acid phosphatase, citric acid, prostate specific antigen (PSA), zinc and calcium (Aumuller, 1990). The alkalinity seminal fluid helps to neutralize the acidity of the vaginal tract and to support sperm survival (Aumuller, 1990).

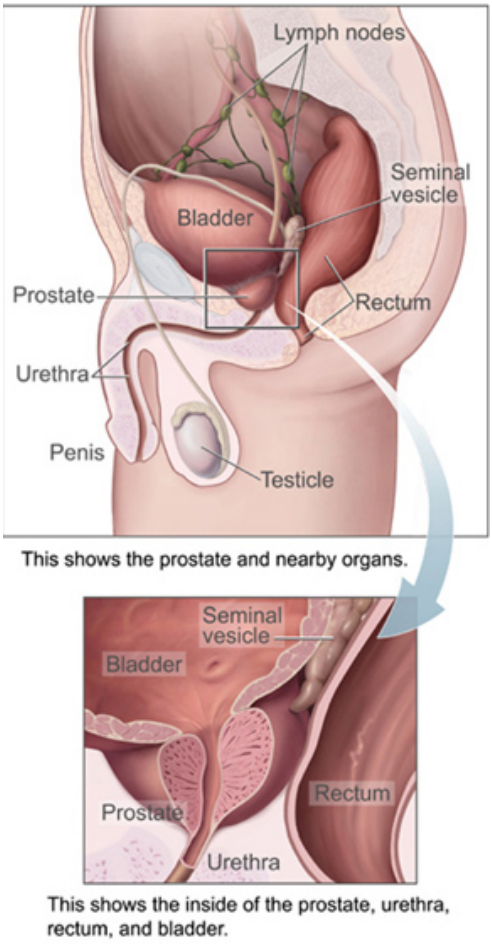

Figure 2. Anatomy of human prostate gland. The human prostate is a walnut shaped gland surrounding the urethra below the bladder (Adapted from http://www.cancer.gov).

Prostate gland development

The prostate develops from the urogenital sinus (UGS). The UGS is an ambisexual embryonic rudiment. The UGS consists of epithelium (UGE) and mesenchyme (UGM). UGS develops into the prostate, prostatic urethra and bulbourethral glands in males, the lower vagina and urethra in females, and into the bladder in both sexes (Cunha, 1987; Staack, 2003). The UGS becomes the sexually dimorphic in response to androgens around 13.5 days post-coitum (dpc) in mice and 8 weeks in human fetus. Around 15.5 dpc in the mouse and 10 weeks in the human, the androgen responsive

UGM signals to the epithelium, inducing it to form epithelial buds. Then epithelial buds grow as solid cords into the surrounding mesenchyme. The solid cords canalize and progress into the branching network (Figure 3) (Kurzrock; 1999; Hayward, 2000; Staack, 2003; Meeks, 2010). In a reciprocal manner, prostate epithelial cells induce mesenchymal cells to differentiate into smooth muscle (Cunha, 2008). In humans, the gland enters a quiescent state after birth until the circulating androgen levels increase at puberty. After puberty, the gland begins to grow slowly and epithelium proliferates, resulting in the branching structures seen in the mature gland (Cunha, 1987). This growth phase continues until adulthood. At age of 45 to 50 years in humans, androgen levels decline and the prostate undergoes a period of involution. While man becomes older, benign prostatic hyperplasia commonly appears (Berry, 1984)

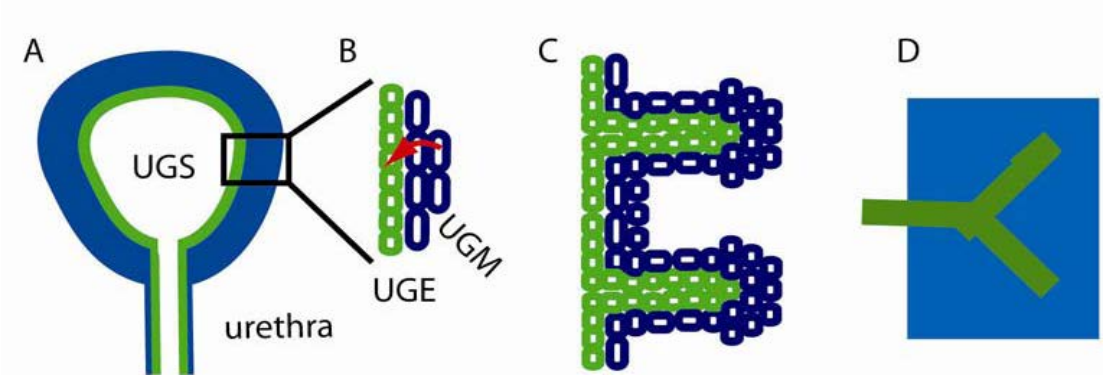

Figure 3. Schematic representation of phases of prostate development. ***A***, The prostate develops from the UGS in response to androgens. The UGS consists of both epithelium (green) and mesenchyme (blue). ***B,*** Androgens engage androgen receptor in the mesenchyme and induce epithelial budding. ***C,*** Epithelial buds elongate into solid cords of tissue that eventually canalize into ducts. ***D,*** Latter phases of prostate development include epithelial branching (Adapted from Meeks J, 2010.)

Prostate structure

The mouse prostate comprises four pairs of lobes depending on the regions relative to the urethra: the anterioral (AP), ventral (VP), dorsal (DP) and lateral prostates (LP) (Figure 4).

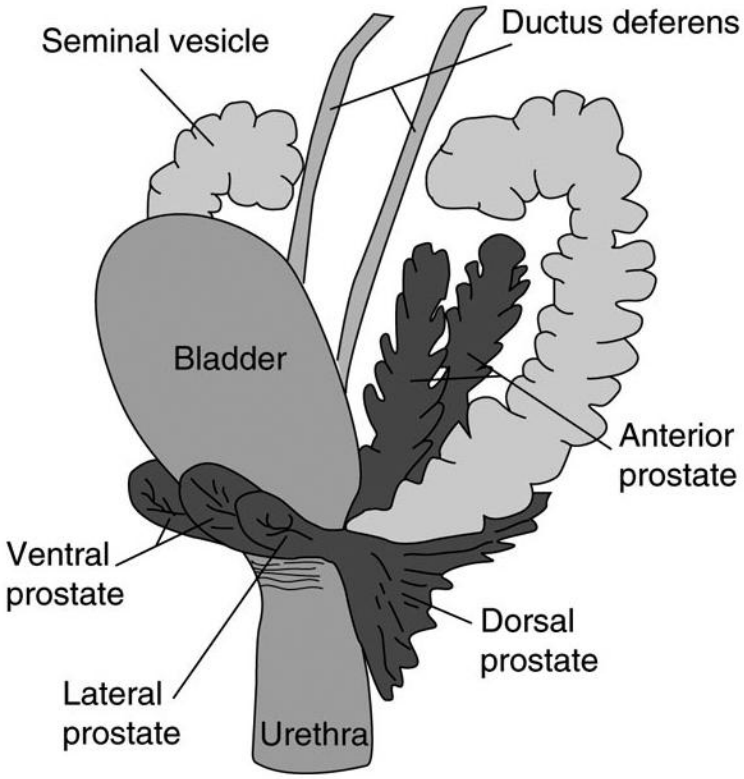

Figure 4. Schematic diagram of the adult mouse genitourinary tract (lateral view). The mouse prostate consists of four pairs of distinct lobes (Adapted from Sugimura, 1986).

Lowsley identified several lobes of prostate in fetuses and newborns including a dorsal or posterior lobe, a median and two lateral lobes, and a ventral lobe (atrophied after birth). In the adult human, these lobes are fused and grow as a single lobe with distinct glandular regions (Lowsley, 1912). In McNeal's model, human prostate is divided into 4 glandular zones: the peripheral zone, transition zone, periurethral zone and central zone (McNeal, 1981) (Figure 5). Majority of prostate cancers arises in the peripheral

zone, while benign prostatic hyperplasia usually originates in transition zone, and a significant subset of prostate cancer (approximately 20%) also occurs in this zone (McNeal, 1981).

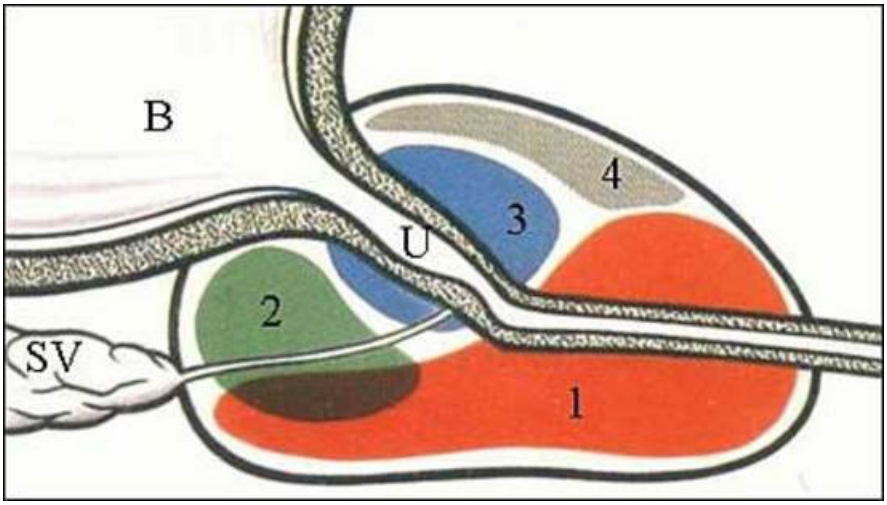

Figure 5. McNeal's model of the prostate

1, Peripheral Zone; ***2,*** Central Zone; ***3,*** Transitional Zone; ***4,*** Anterior Fibromuscular Zone. B= Bladder, U= Urethra, SV= Seminal Vesicle (adapted from Algaba, 1991)

Although these are anatomic differences between the single lobed prostate in human and the multi-lobed prostate in mouse, the early histological events and the steroid hormones that drive prostate development are similar in both mice and humans during the prostate development (Meeks, 2010). Certain similarities in prostate anatomy, clinical and pathological features between the mouse and human prostate gland support the use of mouse models for studying the mechanisms of prostate cancer initiation and progression (Shappell, 2004).

Cell types in prostate gland

Adult prostate epithelium consists of flattened basal cells, tall columnar luminal cells, and rare neuroendocrine (NE) cells (Figure 6). Luminal cells express AR, cytokeratin (CK) 8 and 18. Basal cells express p63, CK5 and CK14. Neuroendocrine cells express chromogranin A and synaptophysin. There are cells with intermediate phenotypes expressing a mixture of basal and luminal markers (CK5, CK8, CK14, CK18, and PSA) (Wang, 2001). The majority of stroma is smooth muscle. Fibroblasts, nerves, endothelial cells and vascular smooth muscle are also located in the stroma (Abate-Shen, 2000; Wang, 2001) (Figure 6).

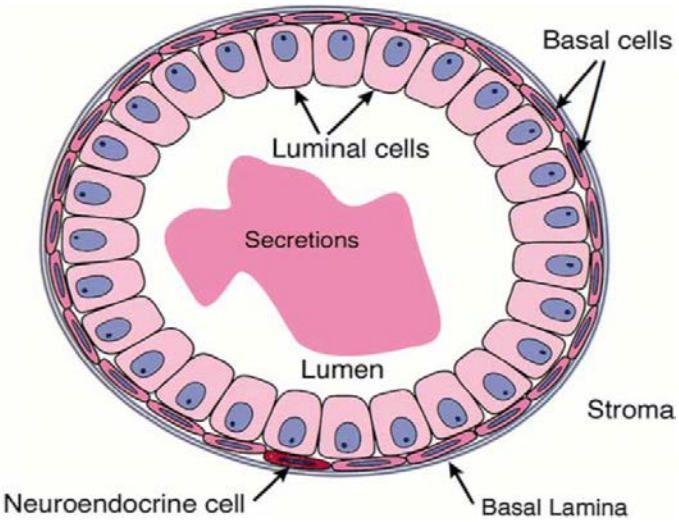

Figure 6. Cell types within a human prostatic duct. Prostatic epithelial cells are composed of luminal, basal and rare neuroendocrine cells (Adapted from Abate-Shen, 2000).

Prostate cancer

Prostate cancer diagnosis

The primary method of screening for prostate cancer is serum analysis for prostate-specific antigen (PSA) (Catalona, 1991). PSA is a kallikrein serine protease that is secreted by prostate epithelial cells. PSA is released into blood due to morphological and pathological changes in the prostate. The major function of PSA is breakdown of semenogelins and fibronectin in coagulated semen, causing liquefaction of semen and facilitating fertilization (Lilja, 1985; 1987). Prostate cancer is associated with elevated level of serum PSA. However, elevation of PSA may indicate other prostatic diseases, such as benign prostatic hyperplasia (BPH) and inflammation of the prostate (prostatitis). A digital rectal examination (DRE) is also used to detect a problem in the prostate. An irregular or hard lump may indicate the presence of a tumor. Rectal examination also determines whether the tumor remains within the borders of the prostatic capsule or goes beyond it. However, enlargement of the prostate gland can be found in BPH patients. Not all of prostatic abnormalities can be found through the rectum. Both DRE and PSA test are also associated with high false-positive rates because they cannot tell whether the problem is cancer or a benign condition. They cannot predict progression of cancer, and may detect some indolent prostate cancer that would never have caused clinical problems. If an abnormality is found after PSA or DRE test, patients will be subjected to transrectal biopsy to diagnose prostate cancer. However, prostatic biopsies are associated with complications, including fever, pain, hematospermia/hematuria, positive urine cultures, and rarely sepsis (Rietbergen, 1997).

Next, the pathologist examines biopsy samples under a microscope and gives a grade. One system of grading uses Gleason score. The most common pattern is given a

grade of 1 (like normal cells) to 5 (most abnormal). If there is a second most common pattern, the pathologist also gives it a grade of 1 to 5, and adds the two most common grades together to make the Gleason score (Gleason, 1974). Another system of grading prostate cancer uses the TNM system, which evaluates the size and invasive of the tumor (T1-T4), the extent of involved lymph nodes (N0 or 1), any metastasis (M0 or M1a-c) also takes into account cancer grade. These are often grouped into four stages (I–IV). (Denoix, 1944; 1950).

Research has shown an increased risk of prostate cancer among men age over 65. The risk is higher if a family member had prostate cancer. The risk is also related to race, most common in African man, and lowest in Asians. Many other possible risk factors are under study. Researchers are also studying how to prevent prostate cancer. Certain chemo-preventive agents including, vitamin E, selenium, green tea extract seem to help in preventing prostate cancer (American Cancer Society).

Prostate cancer treatment

Prostate cancer can be treated by surgery, radiation therapy, hormone therapy, and chemotherapy. It has been reported that current prostate cancer treatments cause permanent side effects in men, such as erectile dysfunction and urinary incontinence (Harris, 2002; Litwin, 2000; Steineck, 2002). Prostate cancer cells depend on androgen receptor for growth and survival. The most common treatment for prostate cancer is hormone therapy (androgen ablation). Although this treatment is effective initially, over time Castration-Resistant Prostate Cancer (CRPC) occurs, which is refractory to current therapeutic modalities. Therefore, one of major challenges in prostate cancer research is to find efficient therapeutic approaches for treatment of CRPC. Notably, some types of

prostate cancer are indolent and may need minimal or no treatment, other types are aggressive and can spread quickly (American Cancer Society). However, the accuracy of identifying indolent versus aggressive cancer was poor, which leads to over-diagnosis and over-treatment of prostate cancer. Because of these, controversy exists in regard to the value of screening, the most appropriate staging evaluation, and the optimal treatment of each stage of the disease (Garnick, 1993.) Therefore, one of major challenges in prostate cancer research is to design reliable screens for distinguishing indolent versus aggressive cancer, and to elucidate the factors that contribute to disease progression.

Model of prostate cancer progression

Human prostate tumorigenesis involves a series of genetic and epigenetic alterations that transform benign prostatic epithelial cells into precursor lesions prostatic intraepithelial neoplasia (PIN) with progression to invasive carcinoma and ultimately to metastastic disease. The skeletal bone, lymph nodes and lung are major metastastic sites for prostate cancer (Shen, 2000) (Figure 7). During this process, many oncogenes such as Myc, Pim1, EZH2 and Egr1 are upregulated, while tumor suppressor genes such as Nkx3.1, p27, PTEN and p53 are downregulated (Abdulkadir, 2005). The long term goal for prostate cancer research is to identify genes and molecular pathways that are involved in the initiation and progression of prostate cancer. These studies will provide insight into new strategies for treatment and prevention of prostate cancer (Abate-Shen, 2000, Shen, 2010).

Neuroendocrine (NE) cells are a minor population in normal prostate epithelium. During the progression of prostate cancer, the number of NE cells in malignant lesions increases especially in advanced prostate tumor, which is correlated with its

tumorigenicity and hormone-refractory growth (Jiborn, 1998; Ito, 2001; Ismail, 2002; Hirano, 2004). The biological basis for androgen insensitivity is not well understood. Recent studies have focused on finding the determinants of metastasis and CRPC for identifying specific therapeutic targets.

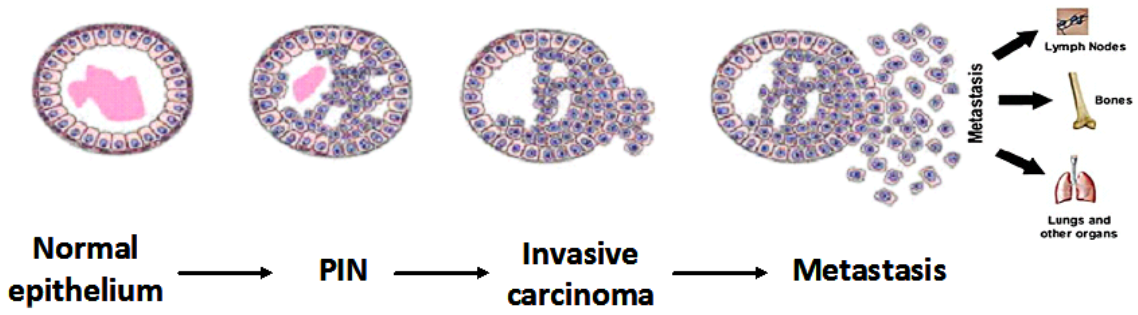

Figure 7. Development of prostate cancer. Diagram showing transformation of normal prostate epithelium to PIN, invasive carcinoma and metastasis with the progression of disease (Adapted from Abate-Shen, 2000).

Mouse models of prostate cancer

Prostate cancer involves a series of genetic and epigenetic alterations that transform normal prostate epithelial cells into cancer cells. Therefore, the development of useful models is necessary for understanding of the molecular mechanisms of prostate cancer.

Mice are used for modeling human cancer due to a relatively short gestation period and lifespan. Importantly, the essential functions of most mouse genes implicated in cancer are structurally homologous to those in humans. Furthermore, another advantage is that mice are susceptible to cancer as humans (Rangarajan, 2003). Indeed, mouse models have provided valuable information about exploring mechanisms of tumorigenesis and testing new therapies.

Genetically engineered mouse models

Transgenic models

Androgen responsive prostate-specific promoters are used to drive the expression of transgenes in prostate epithelial cells. These include elements derived from rat probasin, rat C3 (1) prostate steroid binding protein, human prostate specific antigen (PSA), and mouse mammary tumor virus (MMTV) long terminal repeat (LTR). The probasin promoters (PB) are commonly used, which includes the minimal probasin promoter (Rennie, 1993), long probasin promoter (Yan, 1997) and minimal probasin promoter containing multiple androgen response regions (Kasper, 1994). Probasin and PSA promoters have been shown to essentially drive prostate-specific expression. ARR2PB promoter links two androgen response regions (ARRs) to the PB promoter, which drives high levels of transgene expression in transgenic mice (Ellwood-Yen, 2003; Zhang, 2000). MMTV LTR is responsive to androgen and several other steroid hormones. However, it has been shown that hormone sensitivity is not the only criterion for MMTV expression (Stewart, 1998). C3 (1) regulatory sequence induced gene expression can be found in other tissues in addition to the prostate (Green, 1998).

TRAMP and LADY mice are two well characterized models of prostate cancer. They rapidly form invasive tumors with neuroendocrine features metastases (Greenberg, 1995; Gingrich, 1996; Kaplan-Lefko, 2003; Kasper, 1998; Masumori, 2001). The TRAMP mice contain a minimal probasin promoter to drive the expression of SV40 large T and small t tumor antigens (Greenberg, 1995). SV40 large T antigen functions to inactivate the tumor suppressor p53 and retinoblastoma (Levine, 1990). Small t antigen blocks the function of protein phosphatase 2A (PP2A) and thus activates MAPK signaling pathway (Sontag, 1993). TRAMP mice develop high-grade prostate

intraepithelial neoplasia (HGPIN) within 12 weeks of age and adenocarcinoma and finally, metastatic spread to the lymph nodes, adrenal glands, lung and bone at 18 to 30 weeks of age (Gingrich, 1999). LADY mice contain long probasin promoter to direct expression of only SV40 large T antigen (Kasper, 1998; Masumori, 2001). The LADY mice display the variable pattern of tumor formation depending on different sites of transgene integration, ranging from 12 weeks to over 20 weeks of age, following the development of poorly or undifferentiated carcinoma with NE differentiation (Kasper, 1998; Masumori, 2001).

Knockout models

Deletion of a gene of interest is another way to study gene functions. Due to embryonic lethality of some ablations, conditional knockout mice are made by using prostate specific promoter to drive Cre expression, such as Pb-Cre, Nkx3.1-Cre. The targeted gene was inactivated by Cre deletion of the floxed region and the expression of a truncated protein with loss of gene function. Some knockout models are shown in table1. However, these conditional knockout mice come from constitutively loss of function in early stages of prostate development. An inducible gene targeting system can overcome this limitation by inducing the expression of the gene of interest in adult prostate epithelium. Tamoxifen-inducible Cre driven PTEN null mice is an example of inducible mouse models (Luchman, 2008; Ratnacaram, 2008; Birbach, 2009).

Some other engineered mouse models are shown in Table 1 (Adapted from Shen MM, Abate-Shen C. Molecular genetics of prostate cancer: new prospects for old challenges. Genes Dev. 2010 Sep 15; 24 (18):1967-2000).

Table 1. *Representative genetically engineered mouse models of prostate cancer*[a]

Type	Name	Description	Reference
Gain-of-function transgenic models	*TRAMP (rPB-SV40)*	SV40 large tumor antigen (Tag) driven by a minimal rat probasin promoter (rPB). Phenotype: PIN, adenocarcinoma, neuroendocrine differentiation, and metastases; castration-resistant prostate cancer.	Greenberg et al. 1995
	Lady (LPB-Tag)	SV40 large tumor antigen driven by large probasin promoter. Phenotype: PIN, adenocarcinoma, neuroendocrine differentiation, and metastases.	Masumori et al. 2001
	TgAPT$_{121}$ (ARR2PB-APT$_{121}$)	Truncated SV40 T antigen (without small t antigen) driven by minimal probasin promoter with androgen-regulated sites (ARR2PB). Phenotype: PIN and adenocarcinoma.	Hill et al. 2005
	Hi-Myc/Low-Myc (ARR2PB-c-Myc)	c-Myc driven by ARR2PB promoter; Hi-Myc and low-Myc differ in latency. Phenotype: PIN and adenocarcinoma.	Ellwood-Yen et al. 2003
	MPAKT (rPB-myr-HA-Akt1)	Myristoylated Akt1driven by the rPB promoter. Phenotype: PIN.	Majumder et al. 2003
	iBraf (Tet-BRAF^{V600E}; Tyr-rtTA; Ink4a/Arf$^{-/-}$)*	A human mutant B-RAF (V600E) driven by the Tet promoter and crossed with mice having a tet-regulatable tyrosinase promoter. Phenotype: PIN and adenocarcinoma.	Jeong et al. 2008
Loss-of-function models	*Nkx3.1$^{+/-}$* and *Nkx3.1 $^{-/-}$* and *Nkx3.1$^{flox/flox}$*	Germline deletion of *Nkx3.1* (or conditional deletion of *Nkx3.1* in the germline). Phenotype: PIN.	Bhatia-Gaur et al. 1999; Abdulkadir et al. 2002; Kim et al. 2002b
	Pten$^{+/-}$	Germline deletion of *Pten*. Phenotype: PIN and high-grade PIN; castration-resistant prostate cancer. Phenotypes not restricted to prostate.	Di Cristofano et al. 1998b; Podsypanina et al. 1999
	Nkx3.1$^{+/-}$; Pten$^{+/-}$	Compound germline mutant mice; Phenotype: PIN, adenocarcinoma, metastases to lymph nodes; castration-resistant prostate cancer. Phenotypes not restricted to prostate.	Kim et al. 2002c; Abate-Shen et al. 2003
	Pten$^{+/-}$; p27$^{-/-}$ and *Nkx3.1$^{+/-}$; Pten$^{+/-}$; p27$^{+/-}$*	Compound germline mutant mice. Phenotype: PIN, adenocarcinoma. Phenotypes not restricted to prostate.	Di Cristofano et al. 2001; Gao et al. 2004b
	TMPRS-Erg; Pten$^{+/-}$	Germline loss of function of *Pten* combined with gain of function of the TMPRS-Erg transgene. Phenotype: PIN, adenocarcinoma.	Carver et al. 2009; King et al. 2009
Conditional loss-of-function models	*PB-Cre; Pten$^{flox/flox}$*	Conditional deletion of *Pten* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma; castration-resistant prostate cancer.	Trotman et al. 2003; Wang et al. 2003
	PSA-Cre-ER^{t2}; Pten$^{flox/flox}$	Conditional deletion of *Pten* in the prostate driven by a PSA promoter driving an inducible Cre-ER^{T2} recombinase. Phenotype: PIN, adenocarcinoma.	Ratnacaram et al. 2008
	PB-Cre; Pten$^{flox/flox}$; p53$^{flox/flox}$	Conditional deletion of *Pten* and *p53* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma.	Z Chen et al. 2005
	PB-Cre; APC	Conditional deletion of *APC* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma; castration-resistant prostate cancer.	Bruxvoort et al. 2007
	PB-Cre; Pten$^{flox/flox}$, Z-Myc	Conditional activation of Myc plus conditional deletion of Pten in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma.	Kim et al. 2009
	PB-Cre; p53$^{flox/flox}$; Rb^{flox}	Conditional deletion of *p53* and *Rb* in the prostate driven by a minimal probasin promoter driving Cre recombinase. Phenotype: PIN, adenocarcinoma, neuroendocrine differentiation.	Zhou et al. 2006
	AhCre; LKB$^{flox/flox}$	Conditional deletion of LKB by activation of a p450 CYP1A1-driven Cre recombinase transgene (AhCre). Phenotype: PIN.	Liao et al. 2007
AR signaling	*PB-AR*	Mouse AR transgene driven by a probasin promoter. Phenotype: PIN.	Stanbrough et al. 2001
	rPB-AR-T877A and *rPB-AR-E231G*	Mouse AR transgene with a mutation in either T877A or E231G driven by a minimal probasin promoter. Phenotype (of E231G): PIN, adenocarcinoma.	Han et al. 2005
	Pes-ARKO	Conditional deletion of a floxed AR allele driven by a probasin promoter driving Cre recombinase. Phenotype: hyperproliferation.	Wu et al. 2007
FGF signaling	*PB-FGF7 (PKS) PB-FGFR2iib (KDNR)*	FGF7(PKS) or a dominant-negative FGFR2iib transgene driven by minimal probasin promoter. Phenotype: PIN; KDNR develops neuroendocrine differentiation.	BA Foster et al. 2002
	ARR2PB-FGF8	FGF8 transgene driven by ARR2PB promoter. Phenotype: PIN.	Song et al. 2002
	PB-FGF-R1(K656E) PB-FGFR1; KDNR	Enforced expression of a mutant (activated) form of FGF receptor R1 by a minimal probasin promoter, alone or with a dominant-negative FGFR2. Phenotype: PIN.	Jin et al. 2003; Wang et al. 2004
	iFGF-R1	Chemically inducible FGFR1 in prostate. Phenotype: PIN, adenocarcinoma.	Freeman et al. 2003; Acevedo et al. 2007

[a]Selected models represent the range of phenotypes, transforming events, and allelic alterations that are available for studying prostate cancer in GEM mice.

