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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/

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ING4 negative and ING4-only negative tumors may reflect two distinct subtypes of prostate cancer.

Keywords

Prostate; ING4; Myc; Cancer; Differentiation; Chromatin; Integrins

INTRODUCTION

The 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 downregulation 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 $\alpha 6$ are increased, while basal cell keratin is diminished and AR is expressed (8, 9). Additionally, overexpression of *Erg* up-regulates *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 post-mitotic 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 $\alpha 6 \beta 1$ are co-expressed in the tumors, where integrin $\alpha 6 \beta 1$ cooperates with AR to promote the survival of prostate cancer cells (15). Other markers typically associated with basal or intermediate cells, such as receptor tyrosine kinases EGFR and Met, bcl-2, and co-expression of basal and secretory keratins K5 and K8, are also found in tumor cells 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 Plant Homeodomain-containing transcriptional regulator, which binds trimethylated histone H3 and recruits the HBO1 acetyltransferase to increase histone acetylation (21). ING4 was shown to block Myc-induced anchorage-independence and mammary hyperplasia in a mouse model of breast cancer, suggesting ING4 may function to suppress Myc (22, 23). We hypothesized there would be an interplay between Myc and ING4 in prostate epithelial cell differentiation that would be disrupted in prostate tumorigenesis. In this study, we determine how Myc, Pten, and ING4 are involved in normal prostate epithelial differentiation and demonstrate the importance of ING4 loss in promoting prostate oncogenesis through suppressing differentiation.

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 μ g/ml neomycin for three days, and the resulting population pooled. Cells retain all the basal markers of primary cells. Immortalized cells (iPrECs) 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 μ g/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 confluency were treated in complete growth medium with 10ng/ml keratinocyte growth factor (KGF) (Cell Sciences) and 5nM 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).

Constructs

The wild-type retroviral pBabe-Myc construct was obtained from Dr. Beatrice Knudsen. pLPCX-Erg was generated by subcloning the Erg cDNA NotI/SpeI fragment from pMax Dest N-Erg (9), supplied by Dr. Vasioukhin, into NotI/XhoI of pLPCX. The wild type (pMIG-ING4) and C-terminal deletion mutant (pMIG-ING4- C1) of ING4 were described previously (23). The ING4 shRNA construct was generated by subcloning the oligo 5'-CCGGGCTAGGTGTGATCAACACTTTCTCGAGAAAGTGTTGATCACACCTAGCTT TTTTG-3', complementary to the 3'-UTR of ING4, into a lentiviral vector to generate pLKO.1-shING4. The pLKO vector containing Pten shRNA was generated by first creating a pCR8-GW-TOPO-shLEGO shuttle vector. A 344bp PCR product containing a multi-cloning site, EcoRV/XbaI/SalI/PstI, the pLKO U6 promoter, an AgeI site, a HindIII site, followed by a reverse multi-cloning site, PstI/SalI/XbaI/EcoRV, and an EcoRI site was TA cloned into pCR8-GW-TOPO. Oligo shPten2, 5'-CCGGTCCACAGCTAGAACTTATCAAACCTCGAGTTTGATAAGTTCT AGCTGTGGTTTTTA-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 0.35µg/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 0.35µg/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 non-targeting sequence were purchased from Origene. ON-Targetplus SMARTpool targeted to Bnip3, came from Dharmacon. Differentiated cultures were serially transfected every two 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 (Kip1) from Cell Signaling, and ING4 (EP3804) from GeneTex. Tubulin antibody (DM1A) was purchased from Sigma and GAPDH (6CS) from Millipore. Polyclonal integrin $\alpha 6$ (AA6A) antibody was a gift from Dr. Anne Cress (University of Arizona, Phoenix, AZ) (27).

Immunostaining and Microscopy

Differentiated cultures 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 diluted in 1% BSA/PBS were incubated for 1–2 hours. Nuclei were stained with Hoechst 33258 (Sigma) for 10 minutes at room temperature. Coverslips were mounted using Fluoromount-G (SouthernBiotech). 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, 30–50µg 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, 0.5µg 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⁻ Ct method (28). Primers for ING4 and Myc were as follows: ING4: 5'-TCGGAAGTTGCTTTGTTTTGC-3', Myc: 5'-TTCGGGTAGTGGAACCAG-3',

Mouse Tumorigenesis

Half a million iPreC, EM, EMP, 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 16–18 weeks and prostate glands analyzed histologically for tumors. In one cohort of EMPs, 5 mice with tumors were castrated 16 weeks post-orthotopic transplantation and measured by ultrasound for regression of tumors. All animal work was carried out following IACUC approval at an ALAAC-accredited facility.

