Cancer Cell
Previews

Suppression of Tumor Suppressors in Prostate Cancer: The Emergence of a Novel Oncogenic Pathway

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DOI 10.1016/j.ccr.2007.10.025

Elucidating oncogenic pathways in prostate cancer is an arduous task. In this issue of *Cancer Cell*, Yu and colleagues show that repression of Adrenergic Receptor Beta-2 gene expression by Enhancer of Zeste 2 results in acquisition of transforming activities. It is interesting that these activities point to a role for GTPases as important regulators of transformation in prostate cancer cells. They further implicate epithelial-mesenchymal transition as a biologic end point in this process. Overall, on the basis of their findings, the authors nominate a novel oncogenic pathway for prostate cancer that deserves critical thought and attention.

The identification and functional analysis of oncogenic and tumor suppressor proteins continue to drive progress in basic and translational oncologic research. Recent studies have shown that oncogenic transcription factors are complex oncoproteins that function primarily by recruiting inhibitors of gene transcription to target promoters, resulting in transcriptional repression of selected genes (Hormaeche and Licht, 2007). Enhancer of Zeste 2 (EZH2), a member of this group of transcriptional repressors, has recently gained attention as an important and uniquely acting oncogenic protein (Sparmann and van Lohuizen, 2006). EZH2 is a member of the polycomb group of proteins, which form up to four different multiprotein Polycomb Repressive Complexes (PRCs). PRCs bind to specific target genes and alter their expression through covalent modification of chromatin. These transcriptional regulators play important roles in normal development (Sparmann and van Lohuizen, 2006). As an HMTase specific for histone H3 K27, EZH2 is a catalytically active member of three of these complexes: PRCs 2, 3, and 4 (Kuzmichev et al., 2005). Specific components of these three PRCs are expressed at high levels during embryonic development, and EZH2, specifically, has been shown to prevent stem cell exhaustion and block muscle myoblast differentiation (Hormaeche and Licht, 2007). Overexpression of EZH2 has been associated with various malignancies, including melanoma and endometrial, prostate, breast, and lymphoid cancers (Sparmann and van Lohuizen, 2006).

In their article in this issue of *Cancer Cell*, Yu et al. demonstrate that the creative use of integrated genomics combined with effective broad-based laboratory testing can reveal important and unanticipated molecular pathways in malignant progression (Yu et al., 2007). Previous work from their laboratory demonstrated that EZH2 is overexpressed in prostate cancer and is associated with biochemical recurrence after radical prostatectomy (Varambally et al., 2002). In their current report, these investigators describe their use of gene expression arrays and public gene expression data sets from human tumors to define an EZH2 repression signature for prostate and breast cancer. Promoter occupancy mapping studies restricted the focus to an interesting candidate for EZH2 repression in prostate cancer cells, Adrenergic Receptor Beta-2 (*ADRB2*).

Additional genetic studies showed that EZH2 expression suppressed ADRB2 transcript and protein levels and that, in general, EZH2 levels were inversely associated with ADRB2 expression in prostate cancer cells. Extensive molecular analyses and chemical inhibitor studies confirmed that EZH2 overexpression stimulated PRC2 occupancy of the *ADRB2* promoter.

The authors extended these observations through direct testing of the biologic importance of EZH2-ADRB2 targeting. Those studies revealed that inhibition of ADRB2 had no effect on proliferation yet significantly increased the invasiveness and motility of prostatic epithelial cells in vitro. Conversely, overexpression of ADRB2 or treatment with the agonist isoproterenol reversed the invasive phenotype in multiple models. An interesting note was that downregulation of ADRB2 also led to expression of cell biomarkers consistent with an epithelial-mesenchymal transition (EMT), i.e., increased vimentin and N-cadherin and decreased β-catenin and integrin β4 expression. It is notable that levels of E-cadherin, a critical regulator of EMT, were not significantly affected by ADRB2 knockdown in benign RWPE prostatic epithelial cells. Further studies using prostate cancer xenograft models showed that EZH2 knockdown or isoproterenol treatment suppressed tumor growth in vivo. Finally, ADRB2 immunostaining was considerably reduced in metastatic prostate cancer specimens, and this was inde-

pendently predictive of biochemical recurrence after radical prostatectomy.

