Collaboration of Kras and Androgen Receptor Signaling Stimulates EZH2 Expression and Tumor-Propagating Cells in Prostate Cancer

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Abstract

Elevation of the chromatin repression factor enhancer of zeste homolog (EZH2) is associated with progression and poor prognosis in several human cancers including prostate cancer. However, the mechanisms driving EZH2 expression are not fully understood. In this study, we investigated the functional synergy in prostate cancers in mice resulting from activation of the androgen receptor, Kras, and Akt, which drives three of the most frequently activated oncogenic signaling pathways in prostate cancer. Although, any two of these three events were sufficient to promote the formation and progression of prostate cancer, only the synergy of androgen receptor and Kras signaling could elevate EZH2 expression and expand prostate cancer progenitor cells in vivo. Our findings have revealed a genetic mechanism resulting in enhanced EZH2 expression during the progression of aggressive prostate cancer, with important implications for understanding how to target advanced disease where cancer progenitor cells may be critical. Cancer Res; 72(18); 1–10. ©2012 AACR.

Introduction

Prostate cancer is a heterogeneous and multifocal disease. Disease progression is believed to develop through defined pathologic states, beginning with prostatic intraepithelial neoplasia (PIN), progressing to invasive carcinoma, and metastatic cancer representing the most aggressive phase (1). During progression, genetic and epigenetic alterations and the tumor microenvironment play important roles in the etiology of prostate cancer. Numerous reports suggest that multiple genetic changes occur in advanced prostate cancer, but no single major oncogenic event determines a large fraction of the disease (2). Therefore, a detailed understanding of how commonly occurring oncogenic events may synergize will be helpful for elucidating the mechanistic basis of tumor progression, as well as for the identification of therapeutic targets. Aggressive prostate cancers exhibit a multiplicity of genetic mutations (3) and a high incidence of genetic aberrations (4). A common theme among these genetic alterations is the events contributing to the expression or activation of the androgen–androgen receptor signaling axis (5). This trend is highlighted in that more than 80% of patients with castration-resistant disease maintain high androgen receptor expression or exhibit androgen receptor signaling (6, 7). Targeting the androgen–androgen receptor signaling axis remains an effective strategy in the treatment of prostate cancer (5, 8, 9). In addition to androgen receptor signaling, loss of PTEN or activation of AKT and activation of Ras/Raf pathways are among the most frequently occurring genetic changes in prostate cancer (4). Enhanced PTEN/Pi3K/Akt signaling occurs in almost all prostate cancers, and aberrations in the Ras signaling pathway occur in more than 40% of prostate primary tumors and 90% of prostate metastases (4). However, how these signaling pathways collaborate to promote prostate tumor progression is not well understood.

The progression of metastatic prostate cancer is coupled with enhanced expression levels of enhancer of zeste homolog (EZH2) (10). EZH2 is one of the essential components in polycomb repressive complex 2 protein complex (11). Together with suppressor of zeste 12 and embryonic ectoderm development protein, EZH2 catalyzes the methylation of histone H3 via its methyltransferase activity (12). EZH2 further recruits DNA methyltransferases to hypermethylate the promoter region of its target genes and subsequently mediates epigenetic transcriptional repression (13). The epigenetic silencing function of EZH2 regulates stem cell pluripotency, early embryogenesis, lymphopoiesis, and other normal developmental events (14–17). Numerous reports have also implicated EZH2 in the regulation of tumor cell growth, proliferation, invasion, and metastasis in a variety of human cancers including prostate cancer (18, 19). The expression of EZH2 is

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upregulated by aberrant expression of Myc, ETS transcription factor ERG, or by suppression of microRNA (20–23). Further investigation of whether activated signaling pathways in prostate cancer may similarly upregulate EZH2 expression will be helpful in identifying potential targets for treating aggressive prostate cancer.

