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Developmental and Androgenic Regulation of Chromatin Regulators EZH2 and ANCCA/ATAD2 in the Prostate Via MLL Histone Methylase Complex

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BACKGROUND. Chromatin regulators ANCCA and EZH2 are overexpressed in prostate cancer and play crucial roles in androgen-stimulated and castration-refractory prostate tumor growth and survival. However, how their expression is regulated in the tumors and whether they play a role in prostate development remains unclear.

METHODS. Prostate tissue from different developmental stages of mouse and human were examined by IHC, qRT-PCR and Western for expression of ANCCA, EZH2, and Ki-67. Animals were castrated and T-implanted for the expression response in normal prostate and tumors. siRNA knockdown and ChIP were performed for the mechanism of ANCCA regulation of EZH2.

RESULTS. In contrast to their very low level expression in adult prostate, ANCCA and EZH2 are strongly expressed in the epithelium and mesenchyme of mouse and human UGS. Their expression becomes more restricted to epithelial cells during later development and displays a second peak during puberty, which correlates with the proliferative status of the epithelium. Importantly, their expression in normal prostate and tumors is strongly suppressed by castration and markedly induced by testosterone replacement. While androgen suppresses EZH2 in CRPC cells, in LNCaP cells, physiological concentrations of androgen stimulate expression of PRC2 genes (EZH2, SUZ12, and EED), which is mediated by androgen-induced ANCCA and involves E2F and histone H3K4me3 methylase MLL1 complex.

CONCLUSION. EZH2 and ANCCA are androgen regulated and strongly expressed in early prostate morphogenesis and during puberty, suggesting their important role in prostate development. Regulation of EZH2 by ANCCA emphasizes bromodomain protein ANCCA as a potential therapeutic target against prostate cancer. *Prostate 73: 455–466, 2013.*

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INTRODUCTION

Androgen plays a pivotal role in normal prostate development and homeostasis as well as in prostate cancer development and progression [1,2]. Acting through androgen receptor (AR), androgen regulates epithelial–mesenchymal interactions in the developing prostate to stimulate prostate growth and morphogenesis, which also involves other transcription factors and signaling events. These include members of the homeobox transcription factors such as the Hox13 genes and Nkx3.1, the fork-head box factors such as FoxA1 and FoxA2, and the Wnt, hedgehog, FGF, and TGFβ signaling pathways. Many of them display temporally and spatially characteristic expression patterns that are often concomitant with prostate growth, morphogenesis and epithelial differentiation. For instance, while FoxA2 expression is restricted primarily to the urogenital sinus (UGS) epithelium at embryonic day (E)18 in the mouse and to the growing prostatic epithelial buds at later embryonic and neonatal days, FoxA1 expression is observed in the prostatic epithelial cells throughout the different stages of prostate development and in adult prostate [3]. Although the exact role of FoxA2 in prostate development is unclear, FoxA1 gene deletion demonstrates a critical function in prostate ductal morphogenesis as FoxA1 deficiency resulted in immature, basal-like epithelium and expanded stroma [4]. Wnt5a is highly expressed at the periductal mesenchymal cells during early prostate morphogensis where it plays an important role in bud outgrowth, ductal elongation and lumenization [5].

AR regulates gene transcription through its interaction with multiple co-regulators such as the p160/SRC family members [6,7]. In mouse prostate, SRC-3 (also known as ACTR/AIB-1/NCoA3) is expressed in the basal and stromal cells and its deficiency delayed the normal prostate growth but did not affect castration-induced regression or androgen replacement-dependent regrowth [8]. We recently identified ANCCA (also known as ATAD2) as a novel AR coactivator that is strongly induced in human prostate cancer cells [9,10]. Importantly, ANCCA is overexpressed in a subset of advanced prostate cancer tumors while little can be detected in normal adult human prostate [9]. ANCCA possesses an AAA-type ATPase domain and a bromodomain that recognize specifically acetylated histones such as H3K14ac. Both domains are critical for ANCCA to mediate gene expression [10–12]. Interestingly, in addition to AR, ANCCA associates with ERalpha, E2Fs and c-Myc and controls a specific subset of androgen induced genes for proliferation and survival [9–11,13].

EZH2 is the enzymatic component of PRC2 complex that contains SUZ12, EED and RbAp46 for H3K27 methylation [14]. EZH2 is overexpressed in many types of cancers including prostate cancers and its increased levels are strongly associated with poor clinical outcomes [15,16]. EZH2 up-regulation was detected not only in metastatic prostate cancer tumors, it was also found in a subset of high grade PIN tissue and primary tumors [15]. High levels of EZH2 promote cancer cell proliferation, invasion and tumor metastasis through suppression of CDKN1C/p57, the CDKN2A gene locus, E-cadherin, RUNX3, and antagonists of signaling pathways [17–22]. EZH2 is required for early stages of mammalian development. In ES cells, EZH2 or components of PcG2 are responsible for control of the expression of developmental regulators including members of Hox, Pax, GATA, Wnt, and Fgf gene family [23,24]. EZH2 plays important functions in maintenance of stem cell pluripotency and differentiation. Although gene silencing is likely a major mechanism of EZH2 function [25], EZH2 can also act as a coactivator for specific transcription factors such as β-catenin [26,27].

Several mechanisms have been described to account for de-regulated EZH2 expression in prostate cancer cells, which include the activation by ERG and c-Myc and the loss of micro-RNA-mediated silencing [25,28–31]. However, how EZH2 expression is regulated in normal prostate and whether EZH2 overexpression in cancer involves other important regulators is unclear. By examining EZH2 and the novel chromatin regulator ANCCA expression in different developmental stages and hormonal milieus, we found that their expression is highly induced at early development of prostate and is strongly regulated by androgen. Interestingly, in androgen-sensitive prostate cancer cells, ANCCA mediates androgen induction of EZH2 expression. Together, these results suggest the existence of an ANCCA-EZH2 chromatin regulator network in development of normal prostate and prostate cancer.