Histology

Prostates isolated from mice were Formalin-fixed and paraffin-embedded. Sections were analyzed following H&E or immunohistochemical staining. Human-specific MHC Class I 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 5µm 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 non-immune 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 0–1 representing complete to major loss of protein, and 2–3 near normal to wild type levels.

RESULTS

Myc and ING4 are transiently expressed during differentiation

When grown to confluency 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 (iPrECs) was established by expressing the E6/E7 viral oncogenes and hTert. Treatment of confluent iPrEC cultures with 10ng/ml KGF and 5nM R1881, a synthetic AR agonist, for 18 days resulted in a distinct top layer of cells that no longer expressed integrin $\alpha 6$, K14, or p63 but expressed AR and AR-dependent targets, such as TMPRSS2 and Nkx3.1 (Fig. 1A, Supplementary Figs. S1,S2). These data indicate iPrECs retain the ability to differentiate.

ING4 expression was low to undetectable in untreated iPrECs, but by as early as eight 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 $\alpha 6$ expression, two hallmarks of differentiation (Fig. 1B, Supplementary Fig. S1B). At no time point were we able to dissociate ING4 expression from changes in AR or integrin $\alpha 6$ expression; nor were we able to separate loss of basal keratin K14 from integrin $\alpha 6$ loss (Supplementary Fig. S2), suggesting ING4 controls a major differentiation switch. While 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 to the immunostaining data, is most likely due to 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 (Figs. 1C and 1D). 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). While ING4 expression levels did not affect Myc expression, most notable was the increase in ING4 expression in the Myc overexpressing cells (Figs. 2A and 2B). These results suggest Myc is responsible for the increase in ING4 expression during iPrEC differentiation.

Overexpression of ING4 or Myc accelerated the emergence of differentiated cells compared to the control iPrECs. The appearance of suprabasal layer cells, loss of integrin $\alpha 6$, and gain in Nkx3.1 expression was more robust between days 8 and 12 in the ING4 or Myc overexpressing cells, whereas the control iPrECs 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 to 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 due to 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 $\alpha 6$ (Fig. 2C) and gain of AR, indicating an absolute necessity for ING4 to suppress integrin $\alpha 6$ 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 2–6 failed to induce ING4 expression (Fig. 2D,F) and completely blocked differentiation (Fig. 2E,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 iPrEC differentiation.

Constitutive Myc and ING4 expression leads to cell death of the differentiated cells via Bnip3

While 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) (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 (ING4 CT) was deleted (Fig. 3C). This is further evidenced by the failure to suppress integrin $\alpha 6$ expression (Fig. 3D) in the cells expressing ING4 CT. Furthermore, ING4 CT 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 iPrEC 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 prostate cancer patient tumors

To determine whether ING4 expression is altered in prostate cancer, a tissue microarray containing 50 malignant prostate tumors and 12 non-cancerous prostates was surveyed for ING4 and AR expression (Fig. 4A). ING4 expression was detected in the nuclei of the luminal cell population of non-cancerous samples (Fig. 4B). ING4 expression levels were scored on a scale ranging from 0 to 3; 0 for no detectable expression and 3 for distinct nuclear expression in accordance with a previous study (30). While 100% of control (BPH or TURP) samples were positive for ING4, only 36% of tumor samples (18/50) were positive for nuclear ING4 expression (Fig. 4A). In contrast, 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 down-regulate 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 iPrECs 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 iPrECs