We have previously shown that primary prostate cells from adult prostate tissue could regenerate into prostate tissue in vivo (24, 25). Using this prostate tissue regeneration system, we have reported that collaboration of some oncogenic signaling pathways recapitulate the progression of invasive prostate cancer in vivo, including the process of epithelial-to-mesenchymal transition (26, 27). Therefore, we used this in vivo regeneration system to determine whether the frequently occurring oncogenic events in prostate cancer, including activation of Kras and Akt, and overexpression of androgen receptor can synergize to enhance EZH2 expression and subsequently promote the progression of prostate cancer. We show that any 2 of these 3 oncogenic events synergize in promoting the progression of prostate carcinoma. However, these 3 related tumors, mAkt+AR, Kras(G12D)+mAkt, and Kras(G12D)+AR, showed a diversity in prostate lineage expansion and tumorigenic cell renewal capacity. In particular, primary Kras(G12D)+AR tumors harbor tumor-propagating cells that maintain the tumorigenic re-initiation capability and primary cancer type in sequential passage. Importantly, Kras(G12D)+AR tumors exhibited higher expression of EZH2, concomitant with their propensity for increased prostate tumor propagating cells. Our study reveals a genetic mechanism of enhanced expression of EZH2 and may have important implications for targeting advanced prostate cancer.

Materials and Methods

Prostate regeneration and prostate epithelial viral infections

The prostate regeneration process, lentivirus preparation, titering, and infection of dissociated prostate cells were described previously in compliance with the safety regulations for lentivirus usage at the University of California (Los Angeles, CA) or Medical University of South Carolina (Charleston, SC; ref. 24). Housing, maintenance, and all surgical and experimental procedures were undertaken in fulfillment with the regulations of the Division of Laboratory Animal Medicine at the University of California or Medical University of South Carolina. Dissociated prostate cell suspensions were prepared from 6- to 10-week-old Kras(G12D)-Loxp mice. Dissociated cells or cells fractionated with surface markers (Lin−CD49f+Scal+) were infected with lentivirus FUCRWW-Cre, FUCRWW-mAkt, FUCRWW-mAkt-Ires-Cre, and FUCGW-AR or co-infected with combinations of lentiviruses according to the experimental setup. Infected cells (1 × 10^3 to 2 × 10^7) were mixed with urogenital sinus mesenchymal cells (1 × 10^7 to 2 × 10^7).rafts were implanted under the kidney capsule in severe combined immunodeficiency (SCID) mice and allowed to regenerate for 8 weeks. The plasmids, mice strains, immunohistochemical (IHC) analysis of regenerated tissues, and the use of antibodies are presented in the Supplementary Materials and Methods.

Prostate sphere assay

The sphere assay was done as previously described (28). To examine whether sphere formation of Kras(G12D)+AR tumorigenic cells was inhibited by suberoylanilide hydroxamic acid (SAHA), or 3-deazaneplanocin A (DZNep), after plating normal primary prostate cells, Kras(G12D) or Kras(G12D)+AR tumorigenic cells in Matrigel, 1 μmol/L of SAHA, 5 μmol/L of DZNep, or the same volume of dimethyl sulfoxide (DMSO) was added to the PrEGM medium. Half of the culture medium was replaced with fresh prostate epithelial cell growth medium (PrEGM) containing the same concentration of SAHA or DZNep every 2 days in the experiment.

Prostate cell culture

Normal prostate basal epithelial cell line (PEB) cell line (29) was maintained in PrEGM with 10% serum. Cells were transduced with lentivirus carrying overexpression of androgen receptor (GFP marker), or Kras(G12V) red fluorescent protein (RFP) marker, or in combination. To exclude the uninfected cell population, cells were sorted by fluorescence-activated cell sorting on the basis of GFP and/or RFP markers and were further cultured for protein analysis. The AR+Kras(G12V)-transduced PEB cells were also treated with 10 μmol/L U0126 or 5 μmol/L DZNep or the same volume of DMSO for 1 day. The treated cells were lysed for analysis of protein expression.

Subcutaneous implantation to passage the Kras(G12D)+AR tumor

The regenerated tumor tissues generated from the prostate regeneration assay were minced into small pieces and digested under 1 mg/mL of collagenase in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and 1% penicillin and streptomycin (P/S). A total of 1 × 10^6 dissociated tumor cells were preserved in 10% DMSO of FBS and stored at −80°C. When doing experiments, the frozen cells were revived in a 37°C water bath and spun down in 600 rpm. The cells were washed once with DMEM 10% FBS P/S medium and gently resuspended in 20 μL of collagen and then injected into the left flank of SCID mice. The Kras(G12D)+AR tumors were regrown after 2 months of subcutaneous implantation.