Xenograft models

Human prostate cancer cell lines or primary prostate cancer tissue can be injected subcutaneously or orthotopically into the severe combined immune deficiency (SCID) mice or nude mice (van Weerden, 2000). Xenograft models can monitor tumor progression under different experimental conditions and resemble *in vivo* situation of the original human cancer. In addition, some cancers are able to metastasize when injected into the organ of origin, providing a useful tool for evaluation of new therapies (Thalmann, 1994; Stephenson, 1992). However, this model has several limitations including impaired host immune system, low tumor take rate, incomplete tumor-stroma interactions, and heterogonous microenvironment in host-donor background. In addition, it is difficult to examine the multi-step cancer development since most xenografted tumors or tumor cell lines represent advanced tumor (Frese, 2007; Becher, 2006). There is a limited number of existing human prostate cancer cell lines and most of them lack wild type AR expression. Therefore, all of these limit their use to the study of prostate cancer initiation and progression.

Tissue recombination models

Engineered mouse models can be manipulated to investigate the functions of target genes in development, physiology and disease. However, it takes as long as 1-2 years to make transgenic mice. Current engineered mouse models use androgen-dependent promoters to drive expression of gene of interest. They are not suitable for studying the effects of modulating androgen levels, since androgen deprivation will simultaneously change transgene expression. Tissue recombination is a reliable method of inducing prostate differentiation, which drives to important advances in our

understanding of prostate biology during development and disease. A tissue recombination model can be made by mixing epithelial cells and stromal cells in a collagen gel, and then grafting under kidney capsule of SCID or nude mice. In detail, rat urogenital sinus mesenchyme (UGM) is prepared from 18-day embryonic fetuses. In this stage, the interface of urogenital sinus epithelium (UGE) and UGM is relatively simple for separation. UGE or pieces of prostatic epithelium, or dissociated prostate epithelial cells are combined with UGM cells, and then are suspended in collagen. This tissue recombinant is subsequently grafted under the renal capsule of SCID mice. After a period of growth and development the host is sacrificed and the graft is removed (Figure 8). Surgery to the renal capsule is slightly more difficult than subcutaneous grafting but the graft take is much more efficient. The function of the gene can be examined by the size and histology of the graft (Hayward, 1998; 2002). Tissue recombinants can recapitulate the pathologic features of the neoplastic prostate seen in transgenic mice (Ishii, 2005). It takes just a few days to regenerate prostatic branched network (Cunha, 1983). In order to minimize cell culture artifacts and investigate the function of target genes *in vivo*, genes of interest can either be overexpressed or knocked down by retroviral or lentiviral transduction of dissociated primary prostate cells (Xin, 2003; 2005).

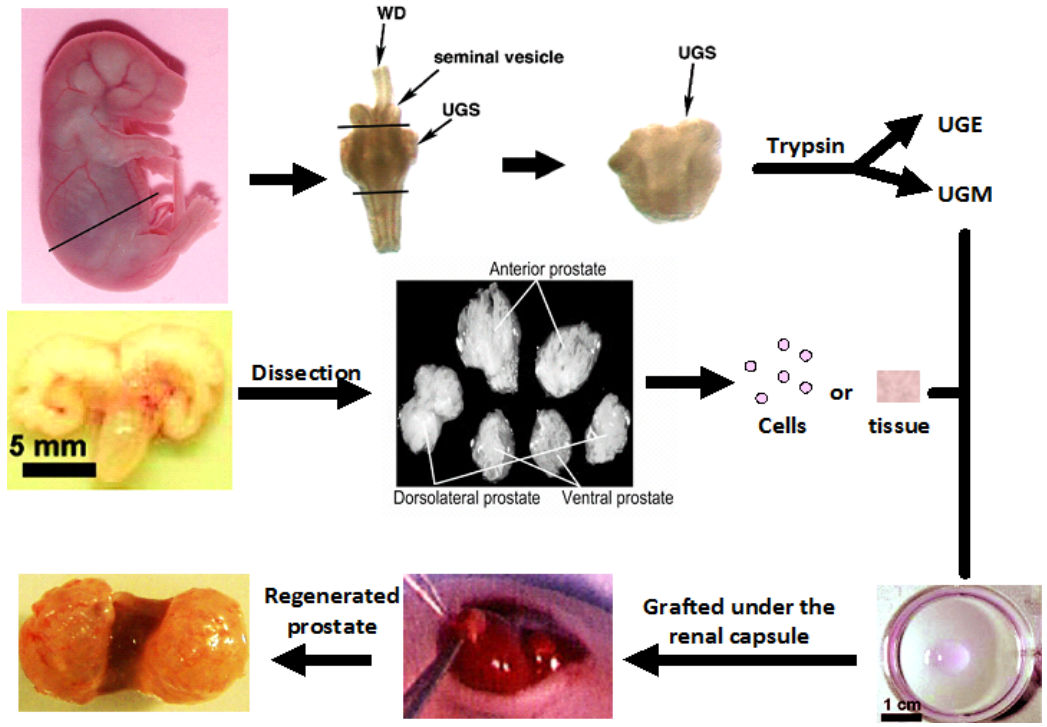

Figure 8. Diagram of tissue recombination approach. Fetal rat urogenital mesenchyme (UGM) cells are combined with adult prostate cells or pieces of tissue and collagen, and then implanted under the renal capsule to regenerate prostates.

Compared to transgenic mice that contain the transgene in every cell or in organ-specific subsets of cells under specific promoter, the tissue recombination model involves a small percentage of "transgenic" cells by retroviral or lentiviral infection surrounding normal uninfected cells within the microenvironment. These conditions more appropriately mimic those seen in the initiation of human cancer, where genetic alterations initially occur in only a few cells and not simultaneously in all cells of a given lineage. Tissue recombination model facilitates *in situ* study of gene specific effects in context of the prostate tissue. Another advantage is the elimination of culture-related artificial selection of cell populations because isolated epithelial or stromal cells can be directly used for tissue recombination. The generation of prostate cancer in the tissue recombination model not only defines a role for a specific transgene or combination in

prostate cancer, but also leads to establishment of many cell lines from cancerous and premalignant tissue grafts, which are subsequently used to further define the molecular basis of prostate cancer. This technique also allows to generate adult tissues of interest from transgenic or knockout mice that are embryonic lethal.

Instead of implanting into subrenal capsule, cells or explants can also be put into pocket created under anterior lobe of the prostatic capsule, which is called orthotopic (prostate) xenograft (Figure 9) (Wang, 2005a). This technique is useful for studying metastatisis and provides insights to develop models to test new therapies (Wang, 2005b).

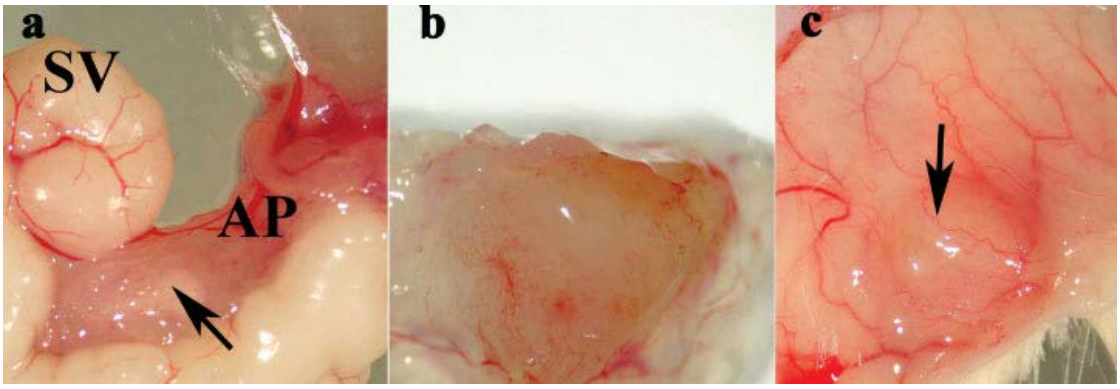

Figure 9. Orthotopic xenografts of human prostate tissue in a SCID mouse. *A,* Whole mount showing the anterior prostate orthotopic graft in place. The anterior prostate is seen in the "crook" of the seminal vesicle (SV).The graft (arrow) can be clearly seen nestled between the two main ducts of the anterior prostate (AP). ***B,*** Gentle microdissection reveals the graft which has clearly become extensively vascularized. ***C,*** Whole mount view of a sub-cutaneous graft beneath the skin of a SCID mouse (Adapted from Wang, 2005a).

Prostate stem cell and cancer stem cell

Normal prostate stem cell

Stem cells are undifferentiated cells that are able to self-renew through asymmetric cell division and undergo multi-lineage differentiation (Becker, 1963; Siminovitch, 1963). Prostate stem cells (PSCs) are capable of giving rise to differentiated

basal, luminal and neuroendocrince cells during prostate development and regeneration (Taylor, 2010).

The existence of prostate stem cells was initially implied by normal prostate regeneration after repeated cycles of androgen deprivation and restoration in rat (English, 1987). It is known that each lobe of mouse prostate contains three regions: distal, intermediate, and proximal regions. The proximal region of glands has been suggested to contain the mouse prostate stem cells (Figure 10) (Tsujimura, 2002; Burger, 2005). Tissue recombination approach also demonstrates the existence of prostate stem cells (Xin, 2003).

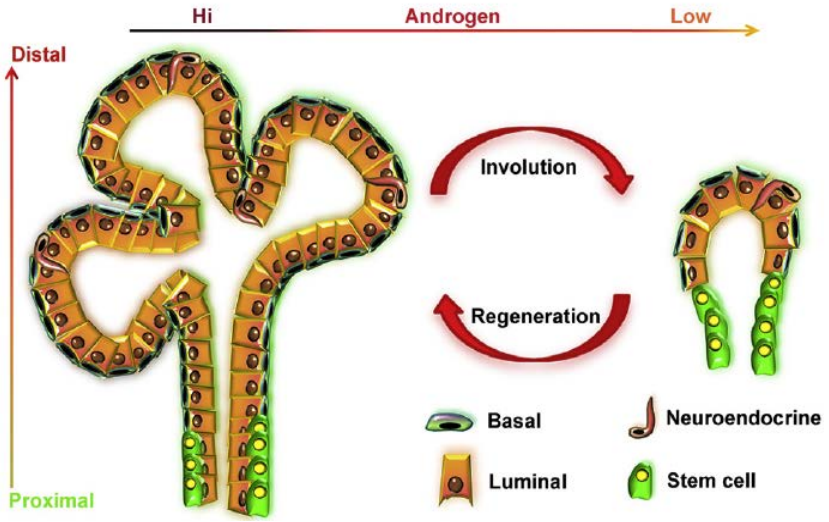

Figure 10. **Response of prostate epithelium to castration and androgen addition.** Androgen withdrawal causes massive apoptosis in the prostate epithelium, leaving behind only castration-resistant cells. Upon addition of androgen, castration-resistant cells are capable of regenerating the gland. The cycle of involution and regeneration can be repeated in the rodent prostate almost indefinitely (Adapted from Goldstein, 2010).

A lot of evidence supports the idea that PSCs reside in basal cells. For example, putative stem cell markers, Sca-1, CD44, CD49b, CD133, Bcl2, p63, CD117, Trop2, belong to prostatic basal markers (Liu, 1997; Signoretti, 2000; Collins, 2001; Richardson, 2004; Xin, 2005; Lawson, 2007; Tsujimura, 2007; Goldstein, 2008; Yao, 2010). In

contrast, some evidence supports the concept that PSCs reside in luminal cells. It has been suggested that both basal and luminal epithelium contain putative stem cells as shown by the label-retaining methods (Tsujimura, 2002). It has been shown that p63 null cells have regenerative capacity of prostate in absence of basal cells (Kurita, 2004). A recent report shows that a small population of castration-resistant luminal cells expressing Nkx3.1 (CARNs) can regenerate prostate (Wang, 2009). Leong et al. isolated a single stem cell defined by $Lin^{-}Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$ that can generate a prostate gland using tissue recombination method (Leong, 2008). Sca-1 and CD133 expression has been found in both stem and non-stem-cell types, including stromal and differentiated epithelial cells (Xin, 2005; Shmelkov, 2008). In addition, CD117 cannot be found either in basal layer or luminal layer, it remains to determine if $Lin^{-}Sca1^{+}CD133^{+}CD44^{+}CD117^{+}$ cells have epithelial origin (Goldstein, 2010). These data suggest there may be multiple independent stem cell populations within the adult prostate, which respond to different stimuli.

Cancer stem cell

Cancer stem cells (CSCs) are cancer cells that have features of normal stem cells, specifically the ability to self-renew and differentiate into multiple cell types in a specific tumor. Such cells are tumorigenic and proposed to exist in tumors, which lead to cancer relapse and metastasis by giving rise to new tumors. It is possible that cancer stem cells come from transformation of normal stem cells or progenitor cells. It is also possible that cancer stem cells can be derived from transformed differentiated cells, resulting in dedifferentiation to acquire characteristics of stem cell. There might be some factors in

the microenvironment that cause the formation of cancer stem cells and initiate tumor formation (Figure 11) (Gupta, 2009; Maitland, 2008; Bjerkvig, 2005).

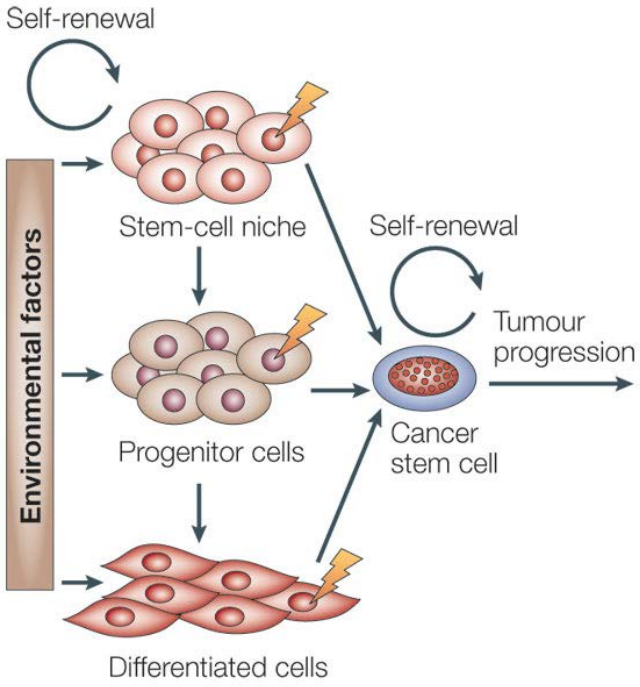

Figure 11. Potential cell origins of cancer stem cell. The diagram shows potential relationships between cancer stem cells and normal stem cells, progenitor cells, or differentiated cells. Mutations (lightning symbol) in a stem cell, progenitor cell or differentiated cell may give rise to a cancer stem cell that has self-renewal potential and form new tumors. The microenvironment may be involved in the formation of a cancer stem cell (Adapted from Bjerkvig, 2005).

At present, the origin of cancer stem cells is still not well understood. It is speculated that cancer stem cells may have luminal cell of origin since the luminal compartment expands and basal cells are lost during prostate cancer progression. It has been found that the putative prostate cancer stem cells exhibit basal cell features although prostate cancer is absence of basal cell (Collins, 2005). Transformed basal cells can generate prostate cancer with luminal phenotype (Wang, 2006; Lawson, 2007; Mulholland, 2009; Goldstein, 2008; 2010). Other studies have shown that prostate cancer

can be derived from transformed luminal cells (Ma, 2005; Korsten, 2009; Wang, 2009). Neuroendocrine cells may possibly represent a stem/progenitor cell population in prostate cancer (Palapattu, 2009). On the other hand, cancer stem cells may be derived from fusion between cancer cells and normal cells, stem cells and differentiated cells (Bjerkvig, 2005). In summary, different genetic alterations may target different cell types and induce different subtypes of cancer. Further study of cancer stem cells will provide the foundation for developing new methods in diagnosis, prevention and treatment of prostate cancer.

c-MYC

c-Myc gene was originally identified as cellular homologue of *v-Myc* which induced myelocytomatosis in birds (Vennström B, 1982; Sheiness D, 1979). Thereafter *N-Myc* and *L-Myc* were found to be amplified in neuroblastoma and small cell lung cancer, respectively (Kohl, 1983; Nau, 1985). These genes are homologous with same general topography. c-MYC is localized at chromosome 8q24.21, a region that is translocated in Burkitt's lymphoma (Dalla-Favera, 1982). The MYC genes have a short half-life of 20–30 min (Gregory, 2000). All Myc isoforms contain two independently functioning domains: a N-terminal transcriptional activation domain and a C-terminal DNA binding domain containing basic-helix-loop-helix-leucine zipper (bHLHZ) segment. Most biological functions of c-Myc require heterodimerization with its activation partner Max to the E-box sequence of c-Myc target genes (Blackwood, 1991). c-Myc also negatively regulates the transcription of genes which function to arrest the cell cycle, primarily through the c-Myc associated zinc finger protein, Miz1 (Schneider, 1997).

c-Myc (Myc, hereafter) is a multifunctional transcription factor that regulates cell cycle, growth and metabolism, differentiation, apoptosis, transformation, genomic instability, and angiogenesis (Figure 12). MYC is overexpressed in many types of tumors in human. Myc is required for embryonic stem (ES) cell pluripotency and reprogramming. Expression of Myc is generally high during early embryonic development (Cartwright, 2005; Takahashi, 2006). Myc expression is low or undetectable in differentiated adult tissues, and is dramatically increased by growth factor-induced stimulation. Gardner, Lee & Dang summarized that "Myc expression persists into the cell cycle, but then returns to its basal level in resting cells. Abnormal or ectopic overexpression of Myc in primary cells activates a protective pathway through the induction of p19/p14ARF and a p53-dependent cell death pathway. Hence, normal cells that overexpress Myc are eliminated from the host organism through apoptosis, thereby protecting the organism from lethal neoplastic changes" (Garden, 2002). Myc also inhibits cellular differentiation (Coppola, 1986), shortens the cell cycle phases G1 and G2 (Karn, 1989), Myc has been implicated in inducing cyclin D1 and D2, cyclin E, CDK4, and Cdc25A, a phosphatase, which activates CDK2 and CDK4 (Bouchard, 1999; Coller, 2000; Hermeking, 2000). Myc has also been shown to reduce the level or inhibit the function of the CDK inhibitors p15, p21, and p27 (Gartel, 2003). A highly regulated cell cycle permits cells to repair DNA damage before replicating, thus protecting genomic integrity. Inappropriate regulated cell cycle results genomic instability. Myc overexpression has been reported to cause gene amplification, aneuploidy and polyploidy (Kuttler, 2006; Prochownik, 2008). Other studies suggest that Myc induces the production of reactive oxygen species (ROS), leading to DNA damage and genomic instability (Ray, 2006). Overexpression of MYC in

human cancers may contribute to enhanced tumor glycolysis, known as the Warburg effect (Figure 12) (Dang, 2010).

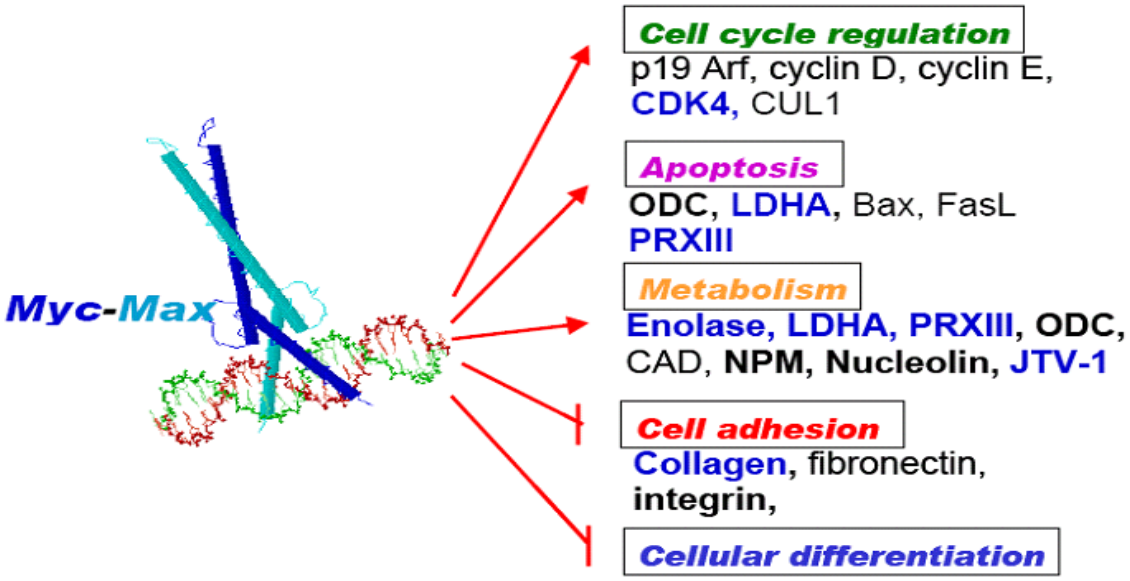

Figure 12. The Myc-Max heterodimer binds and activates E-box of target genes, and regulates downstream of target genes resulting in activation cell cycle regulation, apoptosis, or inhibition of cell adhesion (Adapted from Gardner, Lee & Dang, The Encyclopedia of Cancer, Second Edition, July 2002).

In addition, Myc transcriptionally regulates a number of non-coding RNA transcripts including ribosomal RNAs, tRNAs and microRNAs (Cole, 2008). Myc also has a role in DNA replication, in which directly interacts with components of the replication machinery to positively regulate DNA synthesis. Overexpression of Myc induces inappropriate replication origin firing leading to replication stress and genomic instability evidenced by the activation of a DNA damage response (Dominguez-Sola, 2007).

Pim1 kinase

The *pim1* gene was initially identified as a proviral insertion site of the Moloney Murine Leukemia Virus (MoMuLV) (Cuypers, 1984). *Pim1*, *Pim2* and *Pim3* belong to

Pim family. They are conserved in vertebrates and show sequence and structural similarity (Mikkers, 2004). Pim family is constitutively active serine/threonine kinase that does not require posttranslational modification to be activated. Many cytokines, growth factors, hormones, and hypoxia can induce Pim1 expression (Dautry, 1988; Lilly, 1992; Mui, 1996; Aho, 2005; Wang, 2001; Bachmann, 2005; Magnuson, 2010). Pim1 has a short half-life with 5 to 10 minutes in primary cells (Saris, 1991; Liang, 1996). The *pim1* gene encodes two proteins of 33kD, and 44 kD using an alternative translation initiation at an upstream CUG codon (Saris, 1991). 33kDa Pim1 is primarily in the cytoplasm, whereas 44kDa Pim1 is primarily on plasma membrane (Xie, 2006).

Pim1 structure

Pim1 structure has been solved and demonstrated by several groups (Qian, 2005; Jacobs, 2005; Kumar, 2005). The binding site for ATP is located in a deep cavity formed by the N-terminal lobe containing anti-parallel β-sheets, and the C-terminal lobe containing α-helices. The active state of kinases is characterized by the presence of the conserved lysine-glutamate salt bridge (Lys67 and Glu89). The replacement of a lysine at position 67 by a methionine causes inactivation of the kinase (Figure 13). The Pim1 is post-transcriptionally regulated by eIF-4E (Hoover, 1997), stabilized by Hsp90 (Mizuno, 2001) and degraded by PP2A (Losman, 2003).

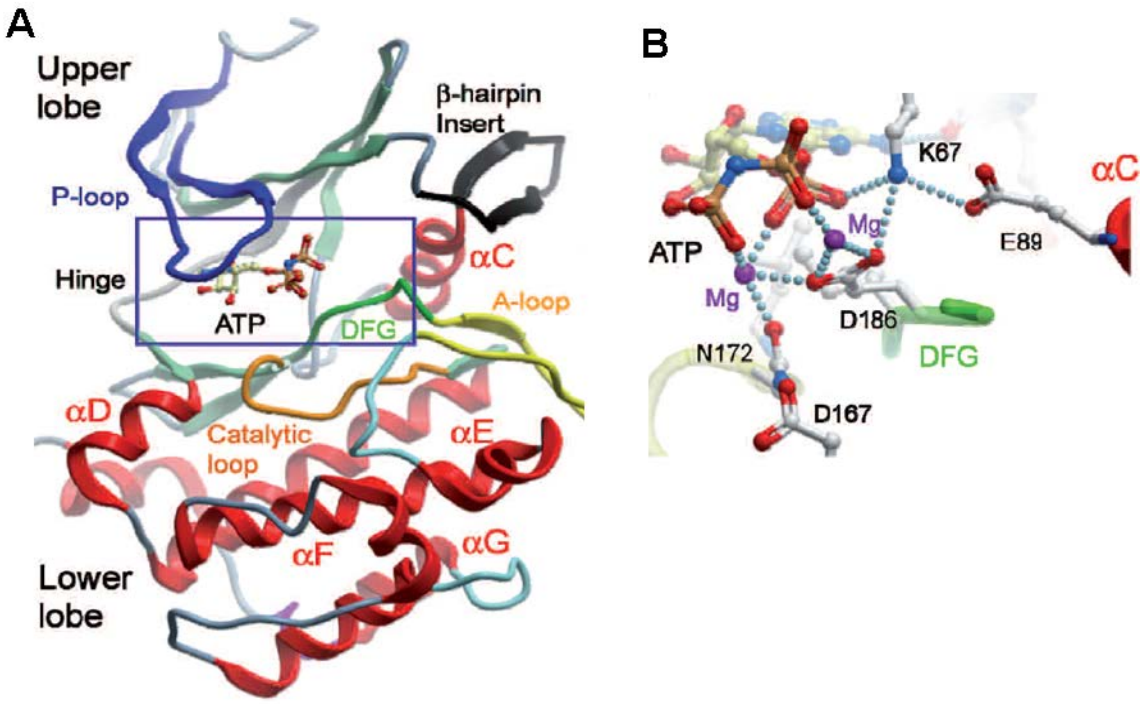

Figure 13. Structural aspects of PIM1. ***A,*** Structural overview of the PIM1 crystal structure in complex with the nonhydrolysable ATP analogue AMPPNP. Regulatory elements (the phosphate binding (P)-loop, the magnesium binding motif DFG, the hinge region, the catalytic loop and the activation segment (A)-loop and the β-hairpin insert) are labelled and highlighted using different colors. ***B,*** Details of the interaction of the phosphate moieties of AMPPNP and Mg^{2+} with the D186FG motif, the conserved lysine (K67) and glutamate (E89) and the catalytic aspartate (D167). The enlarged region corresponds to part of the boxed area in A (Adapted from Brault, 2010).

