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MYC and Prostate Cancer

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Abstract

Prostate cancer, the majority of which is adenocarcinoma, is the most common epithelial cancer affecting a majority of elderly men in Western nations. Its manifestation, however, varies from clinically asymptomatic insidious neoplasms that progress slowly and do not threaten life to one that is highly aggressive with a propensity for metastatic spread and lethality if not treated in time. A number of somatic genetic and epigenetic alterations occur in prostate cancer cells. Some of these changes, such as loss of the tumor suppressors PTEN and p53, are linked to disease progression. Others, such as ETS gene fusions, appear to be linked more with early phases of the disease, such as invasion. Alterations in chromosome 8q24 in the region of MYC have also been linked to disease aggressiveness for many years. However, a number of recent studies in human tissues have indicated that MYC appears to be activated at the earliest phases of prostate cancer (e.g., in tumor-initiating cells) in prostatic intraepithelial neoplasia, a key precursor lesion to invasive prostatic adenocarcinoma. The initiation and early progression of prostate cancer can be recapitulated in genetically engineered mouse models, permitting a richer understanding of the cause and effects of loss of tumor suppressors and activation of MYC. The combination of studies using human tissues and mouse models paints an emerging molecular picture of prostate cancer development and early progression. This picture reveals that MYC contributes to disease 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. These insights pave the way to potential novel therapeutic concepts based on MYC biology.

Keywords: prostate cancer, tumor initiating cells, prostatic intraepithelial neoplasia

Overview of Molecular Aspects of Prostate Cancer

Prostate cancer is the most common noncutaneous malignant neoplasm in men in Western countries, and it is responsible for the deaths of approximately 30,000 men per year in the United States.[1] Risk factors for prostate cancer include advanced age, race, family history, and environmental factors such as diet and inflammation.[2]

Prostate cancer is thought to develop through a stepwise progression by which the benign prostatic epithelial cells transition to high-grade prostatic intraepithelial neoplasia (PIN), invasive adenocarcinoma, distant metastatic disease, and androgen refractory metastatic disease.[3] The transformation of prostate cells from benign to PIN and adenocarcinoma is characterized by several diagnostic morphological features, such as nuclear and nucleolar enlargement and alterations in chromatin structure.[4,5]

The earliest somatic molecular alterations that begin to occur just before or at the onset of PIN include silencing of gene expression through epigenetic changes, such as *GSTP1* promoter hypermethylation, telomere shortening, and the activation of the proto-oncogene MYC.[2,6,7] Oncogenic ETS family transcription factors are activated by gene fusions (the most common results from a fusion between the *TMPRSS2* gene and the *ERG* gene on chromosome 21) at or near the onset of invasive adenocarcinoma in a significant subset of cases.[8-10] Other common genetic changes found in prostate cancers include deletions of regions harboring putative tumor suppressors on chromosome 8p (*NKX3.1*), 10q23 (*PTEN*), 12p13 (*CDKN1B*-p27), 13q (*RB1*), and 17q (*TP53*); gains in regions of oncogenes on chromosome 8q24 (*MYC*) and Xq (*AR*); and point mutations (e.g., in *TP53* and *AR*).[3,11-14]

MYC Overexpression at the mRNA and Protein Levels in Prostate Cancer

In the first study demonstrating an elevation of *MYC* mRNA in prostate adenocarcinoma,[15] Fleming *et al.* compared RNA extracted from prostatectomy-derived prostate tissue from 18 patients, comprising 7 cases of prostate adenocarcinomas and 11 cases of benign prostatic hyperplasia (BPH). RNA was also extracted from normal prostate tissue obtained from autopsy cases. Based on Northern blot analysis and densitometric quantification, there was a significantly higher level of *MYC* expression in the adenocarcinomas than that of both the BPH and the normal prostate tissue. This finding was confirmed by Buttyan *et al.*,[16] who included 9 prostatic adenocarcinomas, 19

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BPH, and 1 normal prostate in their study. Two thirds of the cancer cases were Gleason score 5 and above and showed elevated *MYC* RNA expression compared to that of BPH and normal tissue. The remaining cancers did not show *MYC* elevation and were Gleason score 4 and below. Although these early studies showing *MYC* mRNA overexpression were generally not followed up in larger studies, a relatively large number of whole genome mRNA expression-profiling studies showed that *MYC* mRNA is overexpressed in the majority (80%–90%) of all primary human clinical prostate cancer lesions. For example, work from our institution showed that *MYC* was one of the top genes overexpressed in human prostate cancer tissues, as compared to matched normal-appearing prostate tissue or BPH tissue.[17] Based on the Oncomine database,[18] in which other publications deposited mRNA profiling data using prostate cancer tissues in nonpretreated patients ($n = 7$ separate experiments from the 5 articles),[19-23] *MYC* mRNA was found to be elevated in the cancer tissue as compared to matched benign prostatic tissue in the majority of cases. These results show highly consistent upregulation of *MYC* at the mRNA level in the majority of prostate cancers across a large number of patient samples from multiple institutions.

