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14. ABSTRACT

Prostate cancer (PCa) represents the most frequently diagnosed malignancy of men in the US. Reactivation of androgen receptor (AR) signaling following androgen deprivation is a major driver of the development of castration-resistant prostate cancer (CRPC). Understanding the precise mechanisms underlying aberrant AR-regulated gene activation will advance our knowledge of PCa tumorigenesis, with implementation of new therapeutic strategies. LincRNAs are recently identified novel genetic materials. The role of linRNAs in cancer is being increasingly accepted, both as possible specific biomarkers and as potential therapeutic targets. However, how aberrant expression of these lincRNAs contributes to PCa progression is still not fully understood. This one-year hypothesis-exploratory project was designed to test a novel hypothesis that lincRNAs promote AR reactivation in prostate cancer cells following androgen deprivation through modulation of AR/EZH2-mediated repressive chromatin remodeling. The experiments as proposed were successfully completed during the funding peroid and objectives achieved. Resultant data provide a basis for the identification of novel targets for therapeutic intervention for PCa. The ultimate goal is to define the molecular mechanisms of PCa initiation and development and to develop effective treatments.

15. SUBJECT TERMS

Prostate Cancer; Tumor progression; lincRNAs; PCATs; Androgen receptor; Castration resistance; EZH2; Histone Modifications

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1. Introduction

Prostate cancer (PCa) represents the most frequently diagnosed malignancy of men in the US, with a lifetime incidence risk of 1 in 6, and an expected 241,000 new cases and 33,700 death in 2011. The androgen receptor (AR) is a member of a nuclear receptor superfamily that regulates gene transcription. Many of these AR-regulated genes are key regulators to prostate development and progression. Understanding the precise mechanisms underlying aberrant AR-regulated gene activation will advance our knowledge of PCa tumorigenesis, with implementation of new therapeutic strategies. LincRNAs are recently identified novel genetic materials. The human genome encodes thousands of lincRNAs and each of these molecules can control hundreds of genes[1]. Therefore, these molecules are extremely powerful molecules that regulate almost all aspects of the host's well-being. Recent research indicates that many lincRNAs have been linked to the initiation and progression of human cancer. The role of linRNAs in cancer is being increasingly accepted, both as possible specific biomarkers and as potential therapeutic targets. A subset of lincRNAs, so-called prostate cancer-associated ncRNA transcripts (PCATs), may play an active role in prostate cancer progression[2]. However, how aberrant expression of these lincRNAs/PCATs contributes to PCa progression is still not fully understood. Reactivation of androgen receptor (AR) signaling following androgen deprivation is a major driver of the development of castration-resistant prostate cancer (CRPC). This one-year hypothesis-exploratory project was designed to test a novel hypothesis that lincRNAs/PCATs promote AR reactivation in prostate cancer cells following androgen deprivation through modulation of AR/EZH2-mediated repressive chromatin remodeling. Resultant data provide a basis for the identification of novel targets for therapeutic intervention for PCa. The ultimate goal is to define the molecular mechanisms of PCa initiation and development and to develop effective treatments for this critically important disease.

2. Keywords

Prostate Cancer; Tumor progression; lincRNAs; PCATs; Androgen receptor; Castration resistance; EZH2; Histone Modifications; NF-kB; IKKα; Chromatin immunoprecipitation; RNA immunoprecipitation.

3. Overall Project Summary

Hypothesis and Aims: AR has been known as a transcriptional activator inducing prostate-specific gene expression. Data from integrative genome-wide analysis indicate that AR also acts as a transcriptional repressor. This repression, dictated by EZH2-associated repressive chromatin remodeling such as histone modifications, plays an important role in prostate cancer progression[3]. EZH2 is an RNA-binding protein and can interact with lincRNAs to induce gene repression through H3K27me3. Indeed, EZH2-lincRNA interactions have been demonstrated to direct complex patterns of chromatin remodeling complex, resulting in gene suppression in prostate cancer cells[4]. Nevertheless, the role of lincRNA/EZH2-mediated chromatin remodeling in AR reactivation following androgen deprivation has not been investigated. Although prostate cancer is a heterogeneous disease and its progression involves many pathogenic factors, inflammatory responses elicited by androgen deprivation contribute to the emergence of CRPC[5]. The nuclear factor kappa B (NF-κB) signaling

