Transient Induction of ING4 by Myc Drives Prostate Epithelial Cell Differentiation and Its Disruption Drives Prostate Tumorigenesis

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Abstract

The mechanisms by which Myc overexpression or Pten loss promotes prostate cancer development are poorly understood. We identified the chromatin remodeling protein, ING4, as a crucial switch downstream of Myc and Pten that is required for human prostate epithelial differentiation. Myc-induced transient expression of ING4 is required for the differentiation of basal epithelial cells into luminal cells, while sustained ING4 expression induces apoptosis. ING4 expression is lost in >60% of human primary prostate tumors. ING4 or Pten loss prevents epithelial cell differentiation, which was necessary for tumorigenesis. Pten loss prevents differentiation by blocking ING4 expression, which is rescued by ING4 re-expression. Pten or ING4 loss generates tumor cells that co-express basal and luminal markers, indicating prostate oncogenesis occurs through disruption of an intermediate step in the prostate epithelial differentiation program. Thus, we identified a new epithelial cell differentiation switch involving Myc, Pten, and ING4, which when disrupted leads to prostate tumorigenesis. Myc overexpression and Pten loss are common genetic abnormalities in prostate cancer, whereas loss of the tumor suppressor ING4 has not been reported. This is the first demonstration that transient ING4 expression is absolutely required for epithelial differentiation, its expression is dependent on Myc and Pten, and it is lost in the majority of human prostate cancers. This is the first demonstration that loss of ING4, either directly or indirectly through loss of Pten, promotes Myc-driven oncogenesis by deregulating differentiation. The clinical implication is that Pten/ING4 negative and ING4-only negative tumors may reflect two distinct subtypes of prostate cancer.

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Introduction

Normal prostate glands contain prostatic ducts composed of two distinct layers of epithelial cells: luminal cells that express androgen receptor (AR) and secrete prostate-specific antigen (PSA) and basal cells that express nuclear p63. It is thought that the stem or progenitor cells within or in proximity of the basal layer differentiate and give rise to the luminal cells (1, 2). Prostate tumors are often devoid of the cell layer distinction and express both luminal and basal cell markers, suggesting deregulated cell differentiation. That prostate cancer arises from deregulated differentiation is also supported by mouse models. The most notable example is loss of Nkx3.1, a known prostate-specific differentiation gene, which predisposes mice to develop prostate cancer in the context of additional oncogenic events (3). Two other well characterized oncogenic events linked with prostate cancer are loss of Pten or overexpression of Myc (4, 5). Both of which lead to down-regulation of Nkx3.1 expression, but are also sufficient to induce prostate cancer in mice (6, 7). The prostate-specific oncogene, TMPRSS2-Erg, when overexpressed in mouse prostates leads to prostate intraepithelial neoplasia (PIN), with a corresponding change in differentiation, where progenitor cell markers Sca1 and integrin α6 are increased, whereas basal cell keratin is diminished and AR is expressed (8, 9). In addition, overexpression of Erg upregulates Myc expression and produces an expression profile consistent with a change in differentiation (10). A recent mouse study where Pten was deleted in either basal or luminal cells, demonstrated the appearance of K5+/K8+ intermediate tumor cells, further supporting the idea that deregulated differentiation is a hallmark of prostate cancer (11). However, the mechanism by which differentiation is deregulated is unknown.

We recently reported on an in vitro differentiation model in which AR-negative human basal prostate epithelial cells can be differentiated into AR-positive and androgen-responsive...
postmitotic secretory cells (12). Based on known prostate and epithelial differentiation markers, and the demonstration that PSA can be secreted into the medium from the differentiated cells, this model recapitulates the biology and physiology of the human prostate gland in vivo. A major step in the differentiation process is the loss of integrin expression and cell–matrix adhesion, which is crucial to generate stable AR-expressing cells. This is accompanied by a dramatic shift in survival signaling pathways, whereby basal cells, which survive primarily through integrin-mediated activation of the Erk signaling pathway, give rise to secretory cells that depend on E-cadherin based cell–cell adhesion and activation of Akt for survival.

