Research Article

Reduction of Histone Marks, H3K9me3 and H3K27me3 by Epidrug Induces Neuroendocrine Differentiation in Prostate Cancer1

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Abstract
Neuroendocrine prostate cancer (NE PCa) is an aggressive malignancy, often presenting with advanced metastasis. We previously reported that reduction of histone marks regulated by DNMT1 following epidrug (5-Azacitidine, 5-Aza) treatment controls induction of epithelial to mesenchymal (EMT) and a cancer stem cell (CSC) phenotype, which facilitates tumorigenesis in PCa cells. Here, we use the epidrug 5-Aza as a model for how histone marks may regulate the reprogramming of prostate adenocarcinoma into NE phenotypic cells.

First, we observed that 5-Aza treatment of PCa cells *in vitro* induces a neuron-like phenotype. In addition, significant increases in the expression of the NE markers N-Myc downstream regulated gene 1 (NDRG1), enolase-2 (ENO2), and synaptophysin were observed. Critically, a high density of NE cells with synaptophysin expression was found in tumors generated by 5-Aza pretreatment of PCa cells. Importantly, induction of NE differentiation of PCa cells was associated with an enhancement of NDRG1 expression by reduction of histone marks, H3K9me3 and H3K27me3. Further, more NDRG1 expression was detected in the subset of PCa cells with reduced expression of H3K9me3 or H3K27me3 in the tumors generated by 5-Aza pretreated PCa cells and critically, these biological differences are also observed in small cell carcinoma in advanced stage of human primary PCa tumors.

Our results suggest that reduction of histone marks regulated by the epidrug 5-Aza may control induction of a NE phenotype, which facilitates PCa progression. These studies suggest a strong rationale for developing therapeutics, which target epigenetic regulation.

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer deaths in American males (Pienta and Esper, 1993). Most prostate cancer (PCa) metastasizes or spreads to bones and almost all men who die from PCa have bone metastases at the time of death (Koutsilieris, 1993). Androgen deprivation therapy (ADT) is the primary treatment option for metastatic PCa. Unfortunately, PCa cells frequently become resistant to ADT,

suggesting a critical need for the identification of therapeutic targets and more effective therapies in advanced PCa disease (Borges et al., 2016, Terry and Beltran, 2014).

Neuroendocrine prostate cancer (NE PCa) is an extremely aggressive subtype of PCa that is commonly observed in relapsing individuals particularly after treatment with second-generation anti-androgens (abiraterone and enzalutamide) (Borges et al., 2016, Chan et al., 2010, Conteduca et al., 2014, Nelson et al., 2007, Parimi et al., 2014, Terry and Beltran, 2014, Wang et al., 2014). NE PCa is characterized by neuroendocrine (NE) markers such as chromogranin A, synaptophysin, and neuron specific enolase (NSE), but no or low expression of androgen receptor (AR) and androgen target genes such as prostate specific antigen (PSA/KLK-3) (Borges et al., 2016, Chan et al., 2010, Conteduca et al., 2014, Nelson et al., 2007, Parimi et al., 2014, Terry and Beltran, 2014, Wang et al., 2014). NE PCa typically metastasizes to various organs such as liver, lymph node, visceral, and bone. Diagnosis of NE PCa disease is associated with a poor prognosis with high mortality rates (Borges et al., 2016, Terry and Beltran, 2014). Therefore, understanding the mechanisms underlying the emergence of NE PCa in advanced metastasis is crucial for developing future therapies for cancer treatment.

Epigenetic modifications such as DNA methylation or histone methylation, acetylation, and phosphorylation regulate the phenotypic changes of cells by the alternation of gene expression without concomitant genomic changes and the histone modifications also play the critical roles in transcriptional regulation (Crea et al., 2016, Fuks, 2005). Emerging evidence indicates that the epigenetic alterations play a more fundamental function in NE PCa initiation and development (Beltran et al., 2016, Crea et al., 2016). NE PCa disease is often associated with epigenetic alterations including AR inactivation, the loss of tumor suppressors (RB1, PTEN, and TP53), TMPRSS2-ERG rearrangement, and amplification of proto-oncogenes (Aurka and N-Myc) (Crea et al., 2016, Terry and Beltran, 2014). Recent evidence has been shown that treatment with 5-Aza is associated with histone H3 lysine 9 (H3K9) demethylation in some silenced genes, which results in transcriptional regulation (Kim et al., 2013). We previously reported that the reduction of histone marks regulated by DNMT1 following 5-Aza treatment promoted the induction