Pim1 activation and cytokine signaling

Pim1 is induced by a large set of cytokines and is a downstream target for many cytokine-signaling pathways (Dautry, 1988; Lilly, 1992, Mui, 1996; Aho, 2005). STAT3/STAT5 can bind to the *Pim1* promoter and upregulate Pim1 expression. Pim1 itself can negatively regulate the Jak/STAT pathway by binding to SOCS1 and SOCS3, which are negative regulators of STAT signaling (Chen, 2002; Peltola, 2004). It is proposed that overexpression of Pim1 and other oncogenes, such as Myc, impairs this negative feedback, leading to hyperproliferation and tumor formation.

Pim1 and cell survival

Pim1 is responsible for protecting cells from undergoing cellular apoptosis under adverse conditions (Moroy, 1993; Rahman, 2001). Pim1 phosphorylates Bad on serine 112 to inactivate it and enhances Bcl-2 activity, thereby promoting cell survival (Aho, 2004). Recent reports indicate that other Pim1 substrates are associated with cell survival. One of them is the proapototic transcription factor FOXO3a, which is inactivated by Pim1 (Morishita, 2008). Apoptosis signaling kinase 1 (ASK1) is also phosphorylated and inactivated by Pim1 (Gu, 2009). The other protein is MDM2, which is phosphorylated by Pim1, promotes cell survival (Hogan, 2008).

Pim1 and cell cycle

Pim1 promotes cell cycle G1/S progression by binding and phosphorylating the phosphatase Cdc25A (Mochizuk, 1999), cell cycle inhibitor $p21^{Waf}$ (Wang, 2002; Zhang, 2007) and $p27^{Kip1}$ (Morishita, 2008). Pim1 also phosphorylates Cdc25C associated kinase 1(C-TAK1) and Cdc25C to promote transition from the G2 phase into mitosis (Bachmann, 2004). Nuclear mitotic apparatus (NuMA), responsible for the organization of the spindle apparatus in the M phase, is phosphorylated by Pim1 (Bhattacharya, 2002). Heterochromatin protein (HP) 1γ is identified as a Pim1 substrate (Koike, 2000). Pim1 overexpression in human prostate cells leads to defects in mitotic spindle checkpoints, resulting in polyploidy and chromosome mis-segregation (Roh, 2003; 2005).

Pim1 is a potential diagnostic biomarker

Pim1 is up-regulated in human leukemia, lymphoma, prostate cancer, pancreatic cancer, gastric cancer, and head and neck squamous cell carcinoma (Amson, 1989; Dhanasekaran, 2001; Shah, 2008; Brault, 2010; Magnuson, 2010). The level of Pim1

expression correlates with the clinical outcome of prostate cancer patients despite the absence of Pim1 expression at some advanced metastatic tumor (Figure 14) (Dhanasekaran, 2001). Strong expression of Pim1 has been found to be associated with advanced prostate cancer. Detection of *pim1* mRNA levels in prostate cancer demonstrates similar results with previous findings (Xu, 2005; He, 2009). Some PIN lesions display moderately strong Pim1 staining, which indicates Pim1 expression may be a potential early event in the development of prostate cancer (Valdman, 2004; Cibull, 2006; van der Poel, 2010).

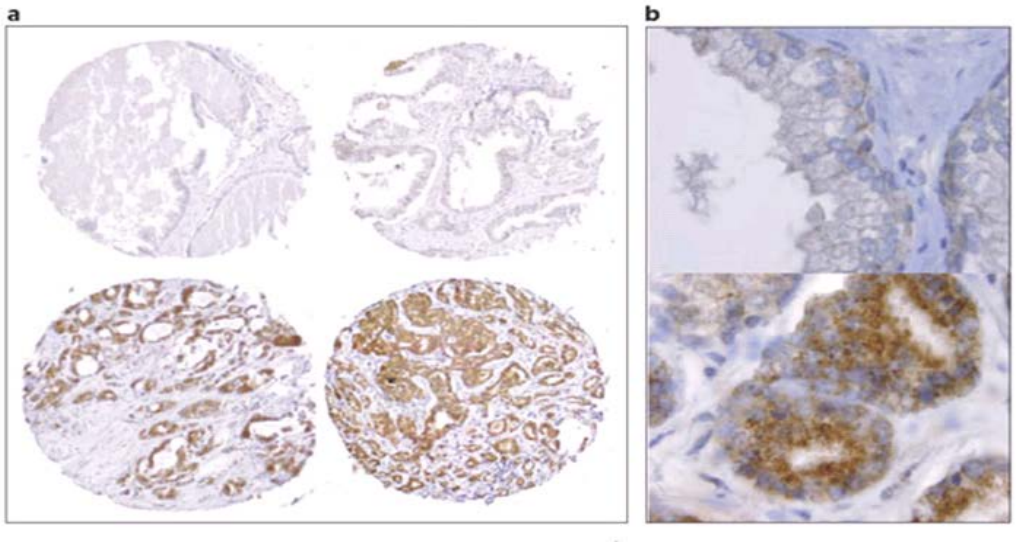

Figure 14. Representative elements of a tissue microarray stained with anti-PIM1 antibody. *a*, Staining is absent or weak in benign prostate (top), but strong in the cytoplasm of localized prostate cancer (bottom). ***b*,** PIM1 expression is absent or weak in the secretory luminal cells of benign prostate glands (top), but strong in infiltrating prostate cancers (bottom) (Adapted from Dhanasekaran, 2001).

Pim1 is a potential therapeutic biomarker

Pim1 knockout mice are viable and fertile with subtle phenotypes in hematopoietic system and cytokine response (Laird, 1993; Domen, 1993). The feature of cell tolerated by Pim1 loss makes Pim1 as an attractive therapeutic target. In addition, Pim1 structure has been solved, which facilitates the development of Pim1 inhibitors.

Pim1 inhibitor quercetagetin is able to inhibit PIM1 activity in prostate cancer cells in a dose dependent fashion (Holder, 2007). SGI-1776 is another Pim1 inhibitor. Prostate cancer cells or B-cell chronic lymphocytic leukemia cells (CLL) treated by SGI-1776 show a concentration dependent induction of apoptosis (Mumenthaler, 2009; Chen, 2009). CLL cells treated with SGI-1776 show decrease phosphorylation of Myc on Serine 62 (Chen, 2009b). Currently, this compound is being used in human clinical trials for prostate cancer and lymphoma. In addition, it has been reported that Pim1 induces multi-drug resistance by increasing expression of two ABC transmembrane proteins - Pgp and BCRP (Xie, 2008; 2010). Therefore, inhibiting Pim1 kinase is a novel approach to abrogate Pgp and BCRP mediated drug resistance. Indeed, SGI-1776 sensitizes prostate cancer cell to chemotherapy treatment (Mumenthal, 2009). In addition to chemical Pim1 inhibitors, anti-PIM1 monoclonal antibodys (mAbs) are reported to function as Pim1 inhibitors. It has been shown that anti-PIM1 mAbs lead to disrupt PIM1/Hsp90 complexes, reduce BAD phosphorylation at S112 and induce apoptosis (Hu, 2009). All of these studies demonstrate that Pim1 can be a valuable therapeutic target.

Pim1 and Myc synergism

Pim1 shows strong synergism with Myc in lymphomagenesis. Pim1 induces lymphoma at a low frequency with a long latency (Cuypers, 1984; van Lohuizen, 1989). However, all mice overexpressing Myc and Pim1 died of leukemia in utero (Verbeek, 1991). In human prostate cancer, both PIM1 and MYC are upregulated and suggest there is cooperation between them (Dhanasekaran, 2001; Ellwood-Yen, 2003). Pim1 is also overexpressed in Myc-driven prostate cancer mouse model (Ellwood-Yen, 2003). However, whether Pim1 and Myc do cooperate in prostate carcinogenesis has not been

demonstrated previously. The possible mechanisms of synergism between Myc and Pim1 kinases have been reported according to *in vitro* studies: (1) Pim1 decreases PP2A activity, thereby decreasing dephosphorylated Myc S62, resulting in Myc stabilization (Chen, 2005). (2) Pim1 binds Myc, thereby increasing phosphorylation of Myc on serine 62 and decreasing phosphorylation of Myc on threonine 58, resulting in stabilized Myc (Chen, 2005; Zhang, 2008). (3) Myc recruits Pim1 to the E boxes of the Myc target genes and phosphorylates histone 3 at serine 10, contributing to the activation of Myc target genes and cellular transformation (Zippo, 2007) (Figure 15). Although those mechanisms can partially explain the cooperation between Pim1 and Myc, the mechanisms of robust cooperation between Pim1 and Myc *in vivo* are still unclear.

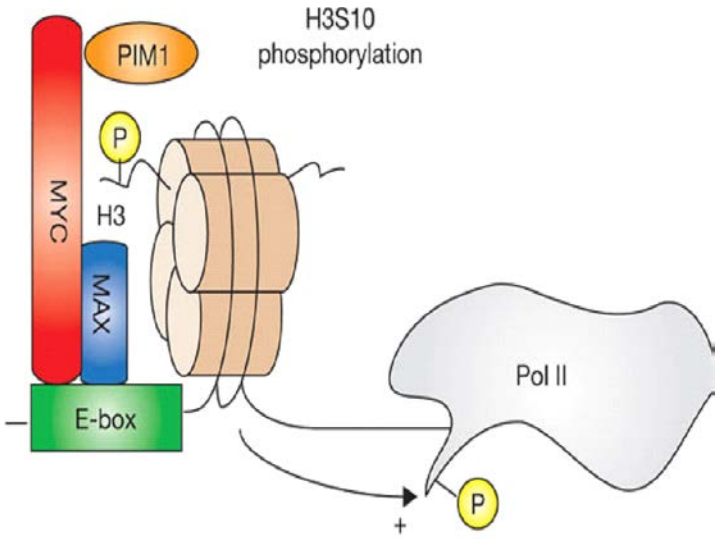

Figure 15. PIM1 is recruited to the E-box element by its interaction with MYC. The MYC–MAX–PIM1 complex brings about the phosphorylation of H3S10, stimulates the binding of RNA polymerase II (Pol II) and contributes to the transcriptional activation of a subset of Myc target genes (Adapted from Zippo, 2007).

CHAPTER II

PIM1 KINASE SYNERGIZES WITH C-MYC TO PROMOTE PROSTATE CANCER PROGRESSION

Introduction

An important area in contemporary cancer research is defining the causative genetic alterations in tumors and their utility as molecular targets. In this regard, gene expression profiling studies have identified overexpression of the serine-threonine kinase PIM1 in a significant fraction of human prostate tumors where its expression is found to be tightly associated with that of MYC (Dhanasekaran, 2001; Valdman, 2004). Pim1 is also overexpressed in Myc-driven transgenic mouse prostate tumors (Ellwood-Yen, 2003). Previous studies in mouse lymphoma models have shown that Pim1 and Myc synergize to promote lymphomagenesis (van Lohuizen, 1989; Verbeek, 1991; Möröy, 1991).

Pim1 is able to interact and phosphorylate several targets that are involved in cell cycle progression or apoptosis. Pim1 can inhibit apoptosis through interactions with the anti-apoptotic molecules, bcl-2 and Gfi-1 (Schmidt, 1998) or by inactivating phosphorylation of Bad at serine 112 (Aho, 2004). Substrates of Pim1 involved in cell cycle regulation include $p21^{Cip1}$ (Wang, 2002), $p27^{Kip1}$ (Morishita, 2008), NuMA (Bhattacharya, 2002), Cdc25A (Mochizuki, 1999), Cdc25c (Bachmann, 2006) and c-TAK1 (Bachmann, 2004). Pim1 overexpression increases the tumorigenicity of human prostate cancer cell lines (Ellwood-Yen, 2003; Chen, 2005; Bhattacharya, 2002; Roh, 2005; 2008; Kim, 2010). Pim kinases may modulate phosphorylation of c-Myc on Ser62

through decreasing PP2A activity (Chen, 2005). Pim1 can increase Ser62 phosphorylation while decrease Thr58 phosphorylation, thus increase c-Myc stability (Zhang, 2008). It has been showed that PIM1 binds MYC, modifys chromatin at at MYC binding sites, and activates MYC target genes (Zippo, 2007). All of these observations suggested the possibility that Pim1 may cooperate with Myc in prostate tumorigenesis. However, whether Pim1 does indeed cooperate with Myc in prostate carcinogenesis *in vivo* has not been conclusively demonstrated.

In this chapter, we first showed that coexpression of MYC and PIM1 in human prostate cancer samples is correlated with high tumor grade. Then we used a tissue recombination model to directly examine cooperativity between MYC and Pim1 in prostate tumorigenesis and the possible role of Pim1 kinase activity in this process. The results revealed a potent synergy between Pim1 and MYC in prostate cancer progression that is critically dependent on Pim1 kinase activity.

Materials and Methods

Lentiviral constructs

Mouse Pim1, the kinase-dead mutant K67M was amplified using pMSCV-Pim1, pMSCV-K67M vector as templates (Roh, 2003). Human c-MYC was amplified using pHAMyc (kindly provided by Dr. L. Lee, Johns Hopkins University). Human c-MYC S62D mutant was cloned by mutating serine at position 62 (TCC) into aspartic acid (GAC) using PCR. Each above PCR product was cloned into the lentiviral transfer vector FM-1 biscistronically expressing YFP (kindly provided by Dr. Jeffrey Milbrandt,

Washington University) and was verified by restriction enzyme digestions and sequencing.

Lentiviral preparation

Lentivirus was produced by cotransfecting the transfer vector containing the gene of interest, the VSVG envelope glycoprotein, and the HIV-1 packaging vector Δ8.9 (kindly provided by Dr. David Baltimore, Caltech) into 293FT cells (Invitrogen) using PEI reagent (Sigma). Virus supernatant was collected at 24, 48 and 72 hrs post-transfection, and then was filtered through 0.45 μm filter. Virus supernatant was concentrated by ultrafiltration using Centricon-70 following manufacturer's instruction (Millipore, UFC710008). Concentrated virus was stored at -80ºC. Viral titers were determined by infecting HT1080 cells with serial dilutions of virus and followed by flow cytometric quantification of YFP-positive cells after 3 days of infection. In parallel, infected cells were visualized under a fluorescence microscope and cell lysates were prepared for Western blot.

Tissue recombination

All lobes of prostates were isolated from 6 week old C57BL/6 mice, minced and digested with collagenase, Trypsin, Dispase, DNase I, and passed through 100μm nylon mesh (BD Biosciences). Dissociated prostate cells were infected with lentivirus at MOI 50-100 in the presence of 8 μg/ml polybrene using the centrifugation method (Xin, 2003). Rat fetal urogenital mesenchyme (UGM) was prepared from 18-day embryos. Urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by tryptic digestion as described previously (Hayward, 1998). Single UGM cells were then prepared by a 90-min digestion at 37°C with 187 units/ml collagenase. 1-

2×10^5 cells were recombined with 2.5×10^5 rat urogenital mesenchyme (UGM) and suspended in rat tail collagen prepared as described (Hayward, 1998). The recombinants were incubated overnight and subsequently placed beneath the renal capsule of male SCID mice. Six or 12 weeks after grafting, the hosts were sacrificed. Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

Histology and immunohistochemistry

Histological and immunohistochemical analyses were performed as described (Abdulkadir, 2001a; Abdulkadir, 2001b). Human tissue microarray was purchased from US Biomax, Inc. The following antibodies were used for immunohistochemical analysis: E-cadherin (BD Biosciences), smooth muscle actin (Sigma), synaptophysin (BD Biosciences), androgen receptor, c-MYC, FoxA2 and Nkx3.1 (all from Santa Cruz Biotechnology), p63 (Biocare Medical), cytokeratin 8 (Covance), Ki67 (Abcam), phospho histone H3 (Millipore), Ascl1 (BD Pharmingen), activated caspase 3 (Cell Signaling), chromogranin A, NSE (kindly provided by Dr. Robert Matusik, Vanderbilt University). For Ki67 and activated caspase3 quantitation, we counted at least 1000 cells per graft. For human prostate tumors samples, tissue arrays from Imgenex were stained by double immunofluorescence for MYC (1:15,000 with Tyramide Signal Amplification, Perkin-Elmer) and PIM1 (Santa Cruz Biotechnology, 1:50) as described (Kim, 2009). Coexpression was scored in samples where at least 50% of the cells coexpressed both antigens. Epithelial staining intensity was scored where $\geq$ 5% of cells show staining on a 4-point scale (0=negative, 1=weak, 2=intermediate, 3=strong) and samples were

categorized as either not overexpressing (scores 0, 1) or overexpressing (scores 2, 3) the antigen. Tissue histology was confirmed by H&E staining.

Western blot analyses

Western blot analyses were performed using the following antibodies: c-MYC, Pim1, AR (Santa Cruz Biotechnology, 1:500); β-actin, Cyclins D1, D2 , E (Santa Cruz Biotechnology, 1:1000); MYC phospho S62 (Abcam, 1:1000).

Statistical analysis

We compared groups by using student's t-test or Chi-square test (http://www.quantpsy.org). Values were considered statistically significant at $P < 0.05$. Quantitative variables are expressed as means $\pm$ SD while categorical variables are expressed as numbers (%).

Results

Co-expression of MYC and Pim1 in human prostate cancer

To examine whether MYC and PIM1 proteins co-expressed in human prostate cancer, we employed double immunohistochemical staining of MYC and PIM1 in tissue microarrays (TMAs) from total prostatectomy specimens. In 91 specimens, MYC staining was observed in 52 (57%) cases and PIM1 staining was present in 58 (64%) tumor samples. There was considerable overlap between samples that express MYC and PIM1 (44.4%) (Figure 16A, B). In addition, among 28 samples with Gleason grade 4/5, 17 (61%) tumor samples were MYC and PIM1 double positive. Coexpression of MYC and PIM1 was significantly correlated to higher Gleason grades (Figure 16C). These results are consistent with, and extend previous findings that MYC and PIM1 mRNA are

frequently coexpressed in human prostate tumors (Dhanasekaran, 2001; Ellwood-Yen, 2003).

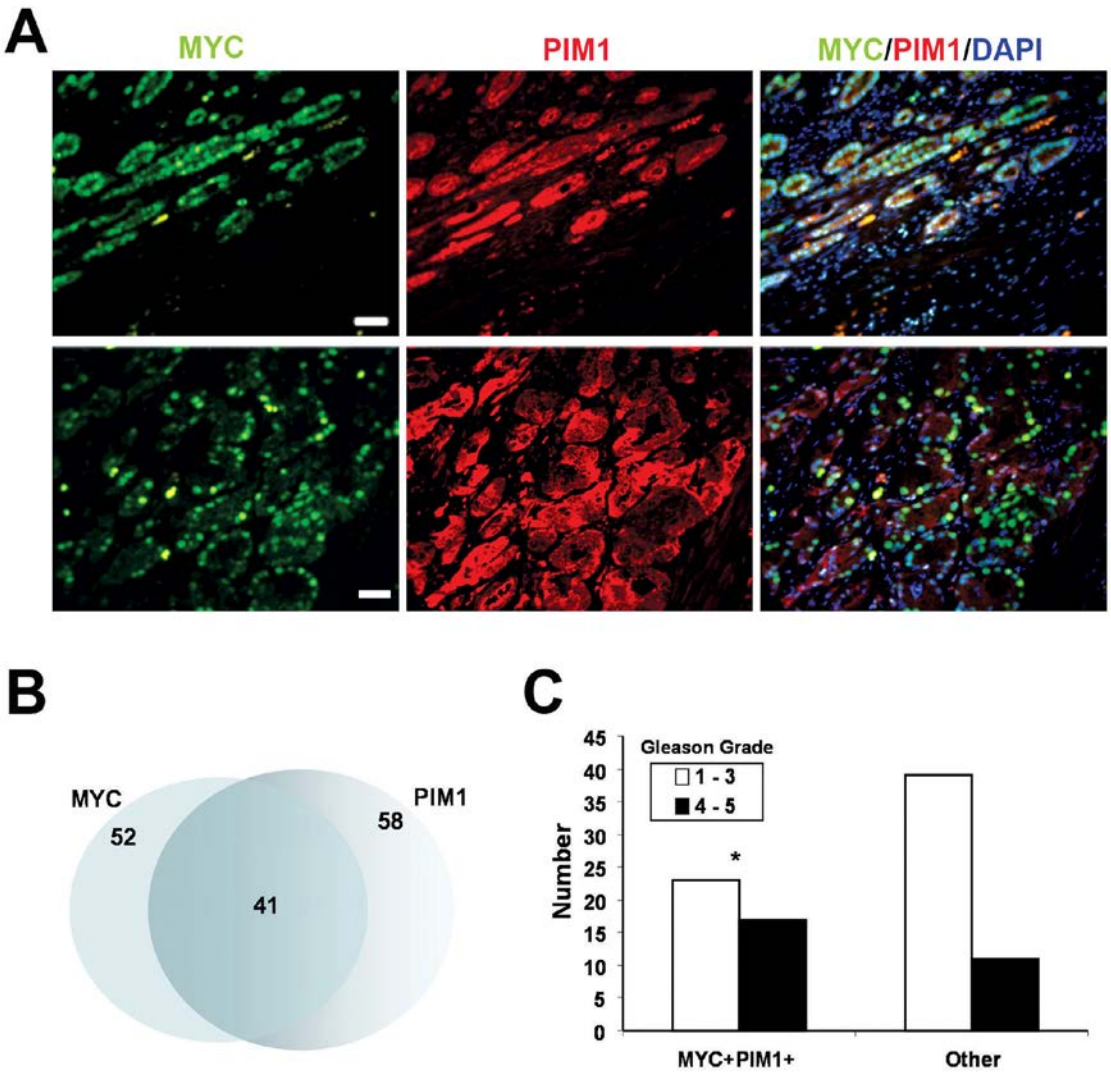

Figure 16. Coexpression of MYC and PIM1 in human prostate tumors. ***A,*** Representative micrographs of human prostate tumor samples showing co-expression of c-MYC (green) and PIM1 (red) by double immunofluorescence staining. DAPI was used as a nuclear counter stain. ***B,*** Venn diagram showing overlap between samples positive for MYC and PIM1 overexpression. ***C,*** Gleason grade distribution of samples coexpressing MYC and PIM1 (MYC+PIM1+) compared to samples without coexpression of MYC and PIM1 (other). MYC+PIM1+ samples are associated with high Gleason grades. *P < 0.05.

Pim1 and MYC synergize to accelerate prostate cancer progression

Lentiviral transfer vector expressing Pim1, or MYC was cloned into FM1 control vector, which bicistronically expresses yellow fluorescent protein (YFP) variant, Venus. To study whether Pim1 and Myc cooperation depends on kinase activity, we also cloned kinase-dead Pim1 mutant K67M. Lentivirus was produced, and all of concentrated virus titer was among 10^7- 10^8 infectious units (I.U.)/µl. The expression of target genes was determined using fluorescence microscopy and Western blot (Figure 17).

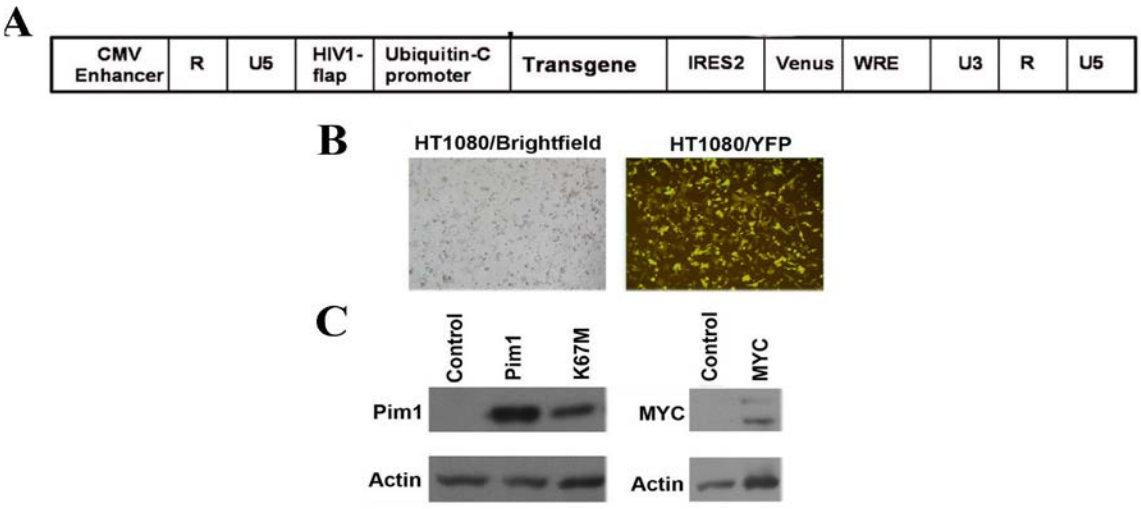

Figure 17. Cloning lentiviral constructs and preparation of lentiviru expressing Pim1, or K67M, or MYC. ***A,*** Schematic of the bicistronic lentiviral vector FM-1 used to target transgene expression together with YFP/Venus. ***B,*** Microscopic detection of YFP fluorescence in lentiviral infected HT1080 cells. ***C,*** Western blot for detection of target protein expression in lentiviral infected HT1080 cells.

To study the effects of MYC and Pim1 on naïve mouse prostate epithelium, we employed tissue recombination with lentiviral-mediated gene transfer (Xin, 2003). We infected dissociated prostate cells from 6-week old C57BL/6 mice with the control, Pim1, K67M or MYC-expressing lentiviruses singly or in combination (MYC/Pim1 or MYC/K67M). Cells were combined with rat UGM and grafted under the renal capsules

of SCID mice (Figure 18A). After six weeks, gross examination showed that the MYC/Pim1 grafts formed large hemorrhagic tumors while control, or Pim1, or K67M, or MYC and MYC/K67M grafts were small and did not differ significantly in their sizes (Figure 18B). Western blot confirmed appropriate transgene expression (Figure 18C). In MYC/Pim1 tumors, MYC and Pim1 proteins levels appear elevated, which may be due to the increased cellularity of tumors or other mechanisms such as increased protein stability. It is known that both Myc and Pim1 have short half-lives (Saris, 1991; Yeh, 2004) and Pim1 inhibits Myc degradation in a kinase-dependent manner (Zhang, 2008).