MATERIALS AND METHODS

Animals and Tissue Collection

All the mice except those younger than 4 weeks were purchased from the Jackson Laboratory. C57B6/J breeder pairs were used to generate mouse embryos at day 18 or 20 of gestation or mice at ages below

3 weeks (n = 6 for each time point). The first day of gestation is considered to be the day when a vaginal plug is confirmed. Mice aged at 18 days or 20 days of gestation and postnatal days 1, 5, 10, 14, 21, 28, 42, 56, 90, 180, 270, and 360 were sacrificed to harvest urogenital sinuses or prostates (n = 5 for each time point). Tissues were fixed with buffered formalin (10% formaldehyde) for 6 hr prior to processing or frozen fresh and then grinded quickly in liquid nitrogen for protein and RNA preparations. C57B6/J mice from the Jackson Laboratory were castrated at 6 weeks old. On day14 after castration, some mice were sacrificed for collection of prostate while others were supplemented with testosterone pellets (Innovative Research, 12.5 mg per pellet per mouse), implanted subcutaneously at the nape of mouse neck. On day1, 3, 5, 7, and14 days after the T implant, prostates were collected and processed for RNA and protein analysis (n = 5 for each group). All the mice were housed in a pathogen-free facility and all animal experiments were approved by IACUC of UC Davis.

LTL311 and LTL313H tumor xenografts of human prostate cancer was developed by grafting tumor biopsy specimen under the renal capsules of SCID mice as described previously [32] (http://www.livingtumorlab.com). The tumor line development protocol and the human specimen use was approved by the Clinical Research Ethics Board of the University of British Columbia, in accordance with the Laboratory Animal Guidelines of the Institute of Experimental Animal Sciences. To collect prostate cancer tissue after castration, fresh LTL-313h tumor tissues from the 5th generation of grafting were cut into 3 mm × 3 mm × 1 mm pieces and re-grafted into the subrenal capsules of male NOD–SCID mice. The animals were maintained for tumor formation for 2 months before castration to remove androgens. Three weeks after castration, when the tumor volume was significantly reduced, the remaining tumor tissues were harvested and prepared as FFPE tissue blocks as reported earlier [33]. Xenograft tissues used in this study were derived from nine tumors from castrated mice and five tumors from five intact mouse hosts supplemented with testosterone that had undergone sham operation. Collection of human prostate rudiments from human fetuses 18 weeks of gestational age was described previously [34].

Immunohistochemistry

Tissue sections from formalin-fixed, paraffin embedded tissue blocks were dewaxed, rehydrated, and blocked for endogenous peroxidase activity. Antigen retrieving was performed in sodium citrate buffer (0.01 mol/L, pH 6.0) in a microwave oven at 1,000 W for 3 min and then at 100 W for 20 min. Nonspecific antibody binding was blocked by incubating with 10% fetal bovine serum in PBS for 30 min at room temperature. Slides were then incubated with anti-ANCCA antibody [35] (at 1:200), anti–Ki-67 (at 1:500; NeoMarker), and anti-EZH2 (at 1:200; AC22, Cell Signaling) at room temperature for 30 min. Slides were then washed and incubated with biotin-conjugated secondary antibodies for 30 min, followed by incubation with avidin DH-biotinylated horseradish peroxidase complex for 30 min (Vectastain ABC Elite Kit, Vector Laboratories). The sections were developed with the diaminobenzidine substrate kit (Vector Laboratories) and counterstained with hematoxylin. Nuclear staining of prostate epithelial cells was scored and the percentage of positive staining was calculated. Images were taken with an Olympus BX51 microscope equipped with DP72 camera.

Cell Culture and Treatments

LNCaP, C4-2B, and PC-3 cells were cultured in RPMI 1640 (Invitrogen) with 10% (for LNCaP and PC-3) or 5% (for C4-2B) fetal bovine serum (FBS; Omega) and maintained at 37°C in a humidified atmosphere of 5% CO_2. For androgen treatment, cells were first plated and maintained in hormone depleted medium (RPMI with 10% or 5% cds–FBS, Hyclone) for 48 hr and then the medium was changed to fresh RPMI with cds–FBS. Twenty-four hours later, the cells were treated with DHT or R1881 and then harvested for protein and RNA analysis at indicated time points. For siRNA knockdown of ANCCA, LNCaP cells were seeded at a density of 2–3 × 10^5 cells per well of six-well plates in hormone depleted medium for 24 hr and then infected with lentiviruses expressing shRNAs targeting different sites of ANCCA mRNA or control TRC2 lentivirus (Sigma) in the presence of 2 μg/ml polybrene for 6 hr. At 24 hr after infection, cells were treated with DHT for 48 hr before harvested for protein analysis. Cells were also infected with shRNA-lentiviruses in regular growth medium and harvested for RNA analysis 48 hr later. For knockdown of EZH2, LNCaP cells were transfected with control siRNA or two different siRNAs targeting EZH2. For treatment with bicalutamide, LNCaP cells maintained in hormone depleted medium for 48 hr were treated with bicalutamide (Casodex, at 10 μM) or DMSO, and 30 min later, treated with 1 nM DHT. Cells were harvested 24 and 48 hr later for protein analysis. For reporter gene assay, PC-3 cells were plated in 24-well plates and cotransfected next day using Fugene-HD (Roche) with 200 ng of pGL3-EZH2-1.9 promoter-luc [36], 50 ng of pCMX-beta-galactosidase, 50 ng of pSh-HCMV-ANCCA [10], and 10 ng of

empty pCMV vector or pCMV-E2F1. Luciferase activity was measured 48 hr later using the luciferase reporter assay system (Promega) and normalized using beta-galactosidase assay.