The separation of AR and integrin functions in the two different epithelial populations is wholly consistent with what is observed in vivo; that integrin expression is limited to the basal cells and AR is only in the secretory cells (13, 14). In prostate cancer this distinction is lost, whereby AR and integrin are coexpressed in the tumors, where integrin cooperates with AR to promote the survival of prostate cancer cells (15). Other markers typically associated with basal or intermedial cells, such as receptor tyrosine kinases EGFR and Met, bcl-2, and coexpression of basal and secretory keratins K5 and K8, are also found in tumors that express AR-dependent differentiation genes (14, 16, 17). Thus, the majority of the primary tumor population in prostate cancer resembles a potential differentiation intermediate. In addition, the unexplained loss of basal cells in prostate cancer points to altered differentiation as a major factor in prostate cancer (18).

Myc is overexpressed in up to 90% of primary prostate tumors, presenting itself as a major driver in prostate cancer (4). Recent studies have unraveled the function of Myc in reprogramming of somatic cells into pluripotent stem cells and the maintenance of self-renewal in stem cells (19), and is consistent with the idea that deregulated Myc prevents full differentiation of prostate epithelial cells, leading to prostate cancer when given additional molecular lesions. ING4 is a tumor suppressor whose expression is lost in several cancers; but whose role in prostate cancer is unknown (20). ING4 is a chromatin-binding protein that binds trimethylated histone H3 and recruits the HBO1 histone acetyltransferase to increase histone acetylation (21). ING4 -

\[ \text{shRNA construct was generated by subcloning the oligo } 5'-\text{CCGGGCTAGGTGTGATCAACACTTTCTCGAGAAAGTGTT}-\text{GATCACACCTAGCTTTTTTG-3'}, \text{ complementary to the 3'-UTR of ING4, into a lentiviral vector to generate pLKO.1-shING4}. \] 

The pLKO vector containing shRNA was generated by first creating a pCR8-GW-TOPO-shLEGO shuttle vector. A 344bp PCR product containing a multicloning site, EcoRV/XbaI/SaI/PmlI, the pLKO U6 promoter, an AgeI site, a HindIII site, followed by a reverse multicloning site, PmlI/SalI/XbaI/EcoRV, and an EcoRI site was TA cloned into pCR8-GW-TOPO. Oligo shPten2, 5'-CCGGTCTAGGTGTGATCAACACTTTCTCGAGAAAGTGTTGATCACACCTAGCTTTTTTG-3', was cloned into the AgeI/HindIII site of the pCR8-GW-TOPO-shLEGO shuttle vector. The AgeI/EcoRI fragment was subcloned into pLKO to generate pLKO-shPten2.

**Materials and Methods**

**Cell lines**

Primary basal prostate epithelial cells were isolated from clinical prostatectomies as previously described (24, 25). Cultures were validated to be Mycoplasma-free and express only basal epithelial cell markers (12, 25). Cells were immortalized with retroviruses expressing HPV E6/E7 and hTert, selected in 150 μg/mL neomycin for 3 days, and the resulting population pooled. Cells retain all the basal markers of primary cells. Immortalized cells (iPrEC) were transformed by retroviruses expressing Erg and Myc (EM), and lentivirus expressing Pten shRNA (EMP) or ING4 shRNA (EMI), then selected and maintained in 0.35 μg/mL puromycin. All lines were maintained and passaged as previously described (24, 25).

**Differentiation protocol**

Differentiation and layer separation protocols were detailed previously (12). Briefly, iPrECs at confluence were treated in complete growth medium with 10 ng/mL keratinocyte growth factor (KGF; Cell Sciences) and 5 nmol/L R1881 (PerkinElmer) every other day for up to 21 days. For biochemical analysis, the suprabasal differentiated layer was separated from the basal layer as previously described (12).