MYC Protein in Prostate Cancer

Although mRNA expression studies clearly indicated *MYC* overexpression in most human prostate cancer lesions, until recently the phase of prostate cancer development in which MYC protein is expressed in humans remained unclear. It was critical to directly ascertain MYC protein levels given that MYC protein is tightly regulated by posttranscriptional and posttranslational mechanisms and that the presence of *MYC* mRNA does not necessarily imply the presence of MYC protein.[24,25]

A number of studies have described MYC protein expression as detected by immunohistochemistry in prostate cancer[26-28] and even one prior study in high-grade PIN.[26] Taken together, these studies are difficult to interpret. For example, in 2 of these studies, MYC staining was localized either exclusively[26] or nearly exclusively[27] to the cytoplasm. This lack of nuclear staining was surprising because (1) most of the known functions of MYC in cellular transformation have been ascribed to actions in the nucleus; (2) endogenous MYC has been localized to the nucleus;[29] and (3) in cells genetically modified to express exogenous MYC, the protein localizes predominantly to the nucleus.[30,31] In the third article that examined MYC staining in prostate cancer, staining was predominantly localized to the nucleus and was positive in 33 of 45 cases.[28] Surprisingly, in that study there was little difference in MYC staining between benign and malignant epithelial cells.[28]

We recently employed a newly developed rabbit monoclonal antibody, in conjunction with genetically defined control experiments that validated the IHC staining, and obtained strong nuclear staining for MYC in human clinical prostate cancer,[6] with much lower expression in benign tissues. Furthermore, we observed that in addition to nuclear MYC protein overexpression in localized primary prostate adenocarcinoma and metastatic disease, MYC protein was frequently overexpressed in PIN,[6] with a stepwise increase from normal to low-grade PIN to high-grade PIN. MYC expression in normal prostate epithelium was generally absent or low, in which case it was most often confined to the nuclei of basal cells. In prostate atrophy, MYC expression was comparable to that of normal prostate epithelium; however, there was a compartmentalization shift, with MYC expression occurring predominantly in the luminal cells.

The Mechanisms Responsible for MYC Overexpression in Prostate Cancer Remain Unclear

1. Gene Amplification and Rearrangement in Prostate Cancer

A number of genetic approaches including comparative genomic hybridization (CGH) strategies have identified numerous allelic losses and gains that are common in prostate cancer.[32-34] The 8q24.21 region, where MYC is located, is contained within a region that is commonly amplified in prostate cancer, especially in advanced and recurrent disease.[26,34-37] By chromosome microdissection, 8q24 amplification was first identified in 2 prostate cancer cases.[35] To verify this, fluorescence in situ hybridization (FISH) was carried out on 44 prostatectomy samples, and the amplification was present in only 9% of the total cases studied but 75% of advanced cases. Accordingly, in the study of patients with recurrent disease, comparative genomic hybridization and FISH analysis showed 8q24 amplification in 8 of 9 cases.[35]

Jenkins *et al*. determined that the gain of whole chromosome 8 was common in PIN, adenocarcinoma, and metastases, whereas the amplification of the MYC locus itself was mostly observed in metastatic disease.[26] This was confirmed by Nupponen *et al*. in a study of 37 hormone refractory prostate cancer cases. They found 8q gain in 72.5% of cases by CGH, but only 29% of these cases showed *MYC* amplification by FISH,[37] which indicated that the whole gain of 8q was more common than the specific amplification of *MYC*.

Because the amplification of the 8q24 region is predominantly observed in late-stage/aggressive tumors, it has been widely held that MYC is involved in disease progression.[26,38-40] Interestingly, the amplification of *MYC* is generally on the order of a few fold; high-level amplifications, such as those seen with *NMYC* in a subset of neuroblastomas, are virtually never seen in prostate cancer. Although 8q24 gain may be responsible for MYC overexpression in a subset of prostate adenocarcinoma cases, the data supporting this are somewhat weak. For example, when we compared MYC protein levels in prostate cancer by semiquantitative image analysis of immunohistochemistry-stained specimens, we did not find a correlation between gain of 8q24 by FISH and MYC levels.[6] Furthermore, as

indicated above, gain of 8q24 is rare in PIN lesions and localized relatively low-grade prostate adenocarcinomas (e.g., Gleason 6-7), yet MYC overexpression is common in these lesions. Further complicating this issue is that other genes, such as *TRPS1*, *EIF3S3*, *RAD21*, *KIAA0916*, and *PSCA*, are known to reside in or near this locus. At times, these are amplified in prostate cancer and have been put forth as potential targets of amplification.[41-46]

2. Wnt-B Catenin/TCF Signaling and MYC in Prostate Cancer

The *APC* gene is frequently mutated in familial adenomatous polyposis, resulting in deregulated Wnt signaling, activation of β-catenin, and transactivation of T-cell factor or leukemia-enhancing factor target genes, including MYC.[47] In prostate cancer, both *APC* and β-catenin mutations occur but are quite rare[48,49] (e.g., ~5% or less in most studies). Despite this, the *APC* gene does appear to be inactivated in most prostate cancers; Yegnasubramanian *et al*. found that *APC* hypermethylation occurred in >85% of 164 primary and metastatic prostate cancers and 7 prostate cancer cell lines studied.[50] This hypermethylation was not observed in 2 normal prostate cells lines and 24 noncancerous prostate tissue samples. Similarly, in a study of 71 samples, Kang *et al*. found that *APC* hypermethylation occurred in 30% of PIN cases and in 56.8% of cancer cases.[51] Interestingly, *APC* hypermethylation was observed more frequently in cases with high Gleason scores and high serum PSA levels. Silencing of the *APC* gene by hypermethylation in prostate cancer may mirror inactivating *APC* mutations in colon cancer, resulting in aberrant Wnt signaling. For example, targeted disruption of both copies of Apc in the mouse prostate results in PIN and invasive adenocarcinoma,[52] although in this study Myc levels were not examined. In a separate study, activation of β-catenin in the mouse prostate resulted in PIN lesions and elevated Myc levels.[53] Whether β-catenin is translocated to the nucleus in human prostate cancer to activate transcription via the classical pathway is currently unclear. For example, Whitaker *et al*. recently showed that nuclear β-catenin was commonly seen in benign prostatic tissue yet was often lost in prostatic carcinoma.[54] In another study, although the relation between β-catenin levels and MYC was not examined, Fiorentino *et al*. found that cytoplasmic β-catenin (after subtracting plasma membrane staining) was elevated in some prostate cancers, indicating that this may be equivalent to nuclear staining in terms of biological significance.[55] Furthermore, they showed that cytoplasmic β-catenin levels were likely regulated by fatty acid synthetase.[55] Despite all these studies, whether MYC activation in human prostate cancer is a result of APC inactivation, by any means, appears at least somewhat unlikely. For example, as opposed to the normal mouse prostate, which appears to constitutively express high levels of APC protein,[52] normal human prostate tissue expresses very little APC protein (S Jobbagy, WG Nelson, S Yegnasubramanian, AM De Marzo, unpublished observations), yet MYC is expressed only at low levels in these tissues.