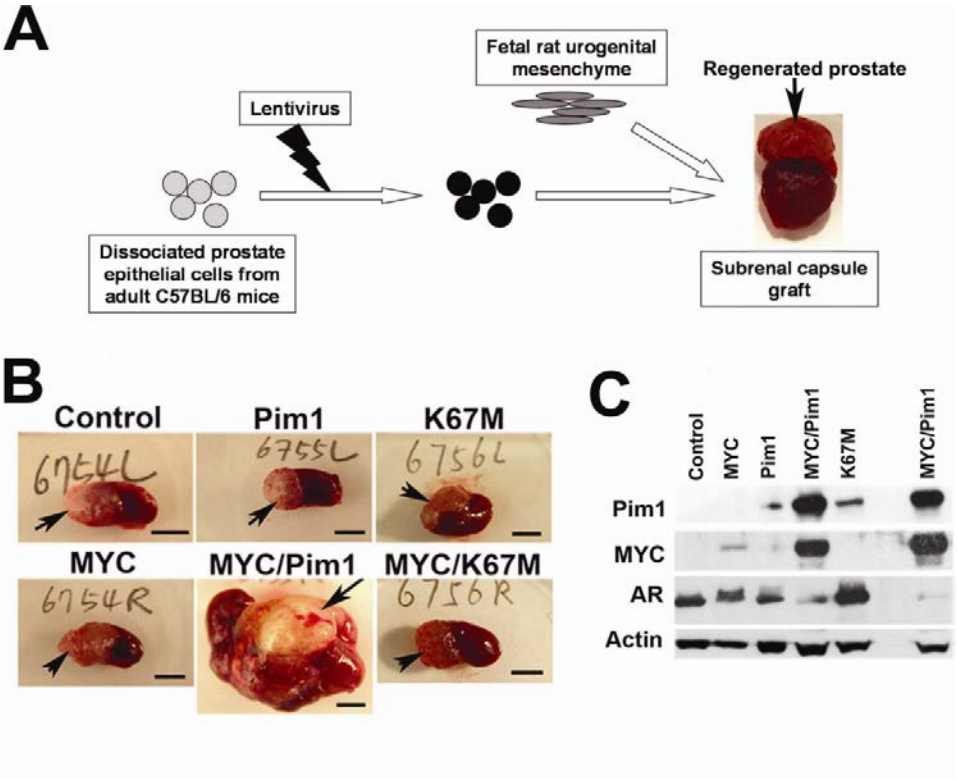

Figure 18. Tissue recombination coupled with lentiviral-mediated gene transfer for expression of MYC and Pim1 in regenerated mouse prostate. ***A,*** Scheme used for prostate recombination. Primary mouse prostate epithelial cells were infected with the indicated lentiviruses and recombined with fetal rat urogenital mesenchyme to regenerate prostates. ***B,*** Representative images of sub-renal capsule grafts (arrows) after 6 weeks. Scale bar, 5mm. ***C,*** Western blot analyses from 6-week graft tissue lysates with the indicated antibodies.

Histologically, all grafts from control (N=7), Pim1 (N=8) or K67M (N=8) group consisted of normal-looking prostatic glands (Figure 19). Two of Pim1 grafts showed very mild hyperplasia (data not shown), consistent with a weak oncogene. In contrast, all MYC (N=19) and MYC/K67M (N=11) grafts showed multiple foci of high-grade PIN (HGPIN), a putative precursor lesion for prostate carcinoma. These lesions are characterized by nuclear pleomorphism, prominent nucleoli, high mitotic activity, apoptotic figures and stromal hypercellularity. None of the MYC alone or MYC/K67M 6-week grafts showed evidence of invasive cancer (Figure 19). Strikingly, MYC/Pim1 grafts (N=17) consisted of prostate tumors growing as sheets of cells with notable rosette formation upon histological examination. The tumor cells had bubbly cytoplasm, vesicular nuclei, high nuclear: cytoplasmic ratios, large nuclei and prominent nucleoli (Figure 19). These tumors were also highly vascular. We observed 3 mice developed metastatic carcinoma and one of them died in the sixth week. These results indicate potent cooperation between MYC and Pim1 in prostate tumorigenesis which is critically dependent on Pim1 kinase activity.

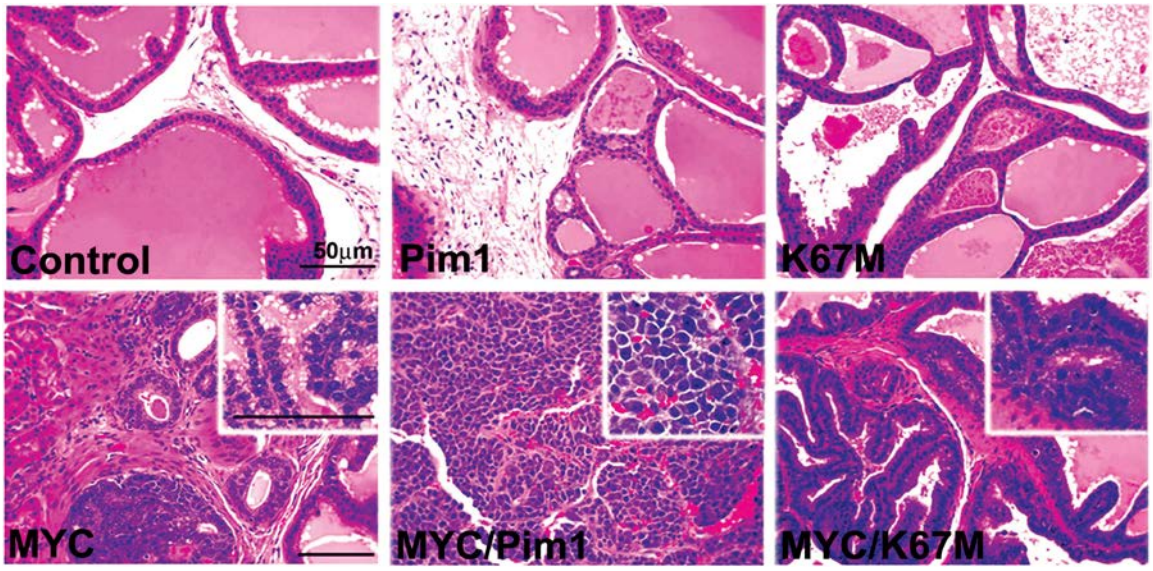

Figure 19. MYC/Pim1 coexpression leads to high-grade prostate cancer within 6 weeks. H&E stained sections of 6-week grafts show normal-looking prostatic glands in control, Pim1 and K67M grafts. MYC and MYC/K67M samples show HGPIN lesions and hypercellular stroma. The MYC/Pim1 samples show high-grade tumor consistent with neuroendocrine carcinoma. Scale bar, 50 µm. Insets: Higher-magnification images.

Evidence of neuroendocrine differentiation in MYC/Pim1 tumors

Normal prostatic epithelium consists of basal and luminal cells, as well as neuroendocrine (NE) cells. To characterize the cell types present in the regenerated grafts, we performed immunohistochemical analyses using a panel of cellular markers: androgen receptor (AR), E-cadherin, CK8, p63, smooth muscle actin (SMA), synaptophysin. Control, Pim1 and K67M, MYC or MYC/K67M grafts showed positive staining patterns for all of these markers (Figure 20). MYC/Pim1 tumors showed loss of AR, E-cadherin, p63, CK8, SMA and tumor suppressor Nkx3.1 (Figure 20, 22). These tumors ubiquitously expressed the NE marker synaptophysin (Figure 20). Further analysis showed that these tumors also expressed the neurogenic transcription factor Ascl1 (Hu, 2004; Vias, 2008) and neuron-specific enolase (di Sant'Agnese, 1987) (Figure 21). However, MYC/Pim1 tumors did not express FoxA2, which is shown in some

neuroendocrine tumors (Mirosevich, 2006) (Figure 22). These results demonstrate that MYC/Pim1 co-expression leads to the development of high-grade cancer with characteristics of NE differentiation within 6 weeks.

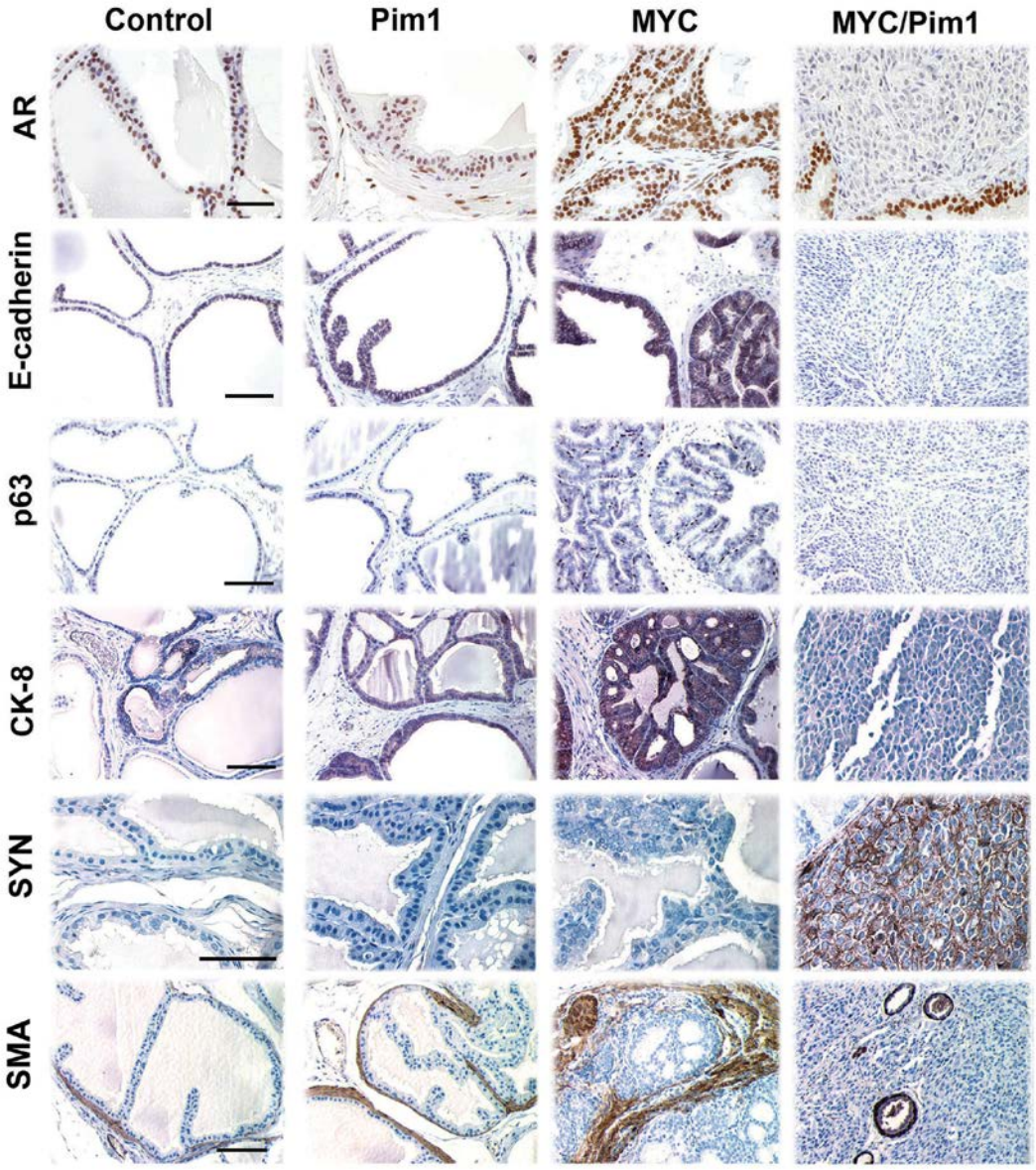

Figure 20. MYC/Pim1 co-expression shows evidence of neuroendocrine (NE) differentiation. Immunohistochemical analysis of 6-week grafts for expression of androgen receptor (AR), E-cadherin, p63, cytokeratin 8 (CK8), synaptophysin (SYN) and smooth muscle actin (SMA). MYC/Pim1 tumors show low or loss of AR, E-cadherin, p63 and CK8 expression and strong expression of synaptophysin. HGPIN lesions in MYC group strongly express SMA in the hypercellular stroma. Note loss of SMA staining in the MYC/Pim1 tumors, consistent with their invasive nature. SMA positive cells surrounding blood vessels in MYC/Pim1 tumor served as internal positive controls. Scale bars, 50 μm.

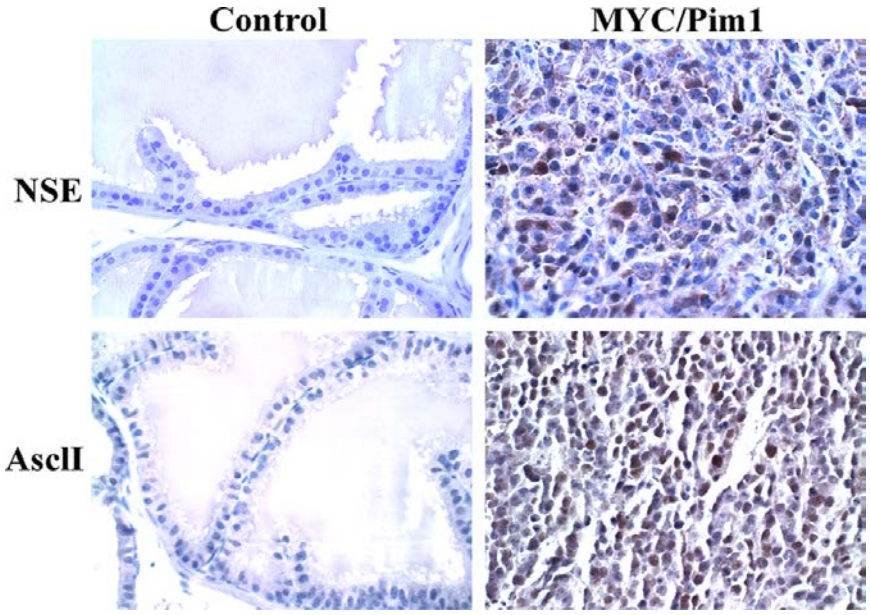

Figure 21. Heterogeneous NSE and AsclI expression (brown) in MYC/Pim1 tumors. Control grafts show negative NSE and AsclI staining (Original magnification: 400×).

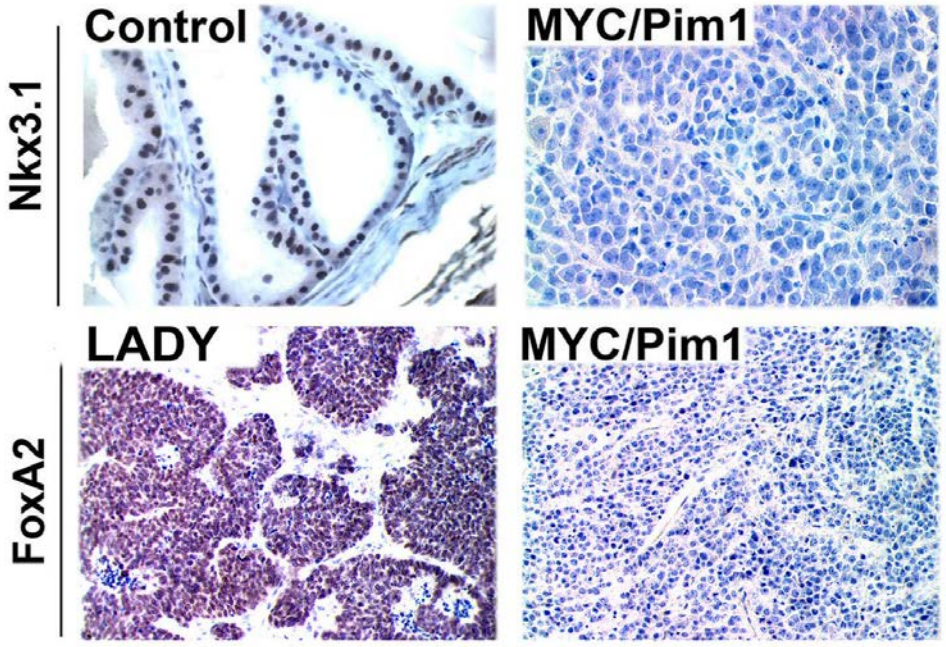

Figure 22. Loss of expression of Nkx3.1 and FoxA2 in MYC/Pim1 tumors. *Upper*, immunochemical staining shows Nkx3.1 expression in control grafts, but loss of expression in MYC/Pim1 tumors (Original magnification: 400×). *Lower*, Staining for FoxA2 shows lack of expression in MYC/Pim1 tumors (Right). Left: positive control from liver metastasis of the LADY transgenic mice (kindly provided by Dr. Xiuping Yu and Dr. Robert Matusik, Vanderbilt University) shows nuclear expression (brown). (Original magnification: 200×). LADY transgenic mice develop prostate cancer due to SV40T antigen expression in the prostate (Masumori, 2001).

Notably, the MYC and MYC/K67M grafts were strongly positive for smooth muscle actin staining in the surrounding hypercellular stroma (Figure 20 and data not shown). Stromal reaction has been noted in association with HGPIN lesions in several mouse models of prostate cancer (Shappell, 2004). Since MYC expression was not found in the stromal part, the hypercellular stroma may result from an invasive phenotype (Figure 23).

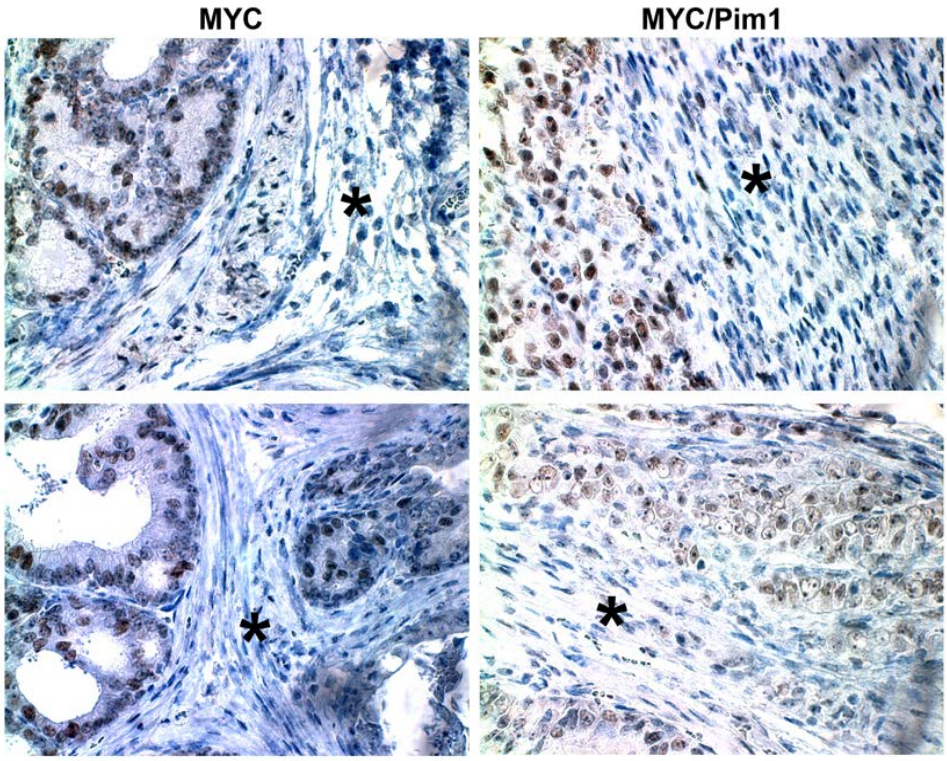

Figure 23. Immunohistochemical analysis of MYC expression in 6 week of MYC and MYC/Pim1 grafts. Note nuclear MYC expression (brown) in HGPIN glands and tumor cells and absence of MYC expression in stroma (*) (Original magnification: 400×).

MYC/Pim1 induced NE tumors arise from transdifferentiation of adenocarcinoma

Neuroendocrine prostate cancer may arise directly from the transformation of rare neuroendocrine cells in the prostate or via the transdifferentiation of adenocarcinoma (Cindolo, 2007). If the neuroendocrine tumor arose from the transformation of

neuroendocrine cells, one would expect to see clusters of neuroendocrine cells in precursor HGPIN lesions. However, we did not observe clusters of synaptophysin positive cells in the HGPIN lesions from any of the MYC or MYC/Pim1 samples (Figure 24 and data not shown). Therefore, the neuroendocrine tumors probably arose via the transdifferentiation of adenocarcinoma to a neuroendocrine phenotype.

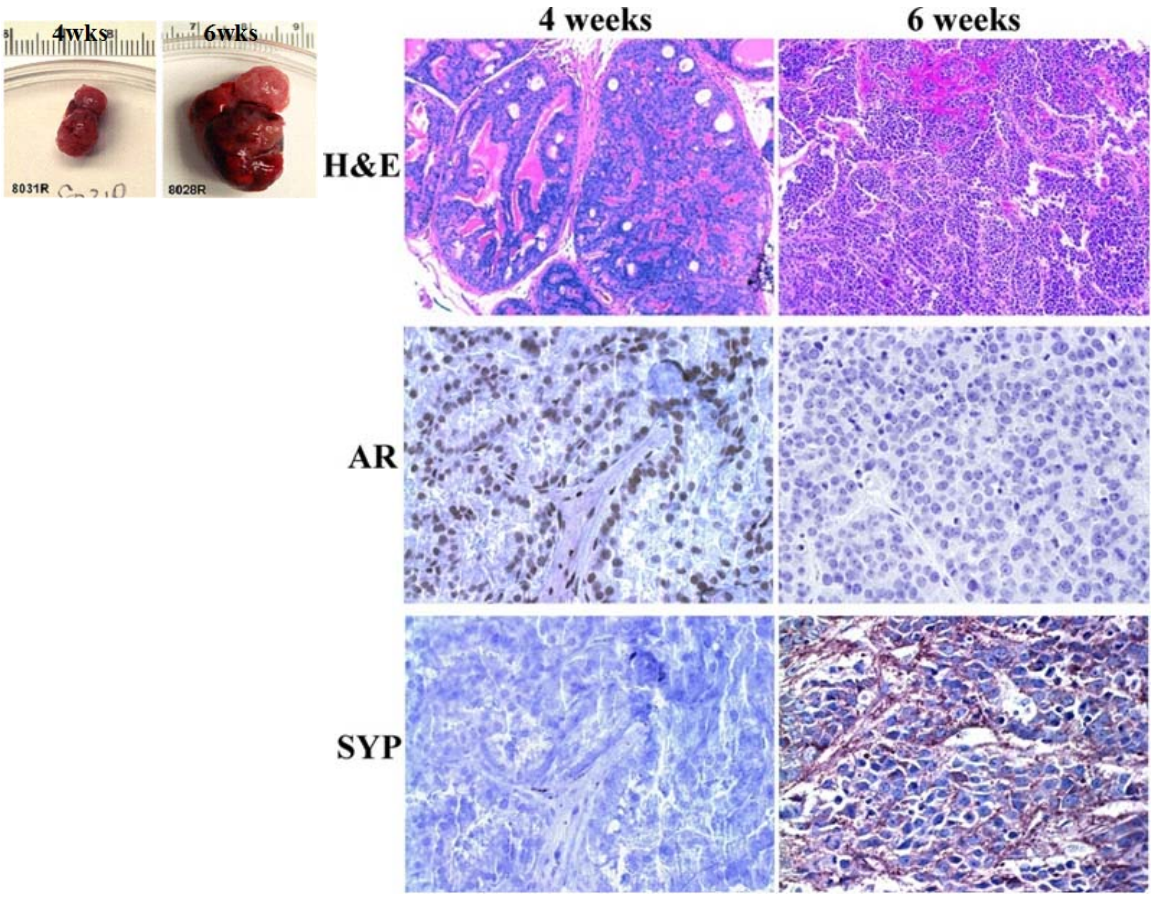

Figure 24. No clusters of NE cells in early lesions of MYC/Pim1 grafts. ***Left:*** A representative image of grafts showing that 4-week MYC/Pim1 grafts were much smaller than 6-week MYC/Pim1 grafts. ***Right:*** Representative H&E image of 4-week graft sections shows HGPIN and adenocarcinoma; that of 6-week graft sections shows high grade cancer (Original magnification: 100×). Immunohitochemical staining shows that 4-week MYC/Pim1 grafts express AR but not express synaptophysin; 6-week MYC/Pim1 grafts do not express AR but express synaptophysin (SYP) (Original magnification: 400×).

Further evidence for this hypothesis was obtained by identification of cells that coexpress AR, MYC and the NE marker synaptophysin in MYC/Pim1 tumors (Figure 25).

These results are consistent with evidence of transdifferentiation of adenocarcinoma to neuroendocrine cancer (Wafa, 2007; Sauer, 2006; Hansel, 2009).