Western Blotting and Real-Time PCR

Protein samples were prepared by lysing cells in modified RIPA buffer. Lysates (50–100 μg) were separated on a 10% SDS–PAGE and transferred to a PVDF membrane. The membrane was probed with antibodies for ANCCA, EZH2 (AC22) and GAPDH (14C10), both from Cell Signaling, E2F1 (KH95), SUZ12 (P-15), EED (H-300), AR (N-20), or beta-actin (C4), from Santa Cruz, followed by incubation with HRP-conjugated secondary antibody. Total RNAs were extracted using Trizol (Invitrogen). Five micrograms of total RNA was used as template for RT. Quantitative real-time PCR was done with cDNA samples using the iQ-SYBR Green Supermix and Bio-Rad platform according to the manufacturer's protocol. The PCR primer sequences are provided in Supplementary Table.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed essentially as described previously [9] with the following modifications. LNCaP cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen) and then maintained in hormone depleted medium for 60 hr before treated with 1 nM DHT for 12 hr and harvested for ChIP. The crude chromatin solutions were first cleared with Protein A beads (Invitrogen) that were pre-coated with pre-immune serum for 2 hr at 4°C. The pre-cleared supernatants were then incubated with pre-immune serum, or antibodies against ANCCA, E2F1 (C-20, Santa Cruz), MLL1 (05–765, Millipore), H3K4me3 (ab8580, Abcam), H3ac (06–599, Millipore) or Pol-II (N-20, Santa Cruz) at 4°C overnight prior to precipitation with Protein A beads that have been pre-blocked with BSA and sonicated salmon sperm DNA. ChIP DNA was analyzed by real-time PCR with SYBER Green on an iCycler. Enrichment of genomic DNA was presented as percentage of recovery relative to the input. The primers are listed in Supplementary Table.

Statistics Analysis

Results were reported as means ± standard deviations. Two sample Student *t*-test was applied to comparisons between groups and a P value <0.05 was considered statistically significant.

RESULTS

EZH2 and ANCCA Are Strongly Expressed During Early Prostate Development

Prostate develops from the outgrowths of urogenital sinus epithelium, which occur around E17 and E18 days of gestation in mice [37]. To explore the potential role of EZH2 and ANCCA in prostate development, we first examined their expression by IHC with mouse prostate tissue collected from E18 day embryos to 12-month-old mice. As shown in Figure 1A and Supplementary Figure 1, at E18 and E20, the majority of urogenital sinus epithelial cells and some of the mesenchymal cells displayed a moderate to strong

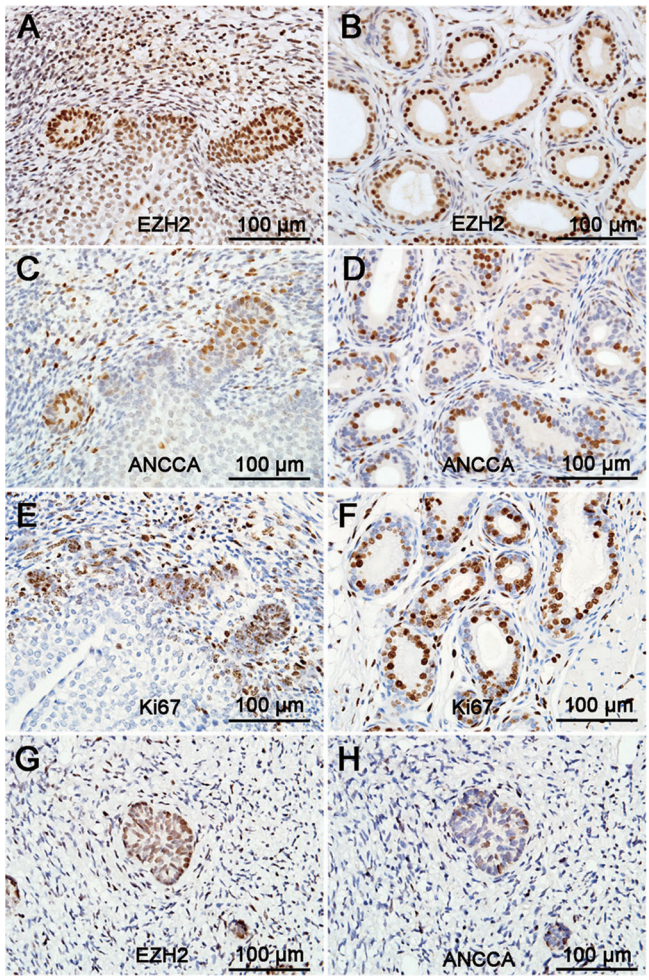

Fig. 1. IHC analysis of EZH2 and ANCCA expression in mouse and human developing prostate. Sections of urogenital sinus from day 20 mouse embryo (**A**,**C**,**E**) and sections of 4-week-old mouse ventral prostate (**B**,**D**,**F**) were subject to IHC using antibodies for EZH2 (A,B), ANCCA (C,D), and Ki67 (E,F). A strong nuclear staining was observed in prostatic bud cells or ventral prostate epithelial cells. Sections of human prostatic buds from EI8 week embryo were analyzed by IHC for EZH2 (**G**) and ANCCA (**H**) expression.

EZH2 immunoreactivity in the nuclei, which persisted until postnatal day 5 (Supplementary Fig. 1). Interestingly, the highest EZH2 immuno-staining was localized in epithelial cells at the epithelial–mesenchymal interface in embryonic urogenital sinus or the developing prostate epithelial buds of neonatal mice (Fig. 1A). Like that of EZH2, ANCCA immuno-stained epithelial cells can be readily detected in the urogenital sinus epithelium and also appear to exist at the epithelial–mesenchymal junction (Fig. 1). Comparing to EZH2, the expression of ANCCA is restricted to a fraction of epithelial and mesenchymal cells in the urogenital sinus. Consistent with previous studies [38], cells of the prostate buds and their adjacent mesenchyme are highly proliferative as indicated by their strong Ki-67 staining (Fig. 1E,F). Interestingly, Ki-67 positive cells are generally in the same areas where EZH2 and ANCCA display strong expression.

Human prostate develops primarily during the second and third trimester [2]. At E16–E20 weeks, human prostate epithelial buds undergo rapid growth and branching morphogenesis. Immuno-staining of E18-week prostatic tissue revealed that EZH2 is strongly expressed in the majority epithelial cells of solid (non-canalized) prostatic ducts and that ANCCA is also expressed in some of the epithelial cells (Fig. 1G,H). Similar to that observed in mouse, the most prominent immuno-staining for EZH2 and ANCCA tends to localize to epithelial cells adjacent to the mesenchyme. Relatively fewer mesenchymal cells were stained positive for either EZH2 or ANCCA.