Results

Synergy among overexpression of androgen receptor, activation of Kras and Akt signaling pathways in the progression of prostate cancer

To investigate the combination of oncogenic events on the progression of prostate cancer, we studied the synergy of 3 frequently occurring oncogenic events in prostate cancer, overexpression of androgen receptor, activation of Akt (mAkt), and activated Kras(G12D), and observed the diversity of tumor characteristics from these combinations. Myristoylated Akt and point mutation (G to D) of Kras at position 12 were used as a surrogate for activation of PTEN/Akt and Ras signaling pathways, respectively. To assess the effects of Kras signaling in tandem with androgen receptor overexpression, we used mice bearing Kras(G12D)-Loxp. Dissociated prostate cells from
these mice were infected with a lentivirus overexpressing Cre and/or androgen receptor (Fig. 1A). Although overexpression of androgen receptor alone inhibited the prostate regeneration as previously reported (27), overexpression of Cre recombinase leads to activation of Kras (30). Tissues regenerated from activation of Kras alone displayed PIN (Fig. 1B and C). In striking contrast, grafts regenerated from activated Kras (G12D) and overexpression of androgen receptor grew larger in size, and tumors invaded into the host kidney tissue (Fig. 1B). The tumor tissue derived from the androgen receptor and Kras (G12D) combination were confirmed by the overlap of RFP and GFP signals (Fig. 1B and C). Tumors derived from Kras (G12D) + AR displayed poorly differentiated or undifferentiated carcinoma (Fig. 1C), and expression of both androgen receptor and phospho-extracellular signal–regulated kinase (Erk) were enhanced in the Kras(G12D) + AR tumor (Fig. 1C). Collectively, our results indicate that activation of Kras synergizes with overexpression of androgen receptor, and the crosstalk of these 2 signaling pathways appears to significantly contribute to the progression of advanced prostate cancer.

We next investigated the potential synergy of the phosphoinositide 3-kinase (PI3K)/Akt and Kras signaling pathways. Prostate cells isolated from mice carrying Kras(G12D)-Loxp were infected with a lentivirus overexpressing Cre, mAkt, mAktires-Cre gene (Fig. 2A). Strikingly, tumor grafts derived from the combination of Kras(G12D) + mAkt weighed approximately 10 times more than those derived from either mAkt or Kras (G12D) alone (Fig. 2B and C). Similar to mAkt, regenerated prostate tissues from overexpression of Cre, and thus activated Kras, showed PIN lesions as described in Figs. 1 and Fig. 2D. In contrast, tissues regenerated from co-overexpression of Kras and mAkt exhibited prostate adenocarcinoma (Fig. 2D). While similar expression levels of androgen receptor were observed in the tissues regenerated from overexpression of Kras and mAkt, activated Kras signaling was confirmed by the elevated expression levels of p-Erk in Kras (G12D) or Kras(G12D) + mAkt tumors (Fig. 2D). Collectively, our results indicate that activation of Kras synergizes with the Akt signaling pathway to promote prostate cancer progression.

We have previously reported that synergy of mAkt and androgen receptor can initiate frank carcinoma in naive adult murine prostatic epithelium (26). Although mAkt-induced tumors showed PIN lesions with secretion in the center of glandular structure, tumor tissues derived from prostate

Figure 1. A, synergy of the activation Kras signaling with androgen receptor promotes prostate cancer progression. Schematic outline for obtaining prostate epithelial cells from Kras(G12D)-loxp mice, lentiviral infection of Cre (with the fluorescent marker RFP) and/or androgen receptor (with the fluorescent marker GFP), and combination with UGSM for prostate regeneration. B, regenerated prostate grafts, RFP/GFP signals (scale bar, 2 mm), and hematoxylin and eosin (H&E) staining of regenerated tissues (low magnification; scale bar, 400 μm) derived from primary prostate cells transformed by androgen receptor, Kras (G12D) or Kras(G12D) + AR. The dashed lines show the regenerated prostate grafts on mice kidney. C, histologic analysis of Kras(G12D) or Kras(G12D) + AR tumor tissues by H&E (high magnification), RFP and/or GFP, and immunohistochemistry for androgen receptor and p-Erk. Scale bar, 50 μm.
epithelia infected with AR+mAkt exhibited sheets of undifferentiated carcinoma cells (ref. 26; Supplementary Fig. S1A and S1B). Taken together, we have shown that any 2 of 3 oncogenic events, activation of Kras and Akt signaling pathways, or overexpression of androgen receptor, synergize leading to the progression of prostate cancer.