of EMT and CSC phenotypes, which facilitates tumorigenesis in PCa cells (Lee et al., 2016a). Emerging evidence indicates that the acquisition of CSC and EMT phenotypes are associated with the development of NE cell differentiation in PCa, leading to more aggressive disease (Borges et al., 2016, McKeithen et al., 2010, Palapattu et al., 2009, Terry and Beltran, 2014). Here, we use 5-Aza to further explore whether reduction of histone marks may regulate the reprogramming of PCa cells into NE PCa cells.

We observed that PCa cells alter their morphological features to a neuron-like phenotype after 5-Aza treatment *in vitro*. We also found that a significant increase in the expression of NE markers, N-Myc downstream regulated gene 1 (NDRG1), enolase-2 (ENO2), and synaptophysin following 5-Aza treatment. Critically, in an animal model it was observed that a high density of NE cells with synaptophysin expression were found in tumors generated by pretreatment with 5-Aza. We further show that enhancement of NDRG1expression by reduction of histone marks is associated in induction of NE differentiation of PCa cells. Our findings suggest that interference of these pathways may be possible ways for the targeting NE development and progression.

MATERIALS AND METHODS

CELL CULTURE

The Human PCa cell lines, PC3 and DU145 were obtained from the American Type Culture Collection (Rockville, MD). PCa cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S).

QUANTITATIVE RT-PCR

Total RNA was extracted from cells using the RNeasy mini kit (cat. 74104, Qiagen, Valencia, CA) and converted into cDNA using a First-Strand Synthesis Kit (Invitrogen). Quantitative PCR (real-time PCR) was performed on an ABI 7700 sequence detector using TaqMan Universal PCR Master Mix according to the directions of manufacturer (Applied Biosystems, Foster City, CA). TaqMan MGB probes (Applied Biosystems) were as follows: N-Myc downstream regulated 1 (NDRG1, Hs00608387_m1), Enolase-2

(ENO2, Hs00157360_m1), Synaptophysin (SYP, Hs00300531_m1). β-Actin (Hs01060665_g1) was used as internal controls for the normalization of target gene expression.

IMMUNOSTAINING

Tumor sections were used on the tumors subcutaneously grown with vehicle or 5-Aza pretreated PCa cells implanted into SCID mice in our previous studies (Lee et al., 2016a). Tumor sections were blocked deparaffinized, hydrated, antigen retrieval, and then blocked with Image-iT FX signal enhancer for 30 min and incubated for 2 hours at room temperature with primary antibodies combined with reagents of Zenon Alexa Fluor 488 (green) (cat. Z25002, Invitrogen) or 555 (red) (cat. Z25305, Invitrogen) labeling kit. Synaptophysin (cat. ab23754, Abcam), Pan-Cytokeratin (cat. ab86734, Abcam), N-Myc downstream regulated 1 (NDRG1) (cat. ab196621, Abcam), H3K9me3 (cat. ab6001, Abcam), and H3K27me3 (cat. ab6147, Abcam) antibodies were used as primary antibody. After washing with PBS, the slides were mounted with ProLong Gold antifade reagent with DAPI (cat. P36931, Invitrogen). H&E stain, Synaptophysin (cat. ab 2375, Abcam), pan-Cytokeratin (cat. ab86734, Abcam) antibodies were used for immunohistochemical (IHC) staining in *s.c.* tumors. Images were taken with Olympus 51Amicroscope. Co-staining of H3K9me3 and NDRG1, H3K27me3 and NDRG1 antibodies were applied to *s.c.* tumors. Images were taken with Olympus FV-500 confocal microscope. Human prostate tissue microarrays (TMAs) were purchased from US Biomax, Inc. (Rockville, MD). Tumors were graded using stage progressing system. Co-staining of H3K9me3 and NDRG1, H3K27me3 and NDRG1 antibodies were applied to TMAs. Staining intensity was ranked on a scale from 1 to 4 (1, negative; 2, weak; 3, moderate; and 4, strong).