EZH2 and ANCCA Expression Increases Significantly During Puberty and Sharply Decreases in Mature Prostate

Prostate ductal growth and branching morphogenesis in mouse occur postnatally and continue until sexual maturity around 6 weeks of age. During this period, the epithelial buds elongate to form ductal cords which then branch and canalize into a complex ductal structure with lobe-specific patterns. During the process, both mesenchymal and epithelial cells proliferate and differentiate into the different cell types found in mature prostate. In contrast to its strong expression in both epithelial and mesenchymal cells during early prostate development, EZH2 expression is restricted to most of the luminal epithelial cells by postnatal day 10. Only a few stromal or basal cells were stained positive for EZH2 (Supplementary Fig. 1). By 3 weeks, about 30% of luminal epithelial cells express EZH2 (Fig. 2A and Supplementary Fig. 1). Interestingly, at puberty (4 weeks old), close to 80% of luminal epithelial cells in the different lobes were stained positive with some of them intensively

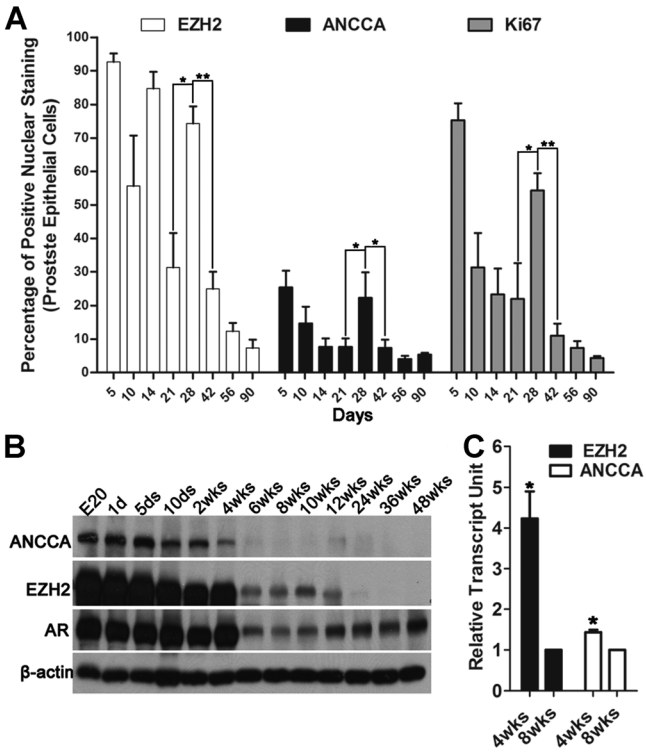

Fig. 2. EZH2, ANCCA, and Ki-67 highly express in developing prostate and their expression decreases sharply in mature prostate. Prostate tissues from indicated ages of mouse were processed for IHC (**A**) or Western blotting (**B**) analysis using specific antibodies for indicated proteins, or for real-time RT-PCR of EZH2 and ANCCA (**C**). At least 500 prostatic epithelial cells from each mouse were scored under the microscope for nuclear positive staining using EZH2, ANCCA or Ki-67 antibody. Values were presented with standard deviation from three mice or more. Western blotting was carried out using protein lysates from mouse UGS or whole prostate tissues at different ages. Quantitative real-time RT-PCR analysis of EZH2 and ANCCA transcript levels was performed using mouse whole prostate at 4 and 8 weeks. RPLI9 was used as the internal control. Data were analyzed with student *t*-test and *P* values are indicated by presence of asterisk: $^{*}P < 0.05$; $^{**}P < 0.001$.

stained (Figs. 1B and 2A). In addition, compared to 3 weeks, more cells in the stroma of 4-week-old prostate appear to express EZH2 (Fig. 1B). Interestingly, ANCCA expression appears to have a pattern of change similar to that of EZH2. Thus, the number of ANCCA positive epithelial cells was reduced from about 25% at neonatal day 5 to <10% by 3 weeks old and increased significantly to over 20% at puberty (Figs. 1D and 2A). Like EZH2, ANCCA also displayed an increase in number of positively stained stromal cells. However, at 6 weeks and later adulthood, very few epithelial cells were stained positive for EZH2 and ANCCA proteins in the prostate lobes examined (Fig. 2A; Supplementary Fig. 2). Western blotting analysis of tissue lysates of UGS and prostate tissue

dissected from different days of mouse demonstrated that EZH2 and ANCCA protein expression were relatively abundant during early and later development until puberty and that their expression sharply decreased by 6-week-old age (Fig. 2B), therefore consistent with the overall pattern revealed by IHC. Furthermore, RNA analysis indicated that EZH2 mRNA level in mature adult mouse prostate (8 weeks old) was decreased at least fourfold when compared to that of prostate at puberty. ANCCA mRNA level in the prostate was also decreased, although less significant (Fig. 2C).

Expression of ANCCA and EZH2 Is Strongly Inhibited by Castration and Robustly Induced by Androgen Replacement

Male puberty onset is associated with a testosterone surge. To address whether the marked increase of ANCCA and EZH2 prostate expression at puberty is due to an induction by androgen, we analyzed their expression during prostate involution and re-growth triggered by castration and subsequent testosterone (T) replacement. While prostate from intact adult animals had a low number (3–5%) of luminal epithelial cells that express the two proteins as described above, prostate from castrated adult mice displayed essentially no positively stained luminal epithelial cells for either ANCCA or EZH2 (Fig. 3A,C and Supplementary Fig. 3). Strikingly, 24 hr after T implant in the castrated animals, approximately 30% and 50% of prostate epithelial cells that are proximal to urethra were immuno-stained positively for ANCCA and EZH2 respectively while prostate cells more distal to urethra showed less significant induction of the two proteins (Supplementary Fig. 4). By 3 days after T implant, strong expression of ANCCA and EZH2 were observed in the majority of prostate epithelial cells in all the lobes (Fig. 3B,D and data not shown). The high levels of ANCCA and EZH2 expression persisted for 5 days and then decreased rapidly. By day 14 after T implant, their expression was limited to a few epithelial cells, similar to that observed in the mature adult prostate of intact animals (Supplementary Fig. 3). Immunostaining of Ki-67 revealed that by day 3 after T implant, prostate cells from castrated mice became highly proliferative. However, by day 14, only a small fraction of the prostate cells were still proliferative (Fig. 3E and 3F and Supplementary Fig. 3). Together, these results suggest that expression of EZH2 and ANCCA is strongly induced by testosterone in the normal prostate.