pathway is the master regulator for inflammatory reactions, and CRPC cells usually exhibit activated NF-κB. Many stimuli activate NF-κB, mostly through activation of IκB kinase α (IKKα) and IKKβ in the cytoplasm, resulting in nuclear translocation of NF-κB subunits and, subsequently, transactivation and transrepression of targeted genes. CRPC cells are usually with the accumulation of activated IKKα in nuclei. The kinase activity of nuclear IKKα mediates phosphorylation of regulatory proteins and is required for IKKα-mediated gene suppression[5]. Intriguingly, phosphorylation of EZH2 can facilitate lincRNA binding to EZH2 and, consequently, chromatin remodeling[6]. Therefore, the **overriding hypothesis** of this exploratory project is that nuclear accumulation of IKKα enhances lincRNA/PCATs-guided chromatin recruitment of EZH2 complex and EZH2/AR-mediated H3K27me3 modification, promoting AR reactivation in prostate cancer cells following androgen deprivation (Figure 1). Two specific aims were proposed to address this central hypothesis: **Aim 1** was designed to define that nuclear translocation of IKKα enhances lincRNA-guided chromatin recruitment of EZH2 complex in prostate cells. **Aim 2** were proposed to clarify that IKKα/lincRNA-induced chromatin recruitment of EZH2 promotes EZH2/AR-mediated H3K27me3 modification and contributes to the reactivation of AR signaling following androgen deprivation in prostate cancer cells.

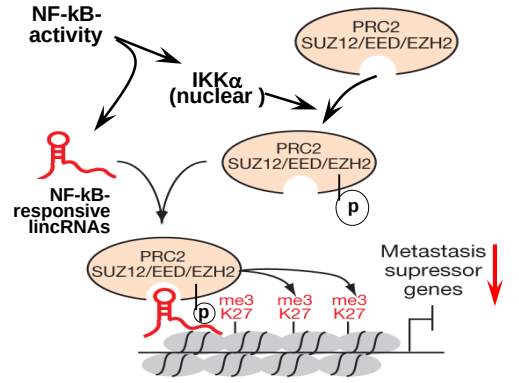

Fig. 1. Our hypothesis on lincRNA/IKKα-mediated gene suppression associated with PCa progression. Aberrant activation of NF-kB signaling will increase expression of NF-kB-responsive lincRNAs and cause nuclear translocation of IKKα. Nuclear translocation of IKKα will promote the chromatin recruitment of PRC2/EZH2/lincRNA complex, contributing to the suppression of metastatic suppressor genes key to PCa progression.

Studies, Results and Significance: During the one-year funding period, we have actively addressed all the key components as proposed, using an array of biochemical and molecular biological approaches, such as chromatin immunoprecipitation (ChIP) analysis to identify chromatin recruitment of EZH2, RIP assay for EZH2-lincRNA interactions, H3K27me3 analysis, and RNA interference for functional manipulation. We have completed all the experiments as proposed in the SOW for both Specific Aims and have not encountered insurmountable technical problems. There were no important modifications to our original plans. Resultant data provide mechanistic insights into the molecular mechanisms that link chronic inflammation to AR reactivation in prostate cancer cells following androgen deprivation.

Specifically, we have screened the expression of lincRNAs in metastatic PCa tissues and cell lines, as well as in PCa cells at the androgen deprivation condition. A panel of NF-kB-responsive lincRNAs was identified to be consistently upregulated in metastatic PCA tissues and cell lines, or PCa cells after androgen deprivation (Figure 2A) and expression levels of selected lincRNAs of the panel by PCR were shown in Figure 2B. These NF-kB-responsive lincRNAs were selected according to the NF-kB binding sites in their gene promoter regions

(Figure 2A). Importantly, induction of these lincRNAs was also detected in LNCaP cells after stimulation with lymphotoxin B (Figure 3). Lymphotoxin B is an activator to stimulate NF-kB signaling and induce nuclear translocation of IKKa in PCa cells and thus, has been used as a positive control in our analysis. Consistent with the concept that aberrant activation of NF-kB signaling in PCa progression, we detected a significant increase of nuclear translocation of IKKa in LNCaP cells at the androgen deprivation condition (Figure 4A). Accordingly, we also detected an increased assembly of a panel of NF-kB-responsive lincRNAs to the PRC2/EZH2 complex in PCa cells after androgen deprivation by RIP analysis. Data showing the assembly of PCAT-1 and Hotair to the PRC2/EZH2 complex in LNCaP cells after androgen deprivation were shown in Figure 4B and Figure 4C, respectively. To identify the target genes that may be associated with PRC2/EZH2 promoter recruitment in PCa cells following androgen deprivation, we measured the expression level of those metastatic suppressive genes. We focused on those genes that have the ORC2-repression signature in their gene loci. Our analysis revealed several of those genes that are downregulated in PCa cells following androgen deprivation (Figure 5A). Downregulation of representative genes in LNCaP cells following androgen deprivation as measured by PCR was shown in Figure 5B. To further test the potential recruitment of PRC2/EZH2 complex and its association with suppressive histone modification, we performed ChIP analysis in LNCaP cells following androgen deprivation and our data demonstrate the recruitment of PRC2/EZH2/lincRNA complex to the gene loci that are downregulated in cells following androgen deprivation. As a representative example shown in Figure 6, using an antibody to EZH2 for the ChIP analysis, we detected an increase in PRC2/EZH2 complex to the KCNA1 gene promoter region. Accordingly, an enrichment of suppressive H3K27me3 was also detected in the nearby region of the KCNA1 gene promoter by ChIP analysis using an antibody against H3K27me3. In addition, we have also measured the involvement of EZH2-lincRNA interactions in EZH2/AR-mediated H3K27me3 modification in LNCaP cells in response to AR activation. All these data are currently in preparation for publication.