**Construc**

The wild-type retroviral pBabe-Myc construct was obtained from Dr. B. Knudsen. plPCX-Erg was generated by subcloning the ERG cDNA NotI/SpeI fragment from pMax Dest AN-Erg (9), supplied by Dr. Vasioukhin, into NotI/XhoI of plPCX. The wild-type (pMIG-ING4) and C-terminal deletion mutant (pMIG-ING4-AC1) of ING4 were described previously (23). The ING4 shRNA construct was generated by subcloning the oligo 5'-CCGGGCTAGGTGTGATCAACACTTTCTCGAGAAAGTGTTGATCACACCTAGCTTTTTTG-3', complementary to the 3'-UTR of ING4, into a lentiviral vector to generate pLKO.1-shING4. The pLKO vector containing shRNA was generated by first creating a pCR8-GW-TOPO-shLEGO shuttle vector. A 344bp PCR product containing a multicloning site, EcoRV/XbaI/SaI/PmlI, the pLKO U6 promoter, an AgeI site, a HindIII site, followed by a reverse multicloning site, PmlI/SalI/XbaI/EcoRV, and an EcoRI site was TA cloned into pCR8-GW-TOPO. Oligo shPten2, 5'-CCGGTCTAGGTGTGATCAACACTTTCTCGAGAAAGTGTTGATCACACCTAGCTTTTTTG-3', was cloned into the AgeI/HindIII site of the pCR8-GW-TOPO-shLEGO shuttle vector. The AgeI/EcoRI fragment was subcloned into pLKO to generate pLKO-shPten2.

**Virus generation and infection**

Lentivirus shRNAs were generated by transfecting a packaging cell line, harvesting virus 3 days later and immediately infecting iPrECs. Cells were selected and pools maintained in 0.35 μg/mL puromycin. Retroviruses expressing ING4 or Myc were generated by transfecting Phoenix cells (National Gene Vector Biorepository), harvesting 2 days later and immediately infecting iPrECs. Myc expressing cells were selected and maintained in 0.35 μg/mL puromycin. ING4 construct has no selectable marker and cells were generated de novo as needed.

**siRNA transfection**

A pool of siRNAs against Myc and a nontargeting sequence were purchased from Origene. ON-Targetplus SMARTpool targeted to Bnip3, came from Dharmaco. Differentiated cultures were serially transfected every 2 days with Myc or
control siRNA using siLentFect Lipid reagent (Bio-Rad) following manufacturer’s directions. Cells were placed in differentiation medium 18 hours after transfection.

**Antibodies**

**Immunofluorescence.** AR (C-19), Nkx3.1 (H-50), and TMPRSS2 (H-50) were purchased from Santa Cruz. ITGα6 (GoH3) was purchased from BD Pharmingen, and PSA (18127) from R&D Systems. Keratin 8 (M20) came from Abcam and Keratin 5 (AF-138) came from Covance. ING4 monoclonal antibody was generated as previously described (26) and a polyclonal antibody was obtained from ProteinTech. Cleaved caspase-3 (Asp175)(5A1E) was purchased from Cell Signaling.

**Immunoblotting.** Myc (o6–340) was purchased from Millipore, Erg (C-20) from Santa Cruz, Pten (138G6) and p27 (Kipl) from Cell Signaling, and ING4 (EP3804) from GenTex. Tubulin antibody (DM1A) was purchased from Sigma and GAPDH (6CS) from Millipore. Polyclonal integrin α6 (AA6A) antibody was a gift from Dr. A. Cress (University of Arizona; ref. 27).

**Immunostaining and microscopy**

Differentially cultured were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton-X 100. After washing, cells were blocked with 2% normal goat serum for 2 hours. Primary antibodies, diluted in 1% BSA/PBS, were applied to samples overnight at 4°C. After washing, secondary conjugated antibodies were diluted in 1% BSA/PBS were incubated for 1 to 2 hours. Nuclei were stained with Hoechst 33258 (Sigma) for 10 minutes at room temperature. Coverslips were mounted using Fluoromount-G (SouthernBiotec). Epifluorescent images were acquired on a Nikon Eclipse TE300 fluorescence microscope using OpenLab v5.5.0 image analysis software (Improvision). Confocal images were acquired by sequential detection on an Olympus Fluoview 1000 LSM using FluoView software v5.0.