3. FOXP3 Deletion on X-chromosome and Prostate Cancer

FOXP3, well known for its role in regulatory T-cell function, was recently shown by Wang *et al*. to regulate MYC expression in the prostate.[56] *FOXP3* mRNA and protein levels were reduced in prostate cancer, as compared to normal prostate tissue, as a consequence of gene deletion and somatic inactivation of the *FOXP3* locus. There was a correlation between FOXP3 downregulation and MYC overexpression. FOXP3 depletion in human primary prostate cells resulted in increased *MYC* mRNA and protein levels. In a murine model, prostate-specific deletion of the *Foxp3* locus also resulted in a similar increase in *MYC* mRNA and protein. The authors found that FOXP3 binds directly to the promoter region of *MYC*, which contains a conserved forkhead binding site, thus repressing its transcription. These intriguing results suggest that FOXP3 may be mediating MYC overexpression in a subset of prostate cancer cases in which *FOXP3* is deleted or inactivated, although these findings need to be validated in additional studies.

4. Germline Variants on 8q24, MYC, and Prostate Cancer

Several genome-wide association studies have shown that the 8q24 region contains several risk loci that are linked to an increased risk of prostate cancer.[57-61] The 5MB locus, which harbors all the known risk alleles, does not contain any well-annotated genes or miRNAs, although some pseudogenes and other noncoding RNAs are present. Interestingly, it was recently shown that multiple enhancer elements are present within this region and that they can regulate transcription.[62,63] Specifically, one such enhancer element physically interacts with the MYC promoter via transcription factor Tcf-4 binding and acts in an allele-specific manner to regulate MYC expression.[62] However, a recent study by Pomerantz *et al*., which evaluated 280 prostatectomy specimens, did not find an association between the 8q24 risk locus and steady state *MYC* mRNA expression.[64] Hence, the effects of these germline variants on MYC expression remain unclear.

Transformation of Prostate Cells by MYC

Gil *et al*. utilized retroviral-mediated gene transfer to overexpress MYC in human prostate epithelial cells obtained from benign prostate tissue specimens.[65] They found that the single-step overexpression of MYC—but not hTERT, MDM2, or E7—was sufficient to immortalize the primary cells *in vitro*. This occurred in part by telomere length stabilization achieved via the upregulation of hTERT and maintenance of telomerase activity, and by bypassing the Rb/p16 checkpoint. Additionally, these cells were able to form colonies in soft-agar assays, indicating that they had been transformed.

Williams *et al*.[66] transduced isolated prostatic epithelial cells with a retroviral vector expressing MYC and also found that the cells could be immortalized in a single step. These immortalized cells formed tumors when recombined with urogenital sinus mesenchyme and engrafted under the renal capsule. The tumor cells possessed the characteristic morphological features of transformed cells, including prominent and irregular nuclei and nucleoli and dense cytoplasm. Additionally, these cells expressed androgen receptor (AR), prostate-specific antigen (PSA), and a number of markers consistent with the luminal phenotype, with an absence of basal cell–specific markers. The resulting tumors had a high proliferative capacity, with numerous abnormal mitotic figures present. Unfortunately, at this time there are no follow-up studies to these exciting findings.

MYC-Based Mouse Models of PIN/Prostate Adenocarcinoma

Early work by Thompson *et al*. found that MYC and RAS cooperated in an oncogene-induced multistage carcinogenesis model.[67] In this study, mouse urogenital sinus cells were infected with replication incompetent retroviruses expressing RAS and MYC singly and together. These cells were then used to reconstitute a prostate gland after transplantation under the renal capsule of isogenic animals. Retroviral infection of cells with MYC resulted in hyperplasia, which resembled a premalignant phenotype, with a 2- to 3-fold increase in the size of the reconstituted organs, as compared to the controls. This suggested a role for MYC in the initiation of prostate cancer. Retroviral coinfection of cells with RAS and MYC resulted in carcinomas that were pleomorphic, undifferentiated, and anaplastic, with invasive properties. The tumor cells were characteristic of prostate luminal cells. Interestingly, when similar experiments were performed with mouse urogenital sinus cells from mice heterozygous or homozygous for mutant *Tp53* alleles, prostatic cancer was found in 100% of the *Tp53* mutant–reconstituted animals, with metastatic deposits in 95% of the mice.[68] These results suggest that Myc and Tp53 can cooperate in driving advanced prostate cancer formation in mice. Interestingly, in humans, *TP53* mutations rarely occur in primary prostatic carcinomas, yet they are found much more frequently (upwards of 50%) in advanced metastatic and hormone refractory metastatic prostate cancer.[69]