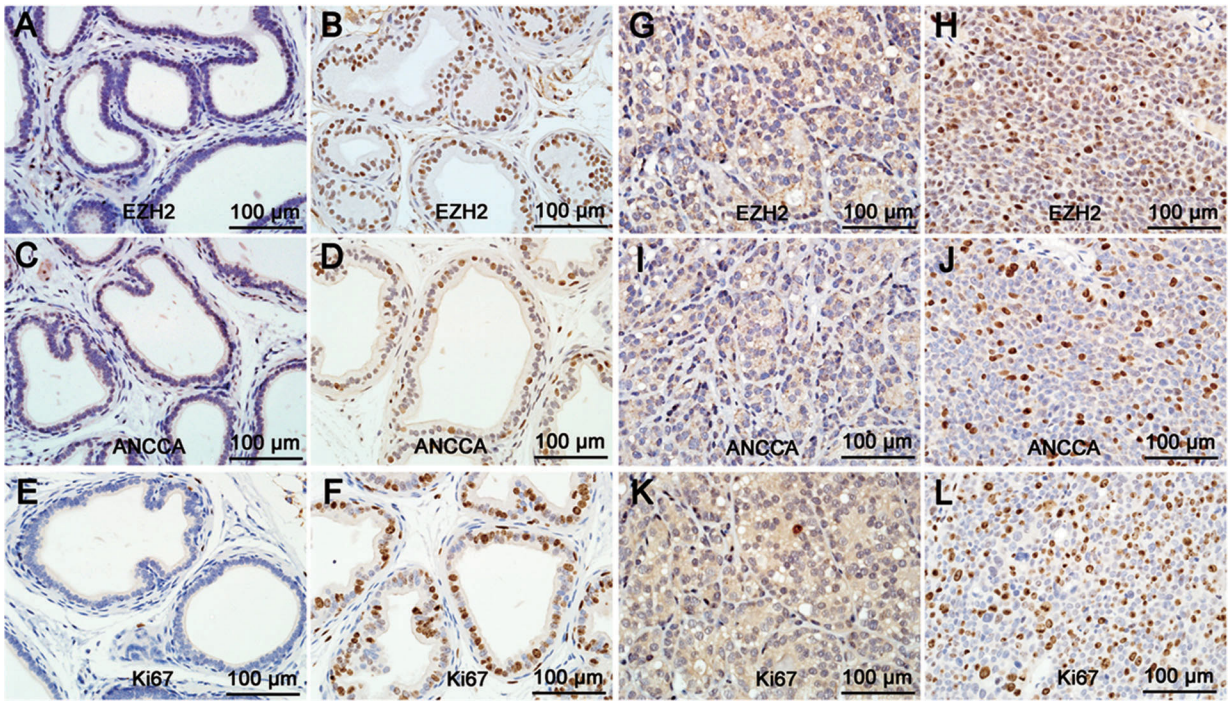

Fig. 3. Androgen regulation of EZH2 and ANCCA expression in mouse prostate and in human prostate cancer xenograft tumors. IHC analysis of EZH2, ANCCA, and Ki-67 expression was performed with ventral prostate tissue from mice castrated for 2 weeks (**A**,**C**,**E**) or from mice that were implanted with testosterone pellet for 3 days after the 2 weeks castration (**B**,**D**,**F**). IHC analysis of EZH2, ANCCA, and Ki67 expression was performed with human prostate cancer xenograft tumor line LTL311 from tumor bearing mice castrated for 7 days (**G**,**I**,**K**) or from intact mice with testosterone pellet implantation (**H**,**J**,**L**).

Androgen Induces EZH2 and ANCCA in Prostate Cancer Cells and Tumors But Suppresses EZH2 in CRPC Cells

We found previously that ANCCA is highly induced by androgen in prostate cancer cells and in xenograft tumors [9]. As described above, ANCCA and EZH2 are stimulated by androgen during normal prostate development. Together these observations suggest that prostate cancer development may involve co-option of developmental mechanisms controlled by the chromatin regulators. To examine whether EZH2 is also stimulated by androgen in prostate cancer, we analyzed EZH2 and ANCCA expression in patient-derived prostate cancer xenograft models. LTL311 and LTL313H xenograft tumors were developed from biopsies of high grade prostate adenocarcinoma when grafted under the renal capsules of SCID mice and display androgen-dependent growth [32] (http://www.livingtumorlab.com). As shown in Figure 3G–J and Supplementary Figure 5, EZH2 and ANCCA are strongly expressed in the LTL311 and LTL313H tumors from T implanted mice. However, the expression of EZH2 and ANCCA was significantly reduced in the two xenograft tumors from castrated mice. Ki67 immunostaining indicated that cells in tumors from castrated mice are largely non-proliferative (Fig. 3K). Similar results were obtained from CWR22 tumor xenogafts (data not shown). These results prompted us to examine whether EZH2 is also induced in other prostate cancer models such as LNCaP and its derivative C4-2B, a CRPC cell line. Treating LNCaP cells with increasing concentrations of androgen revealed that EZH2 protein was significantly induced by 1 or 10 nM DHT treatment (Fig. 4A, 2.4- and 4-fold at 48 hr). Similar induction was seen with LNCaP cells that were cultured for more than 40 passages, more interestingly, with the other components of the PRC2 complex, including SUZ12 and EED, and with synthetic androgen R1881 (Fig. 4A and Supplementary Fig. 6). RNA analysis indicated that EZH2 mRNA was significantly induced after 6 hr DHT treatment. Further increase was observed at 24 hr (Fig. 4B). Interestingly, supraphysiological concentrations of DHT (1,000 and 10,000 nM) did not result in any significant increase. Consistent with previous reports [39], androgen regulation of E2F1 was also concentration-dependent, while PSA was induced by all concentrations of DHT tested. Intriguingly, treatment of CRPC cell C4-2B with 0.1 nM or lower concentrations of DNH showed no significant induction of EZH2, SUZ12, and EED, while higher concentrations of DHT (1–1,000 nM) significantly suppressed the expression of EZH2 and SUZ12 (Supplementary Fig. 7). To further characterize