**Immunoblotting**

Total cell lysates were prepared for immunoblotting as previously described (24). Briefly, cells were lysed in RIPA buffer, 30 to 50 μg of total cell lysates were run on SDS polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in 5% BSA in TBST overnight at 4°C then probed with primary antibody, and HRP-conjugated secondary antibodies (Bio-Rad) in TBST + 5% BSA. Signals were visualized by chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

**RT-PCR**

Total RNA was isolated using Qiagen’s RNeasy Kit. RNA was purified with RNase-free DNase and RNeasy Mini Kits (Qiagen). For qRT-PCR, 0.5 μg RNA was reversed transcribed using a reverse transcription system (Promega). Synthesized cDNA was amplified for qRT-PCR using SYBR Green Master Mix (Roche) with gene-specific primers and an ABI 7500 RT-PCR system (Applied Biosystems). Gene expression was normalized to 18s rRNA by the 2-ΔΔCt method (28). Primers for ING4 and Myc were as follows: ING4: 5’-TCCGAAGTTGCTTTTGGTTGC-3’, Myc: 5’-TTCGGGATGTTGAAAAACAG-3’.

**Mouse tumorigenesis**

Half a million iPES, EM, EMM, EMI, or EM-vector cells were injected orthotopically into the prostates of 8-week nude mice. Mice were monitored by ultrasound between 8 and 18 weeks for the development of tumors. Mice were sacrificed between 16 and 18 weeks and prostate glands analyzed histologically for tumors. In one cohort of EMPs, 5 mice with tumors were castrated 16 weeks postorthotopic transplantation and measured by ultrasound for regression of tumors. All animal work was carried out following Institutional Animal Care and Use Committee approval at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

**Histology**

Prostates isolated from mice were formalin-fixed and paraffin-embedded. Sections were analyzed following hematoxylin and eosin or immunohistochemical (IHC) staining. Human-specific MUC class 1 was purchased from Abcam, polyclonal ING4 was purchased from ProteinTech, and AR (N-20) was purchased from Santa Cruz. IHC was performed using automated immunostaining (Ventana Discovery XT). A human prostate tumor survey tissue microarray (TMA) was constructed as previously described (29). The prostate survey TMA contained 52 de-identified unique prostate carcinomas ranging from Gleason 6 to 9 and 23 control cores, including 14 cases of benign prostatic hypertrophy (BPH). TMA sectioned at 5 μm thicknesses was stained using standard DAB. IHC was performed with ING4 antibody as previously described (26, 30). For validation, sections were also stained with a commercial ING4 antibody (ProteinTech). Negative control was nonimmune rabbit antiserum without primary antibody. TMA staining was scored manually with IHC assigned to each core as composite scores of 0, 1, 2, or 3 with 0 to 1 representing complete to major loss of protein, and 2 to 3 near normal to wild-type levels.

**Results**

**Myc and ING4 are transiently expressed during differentiation**

When grown to confluence and treated with KGF plus androgen, primary cultures of basal prostate epithelial cells (PrEC) undergo differentiation such that a second suprabasal layer forms on top of the basal layer in about 2 weeks (12). An immortalized primary prostate epithelial cell line (iPrEC) was established by expressing the E6/E7 viral oncogenes and hTert. Treatment of confluent iPrEC cultures with 10 ng/ml KGF and 5 nmol/L R1881, a synthetic AR agonist, for 18 days resulted in a distinct top layer of cells that no longer expressed integrin (6, K14, or p63 but expressed AR and AR-dependent targets, such as TMPRSS2 and Nkx3.1 (Fig. 1A and Supplementary Figs. S1 and S2). These data indicate iPrECs retain the ability to differentiate. ING4 expression was low to undetectable in untreated iPrECs, but by as early as 8 days of differentiation, distinct nuclear staining was detected in the newly forming suprabasal layer of differentiated cells (Fig. 1B). The initial increase in
ING4 expression was coincident with the increase in AR expression and the loss of integrin α6 expression, two hallmarks of differentiation (Fig. 1B and Supplementary Fig. S1B). At no time point were we able to dissociate ING4 expression from changes in AR or integrin α6 expression; nor were we able to separate loss of basal keratin K14 from integrin α6 loss (Supplementary Fig. S2), suggesting ING4 controls a major differentiation switch. Although AR persisted in the differentiated layer, ING4 expression was transient and no longer nuclear at later time points.