In terms of genetically engineered mouse models, Zhang *et al*. described a rat C(3)1 promoter-driven MYC transgenic mouse.[70] In these mice, forced overexpression of mouse Myc was observed in the ventral prostates as well as the testes, the latter because of an insufficiency in promoter specificity. These mice developed PIN but did not progress to carcinoma, likely owing to inadequate transgene expression. Two additional strains of transgenic mice expressing human MYC specifically in the mouse prostate were recently developed by Ellwood-Yen *et al*.[71] In the Lo-MYC mice, MYC expression was driven by a modified rat probasin promoter and in the Hi-MYC mice, by the ARR_2/probasin promoter. Both strains of mice developed PIN, which progressed to invasive adenocarcinoma, although at different rates. Importantly, in these mouse models, there was no evidence for the neuroendocrine carcinoma phenotype observed in tumor models based on T-antigen overexpression (e.g., TRAMP and LADY).[72-75] Microarray expression profiling defined a gene expression signature of MYC-induced prostate cancer in Hi-MYC mice that shared a number of molecular features with human prostate cancer, including *Nkx3-1* downregulation and *Pim1* upregulation.[71,76]

We have recently examined Lo-MYC and Hi-MYC mouse models[77] and have found that MYC overexpression, as analyzed by immunohistochemistry, occurs in only the luminal epithelial cells, and the onset of MYC overexpression coincides precisely with morphological transformation into PIN (Figure 1). This latter observation suggests that overexpression of MYC alone *in vivo* may be sufficient to transform prostatic epithelial luminal cells into PIN cells. We also generated a novel transgenic strain in which MYC was driven by the mouse *Nkx3.1* locus. As in the work by Zhang *et al*.,[70] these mice developed mild PIN lesions that did not progress to carcinoma.[77] These findings are consistent the fact that the levels of MYC induced by this construct in the mouse prostate were quite modest.

Kim *et al*. recently generated a mouse model of focal MYC expression in the prostatic luminal cells.[78] In these mice, overt PIN development was not observed when the authors activated MYC alone. This discrepancy, as compared to the Lo-MYC and Hi-MYC mice that developed PIN and cancer, may be due to mouse strain differences or perhaps failure to induce high-enough levels of MYC protein using this construct. However, when these mice were crossed to mice that had prostate-specific targeted inactivation of *Pten*, they developed high-grade PIN, which progressed to microinvasive cancer. These results raised the intriguing possibility that there is cooperation between loss of PTEN and MYC activation in prostate cancer. *PTEN* is a well-known tumor suppressor in prostate cancer that is inactivated more commonly in more aggressive lesions and is associated with prostate cancer progression.[79]

Hepsin is a cell surface protease commonly overexpressed in human prostate cancer.[80,81] When Nandana *et al*. overexpressed hepsin in a transgenic mouse model, the mice showed prostate basement membrane disorganization but otherwise normal cell proliferation and differentiation.[82] This indicates that hepsin alone is insufficient to induce prostate cancer. However, when these mice were crossed with the Hi-MYC mice (described above) to generate a double transgenic mouse that overexpressed both hepsin and MYC in the prostate, the 2 genes appeared to cooperate. The incidence of adenocarcinoma in these mice was observed 1.5 months before the incidence of adenocarcinoma in the

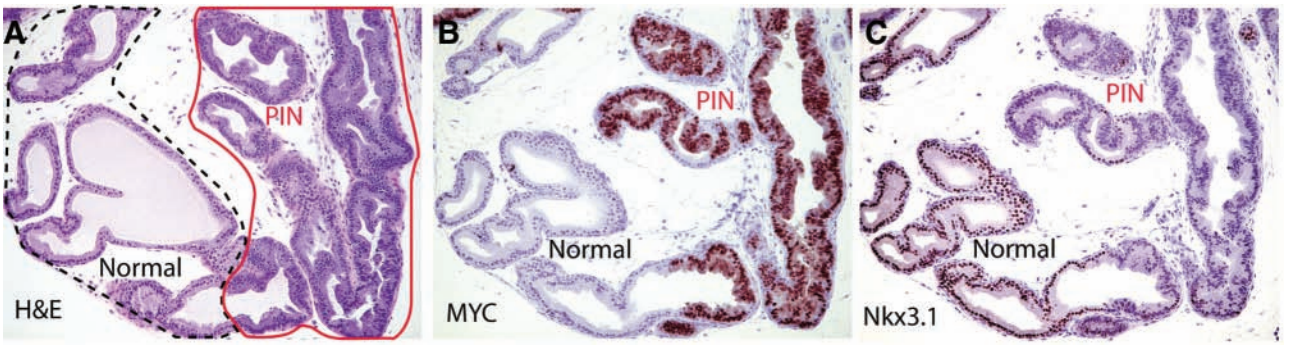

Figure 1. Photomicrographs from Lo-MYC mouse at 4 weeks of age. (**A**) H&E showing 2 populations of acini. Left shows normal histology; right shows high-grade PIN. (**B**) Immunohistochemical staining for MYC shows that morphologically transformed cells all stain positively. (**C**) Prostatic intraepithelial neoplasia cells show reduced Nkx3.1 protein staining. 200x.