overexpressing Erg and Myc (EM) with or without shING4 (EMI), or a non-targeting shRNA (EMshCV) into prostates of nude mice. Cells overexpressing the two oncogenes Myc and Erg (EM) or in conjunction with a non-targeting 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 to adjacent normal tissue (Fig. 5C and 5D). 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 $\alpha 6$ was increased while 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 iPRECs, into the prostates of nude mice produced tumors that were detectable by ultrasound imaging as early as 8 weeks after injection (Fig. 6B). At 16 weeks, the tumors averaged 2.85 mm in diameter, ranging from 2.11 to 3.68 mm. The tumor penetrance was 60%, as 17 out 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 $\alpha 6$ 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 $\alpha 6$ 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 $\alpha 6$ expression in the basal layer (Fig. 7A). Elevated integrin $\alpha 6$ expression in EMP cells was also observed by immunoblotting (Fig. 6A). This resulted in a population of cells co-expressing AR and integrin $\alpha 6$; 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,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 co-express both basal keratin K5 and secretory keratin K8 (16) compared to 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 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)(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, since removal of ING4 prevents Myc from suppressing integrin expression.

Myc or ING4 overexpression in basal cells is sufficient to accelerate differentiation towards luminal cells; however, improper prolonged expression of Myc or ING4 leads to cell death. Thus, the temporal, i.e. Myc expression preceding ING4, and transient nature of Myc and ING4 expression is crucial for normal cell differentiation. The molecular mechanism of this concerted transcriptional relay is currently unclear. Previous ChIP analysis identified Myc bound to the ING4 promoter, suggesting ING4 is a direct target of Myc (36). However, we failed to detect an increase in ING4 mRNA in undifferentiated basal cells overexpressing Myc (not shown). Similarly, Myc overexpression in breast epithelial cells also did not increase ING4 expression (unpublished results). Thus, our results point to an indirect action of Myc in ING4 induction, or requiring additional factor(s) during the course of differentiation.

Differentiation is dependent on the ING4 C-terminal domain containing the PHD motif required for H3K4me3 binding (21). ING4 overexpression alters chromatin modifications (not shown) suggesting ING4 association with chromatin is 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 cell differentiation.

Myc overexpression alone often fails to transform normal human cells due to 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, since the iPrECs express E6 which blocks p53 function. In iPrECs, Bnip3 is responsible for the observed cell death. While 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 demonstrates 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 over 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 co-expressing 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 10–20% of primary and up to 45% of metastatic prostate tumors (47, 48). Our data demonstrate loss of Pten is another mechanism which 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ING4 is transiently expressed in differentiated immortalized prostate epithelial cells
 Confluent immortalized prostate epithelial cells (iPRECs) were induced to differentiate with 10ng/mL KGF and 5nM R1881 for 4–21 days. **A)** Terminally differentiated iPRECs were immunostained (red) and counterstained with Hoechst (blue), then imaged by confocal microscopy. Suprabasal cells (S) express NKX3.1, while only basal cells (B) express integrin α 6. Dashed lines demarcate suprabasal and basal layer cells. **B)** iPRECs were immunostained 4–17 days after differentiation to detect ING4 (green), AR (red), or integrin α 6 (green). **C,D)** Protein or RNA was isolated from whole (W) differentiated cultures at

days 4–8, or only the suprabasal (T, top) cells at days 12–21. **C)** ING4, Myc, and GAPDH (GDH) were detected by immunoblotting. **D)** ING4 and Myc mRNA were measured by qRT-PCR. Data are normalized to 18S rRNA and are mean $\pm$ standard deviation.

Figure 2. Myc-induced ING4 expression is required for differentiation

iPrECs were engineered to overexpress ING4, Myc, ING4 and Myc, ING4 shRNA (shING4), or Myc with shING4. **A)** ING4, Myc, and Tubulin (Tub) expression in basal cells were measured by immunoblotting. **B)** Cells differentiated for 8 days were immunostained for ING4 (green) and imaged by fluorescent microscopy. **C)** Cells differentiated for 12 days were immunostained (red) to detect NKX3.1 or ITGa6, counterstained with Hoechst (blue), and imaged by fluorescent microscopy. **D,E,F)** One day after inducing differentiation, iPrECs (iPr) or iPrECs overexpressing ING4 (ING4) were serially transfected with Myc

siRNA (siMyc) or a scrambled sequence (Scr) on days 2, 4 and 6. **D)** ING4 and Myc expression were detected in undifferentiated (iPr) or in siRNA-treated differentiated cells on day 8 by immunoblotting. **E,F)** Differentiated iPrECs (iPr) or ING4-overexpressing cells were immunostained for ING4 (red), AR (red), or ITGa6 (green) on day 9.