Once a sufficient number of cells have differentiated, typically between day 12 and 14, it is possible to separate the top layer of differentiated cells from the bottom layer (12). Immunoblot analysis of whole cultures from days 4 and 8, and the top layers from days 14 and 17 indicated a transient increase in ING4 protein expression at day 14, which returned to basal level expression by day 17 (Fig. 1C). ING4 mRNA expression also peaked at day 14 (Fig. 1D). The apparent lag in ING4 expression seen biochemically, compared with the immunostaining data, is most likely because of the low number of differentiated cells within the culture relative to the basal cells at early time points.

Over the same time course, Myc protein and mRNA expression were also transiently elevated (Fig. 1C and D). Myc expression preceded that of ING4 expression, suggesting a concerted temporal regulation of Myc and ING4 during iPrEC differentiation.

Myc-induced ING4 expression is required for differentiation

Cells were engineered to overexpress ING4, Myc, and/or ING4 shRNA (Fig. 2A). Although ING4 expression levels did not affect Myc expression, most notable was the increase in ING4 expression in the Myc overexpressing cells (Fig. 2A and B). These results suggest that Myc is responsible for the increase in ING4 expression during iPrEC differentiation.

Overexpression of ING4 or Myc accelerated the emergence of differentiated cells compared with the control iPReCs. The appearance of suprabasal layer cells, loss of integrin α6, and gain in Nkx3.1 expression was more robust between days 8 and
12 in the ING4 or Myc overexpressing cells, whereas the control iPTECs do not robustly express the same set of differentiation markers until days 14 to 16 (day 12 shown in Fig. 2C). Combined overexpression of Myc and ING4 did not exert an additive effect on accelerating differentiation compared with cells overexpressing either Myc or ING4 alone (Fig. 2C). However, it should be noted that the higher levels of ING4 expression in the Myc+ING4 cell line (Fig. 2A) was not always observed; most likely it is not tolerated because of enhanced cell death (see Fig. 3). Thus, it is possible we did not achieve levels of ING4 overexpression required for an additive effect. Downregulation of ING4 expression by shRNA (shING4) severely retarded the emergence of differentiated cells (Fig. 2C). Reduced ING4 expression prevented cells from appearing in the suprabasal layer, and the concomitant loss of integrin α6 (Fig. 2C) and gain of AR, indicating an absolute necessity for ING4 to suppress integrin α6 and permit AR expression.

The ability of Myc to accelerate differentiation was blocked by shING4 (Fig. 2C), indicating ING4 functions downstream of Myc during differentiation. This epistatic relationship is further supported by the fact that transient inhibition of Myc expression between days 2 to 6 failed to induce ING4 expression (Fig. 2D and F) and completely blocked differentiation (Fig. 2E and F). Furthermore, ING4 overexpression rescued the differentiation blocked by siMyc (Fig. 2F). Taken together, our results indicate that a temporal peak in Myc expression is required for the subsequent induction and transient expression of ING4 during iPTEC differentiation.
Constitutive Myc and ING4 expression leads to cell death of the differentiated cells via Bnip3

Although Myc or ING4 overexpression initially accelerated iPrEC differentiation, the differentiated cells eventually became disorganized and dissociated from the basal cells, resulting in the loss of the differentiated cell layer (not shown). Differentiated cells from the control iPrEC cultures remained healthy and viable. At day 12, many more apoptotic cells were detected in the differentiating Myc or ING overexpressing cultures as evidenced by increased cleaved caspase-3 (Fig. 3A) and TUNEL staining (not shown) specifically in the suprabasal layer. The basal cell layer remained intact and displayed no evidence of cell death. Thus, sustained overexpression of Myc or ING4, specifically in the differentiated cells, ultimately causes their death.

A qRT-PCR screen for cell death effectors identified elevated expression of Bnip3 (not shown), which encodes a BH3-only proapoptotic protein. Inhibiting Bnip3 expression with siRNA blocked the death induced by ING4 or Myc overexpression, as measured by a reduction in caspase-3–positive cells (Fig. 3B). Blocking Bnip3 expression did not inhibit differentiation (Supplementary Fig. S3), indicating death occurs after differentiation. Thus, the death induced by Myc and ING4 overexpression in differentiated cells is mediated by elevated Bnip3 expression, leading to apoptosis.