Hi-MYC mice, and at 17 months, the tumors from the double transgenic mice were of a higher pathological grade than those from age-matched Hi-MYC mice. This suggests that once the cells have undergone an initiating transforming event, such as that induced by MYC, hepsin may contribute to the progression of prostate cancer.

MYC as a Therapeutic Target in Prostate Cancer

1. Vitamin D, MYC, and Prostate Cancer

A number of epidemiological studies have suggested an inverse correlation between serum vitamin D levels—as well as the exposure to sunlight, a major source of vitamin D production—and risk for prostate cancer.[83-86] However, other studies have found no correlation or even increased risk of more aggressive disease,[86] although the latter may relate to the possibility that that high levels of vitamin D can result in vitamin D resistance locally in tissues.[87] Regardless of the epidemiological literature, consistent results have been found showing that 1,25-dihydroxy vitamin D_3 (the active form that binds to the vitamin D receptor) and its analogs inhibit the proliferation of various prostate cancer cell lines *in vitro* and in prostate cancer xenograft models *in vivo*.[88-91] This growth suppression appears to be the result of cell cycle arrest in G1, mediated by p27 stabilization, reduced Cdk2 activity, reduced Cdc25A expression, increased p21 expression, and altered tyrosine kinase activity. Weigel and colleagues have shown that these effects of vitamin D on prostate cancer proliferation occur via reduced MYC mRNA and protein levels in various prostate cancer cell lines *in vitro*.[88] Furthermore, 1,25-dihydroxyvitamin D_3 increases the phosphorylation of MYC on T58, hence targeting it for ubiquitin-mediated proteolysis.[88] A more recent study examined the effects of 1,25-dihydroxyvitamin D_3 and a synthetic analog in another human prostate cancer cell line, VCaP.[92] This cell line has the stereotypic gene rearrangement in which the androgen-regulated *TMPRSS2* gene is fused to the oncogenic transcription factor *ERG*, resulting in a fusion transcript.[12] Interestingly, despite the fact that 1,25-dihydroxyvitamin D_3 or a synthetic analog resulted in increased levels of the TMPRESS-ERG fusion gene expression in these cells, the cells were still growth inhibited as a result of repression of MYC by the vitamin D receptor.[92]

2. Targeting with Antisense Oligonucleotides

Antisense technology utilizes sequence-specific oligonucleotides to inhibit gene expression. These oligonucleotides can block mRNA transcription and translation and affect nuclear export, stability, and splicing of mRNA. Goodyear *et al*. evaluated the *in vivo* and *in vitro* tumorigenic capacity of the hTERT-immortalized primary prostate cell line IBC-10a.[93] This cell line comprised $CD133^{lo}$ and $CD133^{hi}$ subpopulations, the latter of which are purported to harbor the putative prostate stem cell compartment. Inhibition of MYC using antisense oligonucleotides in the $CD133^{hi}$ population resulted in reduced cell viability, proliferation, and prostasphere formation. Additionally, these antisense-treated cells failed to generate dysplastic lesions or tumors in NOD-SCID mice. Balaji *et al*. showed that antisense oligonucleotides decreased prostate cancer cell viability *in vitro* and suppressed their proliferation.[94] An antisense phosphorodiamidate morpholino oligomer directed against MYC, AVI-4126, was shown to inhibit the translation of MYC. When tested for efficacy in a murine xenograft model of prostate cancer, there was a reduction in size of the tumor xenografts, resulting from growth inhibition and apoptosis.[95] When tested for safety in a phase I human clinical study, intravenous administration of AVI-4126 did not show significant toxicity or serious adverse events.[96] These preliminary findings suggest that antisense-based approaches targeting MYC could be a useful clinical therapy. Certainly, targeting of MYC by

other mechanisms, such as delivery of siRNA (or analogs) to knockdown MYC or some of its effectors (e.g., mir26a)[97] *in vivo* is a highly promising future approach for potential novel therapies in prostate and other cancers.

3. Targeting MYC with Cardenolides

Cardiac glycosides target and inhibit the Na^+/K^+-ATPase pump, disrupting ion homeostasis and signal transduction pathways. Cardenolides such as ouabain, digitoxin, and oleandrin belong to the cardiac glycoside family and have been shown to promote prostate cancer cell apoptosis and inhibit proliferation.[98-101] Mijatovic *et al*. generated a novel hemisynthetic cardenolide, UNBS1450, by chemical modifications of 2′′-oxovoruscharin.[102,103] This compound displayed *in vitro* antiproliferative effects and *in vivo* antitumor activity on PC3 cells, which were uncoupled to a rise in calcium concentrations or the induction of apoptosis. Instead, the authors noted a reduction in the expression of MYC and MYC-related proteins such as Max, cyclin dependent kinase 1, which is MYC regulated, and phospho-Rb. Interestingly, this was accompanied by impaired nucleolar organization and reduced expression of several nucleolar proteins, some of which, such as UBF, are known MYC targets. This effect was not observed in normal human fibroblast cell lines, indicating a differential toxicity for cancer cells. Although it is not known exactly how UNBS1450 treatment reduces MYC mRNA and protein expression, the authors postulate that this may be mediated through ROS-mediated oxidation of SP1 or through downregulation of STAT3 expression and signaling.[102]