**Phenotypic diversity in expansion of epithelial lineages in Kras(G12D)+AR, Kras(G12D)+mAkt, and mAkt+AR prostate tumors**

We further analyzed the phenotypic diversity of epithelial lineages in Kras(G12D)+AR, Kras(G12D)+mAkt, and mAkt+AR prostate tumors tissues. Tissues derived from mAkt or Kras(G12D) alone displayed predominant proliferation of CK8+ cells, a marker of luminal cells, in PIN lesions (Supplementary Fig. S2A). Similarly, tissues derived from Kras(G12D)+mAkt tumors showed predominant proliferation of CK8+ cells with CK5+ cells, a marker of basal cells, or p63+ cells, a marker of basal and/or progenitor cells, localized at the basal membrane of the glandular structure (Fig. 3A). In some cases, a glandular structure contained predominantly CK5+ and p63+ with fewer of CK8+ cells, and some cells were both CK5- and CK8-positive. Alternatively, the tumor cells from the mAkt+AR tumor displayed weak expression levels of CK8 with no expression of CK5 and p63 (Fig. 3A). Expression of E-cadherin in mAkt+AR-transformed cells suggests these cells were derived from the epithelia (Supplementary Fig. S1B). In striking contrast, tissues derived from Kras(G12D)+AR tumors exhibited multiple layers of CK5+ cells in the basal compartment with fewer CK8+ cells located at the luminal layer (Fig. 3A and B). In addition, CK5+ cells localized on the top of a distinct p63+ basal layer in tumorigenic tubules (Fig. 3A and B; Supplementary Fig. S3). Collectively, our data suggest dramatic diversity of lineage expansion in 3 related tumors. Particularly, synergy of Kras(G12D) with androgen receptor promotes proliferation of progenitor cells in prostate tumors.

Tumorigenic cells derived from Kras(G12D)+AR prostate tumors, but not Kras(G12D)+mAkt or mAkt+AR, maintain self-renewal potential to re-initiate secondary tumors.

As Kras(G12D)+AR primary tumors harbor an expansion of progenitor cells, we asked whether dissociated tumorigenic cells had self-renewal potential, exemplified by their ability to re-initiate secondary tumors. Kras(G12D)+AR, Kras(G12D)+mAkt, and mAkt+AR tumors were dissociated into single cells.
After mixing with collagen, the same number of tumor cells was re-implanted subcutaneously into the flank side of SCID mice (Fig. 4A). Only dissociated tumor cells derived from Kras(G12D)+AR primary tumors gave rise to a visible secondary tumor after 2-month incubation (Fig. 4B). We further examined whether properties of the secondary tumor tissues recapitulated characteristics of parental tumors. Indeed, lineage analysis showed that the secondary tumor resembled the primary tumor, which contained glandular tubules with an expansion of CK5+ and p63+ cells located at the prominent basal layer, and CK8+ cells localized toward the luminal layer (Fig. 4C). In addition, expression levels of both p-Erk and androgen receptor were increased in the secondary tumor (Fig. 4C). In summary, our results show that cells from Kras(G12D)+AR primary tumors exhibited the capacity to regenerate secondary tumors with characteristics resembling the parental tumors.