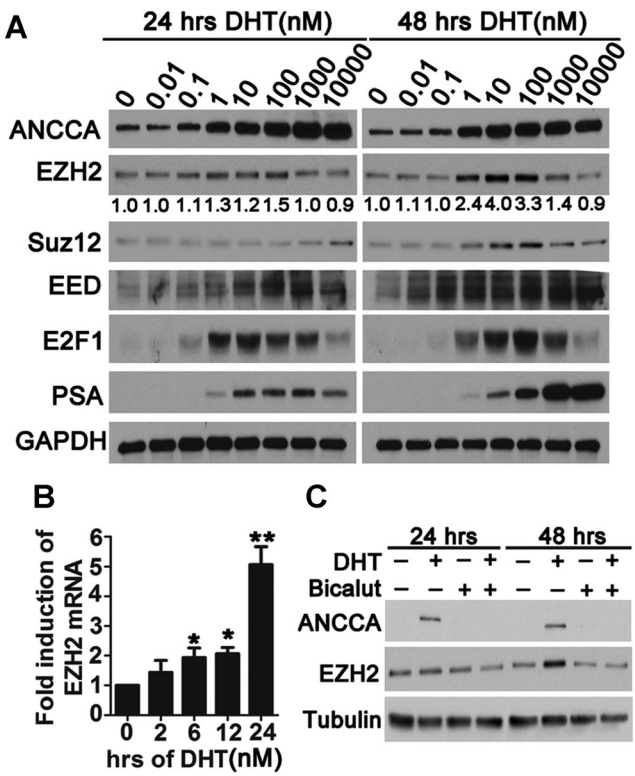

Fig. 4. Androgen induces expression of EZH2 and other components of PRC2 complex in LNCaP cells. Western blotting (**A**,**C**) or real-time RT-PCR analysis (**B**) were performed with LNCaP cells treated with different concentrations of DHT as indicated and harvested at the indicated hours. For androgen treatment, LNCaP cells were first plated and maintained in hormone depleted medium for 48 hr and then the medium was changed to fresh hormone depleted medium. Twenty-four hours later, the cells were treated with DHT and then harvested at indicated time points. For EZH2 transcript analysis in (B), human GAPDH was used for normalization. The relative EZH2 transcript unit from cells without DHT treatment was set as 1. For bicalutamide treatment, LNCaP cells maintained in hormone depleted medium for 48 hr were treated with bicalutamide at 10 μM, and half an hour later, they were treated with 1 nM DHT as indicated. Data were analyzed with student *t*-test and *P* values are indicated by presence of asterisk: $^{*}P < 0.05$; $^{**}P < 0.001$.

androgen induction of EZH2, cells were treated with anti-androgen bicalutamide. Bicalutamide alone did not affect androgen-independent expression of EZH2 or ANCCA. Simultaneous treatment with bicalutamide and DHT effectively abrogated DHT induction of EZH2 or ANCCA (Fig. 4C). To examine whether androgen receptor plays a role in mediating EZH2 expression, we knocked down AR in LNCaP cells as previously [9] and found that AR inhibition strongly suppressed EZH2 expression (Fig. 4C and supplementary Fig. 6E). Together, these data suggest that androgen induction of EZH2 is mediated by AR.

ANCCA Mediates Androgen Induction of EZH2 Expression in Prostate Cancer Cells Through Promoting Recruitment of MLLI and Histone Modifications

ANCCA plays an important role in androgen induction of genes involved in cell proliferation and survival including IGF1R, SGK1, and survivin/BIRC5 [9]. Remarkably, ANCCA depletion by either of the two shRNAs completely blocked DHT induction of EZH2 protein and mRNA (Fig. 5A,B), therefore suggesting that ANCCA mediates androgen stimulation of EZH2. Consistent with previous studies [15,17,40], EZH2 depletion strongly inhibited the proliferation of LNCaP cells (Supplementary Fig. 8). To further investigate the function of ANCCA in control of EZH2, ChIP assay was performed. Consistent with the notion that ANCCA directly regulates EZH2, an increased amount of ANCCA occupancy at EZH2 proximal promoter region was observed after 4 hr of DHT treatment and the androgen induced ANCCA recruitment persisted for at least 24 hr (Fig. 6A). EZH2 was shown to be an E2F target gene in hormone nonresponsive fibroblasts [41]. Interestingly, androgen stimulation also resulted in increased recruitment of E2F1 and E2F coactivator MLL1 which can tri-methylate histone H3 at lysine 4 [42,43]. Consistent with androgen induction of EZH2 transcription, DHT treatment also led to a marked increase in levels of histone modifications that associate with active gene transcription such as H3K4me3 and H3 acetylation and an increased recruitment of RNA polymerase II at EZH2 promoter (Fig. 6A). More importantly, knockdown of ANCCA not only diminished its own occupancy at EZH2 promoter, it also strongly inhibited histone methylase MLL1 and RNA polymerase II recruitment and histone acetylation and methylation stimulated by androgen (Fig. 6B). In a reporter gene assay, ectopic expression of ANCCA in conjunction with E2F1 strongly stimulated the activity of transfected EZH2 promoter reporter gene (Fig. 6C). Together, these data suggest that ANCCA mediates androgen induction of EZH2 through its function as an E2F coactivator and through promoting recruitment of histone modifying complexes such as MLL1 and assembly of polymerase II at EZH2 promoter.