MYC Target Genes and Gene Modules in Prostate Cancer

MYC is known to directly and indirectly regulate the transcription of numerous genes and pathways. By unsupervised clustering of microarray gene expression data from Hi-MYC mice and their wild-type counterparts, Ellwood-Yen *et al*. identified a distinct MYC-driven gene expression signature.[71] The differentially expressed genes included *L-MYC*, *NKX3-1*, *PIM1*, *TMPRSS2*, *SPARC*, *EGF*, and prostate stem cell antigen (*PSCA*) family genes, including *Ly6*. Additionally, a cross-species bioinformatics comparison of the MYC-driven gene expression signature from Hi-MYC mice and human prostate cancers identified several "MYC signature" genes, which were consistently regulated, including *PIM1*, *GNAS1*, *PTOV1*, and *ID3*.[71]

PIM1

PIM1, which is a serine/threonine kinase, was recently shown to be elevated in a subset of human prostate cancers, and its overexpression correlated with poor clinical outcome.[104,105] Additionally, PIM1 has been shown to regulate androgen-dependent survival signaling in prostate cancer cells.[106] Cooperativity between MYC and PIM1 has been demonstrated *in vitro*, as well as in murine lymphoma models.[107,108] Zippo *et al*. showed that PIM1-dependent phosphorylation of histone H3 at MYC-target loci is necessary for MYC-dependent transcriptional activation and oncogenic transformation.[109] A similar functional synergy has been observed in prostate cancer cells. Using a tissue recombination *in vivo* model, Wang *et al*. found that PIM1 alone was only weakly oncogenic. However, coupled with MYC, the grafts formed tumors with high proliferative capacity.[110] In a separate study, PIM1 was found to enhance the transcriptional activity of MYC *in vitro*, and a large fraction of MYC target genes were regulated by PIM1 expression in prostate cancer cells.[111] MYC inhibition also reduced the tumorigenicity of PIM1-expressing prostate cancer cells.[110,111]

NKX3.1

NKX3.1 is a prostate-restricted homeodomain containing transcription factor that is involved in prostate morphogenesis and differentiation.[112-114] Its expression is often reduced in PIN lesions and invasive and metastatic adenocarcinomas, and it is thought to have tumor suppressor functions in prostate cancer.[115-118] However, recent reports have found that NKX3.1 expression is maintained in most high-grade, invasive, and metastatic prostate cancers, indicating that NKX3.1 is dynamically regulated during disease progression.[115,119,120] Reduced NKX3.1 expression is often, but not always, related to single-allelic loss of chromosome 8p; hence, other mechanisms may regulate NKX3.1 expression.[115,121-123] In the Hi-MYC mice, reduced NKX3.1 expression was seen in PIN, and NKX3.1 was almost completely lost in invasive adenocarcinomas.[71] Microarray-based gene expression analyses determined that there was a reduction in NKX3.1 mRNA in the Hi-MYC mice. Iwata *et al*. recently reported that in the Lo-MYC and Hi-MYC mice, MYC overexpression was precisely coincident with the development of PIN and decreased expression of NKX3.1 (Figure 1), suggesting that MYC may be repressing NKX3.1 in PIN.[77] This suggests a novel molecular explanation for NKX3.1 downregulation in PIN and perhaps prostate cancer. NKX3.1 expression is high only in prostatic luminal cells, and it is considered to be a differentiation-related gene. These results indicating that MYC downregulates Nkx3.1 in PIN luminal cells are consistent with the hypothesis that, as in a number of other tumor types,[124] MYC overexpression prevents "terminal" differentiation during prostatic cancer development.

Nucleolar Genes

Nucleolar enlargement is a diagnostic feature of human PIN and prostate cancer, although a molecular mechanism for this morphological change is unknown. In the Lo-MYC mice, MYC expression is concurrent with nucleolar enlargement[77] (Figure 2). Additionally, we observed that MYC depletion in prostate cancer cells results in altered nucleolar architecture, size, and distribution (C Koh, S Yegnasubramanian, M Aryee, WG Nelson, CV Dang, AM De Marzo,

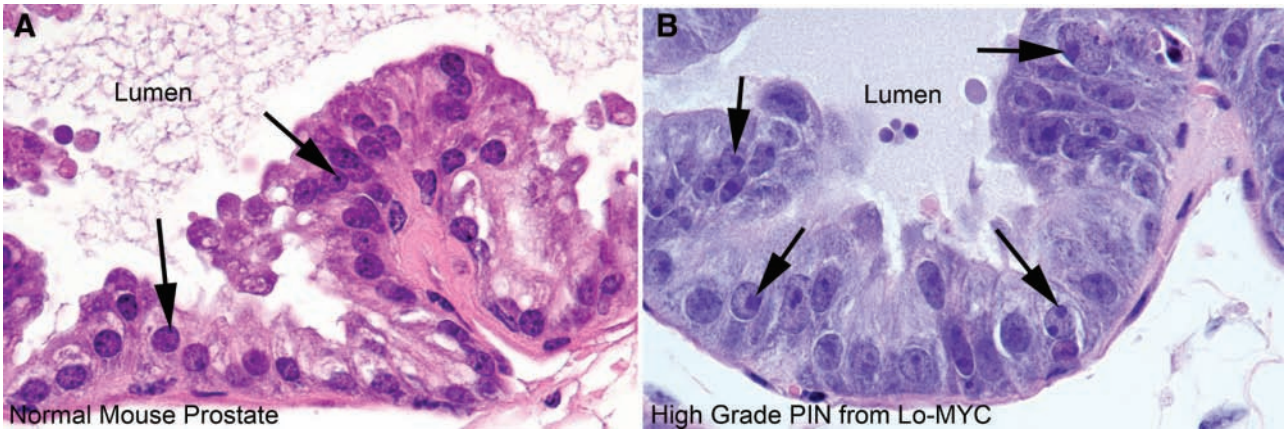

Figure 2. Photomicrograph comparing normal (**A**) and Lo-MYC (**B**) mouse prostate. Arrows indicate nucleoli. Note nuclear enlargement and marked enlargement of nucleoli in Lo-MYC mouse, as compared to age-matched wild-type mouse. 600x.

manuscript in progress). Since it is known that MYC can directly activate the transcription of a large number of genes whose protein products localize to and function primarily in the nucleolus,[125,126] MYC-mediated changes in nucleolar protein expression may account for nucleolar enlargement in prostate cancer.