WESTERN BLOT

PCa cells were cultured in RPMI 1640 with 10% FBS and 1% P/S. The cells were treated with vehicle or 5-Aza (5μM) for 10 days at 37^{o}C. Whole cell lysates were prepared from cells, separated on 4-20% Tris-Glycine gel and transferred to PVDF membranes. The membranes were incubated with 5% milk for 1 hour and incubated with primary

antibodies overnight at 4^{o}C. Primary antibodies used were as follows: N-Myc downstream regulated 1 (NDRG1, 1:1,000 dilution, cat. 9485, Cell Signaling,), Synaptophysin (1:1,000 dilution, cat. 4329, Cell Signaling,), Enolase-2 (ENO2, 1:1,000 dilution, cat. 9536, Cell Signaling,), H3K9me3 (1:1,000 dilution, cat. 13969, Cell Signaling), and H3K27me3 (1:1,000 dilution, cat. 9733, Cell Signaling). Blots were incubated with peroxidase-coupled anti-mouse IgG secondary antibody (cat. 7076, 1:2,000 dilution, Cell Signaling) or peroxidase-coupled anti-rabbit IgG secondary antibody (cat. 7074, 1:2,000 dilution, Cell Signaling) for 1 hour, and protein expression was detected with SuperSignal West Dura Chemiluminescent Substrate (cat. Prod 34075, Thermo Scientific, Rockford, IL). Membranes were reprobed with monoclonal anti-β-Actin antibody (1:1,000 dilution, cat. 4970, Cell Signaling) to control for equal loading.

IN VITRO CHROMATIN IMMUNOPRECIPITATION (CHIP)

CHIP assays were performed on the vehicle or 5-Aza (5μM) treated PCa cells (PC3 or DU145) (4 x 10^{6}) by following the directions of the manufacturer (cat. 334471, EpiTech CHIP OneDay Kit, Qiagen). PCa cells were treated with 1% formaldehyde to crosslink histones to DNA. The crosslinking was stopped by treating the samples with stop buffer for 5 min. The chromatin was extracted and fragmented by sonication, and the lysate was used to immunoprecipitation using Protein A+G beads and the following antibodies; H3K9me3 (cat. GAH-6204, Qiagen), H3K27me3 (cat. GAH-9205, Qiagen), or Rabbit control IgG (cat. GAH-9205, Qiagen). Immunocomplexes were pulled down, washed, and DNA was isolated to run SYBR qPCR with NDRG1 (assay tile: -0.4Kb; assay position: -3262) (cat. GPH1026403(-)04A, Qiagen) specific primer on promoter site.

STATISTICAL ANALYSES

Results are presented as mean ± standard deviation (s.d.). Distribution of the data was determined by skewness. Values within a 1.95 (5%) confidence interval were accepted as a normal distribution and evaluated further using parametric tests for significance. Unpaired Student's t-tests were conducted on data sets of 2 comparisons, and significance of data with multiple comparisons was evaluated using an ANOVA. Values of $P < 0.05$ were considered significant.

RESULTS

EPIDRUG INDUCES A NEUROENDOCRINE-LIKE PHENOTYPE OF PCA CELLS *IN VITRO*

To explore the role that the epigenetic alterations play in the development of neuroendocrine (NE) differentiation, we examined the morphological changes in PCa cells following 10 days of 5-Aza treatment. We found that 5-Aza treatment of PCa cells (PC3, DU145) results in the development of a neuroendocrine-like phenotype (**Fig. 1**). Specifically, 5-Aza treated cells were developed a neuronal-like appearance, which is characterized by bipolar or multipolar cells with small cell bodies, long-projecting axonal processes. In contrast, control treated cells were maintained their epithelial morphology and tended to grow in clusters (**Fig. 1**). The data suggest that 5-Aza treatment alone may be a sufficient stimulus to induce epigenetic alteration in PCa cell lines to induce a neuroendocrine-like phenotype.