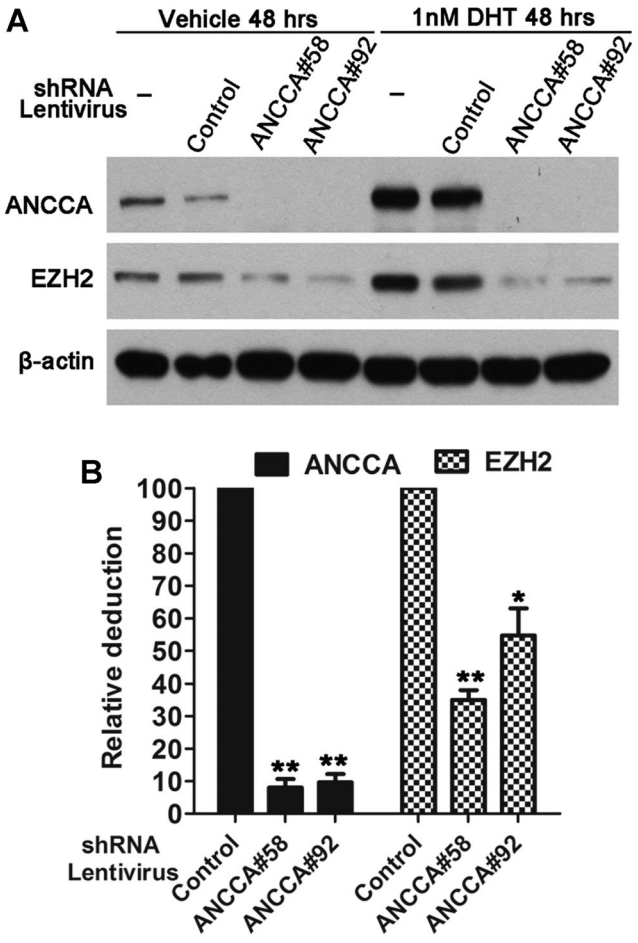

Fig. 5. ANCCA mediates androgen induction of EZH2. LNCaP cells in hormone depleted medium were infected with shRNA expressing lentivirus and then treated with 1 nM DHT for 48 hr before harvested for Western blotting (**A**) or RNA analysis (**B**) as described in Figure 4B with human GAPDH used as the internal control. Data were analyzed with student *t*-test and *P* values are indicated by presence of asterisk: $^{*}P < 0.05$; $^{**}P < 0.001$.

DISCUSSION

Developmental regulatory mechanisms are likely co-opted in different cancers including prostate cancer [44]. AR-mediated androgen signaling plays a dominant role in prostate development by stimulating mesenchymal-to-epithelial paracrine signaling [45–47]. It is also the most important pathway for development and progression of prostate cancer where AR exerts its direct action in the cancer epithelial cells to stimulate their proliferation, instead of a pro-differentiation function in normal prostate [48–50]. The function of histone methylase EZH2 in prostate cancer is well documented. ANCCA, a unique AR coactivator, is also likely to be an important mediator of AR function in prostate cancer. We demonstrate here that ANCCA and EZH2 are highly expressed during the critical stages of prostate development with expression patterns that strongly suggest important functions in prostate development.

We found that ANCCA and EZH2 are prominently expressed in the prostate buds epithelium in both human and mouse embryonic prostatic rudiment. This finding makes the two proteins the earliest

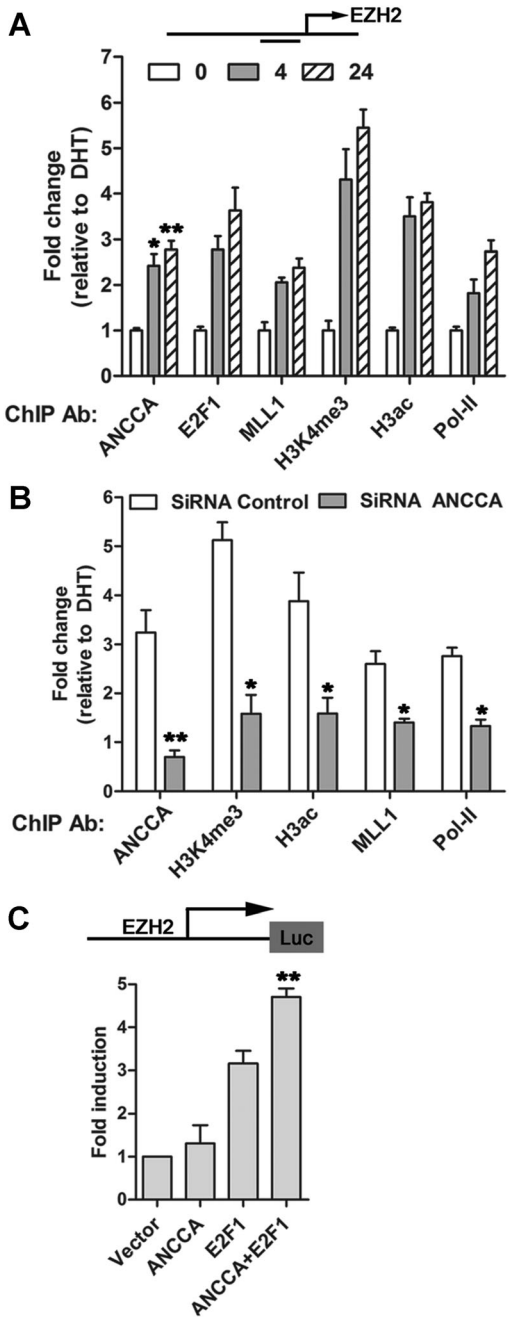

Fig. 6. Androgen induction of EZH2 involves ANCCA-mediated assembly of MLLI histone methylase complex and recruitment of RNA polymerase II. LNCaP cells were treated with I nM DHT as in Figure 4A for indicated hours (**A**), or transfected with siRNA and then treated with DHT (**B**) and harvested for ChIP with indicated antibodies. ChIP DNA was analyzed by real-time PCR with primers amplifying a promoter region of EZH2 indicated in the diagram. ChIP PCR data were normalized to input DNA and presented as androgen-stimulated fold change. Reporter gene assays were performed in PC-3 cells by cotransfection of EZH2 promoter reporter construct with ANCCA and/or E2FI expression plasmids (**C**). Data were analyzed with student *t*-test and *P* values are indicated by presence of asterisk: $^{*}P < 0.05$; $^{**}P < 0.001$.