This article highlights the successful use of cell line and tumor gene expression profiling data together with genome-wide location data to identify EZH2-repressed candidate genes that have potential biologic and clinical importance. The selection and further pursuit of ADRB2 as an important EZH2 target resulted in noteworthy new information about the oncogenic properties of EZH2 in prostate cancer.

An important and intriguing aspect of this study is the authors' observation that the oncogenic activities of EZH2-stimulated ADRB2 repression involve induction of an EMT. As pointed out by the authors, this observation is consistent with inhibition of ADRB2-stimulated, cAMP-mediated induction of Rap1 activities. Rap1 is a Ras-like small GTPase that is activated by many extracellular stimuli and is involved in cell adhesion (Kooistra et al., 2007). Specifically, activation of Rap1 by cAMP has been shown to promote cell-matrix and cell-cell adhesion through regulation of β1-3 integrins and E-cadherin, maintaining the differentiated epithelial phenotype. Overall, these data suggest the existence of a transformation pathway that involves abrogation of cAMP-mediated Rap1 maintenance of epithelial phenotypic properties by EZH2's repression of ADRB2. This would result in an EMT (Figure 1).

An interesting note is that a recent study identified another putative tumor suppressor protein that is repressed by EZH2-DOC-2/DAB2-interacting protein/ASK-interacting protein1 (hDAB2IP/AIP1), which also regulates GTPase activities (Chen et al., 2005). This GTPase-activating protein stimulates Hras, Rras, and TC21 but not Rap1 GTPase activities and suppresses epidermal growth factor-stimulated prostate cancer cell growth (Wang et al., 2002).

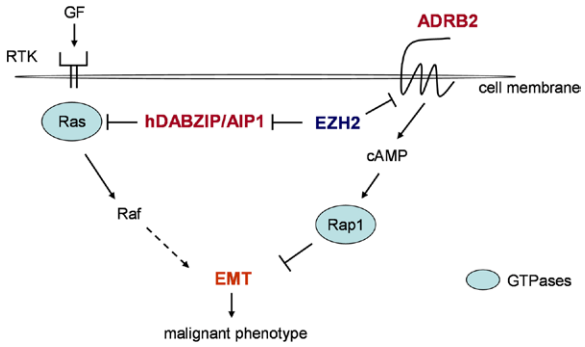

Figure 1. EZH2-Stimulated Repression of GTPase-Regulated Signaling Pathways Involves Induction of EMT

EZH2-stimulated ADRB2 repression involves induction of gene expression activities and induction of an EMT in prostate cancer cells (Yu, et al., 2007). These activities are consistent with downregulation of a signaling pathway involving ADRB2-stimulated, cAMP-mediated Rap1 activation. Stimulation of Rap1 by cAMP has been shown to maintain the differentiated epithelial phenotype through regulation of cell adhesion, and thus suppression of this signaling pathway is consistent with EMT. Recent studies also show that EZH2 downregulates hDAB2IP/AIP1, a GTPase-activating protein and putative tumor suppressor that inhibits EGF-Ras signaling in prostate cancer cells. Thus, EZH2 activities may sustain RTK-stimulated EMT. Overall, EZH2 may induce EMT and malignant activities through subversion of multiple pathways that involve GTPases.

The effects of EZH2's repression of hDAB2IP/AIP1 on the epithelial phenotype have not been reported. Yet it has been previously established that receptor tyrosine kinase signaling through Ras and small GTPases can result in EMT (Guarino et al., 2007). Under some conditions, Rap1 can be affected by upstream tyrosine kinase stimulation (Kooistra et al., 2007). This points to the possibility that signaling from both tyrosine kinase activation and ADRB2 activation converges on Rap1, providing additional regulation of EMT and the transformation state. Although it is premature to make generalizations, the biologic activities related to EMT regulated by these two EZH2-targeted, GTPase-mediated signaling pathways are provocative.