Using microarray analyses following MYC depletion of human prostate cancer cells, we recently found coordinate change in gene sets associated with the nucleolus (GO:0005730) and rRNA processing (GO:0006364) (C Koh, S Yegnasubramanian, M Aryee, WG Nelson, CV Dang, AM De Marzo, manuscript in progress). Specifically, we showed that MYC controls the expression of numerous nucleolar proteins, such as fibrillarin, nucleolin, UBF, and nucleophosmin, in prostate cancer cell lines. We also found that fibrillarin—a nucleolar protein that is part of the C/D nucleolar small nuclear ribonucleoprotein particle and which is required for multiple steps in rRNA processing—is required for proliferation and self-renewal of prostate cancer cells. FBL is directly bound by MYC in its presumptive promoter region, suggesting that it is a direct MYC target gene. Furthermore, fibrillarin mRNA and protein are overexpressed in human PIN and adenocarcinoma lesions, and levels of fibrillarin protein correlate with levels of MYC protein *in vivo*. Taken together with the findings that activation of MYC in mouse prostatic luminal cells results in nucleolar enlargement and that MYC is activated in most human PIN lesions, it is likely that the hallmark diagnostic finding of nucleolar enlargement in human PIN and prostate cancer may reflect activation of MYC, at least in a large subset of cases.

Telomerase/hTERT

Telomerase is a ribonucleoprotein enzyme made up of an RNA template and a protein component with reverse transcriptase activity (hTERT).[127-130] Telomerase adds TTAGGG repeats to chromosome ends, preventing telomere shortening and consequent chromosomal instability.[127,130-132] Telomerase activity is detectable in prostate cancer but not in benign prostate tissue.[133-137] Additionally, hTERT expression correlates with MYC overexpression in prostate cancer.[138] MYC has been shown to activate hTERT transcription as well as telomerase activity.[139] Specifically, the E-box-containing promoter of hTERT can be occupied by MYC or Mad1, resulting in transcriptional activation or repression, respectively.[140] This MYC-driven telomerase activation may confer unlimited replicative potential to prostate epithelial cells and, in combination with other genetic lesions, provide the selective advantage for the development of prostate cancer.[141]

EZH2

EZH2 is a histone lysine methyltransferase involved in chromatin remodeling as part of the PRC2 polycomb repressive complex that is overexpressed in all phases of prostate cancer including the precursor lesion, high-grade PIN.[142-146] EZH2 promotes proliferation, invasion, and tumorigenicity of prostate cancer cells. Upregulation of EZH2 in prostate cancer can result from gene amplification,[147] by deletion of its negative regulator mir-101,[142] or by transcriptional regulation by ETS gene family members.[123,148] However, none of these alterations are thought to commonly occur in PIN lesions. Thus, EZH2 overexpression in prostatic neoplasia may be induced by additional mechanisms, especially early in the disease process. In additional preliminary work, we found that MYC positively regulates EZH2 early during prostatic carcinogenesis by repression of mir26a (CM Koh, Q Zheng, C Bethel, T Iwata, AM De Marzo, in process), a known MYC target for repression,[149] that has separately

been shown to negatively regulate EZH2 in muscle and lymphoma cells.[150,151] These findings reveal an additional molecular mechanism by which EZH2 is overexpressed during prostate cancer initiation and maintenance.

Prostatic Tumor–Initiating Cells, Embryonic Stem Cell–Like Programs, MYC, and Prostate Cancer

Prostatic epithelium is composed of 2 cell layers. The luminal cells express high levels of prostatic differentiation markers, such as AR, PSA, prostatic specific acid phosphatase, and NKX3.1, whereas the basal cells express low levels of these proteins and express high levels of nuclear p63. Although there is still uncertainty regarding the identity of prostatic epithelial stem cells in adult tissues, substantial evidence indicates that cells that reside in the basal compartment and that have a basal phenotype can possess some stem cell–like behavior in terms of the ability for some self-renewal and to differentiate into luminal cells.[152-158] Furthermore, in at least one mouse model of prostate cancer, the tumors appear to arise in the basal-like stem cells located proximately along the ducts.[157,159] However, human prostate cancer and PIN cells resemble cells from the luminal compartment much more than cells from the basal compartment in terms of morphology and phenotypic markers.[160,161] For example, although the overall levels tend to be lower than in most normal-appearing luminal cells, human PIN cells express fairly high levels (as compared to basal cells) of AR, PSA, and NKX3.1.[115,160] Furthermore, only luminal cells in PIN lesions show the characteristic somatic DNA alteration of telomere shortening.[162] Finally, although ETS family gene rearrangements appear to occur infrequently in PIN lesions (~15%),[8,9] when they do occur, only luminal cells show the characteristic FISH abnormalities[163] (M Haffner, R Albadine, S Yegnasubramanian, personal communication). Taken together, these data suggest that luminal-like cells, perhaps at times with a partially differentiated phenotype intermediate between basal and luminal cells,[161,164] may be tumor-initiating cells. Overexpression of MYC in mouse prostatic cells using a number of promoters active predominantly in luminal cells results in the development of PIN, with a luminal phenotype,[71,77] and it appears that overexpression of MYC alone can transform mouse prostatic luminal epithelial cells into PIN cells.[77]