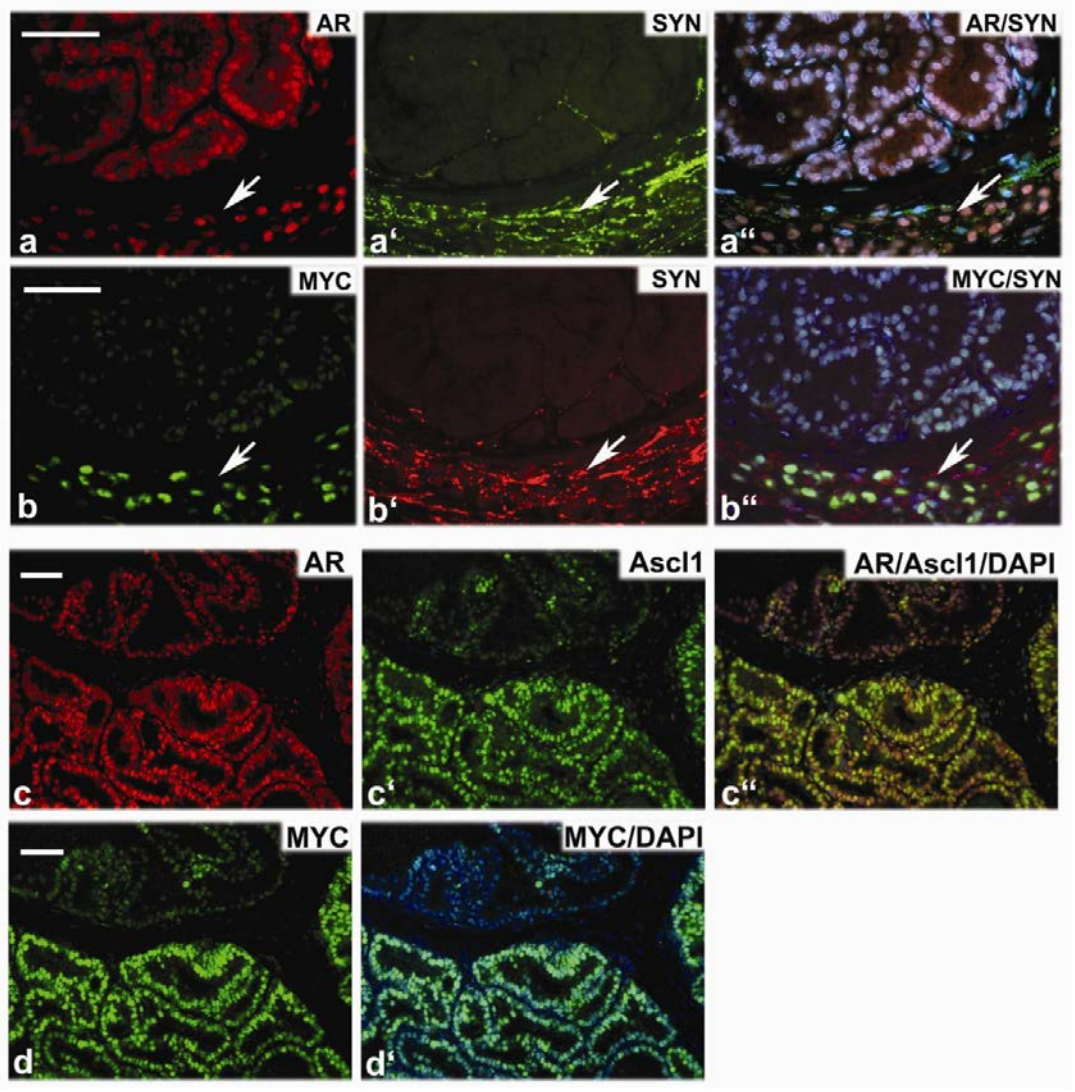

Figure 25. Evidence that c-MYC/Pim1-induced neuroendocrine tumors arise by transdifferentiation. *a-a"*, MYC/Pim1 graft co-stained for androgen receptor (AR, red), synaptophysin (SYN, green) and nuclear stain DAPI (blue). *b-b"*, An adjacent section to that in '*a*' stained for MYC (green), synaptophysin (SYN, red) and DAPI (blue). Arrows point to nest of tumor cells coexpressing AR, SYN and MYC. *c-c"*, MYC/Pim1 graft co-stained for AR (red), Ascl1 (green) and DAPI (blue). Note coexpression of AR, Ascl1 and MYC. *d* and *d'*, An adjacent section to that in '*c*' stained for MYC (green), and DAPI (blue). Scale bars, 50 µm.

Chronic Pim1 overexpression leads to the development of low grade PIN lesions

To examine the effects of chronic overexpression of Pim1, we allowed grafts to grow for 12 weeks. While grafts from the kinase-dead mutant K67M consisted of normal

glands, Pim1-expressing grafts showed focal epithelial hyperplasia and dysplasia consistent with low grade PIN (Figure 26).

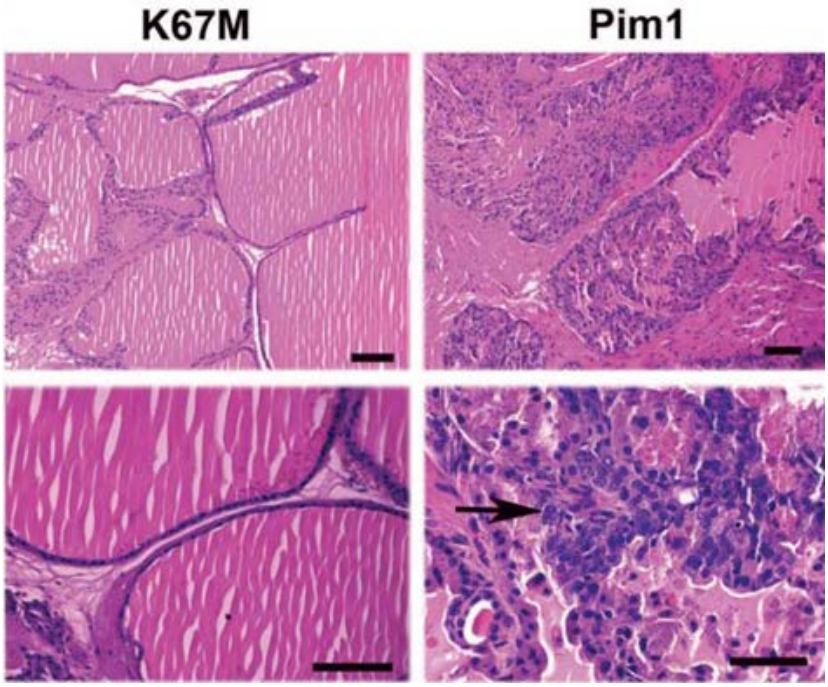

Figure 26. H&E sections show epithelial hyperplasia and LGPIN (arrow) in 12-week Pim1 grafts, while normal-looking prostate glands in K67M grafts. Scale bars, 50 µm.

Pim1 grafts showed a slight elevation of the Ki67-index compared to K67M grafts, but did not reach statistical significance. Both the Pim1 and K67M grafts showed low levels of apoptosis (Figure 27). Pim1 has been reported to interact with and phosphorylate several cell cycle and apoptotic molecules, including Cdc25A/C, C-TAK1, $p21^{cip1}$, $p27^{kip1}$ and Bad (Bachmann, 2005; Morishita, 2008). Thus, Pim1 is expected to promote proliferation and inhibit apoptosis. The lack of a discernible phenotype in 6 week-Pim1 grafts suggests that the homeostatic mechanisms operating in prostatic cells are able to buffer the effects of Pim1 overexpression. At 12 weeks, we speculate that a small reduction in the rate of apoptosis in Pim1 grafts coupled with a modest increase in

proliferation could account for accumulation of epithelial cells that manifests as hyperplasia.

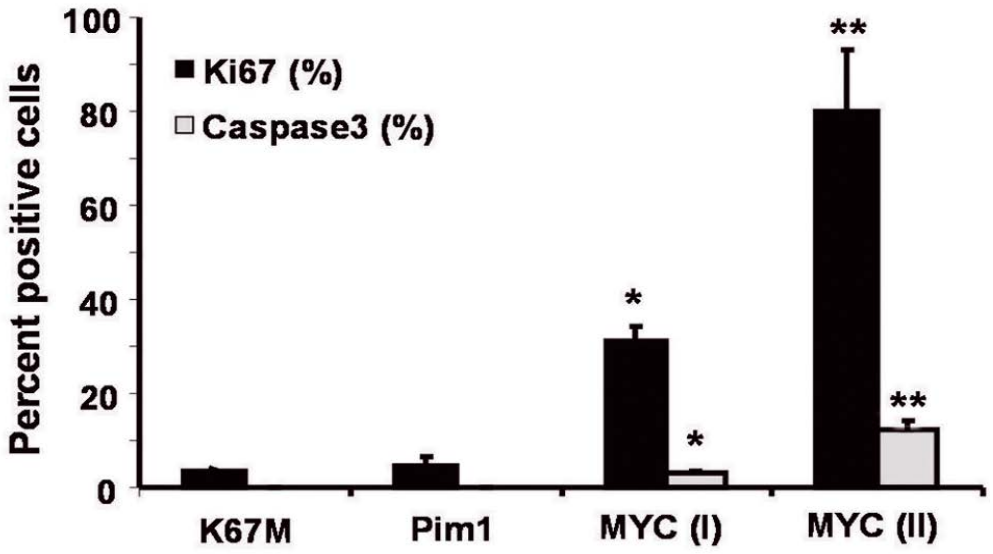

Figure 27. Proliferative (Ki67) and apoptotic (activated caspase 3) indices in 12-week grafts. Data shown as mean ± SD.*, P <0.05 relative to K67M or Pim1. **, P < 0.05 relative to all other groups. N=3.

Chronic MYC overexpression results in adenocarcinoma and carcinoma with neuroendocrine differentiation

Overexpression of Myc in prostate gland of transgenic mice demonstrated that the dosage of Myc expression is correlated to the rate of prostatic tumor progression (Ellwood-Yen, 2003; Zhang, 2000). In addition, based on the notion that Pim1 may cooperate with MYC by amplifying MYC activity, we speculated that chronic expression of MYC alone may result in tumors that resemble 6-week MYC/Pim1 tumors (Williams, 2005). Actually, two different sizes of MYC grafts were observed 12 weeks postgrafting (Figure 28A). Histologically, smaller size of MYC grafts contained HGPIN lesions, while larger grafts were comprised of high grade adenocarcinoma consistent with neuroendocrine differentiation (Figure 28B). Indeed, grafts with MYC overexpression for 12 weeks produced synaptophysin-positive neuroendocrine tumors and invasive

adenocarcinoma with focal synaptophysin-positive cells (Figure 29), which further illustrates that neuroendocrine phenotype arises via the transdifferentiation of adenocarcinoma.

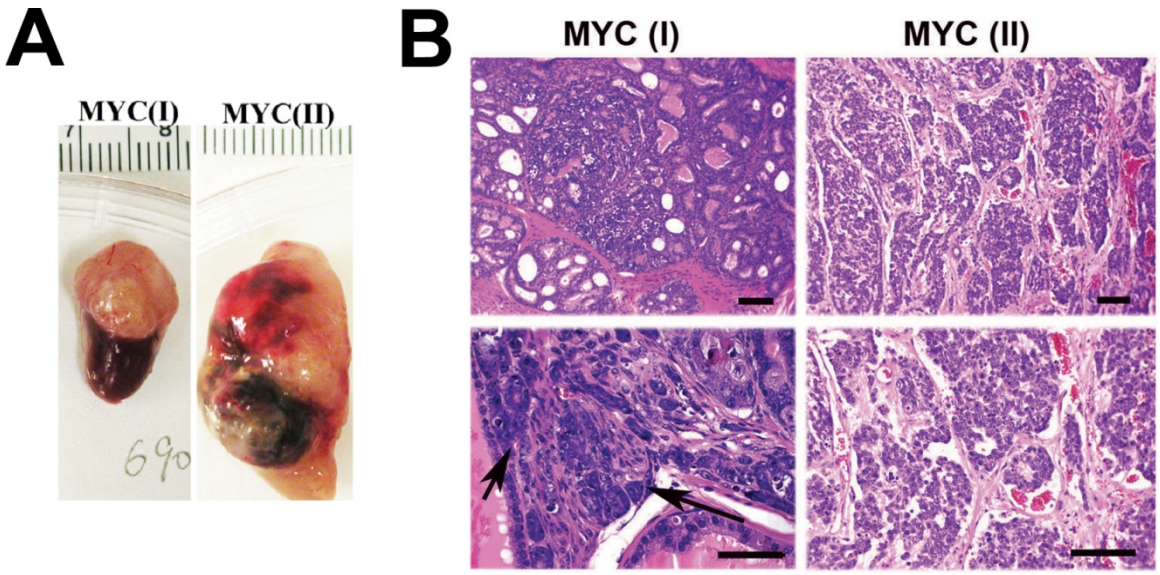

Figure 28. **The size and histology of twelve-week MYC grafts.** ***A,*** MYC grafts show two different sizes. MYC (I) is much smaller than MYC (II). ***B,*** H&E staining shows two types of lesions: MYC grafts (I) display HGPIN with invasive adenocarcinoma (arrows), whereas MYC grafts (II) consist of high-grade tumor. *N*=2 each. Scale bars, 50μm.

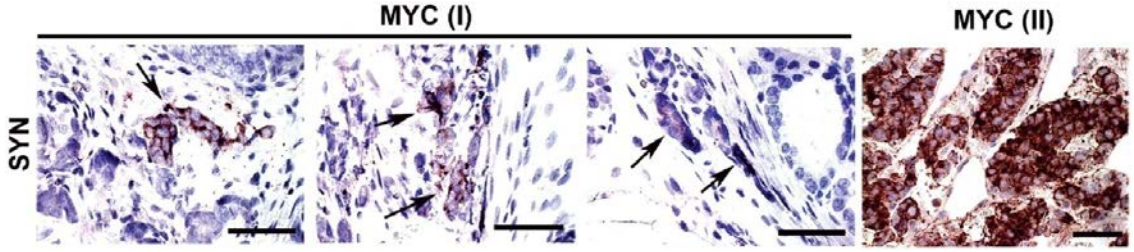

Figure 29. Immunohistochemical staining for synaptophysin (SYN) in 12 week-MYC grafts shows isolated focal expression (arrows) in MYC (I) adenocarcinoma and strong expression in MYC (II) neuroendocrine tumor. Scale bars, 50μm.

The 12-week MYC neuroendocrine tumors expressed low levels of AR using immunohistochemistry (Figure 30) and Western blot analysis (Figure 31)

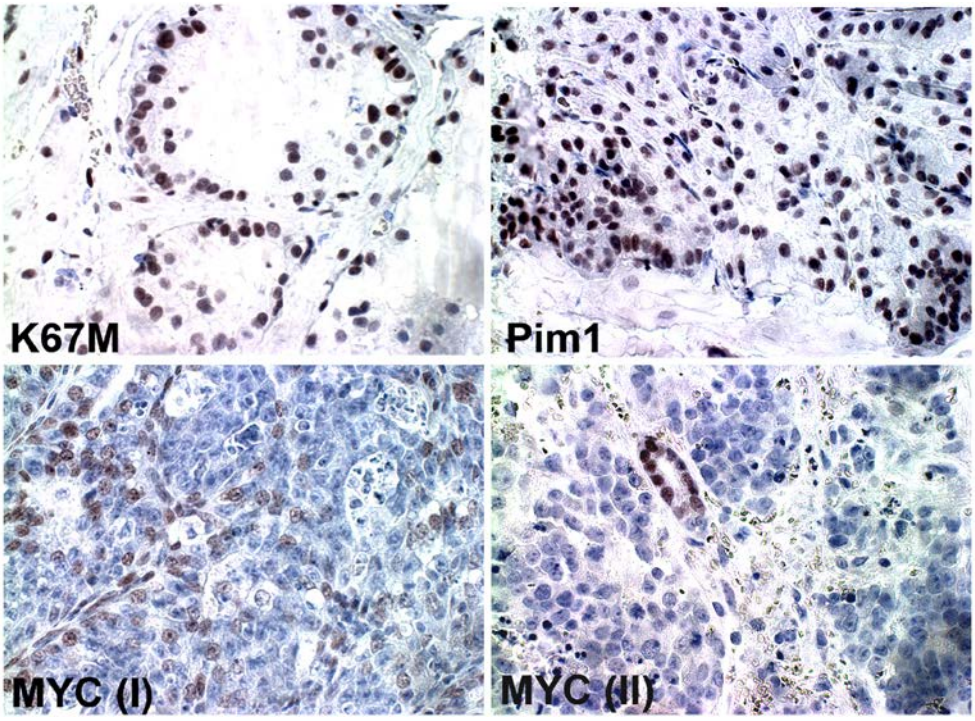

Figure 30. Immunohistochemical staining of androgen receptor in 12 -week grafts. Note uniform nuclear expression (brown) in glands from K67M and Pim1 grafts and examples of heterogeneous or nearly total loss of expression in two different MYC tumors (Original magnification: 400×).

Interestingly, Western blot analysis of the 12-week grafts showed that slightly increased endogenous Pim1 in synaptophysin-positive MYC tumors (Figure 31), suggesting that with progression, MYC tumors select for Pim1 overexpression, or Pim1 may be induced by neuroendocrine tumor secreted factors. These results are consistent with previous observations of Pim1 mRNA overexpression in probasin-Myc transgenic tumors although those tumors were not reported to express markers of NE differentiation (Ellwood-Yen, 2003).

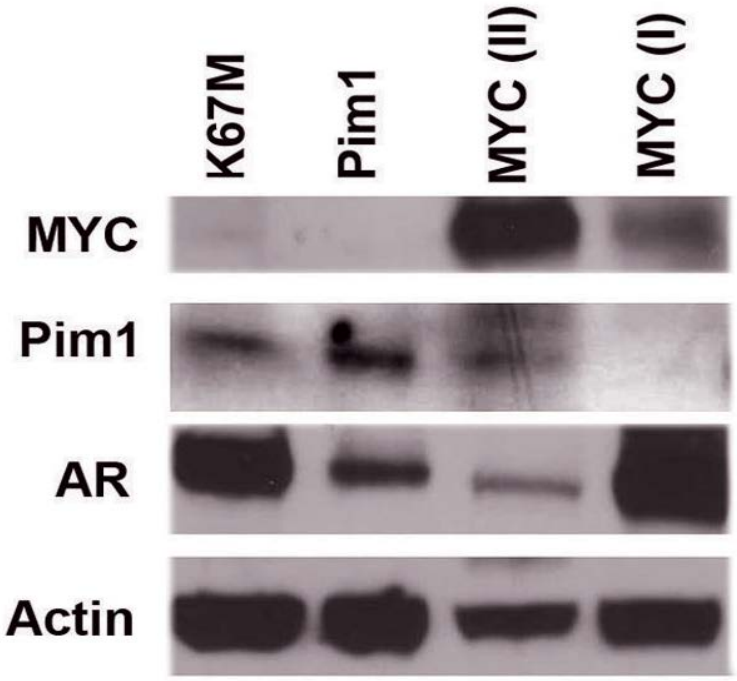

Figure 31. Western blot shows that Pim1, MYC and AR expression in 12-week grafts. Note increased Pim1 and reduced AR expression in MYC (II) sample.

Analysis of 12-week MYC grafts showed levels of proliferation and apoptosis consistent with tumor grade, with the neuroendocrine tumors showing the highest rates of proliferation and apoptosis (Figure 27).

Pim1 may inhibit MYC-induced apoptosis

Myc is known to induce proliferation as well as apoptosis. The Pim1 kinase inhibits apoptosis via interactions with the anti-apoptotic molecules, Bcl-2 and Gfi-1 (Schmidt, 1998) or by phosphorylation and inactivation of the pro-apoptotic proteins, Bad (Aho, 2004), FOXO3a (Morishita, 2008) and ASK1 (Gu, 2009). On the other hand, Pim1 has also been reported to promote Myc mediated apoptosis in serum-deprived Rat-1 fibroblasts (Mochizuki, 1999). We speculated that Pim1 cooperates with MYC by inhibiting the apoptotic effect of Myc. Therefore, we analyzed proliferation and apoptosis

using immunohistochemical staining. Notably, the rates of proliferation (Ki-67 index) and apoptosis (activated caspase 3 index) were similarly low in the 6-week control, Pim1 and K67M grafts, consistent with the absence of histological alterations in these tissues. However, there were significant increases in proliferation and apoptosis in the MYC grafts and similar increases were noted in the MYC/K67M group. The Ki67-index was dramatically increased in the MYC/Pim1 tumors consistent with the nature of high-grade tumors. The apoptotic index was also elevated indicating high turnover of the tumor cells (Fig. 32A, B). The increased apoptosis in the MYC/Pim1 tumors may be due to the markedly elevated rates of proliferation. Although there were many more cells undergoing proliferation than apoptosis, the net effect was increased tumor growth.

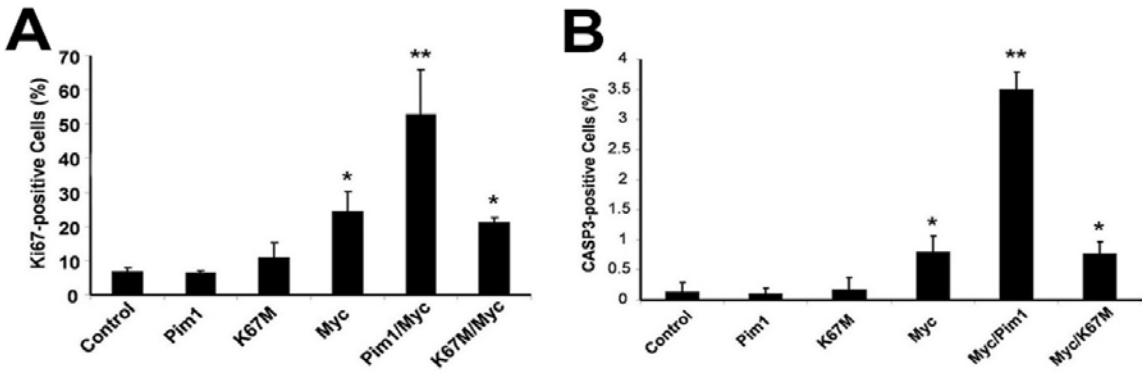

Figure 32. **Increased cellular proliferation and apoptosis in MYC/Pim1 tumors.** The increased apoptosis may be due to the dramatically increased proliferation in the MYC/Pim1 tumors. ***A***, Ki67 proliferative index in graft tissues. ***B,*** Apoptotic index in graft tissues determined by staining for activated caspase 3. Data shown as mean ± SD, N=3. *, P <0.05 relative to control. **, P < 0.05 relative to all samples.

It is speculated that proliferative and apoptotic rates were not comparable between PIN (MYC grafts) and cancer (MYC/Pim1 grafts). Therefore, proliferative and apoptotic rates were analyzed between 6-week MYC/Pim1 tumors and 12-week MYC (II) tumors. The result showed that MYC (II) tumors had higher apoptotic rates than MYC/Pim1

tumors. Furthermore, apoptosis normalized to proliferation was higher in MYC (II) tumor than MYC/Pim1 tumor (Figure 33).

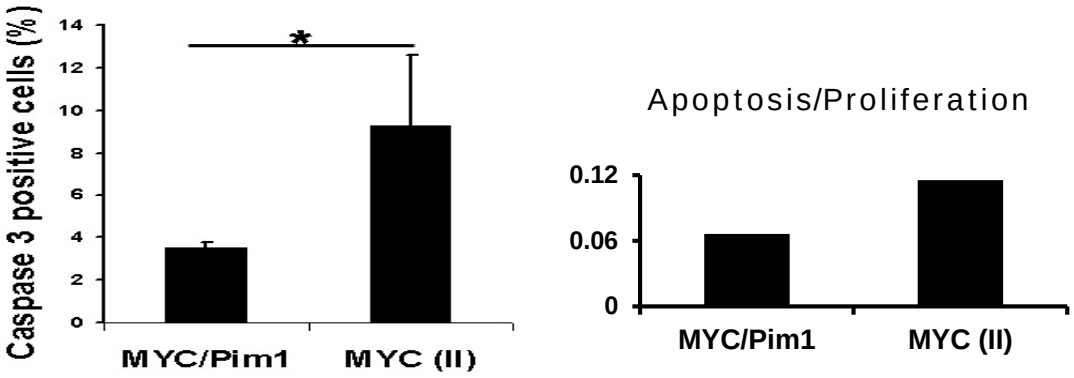

Figure 33. Pim1 suppresses MYC-induced apoptosis. ***Left:*** 12-week MYC (II) tumors show higher apoptotic rates comparing to MYC/Pim1 tumors. Data shown as mean ± SD, N=2-3. *, *P* <0.05. ***Right:*** Apoptotic rate normalized to proliferative rate is higher in MYC (II) tumors than MYC/Pim1 tumors. Data shown as average ratio of apoptosis to proliferation. N=2-3.

Pim1 may increase MYC target gene expression and MYC protein stability

It has been reported that PIM1 may enhance MYC stability and activity by increasing the phosphorylation of MYC on serine 62 (MYCS62P) (Chen, 2005; Zhang, 2008). Other studies have shown that PIM1 and MYC binding induces phosphorylation of histone H3 on serine 10 and facilitates transcriptional activation of MYC target genes (Zippo, 2007). Based on these studies, it is expected that MYC activity will be enhanced in MYC/Pim1-expressing tumors. Consistent with this notion, we found that relative levels of MYC and MYC targets, cyclin D1, cyclin D2 and cyclin E, were elevated in MYC/Pim1 tissues comparing those in MYC or MYC/K67M grafts (Figure 34A, B). Furthermore, phosphorylation of MYC on S62 normalized to total MYC protein level was higher in MYC/Pim1 tumors than MYC or MYC/K67M grafts (Figure 34C).

Increased MYC target gene expression and MYC protein stability may due to the cooperation between Pim1 and MYC, but also may due to indirect gene activation as consequences of tumorigenesis.

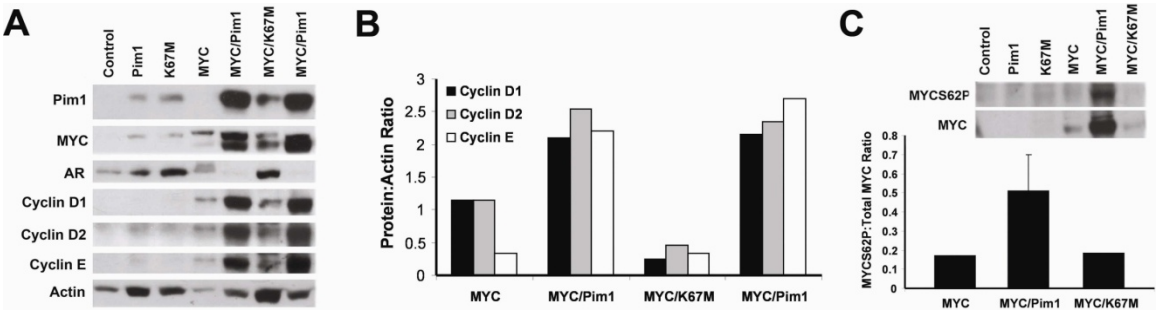

Figure 34. Western blot for detecting Cyclin D1, Cyclin D2, Cyclin E and phosphorylation of MYC on S62 in graft lysates. ***A,*** Western blot for the indicated proteins in graft lysates. ***B,*** Quantitation of Cyclins D1, D2 and E levels from "C" normalized to actin level. ***C***, Upper panel: Western blot for serine-62 phosphorylated MYC (MYCS62P) and total MYC. Lower panel: Quantitation of Western blot data. Data shown as mean ± SD, N=2.

To directly assess the contribution of MYC phosphorylation on serine-62 to tumorigenic activity in vivo, we used the same approach to generate grafts expressing MYC phosphomimetic mutant MYC S62D (serine-to-aspartic acid). The size and histology of grafts revealed no significant difference between MYC and MYCS62D grafts (n=4) (Figure 35A, B). Like the MYC grafts, MYC S62D grafts showed HGPIN with no evidence of invasive cancer as confirmed by SMA staining (Figure 35B). To precisely assess proliferative rates only in MYC-expressing cells, we co-stained the graft sections with phospho-histone H3 (a mitotic marker) and MYC. This analysis revealed a higher mitotic index in the MYC S62D grafts than MYC grafts (Figure 35C). Although MYC S62D phosphorylation has some positive effects on MYC tumorigenicity, it does not account for the bulk of the cooperativity between MYC and Pim1. The functions of Pim1 on MYC transforming activity needs to be further explored.

