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PRINCIPAL INVESTIGATOR: Kathleen A. Cooney, M.D.
Colin Duckett, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109-1274

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**Prostate Cancer Aggressiveness Genes in Hereditary Prostate Cancer**

6. AUTHOR(S)

Kathleen A. Cooney, M.D. and Colin Duckett, Ph.D.

E-mail: kcooney@umich.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Michigan
Ann Arbor, Michigan 48109-1274

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**Introduction:** The *EZH2* transcripitional regulator, recently shown by to be overexpressed in prostate cancer specimens that are more likely to recur, maps to 7q35 and represents a candidate gene for inherited prostate cancer susceptibility. This statement is based on the identification of prostate cancer linkage to distal 7q markers in a recently completed genome-wide scan using hereditary prostate cancer families from the University of Michigan Prostate Cancer Genetics Project (PCGP). Relatively little is known about the molecular basis of *EZH2* function or its DNA specificity. The major hypothesis of this proposal is that germline mutations in the *EZH2* gene will predispose to more clinically aggressive forms of prostate cancer and the characterization of these mutations will provide more information about the function of the *EZH2* molecule in prostate cancer and metastasis. To address this hypothesis, the following two specific aims were proposed: 1) to identify germline mutations in *EZH2* that predispose to aggressive prostate cancer in prostate cancer families, and 2) to characterize the functional consequences of *EZH2* mutations specifically focusing on the role of *EZH2* in transcriptional regulation.

**Body:** To address specific aim 1, we had initially planned to perform a resequencing experiment to identify *EZH2* germline variants, particularly single nucleotide polymorphisms (SNPs), that are present at a frequency of 10% or greater and to use these variants to identify the important *EZH2* haplotypes. The haplotype-tagging SNPs would be used to determine whether one or more SNPs are associated with either prostate cancer incidence or aggressiveness using the DNA samples from the PCGP. Prior to performing the resequencing, we set out to determine whether there was sufficient published data or information in any of the publicly-available SNP databases that we could utilize to understand the genetic variation in *EZH2*. Over the past 5 years, these databases (e.g. HAPMAP) have become very complete and have provided population-based data that can be used for research purposes. In the January 2006 version of the International HAPMAP project, we have discovered that 64 SNPs had been typed in ~77 kb of DNA that encompasses *EZH2*. Of these 64 SNPs, 47 were determined to be polymorphic in the Caucasian sample (CEPH individuals). Using a pairwise tagging strategy, we determined that 12 SNPs (indicated by asterisks in Table below) would be necessary to tag all 47 SNPs at an $R^2$ level of greater than 0.8. Note that the SNP indicated by rs2302427 is the Asp146His *EZH2* variant described in the revised statement of work. It is the only nonsynonymous variant that has been reported for this gene.

<table>
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<tr>
<td>rs2072408</td>
</tr>
<tr>
<td>rs887569</td>
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<tr>
<td>rs734004</td>
</tr>
<tr>
<td>rs734003*</td>
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<td>rs734002</td>
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<td>rs740949</td>
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Also in the past year, Bachman et al. \(^1\) reported on an *EZH2* mutation screen using DNA samples from 24 men with prostate cancer derived from 10 families that demonstrated evidence of prostate cancer linkage to 7q31-33 markers. In their screen of all 20 *EZH2* exons as well as the 5' and 3' UTRs, only two variants were identified in the open reading frame: Asp146His and a silent nucleotide substitution in exon 15 (1731G>A). Thus these authors concluded that deleterious mutations in *EZH2* likely did not account for the distal
chromosome 7 linkage signal. However, several suggestive associations were seen between specific EZH2 haplotypes and prostate cancer which supports the plan of using a haplotype-tagging approach for the completion of aim 1.

We plan to genotype of a set of haplotype-tagging SNPs in a set of 925 brothers derived from 361 families in which there is both a brother with prostate cancer and a brother without prostate cancer. As described in Douglas et al., we will use both family-based association tests as well as conditional logistic regression to determine if any single SNP or haplotype is associated with prostate cancer. The data will then be reanalyzed to determine if any single SNP or haplotype is associated with aggressive prostate cancer. Although we had initially proposed to test the Asp146His EZH2 (rs2302427) in year one of this grant followed by a haplotype tagging strategy, we were significantly delayed in starting aim 1 due to the regulatory concerns raised by the Office of Research Protections for the Department of Defense Prostate Cancer Research Programs. Thus we have determined that it would be most efficient to perform all of the genotyping and analysis for Aim 1 during the last year of the award.