**Kras(G12D)+AR, but not Kras(G12D)+mAkt or mAkt+AR, prostate tumors enhances expression levels of EZH2**

EZH2 plays an important role in suppression of epithelial differentiation and maintenance of stem cell pluripotency by epigenetic silencing (14). We have observed that epithelia in Kras(G12D)+AR primary tumors and secondary tumors were composed of more undifferentiated progenitor cells. We hypothesized that the expansion of prostate primitive cells, such as increasing CK5+ basal cells and p63+ cells in Kras(G12D)+AR primary and secondary tumors, may be correlated with inhibition of the ability to differentiate due to epigenetic silencing. We therefore examined the expression levels of EZH2 in mAkt+AR, Kras(G12D)+mAkt, and primary and secondary Kras(G12D)+AR tumors and found enhanced expression levels of EZH2 only in Kras(G12D)+AR primary and secondary tumors (Fig. 5; Supplementary Fig. S2B). EZH2, an essential component of the polycomb group (PcG) protein complex 2, catalyzes the trimethylation of histone H3 (Lys27; H3K27me3) (13). Consistently, Kras(G12D)+AR primary and secondary tumors displayed higher levels of H3K27me3 (Fig. 5), whereas mAkt+AR showed only basal expression of H3K27me3 similar to mAkt, Kras(G12D), or normal regenerated tubules (Supplementary Fig. S2B). In contrast, Kras(G12D)+mAkt tumors exhibited a downregulation of H3K27me3 expression (Fig. 5). In addition, tissues derived from Kras(G12D)+mAkt and Kras(G12D)+AR primary and secondary tumors exhibited increased expression of cyclin D1. Expression levels of p-androgen receptor were elevated in mAkt+AR and dramatically increased in Kras(G12D)+AR primary and secondary tumors, but, to a less extent, in Kras(G12D)+mAkt tumors than in Kras(G12D) or mAkt tumors (Fig. 5). Collectively, our results have shown that Kras(G12D)+AR, Kras(G12D)+mAkt, and mAkt+AR prostate tumors exhibited distinct molecular features. In addition, epigenetic silencing by increased EZH2 activity may inhibit tumorigenic cell differentiation and enhance cell renewal capacity in Kras(G12D)+AR tumors.

**The expression of EZH2 is important for the enhanced sphere-forming capacity in tumorigenic cells derived from Kras(G12D)+AR prostate tumors**

Sphere formation capacity has been used to measure primitive prostate cell activity in vitro (25). To examine whether cells derived from Kras(G12D)+AR tumors possess enhanced regeneration capacity, we generated Kras(G12D)+AR tumors from enriched basal prostate cells. Prostate basal cells were sorted on the basis of Lin−−CD49f−−Sca1− markers (Supplementary Fig. S4A) from prostate tissues of transgenic mice
carrying Kras(G12D)-loxp (27). The isolated basal cells were infected with either FUCRW-Cre and/or FUCGW-AR. The basal cells transformed by Kras(G12D)þAR showed increased tumor size and advanced adenocarcinoma in comparison with that of Kras(G12D) alone, which showed PIN lesions as previously described (Fig. 1D; Supplementary Fig. S4B–S4D). To further examine whether tumorigenic cells of Kras(G12D)þAR had enhanced capacity to form spheres, the regenerated Kras(G12D)þAR and Kras(G12D) tumors or primary prostate tissue from adult mice (as a control) were subjected for sphere formation assay (28). Oncogene-transformed cells usually become more differentiated (31) and subsequently could lose the capability to form spheres. Tumorigenic cells derived from Kras(G12D) did not form spheres, however cells from Kras(G12D)þAR tumors grew spheres that were approximately 4 times larger in diameter than spheres derived from normal prostate cells (Fig. 6A and B). Interestingly, in some cases, daughter spheres divided out from parental spheres (Supplementary Fig. S5). These results further support our finding that tumorigenic cells from Kras(G12D)þAR show enhanced cell renewal capacity.

Histone deacetylase (HDAC) activity is important for EZH2 function and DNA methylation (19). To examine whether EZH2 plays a critical role in the increased cell renewal capacity in Kras(G12D)þAR tumorigenic cells, we examined whether the HDAC and EZH2 inhibitors, SAHA, and DZNep, can suppress the elevated sphere formation capacity. As expected, DZNep inhibits prostate sphere formation of normal prostate cells (Supplementary Fig. S6; ref. 32). The size or number of spheres derived from Kras(G12D)þAR tumorigenic cells was similarly suppressed by the SAHA or DZNep treatment (Fig. 6C–F), suggesting that enhanced sphere formation capacity in Kras(G12D)þAR tumorigenic cells is likely mediated by EZH2 activity.