The C-terminal domain of ING4 is required for iPrEC differentiation

Myc promotes the trimethylation of H3 at K4 (H3K4me3; ref. 31). ING4 functions in chromatin remodeling complexes by binding to histone H3K4me3 sites via its C-terminal PHD motif and recruiting the HBO1 acetyltransferase via the N-terminal domain (21, 32). Deletion of the PHD motif generates a dominant inhibitory mutant (23). The ability of ING4 to accelerate differentiation was abrogated when the C-terminal domain of ING4 (ING4ΔCT) was deleted (Fig. 3C). This is
further evidenced by the failure to suppress integrin α6 expression (Fig. 3D) in the cells expressing ING4ACT. Furthermore, ING4ACT blocked the ability of Myc to induce differentiation. Cells that did appear in the suprabasal layer were dying as determined by caspase-3 immunostaining (not shown). Thus, the C-terminus of ING4 containing the PHD domain is required for iPPrEC differentiation and survival of the emerging cells, suggesting that the Myc-ING4 differentiation program depends on ING4-dependent chromatin remodeling.

**ING4 expression is lost in patient with prostate cancer tumors**

To determine whether ING4 expression is altered in prostate cancer, a tissue microarray containing 50 malignant prostate tumors and 12 noncancerous prostates was surveyed for ING4 and AR expression (Fig. 4A). ING4 expression was detected in 83% benign lesion sample (10/12) and 90% of the tumors were positive for AR (Fig. 4A). These results demonstrate that more than 60% of prostate tumors downregulate ING4 expression and this loss occurs in AR-positive cancer, indicating that ING4 loss may be a main event in prostate tumorigenesis.

**Loss of ING4 expression cooperates with Myc/Erg in prostate tumorigenesis**

As reported previously, Myc overexpression alone in human iPPrECs was not sufficient to generate a cell line that is tumorigenic in mice (33). Combined overexpression of Myc and the prostate-specific oncogene, Erg (10), was also not sufficient to generate human tumors. To test whether loss of ING4 is also required, we orthotopically injected iPPrECs overexpressing Erg and Myc (EM) with or without shING4 (EMI), or a nontargeting shRNA (EMshCV) into prostates of nude mice. Cells overexpressing the two oncogenes Myc and Erg (EM) or in conjunction with a nontargeting shRNA (EMshCV) did not produce tumors in the mice 18 weeks following orthotopic injection. However, EMI cells produced tumors in 60% of the mice (Fig. 5A). Ultrasound imaging of tumors in mice 18 weeks following orthotopic injection is shown in Fig. 5B. Tumors were positive for AR, but negative for ING4 expression when compared with adjacent normal tissue (Fig. 5C and D). Thus, loss of ING4 is required in human cells to cooperate with Myc and Erg to produce prostate tumors.

**Pten loss prevents ING4 expression**

To further develop prostate cancer models, Pten expression was silenced by overexpressing Pten shRNA in the EM cells (EMP). Overexpression of Myc and Erg and knockdown of Pten was verified in EMP cells by immunoblotting (Fig. 6A). In EMP cells, the expression of integrin α6 was increased whereas the expression of the p27 cell-cycle inhibitor was reduced (Fig. 6A), consistent with changes observed in prostate cancer (13, 34).

Orthotopic injection of EMP, but not iPPrECs, into the prostates of nude mice produced tumors that were detectable by ultrasound imaging as early as 8 weeks after injection. At 16 weeks, the tumors averaged 2.85 mm in diameter, ranging from 2.11 to 3.68 mm (Fig. 6B). The tumor penetrance was 60%, as 17 of 30 injections resulted in prostate tumor formation (Fig. 6C). IHC with human-specific MHC class I antibody revealed the presence of human cells demarcating the tumorigenic foci. The EMP tumors stained positive for AR (Fig. 6D) and castrating the mice 16 weeks after the tumors were established resulted in complete tumor regression, indicating a dependence on androgen for tumor maintenance (Fig. 6C).