We have also made significant progress in achieving the goals of Aim 2. We have validated the interaction between EZH2 and REA, primarily by taking an ectopic expression and communoprecipitation approach (Figure 1A and B). In the previous reporting period, we have additionally focused on the development of approaches to examine the nature and biological significance of these interactions. To this end, we begun to analyze the subcellular localization of interaction, using epitope-tagged versions of these proteins (Figure 1B). While the studies are still underway, we have also developed reagents to use biomolecular fluorescence complementation (BiFC) in order to study the interaction between these two proteins in greater detail. Briefly, this approach involves the generation of empirically derived protein chimeras in which the two proteins of interest (in this case EZH2 and REA) are fused to separate moieties of the fluorescent molecule, YFP. Upon fluorescent excitation, YFP emission will only be reconstituted if EZH2 and REA are physically associated, and thus this approach is a significant enhancement over standard communofluorescence. While the imaging studies are currently underway, our preliminary experiments (Figure 2) reveal that, at least by flow cytometry, YFP fluorescence can be readily detected upon coexpression of the appropriate EZH2-REA chimeras.

We have also begun to make inroads in to understanding the functional consequences of the EZH2-REA interaction. While the readouts for EZH2 biological remain complex, we are continuing to develop systems such as chromatin immunoprecipitation to examine the effects of REA and EZH2 on endogenous gene activity, we have also considered the possibility that EZH2 might affect the activity of REA-responsive genes. As shown in Figure 3, we have found that RNAi-mediated suppression of EZH2 (Figure 3A) can enhance the estradiol-induced expression of the REA-regulated reporter gene, ERE-luc. To date we have observed this effect both in HEK293 cells (Figure 3B) and MDA-MB-231 cells (Figure 3C), and are extending these findings to bother androgen-dependent and –independent prostate cancer lines including PC-3 and LnCaP.

Legend to Figures:

Figure 1: Association between REA and EZH2. (A) Coprecipitation analysis of EZH2 and REA proteins. Expression vectors encoding HA-tagged REA and FLAG-tagged EZH2, or the indicated control plasmids, were transfected into HEK293 cells. Lysates were immunoprecipitated with FLAG antibody, and analyzed by immunoblot with the REA-specific HA antibody or a directly conjugated FLAG antibody, as indicated. (C) HEK293 cells were transfected as in (A) above, except using a glutathione-S-transferase (GST)-tagged EZH2. Lysates were precipitated with glutathione agarose beads and immunoblot for REA as described in (A) above. (C) Coimmunofluorescence analysis of the EZH2-REA interaction.

Figure 2: Preliminary bifluorescence complementation analysis. HEK293 cells were transfected with empty control vectors (upper histogram) or expression vectors encoding REA and EZH2 with different permutations of the amino or carboxy terminal moieties of YFP, and fluorescent reconstitution was evaluated by flow cytometry. As shown in the middle histogram, the combination of the amino half of YFP fused to the carboxy terminal of REA efficiently reconstitutes the remaining half of YFP fused to the carboxy terminal of EZH2.
Figure 3: Effects of EZH2 suppression on REA-regulated transcription. (A) Gen suppressive effects on EZH2 expression following transient transfection of EZH2-specific double-stranded RNAi oligonucleotides into HEK293 cells or MDA-MB-231 cells, as determined by immunoblot. (B) Effects of EZH2 suppression on reporter gene activity of the REA-regulated promoter-luciferase construct, ERE-luc, in HEK293 cells and (C) MDA-MB-231 cells.

Key Research Accomplishments: (new accomplishments in bold)

Specific Aim 1  Annual IRB approval of research protocols.
Design and validation of exon-specific primers for EZH2
Analysis of HAPMAP data outlining the genetic diversity of EZH2
Planning for genetic analysis of PCGP discordant sibling pairs using haplotype-tagging SNPS

Specific Aim 2  Establishment of EZH2-TAP system
Purification and identification of EZH2-associated proteins
Validation of REA as a physiologically relevant EZH2-associated protein
Establishment of reporter systems to analyze EZH2 and REA activity
Design and validation of small interfering RNAs (siRNAs) for REA and XIAP
Coimmunofluorescence studies of the EZH2-REA interaction
Establishment of a bimolecular fluorescence complementation system for dynamic analysis of the EZH2-REA interaction
Evaluation of the effects of EZH2 and REA in stimulus-responsive gene reporter assays
Stable suppression of EZH2 and REA levels by lentiviral delivery of short hairpin RNAi’s

Reportable Outcomes: N/A

Conclusions: We have made significant progress toward addressing Specific Aim 1 and have outlined a plan to examine the whether germline genetic variation in EZH2 is associated with prostate cancer using a haplotype-tagging strategy. We have also made major progress toward achieving the goals of Specific Aim 2, particularly in the examination of the functional consequences of the interaction between EZH2 and REA, and additionally we have developed the critical reagents and experimental systems to further address the dynamics and functional significance of the EZH2-REA complex.

References:


Appendices: See attached Figures 1, 2, and 3.
Figure 1

A. Control IP

B. Inputs Pulldown

C. EZH2 REA overlay
Bimolecular fluorescence complementation – FACS analysis

Figure 2
Figure 3