In terms of embryonic stem cells (ESCs), MYC is required for efficient induced pluripotent stem cell (iPSC) formation, ESC self-renewal, and prevention of terminal differentiation in ESC/iPSC.[124] Furthermore, using a number of human genome-wide expression-profiling data sets, Wong *et al*. identified MYC as a potential regulator of an ESC-like signature.[165] In a recent study, we found that siRNA-mediated knockdown of MYC in prostate cancer cell lines decreased their proliferation and clonogenic potential and resulted in the increased expression of prostate lineage-specific genes associated with terminal differentiation, some of which are AR regulated (C Koh, S Yegnasubramanian, M Aryee, WG Nelson, CV Dang, AM De Marzo, manuscript in progress). Furthermore, MYC depletion dampened the expression of a large fraction of the "core" ESC-like gene set, comprising 334 genes shared between human and mouse ESCs. Additionally, there was an overall decrease in expression of the human ESC-like gene set, comprising 1,235 genes. These findings suggest that MYC is required to maintain an already established ESC-like pattern of gene expression. Further analysis of a gene expression data set previously published by Ellwood-Yen *et al*.[71] indicated that the MYC-driven mouse prostate tumors were enriched for the mouse ESC-like gene set (comprising 631 genes), confirming that human MYC can induce a *de novo* ESC-like signature in mouse prostatic epithelium *in vivo*. As noted above, MYC regulates EZH2 levels in PIN and prostate cancer. Because EZH2 has been shown to prevent the differentiation of ESCs (see Reference 148), it is possible that some of the effects of MYC on stimulating and maintaining an ESC-like program on prostatic cells are mediated via EZH2. Interestingly, 2 recent studies indicated that ERG expression appears to abrogate expression of AR-regulated terminal differentiation genes in prostate cancer.[148,166] This raises the possibility that overexpression of MYC and ERG share some similar molecular consequences. Indeed, there is evidence that ERG itself may activate MYC, indicating that these genes can cooperate in prostate cancer.[166]

Given these findings, we propose a model of PIN in which MYC is activated in luminal cells, whether they are partially differentiated (such as in prostatic atrophy), are terminally differentiated, or resemble a newly described tissue stem cell that resides in the luminal compartment.[167] This pathological overexpression of MYC results in the "reprogramming" of these luminal cells, ultimately resulting in the induction of an ESC-like program of self-renewal. Importantly, although tissue stem cells may be the target, there is no need to necessarily invoke transformation of tissue stem cells in this model. In fact, given MYC's ability to help reprogram adult cells into iPSC, it is possible that even "terminally differentiated" prostatic luminal cells could be targets in this model. Because the phenotype of human prostatic tissue stem cells has not been fully elucidated, further studies are required before it can be determined whether they are the "true" target of transformation. However, the model whereby MYC transforms a non–stem cell in prostate cancer fits nicely with a number of studies in other organs where MYC appears to transform partially differentiated progenitor cells and not stem cells.[124] Although one cannot generalize across all stem cell systems, it has been suggested that at least some tumors that express high levels of MYC are unlikely

to be derived from stem cells, because MYC expression in these stem cells (e.g., skin) promotes their exit from their "niche." The molecular basis for MYC-induced nuclear "reprogramming" is still under intense investigation. Interestingly, MYC has been shown to promote widespread chromatin remodeling.[168] Furthermore, the adenoviral EIA oncoprotein has recently been shown to activate a genome-wide reprogramming of chromatin during its transformation of cells in culture, and EIA has been shown to transform cells at least in part by activating MYC.[169] Finally, how the transformed epithelial cells in the luminal compartment in this model relate to the recent suggestions that the early phases of prostate carcinogenesis represent a "reawakening" of androgen programs of prostate organogenesis[76,170] awaits additional studies as well.

Conclusions

We have reviewed data gathered over many years that indicate that MYC plays a critical role in multistep prostatic carcinogenesis. Recent findings have stressed the potential importance of MYC overexpression in the earliest phases of prostate cancer formation, and these finding shed new light on the origin of prostate cancer precursor lesions and tumor-initiating cells. Further studies directed to uncovering the molecular mechanisms responsible for MYC overexpression in prostate cancer are sorely needed—we can no longer assume that gain of chromosome 8q24 is the main mechanism driving MYC expression in human prostate cancer. Furthermore, given the fact that MYC is so commonly overexpressed in prostatic neoplasms, it may well turn out to become an important biomarker in the early detection and diagnosis of this disease. As such, novel mouse models based on MYC overexpression in the prostate, as well as crosses between MYC-based models and other established prostate cancer models, should prove to be highly useful in further studying the molecular mechanisms driving prostate cancer initiation and progression. Finally, the body of literature implicating MYC in this disease should serve to highlight the fact that novel therapeutic strategies designed to target MYC should include prostate cancer as an important disease for testing these approaches.

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Declaration of Conflicting Interests

The authors declare no conflicts of interest with respect to the publication of this article.

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