Erk signaling partially facilitates the enhanced EZH2 expression in PEB cells overexpressing Kras(G12V) and androgen receptor

To study how co-overexpression of constitutively active Kras and androgen receptor promotes the expression of EZH2, we established an in vitro model. Normal PEB cells (29) were transduced with constitutively active Kras(G12V) [similar to Kras(G12D) mutant] and/or androgen receptor. While as expected, PEB cells with co-overexpression of Kras(G12V) and/or androgen receptor exhibited elevated expression levels of androgen receptor and p-Erk, overexpression of androgen receptor alone led to a repression of EZH2 expression (Fig. 7A). This is consistent with reported studies, in which addition of androgen represses EZH2 expression, whereas knockdown of androgen receptor increases EZH2 expression in LNCaP cells (34, 35). However, similar to the previously described in vitro regeneration model, Kras(G12V)þAR–transduced PEB cells displayed increased expression levels of EZH2 and H3K27Me3 and H3K9Me3 (Fig. 7A), suggesting that co-overexpression of
Figure 5. Distinct molecular features of prostate epithelia in mAkt+AR, Kras(G12D)+mAkt primary tumors, and primary and secondary Kras(G12D)+AR tumors. IHC analysis of regenerated tumor tissues for the expression of EZH2, H3K27me3, p-AR, and cyclin D1. Scale bar, 200 μm.

Figure 6. Dissociated tumorigenic cells derived from Kras(G12D)+AR primary tumors form distinctively large spheres and sphere formation is suppressed by SAHA and DZNep. A, the regenerated prostate tumors, Kras(G12D) or Kras(G12D)+AR, or primary mouse prostate tissues (control) were dissociated into single cells and mixed with Matrigel. After 10-day incubation, images of prostate spheres were taken. Scale bar, 50 μm. B, the diameters of spheres derived from normal prostate primary cells, Kras(G12D), and Kras(G12D)+AR tumors were measured by micro-scale. C–F, the Kras(G12D)+AR tumor cells were treated with DMSO, 1 μmol/L SAHA (C and D), or 5 μmol/L DZNep (E and F) for 10 days in sphere assay. Number or diameter of sphere was measured.
Kras(G12V) and androgen receptor in PEB cells leads to an acquired epigenetic alteration.

To determine whether Erk signaling plays an important role in the regulation of EZH2 expression, Kras(G12V)+AR-transduced PEB cells were treated with the Erk signaling inhibitor U0126 or EZH2 inhibitor DZNep (control). As expected, U0126, but not DZNep, significantly suppressed p-Erk levels (Fig. 7B). Expression of EZH2 was suppressed by DZNep treatment, whereas treatment with U0126 attenuated EZH2 expression to a lesser extent (Fig. 7B). The data suggest that the enhanced expression of EZH2 may partially mediate by Erk signaling in co-overexpression of Kras(G12V)+AR tumorigenic cells.

Discussion

In this study, we have shown that any 2 of the 3 frequently occurring oncogenic events, overexpression of androgen receptor and activation of Akt or Kras, synergize to promote the progression of prostate cancer. Using our regeneration model, tumors derived from the combinations, Kras(G12D)+AR, Kras(G12D)+mAkt, and mAkt+AR, show distinctive features in the expression pattern of EZH2, the expansion of prostate lineages and tumor re-initiation capacity (Supplementary Table S1). Although synergy of Kras(G12D) and mAkt promotes tumor progression, we found this combination downregulated EZH2 expression. This may be due to activation of Akt in promoting EZH2 phosphorylation at serine 21 (36), which downregulates its methyltransferase activity by blocking EZH2 binding to histone H3, decreasing trimethylation at Lys 27, and derepressing epigenetic silencing (36). In contrast, synergy of Kras(G12D) and androgen receptor signaling uniquely recapitulates the elevated EZH2 expression profile observed in the combination of the studied oncogenic events.

Despite the complexity of genetic alterations in aggressive human prostate cancer, persistent androgen receptor signaling and activation of Kras signal transduction pathways represent 2 of the most commonly occurring oncogenic events in prostate tumorigenesis (4). Our results show a genetic mechanism by which oncogenic signaling pathways cooperate to increase the expression of EZH2 and drive a feed-forward loop to support the progression of advanced prostate cancer.