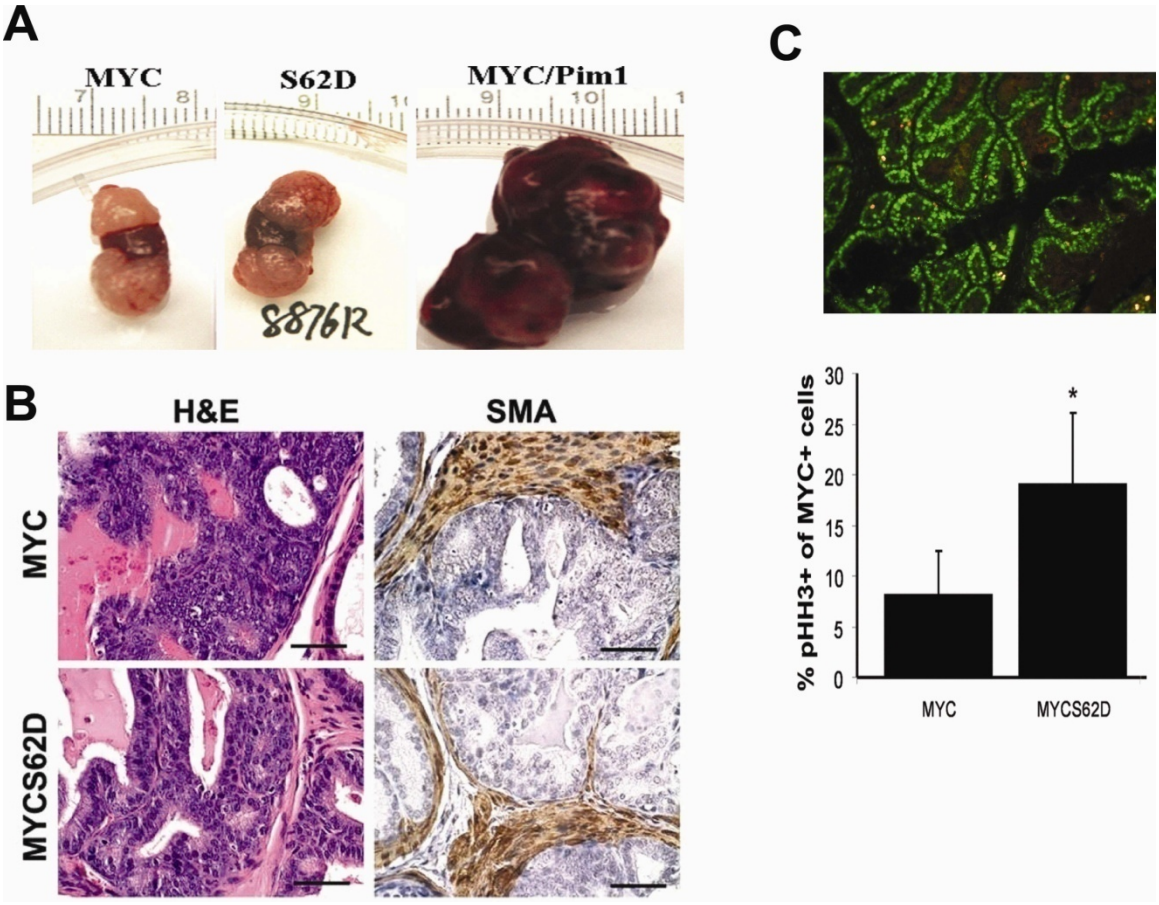

Figure 35. The phenotype of phosphomimetic mutant MYCS62D is similar to that of MYC rather than that of MYC/Pim1 grafts. ***A,*** The size of six-week grafts expressing the phosphomimetic mutant MYCS62D is similar to that of wild type MYC grafts, and much smaller than the size of MYC/Pim1 grafts. ***B,*** H&E and SMA staining show that MYCS62D is similar to wild type MYC grafts. Scale bars, 50 μm. ***C***, Upper panel: representative image of histone H3 (red) and MYC-expressing cells (green). Lower panel: quantization of proliferation. Higher mitotic index (% phospho-histone H3 positive MYC-expressing cells) in grafts expressing the MYCS62D mutant (n=4) compared to those expressing wild type MYC (n=5). Data shown as mean ± SD. * $P < 0.05$.

Discussion

In humans, PIM1 and MYC levels are upregulated, which suggests they cooperate in prostate tumorigenesis. In this study, we found that a significant percentage of the human prostate cancer samples exhibited concurrent overexpression of MYC and PIM1, which is associated with high Gleason grades. Therefore, we examined the effects of MYC and Pim1 overexpression in prostate carcinogenesis using a tissue recombination

model. Our study has provided several insights. We demonstrated that Pim1 by itself is weakly oncogenic, and Pim1 synergizes dramatically with MYC to promote the development of advanced prostate carcinoma. These results are consistent with previous reports in mouse lymphoma models (Van Lohuizen, 1989; Verbeek, 1991). Our results also demonstrated a strict requirement for Pim1 kinase activity for both its oncogenic activity and its ability to synergize with MYC. The precise mechanism by which MYC and PIM1 cooperate in prostate carcinogenesis needs to be further explored.

Tumors derived from the co-expression of MYC and Pim1 show evidence of neuroendocrine differentiation. Pure neuroendocrine or small cell carcinoma of the prostate is rare, and has a poor prognosis. However, partial neuroendocrine differentiation in prostate cancer, defined as expression of one or more neuroendocrine markers such as Chromogranin A, synaptophysin, Neuron specific enolase, L-Dopa carboxylase, is more common and is associated with a poor prognosis (Yuan, 2007; Wafa, 2007; Sauer, 2006; Hansel, 2009). There is also extensive literature on the transdifferentiation of prostate adenocarcinoma to neuroendocrine phenotype. For example, LNCaP human prostate carcinoma cells can be induced to transdifferentiate to NE-like cells by androgen depletion, interleukin-6 treatment or genistein treatment (Zhang, 2003; Pinski, 2006; Deeble, 2001; Kim, 2002). In addition, several observations support the notion that the MYC/Pim1 tumors arise from transdifferentiation of adenocarcinoma cells to acquire neuroendocrine features rather than from the transformation of the rare neuroendocrine cell type in the prostate. First, prostate tissue recombinants are derived from adult mouse prostate cells by prostate regeneration, and if rare neuroendocrine cells were transformed by oncogene expression, one would expect to see clusters of transformed neuroendocrine

cells in early lesions. However, we have never observed clusters of transformed neuroendocrine cells in precursor PIN lesions (Figure 24 and data not shown). This is in contrast to the situation in which neuroendocrine cells are transformed, such as targeted expression of T antigen in the Cr2-TAg model. In Cr2-TAg mice, transformed neuroendocrine cell clusters are readily identified in PIN lesions (Garabedian, 1998; Abdulkadir, 2001b). Secondly, we were able to identify cells coexpressing both AR and synaptophysin, consistent with transdifferentiation of adenocarcinoma cells to neuroendocrine cancer, similar to recent observations in some human prostate tumors (Wafa, 2007) as well as in TRAMP mice (Kaplan-Lefko, 2003). Mouse prostate-specific deletion of *Trp53* and *Rb* induces neuroendocrine tumors in prostate and up-regulation of Pim1 and L-Myc (Zhou, 2006). Tumors from this model were also found to co-express synpatophysin and androgen receptor and to up-regulate the pro-neural transcription factors Ascl1 and Hes6. Interestingly, the expression of pro-neural transcription factors was useful in segregating metastatic form localized prostate cancer (Vias, 2008).

Our findings clearly show that Pim1 kinase activity is important for the synergy between Pim1 and MYC in prostate carcinogenesis. The Pim1 has recently garnered interest as a possible molecular target in multiple cancers including lymphomas and prostate cancer. Mice deficient in Pim1 or all Pim kinases (Pim1/Pim2/Pim3) showed a very mild phenotype, suggesting that therapeutic inhibition of Pim1 may be well tolerated *in vivo.* Therefore, MYC/Pim1 tissue recombination model may be appropriate for testing therapeutic modalities aimed at inhibiting Pim1 kinase activity as it avoids both the drawbacks of xenograft models that use advanced cancer cell lines as well as the cost/time constraints that hamper most transgenic models.

CHAPTER III

PIM1 IS REQUIRED TO MAINTAIN THE TUMORIGENIC POTENTIAL OF PROSTATE CARCINOMA CELLS

Introduction

Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related death in the Western world. Advanced prostate cancer is typically androgen-insensitive and resists conventional chemotherapy, hormone therapy and radiation therapy. Neuroendocrine differentiation has been shown to correlate with hormone deprivation therapy and tumor progression (Abrahamsson, 1999; Hirano, 2004; Vashchenko, 2005; Bonkhoff, 2005).

Pim1 has been implicated in leukemias, lymphoma and some solid tumors such as prostate cancer, pancreatic cancer, and oral cancer (Shah, 2008). Pim1 is a conserved active serine/threonine kinase (Reeves, 1990; Saris, 1991). Transgenic mice overexpressing Pim1 delayed lymphoma at a low frequency (Cuypers, 1984; van Lohuizen, 1989). However, all mice overexpressing Myc and Pim1 died of lymphomas in utero (Verbeek, 1991). Our recent study showed that Pim1 potently synergizes with MYC to accelerate prostate cancer progression (Chapter II; Wang, 2010). The mechanism of cooperation between Pim1 and c-Myc has not been clearly established. Pim1 has been reported to promote tumorigenicity by increasing Myc expression or stability (Chen, 2005; Zhang, 2008). Pim1 also has been shown enhancing the transcription of Myc target genes (Zippo, 2007; Kim, 2010). The phospomimetic mutant of Myc on Ser329 has comparable tumorigenisity to coexpression of Pim kinases and Myc *in vitro* (Zhang,

2008). However, in our tissue recombination model, the phosphomimetic MYC-S62D mutant did not form aggressive tumors as MYC/Pim1 grafts. It is unknown whether other MYC mutation sites that stabilize MYC, such as MYC-S329D, can induce tumor similar as co-expression of MYC and Pim1 *in vivo*. We speculated that other mechanisms may be involved in Pim1 and MYC synergism in prostate cancer addition to increasing MYC-transcriptional activity by Pim1. On the other hand, in tissue recombination model, Pim1 overexpression results in hyperplasia with focal low grade PIN lesions after 12 weeks, while MYC and Pim1 co-expression leads to the development of aggressive prostate cancer within 6 weeks suggesting that Pim1 is a weak oncogene, and strongly cooperates with Myc. Tumors that co-express MYC and Pim1 showed signs of neuroendocrine differentiation, which is observed in advanced stage of human prostate cancer and is associated with poor prognosis (Abrahamsson, 1999; Miyoshi, 2001; Hirano, 2004; Vashchenko, 2005; Bonkhoff, 2005, Taplin me, 2005; Berruti, 2005; Mcwilliam; 1997; Kamiya, 2008). It is needed to be explored whether inactivation of Pim1 in advanced tumor is sufficient to induce tumor regression and prevent tumor formation.

Pim1 overexpression is observed in more than 50% of human prostate cancer, which is correlated with the high Gleason score. Pim1 is also proposed to be a diagnostic and prognostic factor in prostate cancer (Dhanasekaran, 2001; Valdman, 2004; Cibull, 2006; Xu, 2005; He, 2009). In addition, Pim1 appears to be essential for tumor progression and maintenance. Several therapeutic agents have been developed to inhibit Pim1 kinase activity, for example, Pim1 inhibitor Quercetagetin (Holder, 2007); SMI-4a (Lin, 2009); SGI-1776 (Chen, 2009b; Mumenthale, 2009), anti-PIM1 monoclonal antibody (Hu, 2009). Pim1 is constitutively active and its protein levels correlated with

kinase activity Therefore, decrease of PIM1 levels indicates decreased kinase activity, thereby inhibiting tumorigenicity (Qian, 2005). RNA interference can specifically silence gene expression, so it can be used for blocking the expression of oncogenes in cancer cells. To determine whether Pim1 expression is necessary to maintain the malignant phenotype and its potential therapeutic role, we used short hairpin RNA (shRNA) directed against expression of Pim1 to determine the effects of decreasing the levels of Pim1 expression in prostate cancer cell lines. We found that lentivirus-mediated shRNA against Pim1 markedly decreased Pim1 gene expression, and inhibited cellular proliferation and survival of prostate cancer cells. Pim1 knockdown also reduced tumorigenic potential of prostate cancer cells. In addition, phosphoralytion of ERK1/2 was reduced upon Pim1 knockdown. These results indicated that Pim1 is required for tumorigenic maintenance, even in the presence of high MYC level. Targeting Pim1 would be effective for the treatment of prostate cancer, even for advanced stages of cancer.

Materials and Methods

Cell lines and constructs

DU145 cells were cultured in RPMI medium with 10% fetal bovine serum (FBS). MPT cells were isolated from MYC/Pim1 tumors. Briefly, MYC/Pim1 tumors were minced and digested into single cells using collagenase, then plated on collagen-coated dish with DMEM/F12/10% FBS medium. After confluences the cells were split into regular cell culture dishes.

Stable knockdown of Pim1with shRNA

Lentiviral pGIPZ shRNAmir against mouse Pim1 **(**V2LMM_46214) and the sequence-scrambled, non-silencing-GIPZ lentiviral shRNAmir control (RHS4346) were provided by Vanderbilt Functional Genomics shared resource (Open Biosystems). Lentiviral pLKO.1 shRNA targeting human, mouse, rat Pim1 (RHS3979-9631245) and control empty vector were obtained from Open Biosystems. Lentivirus was prepared as described in Chapter II. MPT or DU145 cells were infected by virus in the presence of 8μg/ml polybrene. Puromycin was added to select stably transduced cells 2 days after infection.

Western blot analysis

Cells were washed with PBS, and then RIPA buffer with protease inhibitors was added. Cells were harvested with cell scraper, briefly sonicated and spun down. Lystate was run on SDS-PAGE, transferred to PDVF membranes. Membranes were blot with the following antibodies: c-MYC, Pim1, AR and Actin (Santa Cruz Biotechnology); p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (Thr202/Tyr204) (Cell signaling); β-actin (Santa Cruz Biotechnology); β-tubulin (Sigma).

Proliferation assay

For cell growth curve, MPT Cells were seeded at 20,000 cells per well in a six-well plate. Viable cells determined by trypan blue-excluding ability were counted using a hemocytometer. For MTS assay, cells were quarterly plated on 96-well plate. 3 days later, CellTiter 96® Aqueous One Solution (Promega) was added to each well and absorbance was read at 490 nm in a plate reader according manufacture's instruction.

Active caspase 3 staining

Cells were plated on glass coverslips. Cells were fixed in 2% paraformaldehyde for 10 minutes followed by permeabilization in 1% Triton X-100 for 10 minutes. After washing in PBS, cells were blocked in 10% goat serum, stained with activated caspase-3 (Cell Signal) at 1:500. Coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories). 1000 cells were counted per cover slip.

Colony formation assay

5,000 of MPT cells were plated in 10 cm dishes. Triplicate experiments were performed for each cell line. The medium was changed every 3-4 days. After 10-14 days, the cells were fixed and stained with crystal violet.

Soft agar assay

A 2 ml of 0.6% agar in RPMI-10% FBS was placed into each well of 6 well plate. After agar solidified, 2 ml of 0.3% top agarose in RPMI-10% FBS containing 10,000 cells was added to each well. The cells were fed every 3-4 days with RPMI/10% FBS. The plates were incubated for 2 weeks. The colonies that are larger than 100μm were counted. Each soft agar assay was performed in triplicate.

In vivo tumorigenicity assay

1×10^5 control MPT or Pim1 knockdown cells (shControl#1 and shPim1#1) were mixed with 15μl of Matrigel (Becton Dickinson Labware) and injected subcutaneously in both flanks of 8-week-old male athymic nude mice (BALB/c strain; Harlan Sprague Dawley). Grafts were measured weekly. All mice were sacrificed by 12 weeks after injection. Animal care and experiments were carried out according to protocols approved

by the Institutional Animal Care and Use Committee at Vanderbilt University. Grafts were paraffin-embedded, sectioned, stained with H&E, and analyzed by light microscopy. A tumor was defined as a palpable mass that contained carcinoma cells upon histological examination. Immunostaining was performed as described (Abdulkadir, 2001a; Abdulkadir, 2001b) using anti-synaptophysin (BD Biosciences), anti-androgen receptor (Santa Cruz Biotechnology).

Results

Knockdown of Pim1 expression in MYC and Pim1 overexpressing tumor-derived cell lines

In Chapter II, we demonstrated that Pim1 and MYC cooperation leads to advanced prostate cancer with neuroendocrine differentiation 6 weeks postgrafting. In this study, we isolated cells from MYC and Pim1 induced tumors and established a cell line named MPT, standing for MYC and Pim1 overexpressing Tumorigenic cells (Figure 36A). These cells expressed MYC and Pim1 as they contain exogenous plasmids bicistronically expressing MYC or Pim1 as well as YFP (see Chapter II, Figure 17). MPT cells expressed neuroendocrine cell marker neuron-specific enolase (NSE), consistent with neuroendocrine differentiation in MYC/Pim1 tumors (Figure 36B). To test whether reducing Pim1 expression impacts oncogenesis, and whether Pim1 can be a potential therapeutic target, we used a shRNA approach to inhibit Pim1 expression. MPT cells were transduced with lentiviral shRNAmir against mouse Pim1 or nonsilencing control (named shPim1#1 or shControl#1, respectively). To exclude off-target effects, MPT cells were transduced with another shRNA targeting Pim1 or control lentivirus (named shPim1#2, or shControl#2, respectively). shPim1#1 targets mouse Pim1 only, while shPim1#2 targets both mouse and human Pim1. Stable knockdown and corresponding

control cells were used for the following experiments. Western blot analysis showed that Pim1 expression was efficiently knocked down by two sets of shRNA (Figure 36B). Notably, Pim1 knockdown did not change the expression level of MYC in MPT cells.

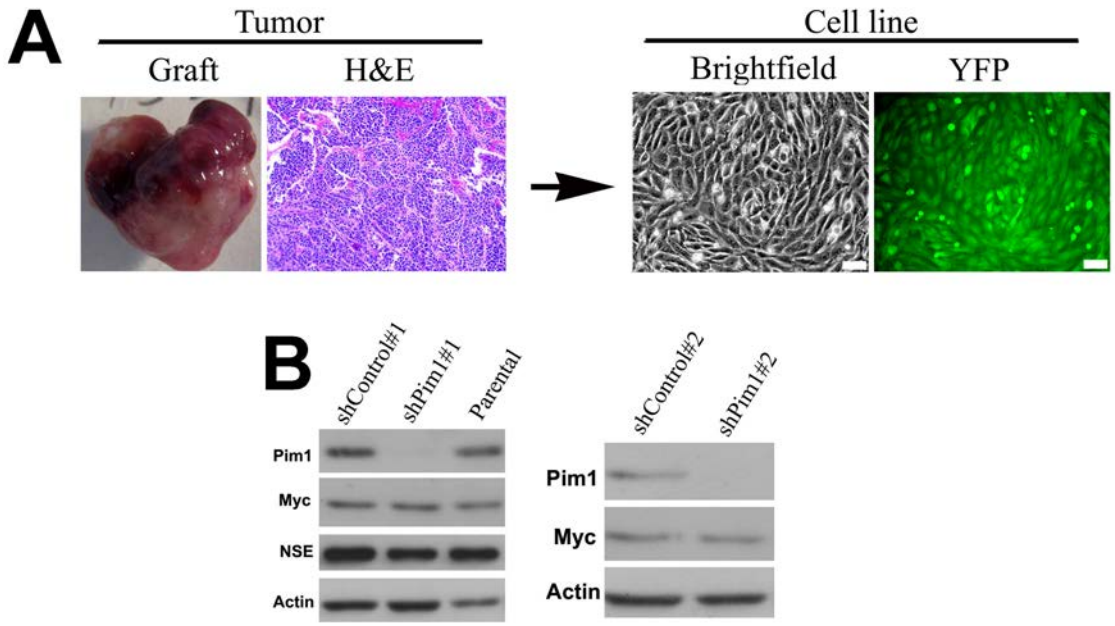

Figure 36. Knockdown of Pim1 expression in 6-week MYC/Pim1 tumor derived cell lines. ***A,*** *Left:* Over-expression of MYC and Pim1 results in large tumors 6 weeks postgrafting in tissue recombination experiments. H&E staining showed the poorly differentiated tumor. *Right:* The image of established cell line from 6-week MYC/Pim1 tumor. YFP fluorescence can be seen under fluorescence microscope as the cells contain exogenous plasmids bicistronically expressing MYC, and Pim1 as well as YFP (Scale bar: 100 µm). ***B,*** *Left:* Western blot shows that parental MPT cells express MYC and Pim1, and neuron-specific enolase (NSE). MPT cells were infected with lentivirus containing GIPZ shRNAmir against Pim1 (shPim1#1) or Non-silencing-GIPZ shRNAmir (shControl#1). Western blot shows that Pim1 is knocked down by shPim1#1. *Right:* Pim1 expression was stably knocked down using pLKO.1 shRNA against Pim1 (shPim1#2), pLKO.1 empty vector was used as control (shControl#2).

We also established a second cell line, named MPT2, from a 4-week MYC/Pim1 graft (Figure 37A). In our previous tissue recombination experiments, 4-week MYC/Pim1 grafts consisted of HGPIN and prostate adenocarcinomas that were androgen receptor (AR) positive and neuroendocrine cell marker synaptophysin negative (Chapter II, Figure 24). Figure 37B showed that Pim1 was markedly knocked down in MPT2 cells.

MPT2 cells expressed NSE and initially expressed AR but lost it with increasing passages in culture. These cells also expressed NSE, suggesting that MPT2 cells might aquire features of neuroendocrine differentiation (Figure 37B).

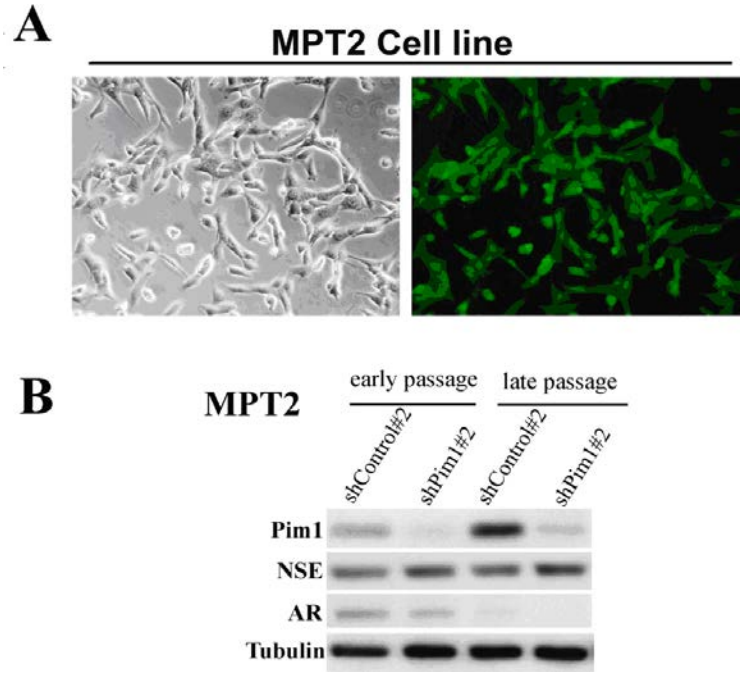

Figure 37. **Knockdown of Pim1 expression in 4-week MYC/Pim1 tumor derived cell lines.** ***A,*** An established cell line from 4-week MYC/Pim1-expressing tumor (named MPT2). A representative image of MPT2 cells in bright field (left) and under fluorescence microscopy (right) (Original magnification: 200×). ***B***, Western blot shows that MPT2 cells express MYC and Pim1. Pim1 expression was stably knocked down in MPT2 cells using shPim1#2. MPT2 cells express androgen receptor (AR) in early passages, but not in late passages. NSE is also expressed in MPT2 cells.

Pim1 knockdown impairs prostate tumor cell proliferation and survival

Although Myc level was not changed in MPT and MPT2 cells, cell growth curves showed that Pim1 knockdown substantially reduced cell growth comparing to the control cells (Figure 38A). MTS proliferation assay also showed that knockdown of Pim1 in MPT and MPT2 reduced proliferation (Figure 38B). Pim1 has been implicated in anti-apoptosis functions via interactions with the anti-apoptotic molecules, bcl-2 and Gfi-1 (Schmidt, 1998); or phosphorylation and inactivation of Bad, FOXO3a and ASK1 (Aho, 2004; Morishita, 2008; Gu, 2009). Under regular cell culture conditions, Pim1

knockdown slightly increased the rate of cell apoptosis but was not statistically significant (data not shown). Both MPT and MPT2 cells are serum sensitive. Both Pim1 knockdown cells showed higher rates of apoptosis than corresponding controls, as determined by activated Caspase 3 staining in low serum condition (Figure 38C). These results suggested that Pim1 plays a role in promoting cell proliferation and protecting from cell death under adverse conditions.

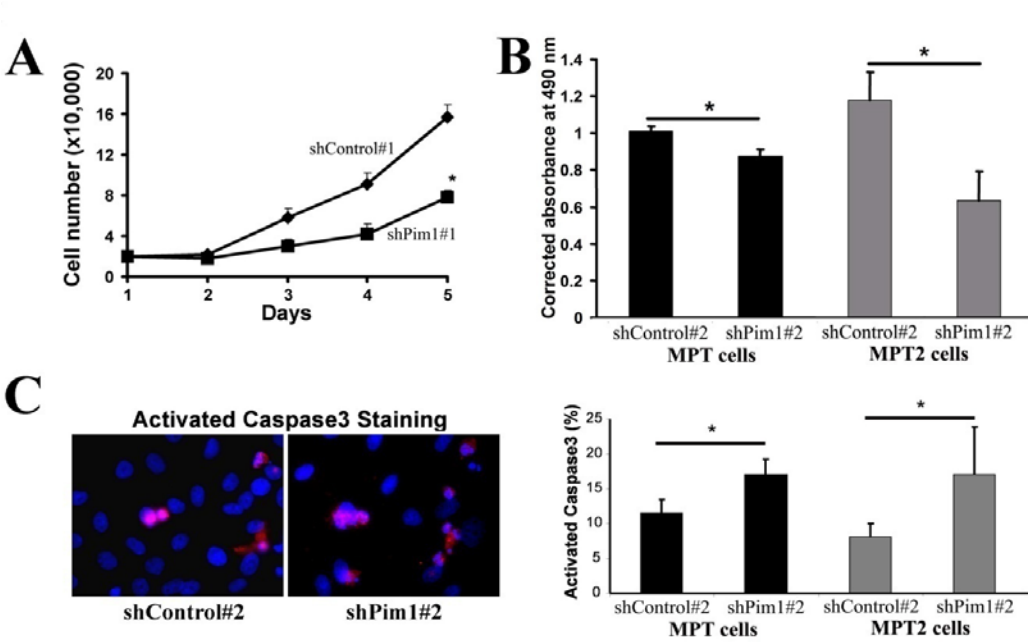

Figure 38, Pim1 knockdown of MPT cells reduced proliferation and survival. ***A,*** Growth curve is generated by counting shPim1#1 and shControl#1 MPT cells for 4 days, duplicated wells for each time points. *P<0.05. ***B,*** Cell proliferation is measured by OD 490 nm using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega). The absorbance is directly proportional to the number of living cells in culture. The results are mean ± SD of quadruplicate wells after 3 days of incubation of 2000 cells. *P<0.05. ***C,*** *Left panel:* Representative image of active caspase 3 staining (red) of overnight serum starved cells (0.5% serum in DMEM/F12). Nuclei are counterstained with DAPI (blue) (Original magnification: 400×). *Right panel:* Quantification of apoptosis rate by counting activated capase 3 positive cells. The results are mean ±SD of two independent experiments *P<0.05.