EPIDRUG INCREASES EXPRESSION OF NEUROENDOCRINE PHENOTYPIC MARKER IN PCA CELLS *IN VITRO*

To determine if the epigenetic alteration by the epidrug 5-Aza regulates NE differentiation, we first examined the expression of NE phenotypes in vehicle- or 5-Aza treated PCa cells by real-time PCR and Western blots. Expression of mRNA for the NE associated transcription factor, N-Myc downstream regulated gene 1 (NDRG1) was increased in 5-Aza treated PCa cells vs. vehicle treated PCa cells (**Fig. 2A**). Likewise, NE markers enolase-2 (ENO2) and synaptophysin were significantly increased by 5-Aza treatment, indicating the induction of a NE phenotype (**Fig. 2B and C**). At the protein level, a significant increase in NDRG1, ENO2, and synaptophysin expression were observed in response to 5-Aza treatment compared to vehicle treatment (**Fig. 2D**). These data suggest that epigenetic alteration by 5-Aza is associated with an NE phenotype in PCa cells.

EPIDRUG INDUCES AN NEUROENDOCRINE PHENOTYPE IN PCA TUMOR CELLS *IN VIVO*

Our previous studies demonstrate that significantly greater tumor sizes were observed in SCID mice implanted with PCa cells pretreated with 5-Aza *in vitro* compared to PCa cells pretreated with vehicle (Lee et al., 2016a). Here, we found that a significantly higher density of NE PCa cells with extensive vascularization (**Fig. 3A and B,** H&E stain) and synaptophysin expression in tumors generated from PCa cells pretreated with 5-Aza *in vitro* compared to PCa cells pretreated with vehicle (**Fig. 3A and B**). Further, to confirm that the tumor cells were of human orign, Pan-Cytokeratin expression was examined (**Fig. 3A and B**). These data suggest that the epigenetic alteration by the epidrug 5-Aza may regulate the induction of a NE phenotype, leading to the emergence of malignant outgrowth.

EPIDRUG INDUCES THE REDUCTION OF HISTONE MARKS ON THE NDRG1 PROMOTER IN PCA CELLS

5-Aza regulates global reductions of di- and tri-methylation of histone H3 lysine 9 (H3K9me2 and H3K9me3) in the many human cancer cells (Kim et al., 2013). We therefore examined whether 5-Aza induces reduction of tri-methylation of histone H3 lysine 9 (H3K9me3) and histone H3 lysine 27 (H3K27me3) in PCa cells treated with 5-Aza or vehicle by Western blots. We found that the levels of both suppressive histone marks, H3K9me3 and H3K27me3 were significantly reduced in PCa cells treated with 5-Aza compared to PCa cells treated with vehicle (**Fig. 4A**).

We further examined whether reduction of suppressive histone marks, H3K9me3 and H3K27me3 on NDRG1 promoter induce NDRG1 expression in the 5-Aza or vehicle treated PCa cells by CHIP qPCR assays (**Fig. 4B and C**). In both cases, we observed that the levels of suppressive histone marks were significantly suppressed in the promotor of NDRG1 site (-0.4Kb (-3262)) in 5-Aza treated PCa cells, which promote the activation of chromatin, resulting in the increase of transcription of NDRG1 (**Fig. 4D and E**). The data suggest that reduction of suppressive histone marks on the NDRG1 promoter is important in the regulation of NDRG1 transcription in the induction of a NE phenotype.

REDUCTION OF HISTONE MARKS, H3K9ME3, H3K27ME3 ASSOCIATES WITH NDRG1 IN PCA TUMORS AND HUMAN PRIMARY PCA TUMORS

To further determine whether NE phenotypic changes by reduction of histone marks *in vitro* reflects the activity *in vivo*, tumors grown *s.c.* were stained for H3K9me3 or H3K27me3 with NDRG1. Tumors generated from PCa cells with pretreated vehicle showed high levels of H3K9me3 or H3K27me3 expression, while more NDRG1 expression was detected in the subset of H3K9me3 or H3K27me3 reduced cell populations in 5-Aza pretreated PCa tumors (**Fig. 5A and B**).

To examine whether our cell line data is indicative of what happens in human tumors, staining for H3K9me3 or H3K27me3 with NDRG1 was performed using tissue microarrays (TMA). TMAs from PCa patients, it was demonstrated that significant levels of H3K9me3 or H3K27me3 expression in high grade tumor cells compare to normal prostate epithelial tissues or low grade tumor cells. Critically, high levels of H3K9me3 or H3K27me3 expression are detected in advanced cancer cells (**Fig. 5C and D**). We further found that high levels of H3K9me3 or H3K27me3 expression with low NDRG1 expression in the luminal type of adenocarcinoma cells, while suppressed H3K9me3 or H3K27me3 expression with an enhanced NDRG1 expression in small cell carcinoma cells in advanced stage of human primary PCa tumors, which correlated with our findings in *s.c.* animal models in this study. The data suggest that reduction of histone marks is associated with more NDRG1 expression in the NE phenotypic cells, which plays a critical role in PCa tumor progression.