markers with chromatin regulatory function in the developing prostate. More importantly, their expression profile coincides with key regulators of prostate development such as Foxa1, Foxa2, and Nkx3.1 and supports the notion that ANCCA and EZH2 play important roles in the early processes of prostate development. It is possible that the two chromatin regulators function in the processes by mediating epithelial–mesenchymal interactions which involves reciprocal signaling by the key morphogenetic factors. From embryonic day 20 to postnatal day 5, they are strongly expressed in the epithelium as well as the mesenchyme of the developing prostate. It has been postulated that tips of prostate buds and elongating ducts are hubs of active paracrine signaling. Interestingly, high levels of EZH2 tend to appear at the leading edge of the buds. Given that both ANCCA and EZH2 are key mediators of cell proliferation, it is conceivable that one major function of ANCCA and EZH2 is to integrate the different signaling to promote the prostate epithelial cell proliferation in response to the paracrine factor stimulation. This conjecture is also consistent with the finding that high levels of ANCCA and EZH2 generally correspond to peaks of epithelial cell proliferation during perinatal and puberty period. However, given that EZH2 is an important player of stem cell pluripotency and cell type identity [51] and that ANCCA acts as a coactivator for several transcription factors including AR, their function in prostate development likely goes beyond being just a proliferation mediator. Future studies will be needed to elucidate their functions in the prostate.

Some of the key morphogenetic factors such as Fgf10, Nkx3.1, and Hoxb13 are androgen regulated during prostate development [2]. Results from this study strongly suggest that ANCCA and EZH2, two putative morphogenetic regulators of prostate, are also downstream mediators of androgen signaling in prostate development. Thus, the expression of the chromatin regulators peaks at the late gestation and neonatal stages and later at puberty when circulating androgen level reaches the high points. While castration causes a strong suppression of their expression, testosterone replacement results in a fast and robust induction. How ANCCA and EZH2 are regulated by androgen during development is unclear at this point. It is possible that androgen stimulates their expression through intermediate factors. This is more likely during the early prostate development (e.g., prior to the branching morphogenesis) when the two proteins are abundantly expressed in the prostate epithelial buds where AR is expressed primarily in the surrounding mesenchyme. Interestingly, in both human prostate cancer cells and tumors, ANCCA and EZH2

are also strongly induced by androgen although the two proteins have distinct induction dynamics with ANCCA induction being immediate and robust and EZH2 induction being a later event. Importantly, we also provided several lines of evidence that ANCCA serves as a mediator for androgen induction of EZH2. Thus, androgen induces ANCCA occupancy at EZH2 promoter. ANCCA depletion in LNCaP cells strongly impedes androgen induction of EZH2 mRNA and protein and the assembly of histone methylase MLL1-E2F1 complex. Therefore, these findings illustrate a previously un-described cascade event of androgen control of epigenetic regulators that involves androgen/AR induction of a histone mark reader ANCCA which in turn, through its cooperation with histone H3K4me3 methylase MLL1, stimulates EZH2 expression.

Different levels of androgens such as DHT and R1881 are known for their dynamic effects on cell proliferation and gene expression. When applied at supra-physiological concentrations (above 10 nM) to androgen-sensitive cells such as LNCaP, androgens can exert inhibitory effects on cell proliferation [39,52,53]. In this study, we observed that while 1 and 10 nM DHT induced EZH2 in LNCaP cells that were maintained in hormone deprived condition for 3 days, concentrations above 100 nM did not. A recent study by Bohrer et al. [54], also found that 1 nM and higher concentrations of R1881, which can be 100 times more potent than DHT [55], suppressed EZH2 expression when applied to LNCaP cells that were hormone-deprived for 24 hr. Therefore, in androgen sensitive cells, the exact androgen response of specific gene expression and cell proliferation can differ depending on the concentrations of androgen applied and the hormonal treatment scheme. Importantly, we found that EZH2 expression is strongly inhibited in three different tumors (LTL311, LTL313H, and CRW22) in castrated animals and highly expressed in T implanted animals, indicating that EZH2 expression in the androgen-sensitive prostate tumors is androgen stimulated in the physiologically relevant systems we examined. Our results are consistent with the previous finding that EZH2 is overexpressed in high grade PINs and primary tumors in addition to metastatic tumors [15]. Interestingly, we also observed that, in CRPC C4-2B cells, androgen did not induce EZH2 and instead it strongly suppressed the expression of EZH2 and its associated protein SUZ12. Therefore, the impact of androgen deprivation therapy on PRC2 expression is likely different for different tumors.

In summary, we demonstrated in this study that the expression of chromatin regulators ANCCA and EZH2 in prostate is developmentally regulated with peak levels displayed in epithelium of UGS and during puberty. Their expression is also stimulated by androgen and correlates with prostate epithelial proliferation, suggesting important roles played by them in prostate development. Our study also showed that in prostate cancer cells androgen stimulates EZH2 expression and that ANCCA mediates the stimulation through facilitating assembly of a histone methylase complex at EZH2 gene. This study illustrates a possible co-option of a key chromatin regulator-based developmental mechanism in prostate cancer development. Although androgen deprivation can suppress EZH2 expression to some extent in androgen-sensitive cancer cells and tumors, significant levels of EZH2 expression remain. Therefore, it is conceivable that a combined treatment with small molecule inhibitors of AR and other regulators of EZH2 can be more effective in blocking the function of EZH2 and tumor progression. In this regard, we also demonstrated previously that ANCCA controls gene expression programs crucial for cell proliferation and survival [9–11,13]. Given the recent discoveries of small molecule inhibitors targeting specific bromodomains with high therapeutic potential [56,57], this study suggests bromodomain protein ANCCA as an attractive therapeutic target for prostate cancer.

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