When subjected to the differentiation protocol, EM cells were completely competent at differentiating as evidenced by the formation of distinct layers, loss of integrin α6, and gain of AR in the suprabasal layer (Fig. 7A). In contrast, the EMI cells failed to differentiate as evidenced by reduced numbers of suprabasal cells, poor AR expression, and retention of integrin α6 in all the cells. EMP cells also failed to differentiate, as evidenced by the lack of a suprabasal layer, and failure to lose
integrin expression (Fig. 7A). However, in contrast to EMI cells, the EMP cells induced high AR and integrin α6 expression in the basal layer (Fig. 7A). Elevated integrin α6 expression in EMP cells was also observed by immunoblotting (Fig. 6A). This resulted in a population of cells coexpressing AR and integrin α6; reproducing the histopathology observed in clinical samples (13). The inability of EMI and EMP cells to differentiate, correlated with a failure of Myc to induce ING4 expression (Fig. 7A and B). The small clusters of AR-positive cells in the EMI culture are cells in which shING4 was poorly expressed, as evidenced by ING4 positivity in those clusters. Analysis of the keratin subtypes further revealed that EMP cells coexpress both basal keratin K5 and secretory keratin K8 (16) compared with normal iPrECs, where each keratin was distinctively expressed in their respective cell types (Fig. 7C). Thus, EMP cells have a dysfunctional differentiation program that prevents ING4 expression in the presence of Myc, resulting in tumorigenic cells with an intermediate differentiation phenotype. Re-expression of ING4 in EMP cells completely rescued the differentiation defect, restoring the suprabasal layer, AR expression, loss of integrin (Fig. 7B), and separation of the K5

**Figure 5.** Loss of ING4 expression is required for tumorigenesis. A, iPrECs were engineered to stably overexpress Myc and Erg, along with Pten shRNA (EMP). Immunoblotting confirmed overexpression of Myc, Erg, integrin α6 (ITGα6), and loss of Pten and p27Kip. B, tumor measured by ultrasound imaging 16 weeks after orthotopic injection of EMP cells into the prostates of nude mice. C, number of mice in which tumors formed compared with control iPrECs 16 weeks postinjection. Sixteen weeks postinjection, 5 mice harboring EMP tumors were castrated and 11 weeks later the number of tumors that regressed was recorded. D, IHC staining of different tumor samples with human-specific MHC class I or AR.
and K8 populations (Fig. 7C). Expression of the ING4\(^{CT}\) mutant in EMP cells did not rescue the differentiation defect (not shown). Thus, the Myc-ING4 differentiation relay is no longer functional in the oncogenic EMP cells and Pten loss is responsible. Together our results support the conclusion that ING4 is required for differentiation of iPrECs and suggest that one of the major oncogenic events in prostate cancer is the uncoupling of the Myc-ING4 differentiation program.

**Discussion**

In immortalized human prostate epithelial cells with the capacity to differentiate in vitro, transient ING4 expression, dependent on Myc, is required for prostate epithelial differentiation. ING4 expression coincides with loss of matrix-based adhesion, downregulation of integrin, and acquisition of AR; blocking ING4 prevents the initiation of these processes. In normal differentiating iPrECs, the acquisition of AR expression and androgen responsiveness is observed only in cells in which integrin expression is lost (12). We found that neither AR nor androgen is required for ING4 expression (not shown), nor were we able to demonstrate any influence of ING4 on AR expression or its ability to activate its transcriptional targets in cells expressing AR. Thus, the role of ING4 in prostate epithelial differentiation lies at least in part within its capacity to target integrins. This is consistent with the observations in the Myc breast cancer mouse model, where overexpression of the C-terminal deletion mutant of ING4 (ING4\(^{CT}\)) restored integrin expression in the tumors (unpublished results; ref. 23). This is also consistent with the established role for Myc in directly suppressing integrin \(\alpha_6\) and \(\beta_1\) transcription during differentiation (35). Our data indicate that ING4 is an essential component of the Myc-dependent effect on integrin expression, because removal of ING4 prevents Myc from suppressing integrin expression.