A

LincRNAs	In metastatic PCa		PCa cell lines at androgen-depr.	NF-kB binding to promoter
	Patients	Cell lines		
PCAT-43	↑↑↑	↑↑↑	↑↑↑	Yes
PCAT-1	↑↑	↑↑	↑	Yes
PCAT-14	↑↑↑	↑↑↑	↑↑↑	Yes
Hotair	↑↑↑	↑↑↑	↑↑↑	Yes
LincMNLK	ND*	↑↑	↑↑↑	Yes
LincROR	ND	↑↑	↑↑↑	Yes
Lincp21	ND	↑↑↑	ND	Yes
LincFOXF1	ND	↑↑	ND	Yes
LincDR1	ND	↓↓	ND	Yes
LincGAR3	ND	↓↓	ND	Yes
LincZFP161	ND	↓↓	ND	Yes

B

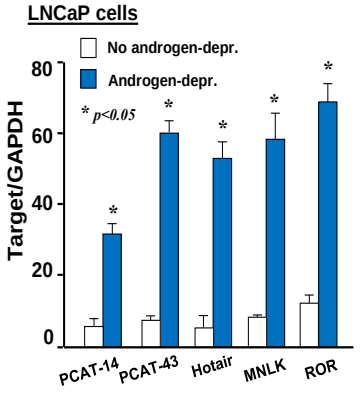

Fig. 2. Aberrant expression of lincRNAs is associated with metastatic and androgen-resistant progression of PCa cells, potentially through NF-kB activity. **A**, a list of lincRNAs was altered in patient PCa tissues, which was further confirmed in metastatic PCa cell lines with potential NF-kB binding sites in their gene promoters. **B**, representative PCR data showing the upregulation of selected lincRNAs in LNCaP cells at the androgen deprived condition.

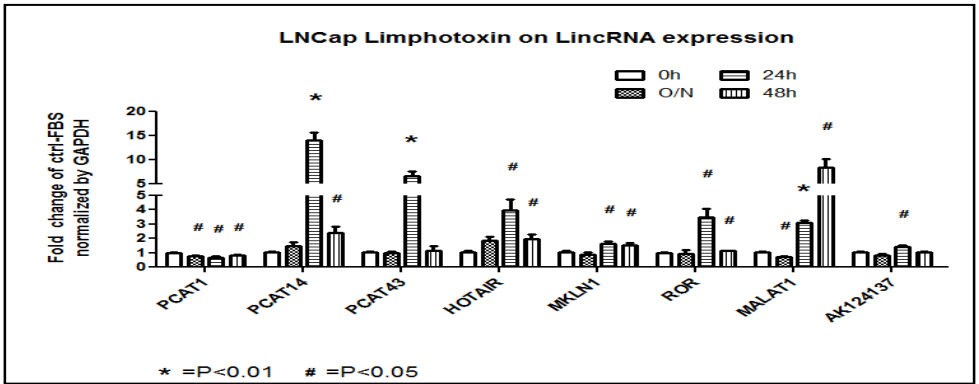

Fig. 3. A list of lincRNAs that was induced in LNCaP cells following stimulation by lymphotoxin B, assessed by real-time PCR. Lymphotoxin B is an activator to induce nuclear translocation of IKKα in PCa cells and thus, was used as a positive control in our analysis.