DISCUSSION

Neuroendocrine prostate cancer (NE PCa) is an aggressive malignancy, often observed in relapsing individuals particularly after second generation of androgen-deprivation therapy in advanced metastasis. Here, we show that epigenetic alteration by the epidrug 5-Aza may associate with the reprogramming of prostate adenocarcinoma cells into NE phenotypic PCa cells. Our studies demonstrate that induction of NE differentiation of PCa cells through reduction of histone marks leading to enhanced NDRG1 expression

suggests that epigenetic regulation of gene expression plays a critical role in the development of aggressive disease.

NE PCa is correlated with prostate tumor progression representing the poor prognosis with lethal events (Borges et al., 2016, Chan et al., 2010, Conteduca et al., 2014, Nelson et al., 2007, Parimi et al., 2014, Terry and Beltran, 2014, Wang et al., 2014). Although androgen deprivation therapy (ADT) provides most patients early clinical responses, this approach remains the limitation for patients with the advanced metastatic PCa, subsequently developing resistance to these treatments (Borges et al., 2016, Chan et al., 2010, Conteduca et al., 2014, Nelson et al., 2007, Parimi et al., 2014, Terry and Beltran, 2014, Wang et al., 2014).

There are at least two possible mechanisms underlying the development of NE PCa disease. One possibility is the emergence of metastatic tumor clones or cells with tumor initiating potential or CSCs that gave rise to at least three distinct components (luminal, basal, and NE), leading extraordinarily aggressive during tumor progression (Terry and Beltran, 2014). NE cells represent a less differentiated phenotype sharing several CSC characteristics, which are distinct from the highly differentiated luminal type of adenocarcinoma cells (Borges et al., 2016). For example, NE cells express high levels of the stem cell surface marker PROM1/CD133 (Borges et al., 2016). In fact, a recent study shows the association NE PCa cells with CSC and/or EMT cells that were confirmed by the injection of CSCs into nude mice, which generates highly vascularized tumors showing high density of NE PCa cells and expressing low levels of E-cadherin and β-catenin and high levels of vimentin (Palapattu et al., 2009). Moreover a further study demonstrates a direct link between EMT and NE whereby the transcription factor snail, which normally induces EMT, also may induce a NE phenotype (McKeithen et al., 2010).

A second pathway for the emergence of NE disease is through transdifferentiation of preexisting adenocarcinoma cells, which acquire several molecular alterations (Terry and Beltran, 2014). In these biologic processes, cell autonomous signaling pathways facilitate

acquisition of a NE state, which in conjunction with microenvironmental clues, promote tumor outgrowth, survival, and therapeutic-resistant properties. As examples, IL-6, epinephrine, and forskolin induce NE differentiation in PCa cells through activation of intracellular cAMP and protein kinase A (PKA) (Bang et al., 1994, Qiu et al., 1998, Zelivianski et al., 2001). Further, CXCL12 and its receptors (CXCR4, CXCR7) are actively expressed in NE cells and signals on mTOR (Circelli et al., 2016). Neuropeptides secreted by NE cells activate Src, ERK, and PI3K/Akt that can act in a paracrine manner to stimulate growth, survival, motility, and metastatic potential of androgen-independent epithelial PCa cells (Terry and Beltran, 2014). Together these studies suggest that multiple signaling pathways are involved in NE differentiation, ultimately contributing to PCa progression. It has also been considered that, the NE transdifferentiation process may be an adaptation mechanism, PCa cells allow to adapt a wide spectrum of therapeutic agents (Crea et al., 2016, Terry and Beltran, 2014). Likewise, accumulation of new genetic alterations such as loss of tumor suppressors (RB1, PTEN, and TP53), TMPRSS2-ERG rearrangements, amplifications of proto-oncogenes (Aurka and N-Myc) under evolutionary pressures or through the natural selection, may facilitate the development of the NE small cell carcinoma phenotype, which promotes extremely tumor outgrowth with rapid proliferative activity (Crea et al., 2016, Lee et al., 2016b, Terry and Beltran, 2014).