To extend our findings beyond MPT and MPT2 cell lines, we studied the effects of Pim1 knockdown in DU145 cells. DU145 is a human prostate cancer cell line that has endogenous PIM1 and MYC expression. We used shPim1#2 that targets both human and

mouse Pim1 mRNA sequence to knock down PIM1 in DU145 cells. Figure 39A showed that PIM1 was substantially knocked down in DU145. Endogenous MYC level was also reduced by PIM1 knockdown. NSE was expressed by DU145 cells, consistent with the previous result of expression of neuroendocrine cell markers in DU145 cells (Leiblich, 2007). NSE expression was not altered by PIM1 knockdown. PIM1 knockdown in DU145 cells reduced proliferation comparing to control cells as shown by MTS proliferation assay (Figure 39B). PIM1 prevented serum starvation-induced cell apoptosis, consistent with results in MPT and MPT2 cells (Figure 39C). These results indicated that Pim1 promotes cell proliferate and survival potential in prostate cancer cells *in vitro*.

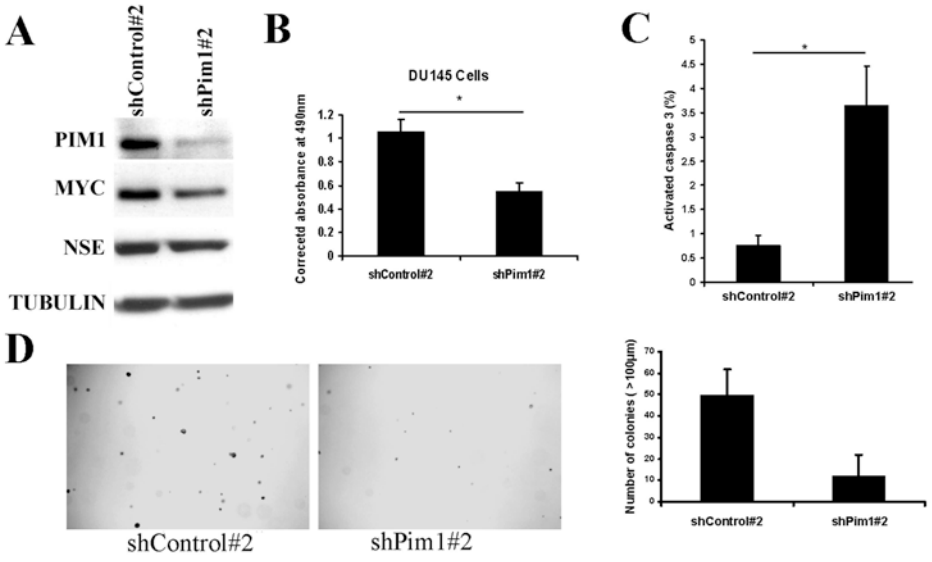

Figure 39. Pim1 knockdown of DU145 cells reduced proliferation, survival and transformation potential. ***A,*** DU145 cells are transduced with shPim1#2 or shControl#2 lentivirus. Stable cell lines selected by puromycin were used for following experiments. Western blots show that PIM1 is knocked down, and MYC level is reduced upon Pim1 knockdown. DU145 cells express NSE. ***B,*** Cell proliferation is measured by MTS assay. The results are mean±SD of quadruplicate wells after 3 days of incubation of 4000 cells. *P<0.05. ***C,*** Quantification of apoptosis by counting active capase 3 positive cells after DU145 cells were treated with serum free medium overnight. The results are mean ±SD of two independent experiments *P<0.05. ***D,*** *Left panel:* reprehensive images of soft agar assay show that Pim1 knockdown in DU145 cells reduce the size and number of colonies (Original magnification: 4×). *Right panel:* Quantification of colonies from soft agar assay of Pim1 knockdown and corresponded control DU145 cells. The results are mean ±s.d. of triplicates (Colony cut-off size: 100 μm). *P<0.05.

Pim1 knockdown impairs prostate tumor cell tumorigenicity

Loss of cell contact-inhibition is one of the hallmarks of cancer cells. We used a focus-formation assay to test whether Pim1 knockdown in MPT cells affected transforming ability *in vitro*. The results showed that control MPT cells lost contact inhibition and formed lots of foci, while Pim1 knockdown cells abrogated focus-forming ability (Figure 40A, B). Anchorage independent growth is another way to test cell transformational potential in vitro. We tested whether Pim1 knockdown in DU145 cells inhibits anchorage-independent growth using a soft agar colony formation. After 2 weeks growing in soft agar, Pim1 knockdown cells exhibited markedly fewer and smaller colonies than control cells (Figure 39D). These results indicate that Pim1 plays a role in maintaining transforming potential of prostate cancer cells.

To investigate whether Pim1 knockdown can reduce tumorigenicity in vivo, Pim1 knockdown MPT cells (shPim1#1) or control MPT cells (shControl#1) were injected subcutaneously in athymic nude mice and graft growth was monitored over 12 weeks (n=10). No apparent tumors were seen in the Pim1 knockdown group. H&E staining of tissue sections from the injected areas indicated that they are composed of fat, muscle, or lymph nodes but no tumor cells were found (Figure 40C, D, E). In contrast, the control group formed large tumors, resulting in 60% tumor incidence (6 out 10 sites of inoculation) (Figure 40C, D, E). In histology, control group consisted of sheets of tumor cells, and expressed neuroendocrine differentiation marker synaptophysin (SYP), but did not express androgen receptor (AR), E-cadherin, p63, cytokeratin 8 and smooth muscle actin, consistent with the features of MYC/Pim1 tumor from tissue recombination experiments (Figure 40F, and data not shown). These results, taken together with our previous data on the effects of Pim1 overexpression on prostate cell transformation (See

Chapter II), indicate that although Pim1 overexpression alone is not sufficient to initiate the development of invasive prostate cancer, continued Pim1 expression is required to maintain prostate cancer cell tumorigenicity.

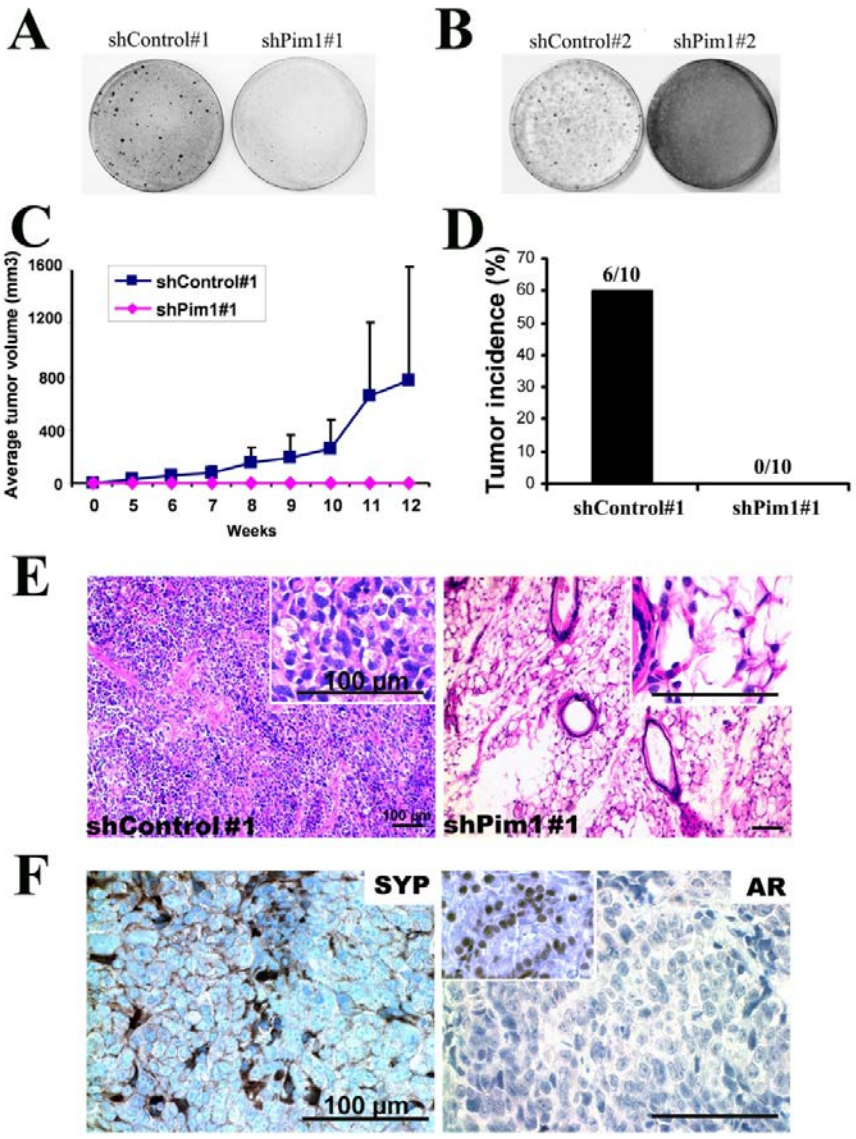

Figure 40. Pim1 maintains the tumorigenic potential of MPT cells. ***A,*** Representative images of focus formation assay by two independent experiments. The result shows that non-silencing control MPT cells (shControl#1) lose cell contact inhibition and form colonies, whereas Pim1 knockdown cells (shPim1#1) keep cell contact inhibition without focus-forming ability. ***B****,* Similar result as *A* is seen in shControl#2 and shPim1#2 MPT as well. ***C,*** Pim1 knockdown (shPim1#1) or non-silencing control MPT cells (shControl#1) were injected subcutaneously into nude mice. No tumor is found in Pim1 knockdown group. Control group forms large tumor after 12 weeks grafting. ***D,*** Pim1 knockdown group (shPim1#1) abrogates subcutaneous engraftment, as compared with a 60% tumor take in the non-silencing control group (shControl#1). Numbers in the parentheses indicate the number of replicates in each group. ***E,*** Representative H&E images of graft sections. shControl#1 group shows high-grade tumor, consistent with features of MYC/Pim1 tumor in tissue recombination experiments. shPim1#1 group shows fat and blood vessel cells without tumor cells. Scale bar: 100 μm. Insets: Higher-magnification images. ***F,*** shControl#1 grafts are synaptophysin (SYP) positive and androgen receptor (AR) negative. Scale bar: 100 μm. Pim1 knockdown grafts are not analyzed because no tumor cells are found by H&E staining. Inset: AR positive control section.

Pim1 knockdown impairs ERK signaling pathway activation

An increasing number of studies have shown that multiple signaling pathways are activated in various human cancers. AKT/mTOR and ERK MAPK signaling pathways are often up-regulated during human prostate cancer progression (Abreu-Martin, 1999; Gioeli, 1999; Malik, 2002; Mulholland, 2006; Shen MM, 2007; Kinkade, 2008). Our results showed that Pim1 knockdown impaired potentials of cell proliferation, survival, and tumorigenicity. We want to detect whether Pim1 knockdown affects AKT/mTOR and ERK/MAPK signaling pathways. We first compared the level of phosphorylation of AKT, mTOR, S6k, PDK1 between Pim1 knockdown cells and control cells using Western blot analysis and results showed no distinct differences in the AKT/mTOR pathways (data not shown). We found that ERK1/2 phosphorylation was consistently reduced by Pim1 knockdown in MPT, MPT2, and DU145 cells (Figure 41A, B, C and D). Phosphorylation of ERK indicates activation of the MAPK pathway. Pim1 knockdown reduced the phosphorylation of ERK, suggesting that Pim1 is essential for MAPK signaling activation. We next examined whether Pim1 expression is sufficient to induce phosphorylated ERK. Since serum can induce ERK phosphorylation, we starved cells with serum free medium and tested ERK1/2 phosphorylation in Pim1 stably overexpressing cell lines LNCaP, PC-3, and DU145 cells (Roh, 2003; Kim, 2010). We found that overexpression of Pim1 did not affect ERK phosphorylation (Figure 41E, F, and data not shown). These results indicated that while Pim1 is necessary for activation of MAPK signaling, it appears that Pim1 alone is not sufficient to mediate this activation *in vitro*. We further tested the phosphorylated ERK1/2 in tissue recombination grafts by Western blot. Compared to the control graft, Pim1 overexpression increased ERK1/2

phosphorylation without affecting total ERK level (Figure 41G), indicating that MAPK pathway can be activated by Pim1 overexpression *in vivo.*

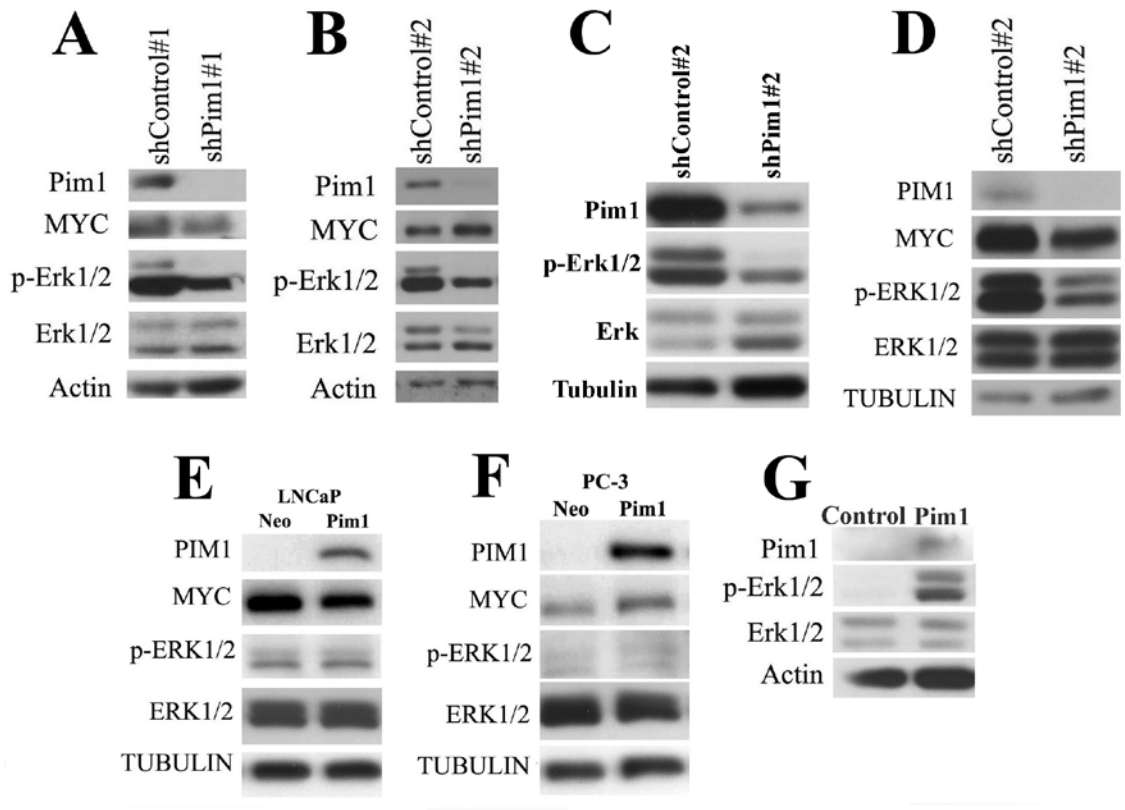

Figure 41. Pim1 is essential for ERK activation. ***A, B, C, D,*** Western blots show that Pim1 knockdown decreases phosphorylation of ERK1/2 compared to control. *A,* shPim1#1 MPT cells; *B,* shPim1#2 MPT cells; *C,* shPim1#2 MPT2 cells; *D,* shPim1#2 DU145 cells with their correspondent controls. ***E, F****,* Western blots showed that Pim1 overexpression did not increase phosphorylation of ERK1/2 compared to control. Neo, empty vector control. ***G,*** Western blot analysis of 6-week graft tissue lysates shows increased phosphorylation of ERK1/2 in Pim1 group compared to control.

Discussion

Although Pim1 is weakly oncogenic, it synergizes dramatically with Myc to induce invasive prostate cancer with characteristics of neuroendocrine cancer (Chapter II, Wang, 2010). The basis for this synergism between Pim1 and Myc is not well understood. To understand the function of Pim1 on MYC/Pim1 cooperation in prostate tumorigenicity, we knocked down Pim1 expression in MYC/Pim1 tumor derived cell lines and a human prostate cancer cell line DU145. We found that, while Pim1 alone is not sufficient to initiate development of invasive prostate adenocarcinoma, depletion of Pim1 expression resulted in decreased growth rate, survival, and tumorigenic potential, suggesting that Pim1 is required for maintenance of the tumorigenic phenotype. In MPT cells, Pim1 knockdown reversed their tumorigenic phenotype even when MYC level was not decreased, which further illustrated that Pim1 and MYC synergism in prostate cancer may depend on other mechanisms in addition to enhancing Myc activity and stability. In addition, Pim1 knockdown cells displayed reduced ERK phosphorylation. Decreased MAPK signaling activation may be correlated with decreased proliferation and tumorigenicity in Pim1 knockdown cells. These results indicate Pim1 is essential for MAPK signaling activation, consistent with a previous report showing that Pim1-depleted and Pim1-inhibitor treated bone marrow cells has impaired ERK phosphorylation (Grundler, 2009). Our data further suggested that Pim1 overexpression did not enhance ERK phosphorylation *in vitro*, but *in vivo*. This is consistent with a report showing that MAPK signaling is activated in cardiac-specific Pim1 transgenic mice but is not activated in Pim1 overexpressing cardiomyocyte cultures (Muraski, 2008). The Raf–MEK–ERK/MAPK pathway is subject to many levels of regulation. Our data

and previous reports indicated that Pim1 may not directly activate ERK/MAPK signaling pathway (Yan, 2006; Muraski, 2008). Constitutive ERK/MAPK activation appears to play a critical role for NE differentiation in prostate cancer cells (Yuan, 2007). It needs to be further investigated whether reduced ERK/MAPK signaling activation by Pim1 knockdown is correlated with decreased NE differentiation and tumorigenicity.

Many studies implicate that targeting Pim1 could be a promising strategy in anti-cancer therapy. Importantly, Pim1 depletion has only subtle effects for normal cells. Pim1 deficient mice are ostensibly normal, healthy and fertile (Laird, 1993). Pim1, Pim2 and Pim3 compound knockout mice are viable and fertile, but show a profound reduction in body size, suggesting Pim kinases might act as sensitizers for growth factor signaling pathways (Mikkers, 2004). Pim kinase inhibition using SGI-1776 in prostate cancer cells or CLL cells (B-cell chronic lymphocytic leukemia) results in a concentration dependent induction of apoptosis (Mumenthale, 2009; Chen, 2009b). Prostate cancer initially responds to androgen ablation therapy, but eventually progresses to an androgen-independent stage. It is still a challenge for effectively treatment of advanced hormone-refractory prostate cancer. The aggressive malignancy of neuroendocrine tumors is believed to be associated with hormonal independence (Vashchenko, 2005; Abrahamsson, 1999). In this study, we knocked down Pim1 expression in androgen independent cell lines and showed Pim1 knockdown abrogated tumorigenesis. Activation of MAP kinase signaling pathway has been implicated in advanced and androgen-independent prostate cancers (Gao, 2006). Pim1 knockdown reduced ERK signaling activation, suggesting a functional role of Pim1 in maintaining tumorigenicity**.** Therefore, Pim1 kinase is required for maintenance of a transformed phenotype, indicating that Pim1 could be an attractive drug target for prostate cancer treatment.

CHAPTER IV

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Although cooperation between Pim1 and Myc was described many years ago in mouse lymphoma models (Verbeek, 1991; van Lohuizen, 1989; Möröy, 1991), the association of Pim1 and Myc in prostate cancer had not been clearly demonstrated in any *in vivo* animal models previously. In addition, it is debatable whether PIM1 is a key gene or a downstream player in cancer development and progression. Our study clearly demonstrated that Pim1 itself was a weak oncogene, but when it cooperated with MYC, dramatically accelerated MYC induced tumorigenicity. Further studies indicated that Pim1 was required to maintain tumorigenicity and ERK/MAPK signaling activity. Thus, Pim1 could represent a therapeutic target for treatment of prostate cancer.

Some limitations regarding a tissue recombination model coupled with lentiviral-mediated gene transfer

In tissue recombination experiments, we used lentivirus to transduce adult prostate cells. This approach enables expression of a transgene in a few cells initially, which mimics the initiation of human cancer. In the current study, we used freshly isolated adult prostate cells and embryonic rat UGM cells in order to reduce undesired differentiation of the prostate stem/progenitor cells and to avoid artificial selection of cell population in the cell culture condition. Using this model we were able to define a role for Pim1 or MYC or their synergism in the development of prostate cancer. Furthermore, we were able to establish many cell lines from MYC/Pim1 tumors. However, there are some limitations in this tissue recombination model.

Lentivral infection is extensively used by many studies since it is very powerful to transduce both dividing and non-dividing cells. High MOI (Multiplicity Of Infection: the ratio of infectious virus particles to target cells) can increase transduction efficiency and transgene expression, but it can also increase the risk of insertion mutagenesis due to integration of multiple copies of transgene per cell (Woods, 2002; Kustikova, 2003). We infected cells for 3 hours using similar MOI and centrifuge method as described (Xin, 2003). Two days post-infection, approximately 20-30% of cells were YFP positive by flow cytometry analysis (data not shown). Since the percentage of cells with the transgene expression was not very high, we did not attempt to evaluate the association of MOI and transgene copy numbers obtained in single cells. Although the risk of multiple and potentially harmful viral integrants per cell might exist due to insertional mutagenesis, we did not find any abnormalities in the regenerated control grafts. Further, we also found similar effects for multiple tissue recombination experiments. Thus, we think our study may not involve insertional mutagenesis.

Another risk for lentiviral transduction is viral vector shuttle via transplanted cells resulting in undesired transduction of recipient cells (Blömer, 2005). In our study, mouse prostate cells infected by lentivirus and UGM cells are combined and incubated overnight, so the risk of viral vector shuttle may exist in our study. In order to test whether lentiviral particles in mouse prostate cells transduced into adjacent rat UGM cells, we carefully examined MYC expression in MYC grafts using immunohistochemical staining. The results showed that MYC expression was found only in epithelial cells, but not in stromal cells (Figure 23). It indicates that viral shuttle might not occur in our study. If YFP

antibody were available to perform immunohistochemistry, it could be further confirmed whether other transgenes, such as Pim1, only expressed in epithelium part.

It is still a debate whether using total prostate cells is sufficient or using isolated prostate epithelial cells for tissue recombination experiments. In our study, transgene expression could be expressed in any cell type because the lentiviral transfer vector contains the ubiquitin promoter (Lois, 2002), but we did not isolate epithelial cells for tissue recombination experiments based on the following considerations. First, tissue recombination requires stem/progenitor cells stimulated by the UGM cells to regenerate prostate glands. Current methods for isolating prostate epithelial cells are mainly based on the size or/and cell surface markers. Since there are no consistent standards for selection of stem/progenitor cells, we might lose the stem/progenitor population after the isolation of prostate epithelial cells. Second, adult stromal fibroblasts could not survive after tissue recombination. Hoechst dye staining confirmed that the stromal cells observed in regenerated glands were of rat origin derived from rat UGM cells. Our data also showed that none of stromal cells expressed MYC transgene and MYC only expressed in luminal cells in the epithelium of regenerated glands. Therefore, we believe it is appropriate to use total prostate cells to perform tissue recombination experiments..

Prostate neuroendocrine cancer

Neuroendocrine (NE) cells are rare in normal prostate epithelial compartments. NE differentiation increases during prostate cancer progression, which is correlated with aggressive disease, tumor grade, hormone deprivation therapy and survival (Abrahamsson, 1999; Miyoshi, 2001; Hirano, 2004; Vashchenko, 2005; Bonkhoff,

2005, Taplin me, 2005; Berruti, 2005; Mcwilliam; 1997; Kamiya, 2008). Although normal NE cells could transform to NE-like tumor cells, more evidence supports that adenocarcinoma cells can undergo a transdifferentiation process to become NE-like cells (Cindolo, 2007). Ta-Chun Yuan *et al.* pointed out that NE cells in prostate cancer originate from cancerous epithelial cells, but not from normal NE cells, and should be defined as 'NE-like cancer cells' (Yuan, 2007). They proposed that adenocarcinoma cells can undergo a transdifferentiation process to become NE-like cells. These NE-like cells acquire a similar phenotype as normal NE cells and express several NE markers, but still retain some epithelial characteristics (Figure 42).

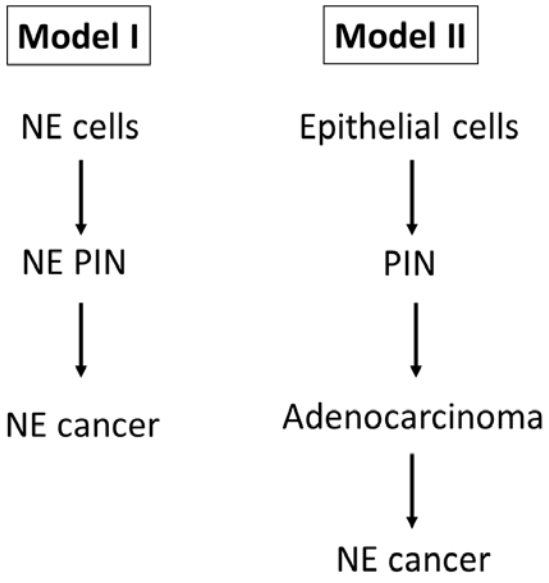

Figure 42. Proposed models of the cellular origins of prostate NE cancer. Model I shows that rare neuroendocrine cells are transformed and give rise to NE PIN, then to neuroendocrine prostate cancer. Model II shows that epithelial cells are transformed and give rise to PIN, adenocarcinoma, and then adenocarcinoma transdifferentiate to neuroendocrine prostate cancer.

Our data suggests that NE carcinoma may be derived from adenocarcinoma (Figure 24 and 25). In addition, Gene Set Enrichment Analysis (GSEA) showed that 6-

week MYC/Pim1 tumors or derived cell lines exhibited similar profiles of gene expression as human prostate cancer or cell lines but not as NE cells in normal tissues (data not shown), consistent with a previous report showing that NE-like tumor cells share identical profiles with non-NE tumor cells (Sauer, 2006). The mechanism of transdifferentiation of prostate cancer into NE-like tumor is unknown. Both TRAMP and Rb/p53-null mice show simultaneous inactivation or knockout of both p53 and Rb genes, which promotes NE like prostate cancer (Perez-Stable, 1997; Zhou, 2006). It has been reported that IL-6 signaling induces neuroendocrine differentiation (Palmer, 2005). ERK/MAPK activation was reported to be a potential mechanism in this transdifferentiation and may serve as a therapeutic target (Yuan, 2007). Proper animal models and cell lines are needed to elucidate the molecular basis of NE differentiation. However, it is claimed that "virtually no animal models of a neuroendocrine/small cell variant of prostate carcinoma are available for experimental studies" (Yuan, 2007). Given that there is limited research on NE tumor, MYC/Pim1 tumors and/or derivative cell lines could be valuable tools for studying the molecular mechanisms for NE transdifferentiation, and for developing and evaluating therapies for NE tumor.