Elevated Ras signaling plays an important role in epigenetic inactivation of multiple genes during cancer progression (37). An involvement for Kras in epigenetic regulation is further supported by a prior report wherein an in vitro genome-wide RNA interference screen showed that the polycomb group proteins EZH2 and BMI1 facilitated Ras-mediated epigenetic silencing in Kras-transformed NIH3T3 cells (38). Our in vivo and in vitro experiments suggest that the collaboration of androgen receptor and active Kras, but not active Kras alone, is required in the regulation of the epigenetic silencing in primary prostate cells or a normal prostate cell line. Although the mutation of Kras leading to constitutive activation is rarely observed in prostate cancer, aberrant Ras signaling could occur via autocrine and paracrine growth factor stimulation or by gene fusions in prostate primary tumor and metastatic disease (39). For example, several gene fusion events, UBE2L3-KRAS (a fusion of Kras with ubiquitin-conjugating enzyme), SLC45A3-BRAF (solute carrier family 45, member3-v-raf-murine sarcoma viral oncogene homolog B1), or ESRP1-RAF1 (epithelial splicing regulatory protein-1-v-raf-1 murine
leukemia viral oncogene homolog 1), have been described in prostate tumors (40, 41). Crosstalk of activated RAS/RAF/MAPK signaling with other oncogenic signaling pathways could dramatically change the tyrosine kinase phosphorylation profile in prostate cancer progression (33). Therefore, dissecting Ras-mediated epigenetic changes will be important in understanding prostate cancer progression.

Elevated Ras downstream Erk signaling may be important in mediating enhanced expression of EZH2 and initiating epigenetic changes. Indeed, we observed increased expression of p-Erk in early-passage PEB cells transduced with Kras and androgen receptor (data not shown). Furthermore, our in vitro data show that suppression of Erk1 signaling modulates the expression levels of EZH2. Erk signaling in the regulation of EZH2 expression has also been reported in a study in which the Mek/Erk1/2/Erk-1 pathway can induce EZH2 overexpression in breast cancer (42), and elevation of Erk phosphorylation by platelet-derived growth factor (PDGF) modulates EZH2 expression levels and, subsequently, regulates adult pancreatic β-cell proliferation (43).

It is still debated as to whether basal or luminal cells are the targets for tumorigenesis in prostate cancer (44). By a lineage tracing approach, Choi and colleagues recently showed that both adult murine prostate basal and luminal cells could serve as targets for tumorigenesis. Although basal cells are more resistant to oncogenesis (such as loss of PTEN), transformed basal cells could differentiate and establish luminal tumor cells (31). Different to PTEN loss or activated Akt induced tumorigenesis, basal cells transformed by the combination of Kras and androgen receptor signaling could elevate the expression of EZH2, and subsequently EZH2-mediated epigenetic silencing could suppress the differentiation of transformed basal cells into luminal cells. This is further supported by the experimental evidence that isolated human prostate basal cells can be transformed to produce luminal cancer with combinations of the most common genetic changes in human cancer (45). Despite the known observation that basal cell carcinoma of the prostate is not common (46), our study implies that tumor-propagating cells might be induced through the synergy of activation of Kras and androgen receptor signaling during prostate cancer progression. Enhanced expression of EZH2 is a marker of prostate metastatic disease (47) and is associated with poor prognosis in a variety of other human cancers (10). We have shown herein that overexpression of androgen receptor alone suppresses EZH2 expression and prostate regeneration (26, 27). This androgen receptor–mediated EZH2 suppression might be regulated by the RB/E2F1 pathway (48) and requires the RB-related protein P130 (35). However, Kras(G12D)+AR tumors are associated with increased expression of EZH2 and possess tumor-propagating cells that highly enhance cancer re-initiation capacity. Aberrant expression of EZH2 may play an important role in maintaining expanded tumorigenic cells in the progenitor stage as confirmed by the proliferation of CK5+/p63+/progenitor cells in the Kras(G12D)+AR primary tumors. EZH2 is an essential component for maintaining stem cell properties (14) and suppressing lineage genes that are required for the differentiation of stem or progenitor cells (18). The enhanced EZH2 expression could further interact with androgen receptor in the process of epigenetic silencing as it has been shown that EZH2 can cooperate with androgen receptor to regulate AR-repressed genes (49). A better understanding of the emerging relationships between cooperating oncogenic events and signaling pathways within the context of EZH2 expression may have important implications for therapeutically targeting aggressive prostate cancer.

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

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Conception and design: H. Cai, O.N. Witte
Development of methodology: H. Cai, O.N. Witte
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Cai, S. Memarzadeh
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References


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