Growing evidence shows that deregulation of cellular signaling by genetic or epigenetic alterations are associated with neuroendocrine differentiation (Crea et al., 2016). Like what we observed in PCa cells, there are growing evidences that the demethylation agent, 5-Aza induces N-myc downstream-regulated gene 1 (NDRG1) expression in pancreatic cancer, breast cancers, and colon cancer, which associated in angiogenesis, metastases, and mechanisms leading to anti-cancer drug resistance (Wang et al., 2013, Angst et al., 2010, Bandyopadhyay et al., 2004, Guan et al., 2000, Kitowska and Pawelczyk, 2010). A recent report also shows that HIF-1α upregulates NDRG1expression through binding to NDRG1 promoter, leading to proliferation of lung cancer A549 cells (Wang et al., 2013) and NDRG1 expression is associated with metastasis and neuroendocrine differentiation in PCa cells through loss of RB in a hypoxia-dependent manner (Labrecque et al., 2016).

Together, these studies support our findings that reduction of histone marks on the NDRG1 promoter by 5-Aza regulates the NDRG1 transcription, which contributes to induction of a NE phenotype in PCa cells.

In summary, these studies evidence that reduction of histone marks by the epidrug 5-Aza regulates induction of NE phenotype, which facilitates the tumor progression in PCa cells (**Fig. 6**). Thus, these studies have important implications for pre-clinical studies of epigenetic targeted therapies. Clinically, 5-Aza-CdR continues to be an effective agent for growth inhibition and induction of apoptosis during the chemotherapy in hematologic malignancies and in several solid tumors including PCa tumors (Karahoca and Momparler, 2013, Thibault et al., 1998). In previous studies, we demonstrated that reduction of DNMT1 by 5-Aza is associated with the emergence of CSC and EMT phenotypes, suggesting that several potential side-effects are possible with the therapeutic use of 5-Aza in prostate cancer. Our data suggest that careful monitoring and evaluation of dose and treatment schedules are warranted for use of 5-Aza-CdR in anticancer therapies.

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AUTHOR CONTRIBUTIONS

E.L. and R.S.T. designed experiments. E.L., J.W, Y.J., and F.C.C. performed experiments and analyzed the data. Dr. Yan Li discussed the results and gave valuable critique on the paper. E.L. and R.S.T. wrote the manuscript.

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FIGURE LEGENDS

***Fig.* 1. Induction of a neuroendocrine-like phenotype by epidrug (5-Aza) in PCa cells *in vitro*.**

PC3 or DU145 cells (1 x 10^5) were treated for 10 days with vehicle or 5-Aza (5µM) and subsequently imaged by bright field microscopy. Bar=100µm.

Fig. **2. Increase of expression of neuroendocrine phenotypic marker by epidrug (5-Aza) in PCa cells.**

PCa cells (PC3 or DU145) (2 x 10^5) were seeded in 10cm culture dishes. The cells were treated by vehicle or 5-Aza (5µM) for 10 days.

(**A-C**) mRNA levels of N-Myc downstream regulated 1 (NDRG1), Enolase-2 (ENO2), and Synaptophysin expression in vehicle or 5-Aza (5µM) treated PCa cells (PC3 or DU145) as quantified by real-time PCR. The data in Fig. 2A-C are representative of mean ± s.d (n=6). *P* values were calculated by Student's *t*-test. (**D**) Protein levels of NDRG1, ENO2, and Synaptophysin following vehicle or 5-Aza (5µM) treatments in PCa cells (PC3 or DU145) as quantified by Western blots.

Fig. 3. Induction of neuroendocrine phenotypic cells by epidrug (5-Aza) in PCa tumors *in vivo.*

Examination of NE markers in tumors grown in SCID mice implanted *s.c.* with PCa cells pretreated with vehicle or 5-Aza *in vitro* (Lee et al., 2016a). High density of NE PCa cells in (**A**) PC3 tumors and (**B**) DU145 tumors were shown by H&E staining (NE PCa: red) with an indication of black arrow. Bar=200μm. Synaptophysin or Pan-Cytokeratin expressing NE PCa cells as detected by IHC staining. Bar=50μm.