Myc or ING4 overexpression in basal cells is sufficient to accelerate differentiation toward luminal cells; however, improper prolonged expression of Myc or ING4 leads to cell death. Thus, the temporal, that is, Myc expression preceding ING4, and transient nature of Myc and ING4 expression is
cell death suggests loss of ING4 is necessary for Myc-dependent overexpression in prostate cancer and its tendency to induce loss to prostate tumorigenesis. The high prevalence of Myc required for differentiation. To our knowledge, this is the first time that the chromatin remodeling properties of ING4 have been linked to differentiation. Once bound, ING4 recruits the HBO1 acetyltransferase (21, 37), facilitates histone H3/H4 acetylation, and activates gene transcription (21, 38). Like ING4, Myc is extensively involved in chromatin remodeling (39, 40). In addition, recent studies have brought to light the chromatin remodeling activity of Myc in the maintenance of pluripotent stem cells (19, 41). Taken together, the relay from Myc to ING4 is likely to install epigenetic changes that govern differential transcription and ultimately prostate epithelial cell differentiation.

Myc overexpression alone often fails to transform normal human cells because of induction of cell death (33, 42). Myc or ING4 overexpression specifically induces death of the differentiated cells, but not the underlying basal cells. This supports the current paradigm that Myc activity manifests in a context-dependent manner such that Myc induces cell death in more differentiated cells, but maintains the proliferative and self-renewal capacity of less differentiated stem or progenitor cells. The death phenotypes induced by Myc overexpression are mediated in part by p53 and ING4 enhances p53 function (43). However, the death induced by Myc or ING4 overexpression in iPReCs is likely p53-independent, because the iPReCs express E6 that blocks p53 function. In iPReCs, Bnip3 is responsible for the observed cell death. Although p53 is reported to regulate Bnip3 (44, 45), our results describe an alternate mechanism of Bnip3 activation that is p53 independent. Nonetheless, ING4 may be part of the mechanism by which p53 regulates Bnip3. In prostate cancer, p53 loss is rare and associated with a small subset of late stage disease (46). Thus, loss of ING4 may be a mechanism by which prostate cancer cells escape the tumor suppressive effects of p53 when Myc is overexpressed. This idea is further supported when contrasting the prostate cancer tissue data, which demonstrate a 60% loss of ING4, with that of breast cancer where p53 loss is more highly prevalent and only 34% of the samples lack ING4.

ING4 expression is lost in more than 60% of prostate tumors, suggesting for the first time a significant contribution of ING4 loss to prostate tumorigenesis. The high prevalence of Myc overexpression in prostate cancer and its tendency to induce cell death suggests loss of ING4 is necessary for Myc-dependent prostate oncogenesis. Indeed, only Myc-overexpressing cells without ING4 are capable of generating tumors in mice. Moreover, loss of ING4 blocked tumor cell differentiation generating cells coexpressing both basal and luminal markers, a phenotype often seen in prostate cancer. The mechanism by which ING4 is lost in prostate cancer needs more investigation, but LOH at 12p13, the genomic region that contains the ING4 gene, has been reported in 10% to 20% of primary and up to 45% of metastatic prostate tumors (47, 48). Our data demonstrate loss of Pten is another mechanism that leads to ING4 loss. The molecular mechanism of Pten in the regulation of ING4 expression is presently unknown and likely to be indirect.

We have established a genetic link between Myc and ING4 in prostate epithelial differentiation and prostate cancer. Our data demonstrate that a Myc-ING4 temporal relay is required for normal prostate cell differentiation and when this relay is missing, it leads to prostate cancer. Whether the Myc-ING4 relay also governs cell differentiation in other cell types, including breast epithelia, needs to be addressed. We propose that ING4 dictates the downstream program driven by Myc toward differentiation, and in its absence Myc is directed toward targets that promote tumorigenesis. Pten loss resulting in the loss of ING4 expression, disruption of the Myc-ING4 relay, a block in differentiation, and susceptibility to tumorigenesis, reinforces the idea that ING4 plays a pivotal role in determining prostate epithelial cell fate.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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