It has long been appreciated that the prostate is a highly innervated contractile organ, capable of responding to various adrenergic stimuli. It is also noteworthy that past studies have shown that ADRB2's activities converge with androgenic stimulation to contribute to prostatic differentiation in vivo (Guthrie et al., 1990). More recent genetic observations also point to the potential of ADRB2 as a tumor suppressor: specifically, polymorphisms in *ADRB2* were shown to be associated with increased risk of breast and colorectal cancers (Takezaki et al., 2001). However, additional genetic and biologic studies that further address the molecular mechanisms underlying ADRB2's tumor suppressor activities and their subversion by EZH2, as revealed by Yu et al., are warranted.

The results of Yu et al. raise some specific questions to begin this process. Are other molecules in GTPase-mediated signaling pathways affected by EZH2/PRC2 gene repression? Are those tumor suppressor pathways and their subversion by EZH2 specific to prostate cancer? What are the endogenous agonists and/or protein/peptide modifiers that regulate ADRB2 signaling in prostate epithelial cells? Can these pathways be exploited in the development of novel cancer therapeutics? These questions and others are now open for discussion and study.

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Nucleosomes at Active Promoters: Unforgettable Loss

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DOI 10.1016/j.ccr.2007.10.024

A variety of chromatin features have been implicated in the regulation of gene expression, including nucleosome occupancy at promoters, histone modifications and variants, and DNA methylation. In this issue of *Cancer Cell*, Lin and colleagues use a powerful single-molecule approach to explore the relationship between nucleosome occupancy and gene expression in cancer cells. They show that nucleosome occupancy is mostly all-or-none at the multiple start sites of the *MLH1* CpG island. After demethylation by drug treatment, nucleosomes are permanently lost as transcription becomes reactivated. Thus, epigenetic maintenance of gene expression may require that promoters are maintained free of nucleosomes.

A growing body of evidence has revealed that chromatin changes correlate with differences in gene expression, leading to the widely held view that transcription factor binding at promoters acts through nucleosomes to activate or repress gene expression (Li et al., 2007). However, it is unclear how the various chromatin differences can lead to changes in the on-versus-off state of promoters. One idea is that histone modifications alter the accessibility of DNA by stabilizing interactions between chromatin-associated proteins and the histones that they bind to (Cosgrove et al., 2004). But then how do these histone tail interactions result in up- or downregulation of gene expression? A paradigm originating from studies of the yeast *PHO5* promoter is that nucleosomes are simply evicted from promoters, and the naked DNA that results allows for transcription factors to gain access to their binding sites and for the basal transcriptional machinery to assemble (Boeger et al., 2003; Reinke and Horz, 2003). Indeed, a distinguishing feature of active promoters in general is that they are depleted in nucleosomes relative to silent promoters (Mito et al., 2005).

A limitation of studies that have been used to map chromatin characteristics, such as histone modifications and variants, is that they provide data that are averaged from large numbers of individual DNA molecules. For example, ChIP-chip and real-time PCR assays can provide sensitive measurements of chromatin features and of nucleosome occupancy, but these are relative measurements that cannot distinguish between a change in the amount of a feature relative to a control and its absolute abundance (van Leeuwen and van Steensel, 2005). Therefore, it has remained possible that the reduction in nucleosome occupancy seen in such studies is not complete eviction of nucleosomes, but rather partial loss or even transient unwrapping. To address this uncertainty, Peter Jones' group introduced a single-molecule modification of the DNA methylation mapping technique for mapping chromatin accessibility (Fatemi et al., 2005; Kladde et al., 1996) (Figure 1A). The M.SssI DNA methyltransferase specifically methylates the cytosines of CG dinucleotide base pairs, making these bases resistant to deamination by treatment with sodium bisulfite. As a result, M.SssI methylation of nuclei followed by DNA extraction and bisulfite treatment results in DNA with CGs intact, but with all other cytosines converted to uracil. The uracil bases are replicated as if they were thymines, so that PCR amplification, cloning, and sequencing of a region using M.SssI- and bisulfite-treated DNA yields sequences from single molecules in which CGs that have been methylated by M.SssI are sequenced as CGs, but those that have escaped M.SssI methylation are sequenced as TGs. In this way, blocking of a CG from the action of M.SssI in nuclei can be quantified by sequencing a collection of PCR-generated clones,