Prostate cancer develops from normal prostate to PIN to adenocarcinoma, and then transits to NE-like cancer, finally to metastasis. We have observed this progression in the Pim1 and MYC tissue recombination model (Table 2). Therefore, our tissue recombination would provide valuable information for the mechanisms of prostate cancer progression, and for developing novel diagnosis and therapeutic approach.

Table 2. The progression of prostate cancer is demonstrated by histology of regenerated grafts

Phenotype	Weeks	Name
Benign	6	Control
	6	Pim1
PIN	6	MYC
	12	Pim1
Adenocarcinoma	4	MYC/Pim1
	12	MYC
NE-like carcinoma	6	MYC/Pim1
	12	MYC

Clinical significance of MYC/PIM1 synergism in human prostate cancer

A major clinical problem is the development of castration-resistant prostate cancer (CRPC) during androgen ablation treatment. The molecular mechanisms underlying the change from androgen dependence to castration-resistance are not well understood and represent a challenge for development of efficient therapies. It has been suggested that Myc may be involved in development of CRPC (Bernard, 2003). On the other hand, Pim1 enhances cell survival at castrate levels of androgen and neuroendocrine functions of PC12 cells (Glazova, 2005). Coexpression of MYC and PIM1 was frequently found in patients under androgen ablation therapy (van der Poel, 2010). In this study, we found that coexpression of MYC and PIM1 is associated with high Gleason grade in human prostate tumors. Using a mouse tissue recombination model we demonstrated that Pim1 strongly cooperates with MYC, resulting in advanced prostate cancer with NE differentiation. NE tumors are correlated with hormonal independence (Vashchenko, 2005; Abrahamsson, 1999). Therefore, our tissue recombination model and its derivative cell lines could be extensively used for evaluation of the mechanisms of CRPC. It remains to be determined whether coexpression of MYC and PIM1 is

associated with neuroendcrine cancer in patients. Of importance in clinic is the need to know if coexpression of MYC and PIM1 can be used for distinguishing indolent versus aggressive prostate cancer.

Potential cancer stem cells in MYC/Pim1 tumors

Takahashi and Yamanaka reported that four transcription factors (Oct4, Sox2, Klf4, and c-Myc) can reprogram mouse and human somatic cells to be pluripotent (Takahashi, 2006). MYC contributes to cancer initiation and progression by stimulating an embryonic stem cell-like signature characterized by an enrichment of genes involved in ribosome biogenesis and by repressing differentiation (Koh, 2010). It is of interest to know whether synergism of Pim1 and MYC enhances stem /progenitor cell-like characteristics.

MYC/Pim1 induced tumor shows evidence of neuroendocrine (NE) differentiation, which arises by transdifferentiation of adenocarcinoma cells with progression. This indicates that MYC/Pim1 tumors have plasticity within a specific time range. At 6 weeks, although the majority of MYC/Pim1 tumor had lost Nkx3.1 expression, there were a few Nkx3.1 positive cells scattered in the tumor (Figure 43). It remains to be determined whether those Nkx3.1 positive cells represent cancer stem cells like CARNs (Wang, 2009). We also studied AR expression in the adenocarcinoma part of MYC/Pim1 tumor (Figure 44). Of note, a small amount of cells did not express AR in those parts. Since there is debate over AR status in prostate stem cells (Shen, 2010), it needs to be determined whether these cells have cancer stem cell properties. It also remains to be studied whether Nkx3.1 positive cells are those AR negative cells in MYC/Pim1 tumor. Furthermore, double staining of stem cell markers (for example Sca-1

Sca-1, CD133, CD117, CD44) and cell lineage markers (for example CK5, CK8, synaptophysin) may facilitate the identification of the cell origin of cancer stem cells in MYC/Pim1 tumors.

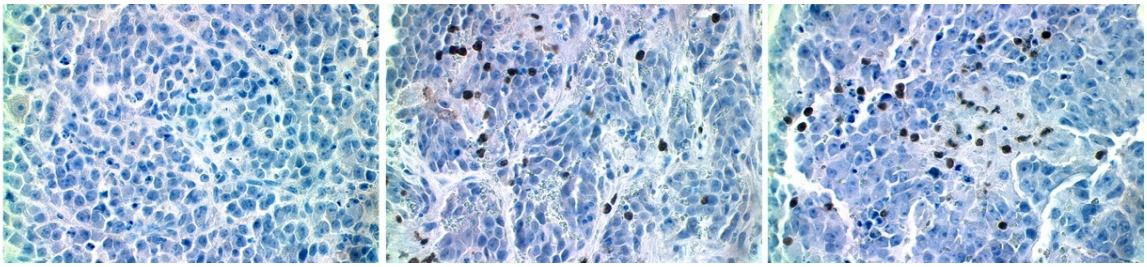

Figure 43. A few areas of MYC/Pim1 tumor section showing Nkx3.1 expression. Immunohistochemical staining shows common loss of Nkx3.1 expression in MYC/Pim1 tumors (left), Nkx3.1 positive cells are seen in a few areas (middle and right) (Original magnification: 400×)..

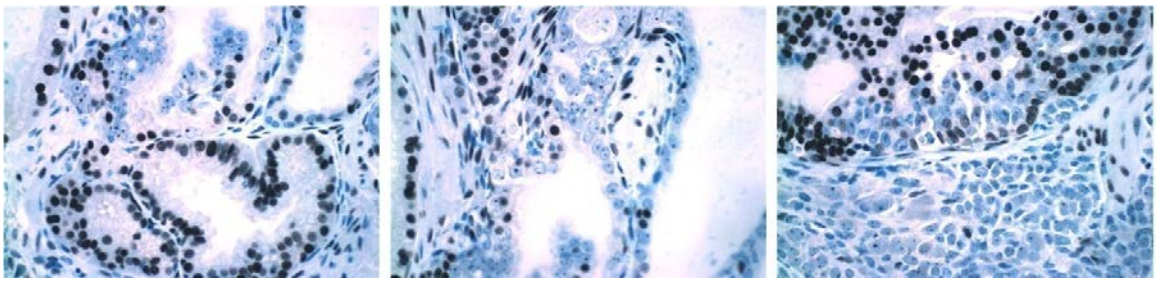

Figure 44. Early loss of AR expression in MYC/Pim1 tumors. Immunohistochemical staining shows some AR negative cells in the early lesions of MYC/Pim1 tumors (Original magnification: 400×).

A sphere forming assay has been developed to study the function of stem cells *in vitro* (Xin, 2007; Garraway, 2010). Our preliminary data showed that earlier passages of control MPT cells were able to form a lot of spheres, while Pim1 knockdown dramatically decreased the number of sphere forming cells (Figure 45). Late passages of MPT cells lost the sphere forming proprieties for unknown reasons. It remains to be determined whether these spheres enrich a stem cell/progenitor population. We have other early passages of MYC/Pim1 cells that may help us further characterize these spheres.

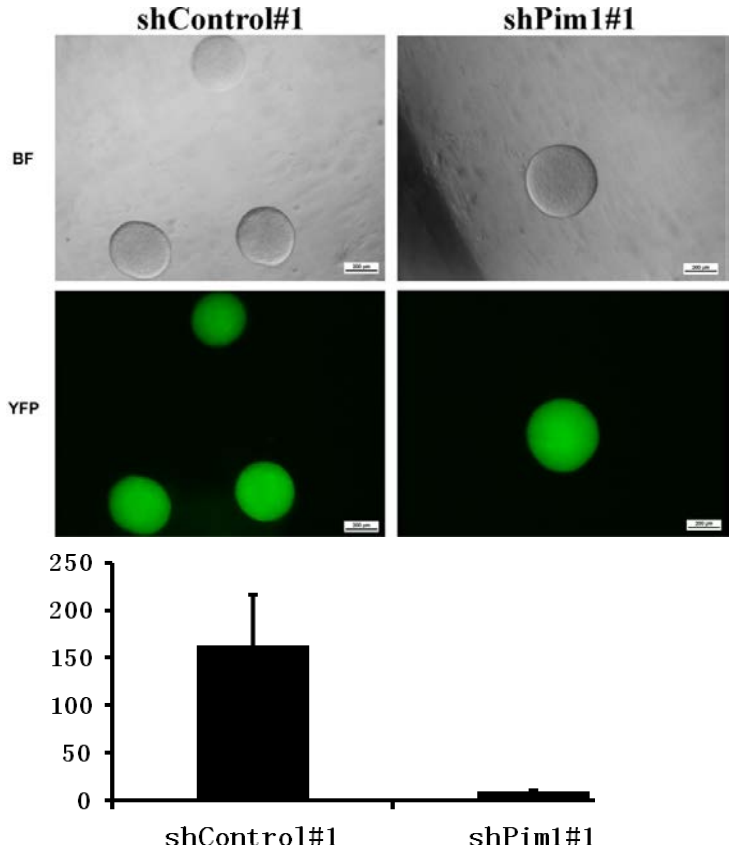

Figure 45, Control MPT cells form more spheres than Pim1 knockdown cells. *Upper panel:* Representative images of solid spheres in Matrigel show that control MPT cells (shControl#1) are enriched for sphere-forming cells, which Pim1 knockdown cells (shPim1#1) lose this potential (Original magnification: 100×). *Lower panel:* Graph shows the numbers of spheres. Results represent the mean ± SD of triplicate wells after 7 days of incubation.

Potential conjunction of PIM1 kinase and ERK inhibitors in the treatment of prostate cancer

Cancer is a multistep process including activation of proto-oncogenes and inactivation of tumor suppressor genes. Extensive evidence shows that inactivation of a single oncogene can be sufficient to induce sustained tumor regression. These observations brought about oncogene addition concept which emphasizes the dependency of some cancers on one or a few genes for the maintenance of the malignant phenotype (Weinstein, 2006).

Oncogene addition has been shown in Myc transgenic mice (Felsher, 1999; Pelengaris, 2002). Targeting Myc could be an effective target in cancer therapy, but may be harmful for normal cells since it is a very important transcription factor for a living cell. Loss of Pim1 expression has only subtle effects in mice (Laird, 1993). In this scenario, we are interested in Pim1 inhibition in prostate cancer treatment. We speculated that Pim1 may establish a preneoplastic state although its overexpression is not sufficient to initiate prostate adenocarcinoma. Since Pim1 alone cannot induce tumorigenesis and there are examples of escaping from oncogene addiction (Ewald, 1996; Plattner, 1996), it remains to be determined whether inactivation of Pim1 is sufficient to induce prostate tumor regression. If Pim1 is not necessary to maintain tumorigenicity, Pim1 inhibitor might not be effective for cancer treatment. Thus, the results of our research are important in light of recent efforts in the development of Pim1 kinase inhibitors. In the current study, we found that depletion of Pim1 expression in established prostate cancer cell lines resulted in reduced cell proliferation, survival and tumorigenicity, indicating that Pim1 is required for tumorigenic maintenance. Because Pim2, Pim3 or other genes can compensate for Pim1 deficiency (Berns, 1999), it remains to be seen whether long-term inhibition of Pim1 leads to a compensatory up-regulation of other PIM family members.

The PIM1 kinase inhibitor quercetagetin is able to inhibit PIM1 activity in prostate cancer cells in a dose dependent fashion (Holder, 2007). In our preliminary study, we also found that Pim kinase inhibition using quercetagetin resulted in dose dependent reduction of MPT cell viability (Figure 46).

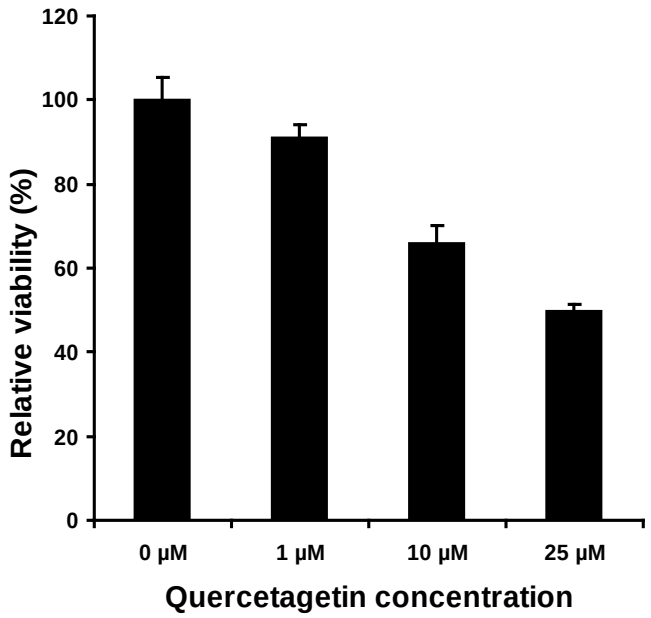

Figure 46. Pim1 inhibitor quercetagetin decreases MPT cell viability. MPT cells are treated with different doses of quercetagetin and cell viability is tested using CellTiter-Glo Luminescence Cell Viability Assay (Promega).

The MYC/Pim1 cancer model may highly appropriate to test the efficacy of Pim1 inhibitors *in vivo*. 4-week MYC/Pim1 grafts displayed HGPIN and adenocarcinoma, while 6-week MYC/Pim1 formed neuroendocrine tumor. It is of interest to determine whether Pim1 inhibitor, such as SGI-1776, is able to abrogate MYC/Pim1 tumorigenicity 6 weeks post-grafting, and to prevent 4-week MYC/Pim1 early lesions progressing to neuroendocrine tumor at 6 weeks using tissue recombination approach.

At present, effective treatment for advanced cancers is still a significant challenge. ERK/MAPK signaling is frequently activated in prostate cancer, especially in advanced cancer and CRPC (Abreu-Martin, 1999; Gioeli, 1999; Mailik, 2002). Pim1 overexpression has been found in prostate cancer and associated with high grade tumor (Dhanasekaran, 2001; Valdman, 2004; Cibull, 2006; van der Poel, 2010). In addition, Pim1 plays an important role in multidrug resistance (Xie, 2006; 2008). Inhibiting Pim1

expression increases the sensitivity of prostate cancer cells to specific chemotherapy (Mumenthale, 2009). Therefore, adjunctive use of Pim1 and ERK inhibitors is expected to increase apoptosis, improve the efficacy and lower drug dosages. This combination therapy is also expected to lower the cytotoxicity and enhance tumor suppression *in vivo*, which would be important in treating hormone-refractory prostate cancer.

Possible mechanisms of Pim1 and Myc cooperation

Pim1 enhances Myc stability and activity

The MYC and Pim1 oncogenes are frequently overexpressed in prostate cancer, and together they are sufficient to induce advanced prostate cancer in a mouse model. The precise mechanism of Pim1 as such an effective partner of Myc in tumorigenicity remains obscure. *In vitro* studies have shown that Pim1 promotes Myc induced tumorigenicity by increasing Myc stability through decreasing PP2A activity (Chen, 2005), through increasing Myc S62 phosphorylation and decreasing T58 phosphorylation (Zhang, 2008), or by increasing Myc transcriptional activity through phosphorylation of histone H3 at Myc target genes (Zippo, 2007, Figure 47).

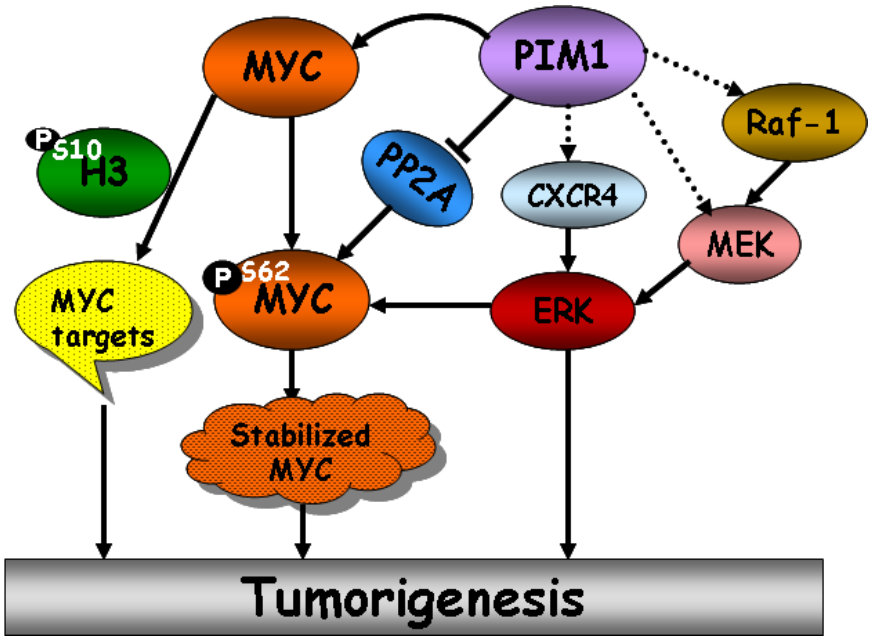

Figure 47. Summary of possibilities regarding Pim1 and Myc cooperation. Solid arrows show published findings; broken arrows show potential interactions with ERK/MAPK pathway (see the text for details and references).

It is well known that phosphorylation of N terminal threonine 58 (T58) and serine 62 (S62) has been implicated in regulating stability of MYC. First, S62 can be phosphorylated by ERK, and then T58 is phosphorylated by glycogen synthase kinase-3β (GSK-3β). Double phosphorylated MYC is isomerized by Pin prolyl isomerase and S62 is then dephosphorylated by serine/threonine protein phosphatase 2A (PP2A), which drives polyubiquitination and proteasomal degradation of MYC (Figure 48) (Sears, 2004).

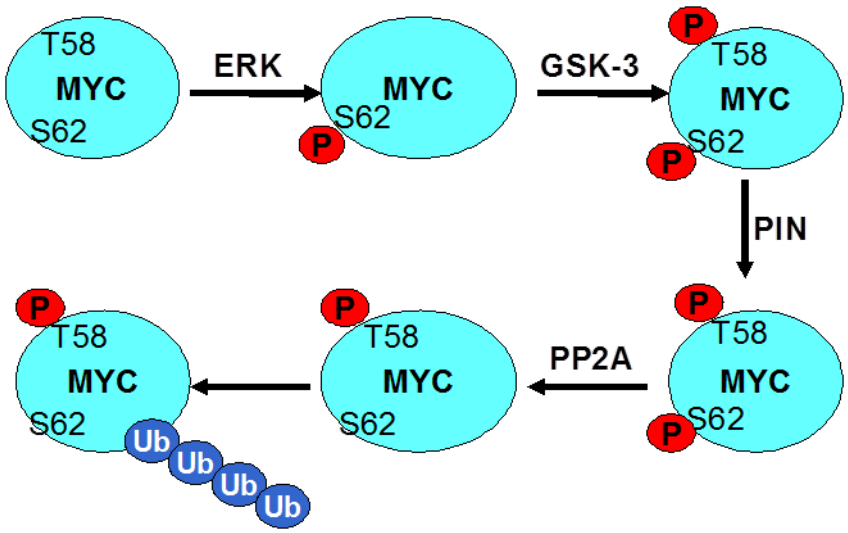

Figure 48. Phosphorylation of N terminal threonine 58 (T58) and serine 62 (S62) regulates Myc turnover (See text for details).

In MYC/Pim1 tumors, both MYC and Pim1 proteins levels appeared elevated compared to Pim1 alone or MYC alone grafts (Figure 18C and 34A). This is likely because both MYC and Pim1 were stabilized. These tumors also showed relatively increased Myc serine 62 phosphorylation, which is claimed to be a stabilized and activate form (Sears, 2004). Whether or how Pim1 stabilizes MYC or vice versa needs to be explored in the future study.

In chapter II, we found Pim1 greatly accelerated MYC induced prostate cancer progression to neuroendcrine tumor within 6 weeks (Figure 19, 20). MYC grafts showed HGPIN, but over time (12 weeks) developed adenocarcinoma with neuroendocrine differentiation (Figure 19, 28). In addition, weak Pim1 expression was observed in 12-week MYC tumors with neuroendocrine differentiation (Figure 31). In summary, MYC stability and activity seems to be enhanced when MYC and Pim1 are over-expressed concurrently.

Although MYC/Pim1 tumors showed increased levels of MYC phosphorylation on serine-62, grafts expressing a phospho-mimicking MYCS62D mutant did not form tumors resembling MYC/Pim1 grafts, revealing that S62 phosphorylation may not be a crucial step for MYC/Pim1 synergism (Figure 35). In addition, MYCS62D mutant is supposed to be more stable than wild-type MYC, but this still needs to be validated. The Myc phosphorylation deficient mutants T58A has been observed in lymphomas and is associated with increased MYC stability (Sears, 2004; Thibodeaux, 2009). Furthermore, the phospomimetic mutant MYC S329D has comparable tumorigenicity as co-expression of PIM1 kinases and MYC *in vitro* (Zhang, 2008). It remains to be determined if these mutants with increased MYC stability, for example, T58A, or S329D, can mimic MYC/Pim1 synergism *in vivo*.

Pim1 seems to enhance MYC functions, showing that higher gene expression of phosphorylated MYCS62 and MYC targets, Cyclin D1, D2, E, in MYC/Pim1 tumors compared to MYC alone and MYC/K67M (Figure 34). However, it is difficult to separate the direct effects of MYC and Pim1 expression on these target genes from indirect gene activation as a consequence of tumorigenesis. Examination of MYC target genes after a few days of introducing transgenes by lentviral infection may clarify whether Pim1 amplifies MYC function.

In chapter III, we showed that Pim1 knockdown abrogated tumorigenicity of MPT cells even when MYC level was not decreased (Figure 40), which also suggests that Pim1 and MYC synergism in prostate cancer may depend on additional mechanisms other than enhancing MYC activity and stability.

ERK/MAPK signaling activation may be involved in Myc and Pim1 synergism

In chapter III, we showed that knockdown of Pim1 expression in MYC/Pim1 derived cell lines decreased phosphorylation of ERK (Figure 41), revealing that Pim1 plays a role in ERK/MAPK signaling activation. Interestingly, Pim1 grafts showed increased phosphorylated ERK comparing to control grafts (Figure 41G), but Pim1 overexpressing cancer cell lines did not show alteration of phosphorylated ERK (Figure 41E, F). It is still unclear whether Pim1 directly or indirectly phosphorylates ERK. In the primary mitogen regulated pathway, extracellular stimuli activate Raf-1, which phosphorylates and activates MAPK kinase (MEK), leading to the phosphorylation and activation of the extracellular signal regulated kinases (ERK). Activated ERK translocates to the nucleus and activates gene expression by phosphorylation of a series of substrates including MYC S62. It remains to be determined whether Pim1 affects Raf-1 or MEK phosphorylation, or interacts with other proteins that are involved in this pathway (Figure 47).

It has been reported that Pim1-deficient bone marrow expresses less chemokine receptor CXCR4 and shows defects in homing and migration because of impaired CXCL12–CXCR4 signaling (Grundler, 2009). CXCL12 is also known as SDF1 (stromal cell derived factor 1), which is the predominant ligand for CXCR4. The CXCL12–CXCR4 pathway has been implicated in promoting prostate cancer cell migration, invasion, angiogenesis and metastasis (Chinni, 2006; Arya, 2004; Singh, 2004; Taichman, 2002; Xing, 2008). ERK/MAPK can be activated by CXCL12–CXCR4 signaling (Tan, 2008, Teicher, 2010). Therefore, we speculate that Pim1 may enhance CXCR4 expression, which interacts with CXCL12 expressed by stomal fibroblast or other cell type in microenvironment, leading to enhanced CXCL12–CXCR4 signaling, and thereby

enhancing phosphorylation of ERK. This may explain why we observed that Pim1 increased phosphorylation of ERK *in vivo*, not *in vitro*, and why Pim1 knockdown decreased phosphorylation of ERK. ERK/MAPK activation can phosphorylate Myc on S62, thereby stabilizing Myc (Figure 48).

Interestingly, our Gene Set Enrichment Analysis (GSEA) of microarray data showed that epithelial-mesenchymal transition (EMT gene) set was down-regulated in Pim1 knockdown cells comparing to control, while EMT gene set was up-regulated in MYC/Pim1 tumor (data not shown). It suggests that Pim1 may play a role in EMT. It is known that MAPK signaling is required for EMT *in vivo* and metastasis (Janda, 2002). A recent report shows that Pim1 promotes cancer cell migration and invasion possibly through a Pim1 substrate NFATc (Santio, 2010). It is important to be confirmed whether Pim1 involved in EMT and differentiation, which may facilitate to study on the mechanisms of Pim1 and Myc cooperation in tumorigenesis.

Other potential mechanisms of Pim1 and Myc cooperation

Using a tissue recombination approach, we found that Pim1 expression resulted in mild pathological abnormalities, suggesting that Pim1 overexpression may establish a preneoplastic state. A recent report showed that Pim1 is induced by DNA damage possibly mediated by the nuclear factor kappa beta (NF-κB) pathway. In addition, Pim1 overexpressing cell lines displayed genomic instability (Roh, 2003; 2005; 2008; Zemskova, 2010). Whether and how Pim1 overexpression induces genomic instability needs be to explored *in vivo*. It will be of interest to know whether Pim1 and MYC cooperate to induce genomic instability.

It is well known that Myc plays an important role in promoting proliferation and apoptosis. Pim1 increases cell proliferation and inhibits apoptosis. Pim1 may disrupt Myc induced apoptotic pathways and cooperate with Myc to transform normal cells. Our microarray data and previous reports implicate that Pim1 and Myc may synergize in promoting cell cycle, possible by activating cdc25 (Mochizuki T, 1999; Bachmann., 2004; Galaktionov, 1996). Pim1 can induce p53-dependent senescence (Hogan, 2008; Zemskova, 2010). Moreover, Myc can induce senescence, which is suppressed by Werner syndrome protein or cdk2 (Campaner, 2010). It needs to be investigated whether and how Myc and Pim1 together can bypass senescence.

Our conventional analysis of microarray data has shown only subtle differential gene expression between Pim1 grafts and control grafts, and between Pim1 knockdown and control MPT cells (data not shown), suggesting that the synergism of Pim1 and MYC may be mediated by epigenetic or/and microRNA regulation.

The major aim of our studies is to establish a mechanistic model for the cooperation between Myc and Pim1. As described, MYC/Pim1 synergy is critically dependent on Pim1 kinase activity. Of importance is the need to know what Pim1's substrates are responsible for its cooperation with Myc, and what downstream targets of Pim1 and Myc are responsible for this cooperation. Those studies will help us better understanding of the molecular basis of human prostate cancer initiation and progression. Such studies could facilitate to development of novel methods for diagnosis and treatment in human cancer.

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