Fig. 4. Reduction of histone marks on the NDRG1 promoter by epidrug (5-Aza) in PCa cells.

(**A**) Examination of global changes of histone silent marks, H3K9me3 and H3K27me3 in PCa cells (PC3, DU145) following in vehicle or 5-Aza treated PCa cells by Western blots. (**B**) Experimental design of CHIP qPCR assays in vehicle or 5-Aza treated PCa cells. (**C**) A scheme shows the primer site at the promoter region from transcription start site of the human NDRG1 genomic locus. (**D**) % respective input of H3K9me3 and H3K27me3 levels at the NDRG1 genomic locus as determined by CHIP qPCR assays in vehicle or 5-Aza treated PCa cells (PC3, DU145). The data in Fig. 4D are representative of mean ± s.d. (n=3). *P* values are calculated by Student's *t*-test. (**E**) A summary diagram shows the activation of chromatin by histone demethylation of H3K9me3 and H3K27me3 following 5-Aza treatment, resulting in the increase of NDRG1 transcription.

Fig. **5. Association of H3K9me3 and H3K27me3 reduction with NDRG1 in *s.c.* PCa tumors and human primary PCa tumors.**

(**A**) NDRG1 (green) expression (white arrow) in H3K9me3 (red) or H3K27me3 (red) reduced cells in vehicle or 5-Aza pretreated *s.c.* PCa tumors as detected by immunofluorescence co-staining. Blue, DAPI nuclear stain. Bar=20μm. (**B**) Quantification of Fig. 5A. Data represent as mean ± s.d. (n=8/group) (Student's *t*-test). (**C**) NDRG1 (green) expression (white arrow) in H3K9me3 (red) or H3K27me3 (red) expressing cells in human primary PCa tumors from the tissue microarray samples (TMA) from PCa patients as detected by immunofluorescence co-staining. Blue, DAPI

nuclear stain. Bar=20µm. TMAs are normal prostate tissue (n=8), Gleason 6 prostate cancer tissue (n=9), and Gleason 9 prostate cancer tissue (n=5). (**D**) Quantification of Fig. 5C. Data represent as mean ± s.d. (Student's *t*-test).

Fig. 6. Experimental Model.

Reduction of histone marks by the epidrug 5-Aza regulates induction of NE phenotype, which facilitates the tumor progression in PCa cells.

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Figure 1

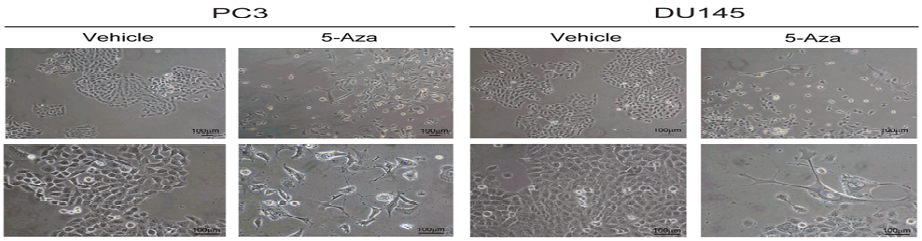

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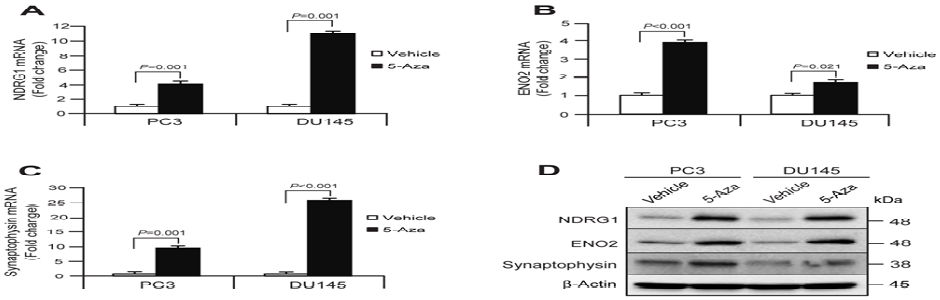
Figure 2