Figure 3. Constitutive Myc and ING4 expression leads to cell death

A) Cell death was measured in cells differentiated for 12 days by immunostaining for Caspase 3 activity (red). Nuclei were counterstained with Hoechst (blue). **B)** After 6 days of differentiation, Myc or ING4 overexpressing cells were transfected with Bnip3 siRNA (siBnip3) or a scrambled sequence (Scr) and immunostained 72 hours later for Caspase 3 activity (red) and counterstained with Hoechst (blue). **C)** The C-terminal truncation mutant of ING4 was overexpressed in iPreCs (ING4 Δ CT) or in Myc overexpressing cells and compared 8 days after differentiation by phase contrast to cells overexpressing wild type

ING4. **D**) After 12 days of differentiation, iPrECs (iPr), ING4 CT, and Myc plus ING4 CT expressing cells were immunostained for integrin $\alpha 6$ (red) and imaged by fluorescent microscopy.

A)	BPH/TURP	1° Tumors
ING4 0-1 (-)	0 (0%)	32 (64%)*
ING4 2-3 (+)	12 (100%)	18 (36%)
AR 0-1 (-)	2 (17%)	5 (10%)
AR 2-3 (+)	10 (83%)	45 (90%)

***p=0.00004**

Figure 4. ING4 expression is lost in prostate cancer patients

A tissue microarray of 50 cancerous and 12 non-cancerous human prostate samples was immunostained for ING4 or AR. **A)** Table of ING4 and AR histological grading (scale 0–3; 3 being highest expression) comparing benign prostate hyperplasia/transurethral resection (BPH/TURP) and primary tumors (1°). $p=0.0004$; $n=50$ **B)** Immunohistochemical staining of ING4 and AR in benign hyperplastic prostate tissue (BPH) and prostate cancer (PCa) tissue.

Figure 5. Loss of ING4 expression is required for tumorigenesis

A) iPrECs were engineered to stably overexpress Myc and Erg (EM) with or without shING4 (EMshING4) or a non-targeting shRNA (shCV). Number of mice in which tumors formed following orthotopic injection of EMshING4 compared to control EM and EMshCV cells 18 weeks post-injection. **B)** Tumor measured by ultrasound imaging 18 weeks after orthotopic injection of EMshING4 into the prostates of nude mice. **C)** H&E and immunohistochemical staining of a tumor sample with AR. **D)** Immunohistochemical staining of ING4 in normal mouse prostate and tumor sample.

Figure 6. Pten loss promotes tumorigenesis

A) iPrECs (iPr) were engineered to stably overexpress Myc and Erg, along with Pten shRNA (EMP). Immunoblotting confirmed overexpression of Myc, Erg, integrin α 6 (ITG α 6) 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 following orthotopic injection of EMP cells compared to control iPrECs 16 weeks post-injection. Sixteen weeks post-injection, five mice harboring EMP tumors were castrated and 11 weeks later the number of tumors that regressed was recorded.

D) Immunohistochemical staining of different tumor samples with human-specific MHC Class I or AR.

Figure 7. Pten-mediated loss of ING4 and altered differentiation in tumorigenic cells

A) iPrECs (iPr), EM, EMI, or EMP cells differentiated for 12–14 days were immunostained for AR (red), ITG α 6 (green), or ING4 (red), counterstained with Hoechst (blue), and imaged by fluorescent microscopy. **B)** ING4, Myc, Erg and Pten expression in undifferentiated iPrECs (iPr), EM, EMP, EMP+ING4 or EMI cells detected by immunoblotting. EMP or EMP-ING4 cells differentiated for 12–14 days were immunostained for ING4 (green), ITG α 6 (red), or Nkx3.1 (red). Dashed lines demarcate supra (T) and basal cell layers. **C)** Cultures of iPrECs, EMP, or EMP-ING4 cells were differentiated for 17 days,

immunostained for keratins K8 (green) and K5 (red), counterstained with Hoechst (blue), and imaged by confocal microscopy.