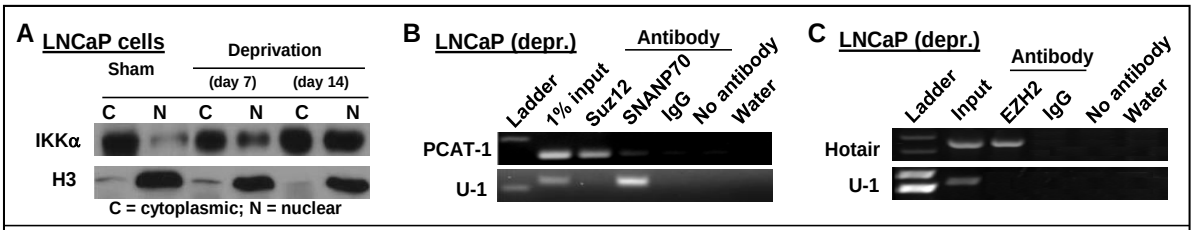

Fig. 4. Nuclear IKKα and assembly of NF-kB-responsive lincRNAs to the PRC2/EZH2 complex in PCa cells at androgen deprivation condition. **A**, nuclear accumulation of IKKα was detected in LNCaP cells after androgen deprivation. **B**, lincRNA PCAT-1 was detected in the PRC2 complex in LNCaP cells at androgen deprivation condition by RIP analysis (Snanp70 and U-1 as Ctrl). **C**, we have also identified an increased assembly of Hotair lincRNA to the EZH2/PRC2 complex in LNCaP cells at androgen deprivation condition.

A

Gene	PRC2-repression signature genes	In metastatic PCa patients	PCa cell lines at androgen-depr.
PTGER3	Yes	↓↓	↓↓
KCNA1	Yes	↓↓	↓↓
HMBS	Yes	↓↓↓	↓↓
CXCL12	Yes	↓↓↓	↓
SOCS2	Yes	↓↓↓	Not tested yet
KRT17	Yes	↓↓↓↓	↓
Maspin	Yes	↓↓↓↓	↓↓↓

B

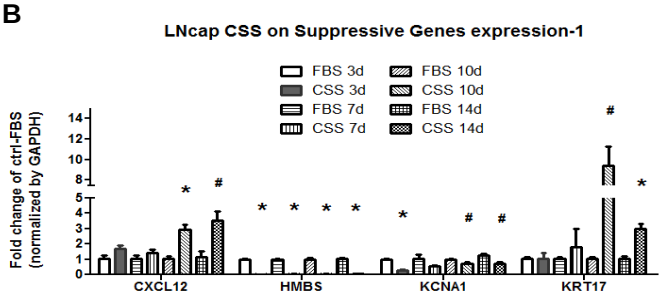

Fig. 5. LincRNA/IKKα-mediated gene suppression may contribute to PCa progression through modulation of PRC2-mediated gene trans-repression. **A**, a panel of metastatic suppression genes was downregulated in metastatic PCa tissues or in PCa cell lines at androgen deprived condition (CSS). **B**, expression of several selected genes, as representative examples for these genes listed above in A, is shown in LNCaP cells at CSS condition.

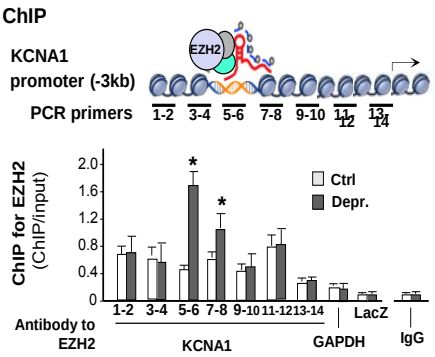

Figure 6. Increased recruitment of PRC2/EZH2 complex to the KCNA1 gene promoter in LNCaP cells at androgen deprived condition.

4. Key Research Accomplishments

- Demonstrating that IKKα induces phosphorylation of EZH2 and enhances lincRNA-guided chromatin recruitment of EZH2 complex in response to AR activation in prostate cells.
- Clarification that IKKα/lincRNA-induced chromatin recruitment of EZH2 promotes EZH2/AR-mediated H3K27me3 modification and contributes to the reactivation of AR signaling following androgen deprivation in prostate cancer cells.

5. Conclusion

In conclusion, this one-year hypothesis exploratory proposal was designed to address the DOD PCRP 2012 focus areas on Mechanisms of Resistance (Understanding primary and acquired resistance to therapy) and Tumor and Microenvironment Biology (Understanding prognosis and progression of prostate cancer). We have actively addressed all the key components and have completed all the experiments as proposed in the SOW during the funding peroid. Resultant data provide not only mechanistic insights into the molecular mechanisms that link chronic inflammation to AR reactivation in prostate cancer cells following androgen deprivation but also a basis for the identification of novel targets for therapeutic intervention for CRPC, addressing the overarching challenge of the DOD PCRP to develop effective treatments for advanced prostate cancer.

6. Publications, Abstracts, and Presentations

Resultant data are in preparation for publication.

7. Inventions, Patents and Licenses

(None).

8. Reportable Outcomes

(None).

9. Other Achievements

(None).

10. References

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11. Appendices

(None).