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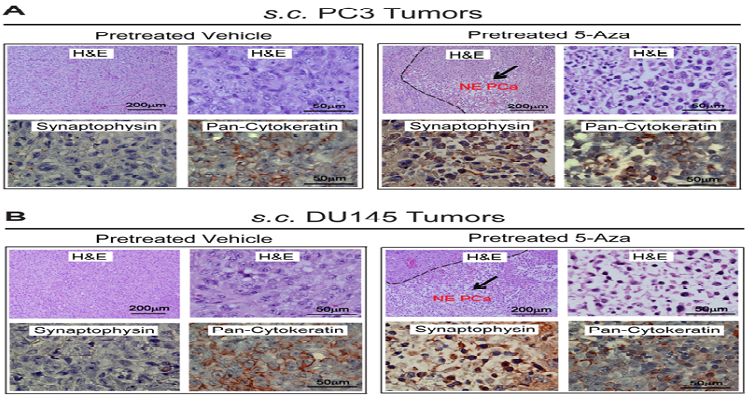

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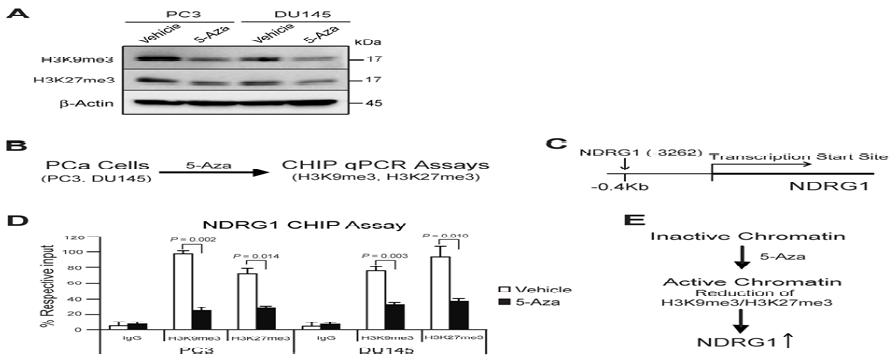

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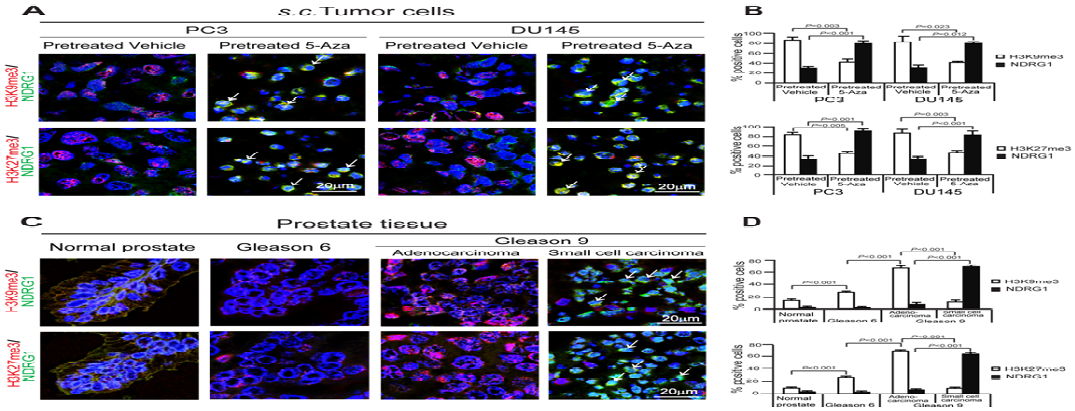

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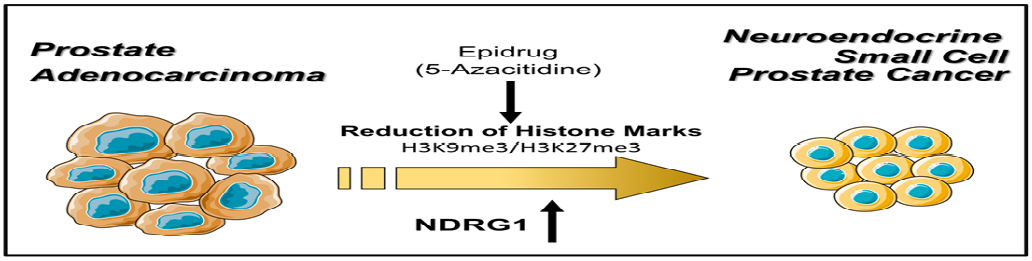
Figure 6



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